Medical Management of Chemical and Biological Casualties

Edited by

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Preface

This book includes mainly reports which were presented at the Symposium with international participation “Medical Management of Chemical and Biological Casualties”, which was held in Military Medical Academy, Sofia, Bulgaria from 27 to 28 April 2009.

This Symposium was organized by MILITARY MEDICAL ACADEMY – SOFIA, BULGARIAN TOXICOLOGICAL SOCIETY, BULGARIAN ASSOCIATION OF CLINICAL TOXICOLOGY, NATIONAL CENTER OF INFECTIOUS AND PARASITIC DISEASES AND UNION OF SCIENTISTS IN BULGARIA – SECTION MEDICAL SCIENCES.

The goal of this symposium was assessment of scientific concepts and practical means for management of Chemical and Biological Casualties. In this book are included the results of both theoretical and practical research for chemical and biological terrorism presented during the symposium.

The main topics of the presentations are:

1. New approaches in organization of emergency response;
2. Characterization and mechanisms of action of chemical and biological agents;
3. Pre-treatment and prophylactics of chemical and biological agents injuries;
4. Diagnosis of exposure to chemical and biological agents;
5. Therapy of chemical and biological agents casualties;
6. Some aspects of national and global defense against chemical and biological terrorism;
7. Threats of terrorist attacks with chemical and biological agents;
8. New approaches in counteraction to chemical and biological terrorism

Different trends of interaction between government agencies, the role of Military Medical Academy in emergency response in Bulgaria and medical support in case of chemical and biological threat are discussed. The problems are analyzed from an interdisciplinary perspective.

This book will be interesting and useful for medical and other university students, medical doctors, specialists in the field of personal and social safety, environmental protection experts, chemists, biologists, and specialists of the army and governmental antiterrorist departments.

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Part 1

NEW APPROACHES IN ORGANISATION OF EMERGENCY RESPONSE AND COUNTERACTION TO CHEMICAL AND BIOLOGICAL TERRORISM
Chapter 1

Military Medical Academy in Emergency Response in Bulgaria

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Abstract: Medical support in disaster situations for the population in Bulgaria by the Bulgarian army and the Bulgarian military medical service is postulated by: - Constitution of Republic of Bulgaria; The law for defense and armed forces; - Military doctrine; - National law for crisis response operations; - Plan of Military Medical Academy for crisis response and other institutional regulations. Under the conditions of reforming Civil Healthcare System, Military Healthcare System, especially Military Medical Academy resolves not only military medical tasks but problems in the country and abroad. The aim of this study is to discuss the capability of Military Medical Academy to provide civilian population with adequate support in disaster situations. Military Medical Academy has specific military units for emergency response: - Military medical detachment for emergency response; - Mobile medical diagnostic complex; - Mobile field hospital that corresponds to NATO role 3; - Center for military epidemiology and hygiene; - Research center for radiological, biological and chemical protection; - Center for treatment of infectious and parasitic diseases. Military Medical Academy is a multi-profile medical facility structured to provide Bulgarian Armed Forces with up-to-date medical care for the soldiers, and to act as a part of the national healthcare system. Its main goals are: - Accomplishment of the health reform in medical support in accordance with military doctrine; and - Achievement of the required capability to provide medical support to the forces according to NATO standards. Military Medical Academy and its temporary and permanent facilities are able to provide an adequate medical support in crisis situations in peacetime and wartime, in our country and abroad.

key words: military medical academy, disaster situations, mass casualty, military medical principles, health care system reform, bulgarian model, medical support

In peacetime in case of natural disaster, industrial accidents and dangerous environmental pollution, the armed forces are involved in prevention, direct protection of civilian population, and search and rescue operations. The following normative acts regulate activities for protection of the population and national economy in crises:
2. The law for local self-government and local administration # 44.
3. The law for defense and armed forces ## 6, 54.
4. The law for administration # 31.
5. The law for safety work conditions # 20.

The basic documents giving right the Military medical service to provide support in disaster situations to the civilian population are:
• Military doctrine
• The law for defense and armed forces – # 67 - In peacetime in cases of natural disasters, industrial accidents and dangerous environmental pollutions, the armed forces are involved in prevention, direct protection of the civilian population, and search and rescue operations;
• # 69 - Military formations of the armed forces can participate in humanitarian assistance and rescue operations abroad in accordance with the Bulgarian constitution and international agreements in which Bulgaria is a side;
• # 70 - Military formations of the armed forces can participate in peace-keeping operations abroad in accordance with the Bulgarian constitution and international agreements in which Bulgaria is a side.
• Plan 2004 and other military regulations;
• National law for crisis response operations (draft);
• Plan of the MOD for crisis response;
• Plan of Military Medical Academy for crisis response.

The aim of this study is to discuss the capability of Military Medical Service, and especially Military Medical Academy to provide civilian population with adequate support in disaster situations and diminish the negative social and health effects on the civil population.

The specific military units for emergency response are: 5 battalions located on territorial basis with special equipment for: Search and rescue operations; Chemical accidents; Nuclear accidents; First medical aid; Camps for displaced people (refugees).

The corresponding military medical units for emergency response are:
• Military medical detachment for emergency response;
• Center for military epidemiology and hygiene;
• Research center for radiological, biological and chemical protection;
• Center for treatment of infectious and parasitic diseases.

Military Medical Academy has specific military units for emergency response:
• 2 helicopters MI – 17 - lifting capability - 6 litters;
• 1 fixed wing aircraft AN-26 - lifting capability - 10 litters + 10 sitting patients.

In case of mass casualty situations Military Medical Academy provides:
• Temporary center for treatment of patients with acute radiation sickness;
• Temporary center for treatment of intoxicated patients;
• Center for treatment of infectious and parasitic diseases;
• Temporary trauma center.
The Military Medical Academy operates in this kind of situations through its Military Medical Detachment for Emergency Response (MMDER) including personnel of three of the hospital facilities of MMA: in Sofia, Plovdiv and Varna. Military Medical Detachment for Emergency Response was founded in Military Medical Academy in 1992 on functional principle initially, later staffed as essential part of MMA. Its purpose is to provide qualified and partly specialized medical care in the event of natural and man-made disasters in peace time for meeting the medical needs of civil population.

Military Medical Detachment for Emergency Response operates in a way and military medical principles as the same military medical units in other countries and has similar main tasks:

• Triage;
• Resuscitation;
• Stabilization;
• Emergency surgical care;
• Evacuation.

• To move in timely manner after the alert to the disaster zone and to deploy;
• To provide qualified triage.
• To provide specialized medical care in accordance with the type of the damaging factor;
• To treat on-site untransportable casualties when necessary;
• To prepare for evacuation all the injured who need comprehensive medical care in specialized hospitals and MMA.

The Military Medical Detachment for Emergency Response has the following capabilities:
• MMDC Sofia - role 2+ (40 beds);
• Unit Plovdiv – role 1;
• Unit Varna – role 1.

The Military Medical Detachment for Emergency Response comprises medical and non medical personnel as follows:
• Doctors – 28;
• Nurses and lab assistants – 32;
• Medical attendants and drivers – 20.
• MMA – 58;
• Military hospital Plovdiv – 12;
• Military hospital Varna – 10 and 3 teams with certificate for SAR and MEDEVAC.

Deployable Elements On Trucks:
1. Triage and admission
2. ICU
3. Operating room /2 tables/
4. Post-operative care
5. X-ray room
6. Lab for blood and urine
7. Therapeutic room
8. Obstetrics and gynecology room
9. Pediatrics’ room
10. Inpatient care room
11. Ambulances
12. Sanitary bus
From 1992 yr. up to now Military Medical Detachment for Emergency Response took part in:

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<tr>
<th>Exercises</th>
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<tr>
<td>“PARTNERSHIP - 96”;</td>
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<td>“COORDINATIVE BEAR 99” POLAND;</td>
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The Military Medical Detachment for Emergency Response (role 2) in MMA allows us an adequate answer in critical situations in western Bulgaria, but in order to cover whole Bulgaria there are medical diagnostic complexes with personnel (role 1) in Plovdiv and Varna. MMDER has medicines, instruments, about 10 life-saving apparatuses including: cardio monitors, defibrillators, aspirators, respirators, oximeters, automatic injectors.

In 2001 year in Military Medical Academy was created Mobile medical diagnostic complex /MMDC/ (including triage room, ICU, operating room, postoperative care room, x-ray room, obstetrics and gynecology room, therapeutic room, laboratory & so on), that was used successfully to ensure medical support in international exercises. It was constructed on a modular principle and the main modules are equipped according to NATO stand-ards in respect of the diagnostic and therapeutic apparatuses, medicines, spare parts, instruments, beds, dresses, etc.
From our experience, the best way to make a Mobile field hospital is on a modular base, as well with autonomous, mobile modules. The latter can easily move to the crisis point function on their own or in connection with one another to form Mobile field hospital – role 3. Military Medical Academy is ready to provide Mobile field hospital with 80 beds and 125 medical personnel that corresponds to NATO role 3. According to us the most appropriate type for the Mobile field hospital is the container type. The basic modules are: operating room, ICU, laboratory, blood transfusion, sterilization and pharmacy. The rest of the Mobile field hospital is in tents with central type air conditioner. Each module is to be a mobile container with its own electricity, water reservoir and air conditioner. (i.e. triage room, ICU, operating room, postoperative care room, x-ray room, obstetrics and gynecology room, therapeutic room, laboratory & so on). For a Mobile field hospital with 80 beds and 125 personnel are needed 45 trucks coherent with the NATO trucks and its own logistic – electric generators, water supply system, kitchen, laundry, ambulances and warehouses. The proposed Mobile field hospital is autonomous (up to 12 wks) and very suitable for long-lasting humanitarian missions and crisis situations, where it is of crucial importance to provide the patient with life-saving measures.

With regard to our experience we can state that the Mobile Medical Diagnostic Complex / Military Medical Detachment For Emergency Response, and the Mobile Field Hospital, which is autonomous (up to 12 wks) and very suitable for long-lasting humanitarian missions and crisis situations as a part of Military Medical Academy, and its temporary and permanent facilities are able to provide an adequate medical support in crisis situations in peacetime and wartime, in our country and abroad. Under the conditions of reforming Civil Healthcare System, Military Healthcare System, especially Military Medical Academy resolves not only military medical tasks and problems in the country and abroad but opens its doors to the civilian population in the whole country.

Military Medical Academy is a multi-profile medical facility structured to provide Bulgarian Armed Forces with up-to-date medical care for the soldiers, and to act as a part of the national healthcare system.

Its main goals are:
- accomplishment of the health reform in medical support in accordance with military doctrine; and
- achievement of the required capability to provide medical support to the forces according to NATO standards.

To fulfill this Military Medical Academy is structured in an appropriate way:
- Multi-profile hospital base for active treatment – Sofia;
- Hospital bases for active treatment – Varna, Plovdiv, Pleven, Sliven;

Hospital Facility For Active Treatment (HFAT) - Varna. A hospital with 280 beds and a diagnostic-consulting block, providing full range of specialized medical services for the military contingent and the civil population of North-Eastern Bulgaria. At HFAT – Varna, there are established: Clinic of Internal Diseases, Department of Surgery, Clinic of Orthopedics and Traumatology, Clinic of Toxicology, Thermal Trauma Ward, ENT Ward and Ophthalmologic Ward. The excellent equipment and the high-qualified specialists allow HFAT – Varna to be a referral hospital for thermal trauma and intoxication treatment for the North-Eastern region of Bulgaria, and an educational base for the Medical University of Varna.
Hospital Facility For Active Treatment (HFAT) – Plovdiv. The Hospital Facility for Active Treatment – Plovdiv is a health establishment with 100-years history and rich traditions in the structures of the Bulgarian Army. The health reform enabled the hospital to sign a contract with the Health Insurance Fund and to render health care to all who wish. The hospital has an Admission-Consulting Block, where 8 functioning rooms (therapeutic, neurological, surgical, orthopedic, ENT, dental, and dermatological), diagnostic laboratories (Clinical Analyses Ward, Pathology Ward, Microbiological Laboratory), as well as Image Diagnostics Ward. The hospital has 120 beds in 8 clinic units: Internal Medicine Clinic, Clinic of Surgery, Clinic of Neurology and Psychiatry, ENT Ward, Dermatology and Venerreal Diseases Ward, Ophthalmologic Ward and Physical Therapy Ward. HFAT - Plovdiv, as a branch of the Military Medical Academy-Sofia uses preferentially the diagnostic and therapeutic capacities of this leading healthcare establishment in the country – nuclear magnetic resonance imaging, computer tomograph scanner, lithotripter, hyperbaric chamber, etc.

Hospital Facility For Active Treatment (HFAT) – Pleven. HFAT is established in 1903 by a Decree of King Ferdinand as a Ninth Division Hospital for the purpose of medical provision of the army in Central North Bulgaria. This is a commitment the hospital has performed till present. The following units are functioning at HFAT - Pleven: diagnostic-consulting block and wards of surgery, internal diseases, neurology, physical therapy, anesthesiology and intensive care. Along with the regulatory assigned contingent, civilians may get healthcare at the hospital under the clinical pathways contracted with the Health Insurance Fund (asthma, diabetes, inguinal hernia, cardiac rhythm disorders, acute appendicitis, pneumonia, heart failure, chronic obstructive pulmonary disease, chronic diseases of tonsils).

Hospital Facility For Active Treatment (HFAT) – Sliven. Hospital Facility for Active Treatment in Sliven is integrated part of the MMA, providing healthcare to the military contingent of Southeastern Bulgaria. It has Diagnostic Consulting Block including: X-ray ward, Functional Diagnostics, Laboratory and Consulting-Reception Rooms. The Stationary Block has 100 beds in 12 wards: Surgery, Emergency and Septic Surgery, Urology, General Therapy, Cardiology, ENT, Anesthesiology Reanimation and Intensive Care Unit, Infectious Diseases, Physiotherapy and Pathology. Civilians have the right to be treated in HFAT - Sliven under the 23 clinical pathways contracted with the Health Insurance Fund - Hospital bases for balneology, rehabilitation and prophylaxis – Hissar, Pomorie, Bankya, Velingrad and Narechen;

Hospital Facility Of Balneology, Rehabilitation And Prophylaxis – Bankya. The major sanative factors of the town of Bankya are the mineral waters (hydrothermal, hydrocarbonate-sulphate-sodium, silicon and lightly fluorized) and the climate (moderate continental with foot-of-mountain influence). The hospital facility of Bankya is specialized in treatment and rehabilitation of cardio-vascular and brain diseases and post-surgery conditions: valvular heart diseases with heart failure up to 1st degree; cardiac defect post-surgery conditions; ischemic heart disease; conditions following myocardial infarction, compensated forms of myocardial dystrophy, 1-2 stage hypertension disease; atherosclerosis in early stage, angioneurosis and 1-2 stage Raynaud's disease; 1-2 stage Burger's disease; atherosclerotic arterial obliteration of extremities - up to the 2 stage; functional disorders of nervous system. A favorable influence is observed in some light forms of thyreotoxicosis, light forms of diabetes,
obesity, chronic neuralgia and neuritis, chronic gastritis and colitis, light forms of arthro-rheumatic diseases.

Hospital Facility Of Balneology, Rehabilitation And Prophylaxis "Kaleroya" – Hissar. Town of Hissar is a famous health resort, located at the foot of the Sredna Gora Mountains, established in the fifth millennium B.C. around the mineral springs by the Tracian tribe of Bessi. Hissar mineral waters have low level of mineralization, being hydrocarbonate-sulphate-sodium waters, containing a small amount of radon, fluoride, hyperthermal and moderately alkaline. They are recommended for the treatment of: kidney stones, chronic pyelonephritis, chronic cystitis and urethritis, post extracorporal lithotripsy state, single kidney, chronic renal failure - initial stage; chronic gastritis, gastro-duodenitis, functional gastric disorders, dyspepsia, chronic non-specific enterocolitis, peptic ulcer disease; gallstones, chronic cholecystitis; biliary dyskinesia, post viral hepatitis' states; metabolic syndrome, obesity, diabetes, gout, arthro-rheumatic diseases; inflammatory gynecological diseases; discopathy, radiculitis, polyneuritis.

Hospital Facility of Balneology, Rehabilitation and Prophylaxis "St. George the Conqueror" – Pomorie. The town of Pomorie is a talasso-, pelo- and climate-therapy resort, located upon a slightly elevated peninsular to the north end of Burgas gulf, established in the VI-V century B.C. The most precious treasure of the town is the world famous Pomorie healing mud, favourable for treatment of: arthrosis, arthritis, states post traumas and fractures; neuritis, radiculitis, disk herniation disease, pediatric cerebral palsy; sterility, dismenorrhea, ovarian hypofunction, parametritis; prostatitis, oligospermia, epididimitis, vesiculitis; psoriasis, psoriatic arthritis, hypophrophic skin lesions; bronchitis, sinusitis. In the hospital facilities a complex treatment is carried out with Pomorie mud, lye treatment, a rich scope of physiotherapeutic procedures, kinesitherapy, acupuncture, body building, aromatherapy, inhalation treatment, laser therapy, underwater jet massage, solarium, anticecellulitis program, sauna, thalassic therapy.

As recognition for good management, the MMA took an efficient management of more medical facilities: two hospital facilities for continuous treatment and rehabilitation in Narechen and Velingrad, and three diagnostics and consultation centers in Haskovo, Stara Zagora and Burgass.
- Center for military medical expertise and aviation medicine;
- Center for military epidemiology and hygiene;
- Research center for radiobiological, biological and chemical protection;
- Military medical detachment for emergency response (urgent military medical unit);
- Troop medical units.
Military Medical Academy has a century old history in providing medical support to a military contingent in peacekeeping operations abroad and humanitarian missions abroad.

Military Medical Academy operates in peacetime and in crisis situations through its permanent and temporary facilities all over the country providing qualified and specialized diagnostics, treatment, rehabilitation and prophylaxis to the Bulgarian population.
The reform of the Bulgarian Healthcare System has both a social and health price for the civilian population and major significance for the development of the Bulgarian military and civil medicine as a whole. Under the conditions of reforming Civil Healthcare System, Military Healthcare System, especially Military Medical Academy resolves not only military medical tasks and problems in the country and abroad but opens its doors to the civilian population in the whole country.

Military Medical Academy’s specific tasks are:
• Diagnosis, treatment and rehabilitation for all who need medical care from the armed forces and civilian population.
• Prophylaxis of diseases and injuries.
• Medical support of combat training and physical training.
• Medical expertise of fitness for military service.
• Scientific research in military medicine.
• Military medical training and postgraduate medical training.
• Readiness for crisis response operations.
• Medical support of missions abroad (interoperability with NATO countries).
• Medical evaluation.
• Medical logistics.
• Elaboration of medical kits and modules.
Personnel And Number Of Beds

- medical doctors – 300
- dentists – 5
- nurses – 460
- other personnel – 555
- professors – 8
- assoc. professors – 44
- assistant professors – 38
- doctors of sciences – 10
- doctors – 64
- MPHBAT – Sofia 581
- HBAT – Plovdiv 120
- HBAT – Sliven 100
- HBAT – Varna 280
- HBAT – Pleven 100
- HBBRP – Bankya 120
- HBBRP – Hisar 215
- HBBRP – Narechen
- HBBRP – Velingrad
- DCC Haskovo, Stara Zagora & Burgass.

To ensure the best possible training of the medical personnel for adequate action on assignments and in case of disaster we share experience with our NATO colleagues. The professional contacts with the Balkan Military Medical Committee have made it possible to compare results with the achievements of the military medical specialists of the member countries of the alliance. A satellite connection with other NATO hospitals, ensures consultations in real time and exchange medical and practical knowledge with our colleagues elsewhere in Europe and the USA.

The MMA has been accredited, with an excellent grade, as a medical and educational establishment in accordance with the Medical Establishments Law and the Higher Education Law. This is indisputable proof of the established authority of the MMA and its national importance in health care, medical science and education. Thanks to the excellent working conditions, modern equipment and competent teaching staff, the MMA provides considerable opportunities for improving the qualifications of our military medical personnel and the successful training of students, interns, residents and physicians from Bulgaria and abroad in all the major medical fields.

Thanks to its history and its staff of eminent scientists and highly qualified specialists, the MMA enters the 21st century proud of its readiness to cope with any kind of emergency for the sake of the Bulgarian people that has already won widespread recognition.

Our success today is based on the achievements of our predecessors!

References:

11. Postanovlenie N 363 na MS ot 29.12.2004 g. za structurni promeni v sistemata na zdraveopazvaneto. Obn. DV, br.3 ot 11.01.2005 g., v sila ot 01.01.2005 g.
13. Protokol N 114 na Komissiata po zdraveopazvane ot 08.09.2004 g. t. 3 Proekt zapromchana na struktura na speshna pomosh – izslushvane na zamestnik-ministura po zdraveopazvaneto prof. Hinkov, po predlojenie na narodna predstavitel d-r Teodora Kostadinova i grupa narodni predstaviteli.
14. Zakon za zdraveto, bn. DV br.70/10.08.2004 g. v sila t 01.01.2005 g. n. 9/2004 g., str.20.
Chapter 2

Specificity of the Interaction between the Bulgarian Ministry of Health with the Other Government Agencies in the Event of Chemical Incidents

Evgeni ZHELEV, Krasimir GIGOV, Slavi KOLEV
Ministry of Health, Republic of Bulgaria

Abstract. The modern economy relies to a great extent on the industrialized production and use of various chemicals. Intensive privatization in Bulgaria has led to difficulties in identifying the owners of industrial facilities and enforcing requirements for the installation and maintenance of physical capital that would reduce chemical emissions. Aging and deterioration of existing industrial facilities raises the risk of chemical spills and associated endangerment of human health. Medical response to chemical incidents is mainly the responsibility of the Ministry of Health. Effective crisis management depends largely on training and equipping specialized medical teams, and coordinating the efforts of the ministries and institutions involved in the crisis response. Currently, the ability to provide adequate medical care in case of chemical incidents is limited. This report analyses these limitations and proposes measures for minimizing the impact of chemical incidents on the population.

Keywords. chemical emissions, environmental damages, healthcare, specialized medical teams

Introduction and Background

The modern economy relies to a great extent on the production and use of various industrial, agricultural, and other chemicals. In the last few decades, we have witnessed many incidents and terrorist attacks involving such chemicals, which have lead to anxiety in the population. Some common examples are the chemical disasters in Sevezo, Italy in 1976; the ammonia leakage incidents in Montreal in 1997 and in Bopal, India in 1984, which caused the exposure and death of thousands of people. [1]
Bulgaria is not an exception in this respect. Examples of industrial incidents include the 1978 incident at Alen Mak factory in Plovdiv, the 1989 and 2008 chlorine leaks in Devnya, the 2007 propane-butane leak at Lukoil Neftochim Bourgas, the 2007 and 2008 incidents at the Arsenal munitions factory, and the 2008 explosion at Chelopechene.

The intense privatization in Bulgaria, which started in 2001 and covered all sectors of the national economy including medicine and science, led to difficulties in the identification of owners of industrial facilities manufacturing and using bulk chemicals and in the regulation of their production processes. Consequently, the unauthorized operation of such facilities has increased the risk of chemical incidents in the country.

One recent example, in which failure to comply with government regulations, lead to the death of three workers is the 2004 blast-furnace gas leak at the metallurgical plant Kremikovtzi near Sofia. Another twenty workers were exposed and required hospitalization. The exposed workers had not received appropriate safety training and the medical first responders lacked any specialized training or experience in this type of situation. The medical teams also did not have appropriate personal protective devices.

Finally, the March 19, 1995 terrorist attack on the Tokyo Metro, which involved the release of the neurotoxic agent Sarin gas is generally considered to be the first act of modern chemical terrorism. This attack brought into focus the need for reliable population protection measures not only in the event of industrial incidents but also in the event of terrorist attacks. [1]

Types of Chemical Incidents

Chemical incidents can be divided into the following two broad categories:

1. Industrial Incidents
   - chemical incidents at industrial facilities;
   - chemical fires resulting from traffic accidents involving hazardous cargo.

2. Terrorist Attacks
   - terrorist attacks at chemical production or warehousing facilities;
   - chemical fires resulting from terrorist attacks involving toxic chemicals.

Because many chemicals spread quickly, proper crisis management in case of chemical incidents or attacks requires quick identification of the toxic substance and adequate provision of first aid. [2] When the magnitude of the incident is large and substantial numbers of individuals are exposed, crisis management becomes a more complex task that requires participation of many government organizations and is managed by the Security Council at the Bulgarian Council of Ministers. [3,4]
Crisis Management Agencies

The key crisis management agencies in Bulgaria are the Ministry of Emergency Management, which supervises crisis management teams from the National Office of Civil Defense; the Ministry of Defense, including the Military Medical Academy; the Ministry of Interior, which supervises the crisis response teams of the National Public Safety Agency and the Directorate General Police; and the Ministry of Health. An organizational chart of these agencies is provided in Figure 1.

Specific Tasks of the Crisis Management Agencies

Ministry of Emergency Management

The main tasks for the Ministry of Emergency Management during chemical incidents are to:
- evaluate the situation and the symptoms of a chemical incident;
- determine the causes and sources of the chemical incident;
- forecast the rate of progress of the chemical incident;
- join efforts with the National Center of Public Health Protection and the Regional Inspectorate of Protection and Control of Public Health to identify the hazardous chemical;
- outline the contaminated geographic area;
- provide immediate medical assistance to exposed individuals;
- perform decontamination activities on people and equipment that were present at the contamination site;
- monitor for chemical contamination; and,
- determine the result from the executed complex restrictive, restoration, rescue and other efforts undertaken by the Ministry of Emergency Management and the Ministry of Health. [5,6]

Ministry of Defense

The main tasks for the Ministry of Defense during chemical incidents are to:
- provide assistance in establishing the causes and sources of chemical damage and in the identification of the hazardous chemical;
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Council of Ministers
Security Council

Ministry of Interior

Ministry of Emergency Management
Directorate General Civil Defense

Ministry of Environment and Water
Regional Inspectorate for Environment and Water

Ministry of Defense

National Public Safety Agency

Directorate General Police

MINISTRY OF HEALTH
Minister

Security and Crisis Management Council

Directorate Administrative Activities and Crisis Management

National Medical Coordination Center

Regional Healthcare Centers

Regional Inspectorates for Public Health Protection and Control

General Hospitals

Specialized Hospitals

Figure 1. Organizational Chart of Crisis Management Agencies

− provide specialized treatment of exposed individuals at the Military Medical Academy;
− allow the use of the toxicological module of the Military Medical Rapid Response Unit;
− monitor for chemical contamination.

Ministry of Interior

The main tasks for the Ministry of Interior during chemical incidents are to:
− limit access to contaminated sites;
− provide access of first response teams to exposed individuals in case of terrorist attacks involving hazardous chemicals;
− provide fast population evacuation from contaminated sites;
− provide fast access of exposed individuals to specialized healthcare facilities;
− if necessary, provide specialized medical care to exposed individuals at the Medical Institute of the Ministry of Interior.
Ministry of Health

Pursuant to the relevant normative documents, the responsibility for providing healthcare to the population in the event of chemical incidents lies with the Ministry of Health. [7] The activities of the Ministry of Health in the event of chemical incidents are regulated by the Crisis Management Act and the Disaster Response Act, as well as the disaster relief and healthcare provision code of the Ministry of Health. [3,4,8,9]
The main tasks for the Ministry of Health during chemical incidents are to:
− join effort with the National Office of Civil Defense to identify the hazardous chemical;
− identify the amount and degree of immediate medical attention required by exposed individuals;
− cooperate with the Ministry of Interior to ensure the fast transportation of exposed individuals to specialized healthcare facilities;
− qualify and approve general and specialized healthcare facilities;
− provide general and specific antidotes for the teams of the First Aid Centers and specialized toxicology clinics and wards.

Organization of the Ministry of Health

The administrative agencies that provide medical response in case of chemical incidents under the guidance of the Minister of Health are:
− the Council for Crisis Security and Control summoned during national emergencies by the Minister of Health;
− the National Medical Coordination Center (NMCC);
− the Regional Health Centers and Regional Inspectorates for Protection and Control of Public Health.

Chemical Incidents Response Steps

A preliminary determination of the magnitude and severity of the chemical incident should play an integral role in determining the necessary treatment for exposed individuals and the overall plan of action for medical response. The initial assessment is made by the NMCC with the aid of the Geographic Information System. To this end, the NMCC has the responsibility of continuously updating information on the location and characteristics of the high risk sites. Using the Geographic Information System, it is possible to rapidly develop information regarding the affected region and population, available healthcare facilities with number of beds, and estimates of the number of exposed individuals.
Immediate medical attention to exposed individuals is provided on-site by the teams of the National Office of Civil Defense and the National Public Safety Agency. Therefore, these teams need to be adequately trained and equipped with modern technical aids. [10]
Additional off-site medical attention is provided by the teams of the First Aid Centers (which are typically not allowed access to the site of the chemical incident). In extreme situations, first aid teams equipped with gas masks, skin protection aids, and antidotes maybe allowed on-site.

Transportation of exposed individuals to healthcare facilities is performed by the teams of the First Aid Centers. If population evacuation and transportation is particularly difficult, additional teams from adjacent First Aid Centers may be directed to the site of a chemical incident.

Qualified medical treatment is provided at the general hospital closest to the site of the chemical incident. Depending on the magnitude of the incident, further medical treatment to exposed individuals may be provided by toxicologists from other healthcare facilities, such as the Multiprofile Hospital for Active Treatment and First Aid Pirogov in Sofia, the Military Medical Academy with branches in Sofia and Varna, the University General Hospital Saint George in Plovdiv, and the University General Hospital in Pleven. [11]

**Recommendations and Conclusions**

Our vision is that specialized medical aid should be provided at the general hospitals for active treatment, allotting suitable wards and assistance from mobile specialized medical teams. A significant obstacle to this approach is the fact that there are currently only 64 toxicologists in Bulgaria, who are heavily concentrated in very few cities such as Sofia, Plovdiv, Varna, and Pleven. (See Table 1)
## Table 1. Register of physicians: toxicologists in the country by region

<table>
<thead>
<tr>
<th>No.</th>
<th>Region</th>
<th>Total Number of Toxicologists Employed at Toxicology Clinics and Wards</th>
<th>Total Number of Toxicologists Employed at Other Types of Clinics and Wards</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Blagoevgrad</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Bourgas</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Varna</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>4.</td>
<td>Veliko Tarnovo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>Vidin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>Vratza</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>7.</td>
<td>Gabrovo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td>Dobrich</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>9.</td>
<td>Kardzhali</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.</td>
<td>Kyustendil</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11.</td>
<td>Lovech</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>12.</td>
<td>Montana</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13.</td>
<td>Paradzhik</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14.</td>
<td>Pernik</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15.</td>
<td>Pleven</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>16.</td>
<td>Plovdiv</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>17.</td>
<td>Razgrad</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18.</td>
<td>Rousse</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>19.</td>
<td>Silistra</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20.</td>
<td>Sliven</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21.</td>
<td>Smolyan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22.</td>
<td>Sofia City</td>
<td>27</td>
<td>6</td>
</tr>
<tr>
<td>23.</td>
<td>Sofia County</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24.</td>
<td>Stara Zagora</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>25.</td>
<td>Targovishte</td>
<td></td>
<td></td>
</tr>
<tr>
<td>26.</td>
<td>Haskovo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>27.</td>
<td>Shumen</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>28.</td>
<td>Yambol</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td><strong>Total:</strong></td>
<td><strong>64</strong></td>
<td><strong>43</strong></td>
</tr>
</tbody>
</table>
The management of the Ministry of Health considers the following tasks as its immediate plan of action for improving the availability of toxicology assistance throughout the country:

1. establish conditions for increasing the number of toxicologists in the country;
2. divide the country into healthcare regions in the event of a chemical incident based on the availability of specialized medical personnel;
3. improve specialized training of the teams from the First Aid Centers;
4. provide specialized equipment (such as individual protection aids) to the teams from the First Aid Centers based on the likelihood of a chemical incident in a given geographic region;
5. provide equipment to mobile specialized medical teams of toxicologists pursuant to Bulletin No. 7 [12];
6. provide adequate supplies of non-specific and specific antidotes;
7. evaluate the magnitude and severity of a potential chemical incident for each region using the Geographic Information Systems;
8. educate the general population on the methods of self-help and assistance to others in the event of a chemical incident;
9. help improve the interaction and coordination between the crisis management agencies by employing national consultants who would work with the crisis management organizations in the event of large-impact chemical incidents; and,
10. further develop the protocols for providing toxicology medical assistance in the event of chemical incidents.

In conclusion, it must be emphasized that the existing healthcare system in the country needs to be improved and continuously upgraded in order to provide adequate response in the event of chemical incidents or terrorist attacks involving toxic chemicals.

References

Chapter 3

Medical Support In Case Of Chemical And Biological Threat

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Abstract - The organization and medical support in case of mass casualties as a result of military and nonmilitary crisis are the most difficult problems that the military healthcare system has to solve. The medical support is a system of closely connected operations for health protection, medical help, evacuation and treatment. The medical support measures could be effective if only they are the result of all the countries and international organizations participation.

Keywords - missions abroad, natural disasters, manmade disasters, terrorist acts, NATO standards; medical support organization.

Globalization is the main cause for the undergoing changes in the world, Bulgaria and the Bulgarian army leading to updating the principles and the priorities of the military medicine and medical support in the contemporary world.

Different kind of missions, natural and manmade disasters are typical for the contemporary society. In such cases the military medical specialist must have not only adequate organization, consistent with the noxious agent but also adequate tools for providing life saving first aid, self first aid and buddy aid. The missions abroad could be peace enforcement, peacekeeping, peace building, humanitarian and disaster relief. The most important thing for the medical support in these operations is the preparatory measures and the supplement of the staff with packages for personal medical defense, according NATO standards.

Non-military disasters include natural and manmade disasters. They are result of a destructive activities of meteorological hurricanes, draught, colds, topological floods, tectonically earthquakes, volcanoes, cosmically, also industrial incidents: chemical, radiation, fires; socio-economical; transport, railway, sea, airway; other kinds of manmade disasters: parasitic and infectious diseases, mass poisoning, ecological and so on. Medical support in such cases has special features: on one hand to individualize the medical help and on the other hand to enforce the principals of military medicine.
In an act of terrorism, the characteristics of the damages depend on resources which are used - explosives; chemical agents; biological agents; radioactive agents.

In case of chemical and biological threat health risk for the military and civilian contingent include death from direct violence; chemical agents; biological agents; radioactive agents; explosives; infectious diseases; other diseases.

In our opinion health measures must be directed to:

- Life saving first aid, as self first aid and buddy aid with medicines and packages for personal medical defense;
- Medical support provided by qualified and specialized medical personal and teams.

The main objective of this study is to analyze the current status of medical support in case of mission abroad, natural and manmade disasters and terrorist acts and offer a decision to the problems connected with the organization of the medical support in coherence with the good medical practice.

The essential fact in the organization of the medical support is that the first aid in such cases is based on first self aid and buddy aid. According to the principles of the military medicine that appear to be quite satisfactory with the medical support in case of mission abroad, natural and manmade disasters and terrorist acts thereafter follow the Role I; Role II; Role III; Role IV, with the included within medical aid division and timely medical evacuation.

Current medical support in missions abroad, natural, manmade disaster and terrorist acts Military Medical Academy has the disposal of: Medical Regiments; Field Hospitals; Military Medical Detachment for Emergency Response MMDER; Mobile Medical Diagnostic Complex /MMDC/.

At this stage of the military reform in the Bulgarian army and the military principles of medical support in missions, crisis and terrorism there is not a structural unit for medical support if NBC agents are used. Therefore we developed an organizational structure of a medical module - Role I and Role II. Medical defense staffs and units provide specialized assistance in chemical, biological or nuclear-related disasters.

The final treatment, the management of medical support organization and the teaching of medical personnel in crisis, missions and terrorist acts are accomplished by Military Medical Academy. Follow by the idea for complex studying and the resolved priorities of medical support in crisis, missions and terrorist acts, including also the unified transition process from diagnostic-treatment, research and development to educational-teaching activity, a new organizational department is founded-Chair of disaster medicine and toxicology.

Chair of Disaster Medicine and Toxicology consists of: Clinic for toxicology and allergy; Scientific-Research Laboratory for Military Toxicology; Scientific-Research Laboratory for Disaster Medicine; Military Medical Intelligence Ward.

As a result, manuals, textbooks, instructions were written, that were used in the training programs for medical, paramedical and non medical personal, trained for the medical support in natural and manmade calamites, terrorist acts and peace enforcement and peacekeeping operations.
Organizational structure of a medical module - Role I (in case of CBRN agents)

<table>
<thead>
<tr>
<th>Role</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Head of examination room – Physician</td>
<td>1</td>
</tr>
<tr>
<td>captain</td>
<td></td>
</tr>
<tr>
<td>Physician – Intensive care and anestheology captain</td>
<td>1</td>
</tr>
<tr>
<td>Technician - decontamination sergeant</td>
<td>3</td>
</tr>
<tr>
<td>Stretch-bearer, Sanitary transport (ambulance for Intensive care) Driver private</td>
<td>2</td>
</tr>
</tbody>
</table>

Medical Ward Equipment:
- Physician bag – 2
- Medical Technician bag – 3
- Hospital attendant bag – 2
- Personal Protective Equipment – 7 /equal to those for in the company/
- Decontamination kit - 1

Organizational structure of a medical module - Role II (in case of CBRN agents)
Products of this newly created structure are the following Bulgarian Armed Forces General Staff approved programs:

Course “Medical Technician Special Training for Missions abroad medical support” (included in „Disaster Medicine, Military Toxicology, Radiology and Psychology Module”);

Course “Military Medical Training for the military personnel assigned for missions abroad” (included in „Disaster Medicine, Military Toxicology, Radiology and Psychology Module”);
Course “Physicians’ Special Training for Missions abroad medical support” (included in „Disaster Medicine, Military Toxicology, Radiology and Psychology Module”).

The following Handbooks contain the main principles of medical support, according, standards of NATO:
- “First Aid”;
- “Medical Assessment – Republic Iraq”;
- “Medical Assessment – Islamic Republic Afghanistan”;
- “Medical Assessment – State of Kuwait”;
- “Crises Medical Support”.

Standard Operating Procedures:
- “Bio-weapon implementation in peace time - Protection Organization and Behavior”;
- “Nuclear weapon implementation in peace time - Protection Organization and Behavior”;
- “Chemical weapon implementation in peace time - Protection Organization and Behavior”.

Tutorials:
- “Missions, Crises and Terrorist acts Medical Support”;
- “Disasters – medical and tactical features”;
- „Terrorism – Medical Risk Assessment and Management”;
- „Crises Medical Support”;
- „Missions Medical Support”.

Based on the world experience and this analysis, it is obvious that the military units in Bulgarian army have to improve or update the preparation and the organization of the medical support in missions abroad, natural and manmade disaster and terrorist acts.

There are some proposals for the forces and resources organization of the medical support in missions abroad, crisis and terrorist act.

Proposals directly related to extraordinary and emergency situations include crises management improvement, financial fund, military means and capabilities at constant readiness, medical intelligence.

Proposals indirectly related to extraordinary and emergency situations include educational measures with the contingent, health financial support, defined diseases indicators, constant prophylaxis.

Proposals related to extraordinary and emergency situations medical preparedness and activities include constant study of medical and geographic characteristics and manpower and equipment readiness maintenance.

In conclusion we could state that at the end of XX century when there is enhanced possibility of originating nonmilitary, natural, industrial crisis, terrorist acts and so on the medical support in combined operations is based on universal rules, principles and operations. The proposed medical modules for Role I and Role II, as well as the newly structured Chair of Disaster Medicine and Toxicology are valuable additive to Military Medical Academy and the Bulgarian Army capabilities in their new priority - the disaster consequences’ management.
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References

2. Demirtchev, V. & N. Tchobanov – Conclusions Drawn from the Medical Support for the Bulgarian Battalion, that took part in the UNTAC, BMMR, vol. 2, N 1, Jan.-June, 1999 yr.
10. UN HQ Medical Support UN, Ch.7, Draft 2, 1995 yr.
Chapter 4

Clinical Particularities and Strategic Complex Advanced Therapeutic Program Against the Toxic Chemical and Biological Traumatism and Terrorism

Alexander MONOV

Toxicology Clinic, MHATEM ‘Pirogov’, Sofia, Bulgaria

Abstract - The author presents proper doctrine about the one complex unified strategy against the contemporary acute toxic traumatism and terrorism. In this doctrine are included universally clinical model-picture of the phenomenons contemporary traumatism and terrorism and unified therapeutic program for treatment of the pathology of these two phenomenons after his doctrine.

Keywords - Toxic traumatism, toxic terrorism, unified therapeutic program, universal clinical model – picture of toxic traumatism and terrorism.

After Second World War a very aggressive pathology, provoked by toxic chemical and biological agents affect very high number of people and cause big humanitarian catastrophes. So is formed the phenomenon toxic chemical and biological traumatism and terrorism when the incident is organized by criminal methods and has terroristic but (1,5,6). The standard medicine was not effective against this pathology. The very high toxic aggression of this pathology is formed by her specific clinical particularities. After the scientific etudes of prof. Al. Monov these particularities are in the following directions: aethiological factors, pathogenesis mechanisms and clinical picture.

The aethiologic agents of the contemporary acuts in toxications are mono- and polytoxic substances with specific physical and chemical properties. They belong to three types of state: gasous, liquid and powder of hard particles. Some times terrorist toxic acts are caused by very toxic substances with quickly mortal effect, prepared in specialized secret laboratories. Very often aethiological agent combinations of more poisons by industrial or nutritives mass intoxications are of chemical and physical
aggressive substances - termo-radioactive and other factors (5,6,7). The group of pathogenesis factors includes intra molecular transformation of the poison molecules and molecule-enzyme interaction in the different cells. This kind toxiquenic forms the model of intracellular toxic destruction by the contempor-ary acute poisonings. By this processes are two important phenomenons: 1. The transmition and deformation of the poison in other metabolit-more toxic products; 2. Intracellular disturbance of the oxidation and the metabolism of the monoschrides and aminoacid products and disfunction of cell membrane and intracellular organells and organel membrane (permoabilitet processes). These microstructure and functional intra-intercellulary deformations are more extremely, when the intoxication is caused by poisons more toxic metabolites caused in very short time, very high aggression with mortal effect. The modern terrorism often uses chemical and biological poisons with the described destruction possibility by terroristic acts.

The standart medicine is not enough effective against the contemporary toxic traumatism and terrorism. That is why it is necessary to be formed one modern complex scientific strategy(1,5,6,8).

The scientific clinical observations and researches of Al. Monov give him the possibility to form modern basic clinical model-picture of the toxic traumatism and terrorism presented in his monographies and in this publication (1,4,5,6,8,9).

Universal clinical model-picture of toxic traumatism and terrorism (Al. Monov)

I. Severe damages of homeostasis
II. Systematic symptoms and syndromes:
   1. Respiratory acute syndromes
   2. Cardiovascular acute syndromes
   3. Febris syndrome
   4. Immunodeficiensy syndrome (3)
   5. Water-salt disbalance syndrome (dehydratation, acidosisy and others)
   6. Hemostasis disbalance syndrome
III. Organ-lesion syndromes:
   1. Monoorganolesion syndrome
   2. Polyorgan lesion syndrome

The kind of the lesion organs depends on the poison, caused the acute intoxication, from his physical and chemical characteristic (5, 6).

The treatment of the contemporary toxic pathology can be effective only by a modern scientific motivated therapeutic program adequated to the presented in this model clinical characteristic. This program must be part of the complex rational strategy against the toxic traumatism and terrorism. The philosophy and the model of this program strategy is formed by Al. Monov and published in his scientific monographies and in this publication (1, 2, 3, 5, 6, 8, 9).

The unified therapeutic program, included in the complex strategy against the toxic traumatism and terrorism and according to the showed over particularities of this aggressive pathology is presented by the following strategic methods:

1. The different kind of reanimation and intensive therapy by all the kinds of severe forms of chemical and biological traumatism and terrorism
2. Antidotes combinations:
Recently the treatment of very severe poisonings caused by toxic agents with only one medicament – antidote in general accepted doses is not effective by some severe intoxications (2, 5).

In these cases effective are antidotes in specific adequate doses or with antidote combinations.

3. Specialized detoxic depuration methods: Forced diuresis; extra renal and extra corporal detoxic depuration; extra renal and extra corporal dialysis – hemodialysis and peritoneal dialysis; plasma feresis. It is very important in the first time of the acute poisoning to depure the entrance area from the poison according to the indication and contra indication.

4. Specialized organoprotective treatment according to the toxic damages of the different organs.

5. Immunity protection in the first days of the poisonings because the very aggressive chemical and biological agents cause very intensive immunity deficiency in first time of the intoxication.

6. Specialized hyperbar oxygenation (with barocamera when is possible in according to indications and contraindications).

7. Symptomatic therapy (5, 6, 8, 9).

All these methods are included from Al. Monov in the complex antitoxic strategy after the specialization and adaptation from him to the particularities of the toxic chemical and biological pathology.

The treatment of the acute intoxications is realized in three phases:

1. The first phase – including the time for rescue actions on the suffering people.
2. The time of the treatment in the transport vehicle
3. The time of treatment in the clinic.

1. In the first it is necessary to use: the reanimation and intensive therapy (by indications), antidotes, symptomatic therapy.

2. In the phase of the transport time – must use the treatment as in the first phase.

3. In the clinic phase it is necessary to use the total therapeutic program according to indications and contraindications.

This program must be included in the instruction program of the student – absolvents of medicine and in social health and in the program, for specialization for all the physisians.

The national strategy against the mass traumatism and terrorism can be successful if this unified medicine program will be included in the permanent anti-crisis readiness of the state.
References

1. Monov, Al. “Acute Intoxications”, 308 p; 1968
8. Monov, Al., Ch. Dishovsky (Editors) “Medical Aspects of Chemical and Biological Terrorism. Biological Traumatism and Terrorism”, 320 p; 2004
9. Monov, Al., Ch. Dishovsky (Editors) “Medical Aspects Of Chemical and Biological Terrorism. Chemical Terrorism and Traumatism” 352 p; 2005
Chapter 5

Military Medical Readiness for Chemical and Biological Terrorists’ attacks

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a Department Disaster Medicine and Toxicology; b Military Medical Detachment for Emergency Response
Military Medical Academy, Sofia, Bulgaria

Abstract - Increased level of biological and chemical terrorist’s threat has created requirement for comprehensive medical structures to cope with the medical support for the chemical or biological attack casualties. The aim of the article is to present established military medical structures and implemented procedures for enhancing military medical readiness for chemical and biological terrorist attack consequences management. By means of descriptive method established in Bulgarian military medical service antiterrorist structures and implemented SOPs were thoroughly analyzed. Comparative analysis was applied in order to define obtained readiness. As a result of the performed analyses could be emphasized that established in Bulgarian military medical service structures provide sufficient military medical capabilities to monitor and assess chemical and biological terrorist threat. These structures and implemented SOPs could be defined as an appropriate basis for medical build up (military and civilian) in case of chemical and/or biological terrorist attack.

Key words - Chemical and biological terrorist threat, Military Medical Detachment for Emergency Response, Department Military Epidemiology and Hygiene, Centre Military Epidemiology and Hygiene, Ward for Medical Intelligence

Introduction:

The past fifty years have seen increasingly rapid advances in the field of chemical and biological related developments in science and technologies. These developments, on one hand led to improvement in humans’ lifestyle, but on the other hand have provided terrorists with powerful weapon. It is becoming increasingly difficult to ignore the possibility terrorists to either obtain chemical and/or biological weapons, or to purchase chemical compounds and/or viral strains and to weaponize them. Terrorists attacks conducted in recent years have spread among citizens all over the world fear of the imminent chemical and biological terrorism. Availability of and easiness of obtaining, not only the required for chemical/bioweapon production materials, but as well the recipes and manuals how to use them, are proofs that widespread fear is not baseless.

Nowadays chemical and biological terrorist threat is becoming concern not only for the ordinary citizen, the security services but for medical services as well. Medical
service is an important component of Unified Rescue System established in Republic of Bulgaria in order to cope with disasters consequences. The terrorist act has all the features of the man-made disaster, which is explanatory why medics have to be familiar with terrorism as a health hazard, and to be prepared to confront consequences of the successful terrorist act.

In the recent years Armed Forces are more frequently, than in the past, involved in disasters’ relief and humanitarian operation not only in their country of origin, but abroad also, alone or as a part of multinational military contingents in military missions abroad. Military medicine with the established Standard Operating Procedures (SOP) for managing great flow of casualties and structures for assessing, planning and conducting medical support in case of Chemical, Biological, Radiological and Nuclear (CBRN) weapons implementation could become a base for further development of Chemical and Biological Terrorist acts’ consequences medical management and support doctrine and policy. Although that there is hardly anyone how can deny the practical and theoretical knowledge of the military medics in the field of CBRN casualties medical management and support, so far, however, there has been little discussion about capabilities and the place of established military medical structures in chemical and/or biological terrorist’s act management.

Purpose

The aim of this article is to present established military medical structures and implemented procedures for enhancing military medical readiness for chemical and biological terrorist attack consequences management.

Materials and Methods

By means of descriptive method established in Bulgarian military medical service antiterrorist structures and implemented SOPs were thoroughly analyzed. Comparative analysis was applied in order to define obtained readiness.

The article has been divided into four parts. First part deals with chemical/biological terrorism threat level assessment. The assessed level is presented in order to highlight the importance of the issue. Second part defines military medicine as a tool for better planning and organizing medical preparedness for chemical and biological attack. In the third part briefly are presented the so called prevention levels. Established medical structures in the Military Medical Academy Sofia are listed in the forth part. Structures are presented comparing their capabilities and requirements imposed by the scope of the particular level of prevention. Capabilities of the established structures are presented and analysed only in relation to chemical/biological terrorist’s act medical management and support. The article is not defining the full spectrum of the analysed structures activities and capabilities.

Results and Discussion

As a result of performed analyzes established structures in Bulgarian military medical service with set objectives to enhance antiterrorism readiness and to confront occurred terrorist’s act consequences were listed.

Chemical/Biological Threat Assessment. While a variety of threat assessments methods have been suggested, this paper will use the assessment developed and im-
implemented by the Ward for Military Medical Intelligence in Military Medical Academy (MMA), Sofia and published 2008 – “Terrorism. Medical Risk Assessment and Management”. [1]. As it is described in the publication terrorist’s threat is function of the intention and capabilities of the terrorists to perform terrorist act towards specific target. In the above mentioned publication the ultimate terrorist target is defined – the public opinion. These clarifications were given, because accordingly to the developed assessment method both chemical and biological threat towards community were assessed as high. Below briefly are given some of the main factors led to the mentioned result [2]:

**Chemical Terrorist’s Threat Level is assessed as high because:**
- Large number historical evidence for terrorist capability to obtain and use chemical weapons – cheap, available substances and recipes for their production and storage, as well manuals for their usage
- Recently declared intention and willingness to produced, purchase and use cheap chemical weapons
- Extremely high consequences severity for affected human combined with enormous and immeasurable psychological affect and social disturbance.

**Biological Terrorist’s Threat Level is assessed as high because:**
- Large number historical evidence for terrorist capability to obtain and use bio-weapons from the onset of warfare
- Recently declared intention and willingness to produced, purchase and use bio-weapons – leaders statements and the trilling plague experiment in Algeria
- Moderate to high consequences severity for human and material goods of society, but with enormous and immeasurable psychological affect and social disturbance
- And there are undoubted indicators for the increasing terrorists’ interest towards biological agents weaponizing.

**Military Medicine in Terrorists attack medical support and management**

As it was mentioned above during its development military medicine has acquired a lot of practical and theoretical experience related to CBRN events medical support. The most important three of them are listed below:
- Military medicine has established and implemented strict process for mass casualty management
- SOPs were designed and countless trained in order to prepare military medics to cope with CBRN events consequences, because of the likelihood of WMD usage during warfare
- Structures for CBRN incidents medical support and management were established. [3, 4, 5]

Based on the requirements derived from the acquired knowledge the following Military Medical Structures were established in MMA, Sofia:
- Research Institute for Radiological, Biological and Chemical Protection
- Center for Military Epidemiology and Hygiene
- Military Medical Detachment for Emergency Response
- Department Disaster Medicine and Toxicology
- Infectious Diseases Hospital
- Department Military Epidemiology and Hygiene
- Department Military Medicine
- Department for physiotherapy and rehabilitation

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Hospital Facilities of Balneology, Rehabilitation and Prophylaxis.

The levels of medical prevention from terrorists’ acts briefly are defined below:

- Primary prevention rests on creating a strong national, international and global norm that rejects development of chemical and biological weapons.
- Secondary prevention implies early detection and prompt treatment of health harm (disease) caused by chemical/biological weapon applied.
- Tertiary prevention limits the disability from the health disturbance/disease, caused by the chemical/biological weapon implementation

Two departments are designed to develop and implement required by the primary prevention normative basis and situational awareness.

- Department Disaster Medicine and Toxicology
  - Military Medical Intelligence Ward
  - Scientific-Research Laboratory for Disaster Medicine
- Department Military Medicine

The main tasks of the Ward for Military Medical Intelligence are:

- To Collect, Analyze, Collate and Disseminate Medical information about scientific and research trends, as well bio-scientific technologies development, related to human health;
- To establish and maintain Close coordination and intelligence product exchange with national and international Intelligence services in order properly to:
  - assess chemical and biological facilities’ terrorist vulnerability
  - assess terrorist’s threat risk
  - recommend measures for risk eradication and minimization.

Main activities of the Research and Scientific Laboratory Disaster Medicine are to:

- Develop theoretical basis for Disaster Medical Management and Support (DMMS)
- Develop DMMS doctrine and policy
- Train medical officers for DMMS activities leaders
- Provide assistance to National Medical Coordination Center, Military Medical Detachment for Emergency Response, Ministry of Health, Ministry of Emergency situation

All data acquired by the Department Disaster Medicine and Toxicology serve as a base for Military Medical Planning purposes. The planning and doctrine development in the military medicine are performed in the Department Military Medicine in MMA.

Some of the main aspects of the military medicine input in the secondary prevention are listed below:

- The military medical community plays an important role in secondary prevention by participating in disease surveillance and reporting and thus providing the first indication of chemical/biological weapons use.
- In addition, continued research to improve surveillance and the search for improved diagnostic capabilities, therapeutic agents, and effective response plans further strengthen secondary prevention measures.

Early detection and casualties’ treatment are performed in the following MMA structures:

- Department Military Epidemiology and Hygiene
- Toxic Chemicals Laboratory
Center for Military Epidemiology and Hygiene
Scientific-Research Laboratory for Military Toxicology
Military Medical Detachment for Emergency Response
Hospital for treatment of infectious diseases
Clinic for toxicology

Early Detection in case of chemical or biological attack could be achieved by the means of the following structures:

- Referent microbiological laboratory Bio-safety Level 3, with capabilities for agent identification on molecular-genetic level
- Toxic Chemicals Laboratory
- Field identification teams and laboratories (part of the Center for Military Epidemiology and Hygiene). The laboratories are mobile and could reach the affected area, if required.
- Epidemiological surveillance by the Center for Military Epidemiology and Hygiene
- New protective kits and antidotes development by the scientific research performed in Department Disaster Medicine and Toxicology and Research Institute for Radiological, Biological and Chemical Protection.

Treatment of the casualties could be commenced near or inside (in case of bioterrorism only) affected area and continued in the MMA:

- On the spot treatment by the Military Medical Detachment for Emergency Response
- Infectious Diseases Hospital
- Clinic for toxicology and alergology

Because of the time constraint in the casualty treatment in case of Chemical or Biological terrorism the established structure for rapid deployment of mobile field diagnostic and treatment facility is of great value for the DMMS.

Military Medical Detachment for Emergency Response main tasks are listed below:

- Keeps constant readiness to provide qualified and partly specialized medical care in the event of natural and man-made disasters in peace time for meeting the medical needs of the civil population in Bulgaria and the Balkan region
- Train and qualify medics for DMMS
- Main Tasks:
  - to move in timely manner after the alert to the disaster zone and to deploy;
  - to provide qualified triage
  - to provide specialized medical care in accordance with the type of the damaging factor;
  - to treat on-site un-transportable casualties when necessary;
  - to prepare for evacuation all the injured who need; comprehensive medical care in specialized hospitals and MMA.

For the tertiary level of prevention MMA has established the following structures:

- Department for physiotherapy and rehabilitation
- Hospital Facilities of Balneology, Rehabilitation and Prophylaxis – in three different Bulgarian resorts.
Conclusion

One of the more significant findings to emerge from this study is that established in Bulgarian military medical service structures provide sufficient military medical capabilities to monitor and assess chemical and biological terrorist threat. These structures and implemented SOPs could be defined as an appropriate basis for medical build up (military and civilian) in case of chemical and/or biological terrorist attack.

References:

Chapter 6

Unified Strategic Doctrine Against the Contemporary Traumatism and Terrorism

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Abstract - The other present proper unified strategic doctrine against the contemporary traumatism and terrorism. The philosophy of this strategy contain the following principles: Foreseeing, preventing, overcoming, prophylaxis. This strategy includes the three unified medical programs: antitoxic, antiinfectioso-antiepidemic and antiphisic – antitraumatic. The positive effect of this strategy is proved by Al. Monov in his use of different toxic, chemical and biological accidents.

Keywords - Unified anti-physic, anti-toxic, anti-biological and anti-crisis strategy and programs

Some times in the different regions of the world the different processes cause the very aggressive pathology and big losses of material and other culture values (1, 3, 4, 8). Very often the standart medicine is not enough effective for the treatment of this pathology. The investigations listed by Al. Monov show that these catastrophic processes form the phenomenons of physical, toxic chemical and biological traumatism and terrorism. The factors, which cause these phenomenons are natural disasters, cataclisms (earth quakes, floods, etc.), accidents in the human environment and random or deliberate human actions. After the origin of these processes there are naturogen and antropogen type of these catastrophes. The proper investigations of Al. Monov form an unified doctrine of the defense against the physical, toxic chemical and biological contemporary traumatism and terrorism.

The mortality and disability caused by the injuries is very high numbered. After complete clinical observations and other scientific investigations Prof. Dr. Al. Monov has formed and published unified medical program in his unified doctrine against toxic, chemical and biological traumatism and terrorism. His unified strategy is based on the specialties and particularities of this new ecological and toxic pathology, caused by physical, chemical biological traumatism and terrorism (1, 2, 3, 4, 7,8). The philosophy of this doctrine is based on these principles: Foreseeing, preventing, overcoming, prophylaxis (8):

Foreseeing - This principle requires the distribution in different regions, the factors and the conditions, which form the possibility for rise of traumatism and terrorism or more casualties by nature crisis.

Preventing - The principle requires action with which the crisis factors will be overcome and this requires anti-crisis structures and actions.
These two principles are effective if they are realized in conjunction.

**Overcoming** - This principle obligates the competent state’s services and institutions to have every time the possibility to overcome the chemical and biological traumatism and terrorism and injuries. In this direction the medical institutions and the medical science include three unified diagnostic-therapeutic systems: antitoxic, antiinfectioso-antiepidemic and antiphisic - antitraumatic program products. All these medical systems contain information for unified medicaments and therapeutic methods and programs – specific treatment for all kinds of affections by physical, chemical and biological traumatism and terrorism (4, 5, 6, 7). In these three therapeutic programs are included reanimation and intensive methods for all the kinds of disturbance of the basic vital functions, affected by physical, biological or chemical agents, specific drugs against chemical and biological aggression. Therapeutic programs of the present unified medical anti crisis doctrine must be used by stage mechanism: 1. In the place of the crisis 2. In the time of the transport 3. the time of the treatment in the hospital, the reanimation and other specific urgent farmacologic treatment is obligated for the three phases of the therapy. In the clinical space will be used the total diagnostic – therapeutic specific system of this unified doctrine according to the clinical indications (2, 3, 6, 7, 8).

**Prophilaxy** - This principle can be realized with the actions in two directions:
1. Forming of the structures and practices against the factors in society and against the conditions in nature, which can produce physical, chemical and biological toxic and eco-traumatic incidents. Preparing and distributing information about the eco-physic-toxic and biological (and including virus and bacterial deseases) pathology, as well as particularity of antitoxic, anti chemical and anti-biological traumatic and terroristic aggression. In this direction it is very important to organize anti-crisis pre-treatment instruction for first aid and permanent anti-crisis readiness of the state and the society, and effective international anti-crisis politic and ecologic collaboration.
2. This principle can be realized by specialized administrative antitoxic, anti-epidemic ecology organization in the state and with the participation and the aid of the international, regional and global political unions and institutions. This program must be included in the instruction programs of the student absolvents of medicine and in the social health and in the clinical program for the specialization of all physicians.

The positive effect of this anti-physic, anti-toxic-chemic and anti-biological anti-crisis unified doctrine from Al. Monov proves its significance in different physic, biologic toxic and biological accidents.

**References**

7. Monov, Al. “Clinical Aspects Of The Main Critical States In The Human Organism”; In “Chemical and Biological Traumatism and Terrorism”(Editors: Al. Monov, Ch. Dishovsky), 2004
Chapter 7

Contemporary Trends in Computer Aided Tools Development for Enhancing Chemical Accidents Medical Support

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Abstract - Nowadays chemical industry became an essential part of contemporary world. The modern society demands increased chemical compounds and products leading to increasing number of chemical installation. Large number of chemical facilities and implementation of highly sophisticated technologies are factors for greater chemical risk level estimation.

The aim of the article is to present contemporary trends in created for chemical incident medical management (CIMM) computer aided tools (CAT) development. By means of descriptive method capabilities of created and established in MMA CAT are analyzed. Comparative analysis was applied in order to define trends in CATs development for better addressing current requirements derived by novel background.

As a result of performed analyses several CAT compounds were defined as appropriate for refining. In order to entirely fulfill adopted in Bulgarian army NATO C4I requirements as well to be fully integrated in unified rescue system (URS) the following priorities were listed:

1. CAT computer base has to be elaborate in order to be compatible with NATO and URS computer systems.
2. Geographic position compound chemical and medical environment compounds have to be refined in order better coordination with CATs implemented in Ministry of Defense and URS, to be obtained.

Developing established in Disaster medicine Department CAT, according to defined trends could improve CAT usage and enhance its utility dimensions, thus timely providing medical planners with required essential data and CIMM meaningful course of action.

Keyword - computer aided tools (CAT), chemical incident medical management (CIMM), unified rescue system (URS), disasters
Introduction

Nowadays chemical industry became an essential part of contemporary world. Large number of chemical facilities and implementation of highly sophisticated technologies are risk factors for a major accident or terrorist attack threat. [1] In such cases it is of a vital importance to make a due time planning of the medical decisions in order to mitigate the consequences. In accident with industrial substances or terrorist acts a sharp disproportion among the great number of medical losses and the available in the region medical resources may occur. In order to keep the requirements in providing timely medical aid the use of computer technology provides fast and vital real time decisions [2].

Materials and methods

Worldwide computer aided tools (CAT) are created for several reasons: because of the increased probability of chemical major incidents occurrence derived by the growing chemicals facilities nowadays, to help Chemical Major Incidents and Medical Management and Support Structures (CMIMMSS), where:

- sharp disproportion among the great number of medical losses and the available in the region medical resources exists;
- medical activities required are time constrained;
- fastest medical evacuation is a must;

The information gathered about the software capabilities usually are related to:

- rapid assessment of a certain accidental situation with involved industrial chemical substances;
- assessment of medical capabilities available in the affected area;
- casualties number estimation in timeline with the chemical incident development
- information about available medical means and capabilities in the vicinity

According to the open literature the proposed worldwide software tools concerning chemical accident consequence prediction and assessment does not incorporate the medical evacuation procedures with the available and needed medical resources in the accident zone.

The created in Disaster Medicine Department computer software offers the possibility of quick assessment of a certain accidental situation with involved toxic industrial substances (TIS) [3]. The computer aided tool can rapidly calculate downwind hazards arising from any release of TIS after major accident in industrial facilities or transport accidents with dangerous loads. The software also provides a capacity for modeling plumes of TIS over complex terrain, and meteorological data, both of which are required for accurate assessment in particular locations and situation. The footprints are also visualized by the user and overlaid upon route and geographical maps of critical facilities. The modeling of a certain accidental situation includes: calculation of plume width, length and persistence, time of arrival to locations with residents, estimates the injuries: quantity of lethal, serious, moderate and minor in the facility and residents overwhelmed by the toxic plume. Concerning the medical evacuation procedures the software estimates:

- number of physician teams required for advanced life support (available and needed);
- number of hospital beds necessary for in-patient hospital treatment (available and needed);
- number of ambulances and courses for patient transportation in order to evacuate chemical casualties (available and needed)

The software provides data and advises about:
- industrial substances’ toxicity;
- personal means of protection for each substance in the dangerous zones;
- main routes of exposure and clinical picture;
- first aid and treatment;
- health care centres (types, available beds and capabilities) in the 15th and 30th km zone around the potentially hazardous facility;
- physicians profile and hospitals profile for medical aid provision;
- patient admission to hospitals by priority;
- number of ambulances in the 15th and 30th km zone and their utilization coefficient;
- time and route length for evacuation of injured from the site to the appropriate hospital with the available sanitary or other transport means;
- medical evacuation – ingress and egress routes, time and direction, means available

The route length from the facility to the above said medical institutions, expected to service in extreme situations, ensures the injured to be admitted by priority according to the time criterion. It is of utmost importance because defines the duly arranged medical evacuation. If a hospital or any medical centre falls within the toxic plume it is automatically rejected as option to be used [4].

The developed in Disaster Medicine Department software tool was used predominantly in conducting preventative exercises in events of major accidents with chemical releases and spills on a local and regional level. It was a principal in high-profile meetings and drills concerning the readiness of university hospitals and regional health centers to cope with mass injuries in such situations.

Results

In order to fulfill adopted in Bulgarian army NATO C4I requirements as well to be fully integrated in unified rescue system were outlined the following priorities:
- software upgrading from DOS to WINDOWS;
- to be in compliance with NATO STANAG 2103 ;
- to be integrated into Military Command and Control software systems (e.g. PRESLAV);
- communication module to be created with the defence and civil emergency services of Ministry of Emergency Situation, National Medical Coordination Centre, Ministry of Environment, National Institute of Hydrology and Meteorology ;
- the map module has to display:
  - vector maps;
  - raster maps;
  - aerial photographs;
- satellite photos;

by means of the both relevant NATO or Bulgarian mapping standards;
- to be able to exchange data about environmental pollution in case of chemical accident with trans-boundary dispersion (Connected to surveillance system of the Ministry of Environment and Ministry of Emergency Situation) in real time;
- to provide assessments for military medical intelligence in military mission preparation and execution [5,6,7];
- additional tool for assessment in case of chemical weapons implementation to be created;
- to be integrated in the assessment and planning process of CMIMMS on district and national leve.

Conclusions

The upgrading of the established in Disaster Medicine Department CAT, according to defined trends could enhance its utility dimensions in timely providing medical planners with required essential data and CMIMMS meaningful course of action. The defined developing trends will improve CAT appliance in CIMM training and preparation in order to enhance national and multinational medical readiness to cope with disasters.

References:

Chapter 8

Bulgarian National Legislation and Biological Weapons Proliferation

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Summary - The Convention for prohibition of proliferation of biological and toxin weapon (CBTW) provisions and their application in the national legislation is barely known to the wide medical community. The control of biological weapon (BW) proliferation covers different areas of human and social activities laying beyond the professional medical interest. Rising awareness amongst medical and scientific communities about CBTW provisions has a critical value for decreasing the risk of intentional or accidental BW transfers. We present relatively comprehensive information on different aspects of national legislation dealing with CBTW provisions. This presentation covers articles from penal and civil code as well as agriculture, veterinary and trade laws and regulations.

Keywords - biological weapon, national legislation, CBTW provisions, application.

Introduction

The Convention on prohibition production, development, storage, distribution and usage of biological and toxin weapon (CBTW) was developed and proposed to the international community by representatives of the former Soviet Union, USA and Great Britain. The CBTW ratification procedure started on April 10-th 1972 and the Convention was empowered on March 26-th 1975 after 22 countries had deposited its ratification files.

The Convention is the first multilateral agreement forbidding development of a whole class of armament. It is a direct offspring of 1925 Geneva Protocol. Bulgaria ratified the CBTW on 30-th of June 1972. (State Gazette [SG] # 54 / 1972). The Convention was empowered on 26-th of March 1975 (SG # 46 / 1975). According to article 5 of the Constitution of the Republic of Bulgaria all international legal agreements, rightfully ratified by the National Assembly and published in the State Gazette are part of the national legislation. These international legal agreements have a priority over the national legislative acts when they contradict them.

Discussion

Brief information on the CBTW contents is necessary to figure out what the Convention stands for. (1) Article 1 declares how far the Convention goes to
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development, production, storage or acquisition of microbial or biological agents or
toxins with no regard to their origin or method of production in type or quantity
unjustifiable by preventive, defensive or other peaceful purposes as well as weapons,
equipment and means for BW delivery with hostile intentions. Article 2 provides
obligations to every member state to destroy or convert to peaceful purposes all
available means no later than 9 months after the CBTW ratification. Article 3 states
that the members are stringed to support encourage or urge directly or indirectly a third
state, group of states or organizations to produce or apprehend BW. Article 4 obliges
all member states to undertake measures for prohibition and prevention activities,
related to article 1 on its territory or under its jurisdiction. Article 5 provides
opportunity to conduct consultations and cooperate in order to solve problems
concerning CBTW. Article 6 gives the right of every member state to comply within
the UNO Security Counsel when there is data available for breeching CBTW
provisions by other member state. All member states are obliged to participate in the
investigations initiated by the UNO Security Counsel. Article 7 the member – states
can assist a third state if the UNO Security Counsel declares that this state is
endangered because of CBTW violation. Article 8 declares that the CBTW provisions
do not limit or exclude obligations taken according to the 1925 Geneva protocol.
Article 9 shows the common will to continue work on effective prohibition of the
chemical weapon. Article 10 obliges the member states to facilitate and participate in
future exchange of equipment, materials and scientific and technological information
for the peaceful use of BA and BW. The Convention must not hamper countries
economic and technological development. Article 11 declares that the Convention
could be supplemented by each member state and the mechanism of addition
ratification is clarified. Article 12 provides ground to carry out CBTW review
conferences in order to monitor the work progress and to equilize discrepancies. Article
13 gives every member – state a possibility to withdraw from the Convention if its
provisions jeopardize its national sovereignty. Article 14 describes ratification
procedure and article 15 selects languages on which the Convention should be
distributed.

Problems with the Convention come from its shortness – five pages of common
language without clear definitions – an acceptable compromise, legacy from the Cold
war. It allows too much freedom in the Convention vocalization, while the lack of
official ways for provisions applications control limits its efficacy. There are contrary
interests by different member-states on the Convention application area as well as the
consolidated measures taken in case of provisions breeches. Restrictions-to-be-imposed
in the field of medicine and biotechnology research are not welcomed by everyone.
Significant part of its provisions can be interpreted in dependence of national, religious,
group or other type of affiliation.

BW / BA diversity could send the legislative measures to a single isolated article
from different legal acts and regulations concerning seemingly unrelated agencies and
organizations of the national executive branch. These legal acts are bind to the penal
and civil codes, trade legislation and safety of the personnel, working with dangerous
microorganisms.

Penal code:

Article 337 covers provisions 1 and 4 on CBTW. Article 337 (SG # 41/85) gives
an imprisonment for 1-6 years to a person who produces, processes, repairs, develops,
storages, trades, carries, imports or exports without legal right or permission by the said administration. Article 337 / 2 (SG # 50/95) provides 2-8 years imprisonment if the offence is made in liaison with the accused official position or it is a reccurent one. Article 337 / 3 (SG # 50/95) declares 3-10 years imprisonment if the offence is on large scale. Article 337 / 4 (SG # 50/95) provides 5-15 years imprisonment if the offence is on large scale and the crime is grave. Article 337 / 5 (SG # 26/04) envisages up to 2 years imprisonment preparation to breech provisions 1-4. (6)

Articles 339, 349, 353, 354 cover provisions 1, 3 and 4 on CBTW. Article 339 (SG # 28/82) forbids acquiring in any way, holding or giving to anybody without a proper permission otherwise imprisonment is up to 6 years. Article 339 / 2 (SG # 41/85) selects 3-6 years imprisonment if the product is acquired in large quantity. According to article 339 / 3 (SG # 28/82) imprisonment is up to 6 years in case of expropriating or delivering to a person not having permission to acquire such products. Article 339 / 4 (SG # 28/82) sentences a person who expropriates or gives armament to anybody without working permission to carry the respective weapon up to 6 years imprisonment. 339 / 5 (SG # 62/87) selects up to 6 years imprisonment for a person covering discovered weapons and ammunition without a proper license of doing so. Article 349 / 1 (SG # 41/85) gives 2-8 years imprisonment for a person who put into or mix hazardous material in well, spring, water supply or other installation for public use from which or by which water is obtained. According to article 349 / 2 (SG # 50/95) if the mentioned above offence leads to severe body injury the imprisonment is for 3-10 years, but if it led to death the sentence varies from 10-20 years to life in prison. Article 349 / 3 (SG # 41/85) sentences to be like those in 349/1 or 2 for people who distribute infectious diseases causative agents in order to infect people. Article 353 / 2 (SG # 62/97) gives 1-5 years imprisonment or 1-3000 levs fine for people who contrary to international agreements carry through the state border biological agents or toxins. According to article 354 (SG # 95/75) the punishment is up to 2 years imprisonment or 1-300 lev fine for those who produce, acquire, hold, expropriate or deliver without permission a poisonous substance which is not placed under license regime. The sentence increases to 3 years imprisonment in case of systematic offence according to article 354 / 2 (SG # 10/93). (6)

Article 415 covers CBTW provision 8. Article 415 / 1 (SG # 62/73) selects 10 years imprisonment for a person or people who contrary to the international rules of war use or order to use bacteriological, biological or toxin weapon. If the offence on the previous article led to severe consequences the imprisonment goes to 10-20 years or life w/o parole according to article 415 / 2 (SG # 153/98). If someone undertakes military preparation to use bacteriological, biological or toxin weapon as method of warfare according to article 415 / a (SG # 92/02) the sentence is 1-6 years imprisonment. (6)

**Trade legislation:**

The Dual Use Materiel Export Control Law (2) deals mainly with CBTW provision 1, but covers also provisions 3 and 4. With article 2 / 1 the Minister Council is authorized to accept a list of double use goods and place them under import supervision. By article 2 / 2 the goods placed under export supervision are set to the EU regulation 1334/2000 annex I.

Chapter III defines the mechanisms of control enabling over export, import or transfer of double use goods, while chapter IV describes the duties of the officials
linked with control activities. Chapter VI defines the mechanisms of control enabling over export, import or transfer of double use technologies. Chapter VII lists the possible violations of the Law and specifies the penalties required. (2)

**Human Pathogens Control Regulations:**

The adopted by the Ministry of Health Standards - Clinical Microbiology (2002), Virology (2002) and Medical Parasitology (2002) - define microorganisms allowed to work with, methods allowed to be used while working with these microorganisms, and items allowed to support usage of these methods by the labs in dependence on their diagnostic level (local, regional, national). The standards cover CBTW provisions 1, 4 and 10. (13, 14, 15)

The Ministry of Health Regulation 13 (11) deals with conditions and order of work in medical laboratories by defining type of labs, labs staff, room hygiene requirements, organization of work covering provisions 1, 4, 10 on CBTW.

Regulation 14 / 2002 (12) is issued by the Ministry of Labor and Social Policy and deals with provision 10 of CBTW. It covers laboratory biological agents exposition safety by defining the minimal requirements for workers health protection wherever a risk of exposure to BA exists or may exists as a result of their occupational activity. It clarifies the know-how of risk assessment by annex 1 which provides BA wide list by type and hazard level, by annex 2 which describes the activities linked with a possible BA exposure, and annex 4 which gives the personnel protection measures in dependence on needed biosafety level.

Instruction 5 / 19.11.2003 deals with causative agents with high medical and epidemiological risk and covers provision 1, 10 on CBTW. Article 2 defines which microorganisms belong to this group. Article 3 says which laboratory can work with these microorganisms. It defines laboratory work organization (sample handling, animals experiments, incidents, etc) and punctiliously prescribes procedures on sample registration, culture storage, movement and sterilization. (5)

**Plants Pathogens Control Regulations**

Basic documents in Plants Pathogens Control are Plant Protection Law (3) and Regulation 1 / 1998 (7) on plant control. They are issued by the Ministry of Agriculture & Forestry and covers provisions 1, 4, 10 of CBTW. Article 3 declares the minister as an official who approves the lists of pests, plants, plant products and other items under control. Annex 1 gives a list of all pests with restricted import / export. Article 4 says that plants and plant products should be imported through previously declared border check points and plant and plant products can not be transported without control authority permission.

Regulation 1 / 2002 about conditions under which pests, plants, plant and other products go scientific work and selection issued by the Ministry of Agriculture & Forestry covers provision 1, 4, 10 on CBTW. (8) The Regulation provides way plant pests to be used for scientific purposes. Article 2 demands every scientific work with plant pests to be permitted by the relevant plant control authority. Through sudden check-ups the regional plant control services  cover the registered scientific activity (article 10). During the scientific trials plant pests must be placed under quarantine under requirements covered by Annex 1 of the Regulation.

**Animals Pathogens Control Regulations**

Veterinary medicine law issued by the Ministry of Agriculture and Forestry (4) covers provisions 1, 4, 10 on CBTW. Chapter 5, part 1 – veterinary requirements for
animals and embryo products – covers the procedures for prevention, localization and elimination of highly infectious diseases. Chapter 6 – animal healthcare control – covers animals contagious diseases diagnostics and prevention. Chapter 9 describes the state veterinary control. The guidelines for veterinary medicine law application (16) which covers provision 10 on CBTW defines laboratory licensing process and laboratory quality control (art. 5-11).

Regulation 4 / 2003 for veterinary medicine products licensing (10) empowers the National veterinary service director the right to control and to license production of veterinary medicine drugs and details the way to control the technology used. It covers provisions 1, 10 on CBTW.

Conclusion

The legislative initiatives are just a small part of the complex of activities linked with the CBTW provisions application. The strict adoption of CBTW depends not only on the quality of the state executive branch acts. The scientists, human and veterinary medicine and plant control workers cautiously participation, combined with the active support of the security services and the corrective supervision of the non-government organizations are necessary elements of the whole process of CBTW application.

References

5. Инструкция №5 / 19.11.2003г за работа с причинители на бактериални, гъбични и вирусни инфекции с висок медицински и епидемиен риск (ИВМЕР), Министерство на здравеопазването, Служебен бюлетин, год. L, бр. 2: 3-15
MEDICAL MANAGEMENT OF CHEMICAL AND BIOLOGICAL CASUALTIES


8. Наредба 1 / 2002 на МЗГ за условията при които вредители, растения, растителни и други продукти се използват за научноисследователски цели и селекция, ДВ бр. 8/2002г.


10. Наредба 4 / 2003г на МЗГ за лицензирането на производството на лекарства и препарати за ветеринарно-медицински цели, ДВ бр. 7/2003г.

11. Наредба № 13 за условията и реда за работа на медицинските лаборатории, ДВ бр.52 / 2 VI.1994г.


13. Медицински стандарт “Клинична микробиология”; 2002г, Заповед РД 09 – 111 / 18.03.2002г, МЗ


15. Медицински стандарт "Медицинска паразитология"; 2002г, Заповед РД 09 – 211 / 16.05.2002г, МЗ

Chapter 9

What are the Risks of WMD by Organized Crime in Southeast Europe

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Key words - CRB terrorism, organized crime, members of secret police and secret services of ex-communist countries

Studies prepared by numerous analysts around the world place organized crime (local and international) as the largest threat today. This primarily refers to illegal drug and human trafficking as well as illegal trafficking of rare plant and animal species, which generate huge financial profits, and in turn, power (hundreds of billions of dollars are in play).

Such illegal organized groups develop much more rapidly than the law-abiding world, in all its aspects (technological, industrial, civilizational). Advanced technologies, with their worldwide expansion, have also been infused in all pores of crime. Thus they facilitate easier travel, making it possible to reach any part of the world quickly, transfer any technological developments and misuse them.

Crime is bound by the law, and laws are made by countries. Political changes on the local, national and transnational level open the door to new opportunities – such as trade in toxic, hazardous waste, CFC’s (chlorofluorocarbons) and other dangerous substances – as well as obstacles i.e. challenges for organized crime. In order to survive, organized and transnational crime constantly adapts to social changes. In the process, poor countries become vulnerable, for example the countries of the former Soviet Union; the disintegration of Yugoslavia and war in that region, poverty in Asia and hardship in Russia – all this creates favourable conditions and a market for illegal immigration.

Organized crime also influences the world economy. Markets are opened and closed. There is no longer a need to illegally import weapons in the territories of former Yugoslavia, but there are increasing opportunities for trade with other countries, like China for example. Successful measures against organized crime can sometimes have unexpected effects. For instance, the accomplishments of the US police in combating drug cartels in Peru and Bolivia have pushed narcotics trade into Mexico, even closer to the United States. On the other hand, pressures on the American and Italian mafia groups have resulted in their weakened positions, but also in increased enclosure and caution. The prevalent issues related to the Republic of Croatia are illegal immigration, ‘white slave’ trade and drug trafficking. Namely, the EU believes that everything pass-
ing through the Balkans ends up in EU streets, which has consequences for its military and political structures, as well as its financial investments in the area, stated Britain’s former Home Secretary David Blunkett. The diversity of Southeast Europe accounts for significant national, religious, cultural and economic differences, but a common bond and unifier is organized crime. Although organized crime in Southeast Europe has always been present to a degree, the escalation of organized crime began in the late 1980s, with the onset of conflict, and later during the war in former Yugoslavia, which opened new markets and created new challenges for organized crime groups that had been developing over a number of years in West and Eastern Europe, i.e. after the return of international criminals of Southeast European origin to their native countries.

Furthermore, Balkan organized crime has attracted the attention of the United States criminal investigation authorities and the US Senate. The Federal Bureau of Investigation (FBI) believes that crime from the Balkans, Eastern and Central Europe and former Soviet Union is expanding and will continue to expand. "In the last year or two, European nations have recognized that Balkan organized crime is one of the greatest criminal threats that they face", said Grant D. Ashley, Assistant Director, Criminal Investigative Division, FBI, in October 2005 in his testimony before the Senate Foreign Relations Subcommittee on European Affairs. He added that European police organizations now estimate that Balkan organized crime groups currently control upwards of 70% of the heroin market in some of the larger European nations, and are rapidly taking over human smuggling, prostitution and car theft rings across Europe. Analysts believe that these criminal groups are involved in all types of criminal activity - drug trafficking, human trafficking, burglary and home invasion robbery rings, money laundering and securities fraud, organized crime gambling and extortion rackets. Albanian organized crime groups have expanded their activities to Italy, Germany, Switzerland, Great Britain and the Scandinavian countries. In recent years, Albanian organized crime groups have reached the United States, forming partnerships with the Gambino, Genovese, and Luchese families. In the meantime, mafia groups in Italy have allegedly taken advantage of their geographical proximity to Southeast Europe to align themselves with Balkan organized crime groups involved in arms and cigarette smuggling, human trafficking and alien smuggling. To fight this growing problem, the FBI has launched a Balkan organized crime initiative, addressing criminal activity emanating from Slovenia, Croatia, Serbia and Montenegro, Bosnia and Herzegovina, Albania, Kosovo, Macedonia and Greece. It is a high profile project launched by the FBI's Organized Crime Section and the Organized Crime and Racketeering Section of the Department of Justice.

Today we know that the former Yugoslavian state security administration UDBA used crime-related individuals for assassinations of enemies of the Yugoslav regime, which at the time had a developed logistics network and infrastructure in Western countries. Several individuals from that crime scene have publicly admitted that they engaged in activities, under orders of the then security-intelligence services, such as espionage, wiretapping, stalking, kidnapping, bribery, insinuations, psychological operations, as well as certain forms of interstate political, financial and diplomatic trade. In carrying out such activities, a range of Yugoslavian state and social structures were
used – from the army and police, foreign affairs resources, educational, cultural institutions and the like to state statistical, media, publishing and other resources. In return, the regime protected the criminals from West European police and provided them with fake documents and identities, under one condition: that they do not perform any criminal acts whilst on Yugoslavian ground. This is perhaps one of the reasons why the former Yugoslavia was, as far as public order and safety were concerned, a considerably safe country with a significantly low crime rate.

Approximately a hundred emigrants are estimated to have been assassinated; several were poisoned with acid and some died of brain haemorrhages, which have been attributed to ricin poisoning. The structure of this security and intelligence system, its role, position and function within the state administration, were grounded in conventional (communist) principles of defending the political regime (subordination to one political authority and the interests of one party). At the beginning of the war, their services were paid in a variety of ways: from permission to pillage in the field, out of which the paramilitary group Serb Volunteer Guard („Srpska dobrotvoljačka garda“) developed an entire industry, to privileges in smuggling oil derivatives, cigarettes and strategic goods, and privileges in foreign currency trading and other financial operations during the period of hyperinflation (scams with pyramid schemes and old foreign currency savings) as well as the most lucrative business of all: heroin trafficking.

Furthermore, we know that Yugoslavia had a program for developing mainly chemical, but also nuclear and biological weapons in the 1970s and 1980s. The program helped acquire knowledge on synthesis, analysis, function, protection, decontamination, medical prevention and prophylaxis, and developed a pool of personnel who possessed the above knowledge, principally amongst the ranks of the then Yugoslav National Army (Jugoslavenska narodna armija - JNA). The project could not have been conducted without strict supervision by the security-intelligence services of the time, and we know now that some participants of the project were themselves members, sympathizers or associates.

JNA avoided using chemical-biological warfare against Croatia, but it did use conventional weapons, based on the knowledge from the program, and attacked, both in Bosnia & Herzegovina and Croatia, every plant with hazardous substances within its range, some of which were attacked several times (Petrokemija Kutina, Rafinerija Sisak, Herbos Sisak...). Croatia and B&H were not prepared to defend themselves from CB terrorism, so the JNA used such weapons several times during the war to ensure victory on the battlefield and achieve their military goals (dissemination of artificial cobweb on several occasions in 1992 and 1993 which was indisputably proven to have originated from military laboratories; the puzzling hemorrhagic fever with renal syndrome (HFRS) epidemic in Eastern Slavonia in 1993 and 1994; the confirmed use of CS in Vukovar and Srebrenica and use of BZ in the Dubrovnik backcountry and Srebrenica - Human Rights Watch, 1998 report; the confirmed use of biological agents with the assistance of organized crime groups during Operation Mač 1 in Western Bosnia and Herzegovina in 1995. Even a Croatian Army prisoner was subjected to test the bio-agent effectiveness).
After the war, and after the states and state administrations in Southeast Europe were stabilised, the operations of organized crime were just slightly dissuaded. Cases of human trafficking (primarily women smuggling), illegal trade in arms, drugs and other goods, as well as assassinations of high ranking state officials, journalists and other individuals who jeopardize the activities of organized crime groups continue to go on in the countries of Southeast Europe. Their mode of operation has been redirected to the realms of financial crime, and a strong connection of political, judiciary and business structures with organized crime has penetrated into a range of business sectors.

Intelligence and political structures played a significant role in the functioning of organized crime (in 1999, drugs worth 60 million DM were discovered in a safe belonging to the Serbian State Security Service (RDB) and RDB officials were indicted with charges of misusing their position and falsifying documents).

When speaking about organized crime groups and their targets, we can say that using CBW substances is a possibility, because using such weapons for individual objectives is a much more subtle method which does not point to clues regarding the organizer or executioner of such attacks. Illegal trade of such substances, technology and knowledge is also possible, as this could generate substantial profit as does trade in dual-use technology.

Both overt and covert methods are equally likely to happen, and war in the form of low intensity conflict can only be the cause, goal or consequence of the new-specialized types of threats, where the most likely targets are the following: Tourist destinations and tourists themselves; Food industry; Agricultural areas; Cattle of the said country; Chemical, petrochemical, oil, pharmaceutical and similar industry with dangerous substances; Critical infrastructure objects, such as airports, sea ports, train and bus stations and their infrastructure for transport, storage and handling goods and passengers; Critical infrastructure objects, such as theatres, concert halls, cinemas, sports halls, shopping centers etc.

References:
Chapter 10

Preparedness Against Chemical Terrorism: Poison Information Centers Roles

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Abstract - Recent advances in dual-use technology may reduce the technological barriers for chemical or biological terrorism. From this view, chemical terrorism is continues threat in modern world. Preparing for a chemical terrorism, especially in pre and post hospital settings have very important role in decreasing of catastrophic dimensions of a chemical terrorism attack. Poison Information Centers (PICs) have very important roles against chemical terrorism attacks. National and regional PICs play a key role in response planning and co-ordination programs and can be a great scientific resource for public and medical staff during chemical terrorism attacks. The aim of this review is to describe the role of PICs in chemical terrorism response planning.

Key words - Poison Information Center, Chemical Terrorism, Preparedness

Introduction

Chemical terrorism is the use of any chemical agents as a weapon, chemical agents such as poisonous vapors, aerosols, liquids or solids that have dangerous effects on humans, animals or plants for to create panic and fear, disrupt the economy or to get a response from the government [1]. Growing awareness and concern about chemical terrorism, as a possible worldwide threat has necessitated a comprehensive review of this problem from several aspects [2]. There is an increased in the use of chemical agents, both in terrorist attacks and in industrial accidents. For example, during the 1995 sarin nerve agent attack on the Tokyo subway system killed 12 persons and injured 5000. In 1984, the accidental release of methyl isocyanate from a pesticide factory in Bhopal, India, injured hundreds of thousands of peoples and killed about 4000 [3, 4, 5]. From this view, chemical terrorism countermeasures are a major priority with healthcare providers, law enforcement agencies and the government [6]. Significant resources are being invested to enhance civilian domestic preparedness by
conducting education at every response level in anticipation of a chemical terrorism attack [2]. The key to a successful response against chemical terrorism incidents, are education, integration of efforts as well as thorough communication and understanding the role that each agency would play in an actual or impending chemical incident. In anticipation of a chemical event, a regional counter-terrorism task force was established to identify resources, establish responsibilities and coordinate the response to chemical terrorism. Members of the task force included first responders, hazmat, law enforcement (local, regional, national), government officials, the health departments, and the Poison Information Center (PIC) [2]. Preparing against for a chemical terrorist attacks have become a very important part of PIC activities [2, 7]. Today, there are the most important roles for PICs in chemical terrorism response planning. The information available through PICs is as unique resource for health care professionals involved in chemical terrorism response planning. Traditionally plan for hazardous materials incidents, and planning for an attack by weapons of mass destruction is no different than a very large-scale hazardous materials incident. The difference is that, in addition to the traditional chemicals involved in a hazardous materials incident. Hazardous materials incidents include gas and vapor releases, spills, explosions, and fires. When planning for a hazardous materials incident, evaluation of threats in area is necessary. In this article, we reviewed the main roles of PIC for preparedness against chemical terrorism attacks and its situation in response planning [2].

1. Special aspects of chemical terrorism attacks

Two major chemicals which used by terrorists in a chemical terrorism attacks are chemical weapons (CW) and hazardous materials (HAZMAT). CW agents are man-made, super-toxic chemicals can be dispersed as a gas, vapor, liquid, and aerosol or absorbed onto a fine powder to create "dusty" agents [6]. Many of the chemical weapons for chemical terrorism attacks can be arranged under the following categories [5]:

- Blistering agents (e.g. Sulfur mustard, Lewisite)
- Pulmonary (choking) agents (Chlorine, Phosgene)
- Blood agents (e.g. hydrogen cyanide, cyanogen chloride)
- Nerve agents (e.g. Sarin, VX)
- Tear agents (e.g. Chlroacetophenon, Chloropicrin)
- Incapacitating agents (e.g. BZ)

The use of CWs has many advantages to terrorists. These include the limited capacity for detecting, relatively low cost, potential of damages and their psychological consequences on the population. The lack of available detection technology makes CW
agents ideal to transport and storage. They can be purchased from legal or illegal markets [9].

Another chemicals that used by terrorists in chemoterrorism attacks are hazardous materials (HAZMAT). This category contains many types of toxic chemicals which used in different industrial processes, such as cyanide, chloroform, chlorine, methylisocyanate, phosgene, benzene, ammonia, Pesticides, industrial acids and bases and etc. Main problem connected with chemical terrorism is that beside chemical weapons, terrorists can use different toxic chemicals from the chemical industry, agriculture or products released from industrial facilities in a terrorist activity. There are some differences between chemical weapons and the chemicals released after destruction of a chemical plant following terrorist act [8]:

- Industrial chemicals are less toxic than chemical weapon, but will be present in much higher quantities for a longer period of time.
- Contamination of hazardous chemical materials eventually covers a bigger area.
- Chemical weapons represent a relatively small numbers of potential agents; against toxic industrial chemicals that have tens to thousands of agents.
- For the known chemical weapons, there are simple methods for detection and quantification. But for hazardous industrial chemicals the qualitative and quantitative process is difficult.
- Military protective filters are suitable for protection against chemical weapons, but some of hazardous chemicals are not very well filtered by these devices.

A chemical terrorism event is likely to be discovered in one of two ways: the local discovery of the environmental release or exposure incident or the diagnosis of the resultant patients [10].

1.2. Epidemiological Clues of a Chemical Terrorism Attack

1. Rapid onset (immediate or minutes to hours) of similar symptoms among victims in close proximity to a hazardous materials release.
2. The chemical release might result from an explosion, fire, spill or release of vapor under pressure or from open containers.
3. The abrupt group onset of symptoms or a sudden release of chemicals in a closed or semi-enclosed non-industrial area (e.g. subway, school, convention center) is suspicious for terrorism.
4. Unprotected rescuers becoming victims they indicate the presence of conditions Immediately Dangerous to Life and Health (IDLH).
None of these clues are pathognomonic of chemical terrorism, but indicate a high likelihood that victims are suffering from chemical exposure [10].

1.3. Guide for Chemical Terrorism Identification Possible Scenarios of Chemical Attacks.

1. Use of chemical weapons or attack on a weapons stockpile
2. Aerosol spraying (handheld devices, crop dusters)
3. Attack on industrial/commercial chemical sites.
4. Intentional hazardous materials transportation mishap (truck, rail car or tanker with chemicals [10].

There are several sources of information available for detection of chemical incidents such as chemical terrorism attacks. These sources include emergency department of local hospitals, regional poison control centers, community and federal initiatives, and state and local health departments. For disaster response planning, it is important to evaluate the resources available in community. This may save response providers from duplicating plans that have already been created. The majority of hospitals have disaster response planning committees, and many of these committees have already started to plan for attacks by weapons of mass destruction. PIC is also an excellent source of information, not only in the planning stages but also while responding to an emergency. A number of community and federal initiatives that address disaster response planning may be active in your city, region, or state. Communication methods, such as a radio, allow health and medical groups to communicate without land and cellular telephones and should be a part of the plan. State health departments work closely with the health care authorities in the government. They coordinates a Health Alert Network (HAN), a nationwide integrated information and communication system established as a platform for the distribution of health alerts and national disease surveillance [2, 10].

2. Poison Information Centers’ Roles and Facilities: At a Glance

After the World War II, Poison Information Centers (PICs) were established for improving the care of poisoned patients and poisoning prevention programs. Four decades later, they are still recognized for their traditional activities, but they are faced to new challenges. The development of numerous toxicological information and progressive in information technology (IT) due to computerized databases and Internet resources, the needs of health professionals to call a PIC has changed. The general concern about the cost-effective utilization of the scarce health care resources will enhance the role of PCs in the reduction of unnecessary referral to emergency departments. Health authorities require the implementation of continuous surveillance system of chemical-related events and a real-time alerting procedure concerning terrorism attacks, child health and other serious events. It has been recognized that PICs were in a unique position to monitor patterns, incidence and severity of exposures and to detect new trends. They could contribute to the accuracy of risk assessment documentation and help to identify the priority list of chemicals that need to be assessed or reassessed. But, in the perspective of using PICs’ data for global risk
assessment and public health, the main challenge is the development of international harmonization of human poisoning data collection to allow their comparability [11].

2.1. Poison Information Centers’ Roles:

- **Patient Information Activities**: PICs have a critical role for public education about poison information. They provide a suitable source of drug and poison information for all of the population. In addition, PICs have accurate assessment and triage of poisoning exposures.

- **Toxicovigilance**: The collected data from regional PICs in collaboration with national PIC make a national database that act as an alert system for the recognition of a variety of public health threats. The statistics obtained from PICs provide an epidemiological map of poisoning categories in the country and can clarify the poisoning pattern in national level.

- **Public Education**: One of the most important roles of a PIC is public education to promote poisoning prevention and awareness of accessibility of PICs services to the general population. From this purpose, PIC can use from many media such as newspapers, regional and national broadcasting, Internet and multi-media products.

- **Professional Education**: PICs provide continuous medical education programs for healthcare professionals in clinical toxicology and poisoning management [12, 13].

2.2. Poison Information Centers’ Abilities:

The most abilities that provide by accredited PICs include:

- A 24-hour emergency and information hotline service for public and healthcare professionals. Calls are directly answered by a poison information specialist who trained in clinical toxicology. The PIC provides a patient assessment and evaluation, medical care recommendations and follows up system. Besides, for each of calls, a standard questionnaire has completed. These recorded forms, have an important role for future assessment and epidemiological evaluations. PICs are staffed by health professionals (pharmacists, nurses, and physicians) with specialized training in clinical toxicology. While most people think that poison control centers only handle calls involving toddlers who are orally exploring their environment, the poison control center's scope of practice is quite extensive. They handle acute and chronic poisonings, environmental and occupational exposures, bites, stings, and much more.

- Continuous follow up of calls for known and suspected poison exposures.
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- Availability to standard poisoned patient management guidelines for health care professionals.

- Public and professional education facilities.

- Accessibility to a communication network via telecommunication systems at local, regional and national levels. This is an important aspect for a PIC that provides an alert communication system for public and health care providers that involved in a disaster response planning.

- Emergency response to information requests received from public, health care professionals, agencies and government at the local, state and national levels. In a natural or manmade disaster, this is a more important facility for designing an effective response planning [2, 12, 13].

3. Roles of poison information centers in preparedness and countermeasure against chemical terrorism

Poison information centers play a key role in chemical disaster response planning and can be a great resource for response providers when establishing plans and responding to disasters. The poison center is one of several critical components of a regional counterterrorism response force. Some of the main roles of PICs for preparedness against chemical terrorism attacks are:

1. PIC may be one of the first agencies notified of a chemical emergency, probably by a call from a concerned citizen, it will be responsible for notifying the proper response agencies. The integrated network of PICs may play the role of an alert system for preparedness against chemical terrorism attacks.

2. PICs must have standard operational guidelines and numerous resources on-site, including references on chemical terrorism and chemical disasters. In certified PIC, classic information resources include tertiary resources (relevant text books to drug and poison information), secondary resources (computerized databases) and primary resources (scientific journals) with Internet accessibility are provided. Response protocols were developed and education was conducted, culminating in all members of the response task force becoming certified NBC instructors. The regional response plan establishes the poison information center as a common repository for all cases in a biological or chemical incident. The development of basic protocols and a standardized staff education program is essential. The use of the RaPiD-T (R-recognition, P-protection, D-detection, T- triage/treatment) course can provide basic staff education for responding to this important but rare consultation to the poison center. They have standard operational guidelines and numerous resources onsite, including references on weapons of mass destruction [14]. The most important resource at the poison center is the specialists in poison information who are experts in toxicology. In addition, each poison control center has a medical director and managing director, many of whom is board certified in toxicology. The staffs of poison control centers have extensive
knowledge of the health care resources in their region and interact with pre-hospital providers to ensure that patients get to the most appropriate health care facility for treatment. They also work with hospitals in the region to ensure that patients get the most appropriate treatment and communicate with the toxicology laboratory to ensure proper collection and testing of laboratory specimens. These experts are familiar with the available antidotes in their region and can assist in identifying an antidote when needed. Poison control centers provide education for both the public and health professionals and play a key role in disaster preparedness and response efforts. Poison control centers also participate in epidemiologic surveillance [14]. Increase the number of available telephone lines, establish communication links with local and state partners, and participate in disaster planning so that each entity works together to ensure the best outcome for the individuals affected. Because a poison control center may be one of the first agencies notified of an emergency, probably by a call from a concerned citizen, it will be responsible for notifying the proper response agencies. For example, in the first anthrax hoax in Utah (USA) the poison control center was one of the first agencies to be notified by the public. The poison control center then notified the proper authorities responsible for handling the incident. Epidemiologic surveillance is another key function of poison control centers in disaster response planning. The data collection system used by poison control centers can assist in the detection of disease outbreaks and track individuals who have been near the site of exposure but may not need to get into the health care system [2].

There are a number of resources available to pharmacists for information about treating people exposed to chemical, biological, and nuclear agents, although a limited number of specific protocols exist. Sources of information about chemical warfare agents include Web sites, military publications, and regional poison control centers. Regional poison control center maintains a list of Web sites that are helpful in providing information on nuclear, biological, and chemical terrorism. For example, CDC's bioterrorism Web site (www.bt.cdc.gov) provides useful information on chemical and biological agents. Regional poison control centers and state or local hazardous materials units are resources for information about handling these threats and may have other resources available to deal with such threats. The most useful resources for information about biological agents are CDC's bioterrorism Web site, military Web sites, regional poison control centers, state and local health departments. The Web site of the United States Army Medical Research Institute on Infectious Diseases (www.usamriid.army.mil) provides useful information about biological agents. State and local PICs are linked directly with health authorities through Wide Area Network (WAN) to provide information and resources during an actual event [2].

3. Co-operation with hospitals in the region to ensure that patients get the most appropriate treatment and communicate with the toxicology laboratory to ensure proper collection and testing of laboratory specimens.

4. PICs play an important role in disseminating basic and clinical toxicology information during a chemical attack to public and medical professionals. In addition, PIC staff
can provide clinical toxicology consultation to health care professionals who are treating victims.

5. PICs can conduct active and passive toxicosurveillance and identify sentinel events [15]. Every call to a poison control center is documented on an electronic medical record [16]. Collected data are compiled nationally to form the National Toxic Exposure Surveillance Database. This database is used to evaluate trends in poisoning and may be useful for real-time surveillance to identify a disease outbreak. Poison centers work with local, state, and federal agencies to ensure that communication lines with various agencies are working properly so that information flows unobstructed during an emergency to be responsive, the poison center staff must be knowledgeable about biological and chemical agents. For example, some calls to poison control centers may be originally identified as food poisoning. However, if a large cluster of the same ailment is identified, it might prompt us to suspect a more widespread incident, such as a bioterrorist attack. Poison control centers play a key role in disseminating clinical toxicology information during a disaster. During a real emergency, there is likely to be mass hysteria. Poison control centers can reduce such hysteria and prevent people from unnecessarily using emergency medical services. This is important in a disaster, as the available medical services will be stretched to their limits. In addition, poison control center staff can provide clinical toxicology consultation to health care professionals who are treating exposed individuals. As a 24-hour service, the public relies on poison control centers for information. Poison control centers can assess individual complaints and refer the patient to appropriate medical care, if necessary [16]. In addition, the poison control center is familiar with road closures, triage points, and hospital status to ensure that the victim gets to the most appropriate health care facility in the shortest period of time. For those individuals who are just concerned but have not actually been exposed to an agent, the poison control center can provide reassurance and recommend shelters. Because CDC recognizes the role that regional poison control centers play in disaster response planning, it issued the CDC Contract for Enhancing Poison Center Preparedness for Nuclear, Chemical, and Biological Warfare Incidents. This contract established consensus panels that identified the most useful references, including texts, videos, journal articles, and Web sites, for responding to chemical, biological, and radiological threats. Key texts and videos were provided to each poison control center. This grant also established a committee to provide recommendations to assist poison control centers in building cooperative relationships with state and local health departments and exploring the use of TESS data for real-time epidemiologic surveillance. This grant also established a panel of experts who were available to provide education on chemical and biological weapons [2].
Conclusion:

Nowadays, chemical terrorism is a major threat with worldwide dimensions. Therefore, planning for preparedness against this problem is necessary for all countries. Preparing an effective response plan for chemical terrorism countermeasure demand to a teamwork planning. PICs have the most important roles against chemical terrorism attacks in collaboration with other response providers. According to PICs facilities and abilities, they are an excellent resource for information about chemical terrorism countermeasure and have a central role for harmonization of activities against chemical terrorism attacks.

References

Part 2

CHEMICAL AGENTS - MECHANISMS OF ACTION, DIAGNOSIS, TREATMENT OF INTOXICATIONS AND PROBLEMS OF CHEMICAL TERRORISM
Chapter 11

Chemical Terrorism, History and Threat Assessment

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Abstract - Nowadays terrorism is becoming not an abstract but an imminent threat for the society security. There is a great array of terrorists' activities, biggest concern of which is the increasing probability of applying chemicals as terrorists' weapon. The aim of the article is to present the historical and recent evidence of chemical terrorism as a base of chemical terrorism threat assessment. In order to achieve the set goal thoroughly were analyzed evidence of chemical weapons or chemical substances usage throughout history by means of historical and descriptive analyses. Based on acquired data chemical terrorism threat was assessed. For threat assessment modified RAPEX methodology was applied. Rapid development of chemical industry and widespread chemical facilities and warehouses are providing terrorists with means and capabilities for chemical weapons usage is stated as a conclusion of the performed analyses. The high level probability of chemical terrorism has to be addressed by national and international imposed restrictions and enhanced medical and rescue preparedness.

Keywords - Medical intelligence, chemical terrorism threat assessment, chemical substances, chemical compounds.

Introduction

Nowadays there is hardly anyone who would not agree that more and more people in our community are obsessed with an idea of chemical terrorism imminence. All media coverage related to terrorists intent and possibility of using chemicals – substances and compounds as a weapon just add to growing concern among society. Since the mid 1990s when in a Japan underground thousand people were injured and some killed in a result of chemical weapon usage by the terrorist religious cult Aum Shinrikyo the fear of chemical terrorism has found its firm and undoubted reason. Moreover a lot of experiments were conducted in order to reveal the easiness of purchasing and/or prepare chemical substances sufficient for killing thousands of innocent citizens. A terrorist attack involving life threatening chemical substances or compounds could produce an uncountable and hardly assessed impact on physical and psychological health and economic as well.
This article presents historical evidence for chemical substances weaponizing and usage of chemicals by states or groups and individuals. Because of tremendous psychological effect of chemical weapon implementation authors do not distinguish state’s from groups and individual actors—always chemical weapons produce terror and fear among the survivors, bystanders and affected society so in this reason every usage of chemicals as a weapon should be defined as terrorist act.

The purpose of this research was to examine terrorists’ (states, groups, individuals) intent willingness capability and readiness to imply chemicals in order to achieve their goals. Based on data provided by performed historical analysis chemical terrorist threat assessment was performed in general. Obtained results should impact community, governmental and world awareness of chemical terrorism imminence.

**Methods and Materials:**

In order to achieve the set goal historical and descriptive methods were performed in historical documents analyses. Acquired results were quantified and frame for chemical terrorist threat assessment was defined thus assessing the reality and level of chemical terrorism.

**Results and Discussions:**

Throughout the history harmful properties of the chemicals were used to inflict death, disease and incapability among adversaries. Even before written documents to become a source of information about chemicals usage the primitive tribes were used to take advantage of known natural poisons, poisoning their arrows, lances, swords and axes. In the preserved legends a lot of heroes had died by poisonous arms for example the death of demi-god Hercules.

First written evidence of chemicals implementation in order to defeat the adversaries’ moral and physical strength is given by the Greek historians describing the siege of Cirrha. Around 590 B.C. Solon of Athens poisoned the Pleistrus River with roots of hellebore to cause mass poisoning and psychological weakness among defenders ranks.

[1]

Chinese manuscripts dating back from 4th century B.C. provide us with information about widely implemented use of toxic fumes during the siege of fortresses. Mohist sect used ox-hide bellows to pump smoke from furnaces in which balls of mustard and other toxic vegetable matter were being burnt into tunnels being dug by a besieging army to discourage the diggers. Toxic cacodyl (arsenic trioxide) smoke was also mentioned in early Chinese war manuals.

Spartans used the toxic smoke generated by burning wood dipped in a mixture of tar and sulfur during its periodic wars with Athens, chemicals were applied in most of the ancient wars as well. All the historical data proves that the ancient Chinese were the masters of chemical warfare in the ancient times. The chemical weapons development could be originated from the fumigation of dwellings to eliminate fleas – practice by the Chinese 700 years before Christ. Chinese writings contain literally hundreds of recipes for the production of poisonous or irritating smokes for use in war, and many accounts of their use. Descriptions of weapons may be found in the artillery manuals of the Chinese army. However in the west, the use of toxic materials was generally
viewed with mixed emotions and some disdain (especially when your enemies were doing it). In contrast to China, where the study of chemicals including their military uses was widespread, in Europe the use of chemicals in battle (with the exception of incendiaries of various sorts and, of course, the use of wet vegetable matter to generate smoke screens) was usually the result of an independent local initiative, rather than the result of a logical building on prior experience. As a reaction to the poisoning of wells by their enemies the Romans announced a declaration “ARMIS BELLA NON VENENIS GERI – War is fought with weapons, not with poisons”. In western countries there was also a strong tendency to keep useful knowledge, especially militarily knowledge, from wide dissemination. While there are many reports of the isolated use of chemical agents in individual battles or sieges, there was no general tradition of their use (again, with the exception of incendiaries and smoke). As a result, the use of chemical agents in conflicts in Europe was fairly limited through the end of the Renaissance. There was, however, considerable attention paid to poisons. And in France, the use of "inheritance powders" would be widespread until the Poison Affair brought scandal to the court of King Louis XIV and the formation of the burning court (chambre ardente) forced, if not an elimination of poisoning, at least a greater degree of circumspection in its practice. But, for the most part, chemical poisons were employed against individuals, usually for assassination, but sometimes in battle as when poisoned arrows were used, and not in a manner calculated to produce mass casualties in groups. However, accusations of attempts at mass poisonings, especially when seen as useful to make for political ends or during times of stress (as when Jewish communities bore the brunt of attacks for "poisoning" during the plague years) were commonly heard.

The Middle Ages have also seen the use of poisonous substances. Poisons have very often been used for criminal poisoning of people. In the struggle for power and heritance individuals or groups of people were fighting among themselves by means of various poisonous agents. But, poisons were used for war purposes. There were examples of the use of chemical agents in the Middle Ages and Renaissance periods:

Barrels of blinding quicklime are catapulted by the English fleet on French vessels (middle of the 13th century).

Bombs, grenades and rags containing arsenic are fired by the defenders of Belgrade against the Turks in 1456.

‘Stinking Jars’ and toxic bombs are used in great quantities during The Thirty Years’ War (1618-1648).

During the American Civil War (1861-1865), Patrick Gilmora, the Northern general, used incendiary and chemical ammunition against the Confederate units of Pierre de Beauregard who calls it "the most destructive ammunition used in a war". Napoleon III used hydrogen in 1865 for military purposes. Also, during the Crimean War a sulphuric smoke was used against the Russian garrison in Sevastopol. During the Boer War in 1900 explosive shells filled with a poisonous gas were used. The aforementioned cases of the use of chemical agents cannot be considered a usual form of warfare, but accidental and periodical events.

World War I has really marked the coming on the scene and the full use of chemical weapons. In no war prior to and after World War I had such quantity of chemical agents been used nor there were so many victims due to their use. Chemical weapons were used by both belligerent parties, Germany and the Allies. The first
significant use of chemical agents took place on 22 April, 1915 at the battle near Ypres in Belgium. On that occasion Germany used bottles filled with chlorine against the Allied forces for the purpose of breaching the Allied front to the length of 6 kilometres. As a consequence of that attack there were 15,000 wounded soldiers on the Allied forces side out of which 5,000 were killed. By war’s end, both sides had used massive quantities of chemical weapons, causing an estimated 1,300,000 casualties, including 91,000 fatalities. The Russian army suffered about 500,000 of these casualties, and the British had 180,000 wounded or killed by chemical arms. One-third of all U.S. casualties in World War I were from mustard and other chemical gases, roughly the ratio for all participants combined. By the war’s end, all the great powers involved had developed not only offensive chemical arms but also crude gas masks and protective overgarments to defend themselves against chemical weapon attacks. Altogether, the warring states employed more than two dozen different chemical agents during World War I, including mustard gas, which caused perhaps as many as 90 percent of all chemical casualties (though very few of these casualties were fatal) from that conflict. Other choking gas agents used included chlorine, phosgene, diphosgene, and chloropicrin. The blood agents included hydrogen cyanide, cyanogen, chlorine, and cyanogen bromide. Arsenic-laced sneeze agents were also used, as were tear gases like ethyl bromoacetate, bromoacetone, and bromobenzyl cyanide.

The horrific casualties of World War I helped persuade many world leaders of the need to ban the use of chemical weapons. A number of proposals were made during the 1920s, and at the 1925 Geneva Conference for the Supervision of the International Traffic in Arms a protocol was approved and signed by most of the world’s states. The 1925 Geneva Protocol made it illegal to employ chemical or biological weapons, though the ban extended only to those who signed the treaty.

Despite the popular reaction against this form of warfare and the international agreement banning the use of chemical weapons, chemical arms were used a number of times in the years between the two World Wars. For example, chemical weapons were employed by British forces in the Russian Civil War (1919), Spanish forces in Morocco (1923–26), Italian forces in Libya (1930), Soviet troops in Xinjiang (1934), and Italian forces in Ethiopia (1935–40). During the Chinese-Japanese War (1937–45), Japanese forces employed riot-control agents, phosgene, hydrogen cyanide, lewisite, and mustard agents extensively against Chinese targets. There is no record of chemical warfare among World War II belligerents other than that of the Japanese. The Axis forces in Europe and the Allied forces adopted no-first-use policies, though each side was ready to respond in kind if the other acted first. Indeed, all the major powers developed extensive chemical warfare capabilities as a deterrent to their use.

After World War II, chemical weapons were employed on a number of occasions. Egyptian military forces, participating in Yemen’s civil war between royalists and republicans, used chemical weapons, such as nerve and mustard agents, in 1963, 1965, and 1967. During the Soviet intervention into the Afghan War (1978–92), chemical arms, such as mustard and incapacitating agents, were used against the mujahideen rebels. In 1987 Libya used mustard munitions against rebels in Chad. The most extensive post-World War II use of chemical weapons occurred during the Iran-Iraq War (1980–88), in which Iraq used the nerve agents sarin and tabun, as well as riot-control agents and blister agents like sulfur mustard, resulting in tens of thousands of Iranian casualties. Chemical weapons enabled Iraq to avoid defeat, though not obtain
victory, against the more numerous Iranian forces. In response to Iraq’s use of chemical weapons, Iran made efforts to develop chemical weapons and may have used them against Iraq, a contention that Iran has denied. Furthermore, Iran claims to have ended its program when it signed (1993) and ratified (1997) the CWC. Iraq also used chemical weapons (thought to be hydrogen cyanide, sarin, or sulfur mustard gas) against Iraqi Kurds who were considered unfriendly to the regime of Saddam Hussein. The most notorious such attack was the killing of 5,000 Kurds, including many civilians, in the city of Halabjah in 1988.

Mentioned above and other examples of chemical substances usage and compounds led terrorist awareness that chemical agents are suitable for fulfillment their goals. The reasons for increased potential use can be grouped into four major categories: the growth of militant groups with political agendas as a percentage of all terrorist groups, the increasing global availability of chemical weapon information and stockpiles, the internationalization of the threat of terrorism, and the clear evidence of terrorist interest and capabilities.

First, there has been a sharp increase in militant religious groups with political aims as a percentage of all terrorist groups. Over the last years of the twentieth century, such groups went from being just over three percent of all identified terrorist groups in 1980 to forty-three percent by 1995. These terrorists may label their victims as heretics or infidels and thus unfit to live. The incentives for such groups to kill large numbers of people may thus be unconstrained by the scruples of earthly constituencies. In combination with this worrisome development, the lethality per terrorist attack went up over the course of the past decade. While there were fewer attacks overall in the 1990s, the number of people killed and maimed per attack increased. This confirmed the fear of many experts that terrorism based on extreme religious beliefs, in association with other developments discussed below, might be even more dangerous than were the left wing, right wing, and ethnonationalist/separatist groups that predominated in earlier years. A larger proportion of the attacks that did occur were executed by persons with religion-based animus.

Second, there is a growing concern about the increasing availability of information and resources for the building of chemical weapons by subnational groups that in former years had been feasible only with the resources of a state. Like the rest of the world, terrorist groups have access to the vast amount of technical data disseminated through the Internet. More and more information that might previously have been difficult to collect is becoming easily accessible. Many groups have reportedly demonstrated interest in acquiring unconventional weapons. The combination of greater movement of people, knowledge and products across borders in a globalized world, and greater availability of materials and expertise in the post-Soviet era, have together led to a potentially serious erosion in state control over chemical weapons (or their ingredients).

Third, the nature of international terrorism has evolved in dangerous ways in recent years. Although many traditional groups carry on in their struggles, the growth of religiously-oriented groups has led to an increased commonality of interests between populations in disparate geographical areas. This internationalization of the threat has often led to a greater distance between groups and targets. The result is not only a removal of moral constraints but also political constraints, with less worry about potentially sullying a homeland or killing potential constituents. Thus, the
internationalization of terrorism may unfortunately imply an increase in just the sorts of incentives that lead groups to consider unconventional weapons.

Fourth, and perhaps most important, there are clear indications of interest in chemical weapons on the part of contemporary terrorist groups, as well as some evidence of actual capabilities. With a long-standing expressed desire to acquire chemical weapons, and a demonstrated willingness to use chemical agents terrorist group cause worrying among international community.

The trilling example for chemical weapon usage by terrorist group is the Tokyo subway attack of 1995. On the morning of 20 March 1995, several members of the Aum Shinrikyo released sarin nerve agent on five different subway trains in Tokyo by puncturing plastic bags containing the agent. The action resulted in twelve deaths, 1,039 injuries, and approximately 4,460 individuals who reported symptoms at local hospitals. It was the first case in which a private group successfully launched a terrorist attack with chemical weapons on a large scale. It raised serious national security questions not only in Japan, but throughout the world. If a little-known, relatively small group such as Aum could secretly manufacture and deploy chemical weapons that could kill thousands of people, then other groups certainly had the potential. Another example for relatively easy production of chemical weapon is the improvised bomb with pesticides used by Hamas in 1997. These events indicate increasing terrorist group’s interest and capabilities to implement large scale chemical attack.

Rapid development of chemical industry with widespread chemical facilities and warehouses are tempting terrorists to use chemical available as weapons. The high level probability of chemical terrorism has to be addressed by national and international imposed restrictions and enhanced medical and rescue preparedness.

All above-mentioned historical evidence was listed in order to provide the required basis for chemical terrorism threat assessment.

Threat is the probability that a specific target is attacked in a specific way during a specific period of time. Thus the threat could be define as function of the intent and capabilities of performing an attack on specific target. In general the specific terrorist target is always the community for creating fear and terror among the society with consequent social unrest. [2] Assessing general chemical terrorism threat could be stated that regardless the great variety chemical substances and compounds used as terrorists’ weapons, the specific method coincides, namely chemicals application in order to cause sickness and death in a large number of victims with set aim to create fear, panic, and paralyzing uncertainty, resulting in disruption of social and economic activity, the breakdown of government authority, and the impairment of military responses. This is the reason while assessing chemical terrorism threat in general to evaluate terrorists’ intentions and capabilities of performing such a deed, only. Analyzed historical data provides with a numerous evidence of terrorists’ intention and willingness to use available chemicals as a weapons. Terrorists’ chemical threat assessment is required for planning medical means and capabilities for the chemical terrorist act consequences management. [3, 4, 5]

The threat assessment is function of possibility and consequences severity. These indicators are measured as in Table 1 and Table 2:
### Table 1

<table>
<thead>
<tr>
<th>POSSIBILITY SCORE - PS</th>
<th>INDICATORS</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>1. Threat existence</td>
</tr>
<tr>
<td></td>
<td>2. Capabilities available</td>
</tr>
<tr>
<td></td>
<td>3. Intention Declared/ Indicators Available</td>
</tr>
<tr>
<td></td>
<td>4. Vulnerable objects is set as a target</td>
</tr>
<tr>
<td>3</td>
<td>1. Threat existence</td>
</tr>
<tr>
<td></td>
<td>2. Capabilities available</td>
</tr>
<tr>
<td></td>
<td>3. Intention Declared/ Indicators Available</td>
</tr>
<tr>
<td></td>
<td>4. No indicators that the object is set as a target</td>
</tr>
<tr>
<td>2</td>
<td>1. Threat existence</td>
</tr>
<tr>
<td></td>
<td>2. Capabilities available</td>
</tr>
<tr>
<td></td>
<td>3. No Intention Declared/ Indicators Available</td>
</tr>
<tr>
<td></td>
<td>4. No indicators that the object is set as a target</td>
</tr>
<tr>
<td>1</td>
<td>1. Threat existence</td>
</tr>
<tr>
<td></td>
<td>2. No Capabilities available</td>
</tr>
<tr>
<td></td>
<td>3. No Intention Declared/ Indicators Available</td>
</tr>
<tr>
<td></td>
<td>4. No indicators that the object is set as a target</td>
</tr>
</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th>SEVERITY SCORE - SS</th>
<th>INDICATORS</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Potential for: Population, military and emergency teams casualties (dead and wounded)</td>
</tr>
<tr>
<td></td>
<td>Infrastructure, Buildings’ Severe damage and Great Material loss</td>
</tr>
<tr>
<td></td>
<td>Environmental damage and serious economic impact (country scale)</td>
</tr>
<tr>
<td></td>
<td>Evacuation required</td>
</tr>
<tr>
<td>3</td>
<td>Potential for: Population, military and emergency teams casualties (wounded are prevailing)</td>
</tr>
<tr>
<td></td>
<td>Partial Infrastructure, Buildings’ damage and Moderate Material loss</td>
</tr>
<tr>
<td></td>
<td>Local impact on environment</td>
</tr>
<tr>
<td></td>
<td>Restriction in the area</td>
</tr>
<tr>
<td></td>
<td>Moderate economic impact</td>
</tr>
<tr>
<td>2</td>
<td>Potential for: Light wounding</td>
</tr>
<tr>
<td></td>
<td>Slight damage on infrastructure</td>
</tr>
<tr>
<td></td>
<td>Local restrictions</td>
</tr>
<tr>
<td></td>
<td>No environmental and economic impact</td>
</tr>
<tr>
<td>1</td>
<td>Few casualties</td>
</tr>
<tr>
<td></td>
<td>Slight surroundings damage</td>
</tr>
</tbody>
</table>
Overall chemical terrorism threat is assessed as high because of:

1. Historical evidence for terrorist capability to obtain and use chemical weapons
2. Recently declared intention and willingness to produced, purchase and use cheap chemical weapons
3. Extremely high consequences severity for affected human combined with enormous and immeasurable psychological affect and social disturbance

Conclusion

Rapid development of chemical industry with widespread chemical facilities and warehouses are tempting terrorists to use chemical available as weapons. The high level probability of chemical terrorism has to be addressed by national and international imposed restrictions and enhanced medical and rescue preparedness.

References

Chapter 12

Structure, Chemical Reactivity and Toxicity of Organophosphorus Compounds

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Abstract - Different scenarios of terrorist attacks with organophosphorus chemical agents and their impact on humans and environment are discussed. The chemical reactivity and biological activity of organophosphorus compounds are explained. Physicochemical and biological processes in the human organism after intoxication, inhibition of the cholinesterase and effective methods and means for its reactivation are described. Methods and devices for decontamination of contaminated objects are presented.

Keywords - organophosphorus compounds, toxicity, biological activity, decontamination

Introduction

Constructive negotiations for prohibition and destruction of nuclear, chemical and biological weapons had been carried out for decades. The signed and ratified conventions and agreements unequivocally show that the mankind approaches the long-awaited dream for total prohibition and liquidation of the gained stockpiles of these weapons. Despite of this, the events during the past years (nuclear power plants accidents, terrorist acts, hidden production of chemical and biological weapons) show that the possibility of use of NBC agent from terrorist organizations still exists. This imposes the need for study of the effects of NBC agents, the appropriate protection devices and the methods and means for their decontamination and removal from contaminated sites.

The chemical weapons have numerous advantages. First of all is the ease of their acquiring. These chemicals could be produce in existing chemical plants. The chemical agents are spread easily and could contaminate large areas. Their detection requires special devices and equipment \cite{1,2}.

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The terrorists expect severe injuries from the use of chemical agents also relying on the shock factor.

Main requirements for high efficiency in use of chemical agents are high toxicity, relative stability in water, air or from light, to have physical and chemical properties which allow forming of aerosols or vapors in the ground layers of the atmosphere.

The phosphorus-containing compounds for a long time were not considered from the military chemists as a potential for further researches. It was considered that the pentavalent phosphorus derivatives could not be highly toxic and suitable for use as chemical weapons.

The first results, served as a basis for further development in the area of organophosphorus compounds was published in 1932 from Lange and Kruger [2]. For the first time the scientists synthesized dimethyl fluorophosphates and diethyl fluorophosphates.

\[
\begin{align*}
\text{H}_3\text{C}-\text{O}-\text{P}-\text{O}-\text{F} \\
\text{H}_3\text{C}-\text{O}-\text{P}-\text{O} = \text{H}_5\text{C}_2
\end{align*}
\]

They noticed that these compounds have strong odor and after inhaling for a few minutes cause difficulties in breathing, lose of consciousness and painful sensitivity to sunlight.

After the first publication from Lange and Kruger, a large-scale research work had started in many countries for synthesis of high toxic substances within the group of pentavalent phosphorus compounds. The researches were made in secret manner and no publications were made before 1946.

After the end of the Second World War it was discovered that during the war Germany organized production of new toxic compounds coded as tabun, sarin and soman.

When the studies on the synthesis of organophosphorus compounds of the German scientists became available after the war, in USA was paid special attention to the production of these compounds using German methods as well as development of new more toxic compounds from this group.

In this way at the end of the 50s were synthesized and the production of new organophosphorus compounds began which were named as V-gases.

The organophosphorus compounds are highly toxic substances with effect on the nervous system. All toxic organophosphorus compounds belong to the group of phosphoric and phosphonic acid and could be described with the following formula:

\[
\begin{align*}
A & - \text{R, RO, R}_2\text{N, RS, etc}; \\
B & - \text{R'O, ArO, R}_2\text{N, RS, etc}; \\
X & - \text{Cl, F, CN, RCOO, etc.}
\end{align*}
\]

The study of the toxicity of these compounds according to their structure show that increased physiological activity have only those derivatives of the pentavalent phos-
phorus, which are capable for phosphorylation in mild conditions (close to the conditions in the living organisms).

The main mechanism of the reactions of phosphorylation could be presented on the scheme below:

\[
R-O: \quad + \quad \begin{array}{c}
\text{O} \\
\text{H} \\
\text{R}
\end{array}
\begin{array}{c}
\text{P} \\
\text{A} \\
\text{X} \\
\text{B}
\end{array}
\quad \rightarrow \quad \begin{array}{c}
\text{R} \\
\text{O} \\
\text{P}^+ \\
\text{A} \\
\text{H} \\
\text{X} \\
\text{B}
\end{array}
\begin{array}{c}
\text{O} \\
\text{R}
\end{array}
\quad + \quad \begin{array}{c}
\text{R} \\
\text{O} \\
\text{P} \\
\text{A} \\
\text{B}
\end{array}
\quad + \quad \text{HX}
\]

It is obvious that the velocity of the reaction of phosphorylation will depend on the partial positive potential of the phosphorus atom. The value of the partial positive potential depends not only on the degree of electronegativity of the halogen substituent, but also on all other substituents, bonded to the central phosphorus atom.

In case of high positive potential of the phosphorus atom, the organophosphorus compounds will become so reactive, that they will be capable to phosphorylate all the compounds they have contact with and will degrade rapidly in water and in other media [1,2]. Such reactive phosphorus containing compounds will lose their selective capabilities towards important ferments and after exposure will hydrolyze in reactions with numerous compounds thus unable to reach the important centrum. Taking this into account for high toxicity it is important to select compounds with optimal capability for phosphorylation, sufficient for interaction with specific ferments like cholinesterase (ChE), but insufficient to phosphorylate other ferments or water.

The change in electron density of the phosphorus atom is not the only method for increase of toxicity of the organophosphorus compounds. Important role also has the presence of defined active centers in the structure of the substituents bonded to the phosphorus atom, as example phosphorylthioholines, which have also anion center in the molecule.

\[
\begin{array}{c}
\text{R}_1 \\
\text{O} \\
\text{P} \\
\text{O} \\
\text{S} \\
\text{N} \\
\text{R}_2 \\
\text{R}_3
\end{array}
\]

Within the compounds which meet most of the requirements for high toxicity are the organophosphorus compounds sarin, soman and VX. Their high toxicity is a result of the specific and selective activity towards limited number of ferments, with high importance of cholinesterase in it.

The main purpose of the cholinesterase is to degrade the enzyme acetylcholine, which acts as a transporter of impulses in the vegetative nervous system. After transmitting of
impulse, the cholinesterase hydrolyses acetylcholine to choline and acetic acid according to the following scheme:

\[
\text{CH}_3\text{COOCH}_2\text{CH}_2\text{N}^+\text{(CH}_3)_2\text{X}^- + \text{H}_2\text{O} \xrightarrow{\text{cholinesterase}} \text{CH}_3\text{COOH} + \text{HOCH}_2\text{CH}_2\text{N}^+\text{(CH}_3)_2\text{X}^-
\]

The process is catalytically conducted and the velocity is colossal – one molecule of cholinesterase could degrade 300000 molecules acetylcholine for a minute. To describe this phenomenon is required to observe the structure of the cholinesterase. It is a protein with large molecular weight, consisting of large number of different amino-acids. It is proved experimentally that the cholinesterase has two sites: esteratic, containing highly-reactive nucleophilic group (e.g. OH) and anion site with increased electron density.

The fluoroanhydride of the isopropyl ester of methylphosphonic acid, sarin is capable for use yearly. The boiling point is 151°C and the melting point -53°C. It is highly volatile \( C^{20} = 13 \text{ mg/l} \). The lethal concentration of exposure is 0.1 mg/l for 1 min. According to its toxic activity, sarin is related to the compounds with nerve and miotic effect. It is a ferment poison, causing inactivation of cholinesterase with severe injury of the nervous system and the whole body [2].
The toxic effect of sarin occurs after intoxication from every route of exposure and causes injuries in vapor and liquid form. Little concentration of sarin causes miosis (Fig. 1) and chest pain.

Fig. 1. Miotic effect of sarin

The lethal dose of sarin in skin exposure is 7-9 mg/kg. Sarin could contaminate large surface areas for continuous time and his high volatility provides spread of vapors to long distances (10-12 km) [4]. The liquidation of consequences after contamination is difficult and hard to solve task (Fig. 2).

Fig. 2. Liquidation of consequences after the sarin attack in Tokyo subway – March 1995
Sarin is relatively stable hydrolytically and could be used for continuous contamination of water resources. The hydrolysis of sarin is carried out with forming of non-toxic products according to the following scheme:

\[
\text{H}_3\text{C}_3\text{O}\text{F} + \text{HOH} \rightarrow \text{H}_3\text{C}_3\text{O}\text{OH} + \text{HF}
\]

The rate of hydrolysis is dependent on the temperature and the alkalinity of the media. The bases increase significantly hydrolysis of sarin to salts of isopropyl ester of methylphosphonic acid and fluorides:

\[
\text{iC}_3\text{H}_7\text{O} - \text{PO}^+\text{O} + 2\text{NaOH} \rightarrow \text{iC}_3\text{H}_7\text{O} - \text{PO}^+\text{ONa} + \text{NaF} + \text{H}_2\text{O}
\]

The decontamination of contaminated with sarin human skin and equipment is carried out with nucleophilic reagents like amino-alcoholic mixtures according to the following reaction:

\[
\text{C}_2\text{H}_5\text{OCH}_2\text{CH}_2\text{ONa} + \text{P} \rightarrow \text{C}_2\text{H}_5\text{OCH}_2\text{CH}_2\text{ONa} + \text{HF}
\]

Garment, contaminated with vapors of sarin is treated with hemisorption sorbent packages. The reaction follows the mechanism described bellow:

\[
\text{Phosphorylthioholines (V-agents) have higher toxicity than sarin. It is explained with the presence dialkylamine esters with anion center, which enhances the more rapid and stable block of the cholinesterase [2].}
\]
As example, the lethal dose of skin exposure for VX is 0.005 mg, while for sarin is 0.5 mg. The main state of V-agents for military use is aerosol due to the insignificant volatility and high boiling point (around 280°C). VX could be with the following systems of delivery: projectiles, rockets, bombs, containers, etc. In practice the aim contamination is to reach concentration of aerosols capable to cause lethal effect, even after one breath. The presence of two centers in the molecule of V-agents predetermines also the ability for forming of unfavorable processes of polymerization:

This causes increase of the viscosity and decrease of toxicity of the compounds. V-agents have high hydrolytic stability and are capable to contaminate water resources for continuous time (in winter for months). Alkaline solutions could be successfully applied for decontamination in combination with high temperatures. To improve the oxidation potential, additives for decrease of pH to 8.5-9 are used with aqueous hypochlorite mixtures [3]. The reaction is carried out with formation of non-toxic products:
This level of pH also ensures rapid decontamination of the toxic compounds like soman, due to the activity of the hypochlorite ion (OCl\(^{-}\)), which makes the mixture multipurpose.

The aqueous hypochlorite mixtures with decreased pH of the media (8.5-9) have improved decontamination efficacy related to the chemical warfare agents and are applied as universal products for decontamination of armament and equipment. Their high potential for oxidation provides effective decontamination of compounds like V-agents and the catalytic properties of hypochlorite ion ensure rapid degradation of toxic agents like sarin and soman. The decontamination substance used in the station for consequence management Sanijet (Fig. 3) is based on Trichloroisocyanuric acid. The presence of catalytic additives and the high temperature of treatment provide more effective and rapid degradation of the toxic compounds to non-toxic products. Highly effective is also the decontamination of garment using vapors of ammonia at temperature around 100\(^{\circ}\)C. The formed products as a result of the reaction are non-toxic [3].
The adequate treatment of the contaminated objectives is a guarantee for rapid removal and decontamination of NBC agents and decrease to minimum of their hazardous effects.

References

4. Зимон А.Д., Дезактивация, Москва, 1976 г.
5. Stanag 2352 NBC (издание 4) – снаряжение за ядрена, химическа и биологична защита
Chapter 13

Biological Markers of Intoxication with Nerve Agents - Biochemical investigations in rats poisoned with tabun

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Military Medical Academy, Sofia, 1606, Bulgaria

Abstract - Nerve agents (OPC) could be used in terrorist acts. Investigation of longer-lived biomarkers for verification of exposure to toxic compounds is important for diagnosis and prognosis of nerve intoxications. We study the effects of toxic agent on some biochemical parameters in rats blood, brain and liver. Three different doses of tabun - 1.0 LD50, 0.5 LD50 and 0.1 LD50 (s.c.) were estimated for toxic effects on the following biochemical parameters: erythrocyte, brain, liver acetylcholinesterase and serum butyrylcholinesterase, albumin, total protein, ASAT, ALAT, ALP, creatinine and β-glucoronidase. There was not antidote treatment after the challenge. The measurements were done 24 hr, 5 and 10 days after the poisoning. Intoxications with different doses of tabun proved that brain and erythrocyte AChE are most sensitive regardless of the dose used. Most of the biochemical parameters determined in this work – ASAT, ALAT, creatinin, total protein and albumin demonstrated a tendency to increase after tabun challenge. β-Glucoronidase activity increase in first day after intoxication.

Key words - biomarkers, diagnosis, intoxications, nerve agents.

Introduction

Organophosphorus compounds (OPC) are widely used like insecticides. They were developed also as warfare nerve agents. The recent use of sarin in the terrorist acts in Matsumoto city and Tokyo underground was removed any doubts about the possibility of using chemical weapons by terrorists. The main problem connected with the chemical terrorism is that in addition to chemical weapons, terrorists can use different toxic chemicals from chemical industry, agriculture or products released from terrorist acts on industrial facilities. Widely used methods to diagnose and biomonitor exposure to OPC, e.g., nerve agents are measurement of enzyme activity of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) in blood and analysis of the intact poison...
or its degradation products in blood and/or urine. Measurement of cholinesterase inhibition in blood does not identify the anticholinesterase and does not provide reliable evidence for exposure at inhibition levels less than 20%. The biochemical examination of human and experimental intoxications with different OPC depends on the severity of poisoning and the kind of the compound. The study of basic biochemical parameters after experimental intoxications with low doses of OPC in different periods after poisoning will be helpful for assess their specificity and sensitivity. In this study we use the nerve agent tabun.

1. Material and methods

**Animals.** Experiments were carried out on male albino “Wistar” rats (180-220 g) obtained from Bulgarian Academy of Science. Prior to the experiments they were housed with 6 animals per cage. Temperature was kept at 18-22 °C, humidity was maintained at 50-65% and 12 hr light-dark cycle was available. Rats were allowed to standard rodent food and tap water ad libitum.

**Nerve agents:** The native Soman (GD, 96%) and Tabun (GA, 86.7%) obtained from the stocks of the Laboratory of Military Toxicology, MMA, Sofia were used in the current study.

**Biochemical investigations.** Biochemical investigations were carried out one day, five days and ten days after soman and tabune poisoning. Intoxications were caused by three doses of OPC - 1.0 LD50, 0.5 LD50 and 0.1 LD 50, injected s.c. in a volume of 0.1 ml/100 g. body weight. For each dose of OPC used rats were divided into 3 groups (n=8 in group). Control group (10 rats) was treated with 0.9% NaCl at the same experimental conditions. Biochemical observations were made 24 hr, 5 and 10 days after the challenge by using Screen Master (Hospitex Diagnostics, Italia).

**Biochemical parameters.** The following biochemical parameters were determined: Aspartate Aminotransferase (ASAT, EC 2.6.1.1) (Kinetic method, U/L); Alanin Aminotransferase (ALAT, EC 2.6.1.2) (Kinetic method, U/L); L-g-Glutamiltransferase g-GT (GGTP, EC 2.3.2.2.) (Kinetic method, U/L); Alkaline Phosphatase (ALP, EC 3.1.3.1) (Kinetic method, U/L); Butyryl Cholinesterase (ChE, EC 3.1.1.8.) (Kinetic method, U/L); Acetylcholinesterase (AChE, EC 3.1.1.7.) in erythrocytes, brain and liver (Ellman method, mkmol/ml.min); Total Protein (Biuret method), Albumin (Colorimetric test, g/l) and β-Glucoronidase (Fishman’s method, Colorimetric, U/L).

**Data analysis:** Statistical significance was determined by using Student’s t-test and differences were considered significant when p<0.05. Statistical evaluation was determined with the relevant computer programs (Basic Statistics, Version 6).
2. Results and discussion

In our work we tried to investigate the toxic effects of different small doses of tabun on some biochemical parameters that are in routine used in laboratory practice, except brain and liver AChE, and are easy to obtain and determine. The most remarkable changes were found for erythrocyte and brain cholinesterase activity which are expectable and reflect the main mechanism of action of nerve agents. The obtained results are presented in Tables 1-3.

Tabl. 1. Changes of biochemical parameters estimated after poisoning with 1.0 LD50 of tabun in rats.

<table>
<thead>
<tr>
<th>Days</th>
<th>Parameters</th>
<th>1 day</th>
<th>5 day</th>
<th>10 day</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Erythrocyte AChE μmol/ml/min</td>
<td>2.773 ± 0.33 (***))</td>
<td>3.590 ± 0.23 (*)</td>
<td>3.994 ± 0.42</td>
<td>4.37 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>Brain AChE μmol/ml/min</td>
<td>0.036 ± 0.007 (***))</td>
<td>0.039 ± 0.011 (***))</td>
<td>0.082 ± 0.013</td>
<td>0.127 ± 0.031</td>
</tr>
<tr>
<td></td>
<td>Liver AChE μmol/ml/min</td>
<td>859.83 ± 129.7 (***))</td>
<td>975.8 ± 276.02</td>
<td>1148.02 ± 101.46</td>
<td>1148.0 ± 90.75</td>
</tr>
<tr>
<td></td>
<td>BuChE (U/l)</td>
<td>359.03 ± 51.38</td>
<td>364.06 ± 49.16</td>
<td>369.4 ± 83.02</td>
<td>403.9 ± 30.22</td>
</tr>
<tr>
<td></td>
<td>Total protein (g/l)</td>
<td>73.33 ± 6.153 (*)</td>
<td>63.40 ± 7.16</td>
<td>74.80 ± 8.75</td>
<td>65.0 ± 3.16</td>
</tr>
<tr>
<td></td>
<td>Albumin (g/l)</td>
<td>38.0 ± 2.52 (***))</td>
<td>32.0 ± 5.33</td>
<td>36.0 ± 3.67</td>
<td>32.3 ± 1.63</td>
</tr>
<tr>
<td></td>
<td>ALP (u/l)</td>
<td>763.46 ± 86.65 (*)</td>
<td>843.32 ± 124.14 (***))</td>
<td>2210 ± 394.37 (***))</td>
<td>568.6 ± 144.8</td>
</tr>
<tr>
<td></td>
<td>ASAT (u/l)</td>
<td>882.50 ± 201.95 (***))</td>
<td>475.80 ± 88.16</td>
<td>448.40 ± 105.3</td>
<td>444.4 ± 72.87</td>
</tr>
<tr>
<td></td>
<td>ALAT (u/l)</td>
<td>1084 ± 48.06</td>
<td>130.2 ± 95.73</td>
<td>93.2 ± 26.68</td>
<td>73.80 ± 13.7</td>
</tr>
<tr>
<td></td>
<td>Creatinine (mg/dl)</td>
<td>0.463 ± 0.039 (*)</td>
<td>0.461 ± 0.049</td>
<td>0.512 ± 0.011</td>
<td>0.517 ± 0.047</td>
</tr>
<tr>
<td></td>
<td>β-glucoronidase</td>
<td>2.567 ± 0.55 (***))</td>
<td>1.795 ± 0.51</td>
<td>1.389 ± 0.51</td>
<td>1.638 ± 0.27</td>
</tr>
</tbody>
</table>

In comparison to the control group: * - P < 0.05; ** - P < 0.01; *** - P < 0.001.
Tabl. 2. Changes of biochemical parameters estimated after poisoning with 0.5 LD50 of tabun in rats.

<table>
<thead>
<tr>
<th>Days</th>
<th>Parameters</th>
<th>1 day</th>
<th>5 day</th>
<th>10 day</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Erythrocyte AChE (μmol/ml/min)</td>
<td>2.804 ± 0.36 (***&lt;br&gt;0.36)</td>
<td>4.330 ± 0.83 (0.36)</td>
<td>3.930 ± 0.88 (0.36)</td>
<td>4.37 ± 0.7 (0.36)</td>
</tr>
<tr>
<td></td>
<td>Brain AChE (μmol/ml/min)</td>
<td>0.037 ± 0.008 (**&lt;br&gt;0.008)</td>
<td>0.049 ± 0.012 (**&lt;br&gt;0.012)</td>
<td>0.006 ± 0.029 (0.006)</td>
<td>0.127 ± 0.031 (0.031)</td>
</tr>
<tr>
<td></td>
<td>Liver AChE (μmol/ml/min)</td>
<td>1189.0 ± 271.8 (0.0)</td>
<td>963.57 ± 136.6 (0.0)</td>
<td>663.68 ± 131.5 (0.0)</td>
<td>1148.0 ± 90.75 (0.0)</td>
</tr>
<tr>
<td></td>
<td>BuChE (U/l)</td>
<td>338.98 ± 30.22 (**&lt;br&gt;30.22)</td>
<td>329.27 ± 41.23 (0.0)</td>
<td>327.70 ± 59.24 (0.0)</td>
<td>403.9 ± 30.22 (0.0)</td>
</tr>
<tr>
<td></td>
<td>Total protein (g/l)</td>
<td>67.25 ± 4.06 (0.0)</td>
<td>70.37 ± 7.80 (0.0)</td>
<td>81.0 ± 5.90 (0.0)</td>
<td>65.0 ± 3.16 (0.0)</td>
</tr>
<tr>
<td></td>
<td>Albumin (g/l)</td>
<td>37.25 ± 1.63 (**&lt;br&gt;1.63)</td>
<td>39.63 ± 1.28 (0.0)</td>
<td>41.13 ± 4.05 (**&lt;br&gt;4.05)</td>
<td>32.3 ± 1.63 (1.63)</td>
</tr>
<tr>
<td></td>
<td>ALP (u/l)</td>
<td>665.70 ± 78.70 (0.0)</td>
<td>621.80 ± 253.50 (0.0)</td>
<td>488.9 ± 103.30 (0.0)</td>
<td>568.6 ± 144.8 (0.0)</td>
</tr>
<tr>
<td></td>
<td>ASAT (u/l)</td>
<td>526.40 ± 72.80 (0.0)</td>
<td>489.80 ± 135.0 (0.0)</td>
<td>508.90 ± 54.20 (0.0)</td>
<td>444.4 ± 72.87 (0.0)</td>
</tr>
<tr>
<td></td>
<td>ALAT (u/l)</td>
<td>85.70 ± 24.60 (0.0)</td>
<td>86.50 ± 24.30 (0.0)</td>
<td>64.50 ± 11.90 (0.0)</td>
<td>73.80 ± 13.7 (0.0)</td>
</tr>
<tr>
<td></td>
<td>Creatinine (mg/dl)</td>
<td>0.588 ± 0.060 (0.0)</td>
<td>0.528 ± 0.06 (0.0)</td>
<td>0.588 ± 0.07 (0.0)</td>
<td>0.517 ± 0.047 (0.0)</td>
</tr>
</tbody>
</table>

In comparison to the control group: * - P < 0.05; ** - P < 0.01; *** - P < 0.001

Tabl. 3. Changes of biochemical parameters estimated after poisoning with 0.1 LD50 of tabun in rats.

<table>
<thead>
<tr>
<th>Days</th>
<th>Parameters</th>
<th>1 day</th>
<th>5 day</th>
<th>10 day</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Erythrocyte AChE (μmol/ml/min)</td>
<td>3.167 ± 0.74 (**&lt;br&gt;0.74)</td>
<td>3.245 ± 0.44 (0.44)</td>
<td>4.448 ± 0.75 (0.75)</td>
<td>4.37 ± 0.7 (0.7)</td>
</tr>
<tr>
<td></td>
<td>Brain AChE (μmol/ml/min)</td>
<td>0.029 ± 0.005 (**&lt;br&gt;0.005)</td>
<td>0.050 ± 0.006 (0.006)</td>
<td>0.093 ± 0.030 (0.030)</td>
<td>0.127 ± 0.031 (0.031)</td>
</tr>
<tr>
<td></td>
<td>Liver AChE (μmol/ml/min)</td>
<td>861.0 ± 219.20 (0.0)</td>
<td>753.4 ± 148.50 (**&lt;br&gt;148.50)</td>
<td>1250.5 ± 481.0 (**&lt;br&gt;481.0)</td>
<td>1148.0 ± 90.75 (90.75)</td>
</tr>
<tr>
<td></td>
<td>BuChE (U/l)</td>
<td>361.62 ± 34.53 (**&lt;br&gt;34.53)</td>
<td>308.16 ± 36.36 (**&lt;br&gt;36.36)</td>
<td>375.38 ± 41.58 (**&lt;br&gt;41.58)</td>
<td>403.9 ± 30.22 (30.22)</td>
</tr>
<tr>
<td></td>
<td>Total protein (g/l)</td>
<td>68.40 ± 7.13 (0.0)</td>
<td>79.90 ± 4.90 (0.0)</td>
<td>78.0 ± 4.80 (0.0)</td>
<td>65.0 ± 3.16 (0.0)</td>
</tr>
<tr>
<td></td>
<td>Albumin (g/l)</td>
<td>38.14 ± 5.10 (0.0)</td>
<td>40.25 ± 4.55 (0.0)</td>
<td>41.28 ± 6.12 (0.0)</td>
<td>32.30 ± 1.63 (1.63)</td>
</tr>
<tr>
<td></td>
<td>ALP (u/l)</td>
<td>909.70 ± 237.80 (0.0)</td>
<td>404.90 ± 95.50 (**&lt;br&gt;95.50)</td>
<td>631.50 ± 113.80 (113.80)</td>
<td>568.6 ± 144.80 (144.80)</td>
</tr>
<tr>
<td></td>
<td>ASAT (u/l)</td>
<td>543.20 ± 85.70 (0.0)</td>
<td>553.90 ± 108.20 (0.0)</td>
<td>531.70 ± 109.70 (0.0)</td>
<td>444.4 ± 72.87 (72.87)</td>
</tr>
<tr>
<td></td>
<td>ALAT (u/l)</td>
<td>85.40 ± 14.80 (0.0)</td>
<td>65.50 ± 11.30 (0.0)</td>
<td>109.96 ± 3.65 (0.0)</td>
<td>73.80 ± 13.7 (13.7)</td>
</tr>
<tr>
<td></td>
<td>Creatinine (mg/dl)</td>
<td>0.647 ± 0.16 (0.0)</td>
<td>0.607 ± 0.083 (0.0)</td>
<td>0.569 ± 0.041 (0.0)</td>
<td>0.517 ± 0.047 (0.0)</td>
</tr>
</tbody>
</table>

In comparison to the control group: * - P < 0.05; ** - P < 0.01; *** - P < 0.001
The results obtained from estimation of brain acetylcholinesterase and erythrocyte AChE reviewed the most profound inhibition for the whole period of observation regardless of the dose of tabun used.

The results obtained after intoxication with different doses of tabun are very close to those described in our previous studies with soman (9). In accordance with the rate of inhibition the susceptibility of cholinesterase to tabun irrespective of the dose used are arranged as follow: brain AChE > erythrocyte AChE > butyrylcholinesterase> liver AChE. The evidence supporting differences between inhibition of AChE and ChE from OPC has been summarised by many authors. Some of them (1) suggest that AChE activity is more important for diagnosis and prognosis of this intoxications than ChE.

Total protein, albumin, ASAT, ALAT and ALP demonstrated tendency to increase as in some cases the differences in comparison to the control group are statistically significant. ALP showed changes particularly after intoxication with the highest dose of tabun.

The results mention above are in agreement with the data received in intoxications in humans and in experiments with OPC (1).

Creatinine showed tendency to decreased in first day after poisoning with the highest dose of tabun (Table 1) and increase after small doses (Table 2 and Table 3). This differ with our finding in experiments with soman where creatinine reacted with increase in all cases of intoxication with high extent of significant for the whole period of observation (9).

(From Samnaliev, Ivanov and Dishovsky [9]).
The increase of β-glucuronidase after intoxication with 1.0 LD50 of tabun was very demonstrative in first day (Fig.2). To the tenth day the level of the enzyme decrease. That differ from our results obtained with soman (Fig. 1) (9). The changes of the values of β-glucuronidase in this experiments were very demonstrative due to their nearly two- and threefold increase at different time points (1, 5 and 10 days) of determination after poisoning with 1.0 LD50 of soman (Fig.1). Some authors conclude that the increase of β-glucuronidase is extremely sensitive biomarker of acute organophosphorus insecticides exposure (2). The same results showed the investigations of severe OP poisoning (10, 4) and in chronic intoxications with OP pesticides (3) in humans. There exist a single clinical cases in which such evidence do not exist (8). Experiments in rats (7, 5) showed specific evaluation of plasma β-glucuronidase activity after treatment with highly toxic organophosphorus compounds.

Our studies showed that soman and tabun differ in his effect on β-glucuronidase. This data are important for the use of this enzyme like biomarker of intoxications with this nerve agent. Some investigation in vitro (6) pointed to existence of structure-activity relationship in OP induced β-glucuronidase release from rats hepatocytes.

3. Conclusions

Intoxications with different doses of soman and tabun in experiments proved that brain and erythrocyte AChE are most sensitive regardless of the dose used. The enzyme inhibition was still observed 10 days after the challenge. In clinical monitoring of intoxications with OPC, determination of erythrocyte AChE could be important marker of severity and differential diagnosis of intoxications. The common use of ChE investigation does not exclude the advantages of measuring of the erythrocyte AChE activity. The current studies showed that tabun applied at different single doses – lethal, sub-lethal and non-lethal is able to cause significant changes not only of
acetylcholinesterase but of some classical biochemical parameters (ASAT, ALAT, creatinine, total protein and albumin) for a period of 10 days after the challenge.

β-glucoronidase could be a sensitive biomarker of acute tabun exposure in first days after poisoning with small doses. There exist differences between soman and tabun on their effect on this biomarker.

Acknowledgements

This work was connected with the joint project “New biological markers for nerve agent exposure and antidote treatment of intoxications” with the TNO Defence Security and Safety (dr. Marcel van der Schans and dr. Herman PM van Helden) and was supported by the MoD in The Netherlands and the MoD of Bulgaria.

References

Chapter 14

Peculiarities of Treating Water Contaminated with Toxic Chemical Substances Using Cold Contact Plasma

Olexandr PIVOVAROV, Olexandr KRAVCHENKO, Ganna TISCHENKO and Valery KUBLANOVSKY

Ukrainian State University of Chemical Engineering, Dnipropetrovsk, UKRAINE

Abstract - Investigation of the process of cyanide compounds’ destruction under plasma action for a number of model solutions and solutions which can be used for arranging the acts of terrorism was carried out. Plasma action results in generation of radicals, ions, excited molecules of water, secondary electrons and other active particles, which contribute to oxidation-reduction reactions running in the solutions. Due to recombination of \( \text{HO} \) and \( \text{H}_2\text{O} \) radicals, highly reactive hydrogen peroxide, also taking part in the reactions, is formed. Investigation of the process of cyanide compounds’ destruction under plasma action for a number of model and technological solutions was carried out. Plasma-chemical sample preparation allows breaking-up of cyanide compounds to non-toxic forms, which may be used for purification of technological solutions and galvanic production wastewaters, posing hazard to people’s health in the event they are purposely used by terrorists.

Keywords - cyanide, potassium cyanide, sodium cyanide, calcium cyanide, destruction,

Introduction.

Cyanide is powerful and rapid-acting poison [1]. Hydrogen cyanide has been used in gas-chamber executions and as war gas. Exposure to large amounts of cyanide can be deadly. The history is well known how Aum Shinrikyo planted a hydrogen cyanide gas generation device in Tokyo subway [2]. Cyanide occurs most commonly as hydrogen cyanide in water although it can also occur as the cyanide ion, alkali and alkaline earth metal cyanides (potassium cyanide, sodium cyanide, calcium cyanide), relatively stable metalloccyanide complexes \([\text{Fe(CN)}_6]^{3-}\), moderately stable metalloccyanide complexes (complex nickel and copper cyanide), or easily decomposable metalloccyanide complexes (zinc cyanide \([\text{Zn(CN)}_2]\), cadmium cyanide \([\text{Cd(CN)}_2]\). Hydrogen cyanide and cyanide ion combined are commonly termed free cyanide. In process solutions of industrial enterprises, which are using these solutions in galvanic production or hydrometallurgy, this kinetics is distinguished by more complicated character, owing to various speed of
decomposition of free cyanide and cyanide-metal complexes. In water, hydrogen and cyanide ion exist in equilibrium with their relative concentrations primarily dependent on pH and temperature. The alkali metal cyanides are very soluble in water. As a result, they readily dissociate into their respective anions and cations when released into water. Unlike water-soluble alkali metal cyanide, insoluble metal cyanides such as are not expected to degrade to hydrogen cyanide. Hydrogen cyanide and cyanide ions in aqueous solution have been found to be very resistant to photolysis by natural sunlight, except under heterogeneous photocatalytic condition.

Plasma-chemical treatment of liquid media, where cyanide compounds are present, gives a possibility to destroy them to non-toxic forms, which method can be used for purification of process solutions and treatment of waste waters posing hazard to human health [3].

Experimental.

Experiments were conducted with the use of compact laboratory and experimental-industrial units including systems of power supply, vacuumization and thermostating of the reactor of discrete and continuous action. This is achieved by positioning two or more pairs of the unlike electrodes into a water layer of between 30 and 100 mm on the opposite sides of the “water-air” border at a distance of between 4 to 15 mm from such level accordingly, said electrodes being made of a material which does not have any catalytic effect on cyanide solution, exposing the water to cold plasma processes, with voltage at each pair of electrodes being 500 - 1500 V, the temperature being below the natural boiling point of water and pressure being between 1.5·10⁴ - 5·10⁴ Pa.

Further investigations proved an assumption stated before, concerning destruction of cyanide complexes and cyanide ions under the action of non-equilibrium plasma [5]. A number of experiments on destructing cyanide ions were conducted both in model solutions and technological solutions containing complex cyanides of various metals (composition of the solutions is given in Table 1.).

Cyanide content was controlled using argentometer and spectrophotometer with barbituric acid and pyridine by means of optimized method [4].

Fig. 1(a) shows dependencies of KCN content in model solutions on time of plasma treatment. They were prepared by means of dilution of the parent solution, containing 21% KCN and 42% KOH. It was shown that at low concentrations of KCN (0,01 - 0,10 %), complete neutralization of solutions was achieved in 2÷8 min. Under cyanide content of 0,3÷1,0 %, this time makes 30 minutes. Solutions with KCN concentration of 1,30 % are not neutralized completely within this time.

Fig. 1(b) represents similar dependencies for technological solutions. Solutions were obtained by means of cyanidation of specified quantities of one metal (Au, Ag, Cu, Zn, curves l’-6’), or the ore concentrate containing all the above stated metals (curve 7’). Figures prove that process of cyanide destruction is determined by the time of plasma action on the solution. For technological solutions, time of treatment required for complete destruction of cyanide ions depends on composition of the solution. The more complex is the composition, the longer time is required for complete degradation of cyanides. Character of the curves is changed as well.
Table 1 Composition of model and technological solutions

<table>
<thead>
<tr>
<th>Solution</th>
<th>$C_{\text{KOH}}$, mol/l</th>
<th>$C_{\text{KCN}}$, mol/l</th>
<th>Metal</th>
<th>$C_{\text{Me}}$, mg/l</th>
<th>$k$, min$^{-1}$</th>
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<td>0.62</td>
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<td>-</td>
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<tr>
<td>6</td>
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</tr>
<tr>
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<td>0.01</td>
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<td>-</td>
<td>0.72</td>
</tr>
<tr>
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<td>0.148</td>
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</tr>
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<td>5'</td>
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<tr>
<td>6'</td>
<td>0.022</td>
<td>0.047</td>
<td>Zn</td>
<td>640</td>
<td>-</td>
</tr>
<tr>
<td>7'</td>
<td>0.039</td>
<td>0.067</td>
<td>Au, Ag, Cu, Zn</td>
<td>*</td>
<td></td>
</tr>
</tbody>
</table>

*Accordingly, 140, 100, 300 & 60 mg/dm$^3$

Calculations show that curves of cyanide ions destruction in the process of plasma treatment for model solutions can be described by first-order kinetic equation

$$k_f = \frac{2 \cdot 303}{\tau} \cdot \lg \frac{a}{a-x},$$

where $k_f$ – speed constant;

- $a$ – concentration of the substance at the starting moment of time $\tau = 0$;
- $x$ – decrease in concentration of the substance on the expiry of time $\tau$.

Linearity of $\lg C$ dependence on $\tau$ proves the first order of the reaction. However, values of the constant of pseudo-first order are varying in time for various initial concentrations of cyanide ions and are actually the same for large enough initial concentrations only (0.203 and 0.267 mol/l of $\text{KCN}$).

It is known that correlation between the substance half-reaction time $\tau_{1/2}$ and its initial concentration $C_0$ is as follows:

$$\tau_{1/2} \approx C_0^{(1-m_0)},$$

where $m_0$ is kinetic order of the reaction with regard to dissolved substance.

Proportionality of dependence of half-reaction time $\tau_{1/2}$ on the initial concentration of $\text{KCN}$ can indicate that the limiting stage, determining kinetics of the process, consists in generation of free radicals in the system. Taking into account that with the increase in the initial concentration $\text{KCN}$ is present in larger excess as compared with...
S. Tonev, K. Kanev, C. Dishovsky

radicals (H, OH, HO₂) being generated, zero order of reaction with respect to dissolved substance is to be expected. For treatment of solutions with the initial concentration of KCN, exceeding 0.203 and 0.267 mol/l, \( \tau_{1/2} \) values are the same. If at low concentrations a stage of radicals’ formation of water is significant, direct plasma action on molecules and ions of dissolved substance has greater impact at higher concentrations. Therefore, the order and mechanism of reaction can change.

Fig. 1. Change in KCN content depending on duration of plasma-chemical treatment of model (a) and technological (b) solutions containing complex compounds: Au (1', 2', 3'); Ag (4'); Cu (5'); Zn (6') u Au, Ag, Cu, Zn (7').

In the event of technological solutions, curves of cyanide destruction have horizontal sections which occurrence is caused by various speed of degradation of free cyanide and cyanide, combined in complex with metal.

Determination of the mechanism of chemical transformations in solutions containing cyanide under the action of non-equilibrium plasma represents a complicated task. Plasma action results in generation of radicals, ions, excited
molecules of water, secondary electrons and other active particles, which contribute to oxidation-reduction reactions running in the solutions. Due to recombination of HO and HO₂ radicals, highly reactive hydrogen peroxide, also taking part in the reactions, is formed. It may be connected with the fact that H₂O₂ formed in the process of plasma treatment is consumed for interaction with KCN and studying dependence of cyanide destruction efficiency on current strength, allow to draw the similar conclusion.

Formation of HO and HO₂ particles is the direct result of electric discharge; therefore, quantity of the particles formed should be functionally connected with electric parameters of plasma flow. This relation was established with the help of regression analysis using the least-squares method, as based on the experimental measurements of hydrogen peroxide generated in the course of solutions’ treatment with plasma. This dependence is expressed by the equation:

\[ C = a + b \cdot \tau + c \cdot I + d \cdot \tau I, \]  

where: \( C \) – hydrogen peroxide concentration; 
\( \tau \) - plasma exposure time; 
\( I \) – set current strength; 
\( a, b, c, d \) – calculated constants;

Equation where the values of current strength and time of plasma exposure are correlated between each other is the most accurate in describing the dependence. This choice is proved by the least value of exact amount - deviation squares \( S \) for the given equation [6, 7].

The paper [8] includes results of investigating electron mechanisms of the impact of active particles, radicals, hydrated electrons artificially generated by plasma on the behavior of cyanide complexes of zinc in water solutions. The above investigation was conducted using quantum chemistry methods. Quantum-chemical calculation of electron structure of the complexes \( \text{Zn(CN)}_4^{2-} \cdot 4\text{H}^+ \cdot 2\text{OH}^- \) with complete optimization of all geometric parameters [9, 10] was performed.

Analysis of calculation results has shown profound reconstruction of links between interacting centers that occurred in the chosen model. The most significant changes are observed in the first coordination sphere of central atom. In the process of interaction of \( \text{Zn(CN)}_4^{2-} \) complex with \( \text{H}^+ \) and \( \text{OH}^- \) particles, there is a tendency to destruction of the first coordination sphere of Zn atom with subsequent forming \( \text{Zn(OH)}_2 \) complex and four molecules of \( \text{HCN} \). Further \( \text{HCN} \) degradation in water solution under plasma action is running in accordance with the pattern below:

\[ \text{CN}^- + 2\text{OH}^- \rightarrow \text{CNO}^- + \text{H}_2\text{O} + 2e, \]  
\[ 2\text{CNO}^- + 4\text{OH}^- \rightarrow 2\text{CO}_2 + \text{N}_2 + 2\text{H}_2\text{O} + 6e, \]  

or

\[ \text{CNO}^- + 2\text{H}_2\text{O} \rightarrow \text{NH}_3^+ + \text{CO}_3^{2-}. \] 

thus ensuring complete decomposition of toxic cyanide.

Efficiency of non-equilibrium contact plasma usage during treatment of solutions in the course of neutralization of complex cyanide compounds of heavy metals was experimentally determined with the use of experimental-industrial unit. The latter is
connected with the fact that in such system, along with heterogeneous processes of electrochemical destruction of cyanide compounds, homogeneous processes of active radicals' reactions of the medium are also possible. In this respect, the method can be rather efficient at relatively low concentrations of cyanides in the solutions, in which, due to running of chemical transformations in diffusion zone, the other methods do not allow achieving high degree of treatment.

Let's consider decontamination of cyanide-containing waste waters by the example of aqueous solution of copper cyanide \[ \text{Cu(CN)}_{\text{aq}} \] under action of contact plasma thereon. In the given case, presence of \( \text{CN}^- \) group plays an essential role.

As a result of our experiments, it was established that during low-temperature plasma electrolysis cyan-group breaks up according to the same mechanism [11].

Breaking-up of cyanides under action of contact plasma was studied by us by the example of cyanide electrolytes of silvering and coppering processes [12]. This being the case, formation of insoluble dispersed compounds of copper oxide is observed.

Typical kinetic curve of copper deposition from waste waters of electroplating processes is shown in Fig 3. The process is described by first-order reaction. Calculated rate constant for the given experimental conditions has the value of \( K = 1,3 \cdot 10^{-3} \text{s}^{-1} \).

![Fig. 2. Kinetic curve of copper deposition from CN\textsuperscript{-}-containing waste waters with initial concentration of copper in the solution being equal to \( C_{\text{init}} = 3,0 \cdot 10^{-5} \text{mol/l}. \)](image)

It was determined that rate of process of \( \text{Ag}^+ \) and \( \text{Cu}^+ \) deposition from aqueous solutions with the use of plasma method was significantly responsive to the influence of the value of initial volume of reaction mixture (Fig. 4); when it decreases, with \( U, I, P \) and \( T \) being constant, growth in the rate of chemical transformations is observed. Kinetic parameters of the process remain the same. Increase in volume leads to reduction of the rate of extraction of ions \( \text{Ag}^+, \text{Cu}^+ \) and other metals \( (\text{Cd}^{2+}, \text{Zn}^{2+}, \text{Fe}^{2+}) \) from the reaction volume. Consequently, the rate of decontamination of waste waters from toxic components is the function \( V/V_0 \), where \( V_0 \) – volume of space, restricted by anode and bottom part of the reactor, \( V \) – volume of treated sample. In case of decrease in the initial reagent volume, the absolute value of contaminating agents is reduced, and im-
Impact of initiating factors has non-linear character, which gives an opportunity to reduce the time of impurities’ deposition from aqueous solution.

In the same manner, solutions containing cyanides of cadmium and zinc can be decontaminated. The Table 2 displays results of experimental investigations, which prove substantially complete extraction of compounds of silver, copper, cadmium and zinc in the form of solid insoluble compounds representing simple chemical forms, not connected with cyan-group. As a result of analyzing solutions upon treatment with contact plasma, no cyanide compounds were found, which confirmed the conclusion about the process running in accord with above mentioned mechanism of chemical transformations.

Table 2. Treatment of cyanide salt solutions using low-temperature plasma method and traditional electrolysis at 0,1 A current value and 5 minutes’ duration of treatment.

<table>
<thead>
<tr>
<th>Metal</th>
<th>Concentration of metals before treatment, mg · dm⁻³</th>
<th>Concentration of metals, mg · dm⁻³</th>
<th>Plasma method</th>
<th>Traditional electrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silver</td>
<td>5,3</td>
<td>0,002</td>
<td>4,8</td>
<td></td>
</tr>
<tr>
<td>Copper</td>
<td>3,7</td>
<td>0,003</td>
<td>3,4</td>
<td></td>
</tr>
<tr>
<td>Cadmium</td>
<td>3,2</td>
<td>0,002</td>
<td>2,9</td>
<td></td>
</tr>
<tr>
<td>Zinc</td>
<td>4,1</td>
<td>0,004</td>
<td>3,7</td>
<td></td>
</tr>
</tbody>
</table>

Kinetic regularities allow to evaluate the rate constants of Ag⁺ and Cu⁺ extraction from solutions containing the said ions. In this case, they can serve as a criterion of qualitative evaluation of represented systems.

Obtained data prove that residual concentration in contact plasma-treated waters of quartet of cyanide solutions has the values of concentration of above metal ions being lower by three orders, than with the use of traditional electrochemical method of treatment. It is an indirect proof of decontamination of such solutions from cyanide compounds.

Conclusion.

Plasma-chemical sample preparation allows breaking-up of cyanide compounds to nontoxic forms, which may be used for purification of technological solutions and galvanic production wastewaters, posing hazard to people’s health in the event they are purposefully used by terrorists.
References

Chapter 15

Difficulties in the Treatment of Poisoning by Carbamates

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Abstract - Carbamates belong to a group of compounds having a broad spectrum of toxicity - from relatively nontoxic to highly toxic compounds comparable with nerve agents. The current treatment of poisoning by organophosphates consists of the combination of cholinolytics like atropine and some oximes. The efficiency of oximes is not, however, satisfactory in the case of carbamates poisoning because oximes are not able to reactivate carbamylated acetylcholinesterase (AChE). It is considered that for treatment of carbamate poisoning administration of different cholinolytics only is effective. In this paper a number of oximes (toxogonine, pralidoxime, dipiroxime, HI-6, HS-3, HS-6, HGG-12), muscarinic (atropine, scopolamine, amysile) and nicotinic (arepalele, pentaphene) cholinolytics have been tested to protect mice against the poisoning by carbamates. The effectiveness of few anticonvulsants (diazepam, phenazepam, clonazepam) was investigated also. The following carbamates were used: insecticides (carbaryl, aldicarb), drugs (physostigmine, aminostigmine, neostigmine, pyridostigmine) and a few pyridile and quinoline carbamates with almost irreversible action on AChE. Oximes alone or with mixture of cholinolytics and anticonvulsants were administered 15 min prior or 1 min after the intoxication. In the experiments in vitro the possibility of oximes to reactivate the carbamylated purified human erythrocyte AChE was studied. By use of different groups of oximes quite different effects were observed. All tested oximes reduced the toxicity of aminostigmine, aldycarb and neostigmine whereas no effect could be determined on the toxicity of physostigmine and pyridostigmine. The toxicity of carbaryl and irreversible carbamates was significantly increased by toxogonine, dipiroxime and pralidoxime. Oxime therapy reduced the protective effect of cholinolytics against poisoning of these types of carbamates. The high efficiency of the mixture of atropine, arepalele and phenazepam for treatment and prophylaxis of carbamate poisoning was shown. As demonstrated by experiments in vitro oximes did not reactivate carbamylated AChE. Thus, these data indicate that the efficiency of oximes against carbamate poisoning is, at best, very limited and unsatisfactory. Therefore, there has been an active search for a broad spectrum antidotes against poisoning by carbamates. Potential threat of terrorist usage of carbamates is connected with the high toxicity of compounds and the absence of universal antidotes.

Keywords - cholinolytics, carbamats, oximes, treatment
Introduction

Carbamates belong to a group of compounds having a broad spectrum of toxicity – from relatively non toxic to highly toxic compounds comparable with the nerve agents (1, 2). There is a real possibility that even more powerful warfare agents that the nerve agents remain to be discovered from the class of carbamates. The basic mechanism of carbamates action is reversible inhibition of cholinesterase. Acetylcholinesterase (AChE) reacts with organophosphates (OP) and carbamates in an analogous manner. In contrast with OP spontaneous decarbamylation occurs relatively rapidly. The clinical picture of poisonings by carbamates is absolutely similar to that for OP, perhaps with more expressed peripheral signs because of the presence of the quaternary nitrogen in toxic carbamates makes penetration through blood brain barrier difficult. Inhibition of enzyme in case of carbamates is based on carbamylation on the active center of AChE. Carbamylated enzyme is resistant to effect of oximes (3, 4). Oximes as a rule is noneffective as drugs for treatment and it is recommend these compounds be excluded from therapeutic scheme (5). Potential threat of terroristic usage of carbamates is connected with the high toxicity of some carbamates and difficulties in the therapy of poisoning by carbamates.

1.Materials and methods

In order to get some new information about the dangerous of carbamates, we studied the toxicity, anticholinesterase activity of compounds and the efficiency of different drugs (cholinolytics, oximes, anticonvulsants) against poisoning by carbamates. The following carbamates were used: insecticides (carbaryl and aldicarb), drugs (physostigmine, pyridostigmine, aminostigmine, neostigmine), piperidile pyridyl carbamates (X-80, X-85), pyridine carbamate (X-83) and few bis-quaternary compounds (X-129, X-130, X-131).

In the first part of investigation, the kinetics of the inhibition of the purified human erythrocytes AChE by different carbamates was studied with the help of bimolecular rate constant (K2) of interaction of the AChE with compounds. AChE activity was assayed by the method of Ellman et al. (6) at pH 7.5, 25°C in the 20 mM of phosphate buffer, containing 100 mM of potassium chloride, 3.1 mM acetylthiocholine iodide as substrate and 0.2 mM of 5’5’- dithio-bis -(2-nitrobenzoic acid). We studied also in the experiments in vitro the speed of carbamylation and the constant of decarbamylation using the graphic method with the determination of relationships between the time and level of enzyme inhibition in diluted mixture AChE-carbamates (7). The toxicity of carbamates in experiments with albino mice following subcutaneous injection of carbamates was determined from the LD50 values.

In the second part of the study, the activity of mice brain AChE, after the i.m. injection of different carbamates was measured by method of Ellman, using acetylthiocholine iodide as substrate.

And finally, we studied the efficiency of drugs against carbamate poisoning. Different muscarinic and nicotinic cholinoreceptors blockers (cholinolytics) alone or with the mixture with some oximes and anticonvulsants were administered i/p 15 –30 min. prior or 1 min. after s/c injection of carbamates. The results of the protection expe-
riments are expressed as the protective coefficient - PC (i.e., the ratio of LD₅₀ value in treated and in untreated animals).

2. Results and discussion

It has been found that the carbamates in the in vitro experiments acted as irreversible inhibitors of AChE - the degree of enzyme inhibition increases with longer incubation time. There is a definite correlation between the value of $K_2$ and toxicity of carbamates. All investigated compounds have been distributed of few groups – with extra high toxicity (X-129, X-130, X-131) and comparatively high toxicity (aminostigmine, aldicarb, neostigmine). The other compounds were with average and low toxicity. The bisquaternary compounds were in 10-50 times more toxic than known nerve agents.

The majority of carbamates acted as reversible inhibitors of AChE. The duration of inhibition of mice brain AChE was about 4 hours after injection of aminostigmine, pyridostigmine and physostigmine. In contrast with these compounds the duration of inhibition of mice enzyme was considerable more than 24 hours after injection of carbamates X-80, X-83, X-85. In contrast with physostigmine and aminostigmine, these carbamates had an almost irreversible action on AChE. In the experiments in vitro acetylcholine decreased the inhibition of AChE by aminostigmine and physostigmine. The compound X-80 acted as irreversible inhibitors of AChE.

With the usage of different groups of oximes quite different effects were observed. Toxogonine and oxime $P_2S$ significantly increased the toxicity of carbaryl and reduced the toxicity of aldicarb whereas no effect could be determined on the toxicity of physostigmine and pyridostigmine.

We studied also the efficiency of different muscarinic cholinolytics with central (atropine, amyzile) and peripheral (metacyn) action and few nicotinic cholinolytics -arpenal, pentaphene and methylaprophen. The highest increase of the mice tolerance to neostigmine was noted following pretreatment by central and perepheric cholinolytics (Table 1).
Table 1. Protective effect of cholinolytics against neostigmine poisoning

<table>
<thead>
<tr>
<th>Cholinolytics</th>
<th>Dose mg/kg</th>
<th>DL50 of neostigmine (mg/kg)</th>
<th>PC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>Prophylaxis</td>
</tr>
<tr>
<td>Atropine</td>
<td>20.0</td>
<td>0.61±0.16</td>
<td>0.82±0.11</td>
</tr>
<tr>
<td></td>
<td>50.0</td>
<td>0.40±0.06</td>
<td>0.96±0.08</td>
</tr>
<tr>
<td>Amyzile</td>
<td>8.0</td>
<td>0.61±0.16</td>
<td>1.61±0.30</td>
</tr>
<tr>
<td>Metacyne</td>
<td>10.0</td>
<td>0.61±0.16</td>
<td>1.52±0.52</td>
</tr>
<tr>
<td>Pentaphene</td>
<td>9.0</td>
<td>0.36±0.10</td>
<td>1.42±0.08</td>
</tr>
<tr>
<td></td>
<td>25.0</td>
<td>0.44±0.03</td>
<td>3.10±0.10</td>
</tr>
<tr>
<td>Arpenale</td>
<td>5.0</td>
<td>0.44±0.03</td>
<td>1.38±0.06</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>0.36±0.10</td>
<td>3.90±0.47</td>
</tr>
<tr>
<td>Methylaprophen</td>
<td>6.0</td>
<td>0.61±0.16</td>
<td>3.94±7.6</td>
</tr>
</tbody>
</table>

The oxime HI-6 was the most effective of 7 reactivators (toxogonine, pralidoxime, dipiroxime, HS-3, HS-6, HGG-12) for the prophylaxis of neostigmine poisoning. The oxime HI-6 strongly potentiated the efficiency of mixture atropine and arpenale (Table 2).

Table 2. Protective effect of cholinolytics and oximes against neostigmine poisoning

<table>
<thead>
<tr>
<th>Drugs</th>
<th>DL50 (mg/kg) of neostigmine</th>
<th>PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.44±0.03</td>
<td>-</td>
</tr>
<tr>
<td>Atropine (20 mg/kg)</td>
<td>1.75±0.12</td>
<td>3.97</td>
</tr>
<tr>
<td>Arpenale (5 mg/kg)</td>
<td>2.61±0.23</td>
<td>5.93</td>
</tr>
<tr>
<td>HI-6 (50 mg/kg)</td>
<td>1.05±0.05</td>
<td>2.38</td>
</tr>
<tr>
<td>Arpenale +arenpale</td>
<td>3.5±0.3</td>
<td>7.95</td>
</tr>
<tr>
<td>Atropine+arpenale+HI-6</td>
<td>6.0±0.62</td>
<td>13.83</td>
</tr>
<tr>
<td>TMB-4 (10mg/kg)</td>
<td>0.48±0.03</td>
<td>1.0</td>
</tr>
<tr>
<td>Atropine+arpenale+TMB-4</td>
<td>3.9±0.3</td>
<td>8.86</td>
</tr>
</tbody>
</table>

As demonstrated in experiments in vitro the oximes HI-6 and TMB-4 did not reactivate neostigmine – inhibited AChE.

The oxime HI-6 was effective for prophylaxis but not for treatment of aminostigmine poisoning. The mixture of atropine, arpenale, HI-6 and phenazepam was most effective for prophylaxis as well as for treatment of aminostigmine poisoning (Table 3).
Table 3. Protective action of mixture of drugs against aminostigmine poisoning

<table>
<thead>
<tr>
<th>Drugs</th>
<th>DL50 (mg/kg) of aminostigmine</th>
<th>PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.22 ± 0.008</td>
<td>-</td>
</tr>
<tr>
<td><strong>Prophylaxis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atropine (15mg/kg)</td>
<td>1.12 ± 0.05</td>
<td>5.1</td>
</tr>
<tr>
<td>Arpenale (5mg/kg)</td>
<td>1.25 ± 0.06</td>
<td>5.68</td>
</tr>
<tr>
<td>HI –6 (5.0 mg/kg)</td>
<td>0.030 ± 0.007</td>
<td>1.36</td>
</tr>
<tr>
<td><strong>Atropine+Arpenale+HI-6+phenazepam (1mg/kg)</strong></td>
<td>6.6 ± 0.3</td>
<td>30.0</td>
</tr>
<tr>
<td><strong>Treatment</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atropine</td>
<td>0.030 ± 0.007</td>
<td>1.36</td>
</tr>
<tr>
<td>HI –6</td>
<td>0.021 ± 0.008</td>
<td>1.0</td>
</tr>
<tr>
<td>Atropine + HI-6</td>
<td>0.074 ± 0.009</td>
<td>3.4</td>
</tr>
<tr>
<td>Atropine+HI-6 +phenazepam</td>
<td>2.28 ± 0.06</td>
<td>10.4</td>
</tr>
</tbody>
</table>

The oxime HI-6 and TMB-4 increased the toxicity of irreversible inhibitors X-80, X-83, X-85. For the first time the high prophylactic effect of reversible inhibitors galanthamine and tacrine as drugs for prophylaxis of poisonings by irreversible carbamates have been shown (Table 4).

Table 4. The efficiency of tacrine and galanthamine were injected i.m. at 0.25 LD50 for prophylaxis of carbamate poisoning in experiments on the mice

<table>
<thead>
<tr>
<th>Carbamates</th>
<th>Control LD50 mg/kg</th>
<th>Galanthamine Efficiency</th>
<th>PC</th>
<th>Tacrine Efficiency</th>
<th>PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-80</td>
<td>12.8±0.18</td>
<td>25.0±1.05</td>
<td>1.95</td>
<td>40.0±2.5</td>
<td>3.12</td>
</tr>
<tr>
<td>X-83</td>
<td>34.0±1.6</td>
<td>105.0±5.9</td>
<td>3.08</td>
<td>120.0±4.1</td>
<td>3.53</td>
</tr>
<tr>
<td>X-85</td>
<td>90.0±2.1</td>
<td>172.0±8.3</td>
<td>1.91</td>
<td>270.0±5.2</td>
<td>3.0</td>
</tr>
</tbody>
</table>

3. Conclusions

Toxicological investigation displayed in our experiments showed that we can not ignore the real possibility that even more powerful carbamates than the nerve agents remain to be discovered. At present we have a new aspect of the chemical threat; known chemical carbamates exist that are many times as potent that are now available for military used. The treatment of carbamates intoxication is very difficult. Oximes are not able to reactivate carboxylated AChE and therefore as a rule are not effective. Our data indicated that the efficiency of oximes against carbamates poisonings is at least very limited and unsatisfactory. Our experiments showed that there no single antidote against carbamates poisoning. At present the administration of different muscarinic and nicotinic cholinolytics and anticonvulsive drugs only is effective and safe. However, further study is necessary to clarify the action of carbamates and antidotes on a molecu-
lar level. Thus the high toxicity of carbamates and difficulties in therapy can be reason for the terrorist use of these compounds.

References

Chapter 16

Synthesis of New Reactivators of Cholinesterase

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Abstract - Irrespective of the fact that the main mechanism of toxicity of the nerve agents (tabun, sarin, soman, cyclosarin, VX) – inhibition of acetylcholinesterase (AChE) in peripheral and central nervous systems is very well known there are some unresolved problems related to the antidote treatment of this type of poisoning. One of the most important question is that many studies have shown that none of the currently available reactivators of cholinesterase, such as 2-PAM, obidoxime (toxogonin), HI-6 and trimedoxime is able to reactivation AChE inhibited by all compounds belonging to the nerve agents. That is way a lot of new cholinesterase reactivators with different chemical structure have been synthesized and tested by using in vitro or in vivo methods for their antidote activity. In our study we describe the synthesis of some new reactivators of ChE and the results obtained for their therapeutic efficacy in rats poisoned with soman and tabun. Our date have shown that two of compounds – BT-05 and BT-07 4M combined with atropine demonstrated very good effectiveness against tabun.

Key words - Cholinesterase reactivators, synthesis, physical and chemical characterization.

Introduction

Organophosphates (OPs) are a large family of compounds with the common main target of action – the enzyme acetylcholinesterase (AChE; EC 3.1.1.7). These compounds irreversibly inhibit this enzyme by a covalent bond on its active site. After the inhibition, the enzyme is not able to fulfill its physiological role in the organism - splitting a neuromediator acetylcholine (Ach) (9). Nerve agents are very important group of OPs. Tabun (GA), sarin (GB), soman (GD), cyclosarin(GF) and agent VX are the best known members of this family. Especially GB, well known after the Tokyo subway attack, has been discussed many times as a potential terrorist threat. Reactivators of cholinesterase (ChE) are pharmacological drugs used as antidotes in intoxications with organophosphorus compounds (OPC). The difficulty in reactivation of the ChE activity and slight antidote effect concerning intoxication with some OPC, are some of the reasons for continuing the examinations for the creation of new reactivators of ChE (3).

Antidote activity of reactivators of ChE is different against the different OPC. Up to now, drugs effective against all the neuroparalitic OPC have not been found.
There are many reports describing the results obtained from in vitro and in vivo experiments carried out with different nerve agents and all available reactivators of ChE – 2-PAM, TMB-4 (trimedoxime), Obidoxime (Toxogonin), HI-6 and HLo 7. It turned out that each of the currently available oximes has disadvantage in reactivating cholinesterase activity inhibited by one or other representatives of the group of nerve agents (2, 5, 6, 9, 1). Additional disadvantage of the last two antidotes is that they are unstable in aqueous solution and need to be administered by wet/dry autoinjector that may cause a delay in administering the drug (12). Due to this, many laboratories are focused on synthesis of broad-spectrum reactivators with the special activity to tabun intoxications. In order to improve the treatment of poisoning with toxic OPC, including nerve agent and pesticides in Department of Experimental Toxicology in Military Medical Academy (MMA), Sofia, Bulgaria, over 50 new compounds have been synthesized since the middle of the 1970s.

1. Materials and methods.

1.1. Chemistry.

Solvents (acetone, DMF, ethanol) and reagents were purchase from Fluka and Sigma-Aldrich and used without future purification. TLC analysis was performed on TLC plates cellulose F pre-coated with dimensions 20 x 20 cm and layer thickness 0.1 mm. The mobile phase was n-butanol: glacial acetic acid: H2O in proportions 9:2:4. The HPLC system used for determination of synthesized reactivator was Gilson (Villers-le-Bel, France) consisting of a 305 master pump, a 306 slave pump, an 805 manometric module and an 811C dynamic mixer. Detector was Spectroflow 757 UV (Applied Biosystems, Ramsey, NJ) and the eluate was monitored at 280 nm. The column was Alltech RP C18, with length 250 mm, internal diameter 4.6 mm, and diameter of particles Dp = 10 µm. The mobile phase was 1 mM sodium cyclohexanesulfamate in DI water:acetonitrile (95:5), pH = 3.0. The column flow was set to 1.0 ml/min.

BT-03 and BT-05 were synthesized by method of Kamil Kuca et al. (2004) which was modified in our laboratory. BT-07, BT-08 and BT-07 4M were synthesized by a modified method of Poziomek and Hackley (11).

2. Results.


Five new compounds from the groups of bispyridinium symmetric and bispyridinium asymmetric oximes have been synthesized:

1,4 bis (4-hydroxyiminomethyl-pyridinium)-butane dibromide (BT-07-4M).
1,4 bis (2-hydroxyiminomethyl-pyridinium)-butane dibromide (BT-08).
1,3 bis (2-hydroxyiminomethyl-pyridinium)-propane dibromide (BT-07).
1-(4-hydroxyiminomethyl-pyridinium)-3-(4-carbamoylpyridinium) butane dibromide (BT-05).
1-(4-hydroxyiminomethyl-pyridinium)-3-(4-carbamoylpyridinium) propane dibromide (BT-03);

2.1.1. Synthesis of BT-07, BT-08 and BT-07 4M.
The synthesis of the compounds was implemented in the following scheme

\[
\begin{align*}
2 + \text{BrBr} & \rightarrow \text{Br}-(\text{CH}_2)_n\text{Br} \\
\text{HC}=\text{NOH} & \rightarrow \text{HC}=\text{NOH} \\
2 & \rightarrow \text{N}+\text{N}+ \\
(\text{CH}_2)_n & \rightarrow (\text{CH}_2)_n \\
\end{align*}
\]

Were:  
\( n = 3; \text{-CH}=\text{NOH} \) on position 2 for BT-07  
\( n = 3; \text{-CH}=\text{NOH} \) on position 4 for BT-08  
\( n = 4; \text{-CH}=\text{NOH} \) on position 4 for BT-07 4

A solution of the hydroxiiminomethylpyridine (10 g, 82 mmol) and (6.92 g, 32 mmol) dibromopropane or dibrombutane in N,N-dimethylformamide was stirred at 80 °C for 30 hours. The reaction mixture was cooled to room temperature and was collected by filtration, washed with acetone (2 x 40 ml) and recrystalized from acetonitrile.

2.1.2. Synthesis of BT-03 and BT-05.
2.1.2.1. Synthesis of intermediate products.
The synthesis of [1 - (4 bromobutyl)-4-carbamoyl pyridinium bromide] (MP-IV) and [1 - (3 bromopropyl)-4-carbamoyl pyridinium bromide] (MP-III) was implemented in the following scheme:
To the solution of 32.4 g. (0.15 M) 1,4-dibromobutane in 100 ml acetonitrile was added solution of 3.66 g. (0.03 M) isonicotinamide in 100 ml acetonitrile. The reaction mixture was stirred at 70 °C for 28 hours. After that the mixture was cooled to room temperature and was collected by filtration, washed with acetone (2 x 40 ml) and recrystallized from acetonitrile.

2.2.2. Synthesis of the end products.

The synthesis of the compounds was implemented in the following scheme:

\[
\begin{align*}
\text{O} & \text{C} \text{NH}_2 \\
\text{N} & \text{C} \text{NH}_2 \text{O} \\
\text{Br}^{-} & \text{Br}^{-} \\
\text{N} & \text{C} \text{NH}_2 \text{O} \\
\text{Br}^{-} & \text{Br}^{-} \\
\text{N} & \text{C} \text{NH}_2 \text{O} \\
\text{Br}^{-} & \text{Br}^{-} \\
\text{N} & \text{C} \text{NH}_2 \text{O} \\
\text{Br}^{-} & \text{Br}^{-}
\end{align*}
\]

Were: \( n = 3 \) for MP-III and \( n = 4 \) for MP-IV.

BT-03 and BT-05 were synthesized in reaction of MP-IV or MP-III with 4-pyridin-aldoxime in molar correlations 1:1,5 in dimethylformamide. The reaction mixture was stirred at 70 – 80 °C for 37 hours. After that the mixture was cooled to room temperature and was collected by filtration, washed with dimethylformamide (2 x 50 ml) and recrystallized from ethanol.

2.3. Physicochemical characterization of the end products.

2.3.1. Thermal analysis.

In thermal analysis were determined melting points as follows: 243-244°C for BT-07 4M; 263-265°C for BT-07; 260-261°C for BT-08; 222-224°C for BT-03; 250-252°C for BT-05.
2.3.2. IR-spectrum.

The IR-spectrum in KBr pellets shows the characteristic absorption in the following wavelengths: BT-07 4M: 1050 cm⁻¹, 1420 cm⁻¹ and 1570 cm⁻¹; BT-07: 1050 cm⁻¹, 1480 cm⁻¹ and 1570; BT-08: 1050 cm⁻¹, 1480 cm⁻¹ and 1570; BT-03: 1630 cm⁻¹, 1010 cm⁻¹, 1420 cm⁻¹, and 1570 cm⁻¹; BT-05: 1640 cm⁻¹, 1050 cm⁻¹, 1420 cm⁻¹, and 1570 cm⁻¹.

2.3.3. TLC methods for determination of end products.

These methods are described above. Rf = 0.43 for BT-07 4M; Rf = 0.44 for BT-07 ; Rf = 0.38 for BT-08; Rf = 0.3 for BT-03 and Rf = 0.35 for BT-05.

2.3.4. HPLC method for determination of end products.

This method is described above. The analysis showed that the purity of all compounds was more than 98%.

4. Discussions.

The synthesis of cholinesterase reactivators started during the fifties of the last century when were synthesized consecutively 2-PAM, trimedoxime, methoxime and later obidoxime which is a structural analogue of trimedoxime. After that in the laboratory of Hagedorn were synthesized hundreds of oximes derivates leading to asymmetric bispyridinium oximes of the H-series to which belong two of the most potent and promising reactivators – HI-6 and HLo 7. Later were synthesized numerous pyridinium, imidazolium and quinuclidinium compounds known as BDB and HGG series. Our experience in the synthesis of cholinesterase reactivators was reported previously (10). In the current work, by using modifying methods, we synthesized five new compounds which belong to the groups of bispyridinium symmetric and bispyridinium asymmetric oximes. For the purposes of their physicochemical characterization we developed TLC and HPLC methods. By means of these methods the purity of new compounds was determined and was proved as more than 98%. Theoretically, the chemical structures of the new oximes should ensured high activity against some nerve agents and particularly tabun. Previously studies have shown that the chemical structure is crucial for the antidote efficacy of oximes and plays a very important role in reducing toxicity of nerve agents (13, 5). By using \textit{in vitro} method for assessment of the reactivating potency of new synthesized and some classic reactivators of ChE Kuca et al. (9) demonstrated that one of the compounds studied in our work was able to reactivate in a significant extent tabun inhibited rat brain AChE. The same study but demonstrated that there is no a wide-spectrum active antidote (oximes) against all nerve agents. In conclusion could be summarized that five new reactivators of ChE were successfully synthesized and their physico-chemical characterization was performed. The next step is these compounds to be evaluated as antidotes against nerve agents and particularly
References.

Chapter 17

Investigation of the Efficacy of New Reactivators of Cholinesterase in Soman and Tabun Inhibited Rat Brain Acetylcholinesterase

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Department of Medicine of Disasters and Toxicology
Laboratory of Military Toxicology

Abstract - Regardless international efforts and agreements such as Convention for Prohibition of Chemical Warfare Agents the most dangerous members of these chemical compounds known as nerve agents are still considered to be threat in case of terrorist act or local conflict. Because of the lack of universal antidote against nerve agents the process of searching of new more effective antidotes is continuing. Reactivators of cholinesterase are aimed to the etiological treatment of poisonings caused by nerve agent through restoration of the enzyme activity. In our study we present the results obtained from in vitro experiments with tabun and soman inhibited rat brain acetylcholinesterase treated with three new reactivators – BT-03, BT-05 and BT-07 4M. Their reactivating potency were compared with currently available antidotes Obidoxime and HI-6. Relationships between the chemical structures and reactivating activity of the new compounds are discussed.

Key words - Brain acetylcholinesterase, soman, tabun, reactivators of cholinesterase, reactivatin potency

Introduction

Highly toxic organophosphorous compounds and particularly so called “nerve agents” (tabun, sarin, soman, cyclosarin, VX) are considered as a real threat in case of usage against unprotected troops during a local warfare conflicts or civil population in domestic terrorist attack. Their toxic effects are due to inhibition of enzyme acetylcholinesterase followed by accumulation of neuromediator acetylcholine in peripheral and central nervous systems (18). The current antidotal treatment of nerve agent induced poisoning usually consists of (a) an anticholinergic drugs (atropine) to antagonize the effects of acetylcholine at cholinergic receptors (b) oximes to reactivate nerve agent-inhibited
acetylcholinesterase and (c) treatment of convulsions with the benzodiazepines (23, 1, 2, 19). Unfortunately, the results obtained from many studies have shown that there is no an antidote with universal activity against all nerve agents. Nowadays for treatment of nerve agent poisoning there are some autoinjectors containing atropine combined with different reactivators of cholinesterase – 2-PAM, Obidoxime (Toxogonin), TMB-4 (trimedoxime) and HI-6 (25, 10, 3). 2-PAM is very effective in reactivating AChE inhibited with sarin or Vx but is inefficient against tabun or soman inhibited enzyme (21, 24, 8). Obidoxime and TMB-4 have been shown to be very effective in experiments against tabun, sarin or VX but at the same time they are ineffective against soman (6, 9, 17, 7). HI-6 is more potent than Obidoxime in protection of various rodent species from intoxication with soman, sarin and VX (20, 9) but it can not reactivate tabun-inhibited AChE (4, 5). Due to the lack of a broad-spectrum cholinesterase reactivators the synthesis of new compounds with different chemical structures and determination of their antidotal effectiveness against nerve agents by using in vitro or in vivo methods are needed.

The goal of this study was to estimate the efficacy of some newly synthesized reactivators of ChE against soman and tabun in “in vitro” experiments and to compare their activity to those of some currently available oximes.

1. Materials and Methods

1.1. Reactivators of cholinesterase.

Three new compounds and two well known reactivators (Obidoxime and HI-6) from the groups of bispyridinium symmetric and bispyridinium asymmetric oximes have been synthesized in our laboratory:

The new compounds were as follow:

1-(4-hydroxyiminomethyl-pyridinium)-3-(4-carbamoylpyridinium) propane dibromide (BT-03);
1-(4-hydroxyiminomethyl-pyridinium)-3-(4-carbamoylpyridinium) butane dibromide (BT-05);
1,4 bis (4-hydroxyiminomethyl-pyridinium)- butane dibromide (BT-07-4M).

The chemical structures of the new compounds are shown on Figure 1.
1-(4-hydroxyiminomethyl-pyridinium)-3-(4-carbamoylpyridinium) butane dibromide (BT-05);

1,4 bis (4-hydroxyiminomethyl-pyridinium)- butane dibromide (BT-07-4M).

**Fig. 1.** Structures of newly synthesized cholinesterase reactivators.

In the current study the reactivating potency of the new compounds were compared to those of two well known reactivators of ChE – Obidoxime and HI-6 given in Figure 2. Obidoxime was kindly given by TNO, Holland and HI-6 was synthesized in Laboratory of Military Toxicology at MMA, Sofia.

1,3-bis(4-hydroxyiminomethylpyridinium)-2-oxa-propane dichloride (toxogonine, obidoxime)

1-(2-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)-2-oxa-propane dichloride (HI-6)

**Fig. 2.** Structures of Obidoxime and HI-6
1. 2. In vitro experiments.

1.2.1. Reactivation of rat brain acetylcholinesterase after tabun inhibition.

Rat brain homogenate diluted in distilled water (2%, w/v) was used as a source of AChE. The homogenate was inhibited with tabun (9.10^{-11} M) for 30 min. Oximes were added for 20 min at 250°C in concentration 1.10^{-3} M, 1.10^{-4} M and 1.10^{-5} M. Enzyme activity was determined by Ellman’s method. Spectrophotometer “Hospitex Diagnostics-Screen Master” was used. Well known HI-6 and Obidoxime were used as referent compounds.

1.2.2. Reactivation of rat brain acetylcholinesterase after soman inhibition.

Rat brain homogenate diluted in distilled water (2%, w/v) was used as a source of AChE. The homogenate was inhibited with soman (1.10^{-10} M) for 30 min. Oximes were added for 20 min at 250°C in concentration 1.10^{-3} M, 1.10^{-4} M and 1.10^{-5} M. Enzyme activity was determined by Ellman’s method. Spectrophotometer “Hospitex Diagnostics-Screen Master” was used.

1.3. Determination of percentage of reactivation.

The percentage of reactivation (%R) was calculated from the measured activities of the intact enzyme (Ao), tabun or soman-inhibited enzyme (Ai) and reactivated enzyme (AR) by using Eq. (1).

\[
\%R = \left[ 1 - \frac{(Ao - AR)}{(Ao - Ai)} \right] \times 100 \quad (1)
\]

Results

The results obtained from experiments with tabun-inhibited rat brain acetylcholinesterase demonstrated that at the lower concentration used (1.10^{-3} M) two of the new synthesized compounds – BT-07 4M and BT-05 are able to reactivate the enzyme activity to a significant extent – 72% and 63.6% respectively. Even at the higher concentration the same compounds showed very good reactivating potency. At the same conditions BT-03 was not so effective as BT-07 4M and BT-05 but the rate of reactivation ensured by it, particularly at the lower concentration, was comparable to those of obidoxime. HI-6 was completely ineffective in reducing tabun inhibition of the brain AChE (Table 1).

In the second part of our study when soman was used to cause inhibition only obidoxime was able to reactivate more than 15% of the enzyme activity. All the rest compounds including newly synthesized oximes did not demonstrate good reactivating potency. Surprising HI-6 again was ineffective, even at the lower concentration, to reanimate soman-inhibited brain AChE.
Table 1. Reactivation of tabun-inhibited rat brain AChE (U/L).

<table>
<thead>
<tr>
<th>REACTIVATOR</th>
<th>A0</th>
<th>AI</th>
<th>AR (%R)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.10-3 M</td>
<td>1.10-4 M</td>
<td></td>
</tr>
<tr>
<td>BT-03</td>
<td>0.257 ± 0.011</td>
<td>0.079 ± 0.012***</td>
<td>0.111 ± 0.018*** (16.4%)</td>
</tr>
<tr>
<td>BT-05</td>
<td>0.197 ± 0.033</td>
<td>0.068 ± 0.009***</td>
<td>0.150 ± 0.011*** (63.6%)</td>
</tr>
<tr>
<td>BT-07 4M</td>
<td>0.359 ± 0.018</td>
<td>0.150 ± 0.0017***</td>
<td>0.302 ± 0.010*** (72.8%)</td>
</tr>
<tr>
<td>Obidoxime (Toxogonin)</td>
<td>0.239 ± 0.021</td>
<td>0.068 ± 0.006***</td>
<td>0.100 ± 0.016*** (18.7%)</td>
</tr>
<tr>
<td>HI-6</td>
<td>0.248 ± 0.025</td>
<td>0.055 ± 0.009***</td>
<td>0.057 ± 0.001*** (1.1%)</td>
</tr>
</tbody>
</table>

*** - P<0.001
The date for BT-03, BT-05 and Obidoxime are in accordance with Samnaliev et al. 2008 (22)

Discussions and conclusions.

In vitro experiments with nerve agent-inhibited acetylcholinesterase are very suitable for assessment of the reactivating potency of the oximes and they are wildly used as a screening method (11, 12). It is well known that the maximal reactivation activity of tested oximes is between 10⁻⁵ and 10⁻² M and therefore the most often used concentrations are between 10⁻⁵ and 10⁻³ M (14). Moreover, the lowest concentration gives an information on reactivation efficacy in general and the highest one is concentration available after administration in vivo (16). In accordance with the results obtained from Kuca et all. (15) the rate of reactivation of nerve agent-inhibited acetylcholinesterase such as 15% and more could be assumed as satisfactorily reactivation.

Table 2. Reactivation of soman-inhibited rat brain AChE (mkmol/ml.min)

<table>
<thead>
<tr>
<th>REACTIVATOR</th>
<th>A0</th>
<th>AI</th>
<th>AR (%R)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.10-4 M</td>
<td>1.10-5 M</td>
<td></td>
</tr>
<tr>
<td>BT-03</td>
<td>0.370 ± 0.008</td>
<td>0.054 ± 0.002***</td>
<td>0.086 ± 0.008*** (10.2%)</td>
</tr>
<tr>
<td>BT-05</td>
<td>0.425 ± 0.027</td>
<td>0.056 ± 0.001***</td>
<td>0.085 ± 0.007*** (7.9%)</td>
</tr>
<tr>
<td>BT-07 4M</td>
<td>0.425 ± 0.027</td>
<td>0.056 ± 0.001***</td>
<td>0.098 ± 0.022*** (11.4%)</td>
</tr>
<tr>
<td>Obidoxime (Toxogonin)</td>
<td>0.238 ± 0.003</td>
<td>0.035 ± 0.004***</td>
<td>0.071 ± 0.014*** (17.7%)</td>
</tr>
<tr>
<td>HI-6 (TOXIDIN)</td>
<td>0.381 ± 0.008</td>
<td>0.059 ± 0.003***</td>
<td>0.065 ± 0.008*** (1.9%)</td>
</tr>
</tbody>
</table>

*** - P<0.001
In our experiments with tabun-inhibited brain AChE all of compounds tested except HI-6 were able to reactivate the enzyme more than 15%. At the same time there was significant difference in reactivating efficacy between BT-07 4M and BT-05 and other two reactivators. The new synthesized compound BT-07 4M administered in both concentrations ensured remarkable rate of reactivation that makes it a potential promising antidote against tabun. This compound possesses two pyridinium rings connected with four methylene bridge and oximes groups on position four in the first and second pyridinium rings. The second new compound demonstrated very good reactivating potency – BT-05 has similar structure except the presence of a carbamoyl group, instead of oxime group, on position four in the first pyridinium ring. It seems that these chemical structures ensured high affinity to the tabun-inhibited enzyme and the high rate of reactivation, respectively. As a support of this presumption is the result for the efficacy of BT-03, the last new compound, that reactivated 16% of the enzyme activity. BT-03 has practically the same structure as BT-05 except the length of the bridge between both pyridinium rings. Obidoxime that is currently available antidote showed, in our study, relatively good reactivation of tabun-inhibited enzyme but its activity was poorly than BT-07 4M and BT-05. In comparison to the new synthesized compound BT-07 4M the chemical structure of Obidoxime has an essential deference, namely, methylene-oxi-methylene bridge between pyridinium rings. Obviously the length and kind of the bridge between pyridinium rings play a very important role for the reactivating potency of the oximes. The importance of the length and the shape of the connecting chain, the number of quaternary pyridinium rings and the position and number of the oxime groups at the pyridinium rings have been discussed previously (13).

In case of soman-inhibited brain AChE only Obidoxime, administered at concentration $10^{-4}$ M demonstrated reactivation more than 15%. The new synthesized compound ensured reactivation between 8 and 11% whereas HI-6 was completely ineffective. At the same time previous results obtained from in vivo experiments have shown that HI-6 is able to reduce soman toxicity in rodents and mammals. This discrepancy between in vitro and in vivo results just confirms how difficult is the treatment of poisoning with soman and that there is no a broad-spectrum oxime effective enough to the all nerve agents.

In conclusion could be assumed that regardless understanding the main toxic mechanism of the nerve agents there are still some unresolved problems related to the treatment of poisoning caused by them. Development of new reactivators of cholinesterase and their investigation by using in vitro and in vivo experiments is the correct approach in searching more effective antidotes.

References

Chapter 18

What are the Clinical Significance of Oxime and Sodium Bicarbonate Therapy for Acute Organophosphate Poisoning?

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Abstract - In order to evaluate the efficacy of oxime and sodium bicarbonate therapy in organophosphate poisoning, and to correlate it with objective endpoints (AChE status, survival, need for mechanical ventilation and atropine consumption) the retrospective study of 109 patients with organophosphate (OP) poisoning was done. The patients were treated in the National Poison Control Centre, Belgrade since January 2003 till June 2007. They were analyzed according to the class of OP and the applied therapy: Group I - atropine and bicarbonate (17); Group II - atropine, oxime and bicarbonate (10); Group III - atropine and oxime (40); Group IV - atropine (42). Pralidoxime methylsulphate (4g/day) and bicarbonate (5mEq/kg/day) were administered as long as OP was present in biological samples.

Results: Majority (59.6%) of patients had severe and fatal poisoning. Based on the degree of AChE reactivation patients were allocated to 4 groups. Reactivation of more than 50% was registered in Group I and Group II, but the difference between groups was not significant. The patients from Group IV required MV more frequently (46%) than patients from other groups (p<0.05). Atropine consumption in Group IV (2330 mg) was significantly higher than in other groups (p<0.01). Eighteen (16.5%) patients died.

Conclusion: Assessment of oxime and bicarbonate therapeutic regimen is difficult when stratification of patients according to the level of poisoning, the poison load, time to therapy is not possible. Reactivation of AChE and survival were not different between therapeutic groups, so one might assume that oxime and bicarbonate should not be used. However the difference in atropine consumption, and the need for mechanical ventilation between the patients on atropine regimen only and other therapeutic groups was significant. Proper evaluation of these regimens requires larger, multicentric study.

Keywords - AChE, organophosphate, oximes, sodium bicarbonate,

Introduction

Pesticide poisoning results from occupational, accidental and intentional exposure. The epidemiological pattern of poisoning shows significant variation in number of deaths
and form of poisoning between developing and industrial countries. According to the World Health Organization, about 1 million accidental and 2 million suicidal poisonings with organophosphorus insecticides are reported per year, with more than 300,000 fatalities. The greatest share of poisonings comes from the developing countries of the Asia-Pacific region, but OP insecticide poisonings are also a problem for countries in the developed world, although their primary concern is defense against terrorist use of these chemicals (1-3).

The clinical manifestations of acute OP poisoning have well established three phasic effect in man: 1) acute cholinergic crisis due to accumulation of acetylcholine at muscarinic, nicotinic and central nervous system synapses, 2) the intermediate syndrome due to pre- and postsynaptic dysfunction at neuromuscular junction, 3) the delayed polyneuropathy due to phosphorylation of neurophatic target esterase. Better understanding of OP mechanism of action – irreversible inhibition of acetylcholinesterase, butyrlcholinesterase and all esterase type enzymes, followed by metabolic dysbalance in the organism, enhanced the development of antidotal treatment (4).

Besides nonspecific therapeutic measures, current therapeutic protocols include atropine, oxime and benzodiazepine. The mainstay of treatment is atropine which efficiently antagonizes the effects of accumulated acetylcholine at the cholinergic synapses, but it is ineffective at the nicotine sensitive synapses. Diazepam is used in counteracting convulsions, and it also improves atropine tolerance, reduces central nervous system damage and central respiratory weakness (1-3). Oximes reactivate phosphorilated acetylcholinesterase which still has not undergone the ageing process, by removing the phosphoryl group, reducing the acetylcholine concentration and cholinergic crisis (1,2). These effects are clearly demonstrated in experimental conditions, but the clinical benefit of oximes remains unclear. The BuChE activity does not reflect the severity of poisoning, but it is a reliable marker of exposure to anticholinesterase and their presence in the body. The best surrogate parameter for synaptic function is AChE and changing concentrations of AChE are assumed to reflect the effects of OPI in target organs. The range of depression of AChE or BuChE seen in patients with identical symptoms may be very large. The sensitivities of AChE and BuChE to OP agent differ and the use of whole blood may thus provide less accurate interpretations. Usefulness of cholinesterase estimations are limited due to physiologic variations that occur within and between individuals and the influence of dieases or medications. Caution is also required as there is no uniformly accepted standard technique and each method has its own „reference range“. Acute mild exposure to anticholinesterases may cause a reduction to 50% of lower normal activity of ChE, moderate exposure causing reduction to 20% of activity, and severe poisoning causes inhibition to to 10% of ChE activity. Clinical recovery correlates with recovery to 30% of normal (1-4).

The usefulness of oximes, has been challenged over the past two decades by physicians in many countries of the world who have failed to see benefit in their clinical practice (1-5). In order to improve the outcomes from acute organophosphate poisoning, alternative treatments have been tested, one of them being plasma alkalninization. Experimental results on animals and limited human research have has suggested benefit from using sodium bicarbonate in acute OP poisoning, leaving the mechanism of improvement unclarified (6).
Objective

The objective of the paper was to evaluate the efficacy of oxime and sodium bicarbonate therapy in organophosphate poisoning, and to correlate it with objective endpoints such as, AChE status, survival, need for mechanical ventilation and atropine consumption.

Methods

Retrospective study of 109 patients with OP poisoning, treated in the National Poison Control Centre, Belgrade (January 2003-June 2007) was performed. The degree of poisoning severity was estimated according to the Poisoning Severity Score (PSS).

The patients were analyzed according to the class of OP (96 had dimethylphosphoryl and 13 diethylphosphoryl compounds poisoning) and the applied therapy: Group I - atropine and bicarbonate (17 pts); Group II - atropine, oxime and bicarbonate (10 pts); Group III - atropine and oxime (40 pts); Group IV - atropine (42 pts). Pralidoxime methylsulphate (4g/day) and bicarbonate were administered as long as OP was present in biological samples. Sodium bicarbonate was administered 5mEq/kg/hour, followed by 5mEq/kg/24 hours. All patients received diazepam. Acetylcholinesterase was determined in 6 hours intervals, and arterial blood gases analysis were performed one hour after sodium bicarbonate infusion and afterwards twice a day. A Kolmogorov-Smirnov test was done for the evaluation of AChE reactivation in poisoning with diethyl and dimethyl OP compounds.

Results

Since January 2003 till June 2007, 109 patients were admitted to the National Poison Control Centre due to deliberate intoxication with OP. Admission interval in majority of patients was less than 6 hours. Dimethylphosphoryl organophosphate poisoning was more frequent and it was confirmed in 96 patients (most of them with malathion and dimethoate poisoning), and 13 patients had acute diethylphosphoryl organophosphate poisoning. The gender distribution in our group of patients with anticholinesterase poisoning was relatively balanced and women outnumbered men for just a couple of percents (54.57% vs 45.43%). Their educational level was similar, and majority of patients was from the agricultural households. The most common clinical signs of poisoning were miosis (61.8%), vomiting and diarrhea (50.8%), bronchorrhoea (51.7%), followed by hypotension (27.8%), acute respiratory insufficiency (25.9%) and coma in 25.54%. Acute respiratory insufficiency was registered in 82 (25.9 %), acute cardiocirculatory insufficiency in 15 (4.7 %) patients and 11 (3.5%) patients presented with the most severe clinical findings such as acute respiratory and cardiocirculatory insufficiency.

The analysis of poisoning severity showed that the difference between the number of severe poisoning (PSS 3), registered in 47 (43.1%), and mild to moderate poisoning registered in 44 (40.3 %) of patients, was not significant. The difference in distribution of patients according to poisoning severity was not significant even for different therapeutic groups (Table 1). There were no significant differences in arterial
blood pH and bicarbonate between the groups at the admission, but after the therapy, the values of arterial blood pH and bicarbonate level were significantly higher in patients from Group I and II (p<0.01) (Table 2).

When Ery-AChE inhibition was analyzed, a high percentage of patients, regardless the chemical structure had no inhibition or it was not significant. Inhibition of Ery-AChE up to the level of 10-20% of lower normal limit was registered in 6 (35,3%) patients from the Group I, and 50% of patients from the Group II and III respectively. The highest number of patients with inhibition of Ery-AChE to the level less than 10% of lower normal limit, was registered in the Group II (30%). Concerning the inhibition of AChE, it was the lowest in Group IV compared to Group II and III (p<0.05) (Table 3).

Gastric lavage was performed in all patients admitted within 6 hours of ingestion, followed by activated charcoal. Sufficient atropine was administered in order to maintain clear lungs and other signs of hyperatropinization. Pralidoxime methylsulphate (Contrathion®) was applied in 50 (45.8%) patients. The average duration of therapy was 4 days, but in one patient it was applied for 7 days. At the end of the treatment the difference in reactivation of Ery-AChE between the therapeutic groups was not significant. There was no reactivation in: 9.1% of the patients from Group I, 11.1% from the Group II, 37,5% from the Group III, compared to 28% of patients from the Group IV which received atropine only. Reactivation of more than 50% was registered in Group I and Group II, but the difference between therapeutic groups was not significant (Table 4).

The average atropine consumption was 252.0 mg ± 116.8 in Group I, 318.7 ± 159.6 in Group II but the highest atropine doses, 1637.1 ± 1125.5 mg were in the Group IV (p<0.01) (Table 5). Obviously pralidoxime methylsulphate and bicarbonate have reduced the need for atropine, and even in patients with dimethyl OP poisoning less atropine was administered. Also the patients from Group IV required mechanical ventilation (MV) more frequently (46%) than the patients from other groups (p<0.05).

There were no significant differences between the groups of patients on oxime or bicarbonate therapy considering the Ery-AChE reactivation, atropine consumption and the need for MV. Complications were registered in 32 (29.3%) patients with OP poisoning: pneumonia in 17 (15%), sepsis in 2 (1.8%), acute renal failure and rhabdomyolysis in 2 pts respectively, urinary infection in 8 (7.3%) and CVI, gastrointestinal bleeding and pulmonary thromboembolism in single cases respectively. Eighteen (16.5%) patients died, but there were no significant differences between the therapeutic groups concerning the mortality rate.

Discussion

The acute effects of OP insecticides are primarily due to the binding and subsequent inhibition of acetylcholinesterase, accumulation of acetylcholine and sustained stimulation of post-synaptic receptors. The AChE may reactivate spontaneously or with the aid of oxime treatment. It may also become irreversibly bound. These pathophysiological processes have implications for development of antidotes. Animal studies have revealed many compounds offering clear benefits, but no treatment has been shown to work in clinical studies during the last two decades. The key factor in
determining the outcomes in OP poisoning appears to be the timing of antidote administration (1-5).

The life-saving effects of atropine are evident within minutes in severely poisoned patients and there is little doubt that any other antidote has the same efficacy. Improvement in survival with atropine in animal studies of OP poisoning is often less than that of oxime, but the clinical effectiveness of oximes such as pralidoxime is unknown (6-9). The lack of universal oxime able to reactivate AChE by all OP has urged the development of new oximes and potential therapeutic agents such as sodium bicarbonate. Experimental studies on animals suggested that sodium bicarbonate favorably decreases mortality rate in OP poisoning (10,11). Bajgar et al examined the role of sodium bicarbonate in rats poisoned with 2 LD50 sc of sarin, dichlorvos and pyridostigmine. It was reported that the administration of bicarbonate had therapeutic effect in OP intoxications, even more when combined with atropine (12). Balali-Mood et al. in clinical study in patients with OP poisoning concluded that high doses of sodium bicarbonate significantly decreased the total atropine dose used in patients with acute OP poisoning (12). Co-administration of sodium bicarbonate significantly increased the protective effects of standard antidotes in rats poisoned with dichlorvos (11).

Out of 109 patients with OP poisoning, 96 (88.0%) had dimethylphosphoryl OP poisoning. Another disadvantage of the study is that the average time elapsed before arrival to hospital was 6 hours, but some of the patients came 12 hours after ingestion, when AChE inhibited by dimethyl-OP became aged and unresponsive to oximes. This is the possible explanation why more than 37.5% of patients from the Group III did not reactivate Ery-AChE at all, although there is no statistic significance comparing with Group IV (28% of patients had no reactivation of AChE). Addition of bicarbonate to oxime (Group II) increased the reactivation of Ery-AChE to significant level (more than 30%) in 55.5% of patients, but the difference between the groups was not significant, which could be explained with small number of patients in different therapeutic groups. There were no significant differences in arterial blood gases between the groups at the admission, but after the therapy, the values of pH and bicarbonate level were significantly higher in patients from Group I and II (p<0.01). Jeevarathiam et al. (13) demonstrated that bicarbonate pretreatment along with standard therapy in rats poisoned with diisopropil fluorophosphates enhanced the therapeutic efficacy of pralidoxime chloride, with the shift of PI from 7.63 to 11.7. In order to explain the possible mechanism, authors also investigated pharmacokinetic properties of pralidoxime proving that bicarbonate application led to a significant increase in oxime distribution into the tissue compartment. One might assume that distribution of oxime in central and peripheral compartments would induce better reactivation of inhibited AChE in target tissues, which was not confirmed. Another mechanism could be the change in the rate of hydrolysis of OP relative to blood pH, thus facilitating elimination rate. But in order to find the exact mechanism of sodium bicarbonate action, further experiments are required (10-14).

In our study the average atropine consumption was the highest in Group IV (p<0.01) which received only atropine, and the lowest in the Group II (p<0.01), so we could conclude that pralidoxime methylsulphate and bicarbonate have reduced the need for atropine. Also the patients from Group IV required mechanical ventilation (MV) more frequently (46%) than patients from other groups (p<0.05). However, there were
no differences between the groups of patients on oxime or bicarbonate therapy considering the Ery-AChE reactivation, atropine consumption or in the need for MV and overall mortality.

Conclusion

It is difficult to evaluate clinical relevance of therapeutic regimens when the stratification of patients according to the level of poisoning, initial AChE, the poison load, time to therapy is not possible. Reactivation of AChE was not different between therapeutic groups, so one might assume that oxime and bicarbonate should not be used. However the difference in atropine consumption, and the need for mechanical ventilation between the patients on atropine regimen only and other therapeutic groups was significant. Proper evaluation of these regimens requires larger, multicentric study.

References

Therapeutic group | PSS 1 | PSS 2 | PSS 3 | PSS 4 | Total
---|---|---|---|---|---
Group I | 2 (12.5) | 7 (41.1) | 7 (41.1) | 1 (6.25) | 17 (100)
Group II | 2 (20.0) | 5 (50.0) | 3 (30.0) | 10 (100)
Group III | 8 (20.0) | 9 (22.5) | 16 (40.0) | 7 (17.5) | 40 (100)
Group IV | 9 (17.4) | 7 (16.7) | 19 (45.2) | 7 (16.7) | 42 (100)

Table 1. PSS in therapeutic groups (n, %)

<table>
<thead>
<tr>
<th>Therapeutic group</th>
<th>pH of arterial blood</th>
<th>Bicarbonate level</th>
</tr>
</thead>
<tbody>
<tr>
<td>On admission</td>
<td>After treatment</td>
<td>On admission</td>
</tr>
<tr>
<td>Group I, II</td>
<td>7.32±0.12</td>
<td>7.48±0.02</td>
</tr>
<tr>
<td>Group III, IV</td>
<td>7.31±0.10</td>
<td>7.32±0.11</td>
</tr>
<tr>
<td>p&lt;0.01</td>
<td></td>
<td></td>
</tr>
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</table>

Table 2. pH and bicarbonate level, p<0.01

<table>
<thead>
<tr>
<th>Inhibition of AChE</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>No significant inhibition</td>
<td>6 (35.3%)</td>
<td>1 (10%)</td>
<td>4 (10.0%)</td>
<td>17 (40.5%)</td>
</tr>
<tr>
<td>AChEe 21-50 %</td>
<td>4 (23.5%)</td>
<td>1 (10%)</td>
<td>15 (37.5%)</td>
<td>16 (38.1%)</td>
</tr>
<tr>
<td>11-20%</td>
<td>6 (35.3%)</td>
<td>5 (50.0%)</td>
<td>20 (50.0%)</td>
<td>7 (16.7%)</td>
</tr>
<tr>
<td>Do 10%</td>
<td>1 (5.88%)</td>
<td>3 (30.0%)</td>
<td>1 (2.5%)</td>
<td>2 (4.7%)</td>
</tr>
<tr>
<td>Total</td>
<td>17</td>
<td>10</td>
<td>40</td>
<td>42</td>
</tr>
</tbody>
</table>

Table 3. Inhibition of AChE, p<0.05
<table>
<thead>
<tr>
<th>AChE reactivation</th>
<th>Group I (9.1%)</th>
<th>Group II (11.1%)</th>
<th>Group III (37.5%)</th>
<th>Group IV (28.0%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No reactivation</td>
<td>1 (9.1%)</td>
<td>1 (11.1%)</td>
<td>12 (37.5%)</td>
<td>7 (28.0%)</td>
</tr>
<tr>
<td>&lt;20%</td>
<td>4 (36.4%)</td>
<td>2 (22.2%)</td>
<td>10 (31.2%)</td>
<td>6 (24.0%)</td>
</tr>
<tr>
<td>20-30%</td>
<td>1 (11.1%)</td>
<td>5 (15.6%)</td>
<td>3 (12.0%)</td>
<td></td>
</tr>
<tr>
<td>&gt;31%</td>
<td>6 (54.5%)</td>
<td>5 (55.5%)</td>
<td>5 (15.6%)</td>
<td>9 (36.0%)</td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td>9</td>
<td>32</td>
<td>25</td>
</tr>
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</table>

Table 4. Reactivation of AChE

<table>
<thead>
<tr>
<th>Therapeutic group</th>
<th>Atropine consumption (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>252.0±116.8</td>
</tr>
<tr>
<td>Group II</td>
<td>318.7±159.6</td>
</tr>
<tr>
<td>Group III</td>
<td>1009.3±505.7</td>
</tr>
<tr>
<td>Group IV</td>
<td>1637.1±760.9*</td>
</tr>
</tbody>
</table>

Table 5. Atropin consumption in different therapeutic groups (p<0.01)
Chapter 19

A Test Battery of Combined Methods to Determine Cytotoxic Effects of Sulfur Mustard and to Investigate Cytoprotective Effects of PARP Inhibitors

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Abstract - Sulfur mustard (SM) is a strong alkylating agent, interacting both with DNA/RNA as well as proteins. As a consequence, apoptotic and/or necrotic cell death is induced. PARP over-activation has been shown as a result of SM-induced DNA damage and may modify the mode of cell death and subsequent inflammation, therefore PARP inhibitors are thought to be beneficial in this situation. Our aim in this study was to distinguish apoptotic from necrotic cell death in HaCaT cells exposed to SM, to quantify cytotoxic events in general and to investigate potential protective effects of PARP-inhibitors. To achieve this, we combined methods to quantify necrosis (ToxiLight BioAssay) with the determination of apoptosis (Cell Death Detection Elisa) and the quantification of the inflammation mediators IL-6 and IL-8.

Adenylate kinase (AK) in the supernatant is an indicator for necrotic cell death. As AK activity can also be quantified from the lysate of surviving cells, the ratio of AK found in supernatant and total AK (lysate and supernatant) serves as an indicator of overall cell survival. Similar reference values can be calculated for nucleosome formation (associated with DNA fragmentation/apoptosis) and the release of inflammation mediators IL-6 and IL-8.

We observed that PARP inhibition reduced necrosis and the release of proinflammatory mediators within the first 4-6 hours post exposure. Apoptosis was amplified after approx. 24 hours indicating a shift from necrosis to apoptosis. This is consistent with recently published results (Steinritz et al. 2007). In summary, the described combination of assays provides more detailed, simultaneous information about cell death and release of proinflammatory cytokines after SM exposure.
Introduction

Sulfur mustard (SM) is a strong vesicating chemical warfare agent that was repeatedly used in military conflicts during the 20th century from World War I to the Iran Iraq War in the 1980s. It is still reason for strong concern as it is easy to produce and handle, creates pronounced toxic effects in case of human exposure and thus, international terror organisations may attempt to use this agent for future attacks. Additional risks persist due to the accidental exposure to SM from World War II ammunitions that had been dumped at sea (Kehe et al., 2005). As of now, no causative therapy exists for SM injuries.

The complex mechanism of SM-induced damage on the molecular and cellular level has only been elucidated in the last decades and even today, some aspects are not fully understood. However, a comparatively early hypothesis that is still valid today had been established by Papirmeister, stating that SM-induced DNA damage may result in an over-activation of poly-ADP-ribose-polymerase or PARP. Under physiological conditions, PARP is an essential enzyme involved in DNA repair, thus maintaining genome stability and cell viability. There is even a demonstrated positive interspecies correlation between physiological PARP activity and life expectancy (Bürkle et al., 2007). However, as NAD is used as a substrate of the reaction catalysed by PARP, excessive PARP activation may deplete the cell of NAD, disabling its capability to produce energy, thus leading to swift necrotic cell death and subsequent inflammation. It was argued that PARP inhibitors may be useful to prevent PARP over-activation and its adverse effects.

The aim of our study was to investigate this hypothesis, possibly identifying candidate substances that might be useful to treat SM injuries and should thus be investigated more thoroughly. To achieve this objective we developed a test battery of combined assays. By doing this, we also aimed to develop and validate a methodology that could be used to investigate SM-induced and other toxic effects in general and to test a much greater variety of therapeutic approaches to counteract cytotoxic effects.

Materials and Methods

Chemicals

SM was obtained from TNO, Rijswijk, The Netherlands. All other chemicals used were reagent-grade products obtained from Sigma (Deisenhofen, Germany).

Cell culture

The HaCaT cells are immortalized human keratinocytes and were kindly provided from Prof. Fusenig (DKFZ, Heidelberg, Germany). The cells were cultured at 37 °C under a 5% CO₂ humidified atmosphere in Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen life technologies, Karlsruhe, Germany) containing 10% fetal calf serum (FCS), glucose, glutamine and sodium pyruvate. Depending on the experimental protocol, cells were either grown in 24-well-plates (100,000 cells per well) or 25 cm² flasks (1.8 million cells per flask). After seeding, cells were cultivated for approx. 20 hours, prior to SM treatment.
Treatment with sulfur mustard and inhibitors

SM was dissolved in ethanol before preparing the final 300 µM dilution in DMEM. No FCS was added to this medium to avoid rapid SM inactivation. Cells were exposed to SM in the absence of FCS at ambient temperature for 30 min. Controls were treated in DMEM, also at ambient temperature for the same duration. Afterwards medium was removed. Immediately after, DMEM, supplemented by 10% FCS was added to the cells. 1 ml of medium was added to each well in 24-well-plates, whereas 8 ml were used in 25 cm² flasks. For PARP inhibition experiments, the medium had been supplemented with 1 mM 3-aminobenzamide (3-AB) or 1 µM 4-aminonaphthylimide (4-AN). Incubation was continued for defined periods of time (2 – 26 hours, depending on the protocol).

ToxiLight BioAssay

The ToxiLight BioAssay (Lonza, Basel, Switzerland) is a reagent kit to detect and quantify adenylate kinase (AK). AK is a ubiquitous enzyme which is essential for cells energy metabolism, i.e. to katalyse the formation of adenosinediphosphate from adenosinetriphosphate and adenosinemonophosphate (ATP + AMP → 2 ADP). Under physiological conditions, AK is never secreted into the extracellular space, thus, a liberation of AK into the supernatant of a cell culture can only occur by membrane damage, i.e. necrotic cell death. In the ToxiLight BioAssay, AK is detected by its physiological reaction as described above. Using the enzyme Luciferase, the chemical energy is used to emit light in a luminescence reaction. The light emitted can be detected and quantified in multiplate reader, such as the Victor 3 (Perkin-Elmer, Wiesbaden, Germany) reader used in our experiments. A dilution series of AK, obtained from cell lysates is used for calibration, allowing the quantification of AK.

Cell Death Detection ELISA plus (CDDE)

To quantify the extent of apoptosis, the Cell Death Detection ELISA plus (Roche, Basel, Switzerland) was used. In the later stages of apoptosis, the chromatine is fragmented into nucleosomes, specific fragments consisting of DNA and a core of histone proteins. Some DNA fragmentation does also occur during necrosis, but it is an irregular process, creating a low number of unspecific, comparatively large DNA fragments. For this reason the formation of mono- and oligonucleosomes within cells is largely specific for apoptotic events. However, DNA released into the extracellular space during a necrotic event is also fragmented into mono- and oligonucleosomes. Thus, in order to obtain information on the extent of apoptosis, the lysate of cells, but not the supernatant had to be assayed.
Interleukine ELISA

Inflammation is an unspecific mechanism of cellular and tissue damage that is also activated in the course of SM injury. To monitor inflammation processes, the interleukines IL-6 and IL-8 were determined from the supernatant of exposed cells using ELISA kits obtained from R&D (Wiesbaden-Nordenstadt, Germany).

Sampling techniques

In 24-well-plates, the entire medium was sampled at the end of the intended incubation time. Cells were washed with phosphate-buffered saline (PBS). PBS was removed, afterwards cells were lysed by a 30 minute treatment with 0.1% Triton-X in PBS. 500 µl of lysis reagent per well were used. Cells were cooled on ice during lysis. Lysates were aliquoted, 250 µl were kept at 4 - 6 °C in a refrigerator for CDDE assays whereas 250 µl were stored at -20 °C for the determination of AK. From 25 cm² culture flasks, 400 µl of supernatant were sampled at any designated point in time. Incubation was continued after sampling. Only after the last sampling (conducted after 6 or 26 hours, depending on protocol), incubation was discontinued and cells were lysed with 5 ml of 0.1% Triton-X in PBS. Again, lysis was conducted for 30 minutes and cells were cooled on ice to prevent AK degradation.

Adenylate kinase as a reference value

When comparing results such as AK liberation, nucleosome formation or interleukine release from different wells or flasks, there is the problem that density of the cell population may vary and influence those results. E.g., a cell population X that has 20% more cells compared to population Y may also release 20% more AK into the supernatant even though the frequency of necrotic events in both populations is actually the same. For this reason, we decided to use the AK, found in the lysate at the specific point in time as a reference value. This amount of AK serves as a parameter for the number of viable cells in a particular population at that specific point in time. When comparing AK liberation, the total AK amount, i.e. AK in the supernatant plus AK in the lysate, was calculated and used as reference value.

Units used

Neither AK nor nucleosomes are available as analytical standards. For this reason, a standard was established and results are given in relation to this standard. For AK, untreated cells were lysed after 24 hours of growth under conditions similar to controls. A dilution series of the lysate was prepared and used for calibration of the ToxiLight BioAssay. As the total AK was used as a reference value, the fraction of AK in the supernatant is used as a parameter for necrotic events. This value (AK in the supernatant divided by total AK) has no dimension.

To obtain a standard for CDDE assays cells were treated with 300 µM SM to induce apoptosis and incubated for 24 hours to allow nucleosome formation. Cells were lysed and a dilution series of the lysate was used for CDDE calibration. AK amounts were used as a reference value. Nucleosome formation and thus apoptotic activity can
only be given in artificial units, e.g. a value of 4 indicates that the amount of nucleosomes, in relation to the AK amount of the same sample, was 4 times higher than the nucleosome amount in the samples used for calibration. In contrast, interleukines IL-6 and IL-8 are available as analytical standards and their precise amount or concentration (in picogram or picogram per milliliter, respectively) can be given.

Results

Anti-Necrotic effect of PARP inhibition in SM exposed cells

Figure 1 depicts the release of AK into the supernatant by SM-exposed HaCaT cells, treated or untreated with 3-AB. Total AK has been used as a reference value, thus the fraction of AK found in the supernatant is shown in Figure 1. SM exposure resulted in an increase of AK liberation after 5-6 h. PARP inhibition by 3-AB reduced that release of AK.

Pro-Apoptotic effect of PARP inhibition in SM exposed cells

Even though we monitored the amount of nucleosomes in cell lysates collected at different points in time, including 1 - 6 hours post-exposure, only in the latter stages of the experiment, i.e. approx. 24 hours post-exposure we observed an increase of that amount in SM-exposed cells. For this reason, only the data nucleosome amount measured 24 hours post-exposure is shown in Figure 2. The amount of AK from the supernatant was used as a reference value. Compared to control values, SM exposure resulted in a pronounced increase of nucleosomes formed compared to controls. PARP inhibition by 3-AB further increased apoptotic activity and thus nucleosome formation. In contrast, 3-AB treatment of controls not exposed to SM has no noticeable effect on apoptotic activity.

Effects of SM poisoning and PARP inhibition on inflammation

As shown in Figure 3 and 4, SM exposure of otherwise untreated cells resulted in a pronounced increase in the liberation of interleukines IL-6 and IL-8, confirming that SM poisoning is associated with inflammation. Our findings, depicted in Figure 3 indicate that, at least 4-6 h after SM exposure PARP inhibition by 3-AB reduced the liberation of IL-6.

The data in Figure 4 were obtained a slightly modified protocol, i.e. the total protein the cell lysate instead of the amount of AK was used as a reference value. Two different PARP inhibitors, 3-AB and 4-aminonaphthyliimide (4-AN) were tested and compared to SM-exposed, but otherwise untreated cells. 3-AB did not reduce the liberation of IL-8, in fact, a slight increase was observed. Treatment with 4-AN, however resulted in a pronounced decrease of IL-8 liberation.
Discussion

Our results clearly indicate an anti-necrotic effect of PARP inhibition, observed in the first 4 - 6 hours post-exposure. Thus, they are in agreement with Papirmeister’s hypothesis that SM-induced DNA damage may lead to PARP over-activation, NAD depletion and subsequent necrotic cell death, a mechanism that can be counteracted by PARP inhibitors. However, as PARP inhibition disables DNA repair, SM-induced damages cannot be repaired and an increase of apoptotic events is observed. Normally, apoptosis is known as a cytotoxic mechanism that is capable to eliminate cells whilst avoiding the release of cytoplasm into the extracellular space or inflammatory reactions. Cells that undergo apoptotic cell death release so-called apoptotic bodies, small portions of cytoplasm still surrounded by an intact membrane. Viable cells in the surrounding are capable to phagocytose and thus eliminate these apoptotic bodies before they can possibly disintegrate. This well-orchestrated mechanism is the reason for the apparent “disappearance” of apoptotic cells, leaving neither fragments nor inflammation as a trace of the apoptotic event. However, if an entire cell population has been exposed to a strong apoptosis inducer such as SM, this useful and protective mechanism is no longer working. When most of the cells in a particular population have been damaged by SM and apoptosis has been induced, they are no longer capable to phagocytose the apoptotic bodies from nearby cells that also undergo apoptosis. When apoptotic bodies cannot be eliminated, their membrane integrity will ultimately be lost and cytoplasm will be released into the supernatant. This event, when despite an initially apoptotic mechanism, membrane rupture cannot be prevented is known as secondary necrosis.

Results indicate that PARP inhibitors also help to reduce inflammation in the early period (1 – 6 hours) post-exposure. This is in agreement with the early anti-necrotic activity of PARP inhibitors described above.

What does that mean with regard to the potential use of PARP inhibitors as active pharmaceutical ingredients to treat SM injuries? One important aspect to understand is that there are two distinct mechanisms of apoptosis, intrinsic due to DNA damage of the cell that will undergo apoptosis and extrinsic, when a specific ligand binds to a “death receptor”, triggering apoptosis in a cell which had been previously intact. Apoptosis, initiated over the intrinsic pathway may even be a useful mechanism, eliminating cells with damaged DNA and thus preventing serious long-term adverse health effects such as cancer formation. In contrast, apoptosis induced over the extrinsic pathway is - in the context of SM poisoning – a rather harmful mechanism as it may trigger the destruction of cell that haven’t been damaged (or at least not been irreversibly damaged) by the primary SM insult. Thus, it would be important to understand whether PARP inhibition also amplifies the extrinsic pathway of apoptosis. If and when the factors involved in the initiation of extrinsic pathway apoptosis could be identified and possibly be inactivated through immunological mechanisms, excessive apoptosis and the related adverse health effects could be avoided whilst the primary effect of PARP inhibitors to prevent necrosis could be used to create a therapeutic benefit. From the data presented in Figure 4 we had also learned that the effect of PARP inhibitors on inflammation may vary from substance to substance. In that experiment, 4-AN reduced IL-8 liberation (at least, in the short term) whilst 3-AB did not. Thus, a greater variety of PARP inhibitors should be tested, possibly identifying a substance that has strong
and sustained anti-inflammatory effects. Perhaps the most important aspect is that whilst PARP over-activation needs to be prevented in order to prevent swift necrotic cell death, in the interest of a beneficial therapeutic outcome PARP should not be totally disabled. The ideal PARP inhibitor would prevent excessive PARP activation and its adverse effects whilst allowing a residual PARP activity necessary for DNA repair and cell survival. In this case, excessive apoptosis, including apoptosis initiated on the intrinsic pathway could be avoided and the initial effects of PARP inhibition could be used to create an overall therapeutic benefit.

**Conclusion**

We have successfully established a test battery of combined assays that is able to produce valid data on cytotoxic effects in skin cells and can be used to assess the protective effects of potential Active Pharmaceutical Ingredients. The system is versatile as additional assays can be integrated into the test battery and the effects of different toxic and/or protective substances can be investigated. Different cell lines may also be used in similar investigations, however this may necessitate changes of the cell culture medium and additional validation efforts.

Regarding the effect of PARP inhibitors, an early anti-necrotic effect could be confirmed, however, due to the pro-apoptotic effect that has also been observed, the overall benefit is still in question. Further investigation is needed to identify candidate substances which have an overall beneficial effect and may be suitable for preclinical, i.e. animal studies.

**Reference**

Figure 1: AK in supernatants, indicating necrotic cell death in SM-exposed cells and controls with/without PARP inhibition

Means ± SD from three independent samples

ut-C: untreated control; i.e., no PARP inhibitor, no SM exposure
ut-SM: untreated, SM-exposed; i.e., no PARP inhibitor, 300 µM SM exposure
3-AB-C: 3-AB treated control; i.e., 1 mM 3-AB, no SM exposure
3-AB-SM: 3-AB treated, SM-exposed; i.e. 1 mM 3-AB, 300 µM SM exposure
Figure 2: Nucleosomes in lysates, indicating apoptosis in SM-exposed cells and controls with/without PARP inhibition

Means ± SD from three independent samples

- **ut-C**: untreated control; i.e., no PARP inhibitor, no SM exposure
- **ut-SM**: untreated, SM-exposed; i.e., no PARP inhibitor, 300 µM SM exposure
- **3-AB-C**: 3-AB treated control; i.e., 1 mM 3-AB, no SM exposure
- **3-AB-SM**: 3-AB treated, SM-exposed; i.e. 1 mM 3-AB, 300 µM SM exposure
Fig. 3: IL-6 in supernatants, indicating inflammation in SM-exposed cells and controls with/without PARP inhibition

Means ± SD from three independent samples
ut-C: untreated control; i.e., no PARP inhibitor, no SM exposure
ut-SM: untreated, SM-exposed; i.e., no PARP inhibitor, 300 µM SM exposure
3-AB-C: 3-AB treated control; i.e., 1 mM 3-AB, no SM exposure
3-AB-SM: 3-AB treated, SM-exposed; i.e. 1 mM 3-AB, 300 µM SM exposure
Figure 4: IL-8 in supernatants, indicating inflammation in SM-exposed cells and controls with/without PARP inhibition

Means ± SD from two independent samples

ut-C: untreated control; i.e., no PARP inhibitor, no SM exposure
ut-SM: untreated, SM-exposed; i.e., no PARP inhibitor, 300 µM SM exposure
3-AB-C: 3-AB treated control; i.e., 1 mM 3-AB, no SM exposure
3-AB-SM: 3-AB treated, SM-exposed; i.e. 1 mM 3-AB, 300 µM SM exposure
4-AN-C: 4-AN treated control; i.e., 1 nM 3-AB, no SM exposure
4-AN-SM: 4-AN treated, SM-exposed; i.e. 1 nM 3-AB, 300 µM SM exposure
Chapter 20

Delayed Complications of Sulfur Mustard Poisoning in Iranian Veterans

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Abstract - Sulfur mustard (SM) is a chemical warfare agent that was widely used during the World War I and in the Iran-Iraq conflict in 1983-1988. It may also be used as a chemical terrorism. SM is an alkylating agent that affects DNA synthesis. Its delayed complications have been considered since the World War One. Delayed complications of SM poisoning in different organs and their severity correlations were studied in Iranian veterans. Haematological and immunological investigations were also performed on a control group of 35 healthy male subjects. Forty male patients (aged 43.8 ± 9.8 years) with confirmed SM poisoning 16–20 years after SM exposure were studied. The most common complications were found in the lungs (95%), peripheral nerves (77.5%), skin (75%), and eyes (65%). WBC, RBC, hematocrit (HCT), IgM, C3, and the percentages of monocytes and CD3+ lymphocytes were significantly (P < 0.042) higher and the percentage of CD16 + 56 (natural killer) cells was significantly (P = 0.006) lower in the patients than in the control group. The severity of respiratory complications revealed a significant correlation with the severity of ocular complications (r = 0.322, P = 0.043), as well as with the haemoglobin (r = 0.369, P = 0.024) and HCT (r = 0.470, P = 0.003). Although late complications of SM poisoning in the skin, eyes, and respiratory system are mainly due to its direct toxic effects, the neuromuscular, haematological and immunological complications are probably the result of systemic toxicity. Cardiovascular complications as myocardial perfusion abnormalities and coronary ectasis have recently been observed in these patients. Further studies on larger groups of veterans and controls with more details particularly on cardiovascular complications are now undertaking.

Key words - Sulfur mustard, chemical warfare agent, poisoning, complication.
Introduction

Brief chemistry and physical characteristics:
Sulfur mustard (SM) is bis(2-chloroethyl) sulphide which is most commonly referred to as mustard gas, was first synthesized in 1822 by Despretz. SM is a colourless oil if pure but normally ranges from pale yellow to dark brown with light garlic or horseradish type odour as the Iranian veterans also described. Its density is 1.27 g/ml, melting point of 14.4 °C and boiling point of 217 °C. SM is only 0.05% soluble in water (1,2).

SM is generally regarded as a “persistent” chemical agent because of its low volatility. In cool weather there is little vapour; however, at higher temperatures, such as those in the Middle East during the hot season (38°C to 49°C), mustard vapor becomes a major hazard (1-3).

Brief historical uses
SM has been the most widely used chemical warfare agent (CWA) in the past century. It was first employed extensively in WWI between 1914 and 1918. In spite of the Geneva Protocol in 1925 on the prohibition of CWA, SM was used by Italian troops in Ethiopia (1935–36) and by Egyptian forces in Yemen (1963–67). The greatest military use of SM was by the Iraqi Army against Iranian soldiers and even civilians in Sardasht and Halabjah between 1983 and 1988, resulting in over 100,000 chemical casualties (4,5).

Types and routes of exposure
Based on the situation involved, several types of exposure including single, multiple, secondary, sub-clinical and chronic may occur.
Most human cases of SM poisoning have occurred during armed conflicts and most accidents were a single exposure (1-5). Multiple low SM exposure occurred occupationally and during the WWI and in the Iran-Iraq conflict (4, 5). First aid workers, nursing and medical staff who were looking after SM casualties in the field clinics and hospitals during the Iraq-Iran war without proper personal physical protection, have become intoxicated. Some of them are now suffering from the delayed toxic effects of SM and have disabilities of 5 to 25% (4).

Low level SM exposure with or without symptoms, but with delayed or long term health effects has been described in details (6-9). Sub-clinical exposure to SM in some Iranian combatants induced delayed toxic effects. A study on 77 subjects, who were present in a contaminated area and had no acute symptoms or signs at the time of exposure, are now suffering from respiratory disorders such as bronchiectasis and bronchiolitis obliterans (10).

Chronic SM exposure is usually occupational. Some factory workers in Japan and in the UK were reported to have had SM poisoning and even malignancies due to SM (11,12).

Inhalation is the major route of exposure which induces respiratory and systemic toxicity.
following absorption across the lung surface (3, 4, 13). However, SM is a vesicant or blistering agent that has direct toxic effects on the skin producing erythema, blistering, epidermolysis and necrosis. It is a lipid soluble compound and thus can be readily absorbed across the skin (4, 14).

The eyes are the most sensitive organs to SM. This marked susceptibility is attributable to several ocular features including the aqueous–mucous surface of the cornea and conjunctiva as well as the high turnover rate and intense metabolic activity of the corneal epithelial cells (15, 16).

SM may also enter the body by oral ingestion. We had observed a few Iranian combatants during the war who had ingested food contaminated by SM and that subsequently became intoxicated. They experienced nausea, vomiting, haematemesis, abdominal pain and dyspnea. SM may also be absorbed through the lower gastrointestinal tract (4). Injection is a very rare route of SM intoxication and has not been reported in man.

_Human Toxicity:_
Exposure to very high doses of SM in the field may induce convulsions and death in less than one hour (3, 4, 18, 19). Such observations have not been reported during the Iraq-Iran war. Acute toxic effects generally appear after variable periods of latency depending on the dose, mode of exposure, the environmental temperature, and on each individual (2, 4, 17-19).

Sub-acute exposure occurred during the Iran-Iraq war and in the workers in SM munitions factories. However, this type of exposure may present as a mild acute SM intoxication or as a complication in the respiratory tract or even as malignancy (20, 21).

Delayed toxic effects of SM have been documented. The first report of delayed toxic effects in Iranian veterans was reported in 1986 (22). Several articles on the delayed toxic effects and complications of SM in Iranian veterans have been published since then (8, 10, 13, 14, 16, 18, 22).

Several studies suggest that workers who were chemically exposed to mustard agents in British and Japanese munitions factories developed chronic respiratory effects. In a cohort mortality study of 3500 workers at a manufacturing plant in England, a statistically significant increase in the number of deaths due to influenza, pneumonia, bronchitis and asthma were reported. This was present even among those with less than three years of employment at the plant (21).

A 25-year follow-up study of workers exposed to SM in a Japanese production plant revealed that more highly exposed workers had more chronic bronchitis and a slightly lower FEV1/FVC ratio than either the less-exposed or an unexposed group of their co-workers (12). In another study, Brown reported on a large number of employees, who were working at the Huntsville Arsenal in Alabama. They were continuously exposed to SM gas over long periods of time and developed bronchiectasis with progressive emphysema and narrow attenuated bronchioles (11).
Mechanism of toxicity
The mono-functional mustards have one alkylating site that can be attacked by electron-rich nucleobases of DNA. The major alkylating site of nucleic acids of mammalian origin is the nitrogen residue of guanine (23). Although SM reacts with RNA, proteins, and phospholipids, the consensus view is that alkylated DNA, plays an important role in delayed toxic effects (24, 25). Cell death from DNA cross-linking is delayed until the cell replicates its DNA or undergoes division. At higher cellular exposures, however, mechanisms other than DNA cross-linking become important and produce more rapid cell death. The acute damage to the cornea, mucous membranes, and skin seen following SM exposure is possibly induced by different other mechanisms. One of these mechanisms that may be involved in acute damage is nicotinamide adenine dinucleotide (NAD) depletion. Other potential mechanisms of cell death are related to rapid inactivation of sulfhydryl containing proteins and peptides, such as glutathione. These sulfhydryl compounds are critical in maintaining the appropriate oxidation-reduction state of cellular components. Glutathione is also thought to be critical in reducing reactive oxygen species in the cell and preventing peroxidation and loss of membrane integrity (26, 27). Tumor necrosis factor-α is involved in SM induced skin lesion (28).

Target organs
Acute toxic effects of SM on the eyes, respiratory tract and the skin are more prominent than the others. Eyes are the most sensitive organs to SM. The first symptoms of SM exposure are usually those on the eyes. (4, 18-20, 29). Next to the eye lesions, the greatest discomfort produced by mustard gas results from irritation and toxicity of the respiratory system. Respiratory effects occur in a dose dependent manner from the nasal mucosa to the terminal bronchioles (18, 20). The most apparent consequence of SM exposure is on the skin which is why SM is also referred to as vesicant or blistering agent. A German and an Iranian medical toxicologist (first author) classified the cutaneous mustard gas lesions which describes under the clinical manifestations (30).

Gastrointestinal (GI) effects following SM exposure have been documented in some studies. Destruction of the mucosa and shedding of the epithelial elements, however, begin days after exposure, resulting in loss of large volumes of fluid and electrolytes. (31). Acute gastroduodenitis with hemorrhagic erosions, acute desquamative enteritis, and severe hemorrhagic necrotic colitis were reported in First World War veterans (32) but not observed in the Iranian veterans.

Extremely heavy exposure to SM can cause central nervous system (CNS) excitation leading to convulsions in animals (17). Balali-Mood and Navaeian (1986) reported convulsions in six Iranian veterans who were hospitalized during the early stages of their intoxication (20). Most casualties from WWI and from the Iran-Iraq conflict, however, revealed mild and very nonspecific neurological effects such as headache, anxiety, fear of the future, restlessness, confusion, and lethargy (20, 32). A frequent long-term complication in patients exposed to SM is delayed neuropathic symptoms, which are underrepresented in most previous studies (33).
Haemato-immunological effects
SM as an alkylating agent, is particularly toxic to rapidly proliferating cells such as lymphoid and bone marrow cells. Leukocytosis is common within the first few days after exposure. White blood cell (WBC) counts then begin to drop on the third and fourth days after exposure and reach their minimum level around the ninth day. This leukopenia is followed by a decrease in megakaryocytes and finally in the erythropoietic series (34-36). Bone marrow biopsies have shown hypocellular marrow and atrophy involving all elements (36). If cytopenia is not marked and there are still remaining stem cells, recovery will take place as the patient recovers (35-39). The bone marrow studies reveal a severe decrease in cellularity and fat replacement, and nuclear changes, such as budding, double nuclear, and kariorrhexis in erythrocyte precursors. The toxic effects of SM on the haematopoietic system are dose dependent and it is concluded that SM causes aplastic or ineffective haematopoiesis (38). Severe leukopenia, however, is an ominous sign, leading to secondary infections and higher mortality rates in these patients. SM victims with WBC counts of 200 cells/mL or less died during their initial admissions (34).

SM poisoning could result in the impairment of both humoral and cellular immune functions (40-42). Along with the appearance of clinical disorders, both C3 and C4 titers showed an increase, followed by a gradual decrease over one year. The majority of SM-exposed patients had increased levels of IgG and IgM during the first weeks and up to the 6th month after exposure (42). Depression of cell-mediated immunity has been observed in the Iranian veterans one, two, and three years after exposure (41). Natural killer (NK) cells, which are known to be one of the most important components of the cellular immunity, have been found to be significantly lower in patients with severe respiratory complications 10 years after exposure (42). In a controlled study, the number of NK cells was still significantly lower 16 to 20 years after exposure (8,37).

1. Patients and methods
The Veteran Foundation of Mashhad provided us with the files of all CWA poisoned patients in the province of Khorasan Razavi, Iran. We reviewed the files and selected the patients who met the following criteria: (a) documented exposure to SM, as confirmed by toxicological analysis of their urine and vesicular fluid during the war, (b) severe clinical complications due to SM poisoning in at least one of the target organs of lungs, skin or eyes. SM was used as a bomb or shell by the Iraqi army against the Iranian troops and thus the veterans exposed mainly to SM vapour. Severe respiratory complications were defined as a forced expiratory volume in the first second (FEV1) of <40% of predicted value or forced vital capacity (FVC) of <50% of predicted value on spirometry. Severe cutaneous complications were defined when >36% of total body surface area revealed burn scars. Severe ocular complications were defined when there was evidence of corneal lesions due to SM exposure. Patients with proven systemic illnesses before exposure to SM (one patient) and cigarette smokers (three patients) were excluded from the study. Forty-seven male subjects fulfilled the above criteria. Of these, 40 patients volunteered to participate in the study and signed the informed written consent. After approval by the medical ethics committee of the university, the patients were hospitalized in groups of 4 or 5 in the Toxicolo-
S. Tonev, K. Kanev, C. Dishovsky

gy Ward of Imam Reza Hospital, where they underwent a thorough history and physical examination by the experienced physicians.

Haematological and immunological studies were also performed on a control group consisting of 35 age-matched healthy men who had no history of exposure to SM. Severity of complications in the skin, eyes and respiratory system of all the patients were classified into four grades, using the criteria shown in Table I. Grading was not performed for the neuromuscular complications due to lack of a generally accepted grading system in this field.

Pulmonary investigations

Pulmonary function test (PFT) parameters were measured, using a flow-sensing spirometer (FUDAC 50; FUKUDA Sangyo, Chiba, Japan) [16]. PFT variables were recorded before and 5 minutes after two puffs of salbutamol (100 mg/puff) and expressed as percentages of predicted values. Arterial blood gas (ABG) analysis was performed with AVL 995 Blood Gas Analyzer (Biomedical Instruments, Graz, Austria). High resolution computed tomography (HRCT) scanning of the chest was obtained from each patient, using a High Speed General Electric CT unit (General Electric Medical Systems, Milwaukee, WI, USA). Patients were scanned at fully suspended inspiration with 1.5–2.0 mm sections taken ABG and PFT results were interpreted by the pulmonologist, using the normal standards described by Andreoli et al. [17] and Boskabadi et al. [18], respectively. HRCT of the lungs were interpreted by a chest radiologist and assessed for reticular densities and honeycombing, as well as for the radiologic evidence of bronchiectasis [19]. Patients who had a history of cough and sputum production in the absence of bronchiectasis were diagnosed as COPD if they revealed an irreversible obstructive pattern on spirometry and as simple chronic bronchitis when there was no obstructive pattern on spirometry. Patients in the asthma group had typical attacks of dyspnoea, wheezing and nocturnal cough with >15% increase in the FEV1 after bronchodilator inhalation. Large airway narrowing was diagnosed according to the bronchoscopic and spirometric findings.

Dermal histopathological procedures

Skin biopsy was performed by the dermatologist for only eight patients who had more distinct dermal lesions. For light microscopy, tissues were fixed in 10% phosphate buffered (pH 7.0) formalin, dehydrated and embedded in paraffin. Serial sections of 3-μm thickness were made and stained with haematoxylin and eosin. For electron microscopy, specimens were immediately fixed in 2.5% glutaraldehyde and 0.1 mol/L of phosphate buffer (pH 7.2). After fixation in 1% osmium tetroxide and 0.1 mol/L of phosphate buffer, the samples were dehydrated in graded series of alcohol (30–100%) and embedded in epoxyresin. Ultrathin (70 nm) sections were collected on 200-mesh copper grids for staining with 2% uranyl acetate and 5% lead citrate. Examinations were undertaken by the consultant clinical pathologist, using LEO 910 transmission electron microscope (Zeiss, Oberkochen, Germany).

Electrophysiologic procedures

Electromyography (EMG) and nerve conduction velocity (NCV) were performed by the neurophysiologist, using MEDELEC MS6 electromyograph (MEDELEC, Oxon, UK).
EMG of the abductor digitorum, opponens pollicis, extensor digitorum communis, tibialis anterior, and gastrocnemius, extensor and flexor digitorum brevis muscles of both extremities were performed. NCV of the median, ulnar, tibial (medial plantar) and deep peroneal motor nerves and relevant sensory nerves of both extremities were also carried out. The results were interpreted according to the normal standards [20].

Haematological and immunological procedures
Peripheral venous blood samples were taken using standard procedures. Haematocrit (HCT) and haemoglobin (Hb) levels, as well as total counts for white blood cells (WBC), red blood cells (RBC), and platelets (PLT), were measured, using autoanalyzer Technicon H1 (Bayer Medical Systems, New York, NY, USA). Flowcytometry was used to quantify leucocyte populations. Sample processing was carried out directly from whole blood and the percentages of lymphocytes, monocytes, and neutrophils, as well as CD3, CD4, CD8, CD19 and CD16 + 56 positive lymphocytes were determined, using FACS Calibur cytometer (Becton Dickinson, San Jose, CA, USA), equipped with Cellquest software. Serum immunoglobulin concentrations of IgA, IgG, IgM, and complement factors of C3 and C4 were measured, using SRID quantification kits (Biogene, Mashhad, Iran). Serum IgE level was determined using ELISA (Radims, Roma, Italy).

Statistical analysis
All data were expressed as mean ± SD. Mann–Whitney U-test was used for comparisons involving two groups. The severity of dermal, ocular and respiratory complications were compared with each other as well as with the haematological and immunological parameters, using Spearman’s rank correlation test. SPSS version 13.0 (SPSS Inc., Chicago, IL, USA) was used throughout with the minimum level of significance at \( P = 0.05 \).

2. Results
The age range of the patients was 32–76 (43.8 ± 9.8) years and that of the control group was 26–55 (42.1 ± 8.8) years (\( P = 0.479 \)). The patients were studied 16–20 (18.0 ± 1.5) years after their initial exposure. Except for the six patients who had two documented history of SM exposure, all the other patients were exposed to the agent only once. The most common complications were found in the lungs (95%), peripheral nerves (77.5%), skin (72.55%) and eyes (67.5%) as shown in table 1.

<table>
<thead>
<tr>
<th>Organs</th>
<th>Number of patients</th>
<th>Percentages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Respiratory tract</td>
<td>38</td>
<td>95</td>
</tr>
<tr>
<td>Peripheral nerves</td>
<td>30</td>
<td>75</td>
</tr>
<tr>
<td>skin</td>
<td>29</td>
<td>72.5</td>
</tr>
<tr>
<td>Eyes</td>
<td>27</td>
<td>67.5</td>
</tr>
</tbody>
</table>
Respiratory complications
All patients complained of coughing and expectoration, 85% of dyspnoea and 60% of haemoptysis. The main objective clinical findings were wheezing (95%), crackles (50%) and stridor (10%). Mild (60 ≤ PaO2 < 85) and moderate (45 ≤ PaO2 < 60) hypoxaemia were found in 67.5 and 27.5% of the patients, respectively. PaO2 was normal (≥85) in other patients (5%). Pulmonary function tests revealed obstructive pattern in 23 (57.5%), restrictive in nine (22.5%), mixed in six (15%), and a normal pattern in two (5%) patients.
HRCT revealed bronchiectasis and pulmonary fibrosis in 37.5 and 7.5% of the patients, respectively. Bronchiectatic lesions were most commonly observed in the left and right lower lobes (eight and seven patients), followed by the right middle lobe (six patients) and the lingula segment (four patients). Right and left upper lobes were involved in only one patient.
From the 24 patients who underwent bronchoscopy, seven patients revealed large airway narrowing. Sites of narrowing were found in the main bronchus of the right middle lobe (four patients), trachea (two patients) and lingula (two patients). Based on the clinical and paraclinical findings, common respiratory complications were diagnosed as COPD in 14 (35%), bronchiectasis in 13 (32.5%), asthma in 10 (25%), large airway narrowing in six (15%), pulmonary fibrosis in three (7.5%), and simple chronic bronchitis in two (5%) patients. Severity grading of respiratory complications was determined as two (5%) patients in grade 1, 11 (27.5%) in grade 2, 14 (35%) in grade 3 and 13 (32.5%) in grade 4. As there were only two patients in grade 1 of the respiratory complications, we did not consider this group in the correlation testing of the respiratory complications with haematological and immunological parameters.

Neuromuscular complications
The most common motor nerve disorders were found in the left (37.5%) and right (35%) tibial followed by the right (20%) and left (12.5%) peroneal nerves. The most common sensory nerve disorders were found in the left tibial (75%) and right peroneal (72.5%) nerves. NCV disturbances were generally more common in the sensory nerves compared with the motor nerves, and more common in the lower extremities than in the upper extremities. Sensory and motor nerve disturbances in both upper and lower extremities were mostly symmetrical. Electromyographic recording revealed a normal pattern in 24 (60%) patients, incomplete interference with normal amplitude in six (15%) and incomplete interference with low amplitude in 10 (25%) patients.

Dermal complications
Five (12.5%) patients had no skin complaints. The most common symptoms in other patients were itching (65%) and a burning sensation (20%). The signs were recorded as hyper-pigmentation (55%), erythematous papular rash (42.5%), dry skin (40%), multiple cherry angiomas (37.5%), atrophy (27.5%), hypo-pigmentation (25%), hair loss (10%), eczema (7.5%) and hypertrophy (2.5%). None of the patients revealed recurrent blisters. These complications were located on the genital areas (47.5%), the back (47.5%), the front thorax and abdomen (45%), lower extremities (mainly inguinal) (45%), upper extremities (mainly axillary) (40%), and the head and neck (15%).
ity grading was determined as 12 (30%) in grade 1, 10 (25%) in grade 2, eight (20%) in grade 3, and 10 (25%) patients in grade 4.

Cutaneous histopathological findings
Light microscopy of the skin specimens of eight patients revealed atrophic scar characterized by epidermal atrophy, hyperkeratosis, basal layer hyper-pigmentation, atrophy of the appendages, non-specific dermal fibrosis and perivascular mononuclear inflammatory infiltrate within dermis. Electron microscopy also revealed increased number of melanosomes within epidermis and increased collagen fibres with some inflammatory cells within dermis (Figure 1).

Ocular complications
Only one patient had no complaints of eye complications. The symptoms were recorded as itching (42.5%), burning sensation (37.5%), photophobia (30%), tearing (27.5%), reading difficulties (10%), red eye (10%), eye pain (2.5%) and foreign body sensation (2.5%). The most common objective findings were found in the following order: chronic conjunctivitis (17.5%), peri-limbal hyperpigmentation (17.5%), vascular tortuosity (15%), corneal thinning (15%), limbal ischaemia (12.5%), corneal opacity (10%), corneal vascularization (7.5%) and corneal epithelial defect (5%). Severity grading was determined as 14 (35%) in grade 1, 15 (37.5%) in grade 2, five (12.5%) in grade 3, and six (15%) patients in grade 4. The correlation between severity grades of complications in different target organs Comparison of the severity grades of respiratory, skin and eye complications revealed a significant correlation only between the severity of respiratory and eye complications (r = 0.322, P = 0.043) as shown in Figure 2. No significant correlation was found between the severity of dermal complications and either respiratory (r = 0.011, P = 0.947) or ocular (r = 0.068, P = 0.679) complications.

Haematological and immunological findings
Haematological and immunological changes of the 40 patients in comparison with 35 controls are summarized in Table 2. Total WBC and RBC counts and HCT level were significantly higher in the patients than in the control group. The percentages of monocytes and CD3+ lymphocytes were significantly higher and the percentage of CD16 + 56 positive lymphocytes (NK cells) was significantly lower in patients than in the control group. Other haematological and flow-cytometric parameters did not show any significant difference between the two groups. Serum IgM and C3 levels were significantly higher in the patients in comparison with the controls. Other immunoglobulins and complement factors did not show any significant difference between the two groups. Haematological and immunological parameters of the patients were compared with the severity grades of target organ complications. While RBC count and Hb level revealed a significant positive correlation with the severity of respiratory complications, the HCT level and total WBC and PLT counts did not show any significant correlation with the severity of respiratory complications. Except for the significant positive correlation between Hb level and the severity of ocular complications (r = 0.341, P = 0.031). No other significant correlation was found between haematological parameters and the severity grades of dermal or ocular complications.
Table 2. Haematological and immunological changes in 40 patients 16 to 20 years after severe SM intoxication comparing with 35 healthy subjects.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Patient Mean ± SD</th>
<th>Control Mean ± SD</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (1000/mm³)</td>
<td>7.24 ± 1.90</td>
<td>5.79 ± 1.12</td>
<td>0.025</td>
</tr>
<tr>
<td>RBC (Million/mm³)</td>
<td>5.46 ± 0.45</td>
<td>5.19 ± 0.28</td>
<td>0.035</td>
</tr>
<tr>
<td>Hb (mg/dL)</td>
<td>15.9 ± 0.7</td>
<td>15.6 ± 0.7</td>
<td>0.223</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>48.3 ± 3.5</td>
<td>45.5 ± 1.9</td>
<td>0.047</td>
</tr>
<tr>
<td>PLT (1000/mL)</td>
<td>255 ± 99</td>
<td>238 ± 10.1</td>
<td>0.594</td>
</tr>
<tr>
<td>Lymphocyte (%)</td>
<td>31.5 ± 8.4</td>
<td>30.5 ± 10.8</td>
<td>0.651</td>
</tr>
<tr>
<td>Monocyte (%)</td>
<td>4.8 ± 1.6</td>
<td>3.9 ± 1.1</td>
<td>0.013</td>
</tr>
<tr>
<td>Polynuclear (%)</td>
<td>63.8 ± 8.7</td>
<td>65.4 ± 8.7</td>
<td>0.327</td>
</tr>
<tr>
<td>IgA (mg/dL)</td>
<td>302.6 ± 142.1</td>
<td>233.1 ± 59.3</td>
<td>0.154</td>
</tr>
<tr>
<td>IgM (mg/dL)</td>
<td>235.3 ± 84.8</td>
<td>136.8 ± 58.3</td>
<td>0.0001</td>
</tr>
<tr>
<td>IgG (mg/dL)</td>
<td>1438.6 ± 485.1</td>
<td>1140.0 ± 244.2</td>
<td>0.065</td>
</tr>
<tr>
<td>C3 (Micg/dL)</td>
<td>109.8 ± 30.1</td>
<td>90.9 ± 14.8</td>
<td>0.030</td>
</tr>
<tr>
<td>CD3 (%)</td>
<td>71.1 ± 8.6</td>
<td>65.6 ± 10.7</td>
<td>0.037</td>
</tr>
<tr>
<td>CD16+5 (NK cells%)</td>
<td>11.6 ± 5.8</td>
<td>17.5 ± 9.6</td>
<td>0.006</td>
</tr>
</tbody>
</table>

A significant negative correlation was observed between C4 and the severity of skin complications ($r = 0.440$, $P = 0.005$). The other serum immunoglobulins and complements had no significant correlations with the severity of complications in different target organs. No significant correlations were found between flow cytometric parameters and the severity grades of respiratory, skin or eye complications.

3. Discussion
During the Iran–Iraq war, about 100,000 people suffered from SM exposure and now after 20 years, around 40,000 veterans are struggling with delayed effects (4,5,10,74). The first report of delayed toxic effects in Iranian veterans was reported in 1986 (22). The most prominent late clinical effects were observed in the respiratory tract (78%), neuropsychiatrics systems (45%), skin (41%) and eyes (36%) (22,26).

Long Term Complications:
Information on the long-term effects of SM comes from two major sources of investigations: Firstly, the studies of soldiers who were exposed to the agent on the battlefield and secondly the studies of workers who were employed in mustard gas factories (occupational exposure). While long term effects following battlefield exposure are referred to as “late” or “delayed” complications, the term “chronic” seems to be more suitable for the complications caused by occupational exposure. It must also be emphasized that delayed effects generally occur some months or years after a single or brief exposure and are not the same as chronic poisoning, which comes from continuous intake of the poison over a relatively long period of time. The first report on the delayed toxic effects of SM poisoning in 236 Iranian veterans revealed that the most common effects were on the respiratory tract (78%), CNS (45%), skin (41%), and
the eyes (36%). These effects were recorded between 2 and 28 months after exposure (22). Comparison of early (one week after exposure) and late (two years after exposure) toxic effects of SM poisoning in 77 CWA victims indicated that eye lesions do not change significantly, dermal complications tend to decrease, and respiratory complications generally deteriorate over the years (6, 8, 13, 16, 43). In a study (44) on 34,000 Iranians, 13 – 20 years after exposure to SM, the most common complications were found in the lungs (42.5%), eyes (39%), and the skin (24.5%) (23). In a group of 40 severely intoxicated Iranian veterans in Mashhad, 16 – 20 years after their initial exposure, the most commonly affected organs were lungs (95%), peripheral nerves (75%), skin (72.5%), and the eyes (67.5%) as shown in table 1 (8).

Respiratory
Respiratory complications are the greatest cause of long-term disability among people with SM exposure. A triad of cough, expectoration, and dyspnea has been found to be present in more than 80% of Iranian veterans three years after their initial exposure (6, 45). Haemoptysis (mainly streaky), chest tightness, chest pain, and nocturnal dyspnea are also frequent. The main objective clinical findings are generalized wheezing (the most common sign), crackles, decreased lung sounds, clubbing, and cyanosis dyspnea are also frequent. Pulmonary function testing has revealed more obstructive patterns than restriction and about half of these obstructive spirometric results are reversible in response to inhaled bronchodilators. FVC, FEV1, and FEV1/FVC (FEV1%) have all been found to be significantly lower in SM intoxicated veterans in comparison to healthy nonexposed subjects and CWA survivors who had used a gas mask at the time of attack (4, 8, 45, 43). Abnormal spirometric findings in general, and restrictive patterns in particular, tend to increase over time (4, 8, 43). A study on 77 subjects, who were present in a contaminated area and had no acute signs and symptoms at the time of exposure, but now have respiratory disorders, indicates that sub clinical exposure to SM can be responsible for the occurrence of delayed respiratory complications such as bronchiectasis and bronchiolitis obliterans (10). Chest X-ray (CXR) findings in patients with late respiratory complications of SM have been described as increased bronchovascular markings, hyperinflation, bronchiectasis, pneumonic infiltration, and radiologic evidence of pulmonary hypertension (8, 43). However, CXR is not sensitive enough for the detection of respiratory complications in these patients and high resolution computed tomography (HRCT) of the chest may be required as the diagnostic imaging procedure of choice (8, 46, 47). A study (48) of 197 Iranian veterans 10 years after a single heavy exposure to SM revealed the development of a series of delayed destructive pulmonary sequelae such as chronic bronchitis (58%), asthma (10%), bronchiectasis (8%), large airway narrowing (9%), and pulmonary fibrosis (12%). Each of these complications is described in more detail below.

1. Chronic bronchitis: Several studies have reported chronic bronchitis as the most common late complication of the respiratory system resulting from war exposure to mustard gas (8, 10, 45, 48-52). Hypoxemia and hypercapnea are commonly observed in moderate to severe cases, leading to cor pulmonale and respiratory failure in the final stages of the disease (4, 8, 45, 48).
Infection of the respiratory tract, resulting in bronchopneumonia, is also a common problem, often complicated by septicemia (4, 8, 19).

2. Asthma: Airway hypersensitivity, manifested as typical attacks of breathlessness, wheezing, and nocturnal cough, as well as a reversible obstructive pattern on pulmonary function tests, have been reported between four weeks to twenty years after SM inhalation. Patients with chronic bronchitis may also have some degree of bronchospasm, which does not respond to bronchodilators. Attacks of bronchospasm are characteristically triggered by respiratory infections, environmental allergens, and cold weather (48-55). New techniques, such as impulse oscillometry (ISO), has been used for evaluation of airway dysfunction. However, it was found less sensitive than spirometry in spotting small airways obstructions. IOS is a good diagnostic method in the detection of pulmonary involvements in uncooperative patients (56).

3. Bronchiectasis: Direct effects of SM on the bronchial wall mucosa and, more recurrent respiratory infections following SM inhalations are known to be responsible for the development of bronchiectasis. Both the severity and frequency of bronchiectatic lesions tend to increase over the long-term follow-ups, as evidenced by a study of 40 Iranian veterans with severe late complications of SM poisoning. These lesions usually begin bilaterally in the lower lobes and then progress toward the middle lobe and the lingula. In severe cases with extensive bronchiectatic lesions, pulmonary hypertension and ultimately cor pulmonale may occur (8, 57-60).

4. Large airway narrowing: Airway narrowing, due to scarring or granulation tissue, is a late sequel of acute injuries to the trachea and large bronchi, usually developing two years after exposure (58-60). A study of 19 Iranian veterans with large airway narrowing due to SM, revealed stenosis in the trachea (7 cases), main bronchi (8 cases), and lobar bronchi (4 cases) (88). In contrast to stenosis caused by prolonged intubations, there is no predilection in the right main bronchus (55,58). The major problem in these patients is the recurrence of the lesion, which usually occurs six months after treatment (89).

5. Pulmonary fibrosis: Late onset pulmonary fibrosis has been reported in several Iranian veterans with combat exposure to SM (55, 59). The analysis of bronchoalveolar lavage fluid from patients with mustard gas inhalation showed that these patients have an ongoing local inflammatory process of the lower respiratory tract resulting in the development of pulmonary fibrosis years after the initial exposure. Histopathological examination of transbronchial lung biopsies (TBLB) of SM-exposed veterans revealed variegated fibrosis, diffuse fibrosis, and an absence of fibrosis in 86%, 4%, and 10% of the patients, respectively. Usual interstitial pneumonitis (UIP) accounted for 97% of all cases of fibrosis (48). In another study, electron microscopic examination of seven TBLB specimens was carried out in a WHO research center in Japan. Abnormal findings included: (a) proliferation, desquamation, and degeneration of the bronchial epithelial cells, (b) interstitial fibrosis or fibrosing alveolitis; and (3) an increased type I and type II alveolar epithelial cells as well as hyperplasia of ciliated and goblet
cells (60). Inflammation and fibrotic processes in the lung tissue of SM-exposed patients may be progressive (58). Diffusing capacity of the lung (DLCO) could be used as an objective monitor of the degree of fibrosis and also as a good predictor of prognosis (48). A clinical review on the respiratory complications of SM, named mustard lung was published in June 2007 in this journal.

**Dermal**
The occurrence and persistence of lesions following SM exposure is directly related to the duration and severity of exposure. Injury that results in erythema and edema without vesicle formation is almost always followed by a complete healing and noresidual effects (4, 61). Blistering and necrotic wounds, however, cause permanent residual effects. The first report of delayed toxic effects of SM poisoning two years after exposure, in 236 Iranian veterans, revealed late skin effects such as hyperpigmentation (34%), hypopigmentation (16%), and dermal scarring (8%) (22). The most common skin complaint among these patients was itching followed by a burning sensation and desquamation. These symptoms are basically due to dryness of the skin and thus become worse in dry weather and after physical activity. A more recent study of 40 Iranian veterans, who were heavily exposed to the gas 16 to 20 years previously, revealed the most common cutaneous lesions as hyperpigmentation, erythematous papular rash, dry skin, multiple cherry angiomas, atrophy, hypopigmentation, and hypertrophy. These lesions were found on the genital areas (48%), the back (48%), the front thorax and abdomen (44%), lower extremities (mainly inguinal) (44%), upper extremities (mainly auxiliary) (41%), and the head and neck (15%). Dry skin was more prominent in the extremities. Hyperpigmentation in some patients had the appearance of pigmented xerodermoid, which is a diffuse hyperpigmented area with superimposed macular hypo- and hyperpigmentations (8, 14).

In another study, the cutaneous lesions of 500 SM-exposed Iranian veterans were compared with 500 of unexposed veterans. An association was found between SM exposure and late skin lesions such as severe dry skin, hyper- and hypopigmentation, local hair loss, eczema, and chronic urticaria. There was also a higher incidence of vitiligo, psoriasis, and discoid lupus erythematosus among SM-poisoned patients. This could be due to the immunological basis of these disorders and the fact that SM has adverse long-term effects on the immune system. Previously injured sites have been reported to be sensitive to subsequent mechanical injury and showed recurrent blistering after mild injury (62).

Histopathological examination of skin biopsies has revealed nonspecific findings including epidermal atrophy, keratosis, and basal membrane hyperpigmentation. Nonspecific fibrosis and melanophages have also been observed within the dermis (8, 14, 62). Occupational exposure to SM has been demonstrated to cause a variety of skin changes, including pigmentary disorders, skin ulcers, and cutaneous cancers (63).

**Ophthalmologic**
In less than 1% of patients with battlefield exposure to SM, a delayed type of ulcerative keratopathy may develop, leading to late-onset blindness (64-68). The maximum
delayed toxic effects usually occur 15 to 20 years after initial exposure, although latency periods as long as 40 years or as short as 6 years have also been reported (16, 68-69). Patients are usually symptom-free for an indefinite number of years when delayed keratitis develops, characterized by photophobia, lacrimation, and failing vision (67).

In acute stages, the limbal region frequently presents a marbled appearance in which porcelain like areas of ischemia are surrounded by blood vessels of irregular diameter. Later, vascularized scars of the cornea are covered with crystal and cholesterol deposits, leading to a worsening of the opacification, recurrent ulcerations, and sometimes corneal perforation. Opacification of the cornea is seen predominantly in the lower and central portions, whereas the upper part is often protected by the eyelid (67, 69). Surprisingly, lesions even recur after corneal transplantation (68). The exact pathogenesis of this condition is unknown, but degenerative processes and immune reactions against corneal proteins (collagen-mustard compound) have been suggested as the cause of long-term damage (69). Unfortunately, there has been no report on any long-term studies on mustard gas workers to determine their ocular status after prolonged occupational exposure.

Psychiatric Complications
Casualties from WWI and from the Iran-Iraq conflict were noted to have long-term mood and anxiety disorders, as well as posttraumatic stress disorder (PTSD)(22, 70, 71). Debility, loss of vitality, impaired concentration, sensory hypersensitivity, diminished libido, weakened potency, neuralgic complaints, and disorders in autonomic regulation are the common manifestations. Neuropsychiatric evaluation of 1428 Iranian veterans, 3 – 9 years after exposure to SM, revealed anxiety (15%), depression (46%), personality disorders (31%), convulsions (6%), and psychosis (3%) (38). Disorders of consciousness (27%), attention (54%), emotion (98%), behaviour (80%), thought process (14%), and memory (80%) were studied in 70 patients, 3 – 5 years after SM exposure (22). Depression and post-traumatic stress in Iranian survivors of chemical warfare, mostly SM exposure, were also reported (68). In another study, decreased libido and impotence were recorded in 52% and 9% of the patients, respectively. Quite interestingly, 10% of the patients revealed an increased libido. Functional photophobia, functional aphonia, and effort syndrome have also been reported (8).

Neuromuscular Conditions
Electromyography (EMG) and nerve conduction velocity (NCV), on 40 Iranian veterans with severe late manifestations of SM poisoning, revealed abnormalities in the peripheral nervous system of 77.5% of the patients. NCV disturbances were more common in sensory nerves compared with motor nerves and more prevalent in the lower extremities than in the upper extremities. EMG recordings revealed a normal pattern in 24 (60%) patients, incomplete interference with normal amplitude in 6 (15%) patients, and incomplete interference with low amplitude in 10 (25%) patients. NCV and EMG disturbances in both upper and lower extremities were mostly symmetric (55).

Immunologic & Haematopoietic Myelo suppressions are the most serious effect of sulfur mustard. SM can cause long term effects on the immune system in patients with severe intoxication. The impaired immunity is probably responsible for the increased
risk of infections in these patients. Forty male subjects (aged 43.8 +/- 9.8 years), who had confirmed SM poisoning 16 to 20 years prior to the study, were investigated.

Carcinogenicity
Sulfur mustard is genotoxic because of its reactions with DNA, which is an important first step in carcinogenesis. Although most cells possess effective DNA repair mechanisms, these are not always effective in the case of sulfur mustard damage. Alkylation of O6-guanine by sulfur mustard seems to be critical. O6-ethylthioethylguanine is a poor substrate for the DNA repair enzyme O6-alkylguanine-DNA alkyltransferase (72). Therefore, this O6-lesion may be the most important mutagenic lesion. However, only limited data is available on the specific mutations produced by sulfur mustard. Mutations in a tumour suppressor or an oncogene gene can favour a proliferate advantage of a clonal cell. Notably, alterations in the p53 tumour suppressor gene have been described in Japanese mustard gas workers (73). However, most of the lesions in this population were similar to smoking related mutations. Mutations in lymphocytes at the hypoxanthine phosphoribosyltransferase (hprt) gene locus have also been reported (74).

Reproductive
The effects of SM exposure during pregnancy are unknown. Data addressing the productive toxicities of SM in human models are both lacking and contradictory (75).

Cardiovascular complications
Cardiovascular complications as myocardial perfusion abnormalities and coronary ectasis have recently been observed in some of these patients (76). Further studies on larger groups of veterans and controls with more details particularly on cardiovascular complications are now undertaking.

4. Conclusions and recommendations
A wide range of delayed toxic effects of SM can be categorized into two major groups: (1) Direct toxic effects on the skin, eyes and respiratory system with subsequent long-term complications such as COPD, bronchiectasis, pulmonary fibrosis, large airway narrowing, hypo- and hyper-pigmentation of the skin, as well as chronic conjunctivitis and delayed keratitis. (2) Systemic toxicities, particularly the immunohaematopoietic complications, are believed to be responsible for the increased risk of infections and malignancies in these patients. However, there are still major gaps in SM literature and further studies on human subjects who have been exposed to the agent are needed. Immunological and psychological dysfunctions, as well as the relationship between SM exposure and carcinogenesis and teratogenesis are important fields which require specific attention. Recent findings on cardiovascular complications experienced by some of these patients also requires further investigations, particularly on molecular basis to find out the mechanisms of the delayed toxic effects of SM in human beings.

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References

30. Helm UK, Balali-Mood M. Cutaneous lesions produced by sulfur mustard. The First International Medical Congress on Chemical Warfare Agents in Iran. Mashhad, Iran: Mashhad University of Medical Sciences; June 13 – 16, 1988; No. 90.
38. Tabarestani M, Farhoodi M, Balali-Mood M. Stem cell and erythroid precursors disorders in three patients with sulfur mustard poisoning. The First International Medical Congress on Chemical Warfare Agents in Iran. Mashhad, Iran: Mashhad University of Medical Sciences; June 13 – 16, 1988; No. 10.


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70. Tabatabae SM. Study of psychiatric complications of poisoning with chemical warfare agents. The First International Medical Congress on Chemical Warfare Agents in Iran. Mashhad, Iran: Mashhad University of Medical Sciences; June 13 – 16, 1988: No. 66.


Chapter 21

Unfavourable Effects of Dioxins on Environment and Human Health

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Abstract - We present the possibilities of air and soil pollution with dioxins, technological processes with separation of substances belonging to the same group, as well as the nomenclature of plant protection products, containing dioxins. The clinical presentations of acute, sub acute and chronic poisoning, as well as the various distant effects on human health are described. Special attention is paid to the constantly increasing requirements for limiting the admissible standards for dioxins presence in environment. The question about the risks of emerging of acute and chronic dioxin poisoning in our country is discussed.

Key words - dioxins, pollution, acute and chronic effects

Dioxin is the common name used to refer to the chemical 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin or TCDD. In addition to dioxin itself there are other compounds, such as the polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and some polychlorinated biphenyls (PCBs), that have similar structures and activity as dioxin. These are often commonly referred to as dioxin-like compounds or "dioxins" because every PCDD molecule contains a dioxin skeletal structure. The most toxic dioxin, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), became well known as a contaminant of Agent Orange, a herbicide used in the Vietnam War.

Chemical structure of dibenzo-p-dioxins

Dioxins are chemical contaminants that have no commercial usefulness by themselves. They are formed during combustion processes, such as forest fires and backyard trash
burning, and during manufacturing processes such as herbicide manufacture and paper manufacture. e.g. dioxin was a contaminant of the herbicide Agent Orange used as a defoliant by U.S. forces in Vietnam [1, 2].

Sources: metal smelting; diesel trucks; land application of sewage sludge; burning treated wood; trash burn barrels. These sources together account for nearly 80% of dioxin emissions [1].

Toxicity: Dioxins are of concern because of their highly toxic potential. Experiments have shown they affect a number of organs and systems. Once dioxins have entered the body, they endure a long time because of their chemical stability and their ability to be absorbed by fat tissue, where they are then stored in the body. The estimated elimination half-life for highly chlorinated dioxins (4-8 chlorine atoms) in humans ranges from 7.8 to 132 years [3]. In the environment, dioxins tend to accumulate in the food chain. The higher in the animal food chain one goes, the higher is the concentration of dioxins.

Health effects in humans
Dioxins build up primarily in fatty tissues over time (bioaccumulate), so even small exposures may eventually reach dangerous levels. In 1994, the US EPA reported that dioxins are a probable carcinogen, but noted that non-cancer effects (reproduction and sexual development, immune system) may pose an even greater threat to human health. TCDD, the most toxic of the dibenzodioxins, is classified as a Group 1 carcinogen by the International Agency for Research on Cancer (IARC). TCDD has a half-life of approximately 8 years in humans, although at high concentrations, the elimination rate is enhanced by metabolism [4]. The health effects of dioxins are mediated by their action on a cellular receptor, the aryl hydrocarbon receptor (AhR) [5]. Exposure to high levels of dioxins in humans causes a severe form of persistent acne, known as chloracne. A case-control study has shown an elevated risk of sarcoma (a type of cancer) associated with low-level exposure (4.2 fg/m³) to dioxins from incineration plants. High levels of exposures to dioxins have been shown by epidemiological studies to lead to an increased risk of tumours at all sites [6]. Other effects in humans may include: Developmental abnormalities in the enamel of children's teeth [7, 8], central and peripheral nervous system pathology [9], thyroid disorders [10], damage to the immune systems [11], endometriosis [12], diabetes [13]. TCDD has been shown to be teratogenic, mutagenic, carcinogenic, immunotoxic, and hepatotoxic. Furthermore, alterations in multiple endocrine and growth factor systems have been reported. The most sensitive effects, observed in multiple species, appear to be developmental, including effects on the developing immune, nervous, and reproductive systems [14]. Dioxins accumulate in food chains in a fashion similar to other chlorinated compounds (bioaccumulation). This means that even small concentrations in contaminated water can be concentrated up a food chain to dangerous levels due to the long biological half life and low water solubility of dioxins.

Studies of dioxins' effects in Vietnam
US veterans' groups and Vietnamese groups, including the Vietnamese government, have convened scientific studies to explore their belief that dioxins were responsible for a host of disorders, including tens of thousands of birth defects in children, that have affected Vietnam veterans as well as an estimated one million Vietnamese, due to their
exposure during the Vietnam War to Agent Orange, a defoliant chemical which was widely sprayed over Vietnamese land and which was found to be highly contaminated with TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin - the most toxic dioxin). Several exposure studies showed that some US Vietnam Veterans who were exposed to Agent Orange had serum TCDD levels up to 600 ppt (parts per trillion) many years after they left Vietnam, compared to general population levels of approximately 1 to 2 ppt of TCDD. In Vietnam, TCDD levels up to 1,000,000 ppt have been found in soil and sediments from Agent Orange contaminated areas, three to four decades after spraying. In addition, elevated levels have been measured in food and wildlife in Vietnam [15].

Dioxin - potent Immune System Poison
Dioxin can modulate the immune system resulting in an inability to fight disease. It is a very powerful immunosuppressant. But it can also upregulate [excite] the immune system so that the people start becoming hypersensitive, developing autoimmunity and allergies. Depending upon the stage [of growth] of the animal and the species, sometimes it could be observed immunosuppression and in other cases it could be observed upregulation [16, 17].

U.S. Environmental Protection Agency's (EPA's) 1994 draft reassessment of dioxin emphasized that dioxin damages the immune system directly and indirectly. From studies of rats, mice, guinea pigs, rabbits, cattle, marmosets, monkeys, and humans, EPA concludes that even low doses of dioxin attack the immune system. Dioxin directly reduces the number of B cells (immune cells that develop in the bone marrow, then circulate throughout the blood and lymph, fighting off invaders). And it reduces the number of T cells (immune cells that develop in the thymus, then circulate throughout the body, attacking invaders), but dioxin's attack on T cells seems to be indirect. One potentially important indirect mechanism is via effects on the endocrine system. Several endocrine hormones have been shown to regulate immune responses, including glucocorticoids, sex steroids, thyroxine, growth hormone, and prolactin. Importantly, TCDD [dioxin] and other related compounds have been shown to alter the activity of these hormones [18].

It is important to consider that if an acute exposure to TCDD even temporarily raises the TCDD body burden at the time when an immune response was initiated, there may be a risk of adverse impacts even though the total body burden may indicate a relatively low average TCDD level. In other words, a single dose of dioxin at the wrong time may damage immune system's ability to protect people. Furthermore, because TCDD alters the normal differentiation of immune system cells, the human embryo may be very susceptible to long-term impairment of immune function from in utero effects of TCDD on developing immune tissue. In other words, dioxin can prevent the immune system from developing properly in an unborn child, with lifelong consequences. Animal studies suggest that some immunotoxic responses may be evoked at very low levels of dioxin exposure [18].

The concentration of serum Ig and the distribution of different lymphocyte subpopulations were studied in peripheral blood samples obtained from 30 human subjects exposed to PCB (polychlorinated biphenyl) and from 23 normal healthy subjects. PCB caused decreased concentrations of IgA and IgM but not that of IgG. By using different rosette techniques to enumerate the percentages of lymphocyte subpopulations, the percentages of total T cells, active T cells and T-mu cells decreased,
while the percentages of B cells and T-gamma cells were not affected. Changes of lymphocyte subpopulations may be responsible for the reported immune deficiency associated with PCB exposure [19].

Effects of postnatal exposure to polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and coplanar polychlorinated biphenyls (Co-PCBs) on lymphocyte subpopulations were investigated in the peripheral blood of 36 breast-fed Japanese babies. Ratios of CD4+ to CD8+ T cells showed significant increasing tendency correlated with organochlorine exposure [20].

Consumption of fatty fish species, like salmon and herring, from the Baltic Sea is an important source of human exposure to persistent organochlorine compounds, e.g. polychlorinated dioxins (PCDDs), dibenzofurans (PCDFs) and biphenyls (PCBs). The high consumers had lower proportions and numbers of natural killer (NK) cells, identified by the CD 56 marker, in peripheral blood than the non-consumers [21].

German researchers published a study of the health of 158 chemical workers who had been exposed to dioxin in 1953 during an industrial accident at a BASF chemical plant [22]. The 158 exposed workers were compared to 161 unexposed workers. The dioxin-exposed workers experienced more frequent infections and parasitic diseases during the 36 years after exposure, consistent with immune system damage. Especially noticeable were increases in respiratory infections, thyroid diseases, disorders of the peripheral nervous system, and appendicitis. Mental disorders were also increased. All together, the highly-exposed group had 18% more recorded episodes of illness than the control group.

Increased levels of dioxins and related compounds (PCBs) correlate with negative changes in lymphocyte subpopulations and CD4+/CD8+ biomarkers; dioxin and related compounds may be related to immunopathy, such as atopic dermatitis; exposures occurred at background levels [23].

Dioxin appears to be a carcinogen in fish, rodents, and other mammals, including humans. Organochlorines have been reported to adversely affect the human immune system, reducing defenses against cancer; organochlorine exposure has been shown to increase risk of non-Hodgkin’s lymphoma; immune damage combined with estrogenic qualities (as with PCBs) have been linked to breast cancer. Prenatal organochlorine exposure could be a risk factor for increased ear infections in infants [24].

Polychlorinated biphenyls (PCBs) are among the most widespread environmental pollutants and a prominent contaminant of the Great Lakes basin. Higher incidence of bacterial infections was reported for breast-fed infants born to mothers who consumed large amounts of Great Lakes fish compared to the incidence in control infants whose mothers ingested low amounts of fish [25].

There have been many incidents of dioxin pollution resulting from industrial emissions and accidents.

**Dioxin exposure incidents**

- In 1949, in a herbicide production plant for 2,4,5-T in Nitro, West Virginia, 240 people were affected when a relief valve opened [26].
In 1963, a dioxin cloud escapes after an explosion in a Philips-Duphar plant (now Solvay Group) near Amsterdam. In the 1960s, Philips-Duphar produced 2250 tonnes of 'Agent Orange' for the US Army [27].

In 1968, an explosion of a reactor with 2, 4, 5-trichlorophenol in Spolana Neratovice plant in Czechoslovakia seriously poisoned about 60 workers with dioxins; after the incident Spolana stopped manufacture of 2, 4, 5-T (most of which was supplied to the US military in Vietnam). Major parts of the Spolana chemical plant were heavily contaminated by dioxins. A large amount of dioxins were flushed into the Elbe and Mulde rivers during the 2002 European flood, contaminating the soils. The consumption of local fish, eggs, poultry and some produce was prohibited because of the post-flood contamination [27, 28].

In 1976, large amounts of dioxins were released in an industrial accident at Seveso, Italy. A cloud of toxic chemicals, including 2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin, or TCDD, was released into the air and eventually contaminated an area of 15 square kilometres where 37 000 people lived. Extensive studies in the affected population are continuing to determine the long-term human health effects from this incident. These investigations, however, are hampered by the lack of appropriate exposure assessments. A minor increase in certain cancers and effects on reproduction have been detected and are being further investigated. Possible effects on the children of exposed people are currently being studied [29].

In 1978, dioxins were some of the contaminants that forced the evacuation of the Love Canal neighborhood of Niagara Falls, New York. Dioxins also caused the 1983 evacuation of Times Beach, Missouri. From 1982 through to 1985, Times Beach, Missouri, was bought out and evacuated under order of the United States Environmental Protection Agency due to high levels of dioxins in the soil. The town eventually disincorporated [30, 31].

In December 1991, an electrical explosion caused dioxins (created from the oxidation of polychlorinated biphenyl) to spread through four residence halls and two other buildings on the college campus of SUNY New Paltz.

In May 1999, there was a dioxin crisis in Belgium: quantities of dioxins had entered the food chain through contaminated animal feed. 7,000,000 chickens and 60,000 pigs had to be slaughtered. This scandal was followed by a landslide change in government in the elections one month later.

Explosions resulting from the terrorist attacks on the US on September 11, 2001 released massive amounts of dust into the air. The air was measured for dioxins from September 23, 2001, to November 21, 2001, and reported to be "likely the highest ambient concentration that have ever been reported." [in history]. The United States Environmental Protection Agency report dated October 2002 and released in December of 2002 titled "Exposure and Human Health Evaluation of Airborne Pollution from the World Trade Center Disaster" authored by the EPA Office of Research and Development in Washington states that dioxin levels recorded at a monitoring station on Park Row near City Hall Park in New York between October 12 and 29, 2001, averaged 5.6 parts per trillion, or nearly six times the highest dioxin level ever.
recorded in the U.S. Dioxin levels in the rubble of the World Trade Centers were much higher with concentrations ranging from 10 to 170 parts per trillion. The report did no measuring of the toxicity of indoor air.

- A few cases of intentional human poisoning have also been reported. The most notable incident is the 2004 case of Viktor Yushchenko, President of the Ukraine, whose face was disfigured by chloracne [32].
- In 2007 in Italy thousands of tonnes of foul-smelling refuse are piled up in Naples and its surrounding villages, defacing entire neighbourhoods. Polychlorinated dibenzodioxins are found in animals and humans over lethal dose. Sources of Polychlorinated dibenzodioxins was identified in refuse and pvc combustion and industrial refuse disposal in uncontrolled industrial waste disposal [33].

**Bulgaria** has a National Environmental Strategy adopted in 2001. One of the priority sectors in the National Strategy is reduction emission of large combustion installations. It sets a target for reduction of the national dioxin emissions according to the 1998 CLRTAP POPs Protocol [34]. The National Environmental Strategy identifies following priority sectors for dioxin emissions reduction:
1. Waste incineration;
2. Metallurgical industry;
3. Solid fuels residential heating.

Current DIOXIN emission inventory in Bulgaria is based on emission factors taken from CORINAIR – 94 methodology, but it is planned to improve the assessment of potential key sources by dioxin emissions measurements [35].

At the moment according to the Bulgarian Regulations on the conditions and requirements for the construction and operation of municipal waste disposal facilities and installations and on the conditions and requirements hazardous waste treatment the concentration of dioxins in the flue gases (from waste incinerators for any type of waste, including hospital) may not exceed 0.1 ng TEQ/m³. Every year Bulgaria provides the UNECE/CLRTAP (Convention on Long-Range Transboundary Air Pollution) with emissions data for SO₂, NO₂, CH₄, NMVOC, CO, NH₃, Cd, Pb, Hg, PAH, PCBs, HCB, PCP, dioxins and furans, from 11 activity sector groups, such as: public power plants, congeneration plants and district heating plants; commercial, institutional and residential combustion; industrial combustion; production processes; extraction and distribution of fossil fuels; solvent use; road transport; other mobile sources and machinery; waste treatment and disposal; agriculture and nature [36, 37].

**Conclusion**

Reducing dioxin exposure is an important public health goal for disease reduction, also with respect to sustainable development. One approach includes source-directed measures to reduce dioxin emissions. Secondary contamination of the food supply needs to be avoided throughout the food-chain. Good controls and practices during primary production, processing, distribution and sale are all essential to the production of safe food.
References

[33.] "Italy's toxic waste crisis, the Mafia – and the scandal of Europe's mozzarella". Retrieved on 2008-03-28.
[35.] Methodology for calculation according to balance methods of the emissions of pollutants discharged in the atmospheric air (under the terms of EMEP/CORINAIR 1997 and 2000, 3-th edition from September - 2004).
Abstract - The knowledge about influence of opioids on the variety of immune mechanisms is not yet broad enough despite multiple studies in this area. The aim of the study is to investigate the changes of some parameters of humoral and cell mediated immunity in a group of heroin addicts. Material and methods: The study involved 55 heroin addicts, with acute heroin and mixed by other psychoactive drugs intoxication, treated in Toxicology Clinic, Emergency Hospital “Pirogov”. In the serum of drug abusers were determined the level of IgG, IgA, IgM antibodies, C4, alfa-2 macroglobulin, alfa-1 antitripsin and haptoglobin. By laser flow cytometry using monoclonal antibodies were determined CD markers of the basic lymphocyte population and sub-population. Results: It was found statistically significant decrease level of IgG, IgA and C4 complement as well as increase of the level of haptoglobin. The study of the lymphocyte receptors showed statistically significant decrease of CD4 and increase of CD8 bearing lymphocytes with lower level of CD4/CD8 ratio. The study reveals the lower level of CD56 lymphocyte population – NK cells. Conclusion: The results of the study show that both humoral and cell immunity are significantly affected by opioids. The mechanisms responsible for the opioid-induced changes in immune function are still unclear. Multifactorial elements might be involved in such dysfunction. Key words - opioid compounds, heroin, immunity

Introduction

Heroin is a highly addictive drug, and its abuse has reflection, that extend far beyond the individual user. The medical and social consequences of drug abuse have a devastating impact on society. Many complications of heroin addiction are related to the unsanitary administration of the drug. Others are due to the inherent properties of the drug, overdose, or intoxicated behavior accompanying drug use. Common complications include pulmonary disorders, hepatitis, arthritic disorders, neurological disorders and immunologic changes. Strongly associated with drug addiction is the spread of blood borne diseases such as HIV and tuberculosis [1, 2, 3].
The immune system plays a critical role in host resistance to disease as well as in normal homeostasis of an organism. The immune dysfunction may take the form of immunosuppression or alternatively, allergy, autoimmunity or any number of inflammatory-based diseases or pathologies.

The immunotoxic consequences of exposure to substances of abuse are difficult to ascertain in most instances as confounding factors, such as intercurrent infections. Secondary to intravenous injection, may contribute to the observed changes. Recent research has provided evidence, however, that substances of abuse can directly affect the immune system. [4, 5, 6]. The knowledge about influence of opioids on the variety of immune mechanisms is not yet broad enough despite multiple studies in this area. [7, 8, 9, 10].

A new information in this regard would be of both theoretical and practical value for the improved treatment and prophylaxis of drug addiction.

The AIM of the study is to investigate the changes of some parameters of humoral and cell mediated immunity in a group of drug addicts with long-standing abuse with heroin.

1. Materials and methods

Examine patients:

The study group included 55 heroin addicts [duration of the abuse with range 1-10 years] hospitalized in Clinic of Toxicology, Emergency Hospital “N.I. Pirogov”, Sofia with acute exogenous intoxications. The age of the patients was between 16 and 43 years. The relation male : female was 4 : 1.

They are divided as follows: 26 – with monotoxic heroin intoxication; 29 – with combined intoxication [heroin and other psychoactive drugs].

1. Humoral immunity study

In the patient sera were determined the quantity of serum IgG, IgA, IgM, C3, C4, alfa 2-macroglobulin, alfa 1 - antitripsin inhibitor and haptoglobin by Manchini’s radial diffusion test in agar/agarose gel using immunoplates by “Imunotest”, Sofia.

2. Study of lymphocyte receptors/markers

On the studied patients was withdrawn blood for a flow cytometry receptor analysis of the lymphocytes through double immunofluorescence using panel with monoclonal antibodies marked with FITC and phycoerythrin. The lymphocyte populations and subpopulations were identified by the following combinations of markers: CD45/CD14, CD3/CD19, CD3/CD56, CD3/CD4, CD3/CD8, CD3/HLA-DR and CD57/CD8. A flow cytometer CALIBUR /Bio-Rad Laboratories/ with an argon laser was used.

The results of investigate patients was compared with results of 25 clinically healthy subjects.

Statistical analysis of data was accomplish by program SPSS 10.00.
2. Results and discussions

1. Humoral immunity study

Table 1 shows the changes of the studied humoral immunity parameters in the included in the study drug abusers. The lower levels of IgG [in about 38%], IgA [in about 20%] and IgM [in about 17%] in the studied patients in comparison with the values in healthy, nonallergic people are impressive \( p < 0.05 \). Only in 3 of the studied patients we observed an increase of the immunoglobuline what doesn’t represent any biological tendency. Rather interesting is the finding that in 31% of the cases the level of haptoglobin is substantially increased. These data may lead to the conclusion that chronic abuse with heroin impairs the synthesis of the major classes of immunoglobulines as well as one of the complement components - C4 [in 31% of the studied patients]. All they play essential protective role in the immune response in the variety of bacterial and viral infections. It may explain to some extent the higher incidence of infections /esp. respiratory/ in drug abusers. The increased haptoglobin level is an index for liver disorders what correlates well with considerable incidence of impaired liver function in the studied patients.

**Table 1. Humoral immunity changes in the studied patients**

<table>
<thead>
<tr>
<th>Studied indexes</th>
<th>Studied patients</th>
<th>Increased</th>
<th>Decreased</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>Number</td>
<td>%</td>
</tr>
<tr>
<td>IgG</td>
<td>heroin</td>
<td>26</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>heroin+</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgA</td>
<td>heroin</td>
<td>26</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>heroin+</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgM</td>
<td>heroin</td>
<td>26</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>heroin+</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3</td>
<td>heroin</td>
<td>26</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>heroin+</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C4</td>
<td>heroin</td>
<td>26</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>heroin+</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alpha 1-antitrip</td>
<td>heroin and heroin+</td>
<td>55</td>
<td>-</td>
</tr>
<tr>
<td>Alpha 2-macrogl.</td>
<td>heroin and heroin+</td>
<td>55</td>
<td>7</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>heroin and heroin+</td>
<td>55</td>
<td>17</td>
</tr>
</tbody>
</table>

\*P<0,05

*Legend: heroin – patients with monotoxic heroin intoxication; heroin + - patients with mixt intoxication [heroin+other psychoactive drugs]*
The same picture we have seen in the studied drug abusers carriers of HCV. On table 2 are shown these results and it is clear that there are not statistical difference in the level of the immunoglobulines in terms of existing HCV infection. Only the level of IgG is much lower in the patients carriers of HCV – in 13 from 22 or 59% of them in comparison with the others – in 8 from 33 or 24.2% [p<0.05].

Table 2. Humoral immunity changes in the studied patients with or without HCV infection

<table>
<thead>
<tr>
<th>Studied indexes</th>
<th>Studied patients</th>
<th>Increased</th>
<th>Decreased</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number %</td>
<td>Number %</td>
<td>Number %</td>
</tr>
<tr>
<td>IgA with HCV</td>
<td>22</td>
<td>-</td>
<td>6</td>
</tr>
<tr>
<td>without HCV</td>
<td>33</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>IgG with HCV</td>
<td>22</td>
<td>-</td>
<td>13</td>
</tr>
<tr>
<td>without HCV</td>
<td>33</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>IgM with HCV</td>
<td>22</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>without HCV</td>
<td>33</td>
<td>-</td>
<td>4</td>
</tr>
</tbody>
</table>

*P<0.05

We didn’t find any statistical significant differences in the level of the other studied serum factors except of the haptoglobin. Its level is statistically significant higher [p<0.05] in comparison with the normal referent values in 13 from 22 or in 59.1% of the heroin abusers with HCV infection.

Table 3. Changes in the haptoglobin level in the studied patients with HCV infection

<table>
<thead>
<tr>
<th>Studied indexes</th>
<th>Studied patients</th>
<th>Increased</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number %</td>
<td>Number %</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>with HCV</td>
<td>22</td>
</tr>
</tbody>
</table>

*P<0.05

2. Study of the lymphocyte receptor markers

Apparently the CD4 lymphocytes are significantly less in the studied patients /p<0.002/. There is a tendency for an increased level of CD8 lymphocytes compared to the value in clinically healthy subjects. Therefore the ratio CD4/CD8 is significantly lower /p<0.05/ what reveals cell-mediated immunity suppression in the studied heroin addicts. Another proof in this respect is the significantly lower level /p<0.042/ of CD56 bearing lymphocytes – i.e. natural killer /NK/ cells.

Our analysis showed a high rate of suppressed cell-mediated immunity in the same drug abusers, determined by intradermal tests with a battery of bacterial and fungal antigens. Their level of CD4 lymphocytes is significantly lower /p<0.005/ and CD8
bearing lymphocytes are significantly higher /p<0.05/ compared to drug addicts with a normal cell-mediated immunity (table 4).

Table 4. Changes of lymphocyte markers/receptors in the studied patients

<table>
<thead>
<tr>
<th>CD4 ↓</th>
<th>CD8 ↑</th>
<th>CD4/CD8 ↓</th>
<th>CD56 ↓</th>
</tr>
</thead>
<tbody>
<tr>
<td>57%</td>
<td>38.9%</td>
<td>54.2%</td>
<td>p&lt;0.042</td>
</tr>
<tr>
<td>p&lt;0.002</td>
<td></td>
<td>p&lt;0.03</td>
<td></td>
</tr>
</tbody>
</table>

The results of our study show that both humoral and cell immunity are affected by opioids.

The similar findings had been reported by other authors. Alterations in a wide variety of immune parameters also have been reported among Rouveix B [9]. There was a profound decrease in the T-helper/cytotoxic T-cell [CD4/CD8] ratio in heroin addicts [8]. McDonough et al. observed the absolute number and percentage of total and active T lymphocytes in the peripheral blood of opiate addicts and T-cell rosette formation were significantly depressed [11].

Opiates have been shown to produce effects on immune function in vivo [12]. Shavit et al. have shown that opiates interacting with opiate receptors in the brain are implicated in the suppression of NK activity. Those authors suggested novel central nervous system mechanisms through which the immune response might be regulated [13]. Reddy et al. and DePaoli et al. observed reduced NK cell activity in HIV-seronegative parenteral drug addicts and a further reduction in HIV-seropositive patients [3, 6]. Our results demonstrate also that the percentage of NK cells was suppressed in heroin addicts.

It has been proposed that the effects of opioids on lymphocyte proliferation may operate via a direct interaction with opioid receptors [6].

From a pathophysiological viewpoint, the ability of opioids to modulate the immune function may have some bearing on the development of the infectious diseases that are often associated with drug abuse. The high percentage of infections among injecting drug users is partly related to injection methods and life-style practices, but it is now accepted that heroin-induced immunosuppression may contribute as a co-factor in the contraction of several microbial and viral infections, such as Hepatitis C virus [HCV] infection [12].

3. Conclusion

The results of the study show that both humoral and cell immunity are significantly affected by opioids. The mechanisms responsible for the opioid-induced changes in immune function are still unclear. They may be mediated directly via opioid receptors present on lymphocytes and/or indirectly via opioid receptors in the central nervous system. However, multifactorial elements might be involved in such dysfunction.
References


Chapter 23

Accidental and Suicidal Self-Poisoning Among Heroin Addicts

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Abstract - Objective: To study the incidence and the particularities of the acute intoxications, come as a result of a suicidal attempt or overdosing among heroin-addicted patients.

Methods: There has been made a research on 90 persons, chosen by lottery method from all of heroin-addicted people, hospitalized in Toxicology Clinic - Emergency Hospital “Pirogov”, with acute heroin and/or combined intoxication in 5-year period.

Results: The age of the studied patients varies between 17 and 32 years. 65% of them are in the age between 20 and 25 years old. Men predominate (72%) than the women (28%). The basic way of taking heroin for 87% of the patients is injecting it into the veins. There is a high incidence of overdosing cases – 82%. Suicidal attempts are commit among persons who are in the beginning of the drug practice, or these one with long standing heroin abuse. Most overdoses occur among heroin users who have used heroin for 2 to 4 years. There is a predomination of the mild forms of the acute intoxications in patient with suicidal attempts and the severe and moderate forms in the overdosing patients. Monotoxic heroin intoxications are higher then mixed poisonings in the overdosing patient. By all the patients with suicidal attempts it refers to combined intoxications. The most common combination is heroin with benzodiazepines into the two groups.

Conclusion: The results of our study show high level of overdosing cases in heroin-addicted patients. We can see the necessity of strategies for reduction the drug overdoses.

Key words - intoxication, heroin, suicidal attempts

Introduction

Drugs of abuse (psychoactive substances) makes central nervous system effects, which produce changes in mood, levels of awareness or perceptions and sensations.

Drug use and abuse is as old as mankind itself. Human beings have always had a desire to eat or drink substances that make them feel relaxed, stimulated, or euphoric. Wine was used at least from the time of the early Egyptians; narcotics from 4000 BC; and medicinal use of marijuana has been dated to 2737 BC in China. In the Middle Ages Paracelsus (1490-1541) introduces laudanum, or tincture of opium, into the practice of medicine. Recreational use of opium was once common in Asia, and from there spread to the West, peaking in the 19th century. Heroin were sold as patent
medicines in the 19th and early 20th centuries, and marketed as treatments for a wide variety of ailments [1, 2, 3, 4, 5].

The use of drugs or psychoactive substances seems to be an almost universal phenomenon which has long been a complex, often highly volatile social concern.

Berman et al found that abuse or dependence on alcohol and other psychoactive substances is often associated with multiple psychosocial problems, psychiatric comorbidity, suicidal ideation, suicide attempts [6].

The predominant view of modern medicine is that suicide is a mental health concern, associated with psychological factors such as the difficulty of coping with depression, inescapable suffering or fear, or other mental disorders and pressures. Suicide is sometimes interpreted as a "cry for help" and attention, or to express despair and the wish to escape, rather than a genuine intent to die. Most people who attempt suicide do not complete suicide on a first attempt; those who later gain a history of repetitions have a significantly higher probability of eventual completion of suicide [7, 8].

Oyefeso A., et al examine suicide trends among registered addicts in the UK over a 25-year period. The findings confirm that addicts are still at higher risk of suicide than the general population and that prescribed drugs, notably antidepressants and methadone, influence this heightened risk [9].

The existing data and longitudinal observation show that in Bulgaria the most problematic psychoactive substance is the heroin. The summary estimate of problem heroin users in Bulgaria is between 20 000 and 30 000 people [10].

**Objective:** To study the incidence and the particularities of the acute intoxications, come as a result of a suicidal attempt or overdosing among heroin-addicted patients.

1. **Material and methods**

There has been a research on 90 persons, chosen by lottery method from all of heroin-addicted people, hospitalized in Toxicology Clinic - Emergency Hospital “Pirogov”, with acute heroin and/or combined intoxication in 5-year period.

The following data were analyzed: age, gender, the length of the drug practice, route of exposure, intent of exposures (suicidal or accidental), conditions and type of the poisoning – monotoxic, or mixed, type of substance and clinical severity of intoxication.

2. **Results**

As it was mention in material and methods the survey investigated 90 subjects (65 males, 25 females), hospitalized in Toxicology Clinic - EMH “Pirogov”, with acute heroin and/or combined intoxication in 5-year period. The age of the studied patients varies between 17 and 32 years (mean age – 21 years). 65% of the investigated people were in the age between 20 and 25 years old. Men predominate (72%) than the women (28%).
1. Basic way of taking heroin

Heroin can be taken in many different ways. Because it comes as a powder, it can be dissolved in water and injected. It can also be smoked. This involves heating the drug on tin foil. The fumes are then inhaled through a small tube - a method sometimes called "chasing the dragon". Injecting creates a more powerful "high". The vast majority of heroin is illegally manufactured and is diluted or "cut" for sale on the street. This is usually done with glucose, but caffeine, flour, chalk, quinine, and even talcum powder are used [11, 12].

The basic way of taking heroin for 87% of the patients from our study is injecting it into the veins.

2. Reason of the acute intoxication

Drug overdoses are sometimes caused intentionally to commit suicide or as self-harm, but many drug overdoses are accidental and are usually the result of either irresponsible behavior. The following definition of suicide attempts from the WHO/EURO multicentre project has been used: “An act with non fatal outcome in which the individual deliberately initiates as non-habitual behavior, that without interventions from others, will cause self-harm, or deliberately ingests a substance in excess of the prescribed or generally recognized therapeutic dosage, and which is aimed realizing changes which the subject desired via the actual or expected physical consequences”. Overdoses were not included as suicide attempts as there had to be an element of intention in the act, e.g. wanting to die or not being able to cope with life any more [8].

In the present study the reason of the acute intoxication in 82% of the researched patients is accidentally overdosing with heroin or combining it with other psychoactive substances (PAS); 18% of them have had a suicidal attempt (Figure 1).

---

**Figure 1.** Reason of the acute intoxication
3. cause for committing suicidal attempts

The most frequent cause for committing suicidal attempts by self poisonings found in both the gender was: various social and economic reasons, isolation from social or family life, lack of prospects, feeling of hopelessness, depression as separate disease.

4. Length of the drug practice

According to the length of the drug practice, the results of the study show that suicidal attempts are commit among persons who are with long standing heroin abuse or these one in the beginning of the drug practice. Most accidental self-poisoning occur among heroin users who have used heroin for 2 to 4 years (Figure 2).

5. Severity of intoxication

Heroin is a member of a class of narcotic analgesic drugs called opioids. Overdose can occur when a dose taken is greater than that you're used to. A tolerable dose for an addict could be fatal to a first-time user. Tolerance to heroin in particular is quickly acquired. Some users have overdosed on their 'regular dose', after just a few week's break. Combined drug intoxication can be caused by interactions between many different drugs.

Interactions between central nervous system depressants such as benzodiazepines and narcotic analgesics may lead to severely depressed breathing or bradycardya, causing the victim to become uncounscious or comatose. While unconscious, the victim may regurgitate and die from asphyxia [13, 14, 15].

According to clinical course of the acute poisoning, and degree of manifestation of symptoms from the central nervous system, respiratory and cardiovascular systems, we differentiate three degree of severity of the intoxication: heavy, moderate and mild.
Figure 3. Distribution according to a reason and a severity of the intoxication

There is a predomination of the mild forms of the acute intoxications in patients with suicidal attempts and the severe and moderate forms in the overdosing patients. No one of the studied patients is dead (Figure 3).

6. Type of intoxication

Overdoses are frequent in subjects who have used heroin with alcohol, benzodiazepines, cannabis, or amphetamines. Usage of illicit drugs that are of unexpected purity, in large quantities, or after a period of abstinence can also induce overdose [16].

Our results show that monotoxic heroin intoxications are higher than mixed poisonings in the overdosing patients with accidental self-poisoning (55.4%).

By all the patients with suicidal attempts it refers to combined intoxications (Figure 4).

On table 1 were presented the other psychoactive substances / medicine mixed with heroin from the two groups patients (suicidal attempts and accidental self-poisoning).

Benzodiazepines are commonly used by polydrug abusers. However, combinations of high doses of benzodiazepines with opiates, alcohol, barbiturates, are particularly dangerous, and may lead to severe complications such as coma or death. The most common symptoms of overdose include central nervous system depression and intoxication with impaired balance, ataxia, and slurred speech. Severe symptoms include coma and respiratory depression. Heroin-related deaths are strongly associated with use of alcohol or other drugs [14, 18, 24]. [14, 16, 17].
Accidental self-poisoning according to the type of the intoxication

Table 1. Other PAS / medicine mixed with heroin

<table>
<thead>
<tr>
<th>Other PAS / medicine mixed with heroin</th>
<th>TS(%)</th>
<th>accidental self-poisoning (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>benzodiazepines (2 or 3 medicine)</td>
<td>66.7</td>
<td></td>
</tr>
<tr>
<td>Barbiturates</td>
<td>33.4</td>
<td></td>
</tr>
<tr>
<td>other opioid compounds</td>
<td>30.4</td>
<td></td>
</tr>
<tr>
<td>alcohol</td>
<td>3.3</td>
<td></td>
</tr>
<tr>
<td>glutethimid</td>
<td>24.2</td>
<td></td>
</tr>
<tr>
<td>other medicine/compounds (phenothiazines, amphetamines, cocaine)</td>
<td>6.0</td>
<td></td>
</tr>
</tbody>
</table>

3. Discussion

Heroin dependence is a serious illness associated with an increased risk of suicidal behaviour. There are many risk factors associated with heroin dependence. The current study examined the sociodemographic and clinical characteristics of a number of young adult heroin-dependent patients who had attempted suicide.

From a phenomenological and pathophysiological perspective the substance dependent lifestyle is a complex mix of many factors including chemical, nutritional, genetic, judicial, congenital, social, familial and environmental factors [7, 13].

According to sociological research conducted in Bulgaria, people consider drug addiction as one of the most important problems of young people at the present day in our country [1].

The investigations of Rossow show that the incidence density of suicide was significantly higher among drug addicts than in the total population, and the excess mortality by suicide was higher for women, higher in the youngest age group [18].
The highest rates of illicit drug use are found among youth ages 18-20 (between 20% and 21%) with marijuana the most commonly used illicit drug [5].

Roy Alec found that patients who had attempted suicide were significantly younger than patients who had never attempted suicide. Significantly more of the patients who had attempted suicide were female; had a family history of suicide; and had a lifetime history of major depression, of having received antidepressant medication, and of alcoholism. Also, patients who had attempted suicide had significantly higher scores for childhood trauma, psychoticism, neuroticism and introversion. These results suggest that social, personality, family, developmental and psychiatric risk factors may predispose to suicidal behavior in drug-dependent individuals [19].

Our results show that 2/3 of the researched patients are males and the highest is the relative part of the 21 years old. The most frequent cause for committing suicidal attempts by self poisonings found in both the gender was: various social and economic reasons, isolation from social or family life, lack of prospects, feeling of hopelessness, depression as separate disease.

James Wines presented data from two studies of clinical populations, one retrospective and one prospective, that highlighted depressive symptoms, drug use, substance use disorders, and previous suicidal behavior as predictors of suicide attempts. He also reported that drug abusing suicide attempters exhibited higher levels of impulsive attempts than attempters who were not substance abusers [20].

E. Johnsson investigate suicide attempts in a group of drug abusers who previously had received treatment of their addiction. Results from the study shows that 39 percent of the drug abusers had attempted suicide during their period of active drug abuse, 60 percent were women. The key situations were: suicide in the teens, mental pane which became unbearable, various coercive interventions linked to the abuse, the negative effects of the addiction such as an increasingly chaotic situation which could be described as "the rock bottom" and finally the fifth situation which reflected the processes of breaking out of addiction and the difficulty of living an "ordinary" social life. The analyses of the interview showed the relation between social, interactive, and individual factors and the significance of the abuse for the suicidal act [21, 22].

Holly Wilcox presented analyses of whether the risk of suicidal ideation and attempts might be greater for early drug users compared to later users or never users. She found that early inhalant use was associated with later suicide attempts [23].

Our results show that suicidal attempts are commite among persons who are in the beginning of the drug practice, or these one with long standing heroin abuse. Most overdoses occur among heroin users who have used heroin for 2 to 4 years. The basic way of taking heroin for 87% of the patients is injecting it into the veins.

Polydrug intoxications are common among substance abusers. The majority of deaths (68%) were associated with drug overdoses. Opiates were the drugs most commonly detected during post-mortem examinations. In the majority of cases, more than one drug was detected. Polydrug use and, heavy drinking, and use of benzodiazepines and amphetamines, were identified as risk factors for mortality [24]. Combined drug intoxication can be caused by interactions between many different drugs. Certain drugs potentiate or amplify the effects of another drug and can lead to much-stronger effects than either drug taken alone would produce; for example, alcohol, a depressant, will potentiate the effects of other depressants and can cause respiratory
depression, unconscious, coma and bradycardia; yet, because it is legal, easy to obtain, and commonly used, it may figure in many cases [17, 25, 26].

Our results show that predominate the mild forms of the acute intoxications in patient with suicidal attempts and the severe and moderate forms in the accidental overdosing patients. Indirectly we may conclude that intoxication is “Call for help”. Monotoxic heroin intoxications are higher then mixed poisonings in the overdosing patient. By all the patients with suicidal attempts it refers to combined intoxications. The most common combination is heroin with benzodiazepines, follow by barbiturate, other opioid compounds and alcohol into the two groups.

The results from our study confirm that suicide attempts among heroin addicts are a major problem, which requires targeted intervention. Prevention of suicidal behaviour demands a treatment programme focusing on addictive behaviour. The most successful prevention of the actual suicide attempt is diagnosis and appropriate treatment of the underlying condition. However, one must be aware that psychotropic medication is often used in an attempt. Therefore, only small amounts should be prescribed at any one time. Family members or close friends should also be involved since they can provide further information, give support to the patient and might be able to watch him/her closely.

4. Conclusions

The use of drugs or psychoactive substances seems to be an almost universal phenomenon which has long been a complex, often highly volatile social concern.

Our findings confirm that addicts are at high risk of suicide and that prescribed drugs, notably benzodiazepines, barbiturates, other opioid compounds, alcohol influence this heightened risk. A first priority for prevention must be to reduce the frequency of drug overdoses. We should inform heroin users about the risks of combining heroin with alcohol and other depressant drugs. Heroin users need to be educated about the potentially dangerous practice of concurrent polydrug and heroin use.

References:

S. Tonev, K. Kanev, C. Dishovsky


Chapter 24

Effect of Number of Cigarettes Daily Consumed on Offspring Sex Ratio

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Abstract - Objective: A number of developed countries have demonstrated a significant decline in male to female ratio of children during the past few decades. The current work assessed the offspring sex ratio (male to female) when the subjects smoked less than 14 cigarettes per day compared with the subjects who smoked more than 14 cigarettes per day in various cities of Pakistan.

Methods: A Cross sectional population based Survey was carried out at four cities of Pakistan. Sample Size of 405 subjects was calculated using Epi-Info 6.0. The subjects were divided into two main groups that were those consuming less than 14 cigarettes per day and those consuming more than 14 cigarettes per day.

Results: For all four groups where subjects were smoking more than 14 cigarettes per day their offspring ratio (male: female) was 0.54 as compared to those who were smoking less than 14 cigarettes per day (1.15). Overall Male/Female Sex ratio among smokers was 0.85. The overall sex ratio among smokers was significantly lower (p=0.006) as compared to nonsmokers.

Multivariate logistic regression analysis showed significant association of offspring sex-ratio with number of cigarettes consumed per day. (p=0.004).

Conclusion: Our study documents that smoking might be a contributing factor to a lower male to female sex ratio of offspring.

Keywords - smoking offspring, sex ratio (male/female)

Introduction

World wide, the sex ratio at birth in human beings has been fairly constant with 106 men born for every 100 women, or some times reported, the male proportion of total births (106 male births divided by 206 total births) is 0.514 (51.4%). In Japan, a study by Misao Fukuda showed that periconceptional smoking might be a contributing factor to a lower male to female sex ratio. Researchers, however, have reported a decreased proportion of male births in Denmark, Netherlands, USA, Canada. Some speculate that exposure to environmental toxins that predominantly affect male and male reproductive system could play a role. Exposure of men to tetrachlorodibenzo-p-dioxin (TCDD) is linked to lowered male to female sex ratio in their offspring. Reduced male
births in major Italian cities interpreted as a signal of increasing exposure to hazardous environmental conditions for male conceptions. Changing sex ratio in United State was seen during Styrene exposure. Stress could also reduce the sex ratio of newborns as witnessed in connection with the Kobe earthquake, which resulted in an abrupt reduction in sperm motility and a significant decline in the sex ratio. Individuals exposed to air pollution from incinerators also showed a decline in sex ratio. Use of tobacco as cigarettes and as smokeless tobacco is increasing day by day in Pakistan and becoming the Global killer. In our study the frequency of smokers was 57%. We assessed the offspring sex ratio (male to female) when the subjects smoked less than 14 cigarettes per day compared with the subjects smoked more than 14 cigarettes per day in various cities of Pakistan.

There have been very few studies examining this issue in developing countries; none have been done in Pakistan.

Smoking rates in this study was determined among males. Data about female smokers in Pakistan is not up to date. Male sex among offspring is only determined by Y chromosome which comes from the male counterpart. It is therefore assumed that smoking among males would have effect on offspring sex ratio. Some researchers suggest that sperm carrying the male sex chromosome are more vulnerable to toxic chemicals in tobacco smoke than sperm carrying the female chromosome such affected Y-sperm cells might be less prone to fertilize or produce less viable embryos.

1. Methodology:

Study Design: Cross-Sectional survey.

Place of study: Karachi-Islamabad-Rawalpindi and Kalarkahar in Pakistan.

Variables:
- Dependent variables: Sex of the offspring.
- Independent variables: Duration of smoking along with number of cigarettes smoked per day.

Sampling: Since there was no sampling frame available for the households, so simple random sampling was not possible. We adopted systematic sampling technique. Starting from the 1st house we picked up every third house in the street till the desired sample size of households was reached in one area of study. The same process was repeated in the other area of study to complete the sample size of 405 subjects.

Sample Size: Sample Size of 405 subjects was calculated using Epi-Info 6.0.

<table>
<thead>
<tr>
<th>Group</th>
<th>Sample Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non Smokers</td>
<td>n=174 (Control group)</td>
</tr>
<tr>
<td>Smokers</td>
<td>n=231</td>
</tr>
</tbody>
</table>

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The smokers were divided into four groups based on the number of years they are smoking as under:

- **Group I**: Less than 15 years  $n=52$
- **Group II**: 16-30 years  $n=100$
- **Group III**: 31-45 years  $n=64$
- **Group IV**: more than 45 years  $n=15$

**Inclusion Criteria**

**Case definition for Smokers:**
Married males with at least one child and with a history of smoking for at least 2 years before their first baby was born.

**Case definition for non-smokers:**
Married males with at least one child and not smoking for at least 2 years before the births of their children.

We did gather data about smoking among males only because of the fact that male gender is determined by Y chromosome which is contributed by father.

**Exclusion Criteria**
Married smokers/non-smokers having no child, persons using other forms of nicotine such as chewing tobacco. We excluded the role of TCDD (Tetrachlorodibenzo-p-dioxin) which is an industrial combustion byproduct, styrene which is related to chemical industry by performing this study in a rural set-up where industrial pollution could not confound the effects of cigarette smoking on off-spring sex-ratio. Similarly workers of incinerators were not included in the study.

**Data collection instrument (Questionnaire)**
Pre-designed, structured questionnaire was used in the survey. The survey was carried out by medical students who were properly trained in the interviewing technique and trained to ask questions in the local language (Urdu/Punjabi). The questionnaire which was initially in English was translated in to Urdu/Punjabi and later translated back in to English language to see it’s validity.

**Duration of the survey**
The Survey was carried out between April 2002 and December 2002.

**Data collection and Analysis**
All the questionnaires were checked at the site of the survey daily for accuracy and completeness by the Research supervisors. If any data were found missing the concerned interviewers were required to go back to the household and get the information if possible.
All data was entered in to SPSS (Statistical package for Social Sciences) version 10.0. The data was re-validated and later analyzed. Smokers were divided into four groups on the basis of their period of smoking in years (<15, 16-30, 31-45, >45). Finally the subjects were divided into two main groups that were those consuming less than 14 cigarettes per day and those consuming more than 14 cigarettes per day. Cigarettes per day and offspring sex ratio were assessed.

**Funding**
No funding is involved in this project.

**Ethical considerations**
An informed consent was taken from each participant of the study and their written consent was kept in record.

2. Results:

Mean age of the study participants was 47.62 ± 13.22 years. Only 7 (6.9%) of the study participants were illiterate while the rest 398 (93.1%) had Primary or higher level of education. Duration of smoking in our study participants was 25.8 ± 12.6 years.

Table 1 depicts the percentage of smokers consuming less than 14 and more than 14 cigarettes per day in each group divided according to the duration of smoking in years, as mentioned in the methodology section.

It is clear from table 2 that in all four groups where subjects are smoking more than 14 cigarettes per day their offspring ratio (male: female) is quite low as compared to those who were smoking less than 14 cigarettes per day. Duration of smoking has been broken down to four categories as already mentioned in the methodology section. Odds ratios and p values calculated for each category of smokers shows significant difference in sex ratio in groups II and III. No other form of tobacco other than smoking was found among the study participants. Offspring sex-ratio among non-smokers was 1.53. Overall male/female sex ratio among smokers was 0.85. The overall sex ratio among smokers was significantly lower (p=0.006) as compared to nonsmokers.

Table 3 shows results of Multivariate logistic regression analysis of the effect of various confounding factors on offspring sex-ratio. All the confounding variables such as age of father, duration of smoking, number of cigarettes smoked per day and socioeconomic status of father were thought to have association with each other and with the outcome of interest i.e sex ratio. Age of father could be related to sex ratio, as it was more likely for the fathers with advanced age to have completed their families, as compared to younger fathers. Similarly duration of smoking in years was thought to be independently associated with sex ratio. Socioeconomic status of father was also thought to be associated with cigarette smoking behavior of the father. All these potential confounding variables were put in the logistic regression model. The only variable significantly associated with offspring sex-ratio was number of cigarettes consumed per day (p=0.004). Offspring sex ratio was significantly low in subjects who were smoking more than fourteen cigarettes per day as compared to those who were smoking less than fourteen cigarettes per day.
Table 4 shows the comparison of offspring sex ratio from various studies. Our study shows a slightly lower sex ratio of 0.85 as compared to other study on smoking fathers, sex ratio 1.0.

3. Discussion

The word ‘nicotine’ is of French origin, named after French Ambassador to Portugal Jean Nicot. Cigarette smoking was introduced in Pakistan by an English ambassador through the court of Akbar the Great as early as 300 years ago. Tobacco use in Pakistan is not limited to cigarette smoking. Chilum, huqqah, chewing tobacco in pan, snuff and niswar are some other common ways of intake. Experts divide tobacco use into two broad categories - smoking and smokeless tobacco. Both uses of tobacco are very common in Pakistan as established by a survey conducted by the PMRC in 1994. Our study was confined to tobacco smokers only as we excluded any other form of nicotine intake. Studies on sex-ratio among other forms of nicotine intakers should be undertaken in the future. Right now, no such data is available from Pakistan. The sex ratio at birth in most countries is between 104 and 106 males to 100 females (1.06). It fluctuates within a narrow range from time to time in some area but the general trend of the last 20 years has been downward. However, when the couples were grouped according to their smoking habits, the ratio changed. Also of interest is the finding that estrogen receptor proteins in human spermatozoa, which like the epididymus, have AH receptors. In addition, antiestrogenic drugs, such as clomifene citrate and tamoxifen, were shown to enhance the functionality of spermatozoa induced by oestradiol. To gain more insight, it would be interesting to know the sex ratio of newborns sired by asthenoazoospermic men who had been treated with an antioestrogen such as tamoxifen. Some researchers suggest that sperm carrying the male sex chromosome are more vulnerable to toxic chemicals in tobacco smoke than sperm carrying the female chromosome such affected Y-sperm cells might be less prone to fertilize or produce less viable embryos. Semen analyses showed that non-smoking men have healthier sperm than smoking men (in terms of sperm viability and longevity). The comparative number of males has been declining in several industrialized countries over the past few decades and researches suspect toxic substances may be partly to be blamed. Our data relates a modification in the expected human sex ratio in a resident population with known smokers. Lowest sex ratio in subjects consuming less than 14 cigarettes per day seen was 0.73. Lowest sex ratio in subjects those consuming more than 14 cigarettes per day were seen and was 0.46. Overall sex-ratio among smokers in this study was 0.85. (Table 2). Table 4 also gives sex-ratios of some other previously published studies. Our study shows a slightly lower male/female sex ratio as compared to the other study among smoking fathers. It is concluded that the smoking might be a contributing factor to a lower male to female sex ratio of offspring. This gives male smokers another reason to kick the habit, and provides another area of emphasis and education for smoking cessation programs.

Study limitations:

Our study did not look in to the familial causes of low sex ratio. Secondly we did not look in to the association of sex-ratio with birth order. In many cultures including ours
there is a tendency of trying to have a son after successive births of daughters in the family. Although this could introduce a bias in the study but it would affect the smokers and non-smokers alike. There was significant difference in sex ratio among smokers as compared to non-smokers.

References


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Table 1. Percentage of smokers consuming less than 14 & more than 14 cigarettes per day. (n=405)

<table>
<thead>
<tr>
<th>Groups</th>
<th>&lt;14 per day</th>
<th>&gt;14 per day</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>69.23%</td>
<td>30.76%</td>
</tr>
<tr>
<td>II</td>
<td>62%</td>
<td>38%</td>
</tr>
<tr>
<td>III</td>
<td>50%</td>
<td>50%</td>
</tr>
<tr>
<td>IV</td>
<td>53.33%</td>
<td>46.66%</td>
</tr>
</tbody>
</table>

Table 2. Sex Ratio in less than 14 & greater than 14 cigarettes per day consumers. (n=405)

<table>
<thead>
<tr>
<th>Duration of smoking in yrs</th>
<th>&lt;15 yrs</th>
<th>16-30 yrs</th>
<th>31-45 yrs</th>
<th>&gt;45 yrs</th>
<th>Non Smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cigarettes per day</td>
<td>&lt;14</td>
<td>&gt;14</td>
<td>&lt;14</td>
<td>&gt;14</td>
<td>&lt;14</td>
</tr>
<tr>
<td>No of smokers</td>
<td>36</td>
<td>16</td>
<td>62</td>
<td>38</td>
<td>32</td>
</tr>
<tr>
<td>No of offspring</td>
<td>n= 71</td>
<td>n= 41</td>
<td>n= 136</td>
<td>n= 177</td>
<td>n= 165</td>
</tr>
<tr>
<td>Male/ Female %</td>
<td>42.25/ 57.74</td>
<td>31.70/ 68.29</td>
<td>64.70/ 35.29</td>
<td>36.15/ 63.84</td>
<td>47.87/ 52.12</td>
</tr>
<tr>
<td>Sex Ratio</td>
<td>0.73</td>
<td>0.46</td>
<td>1.83</td>
<td>0.56</td>
<td>0.91</td>
</tr>
<tr>
<td>Odds Ratio (95%CI)</td>
<td>1.58(0.65-3.84)</td>
<td>3.53(2.14-5.84)</td>
<td>1.59(1.03-2.46)</td>
<td>1.98(0.81-4.85)</td>
<td></td>
</tr>
<tr>
<td>P value</td>
<td>0.268</td>
<td>0.000</td>
<td>0.027</td>
<td>0.09</td>
<td></td>
</tr>
</tbody>
</table>

Overall Male/Female Sex ratio in Smokers = 0.85 (P=0.006 comparing sex ratio among smokers and non-smokers)

n=number of offspring
Table 3. Logistic Regression Analysis of Sex ratio with various confounding factors.

<table>
<thead>
<tr>
<th>Factors</th>
<th>Odds Ratio(95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cigarettes per day (&lt;14 cig/day, &gt;14 cig/day)</td>
<td>1.47 (1.13-1.92)</td>
<td>0.004</td>
</tr>
<tr>
<td>Duration of smoking</td>
<td>0.99 (0.98-1.01)</td>
<td>0.46</td>
</tr>
<tr>
<td>Age</td>
<td>0.82(0.54-1.25)</td>
<td>0.35</td>
</tr>
<tr>
<td>Socioeconomic status (&lt;Rs5000/months, &gt; Rs 5000/month)</td>
<td>1.05(0.80-1.36)</td>
<td>0.73</td>
</tr>
</tbody>
</table>

Table 4. Comparison of Sex-ratios from various studies

<table>
<thead>
<tr>
<th>Name of study</th>
<th>Factor studied</th>
<th>Male/Female Sex-ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Our study</td>
<td>Smoking Fathers</td>
<td>0.85</td>
</tr>
<tr>
<td>Misao Fukuda (Reference 2)</td>
<td>Smoking Fathers</td>
<td>1.0</td>
</tr>
<tr>
<td>Paolo Mocarelli (Reference 7)</td>
<td>Dioxin</td>
<td>0.48</td>
</tr>
</tbody>
</table>
Chapter 25

Epidemiological Research on the Suicidal Attempts and Suicidal Adjustments Due to Intoxications in the Coming Generation

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8 bul. Georgi Kotsctev, Pleven – 5800

Abstract - Modern society is under heavy social stress, which reflects negatively on the minds of the growing generation as well. It was found out that suicide attempts became more frequent and they spread on earlier and earlier age. An epidemiological research on suicide attempts by acute exogenetic intoxication among the children, treated in the Department of Toxicology of the University Hospital of Pleven in the period of 2001-2007, has been made. Autointoxications are 22, 75% of the total number of the intoxications in childhood. Girls predominate in the age group of 14-18. The motives for self-wounding, seasonal and twenty-four-hour dynamic, the most frequent toxical noxa and the seriousness of clinical manifestations have been studied. The data have been compared to the inquiry investigation made among school children on suicide attitude and ways of self-wounding.

Key words - suicide attempts, childhood, intoxication

Introduction

According to Freud /1917/ suicidal attitude could be examined as “directing the aggression against the own EGO and divided position against the other persons”.

It is expressed as sense of guilt, low self-esteem and as a consequence a suicidal attempt or suicide.

Modern society is under heavy social stress, which reflects negatively on the minds of the youngsters and the growing generation with manifestation of aggression and autoagression.

In the recent years as a world tendency it was found out that suicide attempts became more frequent and they spread on earlier and earlier age. It is especially disturbing that intentions for a suicide have been found in pre-school age children. It is assumed that that kind of attitude is of imitative nature resulting from the impact of the
surroundings and the media but it endangers the life and the health of the growing generation /1, 2/. Suicidal attempts by means of autointoxication are a serious toxicological problem as according to the statistical data in Bulgaria it is the most frequent means of autoagression in all age groups.

Thus our **objective** was to carry out an epidemiological research at regional aspect of the suicidal attempts by exogenetic intoxication among pupils and the means of self-wounding.

1. **Material and method**

A prospective study was carried out in regional aspect for a 7-year period /2001 – 2007/ of children, up to the age of 18 with suicide attempts treated in the Department of Toxicology at the Clinic of Pediatrics of the University Hospital of Pleven.

A survey about suicidal aptitude was undertaken among students, age 14 to 18.

It was used:
- Sociological methods – observation, medical documentation, survey
- Screening methodology - GHQ (General Health Questionnaire)
- Data processing statistical methods – software products – SPSS for Windows v. 7 and STATGRAPHICS Plus.

2. **Results and discussion**

For the period 2001 – 2007, 886 children, age 0 to 18, with acute intoxications were treated in the Department of Toxicology at the Clinic of Pediatrics of the University Hospital of Pleven. The percentage of suicidal attempt was 22.75%.

![Fig. 1 – Correlation between the intoxication cases and the suicidal attempts for the period 2001 – 2007](image)
The suicides were distributed per age groups according to the assumed age periods in paediatrics.

- 6 – 11 years old – early school age - 3.55%
- 11 – 14 years old – intermediate school age – 24.37%
- 14 - 18 years old - teenage – 72.08%

The youngest child was 9 years old – a girl suffering for her mother who was working abroad.

Fig. 2 – Age distribution of the cases of suicidal attempts

The percentage in the early school was relatively low but the fact that younger and younger children manifest suicidal aptitude was disturbing. This tendency was pointed out by other authors from various countries. (3, 8, 9)

The children, age 14 – 18, was the prevailing group as the suicidal attempts were a conscious act, affecting mainly the teenage period of growth with its typical behavioral manifestations of the puberty.

Girls showed greater aptitude for depressions and negative emotions. They showed more demonstratively their problems thus the suicidal intoxications were 77.16% for girls compared to 22.84% for boys.

It could be related to the earlier maturity of female gender that aroused striving for independency and self-confidence for maturity.

A greater part of the population in working age and the young people with their families is concentrated in town. The educational institutions and the students are also in towns. It was found out that children with suicidal attempts were mainly from towns – 66.50%, compared to 33.50%. It could be explained by the demographic tendencies in the region.

The suicidal attempts in childhood as well as the acute intoxications had a typical seasonal dynamics, too.
Fig. 3 – Distribution of children with suicidal attempt by acute intoxications per gender
Fig. 4 - Distribution of the cases according to the place of residing

Fig. 5 – Seasonal dynamics of suicidal autointoxications

The problems and the negative emotions are expressed. The time from 6.00 p.m. to midnight is the peak moment of the accumulated mental stress during the day.
The motivation for suicidal attitude in children has a complex genesis and it is related with the experiencing of serious crises. It is an expression of a “cry for help” to the family and society. (4,9)

The destructive family models, traumatic and negative live events reflect on young people, especially on those who are not able to cope with psychotrauma. (2,3) Most frequently these are separated parents, psychopathy of the parents, alcohol and psychoactive substances abuse in the family, very high expectations of the parents from their children, overprotective parents, etc.

School and problems related to it has its important role as a reason for autoagression among the growing generation, followed by intimate dramas and disappointments.

Our data, in regional aspect comply with the data for the entire country. (5)

The main motive for suicidal act in 44.67% of the cases is a family conflict; 28.93% - a conflict at school 19.28% - intimate drama. In 4.06% of the cases it was found that the children endanger their lives when they are deprived of parents’ cares and they suffer for their parents.

The most frequent toxical noxas taken with the purpose of a suicide by the patients were medications – 82.23%, followed by industrial and agricultural preparations – 17.26% and other toxic substances – 0.5%.
Fig. 7 – Distribution of the cases according to the motives for suicidal behavior

Fig. 8 – Types of toxical noxas

The most often used from medications, were tranquilizers – 57%, followed by mixed medication intoxications and drugs for cardio-vascular diseases. These medications are the most widely used at home and are sold without a prescription in the pharmacies.
Depending on the type of the toxical noxa and the time when the medical aid was looked for, 31.98% from the studied children were admitted in severe condition, with conscience impairment to a certain extent, 38.07% in moderate severe condition and 29.44% had slight toxic signs. Intoxication without symptoms was found in a girl that made a demonstrative suicidal attempt to get intoxicated with mercury from body temperature thermometer in order to frighten her parents. She was hospitalized with simulative symptoms.

![Fig. 9 – Degree of condition severity](image)

Two survey investigations were carried out with students from Pleven District in 2005 and 2007 for determining the suicidal aptitudes because of the problem significance. The students were from two high schools in the town of Pleven, age 14-18 (from VIII to XII class). The students in one of the schools were profiled for arts studies and in the other school – for sciences.

The survey was made on voluntary principle with anonymous questionnaire. Totally 233 students took part in it – 120 in 2005 and 133 in 2007; 140 girls and 93 boys.

The results were classified in two main groups comprising two subgroups.

The main groups were: students without suicidal aptitude and students with suicidal aptitude.

The students without suicidal aptitude were further subdivided into two subgroups:
- results within the normal rates;
- anxiety and depressiveness, without suicidal aptitude.

The students with suicidal aptitude were also subdivided into two subgroups:
- suicidal aptitudes, without anxiety and depressiveness, for which we assume that they were connected with demonstrative and manipulative attitude of the type "cry for help";
- suicidal aptitudes, with anxiety and depressiveness.
The results from the summarized questionnaires showed 77.3% children without suicidal aptitude and 22.7% with suicidal aptitude.

- 74.68% were within the normal rates;
- 2.58% were without suicidal aptitude but experiencing anxiety and depressiveness;
- 16.02% were with suicidal aptitude but without anxiety and depressiveness;

The group of students at high risk, showing anxiety, depressiveness and suicidal attempts represented 7.73% from all participants in the survey. They were the potential suicides.
Fig. 11 – Results from the survey investigation on the means of self-wounding among students

The preferred manner of self-wounding in women was medications - 69.8%, followed by other mechanical means. On the contrary, men preferred other mechanical means - 41.3%, as medications took the second order of priority.

The smallest percentage was for the other toxic substances.

It could be concluded that:

- the physical and mental changes in the teenage period form suicidal attitude that was developed on the background of specific personal and social factors;
- the main motive for a suicidal attitude among the growing generation is a conflict in the family;
- epidemiological data showed a tendency of increasing the suicidal attempts by intoxication as medications were the leading toxical noxa.

References

1. Гайдарова Р. – “Самоубийствата”, Монография, Плевен, 1998
5. Цонева-Пенчева Л., Р. Йолова-Йорданова, М. Вуков – “Самоубийствата и човешки потенциал на България”, Сп. Население, БАН, 1-2,
6. Шир Е. – “Суицидалное поведение у подростков”, Жур. Невропатол и псих и мед., Корсаков, 1984, 84, № 10, 1556-15
Chapter 26

Re-evaluation of Some Stages of the Classical Conventional Treatment of Amanita Phalloides Mushroom Poisoning

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² Toxicology Clinic, MHATEM ‘Pirogov’

Abstract - The standard accepted treatment protocol for Amanita Phalloides mushroom poisoning (APMP) consists of: detoxic depuration; reanimation and organo-protective treatment. The aim of our study is the role of partial exchange blood transfusion as one of the stages for treatment of APMP. Two groups of patients with APMP, treated at the Toxicology Clinic in co-operation with the Department for transfusion haematology and immunology at MHATEM ‘Pirogov’ for the period 2004 – 2007 are presented. The first group of patients (n=68) was treated with partial exchange blood transfusion, while the second one (n=42) was treated with contemporary component therapy with blood and plasma components (e.g. erythrocyte concentrate; thrombocyte concentrate; fresh frozen plasma; prothromplex; antithrombin III concentrate and fibrinogen). The data obtained were analysed and the comparative analysis showed that the including of the new plasma substances has prophylactic role for the development of one of the most severe complications of APMP - disseminated intravascular coagulopathy. On the basis of the data and conclusions we propose the exchange blood transfusion to be replaced in the classical treatment protocol by optimal used of blood and plasma components.

Key words - mushrooms poisoning, amanita phalloides, blood transfusion, blood and plasma components

Treatment of amanita phalloides mushroom poisoning (APMP) is extremely difficult because of the acute structural and functional damage of liver. Haemorrhagic diathesis seen in APMP is result of damage of liver synthesis of several clotting factors: coagulation factors II, VII, IX and X, fibrinogen, as well as of the synthesis of some inhibitors of fibrinolysis – antithrombin III (AT III), protein C, protein S, α1 antiproteas inhibitor, α2 macroglobuline.

These conditions require specialised treatment with application of only those blood components which deficiency has leading role in the pathogenesis. The aim of the presented study is to make comparative analysis and re-evaluation of:

- partial exchange blood transfusions as one of the stages in the classical conventional treatment of patients with APMP and
transfusion treatment of these conditions, based on the contemporary component therapy with blood components and plasma products.

Materials and methods:
The patients with APMP, hospitalised and treated at the Toxicology Clinic of MHATEM 'Pirogov' for the period 2004 – 2007 have been studied. The treatment was performed with the active co-operation of the Department for transfusion haematology and immunology at MHATEM ‘Pirogov’. The hospitalised patients were divided into two groups – the first being treated according to the conventional accepted as standard protocol for treatment of APMP while the second group of patients was given component transfusion therapy with blood components and plasma products.

A total of 110 patients with amanita phalloides mushroom poisoning have been treated at the Toxicology Clinic of MHATEM ‘Pirogov’ for the period 2004 – 2007. The First Group consists of 68 patients, who were treated with the accepted standard treatment protocol for APMP, including:

- Detoxic depuration – gastric lavage, enema, etc;
- Humoral detoxic depuration – forced diuresis, extracorporal detoxication, partial exchange blood transfusion, non specific antidotes;
- Detoxic depuration and desintoxication on cellular level (antidotal treatment);
- Organoprotective treatment.

The Second Group consists of 42 patients who were given component therapy with blood and plasma products, e.g. used are only those factors of blood coagulation which deficit is leading in the pathogenesis of haemorrhagic diathesis. Good laboratory diagnostics of the state of haemostasis is in the basis of adequate treatment. That’s why the treatment of all patients was monitored daily, using the following examinations: coagulation time (S); thromboplastin time (%); number of platelets(x 109); fibrinogen (g/l); AT III level (%); haemoglobin (g/l); haematocrit; and total protein.

Clinical signs of APMP are result of complete level down of whole body metabolism of the patient due to the functionally and structurally damaged by the amanita phalloides toxins liver. Various haemorrhagic presentations are seen – in the form of gastrointestinal haemorrhages, subcutaneous haematomas at the application sites, etc. This can easily explained by the decreased synthesis of vitamin-K dependent factors II, VII, IX and X, protein C and S and antithrombin III. The laboratory examinations show typical biochemical constellation of high levels of cytolytic enzymes, serum bilirubin, and low values of serum protein and prothrombin index

Results:
The patients from the First Group were treated with the whole complex of standard accepted protocol for APMP, including also partial exchange blood transfusion with whole blood of approximately 1500-2000 ml isogroup bloos with expiry term of 3 to 5 days.
The patients from the Second Group were given substitution therapy with blood components and plasma products which lasted up to the third day from the beginning of the poisoning. This therapy includes:

1. **Platelet concentrate** – the substitution was performed when expressed thrombocytopenia was present, e.g. it was used for prevention of severe life threatening haemorrhages. Four of our patients were given platelet concentrates, obtained by means of apherese technique.

2. **Fresh frozen plasma (FFP)** - it contains all coagulation factors, plasma inhibitors and plasminogen.

3. **Coagulation plasma products** - the principle of treatment with these products is based on application of only those components that are needed for the given patients in the given circumstances. In this way the patient receives maximum efficacious transfusion therapy and is protected as much as possible against the adverse reactions and complications, which would have occurred if the rest of the blood components would have been given as well.

Plasma products are highly purified substances, containing high specific activity in small volumes. They are prepared from human plasma for quick and efficacious correction of isolated or combined coagulation deficits.

It is preferable to use these products in combination with FFP in order to avoid the risk of thrombotic complications (as ATIII in brought in).

- **The product ‘Protromplex Total Blood Coagulation Factors II, VII, IX and X’** is a highly concentrated preparation, prepared by human plasma and containing coagulation factors II, VII, IX and X in almost equal concentrations. The dosage and duration of treatment is determined by the severity of the haemostasis disturbances and by localization and intensity of haemorrhages (from 20 to 50 U/kg body weight). It is required to apply this treatment together with FFP.

- **The product ‘Abithrombin III concentrate’** is a virally inactivated concentrate of human plasma antithrombin III. Its application is indicated in patients with plasma level of AT III below 70% of the normal values for prophylaxis and treatment of thrombotic and thrombo-embolic complications. The normal value of AT III activity in human plasma is between 8 and 120% and its decrease below 70% leads to high risk for thrombosis. I is recommended that the target value which is aimed to be achieved is at least 70% AT III activity.

- **Fibrinogen** is a plasma product, containing purified fibrinogen; the maximal haemostasis level aimed to be achieved is 1.5 g/l.

Substitution therapy proved to be better because of the well-expressed haemorrhagic diathesis and low levels of haemostasis components. After the application of this substitution transfusion therapy an increase in the monitored lab values was observed in all patients. Clinically these changes are presented by improving of the liver enzymes.
values, decrease in gastrointestinal haemorrhages and disappearing of subcutaneous haematomas, e.g. improvement of the course of the disease and its outcome.

Conclusions:
1. The used transfusion substitutional therapy lead to increase in the values of laboratory indeces and improving of disease course and its outcome.
2. Therapy with coagulation plasma products replaces whole blood and plasma treatment from their use in the clinical practice.
3. High therapeutic effectiveness of the used substitution therapy with plasma products (high survival rate and recovery) justifies the high cost.
4. Based of the conclusions made, we propose partial exchange blood transfusion to be replaced in the classical treatment with optimal usage of blood components and plasma products.

References:
2. Pittiglio D. Modern blood banking and transfusion practices. USA, 2003, 323-345;

Table 1 - Monitoring of coagulation indices

<table>
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<tr>
<th>indices</th>
<th>D 1</th>
<th>D 2</th>
<th>D 3</th>
<th>D 4</th>
<th>D 5</th>
<th>D 6</th>
<th>D 7</th>
<th>D 8</th>
<th>D 9</th>
<th>D 10</th>
<th>D 11</th>
<th>D 12</th>
<th>D 13</th>
<th>D 14</th>
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<tr>
<td>Coagulation time</td>
<td>490</td>
<td>500</td>
<td>520</td>
<td>510</td>
<td>520</td>
<td>540</td>
<td>510</td>
<td>510</td>
<td>480</td>
<td>480</td>
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<td>(s)</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Thrombo-plastin</td>
<td>17</td>
<td>38</td>
<td>35</td>
<td>30</td>
<td>27</td>
<td>16</td>
<td>17</td>
<td>39</td>
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<td>67</td>
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<td>62</td>
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<td>(%)</td>
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<td></td>
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<td>Platelets (x10^9)</td>
<td>80</td>
<td>75</td>
<td>80</td>
<td>60</td>
<td>22</td>
<td>30</td>
<td>40</td>
<td>30</td>
<td>90</td>
<td>70</td>
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<td>Fibrinogen (g/l)</td>
<td>1.8</td>
<td>1.8</td>
<td>1.2</td>
<td>1.2</td>
<td>0</td>
<td>0</td>
<td>1.2</td>
<td>1.3</td>
<td>1.7</td>
<td>1.9</td>
<td>1.9</td>
<td>3.9</td>
<td>3.8</td>
<td>3.7</td>
</tr>
<tr>
<td>AT III level (%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>34</td>
<td>42</td>
<td>50</td>
<td>40</td>
<td>34</td>
<td>42</td>
<td>50</td>
<td>55</td>
<td>70</td>
<td>86</td>
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Chapter 27

Acute methanol intoxications in Varna region during the period 2000 - 2008 year – an important social problem

III announcement on this theme

Yulichka SABEVA, Marieta YOVCHева1, Mariana KOLEVA, Snesha ZLATEVA1, Petko MARINOV1

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Abstract - The aim of this work was to investigate the frequency and etiology of acute intoxications with methyl alcohol during the period 2000 - 2008 in Varna region in order to prevent them after establishing the origin of the toxic substance and eliminating it. The object of the study were 150 patients treated in the Clinic of intensive treatment of acute intoxications and toxoallergy in Naval Hospital - Varna and other hospitals in Varna region and 61 patients with acute methanol intoxication who were an object of forensic medicine and forensic chemistry toxicology expertise. The identification and quantitative analysis of the toxic agent in the blood of the patients were made in Toxicology Laboratory in Naval Hospital - Varna by an own gas-chromatography method for identification of volatile organic solvents, including alcohols. Different liquids with unknown composition, alcohol spirit trade drinks and spirit for technical needs had been analyzed in order to be able to discover the source of the methanol poisoning. These liquids had been drunk by the patients and were brought by their relatives, taken from shops or confiscated by the police. A firm tendency to increasing the number of severe methanol poisonings had been established. More frequently they were incidental and not a result of a suicidal act. On the background of general increasing the alcohol ethanol consumption, the methyl alcohol distributed in drug stores freely as spirit for technical use inevitably leads to wrong ingestion and severe and potentially fatal poisonings. The competent organs should stop the free production and free distribution of this especially dangerous trade product for household use and make strict restrictive rules for its use.

Key words - acute methanol intoxication, socially important problem.
**Introduction**

During the last years an increasing number of acute methanol intoxications after consumption of methylated spirit have been observed, a great part of them ending lethally.

The ethyl alcohol-spirit for technical use, denaturated spirit, popular names- wood spirit, spirit for burning, is 95% ethyl alcohol to which different denaturating substances, depending on the chosen formula, from different kind and in different quantities are added in order to make the fluid not fit to drink. These supplements have unpleasant odour or taste and contain different colouring agents for indication [1, 2]. The methylated spirit in Bulgaria used in households is denaturated by a raw wood spirit (methyl alcohol) in quantity of 1.0 % maximum and is indicated by the colourant methyl violet , that gives the fluid a specific bluish-violet color (Bulgarian state standard 6283-67) [3]. In spite of the fact that methyl alcohol is a compound with especially high toxicity its presence in the standard quantities in the denaturated spirit does not change significantly the total toxicity of ethyl alcohol. We should have in mind that ethyl alcohol is the antidote of methyl alcohol.

**Aim**

An investigation of the frequency and etiology of the acute methanol intoxications on the background of the growing up the frequency of the ethanol intoxications during the period 2000 - 2008 in Varna region; with a view to prevent them through determining the source of the toxic agent and eliminating it.

**Material and methods**

The objects of the study were 150 patients treated in the Clinic of intensive treatment of acute intoxications and toxoallergy in Naval hospital-Varna and in some other hospitals in Varna region with a diagnosis acute methanol intoxication. During the same period 1010 acute ethanol intoxications with blood ethanol level over 0.840mg/ml were treated. The investigation was extended by inclusion of 61 lethal methanol poisonings, object of forensic medicine and forensic chemistry toxicology expertise. Different liquids with unknown composition, alcohol spirit trade drinks and methylated spirit for technical use had been analyzed in order to discover the source of the methanol. These liquids were known to have been drunk by the patients or brought by their relatives because of suspected toxicity or taken in the course of preliminary investigation or confiscated from the shops of the trade network by the police.

The investigation was made by the author’s own gas-chromatography method for identification of volatile organic solvents including alcohols, with use of gas chromatograph GC 5890-series II Hewlett Packard, equipped with FID, Headspace sampler 19395 A and HP 3396-series II Integrator [4].

**Results and discussion**

The distribution of the ethanol and methanol intoxications during the years is presented on Table 1.
Table 1.

<table>
<thead>
<tr>
<th>Year</th>
<th>2000</th>
<th>2001</th>
<th>2002</th>
<th>2003</th>
<th>2004</th>
<th>2005</th>
<th>2006</th>
<th>2007</th>
<th>2008</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>20</td>
<td>25</td>
<td>23</td>
<td>22</td>
<td>35</td>
<td>135</td>
<td>220</td>
<td>218</td>
<td>312</td>
</tr>
<tr>
<td>Methanol</td>
<td>13</td>
<td>9</td>
<td>5</td>
<td>10</td>
<td>17</td>
<td>46</td>
<td>38</td>
<td>42</td>
<td>31</td>
</tr>
</tbody>
</table>

Figure 1 illustrates a tendency of increasing numbers of severe methanol intoxications on the background of the increasing frequency of ethanol intoxications during the last years. Especially high values were reached during 2005. During 2008 a light decrease was registered.

Concentrations of methyl alcohol in the blood in the toxic range (0.08-0.20 mg/ml) were found in 85 of the examined. In all the others the initial values were lethal (over 0.20 mg/ml): in 47 patients they were in the range of 0.21 to 1.00 mg/ml; in 41 patients they were in the range of 1.10 to 2.00 mg/ml; in 23 patients - from 2.10 to 4.00 mg/ml. In 14 cases the found blood methanol concentration was between 4.10 and 6.00 mg/ml [5]. In one case it was 11.57 mg/ml methanol in the blood.

The alarming fact that the number of non-suicidal methanol intoxication was quite high considering the increasing frequency of ethyl alcohol intoxications necessitated the search of the methanol intoxication source.

On Figure 2 we show the results of the analyses of spirit drinks done during the period 2000-2008. The analyzed spirit liquids were different: home made, ethyl alcohol taken from the trade network, liquids with unknown composition, different kinds “spirit for burning” - methylated spirit, taken from the trade network in connection with the poisonings.

It can be seen that only the ethyl alcohol spirit drinks including home made grape - brandy (“rakia”) - 60 objects do not contain any methyl alcohol over the permissible quantity (up to 4.00 g/l according to Bulgarian state standard).
The data from the investigation of 25 samples of liquids with unknown composition showed that 20 of them contained methyl alcohol in dangerous for life concentrations - from 12.7 to 98 volume %. 2 samples were brought to the laboratory by the relatives of two deceased patients. In the first case methyl alcohol had been detected - 18.75 vol. %, ethyl alcohol - 15.75 vol. %. In the second case - methyl alcohol 22.37 vol. %, ethyl alcohol - 14.87 vol. %.

The analyze of different kinds and trade marks of methylated spirit (“spirit for burning”) showed that a number of producers severely violate the standard and replace the ethyl alcohol with the much more toxic methyl alcohol.

84 bottles methylated spirit from different producers were analyzed. Only 22 (26.19 %) fit the Bulgarian state standards for ethanol contents in methylated spirit. 54 bottles (64.28 %) contained only methyl alcohol - from 15.00 to 99.00 vol. %. 4 bottles contained both ethanol and methanol – respectively 17.44 vol. % and 24.11 vol. %; 17.51 vol. % and 24.50 vol. %; 20.92 vol. % and 30.08 vol. %; 75.8 vol. % and 5.31 vol. %. In 2 bottles: 19.00 vol. % methyl alcohol and 66.00 vol. % isopropyl alcohol. Another 2 bottles contain only isopropyl alcohol.

A severe acute lethal intoxication was observed after drinking liquid from a bottle containing 78 vol. % ethanol and 15 vol. % methanol. In this case the methanol concentration found in the blood was 2.70 mg/ml and 3.60 mg/ml in the urine; no ethanol was found in the blood and the urine.

The results from the investigation show that a great part of the methyl alcohol distributed in the trade network, mostly in grocery shops, labelled as methylated spirit, considering the increasing ethyl alcohol drinks consumption inevitably would lead to mistakes, wrong ingestion and therefore, severe and often lethal poisonings.

The so called in Bulgaria “spirit for burning” - technical or denaturated spirit, produced by 8 different firms, is in fact methyl alcohol with concentration between 86 and 98 vol. % One firm produces denaturated spirit containing both methyl and isopropyl alcohol.

For example: Denaturated spirit “for burning” 98 degree MOTOX, produced by the firm “Aromatic” in Kazanlak, has on the label Bulgarian state standard 385-78. It is
a colorless fluid containing only methyl alcohol 98 vol. %. But BSS 385-78 is for ethyl alcohol and it does not permit any methyl alcohol [6].

Another example: Technical denaturated spirit produced by the firm “Rosachim” in Gorna Oriachovitsa- according to the label it fits BSS 6283-67. It is a colourless fluid containing only methyl alcohol with concentration 90 vol. %, while this standard does not permit more than 1.0 % methyl alcohol as denaturant in the denaturated spirit.

The false spirit labeled as “technical”, “denaturated” or “methylated”, lately seen “Spirits for burning-methyl alcohol”, and containing non permitted by BSS concentrations of methanol, is highly toxic not only when ingested but also when inhaled or applied on the skin. This grave falsification practically turns a popular household product into a very dangerous poison not only for the mass consumer but for the workers in this production and also for those who use this product as a solvent often in non-ventilated rooms. A lot of people still use this spirit for massages and compresses, not making difference between medicinal and denaturated spirit. Some poor people in the conditions of social and economic crisis use it as alcohol drink.

Our efforts were directed towards provoking and participation in the police campaigns for large-scale check-up of the trade network, confiscating different probes of methylated spirit from the shops, toxicology examination of them, giving information about the results to the appropriate investigating and judicial authorities as well as to the professional circle through specialized scientific forums, provoking public discussion in order to reduce the unintentional methanol poisonings which resulted from consumption of falsified denaturated spirit, regional and national media information and education. Nevertheless the results are not quite hopeful.

Conclusion

1. During the period 1992-1995 year according to the data of the Testing laboratory – Varna of the Executive agency for certification and testing, the data of Regional Inspection of Public Health Safety – Varna (2006) and our data about the period 1996-2008 the alcohol or so called spirit drinks which were labeled and with a banderol, sold in the trade network, do not contain methyl alcohol above the permitted norms, specified by the state standard.

2. A drastic deviation from the standard has been made by an inadmissible substitution of the formula of the denaturated (methylated) spirit with the much more toxic and dangerous for the health and the life compound methyl alcohol. The possible reason for this is a financial benefit because the sold methanol was non-excisable. The result was appearance of extremely severe methanol intoxications.

3. An important and very alarming fact is that during the last 10 years in Bulgaria the routine control of the various trade household products has been partially or totally insufficient. Some of these products can have potentially minor or severe deteriorating effects on the health of the consumers. Such a non-controlled product is the otherwise harmless when produced properly and used properly original denaturated spirit (so called “spirit for burning” – denaturated technical ethyl alcohol).

4. The competent organs and institutions like Commission of trade and defense of the consumer’s rights should make efforts to restore the controlling functions of the Testing laboratories of the Executive agency for certification and testing.
5. The interference of the competent organs including the Ministry of Health is extremely important in order to urgently stop the production and distribution of these very dangerous for people falsified household trade products.

References


Chapter 28

Use of Derivatized Reference Standards for Determination of Dialkylphosphates/Alkylphosphonates in the Course of Organophosphorus Agents’ Exposure Analysis

Vencislav BARDAROV
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Abstract - Analysis of dialkylphosphates in urine as their pentafluorobenzyl derivatives are used for measurement of exposure to organophosphorus pesticides. The calibration of the instrument used is performed by standard solutions of the pentafluorobenzyl derivatives of dialkylphosphates instead of standard solutions of analytes. This leads to reduction of the time for analysis and higher reproducibility of results. The reference compounds used for preparation of standard solutions are synthesized and purified by preparative HPLC in the lab. The purified pentafluorobenzyl derivatives of dialkylphosphates are available in Toxicochemical laboratory of MMA-Sofia.

Keywords - dialkylphosphates, calibration, biomarkers, pentafluorobenzyl derivatives, reference substances, organophosphates.

Measurement of exposure to organophosphorus agents (OPAs) is of great importance when the origin of toxic action should be identified and procedures for counteraction to be undertaken. Identification by analysis of native intoxicants is often hampered on account of fast irreversible binding of bio molecules and metabolic transformations of the OPAs. Reliable identification of origin of intoxication and assessment its degree, is the carrying out of analysis of OPAs’ metabolites – dialkylphosphates/dialkylphosphonates in biological media, which are acknowledged as biomarkers of OPAs [1,2].
The popular analysis of dialkylphosphates/alkylphosphonates is gas chromatography with phosphorus specific detection after derivatization (pentafluorobenzylolation) [2-4]. Calibration of the GC requires reference standards of analytes. When the reference standards of analytes are used, a longer sample preparation (derivatization) is needed, and an additional charge of budget of uncertainty reflects to lower precision of the quantitative analytical results (Fig. 2).

**Figure 1**
A scheme of the main hydrolytic metabolitc transformation of organophosphorus pesticides

**Figure 2**
Analytical procedures and their distribution to the error in analysis of dialkylphosphates
Reduction of analysis time and improvement of precision can be achieved using derivatized reference substances (RSs), ready for calibration of GC (Fig. 3).

Such of derivatized RSs are synthesized, purified by preparative liquide chromatography (HPLC) and certified by GC/MS as RS (Fig. 4).

Standard solutions of these RSs are used for calibration of GC in the course of dialkylphosphates analysis in Toxicchemical Laboratory of Military Medical Academy - Sofia, and they are available. Their mass spectral, HPLC, GC and purity characteristics are attached. Using them for preparation of standard solutions and calibration in the course of dialkylphosphates analysis, up to 30% reduction of the time for analysis and improvement of precision are obtained [5].
References:

Chapter 29

Organochlorine and Organophosphate Pesticides Monitoring in Non Conventional Biological Samples

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Centre of Toxicology Sciences and Research, Division of Morphology, Medical School, University of Crete, Voutes, Heraklion, 71003, Crete, Greece.

Abstract - Hair is considered as non conventional biomarker of exposure to drugs, pharmaceuticals, metals and pesticides. Recently hair has been used to assess chronic exposure to organophosphorus pesticides as well as to withdrawn organochlorine substances like DDTs, PCBs and HCHs which are currently detected as environmental pollutants. Several epidemiological studies have been conducted in Greece the last years using hair as a biomarker of exposure to organophosphorus and organochlorine pesticides. Many of the examined samples were found positive for HCHs and DDTs despite the fact the use of DDT is banned for over 30 years. Only very few head hair samples were found positive for the organophosphorus pesticide. This could be attributed to the fast and effective metabolism of these organophosphorous pesticides and the original low exposure of the participants to the parent compounds. Lately, new and more sensitive analytical methods have been developed for the determination and quantification of the non-specific metabolites of organophosphates (DAPs) aiming to assess the chronic exposure to organophosphorous pesticides in hair and meconium. More specifically, the dialkylphosphate metabolites under investigation were dimethyl phosphate (DMP), dimethyl thiophosphate (DMTP), diethyl phosphate (DEP), diethyl thiophosphate (DETP) and diethyl dithiophosphate (DEDTP). These methods include preparation of the sample by homogenization where it is necessary, extraction of metabolites followed by liquid-liquid extraction with organic solvent and analysis by gas chromatography-mass spectrometry. DAP metabolites can be considered as a suitable biomarker of exposure for a great number of OP pesticides and can be detected in biological samples at exposure levels below those levels which affect cholinesterase activity.

Keywords - hair, meconium, organochlorines, non specific organophosphate metabolites, organophosphate pesticides, chronic exposure, biomarker.

Introduction

Olive oil, vegetables and fruits are the most widespread agricultural products in the region of Crete and an important source of income of the island. The use of or-
S. Tonev, K. Kanev, C. Dishovsky

ganochlorine, in the past and organophosphate pesticides (OPs) nowadays is widespread. Pesticide sprayers, farmers and population living in rural areas are probably the most highly exposed groups [1]. Recently, scientists have focused their interest in understanding and recording the effects of low level long term exposure to pesticides.

DDT (1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane) has been extensively used all over the world. It is extremely soluble in lipids and organic solvents and metabolized slowly to DDE and DDD, both of them are stored in fatty tissues. Also, lindane, the γ-isomer of the compound hexachlorocyclohexane, is the only one of the five isomers found in the technical formulation possessing insecticidal properties. It is a very cheap and effective insecticide and that explains its extensive use until recently in many countries, including Greece. Like DDT, it is stored in the fatty tissues and metabolized slowly through many different metabolic pathways [2].

On the other hand, most of the OPs are rapidly metabolized in the human body by hydrolysis or oxidative desulfuration, giving the non-specific metabolites dimethyl phosphate (DMP), diethyl phosphate (DEP), dimethyl thiophosphate (DMTP), diethyl thiophosphate (DETP), dimethyl dithiophosphate (DMDTP) and diethyl dithiophosphate (DEDTP), often referred as dialkyl phosphate metabolites (DAPs). These metabolites are polar, water soluble compounds used as biomarkers of OPs exposure in human population [3,4].

Blood and urine samples are usually analyzed in cases of acute intoxications providing information for a short period. Non conventional samples (e.g. hair and meconium) provide important additional information, a wider window of detection, and possess certain advantages (simple, inexpensive and non-invasive sampling protocol) [3, 5, 6].

Hair analysis has already been applied in forensic investigations, historical research, adoption cases, criminal cases, rape cases, doping control [7]. Also, hair analysis has been successfully used to assess chronic exposure to organochlorine pesticides, dioxins and PCBs [5, 8-14] and is currently used for organophosphate and carbamate pesticides [15-18]. On the other hand, meconium is a complex matrix, consisting mainly of water, mucopolysaccharides, lipids, proteins, acids and salts and is mentioned as a repository of endogenous compounds and many xenobiotics like drugs, food additives, heavy metals, nicotine, alcohol and pesticides [19-23].

DDTs and HCHs accumulate in human’s tissues, so hair has been identified as a suitable matrix to assess short or long term exposure to them. Several studies have been published that aim to compare admitted history of pesticide use to detected hair concentrations, while segmental hair analysis may be performed to assess the temporal trends in the chronic exposure to various chemicals [6,17,24-25]. Lately, scientists have reported the possibility of the detection of DAPs in hair and meconium [4,26-27].

1. Materials and Methods

1.1. Sampling

In the last 3 years, several studies have been conducted in the laboratory of Toxicology, Medical School of University of Crete. In these studies, hair and meconium samples have been collected from urban and rural population from different regions of Greece. The biological samples of hair and meconium were analysed for organochlorine pesti-
cides (DDTs and HCHs), organophosphate pesticides and their non specific metabolites (DAPs).

More specifically, thirty eight meconium samples were collected along with data regarding maternal level of potential exposure to pesticides via occupational and residential environment. The participants were classified to two exposure groups (the low risk and the high risk of exposure group). Meconium samples were analysed for DAPs to assess the possible exposure of foetus to organophosphate pesticides [27].

A total of 433 head hair samples were analysed for organochlorine and organophosphate pesticides. Two hundred and eleven (211) head hair samples in the first study (study I) [24] and 222 in the second study (study II) [28] were collected from urban and rural population from two different regions of Greece. The population was divided into three working groups: people working in greenhouses, animal breeders and people working in open cultivations. They were also divided to four age groups and by sex. Also, twenty seven head hair (1 male and 26 female) samples were collected from urban population without occupational exposed to OPs and analyzed for DAPs.

1.2. Hair Treatment

1.2.1. Organophosphate Extraction

Briefly, hair was washed, pulverized and incubated at 37°C in methanol, overnight. The supernatant was evaporated to dryness and the residue was resuspended in 2 ml of water. Liquid-liquid extraction followed with 3 ml of ethyl acetate, twice. The combined organic phases were evaporated to dryness and resuspended in 50 μl of 100 ng/ml 1,2,3,4-tetrachloronaphthalene solution in hexane and analysed by gas chromatography – mass spectrometry [24].

1.2.2. Organochlorine Extraction

Following the decontamination step, the hair was cut in small pieces and incubated in 2 ml of 3M HCl, at 40°C overnight. Liquid–liquid extraction was performed by adding 2*3 ml of hexane-dichloromethane (4:1 v/v) and the organic phases were cleaned up on SPE columns, packed from the bottom with 250 mg deactivated alumina, 500 mg of acidified silica and 250 mg anhydrous Na₂SO₄. The SPE cartridges were activated by adding 2 ml of hexane:dichloromethane (1:1 v/v) and 2 ml of hexane. The elution solvent was hexane (4 ml). The final eluate was evaporated to dryness under a nitrogen stream, resuspended in 50 μl of 100 ng/ml 1,2,3,4-tetrachloronaphthalene solution in hexane and transferred to a GC-MS vial [24,28].

1.2.3. Dialkyl Phosphate Extraction from Meconium

Meconium samples were dried to remove residual water. After the addition of methanol, the suspension was incubated in ultrasonic for 1 hour at room temperature and solid-liquid extraction with mechanical shaking for 30 min followed. Methanol was separated from the solid phase transferred to a clean vial which contained 15 mg of K₂CO₃. Two ml of acetonitrile-diethylether (1:1 v/v) were added to the meconium and
the above procedure was repeated. The organic phase of acetonitrile-diethylether was removed and transferred to the vial containing the initial methanol phase. The combined organic phases were evaporated to dryness, 15 mg of K₂CO₃ was added to the residue, reconstituted in 1 ml of acetonitrile and 0.1 ml solution of pentafluorobenzylbromide (PFBBr) in acetonitrile (1:3 v/v) and incubated in an bath at 80°C for 30 min. After incubation, the mixture was evaporated to dryness under a stream of nitrogen at 35°C. The residue was dissolved in 100 μl of toluene and 1 μl was injected to GC-MS [27].

1.2.4. Dialkyl Phosphate Extraction from Hair

For the removal of the external contamination from the hair matrix, hair was washed twice in water and methanol for two minutes and dried in the oven at 38°C. Subsequently hair was weighed out and pulverized in a ball mill homogeniser. An amount of powder (100 mg) was transferred to a test-tube with 2 ml of methanol and DBP as internal standard was added. Hair was incubated at room temperature for 4 hours and liquid-solid extraction was performed for 0.5 hr by mechanical shaking. The mixture was centrifuged at 4000 rpm for 5 min. The supernatant was transferred to a test-tube containing 15 mg of K₂CO₃ and methanol was evaporated to dryness under a gentle nitrogen stream, at 35°C or in a vacuum evaporator at room temperature. Fifteen (15 mg) of K₂CO₃ was added to the residue, reconstituted in 1 ml of acetonitrile and 0.1 ml solution of pentafluorobenzylbromide (PFBBr) in acetonitrile (1:3 v/v) and incubated in an bath at 80°C for 30 min with occasional swirling. After incubation, the mixture was brought to room temperature and acetonitrile was evaporated to dryness under nitrogen at 35°C. The residue was dissolved in 100 μl of toluene and 1 μl was injected to GC-MS.

2. Analytical Techniques

2.1. GC-ECD and GC-MS Analysis of Organophosphate and Organochlorine Pesticides

The analysis of organochlorine and organophosphate pesticides was performed on a GC-ECD system (ultra Thermo Finnigan) equipped with a NPD and ECD detector and a HP-5 (30m x 0.25mm x 0.25μm) capillary column. Pure helium was used as a carrier gas. Two microliters of the solution were injected into the system in the splitless mode. The column temperature started at 110°C for 1 min, raised to 180°C at 15°C/min, held for 1 min, raised to 250°C at 5°C/min, held for 1 min and was finally raised to 300°C, at 25°C/min, where it remained stable for 7 min. The injector temperature was 220°C. The temperature of ECD was 300°C and the flow rate of nitrogen (make up gas) was 30 ml/min [24]. The confirmation of the positive samples was performed by electron ionisation (EI) mass spectrometric analysis on a GC-2010 Shimadzu system equipped with a HP-5MSI (30m x 0.25mm x 0.25μm) capillary column. The column temperature was initially held at 60°C for 1 min, raised to 180°C at 20°C/min, held for 1 min, raised to 250°C at 4°C/min, held for 1 min and was finally raised to 300°C, at 25°C/min, where it
remained stable for 2 min. The injector temperature was 270°C. The interface temperature was set at 310°C. The ion source temperature was 200°C [24,28].

2.2. GC-MS Analysis of Non Specific Metabolites of Organophosphate Pesticides

Electron ionisation mass spectrometric analysis was performed on a GC-2010 Shimadzu system equipped with a BPX5 (30m x 0.25mm x 0.25um) capillary column (SGE). Pure helium (99.999 %) with a column flow of 1 ml/min was used as a carrier gas. One microliter of the solution was injected into the system in the splitless mode and analysed under the following conditions: The column temperature was initially held at 60°C for 1 min, raised to 180°C at 20°C/min, held for 1 min, raised to 250°C at 4°C/min, held for 1 min and was finally raised to 300°C, at 25°C/min, where it remained stable for 2 min. The injector temperature was 270°C. The interface temperature was set at 310°C. The ion source temperature was 230°C.

Quantitative analysis was performed in selected ion monitoring (SIM) mode with a scan time of 0.2 s, using one target ion for quantification and one qualifier ion for confirmation for each compound. The target ions m/z=306 for DMP, 258 for DEP, 322 for DMTP, 350 for DETP and 366 for DEDTP and the qualifier ions m/z=110 for DMP, 334 for DEP, 211 for DMTP and 274 for DETP and 185 for DEDTP were used.

3. Results and Discussion

3.1. Organochlorine and Organophosphate Findings in Head Hair (study I)

The accumulation of DDTs, HCHs and other organochlorine compounds in human hair has been successfully investigated in the past and hair has been identified as a suitable matrix to assess exposure to these compounds [5,6].

The median concentrations of α-HCH, HCB, lindane, opDDE, ppDDE, opDDD, ppDDD+opDDT and ppDDT in head hair samples collected from the region of Crete (study I) are presented in Table 1. The 24.2% of the head hair samples was positive for α-HCH, 21.2% for HCB, 51.5% for lindane, 45.5% for opDDE, 15.2% for ppDDE, 57.6% for opDDD, 42.4% for ppDDD and opDDT and 9.1% for ppDDT. Methyl parathion, malathion and fenthion were not detected while 0.05% of the total samples were positive for diazinon at mean concentration of 2.8 pg/mg [24].

Table 1. Hair levels of the detected pesticides in the positive samples (pg/mg).

<table>
<thead>
<tr>
<th></th>
<th>STUDY I Median</th>
<th>STUDY I Maximum</th>
<th>STUDY II Median</th>
<th>STUDY II Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lindane</td>
<td>70.2</td>
<td>174.7</td>
<td>124.2</td>
<td>905.9</td>
</tr>
<tr>
<td>α-HCH</td>
<td>7.2</td>
<td>50.3</td>
<td>40.4</td>
<td>687.0</td>
</tr>
<tr>
<td>HCB</td>
<td>2.2</td>
<td>15.9</td>
<td>19.7</td>
<td>323.2</td>
</tr>
<tr>
<td>opDDE</td>
<td>2.7</td>
<td>571.0</td>
<td>6.2</td>
<td>18.4</td>
</tr>
</tbody>
</table>
The median concentration of total HCHs in each population group was 95.0 pg/mg for greenhouse people, 38.2 pg/mg for animal breeders and 24.1 pg/mg for open cultivation group. The median concentration (in pg/mg) of total DDTs in each population group was 8.9 for greenhouse people, 3.3 for animal breeders and 5.2 for open cultivation group. The DDTs burden is similar in the three different occupational groups (p=0.528) while the HCHs burden is significantly higher in the greenhouse workers group (p=0.009) compared to the other two groups (Table 2).

Table 2. Median and maximum (pg/mg) of organochlorine pesticides in selected population groups (study I).

<table>
<thead>
<tr>
<th></th>
<th>Median</th>
<th>Maximum</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sum of HCHs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>/mg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Greenhouse workers</td>
<td>95.0</td>
<td>176.7</td>
<td>0.009</td>
</tr>
<tr>
<td>Animal breeders</td>
<td>38.2</td>
<td>84.3</td>
<td></td>
</tr>
<tr>
<td>Open cultivation</td>
<td>24.1</td>
<td>96.7</td>
<td></td>
</tr>
<tr>
<td>group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Sum of DDTs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>/mg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Greenhouse workers</td>
<td>8.9</td>
<td>2135.0</td>
<td>0.528</td>
</tr>
<tr>
<td>Animal breeders</td>
<td>3.3</td>
<td>28.0</td>
<td></td>
</tr>
<tr>
<td>Open cultivation</td>
<td>5.2</td>
<td>26.3</td>
<td></td>
</tr>
<tr>
<td>group</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.2. Organochlorine Findings in Head Hair (Study II)

The 50.5 % of the hair samples collected from the region of Amaliada, Peloponnesus Greece, were positive for at least one of the three hexachlorocyclohexane isomers examined. Lindane was the most frequently detected isomer (33.3 % of the samples) while α-HCH and HCB were detected less frequently (30.6 % and 26.1% respectively). 32.4 % of the samples were tested positive for at least one of the DDT isomers and metabolites examined. ppDDE was the compound most frequently detected in the hair samples (13.5%) followed by ppDDD and opDDT (9.9 %), opDDD (8.6 %), ppDDT (6.3%) and opDDE (2.7%).

The majority of the examined samples contained pesticide concentrations below the determination limit. The median concentration of lindane was 124.2 pg/mg of hair, for α-HCH were 40.4 pg/mg and for HCB they were 19.7 pg/mg. From the examined DDT isomers and metabolites, opDDE was detected at a median concentration of 6.2 pg/mg in the positive hair samples, ppDDE at 7.8 pg/mg hair, opDDD at 73.1 pg/mg,
ppDDD and opDDT at 8.0 pg/mg and finally, ppDDT at 5.7 pg/mg (Table 1). The total concentration of the HCHs (median 117.8 pg/mg) was much higher than that of the detected DDTs (median 9.4 pg/mg).

There was no statistically significant influence of sex and age on the detected pesticides. 31.2 % and 53.1 % of the female samples were positive for at least one of DDTs and at least one of HCHs. Almost the same frequencies were observed for the male samples. 32.9 % of the male samples were positive for at least one of DDTs and 49.4 % for at least one of HCHs. The above percentages clearly demonstrate that there was no difference in exposure to HCHs and DDTs between males and females.

The concentration trend of each one of the analyzed compounds along the age groups, offers a good record of the history of exposure. The concentration of HCHs remains high and relatively stable across the age groups, suggesting constant exposure until recently. The concentration of the total DDTs presents a statistically significant decreasing trend across the age groups. A similar trend is also presented by the concentration of the parent compound, pp-DDT. Differences in the median values of the rest of the metabolites are not statistically significant across the age groups (Table 3) [28].

Table 3. Hair levels of the detected pesticides in the positive samples per age group (study II).

<table>
<thead>
<tr>
<th>Age</th>
<th>Median (pg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;40</td>
</tr>
<tr>
<td>Lindane</td>
<td>163.4</td>
</tr>
<tr>
<td>α-HCH</td>
<td>47.1</td>
</tr>
<tr>
<td>HCB</td>
<td>16.9</td>
</tr>
<tr>
<td>opDDE</td>
<td>7.6</td>
</tr>
<tr>
<td>ppDDE</td>
<td>4.7</td>
</tr>
<tr>
<td>opDDD</td>
<td>244.8</td>
</tr>
<tr>
<td>ppDDD+opDDT</td>
<td>9.0</td>
</tr>
<tr>
<td>ppDDT</td>
<td>ND</td>
</tr>
<tr>
<td>SUM-DDTs</td>
<td>12.1</td>
</tr>
<tr>
<td>SUM-HCHs</td>
<td>135.5</td>
</tr>
</tbody>
</table>

3.3. DAPs Levels in Hair

Parent organophosphate pesticides could be detected in hair but the frequency of detection and their concentrations were very low. It is probably due to the fact that organophosphate pesticides are rapidly metabolized in human body to non specific metabolites. Ostrea [29] analyzed infant hair samples for the pesticides propoxur, diazinon, lindane, transfluthrin, malathion, chlorpyriphos, bioallethrin, pretilachlor, DDT, cyfluthrin and cypermethrin and reported the presence of chloropyrifos in an infant hair sample. Also, Posecion [17] mentioned the detection of malathion and chloropyrifos in maternal hair with mean concentrations 4.85 and 4.58 μg/g respectively.
DAPs are considered as biomarkers of exposure for a great number of OP pesticides and can be detected in biological samples at exposure levels below the ones affecting cholinesterase activity.

As can be seen from table 4, the median concentration of DMP in head hair samples was 165.00 pg/mg with concentration range 33.08 to 777.05. The median concentrations of DEP, DETP and DEDTP were 51.21, 54.01 and 40.04 pg/mg. The total DEPs (ΣDEPs: diethyl phosphates) and DAPs (ΣDAPs: diethyl phosphate and dimethyl phosphates) median concentrations were 118.02 pg/mg and 301.50 pg/mg. In each head hair at least one DAP was detected.

Table 4. Hair levels and concentration range (pg/mg) of detectable dialkyl phosphate metabolites in urban population without occupational exposure.

<table>
<thead>
<tr>
<th></th>
<th>DMP</th>
<th>DEP</th>
<th>DETP</th>
<th>DEDTP</th>
<th>ΣDEPs</th>
<th>ΣDAPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median value (pg/mg)</td>
<td>165.00</td>
<td>51.21</td>
<td>54.01</td>
<td>40.04</td>
<td>118.02</td>
<td>301.50</td>
</tr>
<tr>
<td>±SD</td>
<td>243.04</td>
<td>101.03</td>
<td>483.55</td>
<td>74.92</td>
<td>504.71</td>
<td>492.22</td>
</tr>
<tr>
<td>Maximum</td>
<td>777.05</td>
<td>540.85</td>
<td>2085.24</td>
<td>346.62</td>
<td>2664.44</td>
<td>2664.41</td>
</tr>
<tr>
<td>Frequency of detection (%)</td>
<td>59</td>
<td>96</td>
<td>67</td>
<td>63</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The frequency of detection for DMP was 59% (16/27), for DEP was 96% (26/27), for DETP was 67% (18/27), for the DEDTP was 63% (17/27) while 96% of the samples were positive for at least one of DEP, DETP or DEDTP.

3.4. DAPs Levels in Meconium

The mean concentration in meconium samples of DMP, DEP, DMTP, DETP and DEDTP were 126.74, 11.46, 215.05, 4.92 and 1.84 ng/g respectively. The 92.1% of meconium samples were positive for DMP, 36.8% for DEP, 60.5% for DMTP, 63.2% for DETP, and 57.9% for DEDTP. The concentration ranges of positive meconium samples were presented in Table 5.

The 97.4% of the meconium was positive for at least one DAP, the 5.3% of samples were positive for all analyzed DAPs, the 55.3% were positive for both DMP and DMTP, 50.0% for both DETP and DEDTP and the 18.4% were positive for DEP, DETP and DEDTP. As it is obvious DMPs are the metabolites with the main contribution to the detected concentration levels compared to those of DEPs. No significant statistically difference in mean levels between the low risk and high risk of exposure group exposure groups of pregnant women living in rural areas in Crete is observed (p>0.05) [27].

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Table 5. Concentrations (SD), range and frequency of detection of dialkyl phosphate metabolites in meconium samples collected from newborns whose mothers live in rural areas in Crete.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Mean values (ng/g)</th>
<th>Range (ng/g)</th>
<th>% positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DMP</td>
<td>DEP</td>
<td>DMTP</td>
</tr>
<tr>
<td>Mean values</td>
<td>126.74</td>
<td>11.46</td>
<td>215.05</td>
</tr>
<tr>
<td>± SD</td>
<td>142.73</td>
<td>20.43</td>
<td>187.34</td>
</tr>
<tr>
<td>Range</td>
<td>10.64-739.45</td>
<td>1.50-79.14</td>
<td>8.54-662.16</td>
</tr>
<tr>
<td>% positive</td>
<td>92.1</td>
<td>36.8</td>
<td>60.5</td>
</tr>
</tbody>
</table>

The detected levels of DAPs in meconium are higher than those reported in general non-exposure population urine samples and similar to, or higher than those in urine from occupationally exposed population. Higher detection frequencies but lower concentrations were detected compared to those previously reported [26]. The observed high incidence of DAPs detection in meconium samples is probably due to the wide use of pesticides in the region of Crete.

DEP in hair and DMP in meconium were the most frequently detected metabolites. Also, the most frequently used pesticides in the region of Crete are azinphos methyl, dichlorvos, malathion, dimethoate, chloropyrifos methyl which produce the non specific dimethylphosphate metabolites (DMP and DMTP) and chloropyrifos which produces the diethylphosphate metabolites (DEP and DETP). Dimethoate is widely used in the cultivation of olive trees, the most prevalent cultivation in Crete. It is well known that olive oil is one of the most important constituents of the Mediterranean diet [30].

Metabolite concentrations found in hair and meconium probably represent chronic occupational exposure to OP pesticides or exposure through diet and drinking water [4]. Because DAPs originate from more than one OP and the parent OPs differ significantly in toxicity, the measured DAP levels do not provide direct measures of toxicity potential [31].

4. Conclusion

Overall, hair analysis could be a suitable tool for assessing low level exposure to organochlorine and organophosphate pesticides. Also, the measurement of non-specific organophosphate metabolites in non conventional biological samples may be used as a valuable biomarker of short or long term exposure to organophosphate pesticides.
References


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Chapter 30

Possible Problems Connected to Alcohol Abstinence in Case of Mass Disasters

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Clinic of intensive treatment of acute intoxications and toxoallergy, Department of Neuropsychiatry*, Laboratory of Toxicology**, Military Medical Academy – Sofia, Naval Hospital – Varna, Bulgaria

Abstract - Ethyl alcohol is the most common abused chemical substance nowadays. The real frequency of ethanol dependence is underestimated as many patients do not realize it and do not seek medical help. In case of mass disaster the ethanol abstinence psychomotor agitation and delirium could create differential diagnostic difficulties with other psychotropic xenobiotics. Abstinent patients, especially when inadequate, could break the quarantine and other restrictive rules, have antisocial and criminal behaviour, increase the panic, etc. As ethanol abstinence is a serious medical condition it requires urgent specialized medical help. Ethanol dependent patients could be more sensitive to chemical agents attacking the CNS or liver. The authors observe 176 patients with toxic ethanol effects treated in Toxicology Clinic during 2007 year. 64 (36.36%) of them had ethanol abstinence. 59 (92.18%) of them were in active age - between 20 and 60 years. The cause of withdrawal was cessation of drinking or a reduction of intake because of illness, surgical operation, isolation, lack of money. 20 patients (31.25%) did not realize they were alcohol dependent. Toxicology chemical analysis showed no ethanol in the blood of 58%; 0.1-0.5‰- in 11%; 0.5-1.0‰- in 4 %; 1.0-2.0‰- in 9 %; over 2‰- in 18 %. All the patients had typical alcohol abstinence syndrome with moderate or severe expression. All of them had toxic ethanol hepatitis. The following complications have been observed: seizures, long lasting severe delirium, severe mono- and polyneuropathy, myelosuppression, pneumonia, pancreatitis, cardiac failure. All the patients were consulted by a psychiatrist. In 16 cases a co-existing psychiatric disorder was found. The duration of hospital treatment was between 2 days and 30 days. The authors suggest that a more thorough investigation and treatment of the existing alcohol dependence would reduce the frequency of severe abstinent states.

Key words - ethanol, abstinence, withdrawal, delirium, toxicology chemical analysis, mass disaster
Introduction

Ethyl alcohol is the most abused chemical substance nowadays. According to the estimation of WHO from 2004 year there are about 2 billion people worldwide who consume alcoholic beverages and 76.3 million with diagnosable alcohol use disorders[1]. In Bulgaria 7.13 l (in litres of pure alcohol) is the estimated total recorded alcohol consumption per capita yearly. There is always unrecorded consumption that is hard to estimate, but for Bulgaria it’s probably another 3.0 l of pure alcohol per capita. Alcohol is related to a wide range of physical, mental and social harms. It affects every organ and system of the human body, but its effect is strongest on the CNS. It is well known that alcohol plays a great role in a number of traffic casualties, accidental or intentional injuries or poisonings, falls, asocial or criminal deeds.

The approximate percentage of alcohol dependence in different countries is between 1.0 and 12.2% of the adult population. Chronic ethanol consumption leads to physical dependence and permanent change of CNS normal processes and activity as well as changes in the other systems. The ICD-10 defines alcohol dependence syndrome as a cluster of physiological, behavioural and cognitive phenomena in which the use of alcohol takes on a much higher priority than other behaviours that once had greater value, persisting use despite harmful effects.[1]. The excessive abuse of alcohol leads gradually to tolerance, physical dependence and physical alcohol withdrawal syndrome.[2]. The cessation or reduction in intake of ethanol in an ethanol-dependent organism results in the so called withdrawal or abstinence syndrome: a complex disorder with craving for alcohol, CNS-over-excitation, α-adrenergic overstimulation, peripheral neurosyndrome, exacerbating of the chronic toxic ethanol effects and diseases. It can start mildly but usually without medical help manifests as moderate or severe, even lethal condition.[3]. In case of mass disaster withdrawal can immitate a warfare psychotropic poisoning. On the other hand in such case alcohol abstinent people can create a lot of problems related to their inadequacy and impaired mental and general state.

Aim. A study of hospital cases treated in Toxicology clinic-Naval Hospital-Varna, during year 2007 with diagnosis: Toxic effects of ethanol-ethanol dependence-ethanol abstinence syndrome. The choice of the observed year was random from several years without great mass disasters. Discussing the potential influence that a mass disaster could have on this contingent and the possible problems that patients with alcohol withdrawal can create in such event.

1. Material and methods

The objects of the study were 64 patients with ethanol abstinence syndrome of total 176 patients who were treated in the Clinic of intensive treatment of acute intoxications and toxaollergy (Toxicology Clinic) in Naval Hospital-Varna for acute or chronic alcohol intoxication during 2007 year. The assessment of alcohol intoxication and abstinence was done according to ICD-10. The toxicology chemical analysis was made by gas-chromatography. All the patients were consulted repeatedly by a psychiatrist. The results are not representative for the total population of Varna district as some of the alcohol abstinent patients were treated in Psychiatry Clinic.
2. Results

During 2007 year the total number of in-patients was 936. 176 of them (18.8%) were with acute or chronic toxic ethyl alcohol effects. 64 patients from this group (36.36%) were treated in Toxicology clinic for moderate or severe alcohol abstinence.

![Percentage of alcohol abstinence of hospital ethanol cases](image)

53 (82.8%) of the abstinent patients were men and 11 (17.2%) - women.

**Figure1.** Percentage of alcohol abstinence of all the hospital ethanol cases.

<table>
<thead>
<tr>
<th>Table 1. Age distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>20-30 years</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>9</td>
</tr>
<tr>
<td>14.06%</td>
</tr>
</tbody>
</table>

It is seen that alcohol abstinence patients are predominantly in active age. 59 (92.18%) patients are from 20 to 60 years old. Only 15 patients declared that they do not work. Some of the regularly working patients had positions of high responsibility and trust.

44 (62.5%) of the patients and their relatives knew they had alcohol dependence. 20 of them had had previous abstinent episodes. Another 20 (31.25%) patients, however, did not realize they had become alcohol dependent until the expression of severe withdrawal. Despite the information during the treatment 8 of them continued to look for other explanations of their delirium.
Figure 2. Abstinent patients who were unaware of their alcohol dependence

The cause of withdrawal was cessation or sharp reduction of ethanol drinking. Different reasons were declared: serious illness that was not connected to alcohol effect (pneumonia, heart failure, myocardial infarction, cerebral insult, duodenal ulcer, acute viral infection, etc.), or a serious complication of chronic alcoholism (ethanol hepatitis, cirrhosis, pancreatitis, gastritis); at the peak of a binge-drinking episode self-limited by toxic gastritis; urgent or planned surgery, traumatic incidents, compulsory isolation, financial problems (lack of money); intentional decision of the patient to stop drinking without medical assistance.

Toxicology chemical analysis of the blood for alcohol was made at the admittance of 45 patients. 8 patients had over 2‰; 1.0-2.0‰- 4 patients; 0.5-1.0‰- 2 patients; 0.1-0.5‰-4 patients and no ethanol-26 patients. The other 19 patients were transferred from other wards or hospitals.

Figure 3

The ethanol blood concentration in cases of ethanol withdrawal can be zero or minimal. Therefore its informative value about the etiology of an abstinent state is quite limited.
The clinical manifestation of the described abstinent cases was moderate in 18 patients and severe in 46 (71 %) patients. The most frequent syndromes and symptoms we have observed are: cerebral abstinent syndrome with confusion, insomnia, periods of inadequate behaviour, mainly in the night - 64 patients (100%), tremours (100%), sweating (100%), gastrointestinal syndrome with loss of appetite, nausea, vomiting - 62 (95%), psychomotor agitation - 45 (70%), short delirious episodes - 45 (70%), long lasting severe delirium - 9 (14%), convulsions - 11 (17%), ethanol polyneuropathy - 12 (18.75%), ethanol mononeuritis - 2 (3%), other alcohol psychoses - 16 (25%), exacerbated alcohol hepatitis - 62 (97%), exacerbated pancreatitis - 8 (125%), myelosuppression - 3 (4.6%), pneumonia - 10 (15.6%).

Table 2. Duration of the ethanol abstinence and hospital treatment

<table>
<thead>
<tr>
<th>Duration</th>
<th>1-3 days</th>
<th>3-7 days</th>
<th>8-15 days</th>
<th>15-30 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>24</td>
<td>22</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>22%</td>
<td>38%</td>
<td>34%</td>
<td>6%</td>
<td></td>
</tr>
</tbody>
</table>

3. Discussion

The alcohol withdrawal cases treated during 2007 were more than one third of all hospitalizations in Toxicology Clinic because of toxic ethanol effects. Men in active age prevailed. 44 patients were aware of their alcohol dependence but 31.25% of the patients did not know it until the appearance of withdrawal. 12 patients were admitted initially in other clinics with other diagnosis. Another group of 7 patients had manifestation of abstinence in the early post-operative period of different surgery. Laboratory findings were not specific and reflected the complications. Toxicology chemical analysis of the blood was negative for ethanol in nearly 58% and from 0.1 to 2.0 ‰ - in another 24 %. A complex combination of CNS symptoms and symptoms from other organs and systems was present. The clinical manifestation was moderate to severe, with prevalence of the severe withdrawal. 1 lethal case was registered. All the patients got intensive and complex treatment in the Intensive Unit of the Toxicology Clinic. The duration of hospital treatment was from 1 to 30 days. In all cases a consultation with a psychiatrist was done repeatedly. All this confirms the data of other authors that ethanol withdrawal syndrome as a serious medical condition partially predictable when the alcohol dependence is known, with specific CNS and autonomic NS symptoms including confusion, psychomotor agitation hallucinosis, delirium and some severe complications like seizures. The initial period of withdrawal is not very specific and diagnostic mistakes can take place. The social effect of abstinence is significant - not only men and women in active age become temporarily incapable but the dramatic clinical expression causes stress to their families, friends. In the stage of alcohol craving 7 patients had asocial behaviour and 2 had committed criminal deeds.

In case of chemical attack with psychotropic warfare any alcohol abstinent reaction, especially when complicated with delirium, can be mistaken for a new chemical attack. The differential diagnosis is quite easy if the possibility of alcohol and other withdrawal reactions is taken in mind. The cases of alcohol delirium are sporadic, their expression and maximum are at different moments and usually late; their clinical ma-
nifestation is variable and probably differs from chemical warfare psychosis. The toxic warfare psychosis will be massive, affecting numerous cases, expressed at one and the same time, with uniform, identical clinical manifestation. The differential diagnosis will be more difficult when the pathogenesis of the psychomotor agitation and delirious state is a complex result of toxic or traumatic injury from a mass disaster, alcohol abstinence and delirium and stress reaction. The understanding of such combined delirious cases is important as the necessary treatment is quite complex - specific and non-specific antidotes, symptomatic treatment, psychiatric and psychological help, sedation, etc.

Discussing the possible effects a mass disaster can have on alcohol dependent people, who have not stopped alcohol drinking beforehand, we should divide them into 4 subgroups. The first group consists of alcohol dependent people who slightly increase their drinking, resulting either in mobilisation, motivation and normal reactions and fulfilment of their duties or in euphoria, panic, depression and inadequate reactions. The second group consists of people with heavy increasing of alcohol drinking, perhaps binge-drinking, which will result in inadequacy, possible development of alcohol delirium or other alcohol psychoses, acute toxic effects or exacerbation of chronic alcohol effects on other organs and systems, other chronic diseases etc. A great part of this group will need specialized medical help and hospital treatment. The third group consists of people who voluntarily or compulsory will decrease slightly their drinking. Some of them perhaps will feel moderate general discomfort but will fulfil their duties; the rest of this group will gradually develop abstinence with partial inadequacy and wrong judgement, depression, panic, psychoses, possible delirium. The fourth group is the most important. It consists of dependant people who have to stop abruptly alcohol drinking and therefore the withdrawal is obligatory – non-complicated or severe with delirium. Partial or total inadequacy, asocial behaviour, dangerous reactions, criminal actions as well as severe delirium, convulsions and eventually death characterise this group. Almost all of the delirious patients will require a long hospital treatment if possible.

On the other hand the confused mental state, agitation, delirium and other alcohol psychoses of the abstinent people can also affect a post- mass disaster situation through inspiring, enhancing or maintaining panic reactions, slow and improper use of protective appliances, breaking the rules, the quarantine, protective isolation, other restrictive safety measures, asocial, aggressive, strange or criminal behaviour, or just by increasing the necessity of hospital beds and specialised medical help. An abstinent alcohol state will always lead to partial or total inadequacy to critical situations [4]. As we have demonstrated almost all of the abstinent patients were in active age and worked, some of them perhaps will feel moderate general discomfort but will fulfil their duties; the rest of this group will gradually develop abstinence with partial inadequacy and wrong judgement, depression, panic, psychoses, possible delirium. The fourth group is the most important. It consists of dependant people who have to stop abruptly alcohol drinking and therefore the withdrawal is obligatory – non-complicated or severe with delirium. Partial or total inadequacy, asocial behaviour, dangerous reactions, criminal actions as well as severe delirium, convulsions and eventually death characterise this group. Almost all of the delirious patients will require a long hospital treatment if possible.

According to a study by Yaneva in 2006 for Bulgaria the registered alcohol dependent people were about 2.5% of the adult population [6]. About 15% were abusing alcohol. About 25% were drinking rarely. Considering the population of Varna city and Varna district in 2007 was reported 495 056, the theoretically estimated alcohol dependent people in the region were 12 376 and of alcohol abusers - 74 258. It is well known that alcohol is widely used in critical situations as mild sedative to cope with the stress.
It is possible in mass crisis some of the abusers to pass into the alcohol dependent group.

Conclusions:

1. The existing significant number of alcohol dependent people creates serious diagnostic and therapeutic medical problems in different fields.
2. Alcohol abstinence syndrome especially when complicated with alcohol delirium can lead to a severe dysfunction of the CNS and other organs and systems. It requires complex and long lasting hospital treatment, compulsory isolation, psychiatric help, caretaking. It was the reason for 36% of all hospital cases connected to alcohol during 2007.
3. 31.25% of the abstinent alcoholic patients treated during 2007 had not realised the existence of ethanol dependence until the appearance of abstinence.
4. The characteristics of alcohol abstinence cases during a random chosen non-disastrous year suggest that these cases have a potential to create some serious medical, social and organizational problems in a mass disaster situation.
5. The authors suggest that more active and thorough investigation and treatment of alcohol dependent people will be a good prevention of these problems.

References:

Chapter 31

Protective Effect of Purified Saponin Mixture from Astragalus Corniculatus on Toxicity Models In Vitro

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Abstract - A Purified Saponin Mixture (PSM), isolated from Astragalus corniculatus Bieb. (Fabaceae) was investigated for its protective effect on different models of toxicity \textit{in vitro} on sub-cellular and cellular level. In conditions of non-enzyme and enzyme lipid peroxidation in isolated rat microsomes, PSM showed statistically significant antioxidative effect, similar to the effect of silymarin. These results correlate with data for antioxidant activity of saponin cycloestragenol-xylozil-glycoside, isolated from extract of Astragalus membranaceus in mice liver. In rat brain synaptosomes, prepared by using Percoll reagent, PMS had statistically significant protective effect, similar to those of silymarin on 6-hydroxydopamine-induced oxidative stress. These results correlate with literature data about protective effects of Astragalus in conditions of stress in rats. In rat hepatocytes, isolated by two-stepped collagenase perfusion, we investigate the effect of PSM on two models of liver toxicity: metabolic bioactivation - carbon tetrachloride (CCl\(_4\)) and mainly mitochondrial induced oxidative stress - tert-butylhydroperoxide (t-BuOOH). In CCl\(_4\)-induced toxicity, PSM had statistically significant cytoprotective and antioxidant activity, near to those of silymarin. These data are supported by literature data about protective effect of saponins, isolated from Astragalus membranaceus and Astragalus sieversianus on hepatotoxicity, induced by CCl\(_4\) and Paracetamol in mice. In model of oxidative stress – induced by t-BuOOH, PSM had statistically significant cytoprotective and antioxidant activity, stronger than the effect of silymarin. These effects of PSM may be due to the influence of the mitochondrial function, which includes the mitochondrial metabolism of t-BuOOH and correlates with data about antioxidant activity \textit{in vitro} of water extract of Astragalus on mitochondria, isolated from rat heart.

Key words - Astragalus corniculatus, microsomes, synaptosomes, hepatocytes, antioxidant, cytoprotection

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Introduction

Chemical studies on Astragalus species reported the presence of triterpenoid saponins, which exhibited a wide range of biological properties, including immunostimulant, hepatoprotective, antiviral, cardiotonic and analgesic activities [1] [2]. There are literature data about antioxidant effect of saponin cycloestragenol-xylosil-glycoside, isolated from extract of Astragalus membranaceus in mice [3]. In some communications is reported that Astragalus is used for the treatment of 43 patients with severe heart attack and is found that Astragalus reduced the production of reactive oxygen species (ROS) in red blood cells, decreased the plasma lipid peroxidation (LPO) and increased the levels of SOD [4]. Bei-Wei et al. report a reduction of noise-stress-induced physiological damage from species Astragalus and Rhodiolae [5]. It is found, that ethyl acetate extract from Astragalus corniculatus showed an antihypoxic activity in mice [6]. Zhang et al. in their experiment, found that saponins, isolated from Astragalus membranaceus and Astragalus sieversianus, had a protective effect against hepatotoxicity, induced by carbon tetrachloride, D-galactosamine and paracetamol in mice [3]. There is communication about antioxidant effect in vitro of water extract of Astragalus on mitochondria, isolated from rat heart [7].

Astragalus corniculatus Bieb. (Fabaceae) is distributed in Southeastern Romania, South Ukraine and Moldova [8]. The plant is a new species for Bulgarian flora and is grown in North Bulgaria [9]. In the Bulgarian traditional medicine Astragalus glycyphyllus is used as an antihypertensive, diuretic and anti-inflammatory remedy [10]. Krasteva et al. report a protective action of Purified Saponin Mixture from Astragalus corniculatus Bieb. in Graffiti-tumor bearing hamsters [11].

Based on the information available, the objective of the following study was to investigate the possible protective effect of a Purified Saponin Mixture, isolated from Astragalus corniculatus on different toxicity models in vitro.

1. Materials and Methods

1.1. Plant material

Astragalus corniculatus Bieb. herbs were collected in July 1999 in Northern Bulgaria. The plant was identified by Dr D. Pavlova from the Department of Botany, Faculty of Biology, Sofia University, where a voucher specimen has been deposited (SO95265).

1.2. Preparation of PSM

Astragalus corniculatus herbs were collected in Northern Bulgaria and a voucher specimen (SO95265) is deposited at the herbarium of Faculty of Biology, Sofia University. The air-dried plant material was powdered and extracted exhaustively with 50% EtOH. The extract was concentrated under reduced pressure and successively treated with CHCl₃ and EtOAc. The aqueous residue was dissolved in MeOH. After filtration and addition of Me₂CO a crude saponin mixture was obtained. The mixture was submitted to column chromatography on silica gel (CHCl₃-MeOH-H₂O, 98:72:9) and fur-
saponins fraction, named PSM. Three new triterpene saponins were isolated from this fraction by preparative TLC (n-BuOH- AcOH-H₂O, 4:1:1). The compounds were identified as 3β-O-[O-4-oxo-pentopyranosyl-(1→2)-β-D-glucopyranosyl]-21α-hydroxyolean-12-ene-28-oic acid (1); 21α-hydroxyolean-12-ene-28-oic acid 3β-4-oxo-pentopyranoside (2) and 19α-hydroxyolean-12-ene-28, 21β-olide 3β-D-xylpyranoside (3) (Fig.1) by chemical and spectral methods [12][13].

For the following *in vitro* study, PSM was used at concentrations of 0.01µM, 0.1µM, 10µM and 100µM [14], which correspond to 19.65ng/ml; 196.5ng/ml; 19.65µg/ml and 196.5µg/ml, respectively. The effects of PSM were compared to those of Silymarin (in concentrations 0.01µM, 0.1µM, 10µM and 100µM, which correspond to 4.8ng/ml; 48.24ng/ml; 4.8µg/ml and 48.24µg/ml, respectively).

![Chemical structures](image_url)

**Figure 1.** Structures of isolated and identified saponins from *Astragalus corniculatus*
1.3. Chemicals

In our experiments, pentobarbital sodium (Sanofi, France); HEPES (Sigma Aldrich, Germany); NaCl (Merck, Germany); KCl (Merck); D-Glucose (Merck); NaHCO₃ (Merck); KH₂PO₄ (Scharlau Chemie SA, Spain); CaCl₂.2H₂O (Merck); MgSO₄.7H₂O (Fluka AG, Germany); Collagenase from Clostridium histolyticum type IV (Sigma Aldrich); Albumin, Bovine serum fraction V, minimum 98% (Sigma Aldrich); EGTA (Sigma Aldrich); 2-Thiobarbituric acid (4,6-dihydroxypyrimidine-2-thiol; TBA) (Sigma Aldrich); Trichloroacetic acid (TCA) (Valerus, Bulgaria); Carbon tetrachloride (CCl₄) (Merck); 2,2’-dinitro-5,5’-dithiodibenzoic acid (DTNB) (Merck); Lactate dehydrogenase (LDH) kit (Randox, UK); FeSO₄ (Merck); Ascorbinic acid (Valerus, Bulgaria); NADPH (Sigma Aldrich); Succrosa (Merck); MgCl₂.6H₂O (Merck); NaH₂PO₄ (Merck); Percoll (Sigma Aldrich) were used.

1.4. Animals

Male Wistar rats (Body weight, 200-250g) were used. Rats were housed in plexiglass cages (3 per cage) in a 12/12 light/dark cycle, temperature 20±2°C. Food and water were provided ad libitum. Animals were purchased from the National Breeding Centre, Sofia, Bulgaria. All performed procedures were approved by the Institutional Animal Care Committee and were in accordance with European Union Guidelines for animal experimentation.

1.5. Isolation of liver microsomes

Liver is perfused with 1.15% KCl. Liver was homogenized with four volumes of ice-cold 0.1M potassium phosphate buffer, pH=7.4. The liver homogenate was centrifuged at 9000 x g for 30 min at 4°C and the resulting post-mitochondrial fraction (S-9) was centrifuged again at 105 000 x g for 60 min at 4°C. The microsomal pellets were resuspended in 0.1M potassium phosphate buffer, pH=7.4, containing 20% Glycerol. Aliquots of liver microsomes were stored at - 70°C until use [15]. The content of microsomal protein was determined according to the method of Lowry using bovine serum albumin as a standard [16].

1.5.1. FeSO₄/Ascorbinic acid-induced lipid peroxidation in vitro

As a system, in which metabolic activation may not be required in the production of lipid peroxide, 20μM FeSO₄ and 500μM Ascorbinic acid were added directly into rat liver microsomes and incubated for 20 min at 37°C [17].

1.5.2. NADPH-dependent CCl₄-induced lipid peroxidation in vitro

In rat liver microsomes, CCl₄ was added and incubated for 20 min at 37°C in the presence of 1mM NADPH [17].

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1.5.3. Lipid peroxidation in microsomes

After incubation of microsomes with the compounds, we added to the microsomes 1 ml 25% (w/v) trichloroacetic acid (TCA) and 1 ml 0.67% 2-Thiobarbituric acid (TBA). The mixture is heated at 100°C for 20 min. The absorbance was measured at 535 nm, and the amount of MDA was calculated using a molar extinction coefficient of $1.56 \times 10^5$ M$^{-1}$cm$^{-1}$ [18].

1.6. Synaptosome preparation

Synaptosomes were prepared by brains from adult male Wistar rats, as previously described by Taupin et al. [19]. The brains were homogenized in 10 vol. of cold Buffer 1, containing HEPES 5mM and 0.32M sucrose (pH=7.4). The brain homogenate was centrifuged twice at 1000 x g for 5 min at 4°C. The supernatant was collected and centrifuged 3 times at 10 000 x g for 20 min at 4°C. The pellet was resuspended in a ice-cold Buffer 1. The synaptosomes were isolated by using Percoll reagent. Synaptosomes were resuspended in ice-cold Buffer 2, containing NaCl - 290 mM; MgCl$_2$6H$_2$O – 0.95mM; KCl – 10mM; CaCl$_2$2H$_2$O – 2.4mM; NaH$_2$PO$_4$ – 2.1mM; HEPES – 44mM and D-Glucosa – 13mM.

1.6.1. Synaptosomes’ vitality, measured by MTT-test, described by Mungarro-Menchaca et al. [20]

After incubation with the compounds, synaptosomes were treated with MTT solution for 1 hour in 37°C. After incubation, they were centrifuged at 15 000 x g for 1 min. The formed formasan crystals were dissolved in DMSO. The extinction was measured spectrophotometrically at $\lambda=580$ nm.

1.6.2. LDH activity in synaptosomal fraction, described by Vasault [21]

Synaptosomes were sedimented by centrifugation at 400 x g. Lactate dehydrogenase activity was determined by lysing these particles in 0.2% Triton X-100. The enzyme activity was measured spectrophotometrically at $\lambda=340$ nm in the supernatant by using LDH kit.

1.6.3. GSH level in synaptosomes, described by Robyt et al. [22]

GSH was determined with the Ellman reagent (DTNB), which forms colour complexes with –SH group at pH=8 with maximum absorbance at 412 nm.

1.7. Isolation and incubation of hepatocytes

Rats were anesthetized with sodium pentobarbital (0.2ml/100g). In situ liver perfusion and cell isolation were performed as described by Fau [23], with modifications [24]. Cells were counted under the microscope and the viability was assessed by Trypan blue exclusion (0.05%) [23]. Cells were diluted to make a suspension of about $3\times10^6$ hepatocytes/ml. Each incubation flask contained 3 ml of the cell suspension (i.e. $9\times10^6$ hepatocytes) [23].
Biochemical determinations in isolated rat hepatocytes

The biochemical parameters were determined by spectrophotometric methods using a Spectro UV-VIS Split spectrophotometer.

1.7.1. Lactate dehydrogenase release

Lactate dehydrogenase release was measured as described by Bergmeyer [25].

1.7.2. GSH depletion

At the end of the incubation, cells were recovered by centrifugation at 4°C, and used to measure intracellular reduced glutathione (GSH), which was assessed by measuring non-protein sulfhydryls after precipitation of proteins with TCA, followed by measurement of thiols in the supernatant with DTNB. The absorbance was measured at 412 nm [23].

1.7.3. Lipid peroxidation (LPO)

Hepatocyte suspension (1 ml) was taken and added to 0.67 ml of 20% (w/v) TCA. After centrifugation, 1 ml of the supernatant was added to 0.33 ml of 0.67% (w/v) TBA and heated at 100°C for 30 min. The absorbance was measured at 535 nm, and the amount of MDA was calculated using a molar extinction coefficient of $1.56 \times 10^5 \text{M}^{-1} \text{cm}^{-1}$ [23].

1.8. Statistical analysis

Statistical analysis was performed by applying the Student’s $t$-test, with $P<0.05$ considered statistically significant. All results ($n=12$) are expressed as mean ± SD.

2. Results

Microsomes incubation with Fe$^{2+}$/AA, resulted in statistically significant increase of the amount of MDA with 138% vs control. In conditions of non-enzyme-induced lipid peroxidation, PSM and Silymarin statistically significant compared to the toxic agent (Fe$^{2+}$/AA), reduced lipid damage with 47% and 48% respectively.

Incubation of microsomes with CCl$_4$/NADPH, resulted in statistically significant increase of MDA level with 236% vs control. In conditions of enzyme-induced lipid peroxidation, PSM and Silymarin statistically significant compared to the toxic agent (CCl$_4$/NADPH), reduced lipid damage with 35% and 39% respectively (Table 1).

The antioxidant activity of PSM and Silymarin is better in conditions of non-enzyme-induced lipid peroxidation.

Synaptosomes incubation with 6-OH-dopamine provokes statistically significant decrease of synaptosomal viability with 33%, increase of LDH activity with 358% and decrease GSH level with 75%, compared to the control.

In conditions of 6-OH-dopamine-induced oxidative stress, PSM and Silymarine show statistically significant protective effect, compared to the toxic agent. PMS pre-
serves synaptosomal viability with 35% and Silymarin – with 29%. The LDH activity is decreased from PSM with 28% and from Silymarin – with 32%. The GSH level is preserved from PSM with 133% and from Silymarin – with 100%. The protective effect of PSM in model of oxidative stress in isolated synaptosomes, is stronger than the effect of Silymarin (Table 2).

PSM and Silymarin, administered alone, showed toxic effects, as statistically significant decreased cell viability and GSH level, increased LDH activity and lipid peroxidation in isolated rat hepatocytes, compared to the control (Tables 3-4).

Hepatocytes incubation with CCl₄ (86µM) [26] – model of metabolic bioactivation, resulted in statistically significant reduction of cell viability by 61%, increased LDH activity by 539%, depletion of cell GSH by 52% and increased lipid peroxidation by 232%, compared to the control.

In combination with CCl₄, PSM and Silymarin statistically significant reduced the damage caused by the hepatotoxic agent - preserved cell viability and GSH level [14], decreased LDH activity and reduced lipid damage (Tables 5-6). The cytoprotective and antioxidant activity of PMS, in conditions of CCl₄-induced hepatotoxicity, is similar to the effects of Silymarin – a classical hepatoprotector.

Hepatocytes incubation with t-BuOOH (75µM) [27] – model of oxidative stress, resulted in statistically significant reduction of cell viability by 77%, increased LDH activity by 601%, depletion of cell GSH by 48% and increased lipid peroxidation by 317%, compared to the control. The toxic effects of t-BuOOH on isolated rat hepatocytes are stronger than the toxic effects of CCl₄.

In combination with t-BuOOH, PSM and Silymarin statistically significant reduced the damage caused by the hepatotoxic agent - preserved cell viability and GSH level [14], decreased LDH activity and reduced lipid damage (Tables 7-8). The cytoprotective and antioxidant activity of PMS, in conditions of t-BuOOH-induced oxidative stress, is stronger than the effects of Silymarin.

### Table 1. Effect of 100µM PSM and Silymarin in conditions of non-enzyme-induced (Fe²⁺/AA) and enzyme-induced (CCl₄/NADPH) LPO in isolated rat liver microsomes

<table>
<thead>
<tr>
<th>Group</th>
<th>MDA, nmol/mg protein</th>
<th>Group</th>
<th>MDA, nmol/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (non-treated microsomes)</td>
<td>1.37 ± 0.01</td>
<td>Control (non-treated microsomes)</td>
<td>1.37 ± 0.01</td>
</tr>
<tr>
<td>Fe²⁺/AA</td>
<td>3.26 ± 0.03 ***</td>
<td>CCl₄/NADPH</td>
<td>4.61 ± 0.05 ***</td>
</tr>
<tr>
<td>100µM PSM + Fe²⁺/AA</td>
<td>1.72 ± 0.02 ***+++</td>
<td>100µM PSM + CCl₄/NADPH</td>
<td>2.98 ± 0.02 ***+++</td>
</tr>
<tr>
<td>100µM S + Fe²⁺/AA</td>
<td>1.70 ± 0.08 ***+++</td>
<td>100µM S + CCl₄/NADPH</td>
<td>2.82 ± 0.06 ***+++</td>
</tr>
</tbody>
</table>

* p < 0.05; ** p < 0.01; *** p < 0.001 vs control

*** p < 0.001 vs Fe²⁺/AA
Table 2. Effect of 100µM PSM and 100µM Silymarin on synaptosomes’ viability, LDH activity and GSH level in 6-OH-dopamine-induced neurotoxicity in isolated rat brain synaptosomes

<table>
<thead>
<tr>
<th>Group</th>
<th>% viability by MTT-test</th>
<th>LDH, nmol/min/mg protein</th>
<th>GSH, nmol/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.859 ± 0.002</td>
<td>0.166 ± 0.002</td>
<td>12 ± 1.2</td>
</tr>
<tr>
<td>150µM DA</td>
<td>0.573 ± 0.001 ***</td>
<td>0.760 ± 0.001 ***</td>
<td>3 ± 1.0 ***</td>
</tr>
<tr>
<td>100µM PSM + 150µM DA</td>
<td>0.775 ± 0.003 *** ***</td>
<td>0.550 ± 0.003 *** ***</td>
<td>7 ± 2.3 **</td>
</tr>
<tr>
<td>100µM S + 150µM DA</td>
<td>0.741 ± 0.004 *** ***</td>
<td>0.520 ± 0.004 *** ***</td>
<td>6 ± 3.4 **</td>
</tr>
</tbody>
</table>

* p < 0.05; *** p < 0.001 vs control
** p < 0.01 vs 6-OH-dopamine; *** p < 0.001 vs 6-OH-dopamine

Table 3. Effect of PSM and Silymarin, administered alone, on cell viability and LDH activity in isolated rat hepatocytes

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell viability, %</th>
<th>LDH, nmol/min/10^6 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>82 ± 4.2</td>
<td>0.132 ± 0.02</td>
</tr>
<tr>
<td>0.01µM PSM</td>
<td>75 ± 6.2</td>
<td>0.234 ± 0.04</td>
</tr>
<tr>
<td>0.1µM PSM</td>
<td>71 ± 4.7</td>
<td>0.310 ± 0.04 **</td>
</tr>
<tr>
<td>10µM PSM</td>
<td>65 ± 6.7</td>
<td>0.435 ± 0.07 **</td>
</tr>
<tr>
<td>100µM PSM</td>
<td>56 ± 5.4</td>
<td>0.601 ± 0.03</td>
</tr>
<tr>
<td>0.01µM S</td>
<td>74 ± 1.2</td>
<td>0.161 ± 0.2</td>
</tr>
<tr>
<td>0.1µM S</td>
<td>73 ± 6.1</td>
<td>0.174 ± 0.2</td>
</tr>
<tr>
<td>10µM S</td>
<td>58 ± 3.1 **</td>
<td>0.198 ± 0.1</td>
</tr>
<tr>
<td>100µM S</td>
<td>56 ± 2.1 **</td>
<td>0.272 ± 0.07 **</td>
</tr>
</tbody>
</table>

* p < 0.05; ** p < 0.01; *** p < 0.001 vs control

Table 4. Effect of PSM and Silymarin, administered alone, on GSH and MDA level in isolated rat hepatocytes

<table>
<thead>
<tr>
<th>Group</th>
<th>GSH, nmol/10^6 cells</th>
<th>MDA, nmol/10^6 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>21 ± 3.9</td>
<td>0.101 ± 0.01</td>
</tr>
<tr>
<td>0.01µM PSM</td>
<td>20 ± 3.2</td>
<td>0.131 ± 0.03</td>
</tr>
<tr>
<td>0.1µM PSM</td>
<td>19 ± 5.0</td>
<td>0.142 ± 0.03</td>
</tr>
<tr>
<td>10µM PSM</td>
<td>16 ± 3.7</td>
<td>0.153 ± 0.02</td>
</tr>
<tr>
<td>100µM PSM</td>
<td>15 ± 1.5</td>
<td>0.174 ± 0.03</td>
</tr>
<tr>
<td>0.01µM S</td>
<td>21 ± 1.0</td>
<td>0.156 ± 0.1</td>
</tr>
<tr>
<td>0.1µM S</td>
<td>20 ± 2.3</td>
<td>0.179 ± 0.05</td>
</tr>
<tr>
<td>10µM S</td>
<td>19 ± 2.5</td>
<td>0.202 ± 0.06</td>
</tr>
<tr>
<td>100µM S</td>
<td>13 ± 2.1 **</td>
<td>0.251 ± 0.08</td>
</tr>
</tbody>
</table>

* p < 0.05; ** p < 0.01 vs control

Table 5. Effect of PSM and Silymarin in CCl4-induced hepatotoxicity on cell viability and LDH activity in isolated rat hepatocytes

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell viability, %</th>
<th>LDH, nmol/min/10^6 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>82 ± 4.2</td>
<td>0.132 ± 0.02</td>
</tr>
<tr>
<td>86µM CCl4</td>
<td>32 ± 2.9 ***</td>
<td>0.843 ± 0.05 ***</td>
</tr>
<tr>
<td>0.01µM PSM + 86µM CCl4</td>
<td>39 ± 6.1 **</td>
<td>0.812 ± 0.05 ***</td>
</tr>
</tbody>
</table>

* p < 0.05; *** p < 0.001 vs control
**MEDICAL MANAGEMENT OF CHEMICAL AND BIOLOGICAL CASUALTIES**

<table>
<thead>
<tr>
<th>Group</th>
<th>GSH, nmol/10^6 cells</th>
<th>MDA, nmol/10^6 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>21 ± 3,9</td>
<td>0,101 ± 0,01</td>
</tr>
<tr>
<td>86µM CCl₄</td>
<td>10 ± 2,9</td>
<td>0,335 ± 0,07</td>
</tr>
<tr>
<td>0,01µM PSM + 86µM CCl₄</td>
<td>11 ± 1,8</td>
<td>0,312 ± 0,03</td>
</tr>
<tr>
<td>0,1µM PSM + 86µM CCl₄</td>
<td>14 ± 2,9</td>
<td>0,304 ± 0,01</td>
</tr>
<tr>
<td>10µM PSM + 86µM CCl₄</td>
<td>16 ± 3,9</td>
<td>0,244 ± 0,05</td>
</tr>
<tr>
<td>100µM PSM + 86µM CCl₄</td>
<td>18 ± 3,7</td>
<td>0,218 ± 0,01</td>
</tr>
<tr>
<td>0,01µM S + 86µM CCl₄</td>
<td>17 ± 2,1</td>
<td>0,300 ± 0,03</td>
</tr>
<tr>
<td>0,1µM S + 86µM CCl₄</td>
<td>18 ± 2,8</td>
<td>0,327 ± 0,05</td>
</tr>
<tr>
<td>10µM S + 86µM CCl₄</td>
<td>20 ± 4,6</td>
<td>0,316 ± 0,02</td>
</tr>
<tr>
<td>100µM S + 86µM CCl₄</td>
<td>21 ± 3,5</td>
<td>0,280 ± 0,04</td>
</tr>
</tbody>
</table>

**Table 6.** Effect of PSM and Silymarin in CCl₄-induced hepatotoxicity on GSH and MDA level in isolated rat hepatocytes

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell viability, %</th>
<th>LDH, nmol/min/10^6 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>82 ± 4,2</td>
<td>0,132 ± 0,02</td>
</tr>
<tr>
<td>75µM t-BuOOH</td>
<td>19 ± 3,9</td>
<td>0,925 ± 0,10</td>
</tr>
<tr>
<td>0,01µM PSM + 75µM t-BuOOH</td>
<td>41 ± 2,2</td>
<td>0,683 ± 0,02</td>
</tr>
<tr>
<td>0,1µM PSM + 75µM t-BuOOH</td>
<td>46 ± 2,2</td>
<td>0,617 ± 0,06</td>
</tr>
<tr>
<td>10µM PSM + 75µM t-BuOOH</td>
<td>50 ± 9,9</td>
<td>0,460 ± 0,10</td>
</tr>
<tr>
<td>100µM PSM + 75µM t-BuOOH</td>
<td>63 ± 5,5</td>
<td>0,297 ± 0,07</td>
</tr>
<tr>
<td>0,01µM S + 75µM t-BuOOH</td>
<td>25 ± 3,6</td>
<td>0,279 ± 0,03</td>
</tr>
<tr>
<td>0,1µM S + 75µM t-BuOOH</td>
<td>33 ± 2,1</td>
<td>0,254 ± 0,04</td>
</tr>
<tr>
<td>10µM S + 75µM t-BuOOH</td>
<td>45 ± 4,5</td>
<td>0,235 ± 0,03</td>
</tr>
<tr>
<td>100µM S + 75µM t-BuOOH</td>
<td>62 ± 2,6</td>
<td>0,230 ± 0,02</td>
</tr>
</tbody>
</table>

**Table 7.** Effect of PSM and Silymarin in t-BuOOH-induced oxidative stress on cell viability and LDH activity in isolated rat hepatocytes

\*\* p < 0,01; \*** p < 0,001 vs control
\*\* p < 0,05; \*** p < 0,01; \**** p < 0,001 vs CCl₄
3. Discussion

In experimental toxicology the in vitro systems play an important role for the investigation of xenobiotic biotransformation and reveal the possible mechanisms of toxic stress and its protection. There are different in vitro systems for investigating metabolism on sub-cellular and cellular level. These systems help for the reduction of the experimental laboratory animals.

One of the most suitable sub-cellular in vitro system for investigation of drug metabolism is isolated microsomes.

The microsomal fraction, which is prepared by differential centrifugation, contents fragments from endoplasmic reticulum and preserve the enzyme activity, mostly Cytochrome P450 enzymes. Microsomes may be used as a model of lipid membrane in experiments, connected with the process of lipid peroxidation. They are convenient model for investigation of possible anti- or pro-oxidant activity of biologically active compounds from natural or synthetic origin [28]. In conditions of non-enzyme and enzyme lipid peroxidation in isolated rat microsomes, PSM showed statistically significant antioxidative effect, similar to the effect of silymarin. These results correlate with data for antioxidant activity of saponin cycloestragenol-xylozil-glycoside, isolated from extract of Astragalus membranaceus in mice liver [29].

The treatment of isolated rat brain synaptosomes with 6-OH-dopamine is a convenient in vitro sub-cellular system for the investigation of processes, which play role in the neurodegenerative diseases, including Parkinson’s and Alzheimer’s disease. The mechanism of 6-OH-dopamine neurotoxicity includes the formation of ROS and reactive metabolites, as a result of its metabolism in mitochondria of the nerve cells [30]. The mechanism of the destruction of the nerve terminals is thought to involve oxidation of 6-OH-dopamine to a p-quinone, the production of a free radical or of superoxide anion. It seems that a reactive intermediate is produced which reacts covalently with the nerve terminal and permanently inactivates it [31]. In rat brain synaptosomes, prepared by using Percoll reagent, PSM had statistically significant protective effect, similar to this of silymarin on 6-hydroxy-dopamine-induced oxidative stress. These results

Table 8. Effect of PSM and Silymarin in t-BuOOH-induced oxidative stress on GSH and MDA level in isolated rat hepatocytes

<table>
<thead>
<tr>
<th>Group</th>
<th>GSH, nmol/10^6 cells</th>
<th>MDA, nmol/10^6 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>21 ± 5,9</td>
<td>0,101 ± 0,01</td>
</tr>
<tr>
<td>75µM t-BuOOH</td>
<td>11 ± 4,7 **</td>
<td>0,421 ± 0,03 **</td>
</tr>
<tr>
<td>0,01µM PSM + 75µM t-BuOOH</td>
<td>11 ± 1,8 ***</td>
<td>0,312 ± 0,03 **</td>
</tr>
<tr>
<td>0,1µM PSM + 75µM t-BuOOH</td>
<td>12 ± 4,1 **</td>
<td>0,298 ± 0,01 ** ***</td>
</tr>
<tr>
<td>1µM PSM + 75µM t-BuOOH</td>
<td>13 ± 2,6 **</td>
<td>0,244 ± 0,05 ** ***</td>
</tr>
<tr>
<td>10µM PSM + 75µM t-BuOOH</td>
<td>16 ± 2,2 ***</td>
<td>0,193 ± 0,05 ** ***</td>
</tr>
<tr>
<td>0,01µM S + 75µM t-BuOOH</td>
<td>11 ± 3,1 ***</td>
<td>0,368 ± 0,03 ***</td>
</tr>
<tr>
<td>0,1µM S + 75µM t-BuOOH</td>
<td>11 ± 2,9</td>
<td>0,316 ± 0,03</td>
</tr>
<tr>
<td>1µM S + 75µM t-BuOOH</td>
<td>12 ± 2,1 ***</td>
<td>0,312 ± 0,06 ** ***</td>
</tr>
<tr>
<td>10µM S + 75µM t-BuOOH</td>
<td>14 ± 1,4 ***</td>
<td>0,247 ± 0,05 *** ***</td>
</tr>
</tbody>
</table>

* p < 0,05; ** p < 0,01; *** p < 0,001 vs control
* p < 0,05; ** p < 0,01 vs t-BuOOH
correlate with literature data about protective effects of *Astragalus* in conditions of stress in rats [5] [32].

Isolated liver cells are used as a suitable model for evaluation of the cytoprotective effects of some perspective biologically active compounds, both newly synthesized and plant isolated.

It’s known that CCl₄ is bioactivated by CYP2E1, as well as CYP2B1 and possibly CYP3A, to form the trichlormethyl radical (•CCl₃), which initiates the chain reaction of lipid peroxidation [33]. Pre-incubation of the hepatocytes with PSM and Silymarin resulted in protection against CCl₄ toxicity. These effects were the most visible at the highest concentration - 100µM [14]. These data are supported by literature data about protective effect of saponins, isolated from *Astragalus membranaceus* and *Astragalus sieversianus* on hepatotoxicity, induced by CCl₄, D-galactosamine and Paracetamol in mice [34].

Another model in isolated rat hepatocytes, used for oxidative stress, is t-BuOOH. The metabolism of t-BuOOH to free radicals undergoes through several steps. In microsomal suspension, in the absence of NADPH, it has been shown to undergo one-electron oxidation to a peroxyl radical (reaction 1), whereas in the presence of NADPH it has been shown to undergo one-electron reduction to an alkoxyl radical (reaction 2). In isolated mitochondria and intact cells, the t-BuOOH has been shown to undergo β-scission to the methyl radical (reaction 3). All these radicals cause lipid peroxidation process [35] [36].

\[(\text{CH}_3)_3\text{COOH} \rightarrow (\text{CH}_3)_3\text{COO}^- + e^- + H^+ \quad \text{(reaction 1)}\]
\[(\text{CH}_3)_3\text{COOH} + e^- \rightarrow (\text{CH}_3)_3\text{COO}^- + \text{OH}^- \quad \text{(reaction 2)}\]
\[(\text{CH}_3)_2\text{CO}^- \rightarrow (\text{CH}_3)_2\text{CO} + \cdot\text{CH}_3 \quad \text{(reaction 3)}\]

In model of oxidative stress – induced by t-BuOOH, PMS had statistically significant cytoprotective and antioxidant activity, stronger than the effect of silymarin. These effects were most expressive in concentration 100µM [14]. The protective effects of PSM might be due to the influence of the metabolism of t-BuOOH on microsomal and mitochondrial level. These data correlate with literature data about antioxidant activity *in vitro* of water extract of *Astragalus* on mitochondria, isolated from rat heart [7].

In our studies on different models of toxicity *in vitro*, Purified Saponin Mixture, isolated from *Astragalus corniculatus*, showed statistically significant cytoprotective and antioxidative effects.

On sub-cellular level – isolated rat microsomes and synaptosomes, PSM showed pronounced antioxidative effect.

Cytoprotective effect of PSM in isolated rat hepatocytes might be due to the influence of the metabolic bioactivation and oxidative stress.

The effects of PSM were similar to the effects of silymarin.
References


[22] D. Fau, A. Berson, D. Eugene, B. Fromentty, C. Fisch, D. Pessayre, Mechanism for the hepatotoxicity of the antiandrogen nilutamide. Evidence suggesting that redox cycling of this


Chapter 32

Effects of Multiple D-amphetamine Administration on Some Hepatic Biochemical Parameters in Spontaneously Hypertensive Rats (SHR)

Rumyana SIMEONOVA, Vessela VITCHEVA, Mitka MITCHEVA

Abstract - Amphetamine is a widely used drug of abuse, known with its hepatotoxic effects both in humans and rats. There are literature data about amphetamine hepatocellular damage, investigated mainly in normotensive Wistar rats. The aim of the following study was to investigate d-amphetamine hepatotoxicity in spontaneously hypertensive rats (SHR) in terms of serum ALAT activity, reduced glutathione (GSH) level, malondialdehyde (MDA) quantity. The total cytochrome P450 quantity and activities of some hepatic microsomal enzymes - ethylmorphine-N-demethylase (EMND), aniline-hydroxilase (AH), were assessed, as well. For the experiments, male SHR (n=12), strain Okamoto Aoki, were used. The animals were divided into two groups: control group and group, treated with d-amphetamine (5 mg/ kg i.p. 5 days). The results of our study showed that d-amphetamine treatment significantly increased serum ALAT activity, which correlated with depletion of GSH level by 28% and an increase lipid peroxidation. The EMND activity and the cytochrome P450 quantity were significantly increased by 35% and by 34%, respectively, compared to the control levels, without any change in AH activity. It is known that in Wistar rats, amphetamine may inhibit cytochrome P 450. Our results, concerning P450 quantity and EMND activity in SHR, are opposite to those in Wistar rats. We supposed, by an observed increased activity of EMND, that this isozyme may play a role in biotransformation and toxicity of D-amphetamine in this pathological strain.

On the base of this study we might suggest that biochemical differences in hypertensive rats, after multiple D-amphetamine administration, are probably due to different pathophysiological characteristics of SHRs.

Keywords - amphetamine, SHR, metabolism

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Introduction

The social use of amphetamines is widespread. The use of these drugs has been associated with numerous reports of toxicity, which are originated mainly from the CNS, liver and heart. Recreational use of these drugs presents an important but often concealed cause of hepatitis or acute liver failure, particularly in young people [1]. The hepatocellular damage, following intake of amphetamines, has increasingly been reported in humans [2]. Amphetamine-induced liver injury has been also observed in experimental animals [3]. D-amphetamine is well known substance of abuse, which cause cardiac problems and an elevation of blood pressure.

On the other hand essential hypertension is associated with oxidative stress, lipid and protein damage [4], which leads to development of some complications, such as cardiovascular disease, arteriosclerosis and fatty liver. Fatty liver disease is a spectrum of liver diseases, ranging from simple hepatic steatosis through steato-hepatitis to cirrhosis and hepatoma [5]. In all these injuries, hepatic biotransformation may be affected.

As for all pathologic conditions, the use of animal models is of a great importance for the study of pathophysiological disturbances of hypertension, including the role of drug metabolizing enzyme systems and toxicity in this state.

The spontaneously hypertensive rats (SHR) are a model of genetic hypertension, sharing common characteristics with primary hypertension in man. There are literature data about the increased activity of some liver microsomal enzymes in SHRs, especially CYP3A [6] and their possible role in hypertension [7]. Some authors report significant differences in drug biotransformation between SHRs and their age-matched normotensive control, which may be genetically determined [8].

The studies about biotransformation and hepatocellular damage of D-amphetamine were carried out mainly in normotensive Wistar rats. It is well known that the liver organelles are more susceptible to some hepatotoxics in hypertensive animals than in normotensive [9].

Thus the aim of this study was to investigate the possible differences of toxic effects and hepatic metabolism of D-amphetamine in SHRs.

1. Materials and Methods

1.1. Experimental Animals

Male SHRs strain Okamoto-Aoki, 220 g ± 10 g, blood pressure 219.8 ± 8.2/ 127.4 ± 11.4 mmHg, were obtained from the breeding colony of department of Physiology in Medical University – Sofia, Bulgaria. All animals were randomly assigned to the treatment groups and housed in groups of six in macrolon cages with standard food and tap water ad libitum. They were kept in an air-conditioned room under controlled environmental conditions at 20 ±2°C, with 12h alternating light/dark cycles. All experiments were performed after at least one week of adaptation to this environment.

Sistolic (SBP) and diastolic (DBP) blood pressure were measured in conscious SHRs and their normotensive controls (NTRs) using a semi-automated tail-cuff indirect blood pressure meter (LE 5002). Before the experimental period, the rats were
conditioned to the restraining cylinders, and were prewarmed for 10 min, using a temperature – controlled warming holder (37°C) to facilitate tail blood flow, before their blood pressure was measured. The mean of three tail-cuff readings was used as the systolic and diastolic blood pressure value.

The experimental procedures were approved by the Institutional Animal Care and Use Committees at the Medical University – Sofia, Bulgaria. All animal procedures were in accordance with the Veterinary Medical Office in Bulgaria, which follow the NIH guidelines of the care and use of laboratory animals. The number of rats was reduced, as much as possible, depending on statistical significance.

1.2. Design of the Experiment

The animals were divided into two groups (n=6). The first group was treated with D-amphetamine - 5 mg/kg i.p., once daily, for 5 days [10]. The second group, included respective controls, untreated animals, which were involved in the experiment from very beginning and housed under the same standard laboratory conditions as treated animals.

All animals were fasted overnight before euthanasia. 24 hours after the last administered dose amphetamine, the animals were weighed, euthanized by decapitation and blood and livers were taken for biochemical assessment.

1.3. Drugs Used

The drug used in this study was D-amphetamine sulphate, purchased from Sigma Chemical Co. (Germany).

1.4. Biochemical Methods

1.4.1. Preparation of Liver Microsomes for Assessment of Cytochrome P450 and Enzyme Activities.

The livers were perfused with 0.15mol L⁻¹ KCl, excised and minced. The latter was homogenized with three volumes of 1.17% KCl solution in a glass homogenizer with Teflon pestle, at 4°C, as described by Guengerich [11]. Nuclei, mitochondria and lysosomes were removed by centrifugation at 9,000 x g for 20 min at 4°C. The supernatant fractions were subjected to ultracentrifugation at 105,000 x g for 60 min at 4°C. The resulting microsomal pellets were stored at -20°C until the essay. Microsomal protein concentration was determined by the method of Lowry [12].

1.4.2. CYP Activity Assays in Rat Liver Microsomes

1.4.2.1. Aniline 4-Hydroxilation [13]

4-hydroxilation of aniline to 4-aminophenol was executed in 500 µL of reaction mixture in shaker at 37°C for 20 min. Aniline (1mM) was used as substrate in a reaction mixture, contained microsomes, potassium phosphate buffer, co-factors. The reaction was stopped in ice by addition of 250 µL of 20% TCA. After centrifugation at
4,000 rpm for 20 min, the supernatant was mixed with 10\% Na\(_2\)CO\(_3\) and 2\% phenol and incubated for 30 min at 37\(^{\circ}\)C. 4-aminophenol was formed with an absorption maximum at 640nm. Enzyme activity is expressed as nmol/min/mg protein.

1.4.2.2. Ethylmorphine-N-Demethylation [13]

Reaction was performed in a reaction mixture, that contained liver microsomes, ethylmorphine, as a substrate, co-factors and potassium-phosphate buffer, pH 7.4 in a shaker at 37\(^{\circ}\)C for 20 min. The reaction was stopped in ice, by addition of 4.5\% Ba(OH)\(_2\) and 15\% ZnSO\(_4\). After centrifugation at 4,000 rpm for 20 min, the supernatant was mixed with Nash reactive pH 5.5-6.5 and incubated at 60\(^{\circ}\)C for 30 min. The enzyme activity was evaluated by the formation of formaldehyde, trapped in the solution as semicarbazone and measured by the colorimetric procedure, at 412 nm. Enzyme activity is expressed as nmol/min/mg protein.

1.4.3. Assessment of Cytochrome P450 Quantity [14]

At the day of assay the microsomal pellets were resuspended and diluted in phosphate buffer + EDTA (pH=7.4). Liver protein concentration was adjusted to 10 mg/ml [10]. Cytochrome P450 quantity was quantified as nmol/mg protein spectrophotometrically as a complex with CO, at 450nm.

1.4.4. Determination of MDA Quantity in Liver Homogenate [15]

Liver was homogenized in four volume of potassium phosphate buffer pH 7.4, and 25\% trichloracetic acid (TCA) and 0.67\% thiobarbituric acid (TBA )was added to them. The sample was then mixed thoroughly, heated for 20 min in a boiling water bath, cooled and centrifuged at 4,000 rpm for 20 min, and the absorbance of supernatant was measured at 535 nm against a blank that contained all the reagents except the tissue homogenate. MDA level was calculated as nmol/g wet tissue.

1.4.5. Determination of GSH Level in Liver Homogenate [16]

Liver was homogenized in four volume of 5\% trichloracetic acid. The homogenates were centrifuged at 4,000 rpm for 20 min. The supernatants were mixed with DTNB reagent and the absorbance was measured at 412 nm against a blank that contained all the reagents except the tissue homogenate. GSH level was calculated as nmol/g wet tissue.

1.4.6. Evaluation of Alanine Aminotransferase Activity (ALAT) in Serum

The blood was taken into a tube containing EGTA. Serum was separated by centrifugation in bench centrifuge (Eppendorf; MiniPlus) (Mettler) at 10 000 rpm for 10 min, 4\(^{\circ}\)C and serum activity of alanine aminotransferase (ALT), were measured using automated, optimized spectrophotometrical method (COBOS Integra 400 plus, Roche Diagnostics).
1.5. Statistical Analysis

The results were presented as ± SD of 6 animals in each group. Student’s t-test was used. Probability values less than 0.05 were considered significant.

2. Results

SBP and DBP were significantly increased by 56% and by 58% respectively (p<0.05) in unanaesthetized untreated SHRs, compared with their age-matched normotensive controls NTRs (Figure 1).

The results on the quantity of hepatic cytochrome P450 and activities of EMND and AH in SHRs are shown in Table 1. D-amphetamine administration (5 mg/kg) to SHRs, induced a significant increase in quantity of total cytochrome P450 by 34% (p<0.05) and an increase in EMND activity by 35% (p<0.05). AH activity remained unchanged by D-amphetamine.

The results on the toxic parameters – ALAT activity, GSH level and MDA quantity are shown in Table 2. ALAT activity showed a significant increase in SHR treated rats by 35% (p<0.05). Administration of D-amphetamine to SHRs resulted in decrease of GSH level, by 28% (p<0.05) and in slight increase in MDA level, by 15% (p<0.05).

Figure 1. Sistolic and diastolic blood pressure in SHRs, compared to NTRs.

<table>
<thead>
<tr>
<th>Group</th>
<th>cytP450 (nmol/mg P)</th>
<th>EMND activity (nmol/min/mg P)</th>
<th>AH activity (nmol/min/mg P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.287 ± 0.03</td>
<td>0.423 ± 0.02</td>
<td>0.035 ± 0.004</td>
</tr>
<tr>
<td>Amphetamine</td>
<td>0.394 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.524 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.042 ± 0.001</td>
</tr>
</tbody>
</table>

<sup>a</sup>p < 0.05 vs control
Table 2. Effect of multiple administration of d-amphetamine on ALAT activity, GSH level and MDA quantity in SHRs

<table>
<thead>
<tr>
<th>Group SHRs</th>
<th>ALAT activity (IU)</th>
<th>GSH level (nmol/g)</th>
<th>MDA level (nmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>71.2 ± 5.19</td>
<td>4.55 ± 0.21</td>
<td>1.38 ± 0.12</td>
</tr>
<tr>
<td>Amphetamine</td>
<td>92.2 ± 6.12(^a)</td>
<td>3.31 ± 0.13(^a)</td>
<td>1.56 ± 0.21</td>
</tr>
</tbody>
</table>

\(^a\) p < 0.05 vs control

3. Discussion

Amphetamines are a good example of drugs of abuse with variable interindividual [17] and interspecies [18] metabolism. The recognition of the pharmacokinetic differences of these drugs is important to understand the susceptibility to their toxic effects. Thus, the aim of this study was to investigate a possible existence of interspecies differences in some biochemical liver parameters, involved in D-amphetamine biotransformation and toxicity in hypertensive model of rats. On one hand, the SHRs were chosen, since it is established that amphetamine provokes cardiovascular injury and hypertension, and on the other hand, because hypertensive humans and SHRs may have an altered blood flow, which may disturb liver function and biotransformation.

Our results showed that multiple D-amphetamine administration increased statistically significant the quantity of cytochrome P450 and activity of EMND, compared to the control group. It is known that in Wistar rats, amphetamine may inhibit cytochrome P450 through a nitroso metabolic intermediate. A number of in vitro [19] and in vivo [20] studies have proved that 4-hydroxylation of d-amphetamine in normotensive rats is favored by CYP 2D. For this isoform it is known to be not inducible [21]. Our results, concerning P450 quantity and enzyme activity in SHR, are opposite to those in NTRs. Some authors suggest a participation [22] and induction [23] of other CYP izoenzymes in D-amphetamine biotransformation, different from CYP2D. In SHRs CYP3A is more expressed and active, than in NTRs and it is partly responsible for the hypertension [24]. Basu et al., [25] demonstrated that troleandomycin (TAO), a selective CYP3A inhibitor, decreased enhanced in vivo corticosterone-6-β-hydroxilation and BP in SHRs. We supposed, by an observed increased activity of EMND, that this izoenzyme might play a role in biotransformation and toxicity of D-amphetamine in this pathological strain.

The results of the present study indicated a significant increase in serum ALAT activity and slight increase of hepatic levels of MDA, in comparison with the control group. These results are in agreement with the results, obtained by Carvalho et al. [26] for NTRs. Experimental studies performed in isolated rat hepatocytes by the same group of Carvalho et al. [27] have proved that in the liver D-amphetamine metabolises to an epoxide, a toxic intermediate, which reacts with glutathione, and forms GSH-adduct (GSH-S-yl-p-hydroxyamphetamine). This partly explains D-amphetamine hepatotoxicity and GSH depletion. In the present study D-amphetamine induced a significant decrease in GSH level in SHRs. We have previously demonstrated that multiple D-amphetamine treatment decreased GSH level and increased MDA formation in both strains, SHRs and NTRs, in similar manner, by approximately 29%.
and by 15% respectively (data not shown). However, the initial GSH level in control SHRs were lower and MDA quantity were higher, compared to NTRs. Thus hypertensive animals would be more sensitive to toxicity, induced by D-amphetamine. In our previous studies, a higher toxicity of paracetamol in SHRs, compared to NTRs were observed. Hsu et al. [9] proved higher sensitivity of SHRs to carbontetrachloride-induced hepatic toxicity.

Many authors reported that the oxidative stress in this animal model is characterized by lower GSH level and higher MDA formation [28].

On the basis of this study we might suggest that the differences in biotransformation and toxicity of D-amphetamine in hypertensive rats are probably due to their special pathophysiological characteristics.

However, in order to understand whether the liver disturbances in essential hypertension in humans could be easily exacerbated by different hepatotoxins, and whether or not they have the ability to repair quickly after liver damage, further investigations are needed.

References


[22] V. Vitecheva, M. Kondeva-Burdiina, M. Mitcheva. D-amphetamine toxicity in freshly isolated rat hepatocytes: a possible role of CYP 3A. *Arh Hig Ind Toxicol* 60 (2009); (2) in press


Chapter 33

Protective Effect of New L-Valine Derivatives on Brain Function in Experimental Model of Aggression in Mice after Social Isolation

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2 Medical University of Sofia, Medical Faculty
3 University of Chemical Technology and Metallurgy – Sofia

Abstract - Two newly synthesized compounds combine amino acid L-valine and pyridinic moiety attached in m-(M) or p-(P) positions. Previous data demonstrated their low toxicity and good therapeutic index. The purpose of this study is to evaluate their influence on the cognitive processes on the model of experimental aggression in mice by social isolation (for period of 6 weeks). Applied in Albino mice (125 mg/kg b.w., i.p., for 3 days) both compounds improved significantly short-term and long-term memory (step-through test) (about 50%) as well as exploratory activity. Neuromuscular coordination and muscle tone were significantly increased by M-6, and even better by P-6. Compound P-6 has significant analgesic effect according acetic acid test. Isolated mice were divided in two groups - aggressive and non-aggressive animals. Surprisingly, isolated non-aggressive animals had even better short-term and long-term memory than control animals living in a group. Aggressive mice demonstrated bad cognitive functions. Both compounds increased significantly damaged long-term memory in aggressive animals (M-6 with 81% and P-6 with 88%) as well as exploratory activity, muscle coordination, stability and concentration of animals. The improving effect of M-6 and P-6 on the cognitive functions of control mice living in groups was commensurate with that of isolated aggressive animals. The mechanism of the stable preventive effect of both compounds on damaged cognitive processes in aggressive animals is still not clear. The variations in the effects of both compounds can be explained with their positional isomery and difference in some physico-chemical parameters.

Keywords - L-valine, aggression, animal models, learning and memory

Introduction

Effective protection with amino acids and pyridine derivatives has been reported against memory deficit occurring in some mental diseases, aggression, trauma, brain
damages, or intoxications. Two newly synthesized isomeric compounds combine amino acid L-Valine and pyridinic moiety attached in m-(M) or p-(P) positions. Previous data demonstrated their low in vitro and in vivo toxicity (intraperitoneal and oral toxicity over 2 000 mg/kg and citotoxicity lower than vitamin C) and good therapeutic index (over 8).

The purpose of the study: To evaluate the influence of the compounds M6 and P6 on the cognitive processes in mice with experimental aggression.

Materials and Methods

Animals: Male ICR mice (18–20g)-10 in group; male Wistar rats (160-180 g)- 6 in group

The experimental model of intraspecies aggression (1,2) was developed via social isolation syndrome (for a period of 6 weeks). Isolated mice were divided in two groups - aggressive and non-aggressive animals.

Both compounds (in single or repeated doses 125 mg/kg b.wt. i.p.,3 days) were applied in mice. The following tests were performed on the 24th hour and on the 7th day after the last injection:

1. Exploratory activity (“Hole board” test).
2. Learning and memory (step-through test)
3. Muscle tone (Horizontal bar test)
5. Analgesic (antinociceptive) effect- test for chemical irritation with 1% acetic acid solution.

In rats treated with a single dose of the compounds (250 mg/kg, b.wt. i.p) some differences in biogenic monoamine levels in hippocampus (dopamine-DA, noradrenaline-NA and 5-hydroxy tryptamine-5-HT) were examined according method of Jacobowitz D., Richardson J.,1978 (3).

ANOVA was used for statistical assessment of the experimental data.

Results and Discussion

Aggressive isolated mice demonstrated seriously damaged cognitive functions- the short- and long-term memory decreased significantly (Fig. 1).
Isolated non-aggressive animals surprisingly, had even better short-term and long-term memory than control animals living in a group (Fig 2 a, b, c)

Figure 1 Learning and memory in grouped and isolated controls

Figure 2a) Learning and memory in grouped and isolated control mice

Figure 2b) Learning and memory in isolated mice treated with M6
In grouped animals the long-term memory was improved (about 50%), as well as the exploratory activity after 3 days of treatment with the compounds (Fig. 3a,b,c)

**Figure 2c)** Learning and memory in isolated mice treated with P6

**Figure 3a)** Exploratory activity after 3 days of i.p. Ol.Helianthi application

**Figure 3b)** Exploratory activity after 3 days of treatment with M-6
Figure 3b) Exploratory activity after 3 days of treatment with P-6

Neuromuscular coordination and muscle tone were significantly increased by $M$ and even better by $P$. (Fig. 4 and 5). The compound $P6$ has significant analgesic effect according to the acetic acid test (Fig. 6)

Figure 4. Neuromuscular coordination in isolated vs. grouped animals after 3 days-treatment

Figure 5. Muscle tone after 3 days of treatment with the compounds
Both compounds did not improve the short-term memory. But on the 7th day the opposite effect was established. M6 and P6 improved significantly damaged long-term memory in aggressive animals (M6 with 81% and P6 with 88%), as well as exploratory activity, and muscle coordination of the animals. The established stable preventive effect on damaged cognitive processes in aggressive animals, together with the analysis of physico-chemical parameters of compounds (water solubility and partition coefficient) suggest a long half-life time and a slow metabolism of compounds in the body.

In single doses both compounds increased 5-HT levels in rat hippocampus (significant only for P-6) and P-6 also increased NA levels.

Conclusions.

1. Significant neuropharmacological potency of the compounds on damaged cognitive processes in aggressive animals was found, but the mechanism is not clear as yet.
2. P6 demonstrated a stronger effect on learning and memory (in normal and esp. in aggressive animals), but M6 had a stronger and persisting analgesic effect. The variations in the effects of both compounds can be explained with their positional isomery and difference in some physico-chemical parameters.
3. The compound P also changed the functional activity of the brain neurotransmitter system, modulating the levels of biogenic monoamines in hippocampus, increasing 5-HT and NA levels.
4. The established stable preventive effect the cognitive processes, together with physico-chemical analysis of compounds (water solubility and partition coefficient) suggest a long half-life time and a slow metabolism of compounds in the body.

References

Chapter 34

In Vitro and in Vivo Studies on Toxicity and Pharmacological Activity of New L-Valine Peptidomimetics

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Abstract - Four newly synthesized peptidomimetics were studied as potential pharmacological agents. The aminoacid L-valine is bound to nicotinic (m-pyridinic) acid [M] or isonicotinic (p-pyridinic) acid [P] on one side, and to alkyl spacer containing 3 or 6 methylene groups on the other side. Our results show that the compounds are neuropharmacologically active agents (especially 6-isomers) with low toxicity (in vivo and in vitro) and moderate antiviral activity against Herpes Simplex. The compounds showed a very low toxicity in vivo (in Albino mice) (intraperitoneal and oral- over 2 000 mg/kg; and cytotoxicity lower than this of vitamin C) and also in vitro (in cell culture); as well as a good therapeutic index (over 8). Established antiviral activity against herpes simplex was moderate and is probably related to their chelating activity. Two of compounds (M6 and P6) increased processes of learning and memory in mice and had significant analgesic effect. Their high lipid solubility is probably responsible for their CNS-affinity. M6 and P6 had a higher log P than M3 and P3 (in system octanol/water) and they had better analgesic and anticonvulsant activity in vivo than compounds with 3 spacers. The compounds had the ability to modify the effects of some CNS-drugs. In acute treatment hexobarbital narcosis was prolonged by P6 and M6, but after 5 days of treatment they shortened it significantly. The mechanism of interaction is probably not only on the CNS level, but on the metabolic level too (affecting hepatic P-450- monooxygenases). The differences and varieties in their effects obviously are due to their positional and structure isomery.

Keywords - L-valine, peptidomimetics, toxicity, biological activity, cognitive enhancing drugs, drug development
Introduction

Four compounds, derivatives of L-Valine, nicotinic (m-pyridinic) acid (M) or isonicotinic (p-pyridinic) acid (P) as well as an alkyl spacer consisting of 3 or 6 methylene groups were studied as potential pharmacological agents. We use shorter names in accordance with their structure, namely M3, P3, M6 and P6, (Fig. 1).

These compounds belong to the group of low molecular weight gelators (LMWG) and have a very high affinity to form H-bonds linking to each other, as well as to include solvent molecules in their supramolecular complexes formation. [1, 2, 3]

The four compounds are constructed by the natural L-α-aminoacid – Valine, bound by amide (peptide) bounds to neighbouring groups which are not natural L-α-aminoacids and thus the compounds are representatives of the peptidomimetic group.

Availability of natural constituents in their structures, like an α-aminoacid, suggests low toxicity and good acceptance by the organism. The other ingredient of the molecule is nicotinic/isonicotinic acid, which we expect will determine specific biological activities. There are a number of reports in the literature for pronounced biological activities of such compounds - derivatives of nicotinic and isonicotinic acids [4-8].

The purpose of the study: To evaluate the compounds’ toxicity and their pharmacological activity (in vitro and in vivo).

1. Materials and Methods

Biological activities experiments:

Materials:

1. Male Albino mice ICR with initial body weight 18–20g (10 in groups) were treated with the compounds in single or repeated (5 days) doses (125 and 250 mg/kg b.w. i.p.).
2. F4N- mouse erythro-leukemic cells, obtained by erythroidal cells, transformed by the Friend virus

Methods:

Toxicological studies
In-vitro: The method is based on the ability of the live cells to throw out the blue dye penetrated through membranes, and remain uncolored while the dead cells stain blue.

In-vivo: For toxicology activities estimation it was necessary to define the following effects in the living body:
1. Parameters of acute toxicity.
   - Limit of acute activity (Limac).
   - No Observed Effect Level (NOEL)
   - Lethal dose 50% (LD50) – according to Bulgarian standards 15380-81
2. Prolonged toxicity – after 5, 7 and 14 days.
3. Reversibility of the toxic damages – till the 14th day after acute administration of the compounds.

II Neuropharmacological studies were performed to establish the substances' activities in the following:
1. Analgesic (antinociceptive) effect- test for chemical irritation with 1% acetic acid solution.
4. Learning and memory (“Passive avoidance step down test”)

III Drug interactions with the central nervous system (CNS) model compound - Hexobarbital (HB), 50 mg/kg applied intraperitoneally (i.p.).

IV. Antiviral activity (against Herpes simplex virus) and chelating activity to Fe (II) in blood plasma by a spectroscope method.

2. Results and Discussion

Toxicity: In vitro (cytotoxicity): The four peptidomimetics were found as nontoxic at concentrations equal to, or lower than 250 μM. We tested Vitamin C at the same experimental conditions and established that Vit. C revealed toxicity at 200 μM, i.e. our compounds are less toxic than Vit. C.

In vivo: As a result of the performed experiments low acute effect and lack of prolonged toxicity were established. Neither of the tested compounds (applied in both ways: intraperitoneal (i.p.) and oral (p.o.s.), provoked any symptoms of intoxication in doses over 2000 mg/kg, i.e. LD50 is over 2000 mg/kg (p.o.s and i.p.). No observed effect level (NOEL) was estimated as 40 mg/kg i.p., and limit of acute toxicity (Limac) was found at 80 mg/kg i.p.

Dissection of the animals on the 5th, 7th and 14th days of the treatment did not show any changes or irreversible toxic damages in the organs, which points to the lack of prolonged toxicity.

Analgesic effect: A significant analgesic effect of M6 and P6 (in dose 250 mg/kg) was found to be mostly pronounced at the end of the 1st hour for P6 and at the end of the 3rd hour for M6 (Fig 2). M3 and P3 did not show significant analgesic effect.
Exploratory activity: The compounds M6 and P6 possess some depressing effect on treated animals, compared with control animals. This effect is most evident in the 1st minute during a 3-minute numbering of peeks over a hole-board (Figure 3).

Neuromuscular coordination: The effect of decrease in the neuromuscular coordination ability is established after 5 days of treatment. P3 has a more pronounced effect than M3. M6 also has such influence in lower doses (125 mg/kg), but at 1000 mg/kg this effect is not revealed. P6 does not show such influence at any concentration.

Learning and memory: A positive dose-dependent effect of M6 and P6 on learning and memory of the treated animals was established (Figures 4a, b, and 5a, b). The compound M6 has a stronger effect than that of P6 (Figures 5a, b vs. Figures 4a, b).
Figure 4 a) Effect of P6 on learning (24th hour)

Figure 4 b) Effect of P6 on memory (7th day)

Figure 5 a) Effect of M6 on learning (24th hour)
Drug interactions with a CNS affecting model compound - Hexobarbital (HB), 50 mg/kg applied intraperitonealy (i.p.). The conducted experiments show that the four compounds influence the hexobarbital effect in different ways. This can be briefly summarized in the following results: The compound P6 prolonged HB narcosis several times in the 1st h and was observable even after 24 hs. The compound M6 had the opposite effect on HB narcosis and antagonized completely the narcotic effect of hexobarbital during the 1st hour (Fig. 6). M3 and P3 prolonged the effect of HB with 80% for M3 and 36 % for P3 as a result of the first dose and after 5 days of treatment they had the opposite effect and antagonized the narcotic effect of HB, 60% for M3 and 53% for P3.

It was found that the effective dose (ED50) for treatment is 250 mg/kg b.wt. i.p. in almost all experiments concerning physiological activities of the compounds. A high therapeutic index can be calculated (LD50/ED50>8), based on the knowledge that LD50 is over 2000 mg/kg.
The compounds interacted with pentylene tetrazole - PTZ (Fig. 7 and 8)

**Figure 7** Tonic seizure thresholds – effect of M-6
![Figure 7](image)

**Figure 8** Tonic seizure thresholds – effect of P-6
![Figure 8](image)

3. Conclusions

1. The four compounds are nontoxic at concentrations ≤ 250 μM. They show citotoxicity even lower than this of Vit C.
2. A high therapeutic index was estimated, > 8.
3. The compounds are neuropharmacologically active agents (especially the 6-isomers) with low toxicity (in vivo and in vitro) and moderate antiviral activity (Herpes Simplex virus).
4. Comparing data in vivo and in vitro allows us to assume that their lipo-solubility and chelating ability are probably important for their pharmacological effects.
5. The differences and varieties in their effects are obviously due to their positional and structure isomery. Some important interactions with other CNS-drugs can be predicted.

References

Chapter 35

Influence of Nifedipine on Morphine Elimination after Multiple Administration in Rats

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Abstract - L-type calcium channel blockers of the dihydropyridine class (Nifedipine) has been reported to modulate morphine effects, including tolerance and dependence development. At the same time, both Nifedipine and Morphine undergo extensive hepatic biotransformation, mediated by cytochrome P 450 CYP 3A isoform. Thus, these two classes of compound could be substrate competitors for CYP 3A. The aim of the following study was to investigate a possible influence of nifedipine, after multiple (7 days) administration along with morphine, on some metabolic biochemical parameters and urinary excretion. Male Wistar rats were divided into four groups: control; treated with nifedipine (5 mg/kg i.p., twice daily); treated with morphine (5-30 mg/kg i.p., twice daily, 7 days); combined group. Quantity of cytochrome P 450 and activity of ethylmorphine-N-demethylase (EMND) were measured spectrophotometrically. Urine samples were collected at the 8th day, 24 hours after the last morphine administration, alone and along with nifedipine. The urinary excretion of morphine was determined, using GC/MS method. Cytochrome P 450 quantity and EMND activity were significantly increased by 13% and 27% and by 56% and 35% respectively. This was accompanied by significant decrease in urinary excretion of morphine, by 57%, when is administered together with nifedipine. At the same time toxic effects, such as suppressed locomotor activity, respiratory depression, cyanosis and increased death, were observed among the animals of the combination group. These results suggest metabolic interaction between morphine and nifedipine that leads to decrease in morphine urinary excretion and increased toxicity in the combination group.

Key words: morphine, Nifedipine, urinary excretion, cytochrome P 450, EMND
Introduction

L-type calcium channel blockers of 1,4-dihydropyridine type, such as nifedipine, have been reported to modulate morphine tolerance and dependence development due to a reduction of Ca\(^{2+}\) influx into the neurons and subsequent inhibition of neuronal nitric oxide synthase (nNOS) [1]. In our previous study we found out that multiple administration of nifedipine along with morphine, resulted in significant decrease of nNOS activity, which was enhanced by morphine after multiple administration in rats [2]. Besides this interaction between morphine and nifedipine on the level of the central nervous system, a metabolic interaction could be suggested, as well. Nifedipine has been reported to be a substrate for one of the most abundant cytochrome P 450 isoenzymes CYP3A [3]. At the same time it is known that nifedipine induces cytochrome P 450 both in rats [4] and humans [5]. According to Projean et al. [6] some of the administered morphine is metabolized to the N-demethylated form – normorphine - in rodents, possibly by the CYP 3A subfamily. This suggests a possible metabolic interactions of morphine with substrates of CYP 3A, such as nifedipine. A possible pharmacokinetic interaction between both compounds, might influence morphine elimination via urine. According to the data of Milne at al. [7], the kidney plays a major role not only in excretion of glucuronide metabolites – morphine-3-glucuronide and morphine-6-glucuronide, but also in the excretion of normorphine.

Taking into account this information, the aim of the following study was to investigate a possible influence of nifedipine on morphine elimination, after multiple administration in rats.

1. Materials and methods

1.1. Animals

Male Wistar rats (body weight 200 g - 250 g) were housed in Plexiglas cages (3 per cage) at 20±2 °C and 12/12-hour light/dark cycle. Food and water were provided ad libitum. The animals were purchased from the National Breeding Centre, Sofia, Bulgaria. All procedures were approved by the Institutional Animal Care Committee and performed strictly following the principles stated in the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123) (1991) [8].

1.2. Design of the Experiment

The animals were divided into 4 groups:

The first group (n = 7) was treated with nifedipine at doses of 5 mg/kg i.p., twice daily, for 7 days [9].

The second group (n = 7) was treated with morphine - 5 – 30 mg/kg i.p., twice daily, for 7 days [9].
The third group was (n = 14) treated with nifedipine (5 mg/kg i.p) and 20 min after with morphine (5 – 30 mg/kg i.p). The drugs were administered twice daily, for 7 days. The fourth group (n = 14) was control non-treated animals, which were involved in the experiment from the very beginning and housed under the same standard laboratory conditions as the treated animals in groups 1-3. From 7 animals of the second and third group 24 hour urine was collected. The evaluation of the biochemical parameters was carried out in the four groups of animals, 24 hours after the last administration of the compounds.

1.3. Drugs Used

The drugs used in this study were morphine hydrochloride, purchased from Sopharma (Bulgaria) and Nifedipine hydrochloride, purchased form Sigma Chemical Co. (USA).

1.4. Urine Sample Collection

For urine collection, animals were housed in metabolism cages, equipped to separate urine and feces, for a 24-h period. Food and water were available ad libitum. The urine samples were analyzed by gas chromatographic/mass spectrometric (GC/MS) method for the detection of drugs of abuse in urine.

1.4.1. Preparations of Standard and Internal Control

Stock solution of morphine was prepared in water/methanol to yield a final drug concentration of 41 μg/mL. The standard curve for morphine was linear over the concentration range 0.49 - 7.50 μg/mL. Linearity was investigated by adding an appropriate volume: 0.4, 0.9, 1.4, 1.7 и 2.2 mL from the stock solution to drug-free urine.

Working solution of the internal standard, Levorphanol, was prepared to obtain a concentration of 0.43 μg/ml

1.4.2. Chemical Treatment of the Samples

In glass tubes (Wheaton, 20 ml volume), a 1.0-ml aliquot of urine was mixed with NaHCO₃:K₂CO₃ (1:2), pH = 9.6. Samples were shaken manually, Na₂SO₄ anhydricus and 20 μL Levorphanol, an internal standard, were added. Sample extraction was performed with 4 mL tert-butylmethylether for 30 min and then samples were centrifugated for 5 min at 2 500 rpm/min. For separation of the organic from non-organic phase, the samples were put in cryostat at -29°C. After the freezing of the water phase, the ether phase was concentrated under a stream of nitrogen.

1.4.3. Derivatisation

The purified dried extract was added to 50 μL MSTFA and 15 μl MBTFA. The derivatisation was performed at 80°C for 20 minutes. Aliquots of 1 μL were injected into the GC–MS equipment (Agilent Technology, USA).
1.5. Preparation of Liver Microsomes for Biochemical Assay [10]

Rats were decapitated and the livers were excised, perfused with 0.15 M KCl and minced. The latter was homogenized with 3 volumes of 1.17% KCl solution in a glass homogenizer. The liver homogenates were then centrifuged at 10,000 x g for 30 min. The supernatant fractions were centrifuged at 105,000 x g for 60 min. The resulting microsomal pellets were stored at -20°C until assayed.

1.6. Evaluation of Phase I of Biotransformation


The enzyme activity was evaluated by the formation of formaldehyde, trapped in the solution as semicarbazone and measured by the colorimetric procedure of Nash, at 415 nm. Enzyme activity is expressed as nmol/min/mg.

1.6.2. Assessment of Cytochrome P450 Quantity [12]

At the day of assay the microsomal pellets were resuspended and diluted in phosphate buffer + EDTA (pH=7.4). Liver protein concentration was adjusted to 10 mg/ml [13]. Cyt P450 quantity was quantified spectrophotometrically as a complex with CO, at 450 nm

1.7. Statistical Analysis

The results were presented as mean values (± SD) of 7 animals. Student’s t-test was used. Probability values less than 0.05 were considered significant.

2. Results

The influence of multiple administration of morphine and nifedipine on the quantity of hepatic cytochrome P 450 and the activity of EMND was shown in Table 1. Independently administered, morphine and nifedipine increased, in a statistically significant manner, as cytochrome P 450 quantity by 27 % (p < 0.05) and by 13 % (p < 0.05), respectively, as well as EMND activity – by 56 % (p < 0.05) and by 26 % (p < 0.05). The results were compared to the control. Co-administration of morphine and nifedipine led to a significant decrease in both parameters. Cytochrome P 450 quantity was decreased by 12 % (p < 0.05), compared to the control group and by 24 % (p < 0.05), compared to the morphine treated group. EMND activity was decreased by 26 % (p < 0.05), compared to the control group and by 52 % (p < 0.05), compared to the morphine treated group.

The influence of nifedipine on urinary excretion of morphine, after multiple administration, is shown in Table 2. In the combination group, the quantity of excreted morphine was decreased by 57 % (p < 0.05), in comparison to the group, treated only with morphine.
In all done experiments, amongst the rats, which morphine and nifedipine were co-administered, an increase toxicity, manifested by changes in locomotor activity, chianosis, respiratory depression and death (4/7), were observed.

**Table 1.** Multiple administration of morphine (5-30 mg/kg, i.p., twice daily, 7 days), alone and along with nifedipine (5 mg/kg, i.p., twice daily, 7 days) – effect on cytochrome P 450 quantity and EMND activity

<table>
<thead>
<tr>
<th>Group</th>
<th>Quantity of cytochrome P 450 (nmol/ mg)</th>
<th>Effect (%) vs control</th>
<th>EMND activity (nmol HCHO/min/mg)</th>
<th>Effect (%) vs control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.346 ± 0.022</td>
<td>-</td>
<td>0.246 ± 0.02</td>
<td>-</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>0.439 ± 0.04*</td>
<td>↑ 27</td>
<td>0.310 ± 0.01*</td>
<td>↑ 26</td>
</tr>
<tr>
<td>Morphine</td>
<td>0.390 ± 0.02*</td>
<td>↑ 13</td>
<td>0.384 ± 0.08*</td>
<td>↑ 56</td>
</tr>
<tr>
<td>Morphine + Nifedipine</td>
<td>0.305 ± 0.016*+</td>
<td>↓ 12</td>
<td>0.182 ± 0.02*+</td>
<td>↓ 26</td>
</tr>
</tbody>
</table>

* p < 0.05 versus control, + p < 0.01 versus morphine

**Table 2.** Urinary excretion of morphine (5-30 mg/kg, i.p., twice daily) after multiple administration (7 days) alone and in combination with nifedipine (5 mg/kg, i.p., twice daily)

<table>
<thead>
<tr>
<th>Group</th>
<th>Urinary concentration of morphine (mcg/mL)</th>
<th>Effect (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>154.25 ± 10.24</td>
<td>-</td>
</tr>
<tr>
<td>Morphine + Nifedipine</td>
<td>65.75 ± 5.85*</td>
<td>↓ 57</td>
</tr>
</tbody>
</table>

*p < 0.001 versus morphine

3. Discussion

It is well known that Ca++-channel blocker nifedipine is a substrate of hepatic CYP 3A in humans. A number of clinically significant drug-drug interactions between nifedipine and other substrates of this isoform, have been reported [14]. The experimental data of Guengerich F.P. et al. [15] proved that nifedipine undergoes hepatic biotransformation in rats, mediated by CYP 3A1. Different experimental studies showed that hepatic CYP 3A plays an important role in the metabolism of morphine to normorphine [16, 6].

In our study, the quantity of cytochrome P 450 and the rate of N-demethylation of ethylmorphine were assessed, after multiple administration of morphine and nifedipine, alone and in combination.

Our results show that morphine and nifedipine per se significantly increased both the cytochrome P450 quantity and EMND activity. The effect of nifedipine observed, correlates with the observations made by Konno Y. et al. [17], that multiple nifedipine administration in rats led to induction of different cytochrome P450 isoforms, including CYP 3A. The detected induction, caused by morphine, could be regarded as an
autoinduction, a process that possibly plays a role in morphine tolerance on a pharmacokinetic level.

In contrast to their own effect, multiple co-administration of morphine and nifedipine resulted in significant reduction as in cytochrome P 450 activity, as well as in EMND activity. Regarding the metabolic pathways, namely N-demethylation, of both compounds, these results might be due to a possible metabolic interaction of the two drugs. Together with this interaction, it is important to note that amongst the rats, which morphine and nifedipine were co-administered, an increased toxicity, manifested by changes in locomotor activity, cyanosis, respiratory depression and death (4/7), were observed.

On the basis of these data, the urinary excretion of morphine was measured. Our results showed that in the group co-administered with morphine and nifedipine, urinary morphine excretion was significantly reduced, compared to the group, treated only with morphine. In the kidney separate transport systems exist for the secretion of organic anions and cations across the membranes of the proximal tubule cells, and each transport system has a broad and overlapping substrate specificity. The renal clearence of morphine, which exists predominantly as a cation at physiological pH, involves glomerular filtration, tubular secretion and possibly reabsorption, that is carrier-mediated [18]. P-glucoprotein exists in proximal cells and this transporter has been shown to be involved in transport of morphine across the luminal membrane of renal proximal tubule cells in mice and humans [19]. Tubular secretion, like any other carrier-mediated transport, has a limited capacity and saturation may occur. At the same time, in one of their studies Shimizu et al. [20] found out that co-administartion of morphine with Ca-cannel blockers leads to marked increases in morphine level in the serum but not in the brain.

Considering this information, the observed decrease in morphine urinary concentration, after its co-administration with nifedipine, might be due to its possibly increased serum levels, as a result of a metabolic interaction with nifedipine and subsequent saturation of the transport system, responsible for its excretion. All this probably resulted in the increased toxicity, detected in the combination group.

References


[7] Projean D., Morin, PE, Tu, TM., Ducharme J. Identification of CYP 3A4 and CYP 2C8 as the major cytochrome P450s responsible for morphine N-demethylation in human liver microsomes. *Xenobiotica* 33 (2003), 841-54.


Chapter 36

Learning, Memory and Biogenic Amine Levels in Rat Hippocampus after Treatment with New L-Valine Derivatives

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Abstract - Memory deficit was documented after brain damages, trauma, intoxications or diseases and aging. Improving effect of some amino acids and nicotinic acid derivatives had been reported in literature. Two newly synthesized compounds combine in their molecule the amino acid L-Valine and nicotinic or isonicotinic acid. They differ in structures only by the position of L-Valine connected by amide bonds to a pyridine residue in m-(M) or p-(P). The compounds (in repeated doses 125 and 250 mg/kg b.wt. i.p. for 3 days) M6 and P6 demonstrated significant neuropharmacological activity on learning and memory in experiments with Albino mice (step through test), exploratory activity (hole board test) and nociception (acetic acid test). The established effects were stable and persisted even after 7 days after treatment. Some differences in biogenic monoamine levels in hippocampus were examined in male Wistar rats treated with a single dose of the compounds (250 mg/kg, b.wt. i.p.). In comparison to the control animals the levels of 5-hydroxytryptamine and noradrenaline were significantly increased by P-6. Our results show that the compounds are pharmacological active agents improving learning and memory and changing the functional activity of neurotransmitter system in rat hippocampus. They can modulate levels of biogenic amines probably via regulation of release of dopamine, noradrenaline and serotonin.

Keywords - learning, memory, biogenic monoamines, hippocampus, L-valine

Introduction

Memory deficits were documented after brain damage, trauma, intoxications or neurological alterations and aging. Two newly synthesized compounds combine in their molecule amino acid L-valine and nicotinic or isonicotinic acid (1, 2). They differ in structures only by the position of L-valine connected by amide bonds to a pyridine residue in m-(M) or p-(P) position.
Previous data showed their low toxicity and good tolerance in animals (3). Some amino acids and nicotinic acid derivatives have improving effect on the memory processes. **The aim of the present work** is to study the effect of the compounds on cognitive functions and to relate to certain changes in the neurotransmitters of hippocampus.

1. **Materials and Methods**

Experiments were performed on ICR Albino mice. The compounds M6 and P6 were applied in repeated doses 125 and 250 mg/kg b.wt. i.p. for 3 consecutive days. Learning and memory were studied by the step-through test, exploratory activity by the hole-board test and nociception - by acetic acid test.

The biogenic monoamine levels in hippocampus (dopamine, noradrenalin and 5-hydroxy tryptamine) were recorded spectroscopically in male Wistar rats, 1 hour after treatment with single dose (250 mg/kg, b.wt. i.p. (4).

2. **Results**

Our experiments established significant neuropharmacological effect of the studied compounds on learning and memory, exploratory activity and nociception. Learning and memory were significantly improved by M6 on the 1st day as well as on the 7th day (by M6 and P6). (Fig.1)
Figure 1. Learning and memory after 3 days of treatment with M6&P6 (125mg/kg, i.p.)

The exploratory activity of animals was not changed significantly by compounds on the 1st day after the treatment. But on the 7th day it was increased significantly (Fig 2a, b).

Figure 2. a) and b) – Exploratory behaviour after administration of the compounds
The neuromuscular coordination (Fig. 3) was increased both on the 1st and better on the 7th day. The muscle tone was also improved significantly by M-6 and better by P-6 (Fig. 4).

![Figure 3. Neuromuscular coordination after 3 days of treatment](image)

![Figure 4. Muscle tone on the 7th day after 3 days of treatment](image)

The observed effects were stable and persisted even on the 7th day. It was established that the compounds M6 and P6 have significant analgesic effect - more pronounced for P-6. (Fig. 5)

![Figure 5. Analgesic effect after 3 days of treatment](image)
Some differences in biogenic monoamine levels in hippocampus were found in treated rats with a single dose of the compounds (250 mg/kg, b.wt. i.p.). Compared with the control animals the levels of 5-hydroxy triptamine and noradrenalin were significantly increased by P-6.

3. Discussion.

Our results show that M6 and P6 are neuropharmacologically active agents. The compound M\textsubscript{6} demonstrated a stronger effect on learning and memory, while the compound P\textsubscript{6} had a stronger and more persistent analgesic effect. We suggest that the established differences and variety in their activity are obviously due to the positional isomery of the compounds.

Taking into account the important role of the hippocampus in cognition, we suggest that the increase of 5-HT level in this brain region by the compound P6 is one of the important neurochemical correlates of its nootropic effects.

A large body of research has shown memory-improving effects of the 5-HT uptake inhibitors, which are thought to increase 5-HT-ergic neurotransmission by increasing 5-HT concentrations in the synaptic cleft (5, 6).

The established changes in NA and 5-HT levels in rat hippocampus by P6 provided evidence for the realization of its CNS effect through changes in monoamine levels. P6 can modulate levels of biogenic amines probably via regulation of the release of noradrenaline and serotonin.

References

Chapter 37

Hepatic Interaction Between Amphetamine and Calcium Channel Blocker Nifedipine after Multiple Administrations in Rats

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Abstract - Amphetamine is an example of a drug of abuse that has extensive hepatic biotransformation. In rats it undergoes mainly \( p \)-hydroxylation of the aromatic ring, process favorably mediated by CYP 2D. In our previous \textit{in vitro} studies, using inducers and inhibitors of CYP 3A, we suggested an involvement of this isoform in amphetamine’s metabolism and toxicity. This poses possible \textit{in vivo} metabolic interactions of the compound with other substrates of this enzyme, among which are the Ca-channel blockers (nifedipine). On the basis of these data, the aim of the following study was to trace a possible hepatic interaction between amphetamine and nifedipine (CYP 3A substrate and inhibitor), after multiple administration (5 days). For the experiment, male Wistar rats were used. The animals were divided into 4 groups: control; treated with amphetamine; treated with nifedipine and combined group. Changes in cytochrome P 450 quantity, ethylmorphine-N-demethylase (EMND) activity and aniline hydroxylase (AH) activity, were measured spectrophotometrically. Administered alone, amphetamine led to significant decrease of P 450 quantity by 25% and EMND activity by 40%, without changing AH activity. Nifedipine, compared to the control group, increased P450 quantity, EMND activity and AH activity, by a statistically significant 28%, 35% and 26%, respectively. In the combination group cytochrome P 450 was decreased by 27%, while EMND activity and AH activity were increased by 34% and 21%, versus controls. According to the results of this study we suggest \textit{in vivo} interactions betweenamphetamine and nifedipine at the metabolic level.

Key words - amphetamine, nifedipine, rats, metabolism, interactions

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Introduction

Amphetamines are indirect-acting sympathomimetic drugs widely abused, due to their physical and psychostimulating effects. However, the use of these drugs has been associated with numerous reports of hepatotoxicity, a result of their hepatic bioactivation [1, 2]. Amphetamine shows variable interspecies biotransformation. In rats its aromatic ring is hydroxylated, and the urinary excretion of p-hydroxyamphetamine is the main elimination pathway (81%) [3]. Animal studies in vitro [4] and in vivo [5] have proved that d-amphetamine 4-hydroxylation in rats is favored by CYP 2D. This metabolic pathway is thought to be responsible for amphetamine hepatotoxicity [6]. It is also known that amphetamine inhibits cytochrome P450 through a nitroso metabolic intermediate, which complexes with the enzyme and causes its inhibition and possible toxic effects [7, 8]. The main cytochrome P450 isoform which catalyses the formation of the nitroso intermediate is CYP 3A [9].

L-type calcium channel blockers of the 1,4-dihydropiridine class, such as nifedipine, nimodipine, amlodipine, have been reported to affected different types of drug dependence and withdrawal, including those to psychostimulants [10]. At the same time they are known to be substrates for one of the most abundant cytochrome P 450 isoenzymes CYP3A that interacts with other substrates of this isoform.

In our previous in vitro studies we investigated a possible involvement of CYP3A in D-amphetamine hepatotoxicity in rat hepatocytes, isolated from nifedipine-treated rats [11]. Using amiodarone, an inhibitor of CYP 3A isoform, we suggested the involvement of CYP 3A in amphetamine metabolism and hepatotoxicity.

On the basis of these data the aim of the following study was to assess a possible in vivo interaction between amphetamine and nifedipine, after multiple co-administration in rats.

1. Materials and methods

1.1. Animals

Male Wistar rats (body weight 200 g - 250 g) were housed in Plexiglas cages (3 per cage) at 20 ± 2 °C and 12/12-hour light/dark cycle. Food and water were provided ad libitum. The animals were purchased from the National Breeding Centre, Sofia, Bulgaria. All procedures were approved by the Institutional Animal Care Committee and performed strictly following the principles stated in the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123) (1991) [12].
1.2. Design of the Experiment

The animals were divided into 4 groups (n = 6). The first group was treated with nifedipine at doses of 5 mg/kg i.p., once daily, for 5 days [13].

The second group was treated with d-amphetamine - 5 mg/kg i.p., once daily, for 5 days [14].

The third group was treated with nifedipine (5 mg/kg i.p) and 30 min after with d-amphetamine (5 mg/kg i.p.). The drugs were administered once daily, for 5 days.

The fourth group was control non-treated animals, which were involved in the experiment from the very beginning and housed under the same standard laboratory conditions as the treated animals in groups 1-3.

The evaluation of the biochemical parameters was carried out in the four groups of animals, 24 hours after the last administration of the compounds.

1.3. Drugs Used

The drugs used in this study were D-amphetamine sulphate and Nifedipine hydrochloride were purchased from Sigma Chemical Co. (Germany).

1.4. Preparation of Liver Microsomes for Biochemical Assay [15]

Rats were decapitated and the livers were excised, perfused with 0.15 M KCl and minced. The latter was homogenized with 3 volumes of 1.17% KCl solution in a glass homogenizer. The liver homogenates were then centrifuged at 10 000 x g for 30 min. The supernatant fractions were centrifuged at 105 000 x g for 60 min. The resulting microsomal pellets were stored at -20°C until assayed.

1.5. Evaluation of Phase I of Biotransformation

1.5.1. Assay of Aniline 4-Hydroxilase Activity [16]

4-hydroxilation of aniline to 4-aminophenol, that is chemically converted to a phenol-indophenol complex with an absorption maximum at 630 nm. Enzyme activity is expressed as nmol/min/mg.

1.5.2. Assay of EMND Activity [16]

The enzyme activity was evaluated by the formation of formaldehyde, trapped in the solution as semicarbazone and measured by the colorimetric procedure of Nash, at 415 nm. Enzyme activity is expressed as nmol/min/mg.

1.5.3. Assessment of Cytochrome P450 Quantity [17]

At the day of assay the microsomal pellets were resuspended and diluted in phosphate buffer + EDTA (pH=7.4). Liver protein concentration was adjusted to 10 mg/ml [18].
Cyt P450 quantity was quantified spectrophotometrically as a complex with CO, at 450 nm

1.6. Statistical Analysis

The results were presented as mean values (± SD) of 6 animals in each group. Student’s t-test was used. Probability values less than 0.05 were considered significant.

2. Results

The results after multiple administration of d-amphetamine and nifedipine on the quantity of hepatic cytochrome P450 and activity of EMND and AH are shown in Table 1. Both compounds, independently administered, influenced the enzyme quantity in a statistically significant fashion, as follows: nifedipine increased cytochrome P 450 by 28% (p<0.05) and the activity of EMND and AH - by 35 % (p < 0.05) and by 26 % (p < 0.05), respectively, versus control, while d-amphetamine decreased the cytochrome P 450 quantity by 27 % (p < 0.05) and by 40 % (p < 0.05) EMND activity, without changing AH activity.

In the combination group the studied parameters were influenced as follows: cytochrome P 450 quantity was decreased by 27 % (p < 0.05) vs control, which is commensurable with the effect of D-amphetamine itself. The activities of EMND and AH in the combination group were commensurable with the effect of nifedipine after its alone administration - EMND activity was increased by 34 % (p < 0.05) vs control and by 125 % (p < 0.05) compared to amphetamine group; AH activity was increased by 21 % (p < 0.05) compared to control and by 15 % (p < 0.05) versus cocaine group.

Table 1. Effect of multiple administration of d-amphetamine and nifedipine on cytochrome P450 quantity, EMND and AH activity in rats

<table>
<thead>
<tr>
<th>Group</th>
<th>cytP450 (nmol/mg)</th>
<th>EMND activity (nmol/min/mg)</th>
<th>AH activity (nmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.345 ± 0.022</td>
<td>0.246 ± 0.02</td>
<td>0.038 ± 0.004</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>0.440 ± 0.022a</td>
<td>0.332 ± 0.02a</td>
<td>0.048 ± 0.002a</td>
</tr>
<tr>
<td>Amphetamine</td>
<td>0.257 ± 0.06a</td>
<td>0.147 ± 0.04a</td>
<td>0.040 ± 0.001</td>
</tr>
<tr>
<td>Nifedipine + Amphetamine</td>
<td>0.251 ± 0.01a</td>
<td>0.330 ± 0.04ab</td>
<td>0.046 ± 0.005b</td>
</tr>
</tbody>
</table>

*a p < 0.05 vs control ; b p < 0.05 vs amphetamine
3. Discussion

Amphetamine is a well-known and wide-spread psychostimulant that causes behavioral activation, tolerance and dependence development. Its multiple administration is related to severe hepatotoxicity. Although the p-hydroxylation of amphetamine is considered to be the main metabolic pathway [3], it is possible that some other cytochrome P 450 dependent metabolic pathways to be involved in its toxicity. There are clinical data revealing significant interactions between recreational drugs, including amphetamine and potent inhibitors of CYP 3A4 such as some of the HIV protease inhibitors [19]. The antiretroviral agents are thought to be capable of causing fatal amphetamine accumulation from normally safe dosages as consequences of metabolic inhibition. Thus, a possible involvement of CYP 3A in amphetamine metabolism and toxicity in rats could be suggested. In our study a possible in vivo interaction between amphetamine and calcium channel blocker nifedipine, a substrate and inducer of CYP 3A [20, 21], was investigated.

Our results show that in the liver, amphetamine and nifedipine exerted opposite effects. While multiple nifedipine administration led to statistically significant increase of hepatic cytochrome P 450 level and EMND and AH activity, amphetamine itself reduced, in a statistically significant manner, the first two parameters, without changing AH activity, compared to the controls. These effects of amphetamine could be explained by a formation of nitroso metabolic intermediate, which complexes with cytochrome P 450 [8] and may cause a possible repercussions on its own metabolism.

In the combination group an interaction between amphetamine and nifedipine was observed. Changes in EMND activity showed that amphetamine was not able to exert its inhibitory effect – the enzyme activity was significantly increased, as the increase was commensurable with the effect of nifedipine per se. Being a substrate of CYP 3A and administered along with amphetamine, it is quite probable that nifedipine interferes with amphetamine metabolism. A substrate competition between two compounds on the level of CYP 3A might be also discussed.

On the other hand, in the combination group, on the level of cytochrome P450 an effect of amphetamine was observed – the quantity of P 450 was significantly decreased. Jönsson KH & Lindeke B. [22], in one of their studies, used three different enzyme systems – liver microsomes from phenobarbital pretreated rats and two reconstituted systems containing CYP 2B1 and 2C11, in order to study formation of metabolic intermediate complexes of phenylalkylamines, including amphetamine. They found out that the highly purified CYP 2B1 system formed the metabolic intermediate complex with amphetamine and this resulted in enzyme inhibition. Thus, the observed decrease in cytochrome P 450 quantity after multiple co-administration of amphetamine and nifedipine, might be partly due to an involvement of cytochrome P 450 isoforms, different from CYP 2D and CYP 3A.

References


Part 3

BIOLOGICAL AGENTS - PROBLEMS OF BIOLOGICAL TERRORISM
Chapter 38

Bioterrorism, History and Threat Assessment

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Abstract - From the onset of human civilization biological agents have been used as weapons because of their abilities to promote terror within adversary and thus to be utilized as a mean to gain the desired political or social result. The aim of the article is to present the historical and recent evidence of bioterrorism as a base of bioterrorism threat assessment. In order to achieve the set goal thoroughly were analyzed bioterrorism evidence throughout history by means of historical and descriptive analyses. Based on acquired data bioterrorism threat was assessed. For threat assessment modified RAPEX methodology was applied. As a result of the performed analyses is concluded that with their features bio-agents are ideal as a terrorists weapons. The high level bioterrorist’s threat has to alarm governments and international organization all over the world for the bioterrorists’ attacks eminence, what could be define as one of the security features of contemporary world.

Keywords - Medical intelligence, bioterrorism threat assessment, bioagents.

Introduction

In recent years a lot of publicity has been given to the problem of increased terrorists’ activities. Globalization has brought interdependence of the whole globe thus increasing need of all societies to confront any instability in order to maintain the obtained development pace. Therefore specific reference to any single source of social unrest has to be examined. Terrorism could be examined as a form of struggle against the governance and political system established in the country, region or even world – what is observed in recent terrorists’ acts.

Historical data provides uncountable evidence where the oppressed and minor social groups had tried to overcome their social status by implementing all available armaments. Clearly the tactics and the means applied were those one obtainable without many efforts and expenses. As the nature provides a lot of poisonous resources, despite of special knowledge required for their extracting and usage, the biological agents were the most appropriate arms for our predecessors. [1]

From the onset of human civilization biological agents have been used as weapons because of their abilities to promote terror within adversary and thus to be utilized as a mean to gain the desired political or social result. As the terrorists’ main
objective is to create panic and terror in the society in order to obtain their political or economic goals, it is rational that they have been using great variety of biological agents and toxins as weapons.

The recent terrorist attacks have raised great concern among all social groups about the public vulnerability in case biological weapon is applied.

In order to better understand biological terrorist’s threat, thoroughly historical research has to be performed. The purpose of the historical research is to describe those biological weapons features that transform them in desirable and obtainable terrorist weapon. Furthermore the historical analysis could provide data about terrorists’ willingness and readiness to apply bio-agents as weapon and their capabilities to weaponize them. Results of the historical evidence of biological weapon usage are basis for biological threat assessment. Threat assessment is function of terrorists’ intention and capability to imply bio-weapons in order to inflict death, disease and terror among the society, as a basis for achieving social unrest and political change. [2]

Authors are perfectly aware of the fact that increasing capabilities of terrorists to purchase and/or produce bio-weapons is becoming issue threatening societies all over the world, especially in last twenty years, and there have been commenced a large number of reasonable steps in order to reduce the bioterrorism threat. [3, 4, 5] Recent evidence opposes expectations for decrease in terrorists activity related to bio-weapons obtaining and implementation.

Aim:

The aim of the article is to present the historical and recent evidence of bioterrorism as a base of bioterrorism threat assessment.

Material and Methods:

In order to achieve the set goal thoroughly were analyzed bioterrorism evidence throughout history by means of historical and descriptive analyses. Based on acquired data bioterrorism threat was assessed. For threat assessment modified RAPEX methodology was applied.

Results:

Throughout history there is uncountable evidence for human intention and readiness to use wide spread natural hazards as a weapon in order to defeat or convince the adversary.

It is hard to determine if disease/epidemic spread was initiated deliberately or was consequence of natural occurrence in the dark pre-historical times, but there are undoubted proofs that from the beginning of the warfare all available sources to inflict casualties were applied. As biological means were found everywhere they were the first one applied in the armament. Recorded are the following:

- Non written, but preserved in mankind memory are the first application of bio-agents in the warfare - place animal and human corpses in water supplies; contaminated swords, arrows and lances with manure or decomposing bodies in order to create fear and desperation among the adversary;
- Holly Bible describes spread of “tumors” among the Philistines related to captured Ark of Covenant (most probably first recorded deliberate usage of bubonic plague in order to create fear and panic among adversary;
- 700 BC- the Assyrians poisoned wells with rye ergot;
- 590 BC – Solon of Athens poisoned water with hellebore roots during the siege of Cirrha;
- 400 BC – Scythian archers dipped arrowheads in blood of decomposing bodies;
- 190 BC – Hannibal’s army threw poisonous snakes into King Eumenes of Pergamon ships in Second Macedonian War;
- 1346-7 – Kaffa under siege by Tatars - catapulted bodies of plague victims (soldiers) over walls. Plague did occur in Kaffa and Black Death spread to Europe from this region – port cities (Sicily, Venice) and spread inland First epidemic in Europe 1348 - 50; four other epidemics in 1300’s may have killed one-third of population in Europe; Also in China – maybe as early as 1331; severe epidemics recorded 1353-54 during unending feudal wars;
- The Spanish, in 1495, infected French wine with blood from leprosy patients; Pizarro reportedly gave smallpox virus-contaminated clothing to South American natives in the 15th century;
- In the mid-1600’s, a Polish military general reportedly put saliva from rabid dogs into hollow artillery spheres for use against his enemies;
- Russian troops may have used a tartar strategy involving corpses of plague victims against Sweden in 1710;
- During the French-Indian War, the British gave blankets used by smallpox victims to the Native Americans and consequently smallpox raged through the Native American community and decimated their numbers. 1763 - Sir Jeffrey Amherst (commander of British forces in North America) inflicted smallpox epidemic at Fort Pitt – June 24 – Capt. Ecuyer gave blankets and a handkerchief contaminated with smallpox;
- Civil War in USA - Dr. Luke Blackburn, the future governor of Kentucky, attempted to infect clothing with smallpox and yellow fever which he then sold to Union troops 1775 – smallpox epidemic among Washington’s troops; Washington orders troops inoculated with smallpox 1777.

Biological science development started 200 years ago multiplied capabilities of bio-agents weaponizing.

Question could be raised – there is not even single evidence above mentioned of terrorist bio-attack, what is the purpose of listing those facts? Answer is found in bioterrorism definition: Bioterrorism is an intentional release of bacteria, viruses and other biological agents with set aim to cause diseases and/or death within population, animals and/or crops or other vegetation. Most of the agents used as bio weapon are widely spread in the nature, but some of their deadly features are intentionally strengthened or modified to increase their capability to inflict diseases, to increase their drugs’ and disinfectants’ resistance, or/and enhance their stability and ways to be spread in the environment. Biological agents could be spread within air, water and/or foods. Analysing in depth listed events could be concluded that states, military commanders or ordinary servicemen were performing bioterrorism in order to achieve their goals. Similar evidence could be listed while analyzing war conflicts in the XX century:
- 1915, Dr. Anton Dilger, a German-American physician, developed a microbiology facility in Washington D.C. where were produced large quantities of anthrax and glanders bacteria, by seed cultures provided by the Imperial German Government. German agents, inoculated 3000 head of horses, mules, and cattle that were destined
for the Allied Forces in Europe at loading docks. As a consequence, several hundred military personnel were secondarily infected.
- 1918, Japan formed a biological weapons section in the Japanese Army (Unit 731). From 1931 Unit performed biological weapons experiments in Harbin, Manchuria on Chinese prisoners of war, directed by Japanese General Ishii, until 1945. It is estimated up to 3,000 more prisoners and Chinese nationals may have died in this facility.
- 1931, During an investigation of Japan’s seizure of Manchuria in, Japanese military officials unsuccessfully attempted to poison members of the League of Nations’ Lytton Commission by lacing fruit with cholera bacteria.
- 1939, Japanese military poisoned Soviet water sources with intestinal typhoid bacteria at the former Mongolian border.
- 1941, Japanese Military released an estimated 150 million plague-infected fleas from airplanes over villages in China and Manchuria, resulting in several plague outbreaks in those villages.
- 1942, Large outbreak of tularemia occurred, on the German-Soviet front, shortly before the battle of Stalingrad. Because of of an intentional release several thousand Soviets and Germans contracted the illness (70% of the victims had pneumonic tularemia, evidence of an intentional release). Later was determined that the Soviets had developed a tularemia weapon previous year.
- 1975-1983, Tricothecene mycotoxins (T-2 toxins) were used in what was called "Yellow Rain" by Soviet-backed forces in Laos, Cambodia, and Afghanistan causing disorientation, illness even death among the exposed people and animals.

Bio weapon is a favorite terrorists’ weapon, because of the arduous detection, as well as postponed appearance – hours and days after the terrorist’s attack. When there is a bio-weapon incident suspicions the main obstacle is to define weather this incident was caused by natural agents or by bio-weapon usage. The possibility of secondary dissemination of the bio-agents is one more terrorists’ usage advantage.

There are many bio-weapon features that transform them in one of the terrorists’ favorite weapons. The most important are listed below:
1. In comparison with chemical and nuclear weapons biological agents that could be used as a bio-weapon are cheapest and more than easy obtainable;
2. All biological agents are very hard detectable prior their usage as a bio-weapon;
3. The biological attacks’ consequences used to be visible days after the attack;
4. The processing of the biological agents into bio-weapons is described and obtainable via Internet;
5. Bio-weapons could be much more deadly in comparison with the other Weapons of Mass Destruction (WMD). For instance to cause mass casualty (MASCAL) event in an area of one square mile 1800 pounds of zarin is required, but the equaling MASCAL effect could be achieved by spreading only ¼ ounce anthrax spores.

The anthrax attacks in USA (2001) are an outstanding example of the terrorists’ willingness and resolve to apply biological agents if available. The criminals performed the attacks are still undiscovered, but there are many indicators that they were deeds of al Qaeda’s (AQ) activists. Despite of the few casualties, the economic and psychological effects were enormous. The high alert status of the coast states gave its negative impact on commerce and common community lifestyle weeks long. For the residual psychological stress observed for longer period indicative were the media,
community and security cervices reactions on the followed pseudo biological attacks with talcum, anthrax instead.

In Table 1 are presented some of the most famous bioterrorists’ attacks occurred in the second half of the XX century:

<table>
<thead>
<tr>
<th>Case</th>
<th>Objective</th>
<th>Ideology</th>
<th>Target(s)</th>
<th>Agent(s)</th>
<th>Delivery</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weather Underground</td>
<td>Temporarily incapacitate U.S. cities to demonstrate impotence of the federal government</td>
<td>Revolutionary movement opposed to American imperialism and the Vietnam War</td>
<td>Urban populations in the United States</td>
<td>Reportedly sought to obtain agents at Ft. Detrick by blackmail of gay soldier</td>
<td>Reportedly planned to put incapacitating CW/BW agents in urban water</td>
<td>Report originated with U.S. Customs informants; case probably apocryphal</td>
</tr>
<tr>
<td>R.I.S.E. (1972)</td>
<td>Kill off most of humanity to prevent the destruction of nature, then star human race over with a select few</td>
<td>Perpetrators were college students influenced by ecoterrorist ideology and 1960s drug culture</td>
<td>Initially entire world population, later narrowed to residents of five states around Chicago</td>
<td>Eight microbial pathogens including agents of typhoid fever, diphtheria, dysentery, and meningitis</td>
<td>Planned BW aerosol attacks dispersed by aircraft and contamination of urban water supplies</td>
<td>Attack aborted when cultures were discovered; the two main perpetrators fled to Cuba</td>
</tr>
<tr>
<td>Red Army Faction</td>
<td>Allegedly planned BW attacks against West German officials and business leaders</td>
<td>Marxist-revolutionary ideology</td>
<td>Specific targets unknown</td>
<td>Group member allegedly cultivated botulinum toxin in a Paris safehouse</td>
<td>Unknown</td>
<td>Probably an erroneous report, later repudiated by German government (BKA)</td>
</tr>
<tr>
<td>Rajneeshee Cult (1984)</td>
<td>Scheme to incapacitate voters to win local election, seize political control of county</td>
<td>Indian religious cult headed by a charismatic guru</td>
<td>Residents of the town of The Dalles and Wasco County, Oregon</td>
<td>Salmonella Typhimurium</td>
<td>Multiple methods, mainly contamination of restaurant salad bars</td>
<td>Plot revealed when the cult collapsed and members turned informant</td>
</tr>
<tr>
<td>Minnesota Patriots</td>
<td>To cause harm to the federal government, obtain personal revenge</td>
<td>Anti-government tax protesters; “right-wing patriot” movement</td>
<td>R.I.S. officials, U.S. deputy marshal, local law enforcement officials</td>
<td>Ricin extracted from castor beans obtained by mail-order</td>
<td>Planned aerosol delivery of skin damage with DMSO and aloe vera, or as dry aerosol</td>
<td>Group was penetrated by FBI informants; four key members arrested</td>
</tr>
<tr>
<td>Shinrikyo (1995)</td>
<td>To alert Americans to the Iraqi BW threat; seeks separate homeland for whites in the United States</td>
<td>Mass civilian populations, individual opponents of cult, judges ruling against and police investigating cult</td>
<td>Biological agents (anthrax, botulinum toxin, Q fever Ebola virus) and chemical agents (sarin, VX, hydrogen cyanide)</td>
<td>Obtained plague and anthrax (vaccine strain), reportedly isolated several other bacteria</td>
<td>Discussed the dissemination of BW agents with crop-duster aircraft and other methods</td>
<td>Multiple CW attacks (in Matsumoto, Tokyo, and assassination campaign) killed at least 20 people and injured more than 1,000</td>
</tr>
<tr>
<td>Larry Wayne Harris</td>
<td>To alert Americans to the Iraqi BW threat; seeks separate homeland for whites in the United States</td>
<td>New Age doomsday cult seeking to establish a theocratic state in Japan, with a charismatic, power-hungry leader</td>
<td>Mass civilian populations, individual opponents of cult, judges ruling against and police investigating cult</td>
<td>Biological agents (anthrax, botulinum toxin, Q fever Ebola virus) and chemical agents (sarin, VX, hydrogen cyanide)</td>
<td>Obtained plague and anthrax (vaccine strain), reportedly isolated several other bacteria</td>
<td>Discussed the dissemination of BW agents with crop-duster aircraft and other methods</td>
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</table>
As the case of the religious cult Aum Shinrikyo was the most frightening, their activities in the period April 1990-March 1995 have to be listed separately:

Shoko Asahara founded Aum Shinrikyo, cult that develop lately into a massive empire with a membership of approximately 10,000 with financial assets of at least US $300 million. Aum was organized into Ministries and Departments, copying the governmental structure of the Japan. Ministry of Science and Technology, headed by Hideo Murai, and the Ministry of Health and Welfare, headed by Seichi Endo were responsible for biological warfare activity. Both ministers had completed university education (Murai had graduated from Osaka University with a degree in astrophysics; Endo had researched genetic engineering at Kyoto University’s Viral Research Center). Efforts of the cult to develop bio-weapons resulted in the following terrorists acts:
- April 1990, the Aum Shinrikyo outfitted three vehicles to disseminate botulinum toxin. Simultaneously were attacked the Japan’s parliament, the town of Yokohama and the Yokosuka naval base, the U.S. Navy’s most important facility in the east Pacific the area of the Narita International Airport, one of Japan’s most important airports.
- June 1993, Specially equipped automobile. spread botulinum toxin in downtown Tokyo in cult attempted to disrupt the planned wedding of Prince Naruhito, Japan’s Crown Prince;
- June 1993, Attempt to spread anthrax in Tokyo using a sprayer system on the roof of an Aum-owned building in east Tokyo.
- July 1993, Attempt to disseminate anthrax by using truck was made, in order to contaminate the area around the parliament in central Tokyo;
- July 1993, Another attempt to disseminate anthrax in the area around the Imperial Palace in Tokyo by truck-based system;
- March 15, 1995, Three briefcases designed to release botulinum toxin were planted in the Tokyo subway. The failure of this attack which led the cult to use sarin in its next attack was caused by the individual responsible for filling the botulinum toxin who had substituted toxin with a non-toxic substance.

All attacks failed because of the fact that Aum may have relied on a strain of C. botulinum that produced little or no toxin, and thus may have been incapable of producing the desired effects. The quantities of toxin disseminated may have been too small to cause lethal effects. Similar is the cause of failure of anthrax usage - Aum was using a vaccine strain of the organism, which was “relatively harmless.”

As far as is known, no one became ill or died from the Aum biological attempts.

In addition to botulinum toxin and anthrax, the Aum also was believed to have experimented with Q fever, spores from a poisonous mushroom and Ebola.

The definition of the threat is given as imminent and highly probable likelihood of danger. When describing the terrorists’ threat, we could define it as declared or existing intentions, capabilities and indicators for activities with set objective – community (population and goods) harm and destruction.

The open sources publication analyses give us an undoubting data proving the bioterrorist’s threat reality and imminence. Just only Al Qaeda (AQ) related evidence for bio-scientific activities for obtaining bio-weapons are listed in the article:

1. There were several declarations on AQ bio-weapon intentions, given by terrorists’ leaders Osama bin Laden, Abu Musab Zarqawi etc. As secretary general of
international policing organisation Interpol Ronald Noble (Nov 25, 2005) said AQ had "openly claimed the right to kill four million people" using biological and chemical weapons, and had posted instructions on how to make these weapons on its website. "In my view, al-Qaeda's global network, its proven capabilities, its deadly history, its desire to do the unthinkable and the evidence collected about its bioterrorist ambitions, ominously portend a clear and present danger of the highest order that al-Qaeda will perpetrate a biological terrorist attack." The threat of an al-Qaeda bioterrorism attack was a "clear and present danger of the highest order" and no region in the world was safe he concluded.

2. The late leader of AQ in Iraq, Abu Musab Zarqawi, was suspected of developing ricin in northern Iraq. Then-Secretary of State Colin L. Powell referred to the poison in his presentation to the U.N. Security Council in February 2003 that sought to lay the groundwork for the U.S. invasion of Iraq.

3. There are several facts about the terrorists’ capabilities to obtain bio-weapon:
The tremendous financial, material and human resources that AQ poses are beyond any doubt and would not be discussed in the article;
In the AQ there are a lot of specialists graduated from world famous universities in biological and biotechnological specialties. What concerns most the terrorism analysts is the AQ continuing process of acquiring more and more bio-specialist. For any pathogen to leave the realm of Mother Nature and enter the sinister realm of bio-weapon, it needs a microbiologist to weaponize it. For instance AQ operative Yazid Sufaat is the microbiologist who once oversaw AQ’s germ warfare programs, graduated from California State University at Sacramento — with a bachelor’s degree in biological sciences.
Another alarming evidence of this trend is the 2002 arrest in Paris, of Menad BenchelalI a terrorist specialized in poisons, who had produced small amounts of ricin and Botulinum, toxin that he intended to release in France.
Then in 2003, British authorities arrested seven individuals accused of also producing ricin. An AQ handbook with recipes for poisons’ production was confiscated in the operation.
But what terrified the word recently is the evidence of the continuing experiments for obtaining bio-weapons from terrorist’s camp in Algeria. A number of AQ militants in training have been killed by plague. At least 40 AQ followers have died since the disease swept through a training camp in Algeria, as was reported on Jan, 19 2009.
As it was stated above the threat assessment is function of possibility and consequences severity. These indicators are measured as follows in Table 2 and Table 3:
Table 2

<table>
<thead>
<tr>
<th>POSSIBILITY SCORE - PS</th>
<th>INDICATORS</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>1. Threat existence</td>
</tr>
<tr>
<td></td>
<td>2. Capabilities available</td>
</tr>
<tr>
<td></td>
<td>3. Intention Declared/ Indicators Available</td>
</tr>
<tr>
<td></td>
<td>4. Vulnerable objects is set as a target</td>
</tr>
<tr>
<td>3</td>
<td>1. Threat existence</td>
</tr>
<tr>
<td></td>
<td>2. Capabilities available</td>
</tr>
<tr>
<td></td>
<td>3. Intention Declared/ Indicators Available</td>
</tr>
<tr>
<td></td>
<td>4. No indicators that the object is set as a target</td>
</tr>
<tr>
<td>2</td>
<td>1. Threat existence</td>
</tr>
<tr>
<td></td>
<td>2. Capabilities available</td>
</tr>
<tr>
<td></td>
<td>3. No Intention Declared/ Indicators Available</td>
</tr>
<tr>
<td></td>
<td>4. No indicators that the object is set as a target</td>
</tr>
<tr>
<td>1</td>
<td>1. Threat existence</td>
</tr>
<tr>
<td></td>
<td>2. No Capabilities available</td>
</tr>
<tr>
<td></td>
<td>3. No Intention Declared/ Indicators Available</td>
</tr>
<tr>
<td></td>
<td>4. No indicators that the object is set as a target</td>
</tr>
</tbody>
</table>

Table 3

<table>
<thead>
<tr>
<th>SEVERITY SCORE - SS</th>
<th>INDICATORS</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Potential for: Population, military and emergency teams casualties (dead and wounded)</td>
</tr>
<tr>
<td></td>
<td>Infrastructure, Buildings’ Severe damage and Great Material loss</td>
</tr>
<tr>
<td></td>
<td>Environmental damage and serious economic impact (country scale)</td>
</tr>
<tr>
<td></td>
<td>Evacuation required</td>
</tr>
<tr>
<td>3</td>
<td>Potential for: Population, military and emergency teams casualties (wounded are prevailing)</td>
</tr>
<tr>
<td></td>
<td>Partial Infrastructure, Buildings’ damage and Moderate Material loss</td>
</tr>
<tr>
<td></td>
<td>Local impact on environment</td>
</tr>
<tr>
<td></td>
<td>Restriction in the area</td>
</tr>
<tr>
<td></td>
<td>Moderate economic impact</td>
</tr>
<tr>
<td>2</td>
<td>Potential for: Light wounding</td>
</tr>
<tr>
<td></td>
<td>Slight damage on infrastructure</td>
</tr>
<tr>
<td></td>
<td>Local restrictions</td>
</tr>
<tr>
<td></td>
<td>No environmental and economic impact</td>
</tr>
<tr>
<td>1</td>
<td>Few casualties</td>
</tr>
<tr>
<td></td>
<td>Slight surroundings damage</td>
</tr>
</tbody>
</table>
Overall bioterrorism threat is assessed as high because of:

1. Historical evidence for terrorist capability to obtain and use bio-weapons
2. Recently declared intention and willingness to produced, purchase and use bio-weapons
3. Moderate to high consequences severity for human and material goods of society, but with enormous and immeasurable psychological affect and social disturbance

Conclusion:

There is no doubt that terrorists are inclined to apply all scientific developments in achieving their objective to terrorise society for political implication. With their features bio-agents are ideal as a terrorists weapons undoubtedly proved by performed bioterrorism assessment. The high level bioterrorist’s threat has to alarm governments and international organization all over the world for the bioterrorists’ attacks eminence, what could be define as one of the security features of contemporary world.

References:

Chapter 39

Dual-Use Goods in the Production of Biological Weapons

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Abstract - Compared to the very complex steps needed to produce some Weapons of Mass Effect, biological weapons can be relatively easy to produce. They can be produced on a very small scale using laboratory equipment or on a mass production (industrial) scale. The technology and equipment can range from very simple to extremely complex and sophisticated. One of the difficulties in detecting and intercepting biological organisms and equipment needed to make biological WMD is the dual use aspect of biological commodities. The main goal of this paper is to help you better understand the dual nature use of these commodities and to give an awareness of the types of export-controlled, biological weapons-related equipment that proliferant countries or groups seek to obtain. We will discuss controlled materials and equipment, concentrating on what the items look like and how they might be packaged. We will also discuss Bio-weapons proliferation and the steps-including an overview of the material and equipment-involved in developing biological weapons. We will also review the international export control agreements, i.e. Australia Group and the Wassenaar Arrangement - and the LISTS of dual-use items these groups of nations have agreed to control.

Keywords - dual-use, biological weapons, control regimes

Introduction:

Biological agents are microscopic organisms (bacteria, viruses, fungi, rickettsiae) or their products (toxins) that can have drastic effects on humans, livestock and food crops. The difficulty in controlling these agents as they are shipped or carried across borders is that agents that are WMD concern look so much like legitimate microorgan-
isms that are vital in many industries. So the shipment and transfer of biological agents is not unusual or controlled unless the agents in question are controlled agents because of WMD concerns.

Thinking about biological agents, small is a key word – the agents themselves are either microscopic or produced by microscopic organisms as toxins. Also, very small quantities can do much harm. Biological weapons can be relatively easy to produce either on a very small scale using laboratory equipment or on a mass production (industrial) scale. The technology and equipment for the production can range from very simple to extremely complex and sophisticated. One of the difficulties in detecting and intercepting biological organisms and equipment needed to make WMD is the dual use aspect of biological commodities used for production and distribution of this weapons that can present legitimate equipment and processes for the manufacture and distribution of biological products.

In this paper we will discuss the steps of bio-weapons proliferation, including an overview of the material and equipment involved in developing biological weapons. We will also review the international export control agreements, i.e. Australia Group and the Wassenaar Arrangement including controls of potential bioweapons and dual-use facilities for its production.

The aim of the study

The main goal of this review is to help better understanding of the dual nature use of the commodities used in the production of biological weapons. One of the objectives is to give inspectors and enforcement officers an awareness of the types of export-controlled, biological weapons-related equipment that proliferant countries or groups seek to obtain that is essential to catch potentially controlled items during inspections.

Bio-weapons proliferation: main steps

Biological agents can be grown and produced very simply (low tech) or they can be produced in sophisticated and complex ways (high tech). Low technology methods would most likely be used by terrorists. Sophisticated methods of production would be used by a government intent on developing a biological weapons program. The high technology pathway is similar whether it is for pharmaceutical or agro-pesticide firms or for state-sponsored bio-weapons programs. The only difference between the two is the intent for the final product. The production processes and quantities are similar – so the commodities are dual use. Intercepting the purchase or transfer of the commodities needed for biological weapon production requires knowledge of the commodities.

The sophisticated production of BW include the following steps (figure 1): obtaining of the inoculums, growing of the organism in large quantities that requires fermentation or cell culture equipment, harvesting or concentrating of the agent or toxin – requires centrifuges or filtration equipment, stabilization of the product – involving freeze drying product or physical and/or chemical means to stabilize the agent, the weaponization, effectiveness testing and delivering methods. This production cycle involves potential exposure to harmful biological agents, and thus biosafety is an integral part of the process. Some commodities are controlled because of the safety protection they provide to workers producing biological weapons.
Because microorganisms are everywhere and because there are many legitimate reasons to grow microorganisms, it is very difficult to recognize and control agents that are being obtained and cultured for WMD purposes. The inoculums can be obtained from culture collections, soil and water, disease vectors, e.g. an insect or a viral vector, infected human or animal carriers, or from natural reservoirs.

The proliferators must obtain a pathogen and culture it, isolate the specific pathogen they are trying to weaponize and make more of it until there is enough to place it in a bio-reactor. The purification, growth, and testing phase generally require small volumes and no specialized equipment.

After obtaining inoculums, standard microbiological methods are used to purify and grow a culture used for WMD. First steps are laboratory/bench scale operations. Small volumes of the culture are grown and tested in cultivating (solid or liquid) media.

Cultures could be genetically modified to increase hardness and antibiotic resistance that would require specialized expertise and commercial equipment.

Agents or toxins can be multiplied through small-scale as it was previously described or in industrial-scale processes using fermenters or bio-reactors with controlled environmental factors (temperature, pressure, oxygen level, pH value).

Biological weapon are enhanced (more effective) when agent is concentrated after growth. Equipment used to concentrate the target agent or toxin includes equipment capable of separating solids from liquids and/or capable of separating liquids by density using different centrifuges or cross-flow systems.

After concentrating, the microbe or toxin must be stabilized by one of several methods in order to maintain the viability and virulence of the agent reducing its vulnerability to environmental degradation, not only in storage but also in application. These include the use of freeze or spray dryers and formulations into a special stabilized solid, liquid or sometimes gaseous form.

The last processes in BW production include weaponization, testing and development of effective delivering methods. The weaponization process include freeze drying in order to enhance stability and remove all of the moisture for further processing, micro encapsulation using commercially available biomaterials to provide more stability to the agent or toxin and to produce a particle capable of being aerosolized and extenders to prevent clumping of particles.

The testing of BW effectiveness can be performed using animal and dispersion models. Animal models include the use of aerosol inhalation chambers or open air-testing as it was done on animals isolated on Vozrozhdeniye Island in the Aral Sea in the former Soviet Union. Dispersion testing models include the use of surrogates (substitutes for real agents or toxins) released over a contained or open air grid and the use of detection equipment.

Delivery system, either sophisticated or simple, can be used to get the agent to its intended victims. Best delivery systems aerosolize the agent so that victims breath in the agent. But other means of spreading WMD would be effective as the use of special munitions for agents such that they don’t destroy the agents in firing, the use of sprayers or vectors (animate-insects, people or inanimate – the letter that delivered anthrax in US in 2001).

Packaging and shipping of biological materials
A biological agent can be interdicted through knowing that the shipper or the receiver are suspect, through documentation that doesn’t jive with the contents or appearance of a container, through suspect material - leaking powder or liquid, if end-user of package doesn’t “make sense” – example - biologicals going to car manufacturer – it may cause you to give package more scrutiny.

Biological materials include Category A infectious substances and Category B Biological Substances. There are specific requirements for the packaging and labeling of biological materials. Proper shipping papers, “Shippers Declaration for Dangerous Goods”, must be completed for shipment of infectious substances, but not for Category B Biological Substances. The shipper is responsible for the safe transport of goods for legal export and for understanding and observing these regulations that govern dangerous materials.

Category B-biologicals that are not considered to be Infectious agents must be labeled with UN/ID number– UN3373. The UN Number for Infectious Substances may be UN 2814 or UN 2900, as appropriate. Assignment to UN 2814 or UN 2900 must be based on the known medical history or symptoms of the source patient or animal, endemic local conditions, or professional judgment concerning the individual circumstances of the source human or animal.

Otherwise, all hazardous materials are classified into 9 categories of dangerous goods. Infectious substances belong to Class 6, Division 6.2. So all packages containing Infectious Substances should be labeled as Infectious substance and should have a Class 6 label.

The shipping of any biological materials should meet the following requirements: plastic inner bottle made of teflon, plastic, glass or corrosion resistant steels, secondary containment (heavy plastic bag) around bottle, adsorbent material and double walled cardboard box to resist breakage. Point out these shipping containers may be OK for shipping diagnostics or biological products – but that shipping infectious substances have different very specific requirements.

Any reliable transportation with safety features and proper materials of construction can be used for transport of hazardous biological materials i.e. ship, rail, truck, car, air, carrier (human/animal/plant). Smugglers (terrorists) don’t always follow the rules but it is supposed they will take precautions to make sure their shipment gets through. “Following the rules” in terms of shipping infectious biological agents would involve packaging, labeling, documenting according to information already presented. Shipping potentially infectious agents as diagnostic biologicals would be a way to get infectious agents to their proper locations without drawing undue attention to the package. The person investigating the package would find it almost impossible to figure out that the agent was NOT a diagnostic biological but a true infectious agent of WMD concern without help of microbiologists. Anyway, if just because a package of biologicals lacks the infectious substance labels, don’t assume that it is not infectious.

If a suspicious package is encountered, check the documentation. Does the product have identification? A properly shipped biohazard must have identification of the contents on the package and a contact number for the person who shipped it. But do the general contents of the package match the identification on the package? If it does not have the documentation declaring it an infectious agent – be cautious – treat it as if it were a known infectious agent. If package containing suspected biohazard is com-

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promised in any way, the package should be isolated and not touched. Only persons with appropriate personal protective equipment should handle or move the package.

Export control regimes:

In response to proliferation developments, the nonproliferation regime has developed guidelines and control lists. The control lists of the multilateral export control regimes cover an enormous range of dual-use materials, components, and equipment used in WMD programs. Member and adherent nations implement the multilateral regime controls through their own national legislation and regulations.

The Australia Group (AG) lists the biological agents by their target i.e. humans, animals, and plants and by type of agent i.e. bacteria, rickettsia, viruses, fungi, and toxins. Many of the agents that are targeted at humans are zoonotic diseases; they cause diseases in animals as well as man. However, as bioterrorism agent the primary concern is the risk to humans. Control list include 32 viruses, 4 rickettsiae, 15 bacteria, 19 toxins (human pathogens), 17 viruses and 1 bacteria (animal pathogens), 5 bacteria, 6 fungi and 2 viruses (plant pathogens). AG list also include dual use biological equipment. The list is permanently updated and can be find on the website www.australiagroup.net

Wassenaar Arrangement Control List includes conventional arms and dual-use goods and technology including biological weapons production facilities.

The EU has developed a “harmonized” list that incorporates all controls for dual-use items and technology in a single document. Many countries around the world have adopted the EU list structure as a model for their own national lists. The US has adopted the basic structure of the EU list for its own Commerce Control List (CCL).

Conclusions:

After this short story it is completely clear that if someone is interested in producing biological agents on a large scale, what would they need in their facility and what commodities would they begin to collect. So that controlling access to these commodities will help prevent proliferation of biological weapons. An improperly shipped agent could pose a threat and could be a cause for concern. It is necessary to understand the appropriate ways to package and ship any biological agent. Without enforcement, all of the international and national efforts at export controls are for nothing.

Export control laws must be effectively enforced- that means inspection for illegal shipments and diversion. The effectiveness of national export controls in preventing proliferation depends on detecting, deterring and interdicting illegal shipments and diversions of controlled commodities

References
1. Handbook of the export control regimes in the arms, military equipment and dual-use goods. Serbian Ministry of economy and regional development, Belgrade, 2007

Figure 1. Biological Processing and Weapons Development

Figure 2. Infectious Substances – proper shipping
Chapter 40

Transborder Cooperation on the Protection, Surveillance and Control of Endemic Diseases

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2 Association “Social Health”, Sofia Bulgaria
3 Ministry of Health, Sofia, Bulgaria

Abstract - This paper discuss some concern and challenges regards the Bulgarian-Greece transborder cooperation with respect the protection, surveillance and control of some endemic for this transborder region diseases like: Q-fever, Brucellosis, Lyme disease, Crimean-Congo hemorrhagic fever and Marseilles fever. The study examines transborder activities, including a background for the infection diseases state for the period 2004-2007, the problems of training and equipment of the specialists for sampling and identification of these diseases, development of strategy and conception for control of spreading of the infectious agents in 4 bulgarian regions / Blagoevgrad, Haskovo, Smoljan and Kardjeli/ and in the corresponding regions in Greece – Seres, Drama, Ksanti and Evro. Additionally, there is presented the role of local governmental representatives to manage these transnational border issues.

Keywords - transborder cooperation, endemic infectious diseases, infectious control and surveillance

Introduction

The analysis of the infectious morbidity in Bulgaria shows a tendency of it’s increasing, which is very important from medical and social point of view. The large cultural and economical connections between Bulgaria and the countries in Asia, Africa, South and Central America as the process of migration of Bulgarian and foreign citizens, create the conditions also for importation of different infectious diseases. All these events as well as the existing of optimal complex of biotic and abiotic factors for their distribution, determinate the risk of origin and spread of local endemic outbreaks, caused by

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important for infectious pathology antroponoses and zoo antroponoses in this local region. / 1 / Because of increasing of endemic areas with the presented infections in Balkan region /Bulgaria, Greece, Romania, Serbia/ as well as the possibility to use these agents as a tool for bioterrorism, it is necessary to establish strong strategy for surveillance and epidemic control of these infections, especially in transborder region between Bulgaria and Greece in the frame of European Community / 4 /.

The aim of the study is to investigate the opportunities for the protection, surveillance and control of five infectious diseases / Lyme disease, Q-fever, Marseilles fever, Crimean-Congo hemorrhagic fever, Brucellosis /, endemic for transborder with Greece region, consisting of four districts – Blagoevgrad, Khaskovo, Smoljan and Kurdzhal.

Background

The retrospective study of infectious morbidity, concerning the infections mentioned above for 2004-2007 period of time show that the most relative part takes Marcelles fever with 50.0%, followed by Lyme borreliosis 41.3% and Brucellosis – 6.7% / Table 1/. The same data, collected according to the district location represent that this problem is more important for Khaskovo district 68.4%, Blagoevgrad 20.7% and Kurdzhal – 9.9% / Fig. 1 /.

Main points of the realization of the study

Establishment of system, promising the uncovering of new threats, demanding the introduction of new approaches, which are accepted to be sure and effective for early diagnostics of patients with infectious diseases, including monitoring of symptoms, death cases, hospital admission and drugs prescription /antibiotic stewardship/. The symptoms are very important point for the characterization of the infections investigated and according to the literature / 2 / the more important from themselves include cough, vomiting, fever, rash, double vision, haemorrhages. Very important moment in this respect is also the information, available for any contacts with animals, information for tick bite and kind of profession. By the way of surveillance, ranging over of number of general practice /GP/ doctors, which can send the information to Public health services or by the “hot” phone, operated by the nurse. In both cases, the information will be analyzed for the specific symptoms confirmation as indicators for risk estimation of possible outbreak beginning.

The estimation of laboratory capacity and harmonization of standards for laboratory diagnostics are important issues, necessary for improvement of this type of diagnostics in Greece and Bulgaria in future time. / 3, 5 / At the moment Public health laboratories in Bulgaria do not have enough capacity /staff and equipment/ for this type of investigation, but according to the standard microbiology procedures, the confirmation of the majority of these infections must do in the reference laboratories. / 5 / The lack of specific programs for post doc education /GP doctors/ and appropriate software restricts the opportunities for early detection of a such of epidemic outbreaks. It is also necessary to promote harmonization of standards for laboratory diagnostics and epidemiological investigation of the infections between Bulgarian and Greek institutions according to the international requirements. In this connection the development of
programs for laboratory diagnostics education, exchange of experts, realization of seminars and courses are very important forms of education' improvement. Additionaly, materials for health promotion and society information with respect of these special infectious diseases by electronic media, newspapers, other printed materials /newssheets, pictures/ and all these materials are putted together in Internet Website: www.infozdrave.net.

Conclusion

In the frame of the project, it was established for first time the model of transborder cooperation between Bulgarian and Greek health institutions. Also, were educated in different courses and seminars 610 specialists from Public Health Services and GP doctors, which is very important issue with respect to the protection, surveillance and control of the endemic diseases studied

Funding. This study was realized on the base of the Project BG 2005/17-454.03.01 supported by European Community, Programme FAR, 2008

References

Table 1.

Table 1. Retrospective study of infectious morbidity in Blagoevgrad, Khashovo, Smoljan and Kurdzhal district 2004-2007

<table>
<thead>
<tr>
<th>Type of infection</th>
<th>Number of cases</th>
<th>Total</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2004</td>
<td>2005</td>
<td>2006</td>
</tr>
<tr>
<td>Lyme borreliosis</td>
<td>96</td>
<td>100</td>
<td>51</td>
</tr>
<tr>
<td>Q-fever</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Marseilles fever</td>
<td>160</td>
<td>101</td>
<td>65</td>
</tr>
<tr>
<td>Crimean-congo hemorrhagic fever</td>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Brucellosis</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>262</td>
<td>204</td>
<td>122</td>
</tr>
</tbody>
</table>

Fig. 1. Infectious morbidity according to the district
Chapter 41

Threats and Counteractions Concerning the Use of Viruses for Biological Terrorism

Zlatko KALVATCHEV
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Abstract - Different viral agents are classified as potential weapons for bioterrorism. Although their massive distribution is difficult, there is still an appreciable threat present as most people are susceptible to those agents, while there are limited or none at all possibilities for vaccination or specific treatment. The clinical course of these particularly dangerous viral infections in early stages is outlined with nonspecific symptoms making the diagnosis difficult or sometime even impossible to define. Therefore, in the case of an act of bioterrorism, quick and exact diagnoses of the first emerging cases are crucial for the subsequent urgent prophylactic measures, in order to prevent the outbreak of infection and to administer correct medication. Considering the lack of effective drugs and vaccines against most of these viruses, counteraction could be achieved only through pedantic execution of the measures for control of infectious diseases. An important part of the control measures are the existing systems for prompt warning of emerging infectious diseases, including these concerning acts of bioterrorism (ProMED-mail and other). The following material presents actual information and discussion about the possible menace to the use of viruses as agents for biological terrorism and the measure that should be taken.

Keywords - very dangerous viral infections, bioterrorism

In the 21st century we are likely to continue to see the emergence of new pathogenic viruses (SARS-CoV, Avian and Swine influenza viruses) and the spread of existing viruses to new demographic areas and hosts (West Nile virus, Chikungunya virus). The possibility of massive epidemic outbreaks due to bioterrorism also exists. In spite of these potential events, the public health services in the different countries have been in a gradual decline during the last decades [1]. The rebuilding of the public health service and a new coordination of the medical infrastructure together with the antibioterrorism initiatives discussed in this review will strengthen our ability to respond with vigor and efficiency against an attack or an introduced epidemic.

According to the Centers for Disease Control and Prevention (CDC), the bioterrorism is the deliberate release of viruses, bacteria, or other germs (agents) used
to cause illness or death in people, animals, or plants [2]. These agents are typically found in nature, but it is possible that they could be changed to increase their ability to cause disease, make them resistant to current medicines, or to increase their ability to be spread into the environment. Biological agents can be spread through the air, through water, or in food. Terrorists may use biological agents because they can be extremely difficult to detect and do not cause illness for several hours to several days. Some bioterrorism agents, like the Smallpox virus, can be spread from person to person and some, like Dengue virus, cannot.

A diverse group of viruses are capable of causing viral hemorrhagic fever or encephalitis syndromes [3, 4, 5, 6]. These include RNA viruses that are members of the Filoviridae (Ebola and Marburg viruses), Arenaviridae (Lassa fever, Junin, Machupo and Guanarito viruses), Bunyaviridae (Hantavirus, Rift Valley fever, and Congo-Crimean hemorrhagic fever viruses), Flaviviridae (Yellow fever, Omsk Virus and Dengue fever viruses), and Togaviridae (Venezuelan, Eastern and Western Equine Encephalitis Viruses). Humans are exposed to these agents by contact with infected animals or via arthropod vectors. The infections caused by this group of viral agents are characterized by vascular damage and altered vascular permeability. Symptoms commonly include fever and myalgias, prostration, hemorrhages in mucous membranes, and shock. Viral hemorrhagic fevers or encephalitis cause high morbidity, and in many cases high mortality rates are observed. Treatment consists mostly of supportive measures, although the antiviral agent ribavirin seems to be useful for treatment of infection with certain agents such as Lassa fever virus, Junin, Bolivian, and Congo-Crimean hemorrhagic fever viruses, and Rift Valley fever virus [6]. Only a handful of reference laboratories (usually BSL 3 or 4 facilities) are equipped to diagnose agents of viral hemorrhagic fevers by culture or nonculture techniques, which include serological, immunohistological, and nucleic acid amplification methods. Contact precautions should be observed for patients suspected for viral hemorrhagic fever or encephalitis, and specimens obtained from such cases should be handled with care. Table 1 summarizes the viruses with potential to be used for biological terrorism.

The CDC has defined and categorized bioterrorism agents according to priority as follows [4, 6]:

**Category A** (high-priority agents) include organisms that pose a risk to national security because they: (a) can be easily disseminated or transmitted from person to person; (b) result in high mortality rates and have the potential for major public health impact; (c) might cause public panic and social disruption; and (d) require special action for public health preparedness. These are biological agents with both a high potential for adverse public health impact and that also have a serious potential for large-scale dissemination. Many of these agents require Biosafety Level 3 and 4 laboratories. The category A viruses are smallpox virus (Poxviridae) and the Filoviridae (genera Marburg and Ebola). Ebola has fatality rates ranging from 50-90%. No cure currently exists, although vaccines are in development. Ebola virus kills its victims through multiple organ failure and hypovolemic shock. Marburg virus was first discovered in Marburg, Germany. No treatments currently exist aside from supportive care.
Table 1. Viruses with a potential to be used for biological terrorism

<table>
<thead>
<tr>
<th>Viral family</th>
<th>Virus</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arenaviridae</td>
<td>Junin-, Machupó-, Guanarito- Virus</td>
<td>Hemorrhagic fever, Encephalitis</td>
</tr>
<tr>
<td></td>
<td>Lassa Virus</td>
<td>Hemorrhagic fever, Encephalitis</td>
</tr>
<tr>
<td>Bunyaviridae</td>
<td>Crimean Congo Virus</td>
<td>Hemorrhagic fever, Encephalitis</td>
</tr>
<tr>
<td></td>
<td>Sin Nombre Virus</td>
<td>Hantavirus pulmonary syndrome</td>
</tr>
<tr>
<td></td>
<td>Hantaanvirus, Puumula Virus</td>
<td>Hantavirus renal syndrome</td>
</tr>
<tr>
<td></td>
<td>Rift Valley Virus</td>
<td>Rift valley fever</td>
</tr>
<tr>
<td>Coronaviridae</td>
<td>Corona Virus</td>
<td>Severe Acute Respiratory Syndrome</td>
</tr>
<tr>
<td>Filoviridae</td>
<td>Ebola Virus</td>
<td>Hemorrhagic fever, Ebola</td>
</tr>
<tr>
<td></td>
<td>Marburg Virus</td>
<td>Hemorrhagic fever, Marburg</td>
</tr>
<tr>
<td>Flaviridae</td>
<td><strong>Tick-borne viruses</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kyasanur Forest Virus</td>
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<td>Omsk Virus</td>
<td>Omsk hemorrhagic fever</td>
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<td>Louping ill-, Tick borne encephalitis virus</td>
<td>Tick-borne encephalitis</td>
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<td><strong>Mosquito-borne viruses</strong></td>
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<td>Dengue Virus</td>
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<td>West Nile Virus</td>
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<td>Yellow fever Virus</td>
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<td>Eastern Equine Encephalitis Virus (EEEV)</td>
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<td>Influenza Virus</td>
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<td>Nipah Virus</td>
<td>Influenza like disease, Encephalitis</td>
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<td>Poxviridae</td>
<td>Smallpox Virus</td>
<td>Variola major, Variola minor</td>
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<td>Monkeypox Virus</td>
<td>Monkeypox Variola</td>
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**Category B agents** are moderately easy to disseminate and have low mortality rates. This group includes viruses that (a) are moderately easy to disseminate; (b) result in moderate morbidity rates and low mortality rates; and (c) require specific enhancements of laboratory diagnostic capacity and enhanced disease surveillance. The category B viruses are Venezuelan Equine Encephalitis Virus (VEEV), Eastern Equine Encephalitis Virus (EEEV) and Western Equine Encephalitis Virus (WEEV).

**Category C agents** include re-emerging viruses that could be engineered for mass dissemination in the future because of (a) availability; (b) ease of production and dissemination; and (c) potential for high morbidity and mortality rates and major health impact (examples: Nipah virus, Hantavirus, Influenza, etc.).

**Laboratory (virology) diagnosis in case of bioterrorism attack**

Virology laboratories play a key role in the detection and identification of biological agents likely to be used in bioterrorist events [5, 6]. In the immediate aftermath of a covert attack, specimens containing critical biological agents might be submitted to the laboratory for routine processing before the presence of the agent was even suspected.

The CDC has developed a plan for bioterrorism preparedness for clinical microbiology (virology) laboratories with various capabilities. According to this plan, laboratories are classified into one of four levels depending on their testing facilities and abilities. Level A laboratories are represented by the majority of clinical microbiology laboratories that culture and identify routinely isolated pathogens. These laboratories would perform a small number of simple rule-out tests on suspected isolates and, depending on test results, refer those organisms to a higher-level laboratory for further testing. Thus, the role of the level A laboratory is summarized as rule out or refer. Level B laboratories are represented by many public health laboratories and should contain biosafety level (BSL) 3 facilities. Level B laboratory activities include tests for rapid presumptive identification (e.g., with fluorescent antibody reagents) and confirmatory identification. Critical biological agents would be referred from level B laboratories (rule in and refer) to level C facilities (BSL 3), which have the capacity for nucleic acid amplification testing, molecular typing, and toxin testing. Level C laboratories (rule in and refer) would include certain public health and other laboratories that can perform strain-typing procedures. Critical biological agents would finally be referred to level D laboratories, which are BSL 4 facilities (maximum containment “hot labs,” like the facility at the CDC). The role of these laboratories is archiving critical biological agents and the performance of other specialized tests, such as culture or molecular identification of highly dangerous viral agents that require BSL 4 facilities.

Most of Bulgarian virological laboratories are not adequately equipped for placing the rapid diagnosis of any of the particularly dangerous viruses, therefore suspicious samples must be packed appropriately (fig. 1) and transported for examination in the specialized laboratories of the National center of Infectious and Parasitic Diseases and/or Military Medical Academy, where the diagnosis can be put or confirmed [6].
All suspected cases should immediately be reported to the public health institutions, which in turn inform the World Health Organization (WHO) and European CDC (ECDC). WHO experts have developed standards for monitoring of acute haemorrhagic syndromes for the purpose of early detection and rapid identification of natural outbreaks, and to mark the case even before the identification of the agent. Besides the immediate reporting of suspicious cases, a patient with suspicious continued fever comes under medical supervision if at least 2 of the symptoms characteristic of viral haemorrhagic fever: purple rash, epistaxis, hematemeza, presence of blood in the urine and/or faeces.

Viruses cause haemorrhagic fever and encephalitis are highly infectious during laboratory work and possible transfer to the laboratory personnel in air-drip time. Risks are associated in particular with aerosol-generating procedures (work with centrifuges). To minimize the possibility of an intra-laboratory contamination, all laboratory staff should be warned of the possibility of diagnosis "particularly dangerous viral infection". Laboratory workers should be well trained for the activities related to suspect patients, storage and processing of the samples. All procedures are conducted in laboratory safety cabinets (Fig. 2).
Laboratory staff must provide reliable protection, and all diagnostic procedures to be carried out under Biosafety level of the 3rd level and virus isolation - on the 4th level (fig. 3).

**Figure 2.** Class III Biological Safety Cabinet. A = stand, B = glove ports, C = O-ring for attaching arm-length gloves to cabinet, D = sash, E = supply HEPA filter, F = exhaust HEPA filter (note the second exhaust HEPA filter required for Class III cabinets is not shown in this diagram), G = double-ended autoclave or pass-through box.

**Figure 3.** Overview of virology laboratory with a level of protection from 3rd grade (BSL3): a) Working conditions in the laboratory; b) Staff equipment.

**Universal precaution**

The universal precautions are derived from prudent laboratory and microbiological (virological) practices and common sense and include the following (in no
particular order or priority): (a) Hands should be washed prior to leaving the facility; (b) Employees should not shear off, break, bend, recap, or remove contaminated needles or other sharps for disposal. (Sharps are defined as any sharp object that can puncture or lacerate the skin. This includes hypodermic needles, razor blades, scalpel blades, and Pasteur pipettes, both intact and broken. These materials must be placed in an approved sharps container); (c) The container for storage must be labeled with the universal biohazard symbol and the words "Infectious waste", or "Biohazard waste"; (d) Eating, drinking, smoking, and applying cosmetics are prohibited in work areas where there is reasonable likelihood of an occupational exposure; (e) Food and drink must not be kept in refrigerators, freezers, shelves, cabinets, or on countertops where human blood is present; (f) All procedures involving human blood must be performed in such a manner as to minimize splashing, spraying, spattering, and generation of aerosols; (g) Mouth pipetting or mouth suctioning of human blood, or other biological materials, is prohibited; (h) Human blood specimens must be stored, transported, or shipped in a solid container that will not allow the contents to leak out; (i) The container for storage, transport, or shipping must be labeled with the universal biohazard symbol as a minimum. If the outside of the primary container becomes contaminated, the primary container must be placed inside a second suitable uncontaminated container; (j) Human blood specimens for disposal must be placed in an approved alternative container.

When there is potential for occupational exposure, the Principal Investigator shall provide appropriate personal protective equipment (PPE) such as gloves, gowns, laboratory coats, face shields or masks, eye protection, or other safety devices. PPE must be used when necessary. PPE must not permit human blood to pass through to the employee's work or street clothes, undergarments, skin, eyes, mouth, or other mucous membranes.

The department must clean, launder, and dispose of PPE at no cost to the employee. If a garment is penetrated by human blood, the garment must be removed as soon as possible. All PPE must be removed and placed in the appropriate container prior to leaving the work area. Gloves must be worn when the employee may have hand contact with human blood. Disposable gloves must be replaced immediately if torn or punctured. Disposable gloves must be discard after use; not washed or decontaminated.

Contaminated laundry shall be handled as little as possible with a minimum of agitation. Contaminated laundry must be placed in a container at the location where it was used and not sorted or rinsed on location. Contaminated laundry must be placed in red bags or bags labeled with the biohazard symbol. Medical waste must be handled in a manner to protect employees and the environment. Masks, eye protection, and face shields shall be worn whenever splashes, spray, spatter, or droplets of human blood may be generated and eye, nose, or mouth contamination is anticipated. Surgical caps or hoods and/or shoe covers or boots shall be worn when gross contamination can reasonably be anticipated.

The employee will ensure that the worksite is maintained in a clean and sanitary condition. There must be a written schedule for cleaning and method of decontamination. All equipment and environmental and working surfaces shall be cleaned and decontaminated after contact with human blood. All contaminated work surfaces shall be decontaminated with a virucidal disinfectant (such as a 10% solution
of household bleach) after completion of procedures, as soon as possible after any spill of human blood, and at conclusion of experiment, if the surface may have become contaminated.

Plastic wrap, aluminum foil, or other protective coverings used to cover equipment and other surfaces, should be removed and replaced as soon as possible if contaminated. All bins, pails, cans, and similar receptacles intended for reuse which may be contaminated, should be inspected and decontaminated on a regular cleaning schedule and when visibly contaminated. Broken glassware which may be contaminated shall not be picked up directly by hand but by mechanical means such as brush and dust pan, tongs, or forceps. Reusable sharps that are contaminated with human blood shall not be stored or processed in a manner that requires employees to reach by hand into containers where these sharps have been placed. All medical waste must be segregated from other solid wastes at the point of origin. At no time may sharps be discarded in the trash. Medical wastes may not be stored longer than seven days without sterilization. Red bags or red containers must be used for all contaminated waste.

**Post-Exposure Follow-up**

Post-exposure follow-up is available to all employees who have had an exposure incident [6]. Employees exposed to human blood or other infectious body fluids will be provided serologic testing, post-exposure prophylaxis as appropriate, and counseling. The post-exposure follow up is maintained in confidential medical records separate from personnel records.

Use of Ribavirin is not recommended for individuals with proven or suspicious contact with haemorrhagic fever viruses, but expressed no symptoms of the disease. The Ribavirin has no effect on Filoviruses and/or Flaviviruses. In case of the Arenaviruses, post-exposure prophylactic with ribavirin may delay the beginning of the disease but not prevent it. In addition, CDC recommended the use of Remantadin for post-exposure prophylactic in cases of Lasa fever.

The recommended by CDC licensed vaccines are: live attenuated vaccine (17D) against yellow fever virus, and the vaccine against Crimean-Congo Haemorrhagic Fever (CCHF), which is produced only in Bulgaria (NCIPD-Bulbio LTD). CCHF vaccine represents a suspension for subcutaneous injection containing inactivated virus-specific antigen with an excellent tolerability and immunogenicity. Administered immediately after infection, these vaccines can not prevent disease due to short incubation period of haemorrhagic fever (from 1 to 6 days) and time of two weeks for the formation of neutralizing antibodies after vaccination, but can result in mild course of infection.

**ProMED-mail: A program for monitoring and early warning of emerging and re-emerging infections**

ProMED-mail is an Internet-based reporting system dedicated to rapid global dissemination of information on outbreaks of infectious diseases and acute exposures to toxins that affect human health [6]. Electronic communications enable ProMED-mail to provide up-to-date and reliable news about threats to human, animal, and food plant
health around the world, seven days a week. By providing early warning of outbreaks of emerging and re-emerging diseases, public health precautions at all levels can be taken in a timely manner to prevent epidemic transmission and to save lives.

ProMED-mail is open to all sources and free of political constraints. Sources of information include media reports, official reports, online summaries, local observers, and others. Reports are often contributed by ProMED-mail subscribers. A team of expert human, plant, and animal disease moderators screen, review, and investigate reports before posting to the network. Reports are distributed by email to direct subscribers and posted immediately on the ProMED-mail web site. ProMED-mail currently reaches over 40,000 subscribers in at least 185 countries.

ProMED-mail was established in 1994 with the support of the Federation of American Scientists and SatelLife. Since October 1999, ProMED-mail has operated as an official program of the International Society for Infectious Diseases, a nonprofit professional organization with 20,000 members worldwide. A central purpose of ProMED-mail is to promote communication amongst the international infectious disease community, including scientists, physicians, epidemiologists, public health professionals, and others interested in infectious diseases on a global scale. ProMED-mail encourages subscribers to participate in discussions on infectious disease concerns, to respond to requests for information, and to collaborate together in outbreak investigations and prevention efforts. ProMED-mail also welcomes the participation of interested persons outside of the health and biomedical professions.

**European Centre of Disease Prevention and Control (ECDC)**

The ECDC was established in 2005. It is an EU agency with aim to strengthen Europe’s defenses against infectious diseases. In order to achieve this mission, ECDC works in partnership with national health protection bodies across Europe to strengthen and develop continent-wide disease surveillance and early warning systems. By working with experts throughout Europe, ECDC pools Europe's health knowledge, so as to develop authoritative scientific opinions about the risks posed by current and emerging infectious diseases.

Within the field of its mission, the Centre shall: (a) search for, collect, collate, evaluate and disseminate relevant scientific and technical data; (b) provide scientific opinions and scientific and technical assistance including training; (c) provide timely information to the Commission, the Member States, Community agencies and international organisations active within the field of public health; (d) coordinate the European networking of bodies operating in the fields within the Centres mission, including networks arising from public health activities supported by the Commission and operating the dedicated surveillance networks; and (e) exchange information, expertise and best practices, and facilitate the development and implementation of joint actions.

**Conclusions**

At this point in the discussion of the complex issue of bioterrorism, we are in a position to raise more questions than answers. However, our intent is to contribute to a fruitful dialogue which will stimulate the process to generate answers that will be the building blocks for our preparedness and the nation's defense against the threat of
bioterrorism. The threat of bioterrorism is real and looming before us. The involvement of virologists and other health care professionals in preparing for a bioterrorist act should extend beyond the institution of protocols and plans to be followed in the wake of such an event. To be prepared in a responsive and responsible way, we need a paradigm of scenario planning in which we look at a range of possibilities and countermeasures rather than construct a linear and reactive strategic plan. This demands that, in our collective imagination, we move the previously unthinkable into the realm of possibility in order to develop a realistic response strategy. We will have to integrate many conflicting issues and satisfy conflicting needs through compromises that seek to find the second-best solutions. This will still be smarter than having no solutions at all. The answer is to reconsider the present rather than prophesy the future based on vague assumptions. The time has come to get prepared and develop an integrated policy.

References:

Chapter 42

Generation of Monoclonal Antibody for Real-time Detection of Chemical and Biological Agents by an Optical Biosensor

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Introduction and purpose

Because a time of exposure is critical for efficiency of medical treatment, the development of methods for fast detection of biological (BA) and chemical (CA) agents is very important. Over the last decade, different antibody based approaches have been described for BA and CA analysis, including immunosensors. For sensitive and specificity of BA/CA detection by immunosensors appropriate antibodies need to produce. The purpose of the present work was production of monoclonal antibodies (MAb), which would detect with high specificity BA (*B. anthracis* spores of vaccine strain as a model) and CA (insecticide pyrethroid as a model) and would serve as biological recognition elements for fast detection in real time of anthrax spores and pyrethroids on a biosensor surface.

To develop of test-system for rapid detection and identification of *B. anthracis* spores the antigens on the spore surface should be characterized. Exosporium consists of two layers-basal and peripheral and has been form by protein, amino- and neutral polysaccharides, lipids and ash. Number of anthrax exosporium proteins was described and identified: glycoprotein BclA, BclB, alanine racemase, inosine hydrolase, glycosyl hydrolase, superoxid dismutase, ExsF, ExsY, ExsK, CotB, CotY and SoaA [1- 4]. The first target-glycoprotein antigen from *B. anthracis* exosporium with molecular weight 95 kDa (SA) was purified. So far no glycosilated proteins other then highly immunogenic glycoprotein’s BclA, BclB were detected in the *B. anthracis* spore extract although several exosporium-specific glycoprotein have been described in other mem-
bers of the *B. cereus* family- *B. thuringiensis* and *B. cereus* (in contrast with 250 kDa of BclA and 205 kDa of BclB antigens from Stern strain) [5, 6]. Use of SA for immunization has allowed to inducing high-affinity polyclonal antibodies in rabbits. The antibodies reacted specifically with the spores of various *B. anthracis* strains and not reacted with vegetative cells of *B. anthracis* and with the spores of other representatives of *Bacillus* [7]. Similar specific antibodies were obtained in the experiments when conjugate KLH with species specific synthetic sugar-anthrose was used as an immunogen [8].

Over the last decade, different antibody based approaches have been described for BA and CA analysis, including immunosensors [9 - 12]. For sensitive and specificity of BA/CA detection by immunosensors appropriate antibodies need to produce. The purpose of the present work was production of monoclonal antibodies (MAb), which would detect with high specificity BA (*B. anthracis* spores of vaccine strain as a model) and CA (insecticide pyrethroid as a model) and would serve as biological recognition elements for fast detection in real time of anthrax spores and pyrethroids on a biosensor surface. Several biosensors have been developed based on the surface plasmon resonance technique (SPR). Lim et al. [13] described a method for atrazine analysis based on the SPR determination of P450 mRNA levels in Saccharomyces cerevisiae, while Nakamura et al. [14] reported the specific detection of atrazine using a self-assembled photosynthetic reaction centre of Rhodobacter sphaeroides on an SPR chip. Furthermore, Shimomura et al. [15] developed a method using SPR based on a competitive immunoassay that gave a limit of detection of 5 μg/L.

SPR has become a well-established tool for the characterization of biorecognition systems for the detection of pathogens [16- 19] and pathogen products, including *Escherichia coli* enterotoxin [20]; virulent *L. monocytogenes* [21]; *E.coli* O157:H7 [22] and [23]; *Salmonella* [24] and biological warfare agents [25]. SPR is rapid and allows the direct, label-free detection of target species.

1. Materials and methods

Bacterial strains and cultivation.

The bacilli of *B. anthracis* (vaccine strain STI-1) and others species of *Bacillus* genera were cultivated at 36 °C for 7 days on a beef-extract agar. The quality of the formed spores was spotted under a phase contrast microscopy. The spores were harvested from plates, washed by centrifugation and suspended in PBS to a concentration of 10⁹ spores/ml.

Analytical procedures.

SDS-PAGE electrophoresis experiments were performed by the method of Laemmli. Western- and dot-blotting performed according to the method Towbin and Gordon. Preparation of antigen.

Antigen from spores was extracted by 1-5% SDS at 90 °C within 5 minutes. The obtained extract was purified by gel filtration on a column with Sephacryl S-200. Conjugation of PBA with carrier’s proteins.

Synthesized ligands on a base of 3-phenoxybenzoic acid (PBA) were covalent immobilized by means of carbodiimide on carrier proteins - soybean trypsin inhibitor and bovine serum albumin. Additionally for conjugates production haptenes were linked.
via spacer with different length and flexibility (ε-aminocaproic acid, β-alanin, diglycin, glycine).

Producing of MAb.

To generate MAb regular hybridoma technology was used. Hybridomas were grown in ascites of mice, and monoclonal antibodies will be isolated by affinity chromatography on Protein A column.

Immunoassay on a chip for spore detection.

A glass slip was biotinylated and then coated with streptavidin. The obtaining MAb were biotinylated and then sorbed on a streptavidin layer. A test sample with spores was pumped along the sensor channels. After washing, the change of the analyte layer thickness in spots will be measured by the Picoscope.

Competitive immunoassay on a chip for PBA detection.

Biotinylated glass slip was coated with streptavidin. The biotinylated ligands were applied in spots. A test sample contained PBA was mixed with anti-PBA MAb and pumped along the sensor channels. After washing, the change of the analyte layer thickness in spots was measured by the Picoscope.

2. Results

The EA1 protein originally described as main component of S-layer from vegetative cells. However EA1 regular observed in different exosporium preparations and beside anti-EA1 monoclonal antibodies able to recognize spore surface. We have revealed that EA1 isolated from spore of vaccine strain STI-1 compare with vegetative cells contain additional carbohydrates. It was shown that carbohydrates determine immunogenicity of EA1 and can be used for generation of MAb to species-specific non-conserved epitope on the spore’s surface. As a result, we selected hybridoma, which produced MAb that reacted specifically with the spores of various B. anthracis strains and did not react with vegetative cells and spores of other representatives of Bacillus (Figure 1).

Another target (main pyrethroid metabolite) was produced by synthesis of hapten - 3-phenoxybenzoic acid (PBA) followed by hapten conjugation with carrier proteins - bovine serum albumin (BSA) and soybean trypsin inhibitor (STI). Hapten conjugated with BSA was used as immunogen for immunization of BALB/c mice and hapten – STI conjugate for hybridoma selection. Selected hybridomas which secreted high affinity antibodies to PBA were used for MAb production. Biosensor based on the spectral-correlation method is a perspective tool for fast real-time detection of pathogens, pathogen products, chemical and biological warfare agents [26]. As the sensor chip, a microscope glass slip is used without deposition of any metal or dielectric film. The chip is integrated into a micro-fluidic unit that allows simple and rapid sample treatment.
Figure 1. A. Western blot. B. Dot blot of the α-95 kDa MAb with different spore-forming *Bacilli*. Line 1-5: 1 – *B.anthracis*, 2 – *B.cereus*, 3 – *B.subtilis*, 4 – *B.megaterium*, 5 - *B.thuringiensis*. Reaction with only anthrax spore was observed (line 1).

To consider the perspectives of such tool for fast multiplex detection of BA and CA we are carrying out experiments on employment of both MAb types as biological recognition elements in the label-free optical biosensor “Picoscope™” (Figure 2).

Figure 2. Optical scheme of 1D-Picoscope: 1 – super-luminescent laser diode, 2 - scanned Fabry-Perot interferometer, 3 - semi-transparent mirror, 4 - glass slip, 5 - optics, 6 – CCD camera, 7 – fluidic system, 8 - recognition spots or wells, 9 - interface
Firstly the sensor specificity was tested using common spore forming bacteria. The detection limit for \textit{B. anthracis} spores was estimated to be $2 \times 10^6$ cfu/ml (Figure 3). Real-time pyrethroid detection in a competitive immunoassay was demonstrated with using of a pair consisted of anti-PBA MAb/immobilized PBA conjugate (Figure 4).

\begin{figure}[h]
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\caption{The anti-95 kDa antibodies reactivity measured by optical biosensor with different spore-forming \textit{Bacilli}. The limit of the anthrax-spore detection is $2 \times 10^6$ cfu/ml.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=0.5\textwidth]{figure4.png}
\caption{Competitive immunoassay curve for pyrethroid detection with using optical biosensor.}
\end{figure}
3. Conclusion

The described approach can be used to generate MAb to any desired target and demonstrates advantages of using the hybridoma technology for production of biological recognition elements for biosensor detection of biological and chemical threat agents.

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References

Chapter 43

Congo-Crimean Hemorrhagic Fever: Spread and Prophylaxis in Bulgaria

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Abstract - Crimean-Congo Haemorrhagic Fever (CCHF) is a severe viral disease with high mortality 30-50%. Following a short incubation period there is an influenza-like illness with fever and haemorrhagic manifestations. The virus is transmitted by ticks of genus Hyalomma, with intermediate vertebrate hosts. Human become infected through tick bites, crushing an infected tick, or at the slaughter of viraemic livestock. In naturally occurring epidemics, cases do not show narrow clustering and person-to-person spread is rare. However, nosocomial spread appears to occur. CCHF could be delivered by aerosol or vectors and is recognized (NATO Handbook) as potential agent of Biological Weapon. The prophylaxis in Bulgaria is complex of general epidemiological and hygiene activities and specific immunoprophylaxis on risky people (military men, forest and field workers) with the Bulgarian licensed, specific and original vaccine, has no analog in the world. The occurred relative reduction of Morbidity and Mortality rate of CCHF recent years in Bulgaria correlates with the adequate prophylactic policy of Ministry of Health, especially the National Program for Control and Prevention of Tick-born Diseases (2004).

Keywords - Crimean-Congo Haemorrhagic Fever (CCHF), vaccine, hyperimmune serum

Introduction

Crimean-Congo Hemorrhagic Fever is an important health problem with broad geographic distribution, epidemics and nosocomial outbreaks. The virus is recognized as agent of Biological weapon because of: short incubation period of the disease with severe clinical course, high mortality rate, transmission human to human and possible aerosol application. The disease was first characterized in the Crimea in 1944 and given the name Crimean hemorrhagic fever. It was then later recognized in 1969 as the cause of illness in Congo, thus resulting in the current name of the disease. Fatality rates in hospitalized patients have ranged from 9% to as high as 50%. Causative organism: (Systematic name in 1997) Crimean-Congo Hemorrhagic Fever Virus (CCHF Virus), a Nairovirus, an enveloped spherical virus with two subgenomic single-stranded RNAs belonging to the Bunyaviruses.
The natural reservoir and vector are several genera of hard-bodied or ixodid ticks including *Hyalomma, Dermacentor, Amblyomma,* and *Rhipicephalus*: the premature tick forms (larva, nymph) live in rabbits, birds and mice, but the adult tick—in livestock and wild animals. Transmission to humans realizes by bite of ticks, contact with infected animal blood or tick, contact with infected human blood or body fluids, aerosols, generated during slaughter of infected livestock (domestic animals become infected but do not have significant disease), aerosol in laboratory (animal models). After Incubation period of 5-10 days the symptoms of CCHF are: sudden onset of fever, headache, malaise, dizziness, myalgias, nausea/vomiting, mucous membrane bleeding, ecchymosis, shock, bleeding, mortality (9-50%). Laboratory confirmation: Nucleic acid hybridization & immunohistochemistry of formalin-fixed tissues; Electron microscopy can provide definitive evidence, virus isolation from acute blood (Biosafety Level 4), Polymerase chain reaction (PCR).

World spread of CCHF: Eastern Europe, particularly in the former Soviet Union, Mediterranean, Northwestern China, Central Asia, Southern Europe, Africa, Middle East, Indian subcontinent with endemic spread in Balkan Peninsula.
CCHF Spread in Bulgaria

In Bulgaria over 700 epidemic foci are reported in 2 Main areas: one in the southeast, second one in northeast. From 1953 to 1974: 1105 CCHF cases reported. The fatality rate was 17%! Of them, 20 cases were nosocomial infections and 52% were fatal. The mean age of patients is 52 years (range 11–79 years). Most patients are men (74%), probably because they are more frequently exposed to ticks bites during outdoor activities. The most of cases are observed from March to July when ticks are more active. From 1997 to 2003: total of 138 cases occurred 29 of them fatal.

The recent 3 Years (2006-2008): 2006- 2 cases, 2007- 1 case, 2008- 6 cases, 1 fatal: 4-exposed to ticks, 2-exposed to blood from a patient, total: 9 cases, 1 fatal.

Prophylactic Complex in Bulgaria:

- **General epidemiological and hygiene activities:**
  - Insect control: treatment with acaricides
  - Break the chain: animals (wild) -----ticks-----animals (livestock, rodent, birds)
  - Use of insect repellent on exposed skin and clothing (agricultural workers and others working with animals).
  - Use of gloves and other protective clothing
  - Avoid the contact with the blood and body fluids of livestock or humans who have infection
  - Healthcare workers use proper infection control precautions to prevent occupational exposure
  - Clinical Laboratory Procedures: strict barrier precautions: gloves, gown, mask, shoe covers, protective eye/faceshield, consider respirator with HEPA filter, handle specimens in biosafety cabinet when possible.

- **Specific immunoprophylaxis:** Immunization program was introduced in 1974 for medical workers and military personnel in endemic areas.
Vaccine

The created Bulgarian vaccine is: specific, original, licensed with no analog in the world.

Indication: The vaccine is designed for the protection against infection with the CCHF virus for persons over 16 years of age: border army units, agricultural workers, medical workers and other persons, living in CCHF endemic regions. Mode of application: Primery immunization: 2 injections of 1 ml (1 dose) subcutaneously with interval of 30-45 days. Reimmunization- 1 year after the first application and every 5 years. Immunization starts in the pre-epidemic period (March-April). Undesired side reactions: local reactions-slight reddening of the place of injection, general reactions-an increase of body temperature up to 37.5°C for up to 48 hours.

Specific immunotherapy CCHF-Bulin

Composition: Human anti-CCHF immunoglobuline, obtained from reconvalescent or immunized persons. Solution for intramuscular injection - 3 ml
Conclusion

The occurred relative reduction of Morbidity and Mortality rate of CCHF in Bulgaria correlates with the adequate prophylactic policy of Ministry of Health, especially the National Program for Control and Prevention of Tick-born Diseases (2004).

References:

6. N. Kalvatchev, I. Christova, ONE STEP RT-PCR FOR RAPID DETECTION OF CRIMEAN-CONGO HAEMORRHAGIC FEVER VIRUS, Biotechnology & Biotechnological Eq, 2008; 22/3: 864-6
Chapter 44

Rickettsioses – Imminent Infections to our Present

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Abstract - During the last years the tick-borne rickettsial infections take more important place in the infectious pathology of the human. New causative agents were found, also new types of rickettsioses which were unknown until now, and new vectors. It is determined that the known areas are widened and rearranged. 

R.felis was found with vector flea, R. typhi was also found. Similar processes are observed, investigated and analyzed in Bulgaria for many years. National program for control and counteraction to tick-born infections is developed. The results and the effect of the performed investigations and the realized methods are represented.

Keywords - tick-borne rickettsial infections, biological agents, counteraction, national program

Several new emerging rickettsioses and their etiological agents are discovered and described during the last decades. At the same time the morbidity in well-known rickettsioses increased also, so they were called re-emerging diseases. Marseille spotted fever and siberian tick typhus are examples.

Different epidemiological sides of rickettsioses and of the ecology of their agents, such as vectors, reservoirs, biology of SFG rickettsioses were researched and clarified. New methods of diagnosis and medical treatment were introduced.

Significant changes in the structure and organization of the world has occurred during this period. Processes of globalization, disturbed ecological balance, changes in the climate, etc., caused influence over the situation of infectious illnesses, including rickettsioses. Serious and dynamic changes are observed.

Leading changes in contemporary conditions are:
- vast spreading of rickettsioses, significant increasing of morbidity in different regions all over the world. There is existing of sporadic morbidity cases as well as thousands of morbidity cases in different areas. The significance of tick fever grows up.
- New unknown febrile diseases, caused by rickettsiae SFG were found. Some of them are astrakhan-, japanese-, african-, israel-, australian- tick fever, Tick limphadenopathy – TIBOLA, rickettsial limphangiit (LAR), etc. Sixteen new tick spotted fevers are described after 1984.
- New species rickettsia SFG were found out, such as *R.slovaca*, *R.helvetica*, *R.japonica*, *R.africae*, *R.conorii*, subsp. *Capiensis*, *R.honey*, *R.massiliae*, *R.ripicephali*, *R.felis* (with vector – cat flea, Ctenocephalides felis).

- Enlarging the frontiers of the areas with TSF is visible as well as a trend to suburbanization and urbanization of the natural foci and also formation of city-type foci.

- Different kinds of TSF and their agents are found out in non-typical geographic regions. African tick fever is proven in Italy, Spain, France, USA, Switzerland and Norway. TSF are observed in Japan, Korea, The Ands – in Northern Peru. Rickettsioses with agent *R.conorii* are diagnosed in USA and Uruguay. Rickettsial lymphangiitis with agent *R.sibirica* is described in Portugal, Spain, France, Africa and China.

- Hosts of Rickettsia SFG are not only the known species of ticks, but also others which are not thought as reservoirs and vectors for specified types Rickettsia SFG, until now. That shows the researches taken through the last years. Agents of Astrakhan fever are found in ticks, which are collected in Kosovo and Chad; agents of TSF which are not connected with *R.rickettsii* are found in ticks living in the area of Mississippi; Rickettsia SFG are isolated different types of ticks in Japan, Spain, France, USA, Italy. Agents of SFG, which are connected with reptiles, are found in Australia and Tasmania.

- Special features in clinics of TSP are observed. The risk factor is erased. All age groups are enveloped; TSP passes harder in contemporary conditions, the number of non typical passing forms increases.

The Balkan region is endemic for a number of rickettsioses. Mediterranean spotted fever takes leading place. MF is described for the first time in Bulgaria by prof. I. Vaptsarov in 1948.

The 60-year history of MF in our country is proposed by three periods:

1. First wave – from 1948 to 1970. 240 cases are registered for a period of 22 years. Researches over clinics and epidemiology of Rickettsia are implemented. Illnesses have had mostly sporadic character. They are concentrated in Southern Bulgaria and in Black sea region.

2. The number of illnesses decreases in 1960s. Cases of MF are not observed in Bulgaria after 1970 for a period of 22 years. It was considered that rickettsioses are “successfully eradication”.

3. The second wave of MF started in 1993. Rickettsia had appeared again. It became a serious health and social problem, as it was “successfully eliminated”. It is characterized with several special features, like sharply increasing of illnesses (over 13 000 cases are registered from 1993 to 2008 – this is 50 times more than the First wave period), new foci are formed out of the known areas, differences in taking TSF are observed in different areas, erasing of the risk factor, changes in age and social structure, in contemporary conditions. MF has become a city population disease.
Researches made during this period by specialists from MMA, NCIPD and NDSRVMI, over natural and agricultural foci of some zoonoses including MF, cause interest. Researches embraced the whole country territory. 380 settlements are examined.

The presence of some types is specified:

Numerous NF and AF, caring of Rickettsia SFG is found at an average 18.40% of the researched ixodic ticks.

Other species from TSF group with antigen structure similar to *R. sibirica* and *R. rickettsii* are found in NF side by side with *R. conorii*, which is considered as an agent of MF in the Balkan region.

- Rickettsia SFG are found not only in ticks *R. sanguineus*, but also in other species: *D. marginatus*, *R. bursa*, *I. ricinus*. Antibodies against SFG rickettsia are found at an average 5.92% of the examined animals.

- Other SFG are proved on the territory of our country except MF.

The results were confirmed in Center of Rickettioses – Marseille, France.

That is with meaning of accurate etiologic diagnosis and right therapy method.

Tick lymphadenopathy is proved in our country.

The fact that the suspect for MF patients are confirmed in 55% of all cases, cause interest. We have to know, that other diseases with similar clinic and epidemiology like TSF exist during the season: ehrlichioses/anaplasmoses, flea rickettioses – endemic spotted typhus, etc.

The necessity of extended researches over these topical, health-social significant questions, improvement of the diagnostic, prophylactics – mainly clarifying the action of the complex of global and local factors over morbidity of TSF and typical cycles, are obvious. The National program for preventing and control of tick-borne diseases takes leading place.

![Figure 1](image.png)

**Figure 1.** Mediterranean spotted fever in Bulgaria 1948-2008y.
Figure 2. Mediterranean spotted fever in Bulgaria 1948-2008y.

References

Chapter 45

Rickettsioses – Emergency Situations – Bioterrorism – Preventive Therapy

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Abstract - Many natural and agricultural foci of Mediterranean spotted (Marseille) fever and other tick-borne infections, Q-fever are found in Bulgaria. The status of these foci is a subject of a constant control and research. There are observed many epidemics of the mentioned infections as a result of action of global and local factors. In the conditions of emergency situations of different kinds the risk of ascending of morbidity and the creation of epidemic situation increases many times. The causative agents of some of these diseases are suitable for using as a biological weapon. All of that define the need of developing effective methods for counter actions and prophylactics including improvement of the methods of diagnosis and treatment, prevention therapy (urgent prophylactics). The results of collaborated researches of these topical for the Health problems are presented.

Keywords - rickettsioses, emergency situations, bioterrorism, preventive therapy

Rickettsioses, which are historically the most significant infectious diseases, use to be a serious health and social problem for many regions in the world, where they are endemic and there are their vectors and agents. They are typical with their cyclic which is determined by complex of factors.

Howard Taylor Ricketts founded the principles of tick-borne spotted fevers (SFG – rickettsioses) more than a hundred years ago with classical researches upon the agent of Rocky Mountain Spotted Fever (RMSF) – R. rickettsii and natural foci of the infection – D. andersoni ticks. During 1909 Stanislaus von Prowazek had established the agent of epidemic typhus R. prowazekii, and later Charles Nicolle described its transmitting mechanism.
The epidemic spotted typhus is again topical especially after the civil war in Rwanda and Burundi. From January till September 1997 a total of 45,345 persons acquired typhus and the mortality was up to 15%. Rocky Mountain Spotted Fever (RMSF) is topical for the American continent. It is characterised with high level of mortality (30%-70%). Q-fever is widespread around the world except in New Zealand. It often passes with atypical symptoms or without any. There are often observed hard Q-rickettsial endocarditis, hepatitis, vessel defeat and other except atypical pneumonia. The risk factor no longer exists. The younger population is affected. The cattle industry is affected too. The diseases among the people and the epidemics are strongly connected with epizootics at animals. Non-malignant tick-borne rickettsioses are important for the social health. R. felis was found in 1990 with vector cat flea. Epidemic typhus is very important, too.

Rickettsioses and their agents are known as appropriate for bioterroristic actions. The epidemic spotted typhus (R. prowazekii) and Q-fever (C. burnetii) are classified in category B of potential biological agents corresponding to a specification of CDC. The other are included in group C. The properties which determine the interest to these agents are:
- Second paragraph. possibility of generating stable aerosols and aerosol-fractions with small size, which ensure penetrating in the lower sections of the breathing ways;
- high infecting of the aerosols in small doze (for some pathogenic Rickettsioses ID50 is 1 or 2 micro-organisms);
- Causing of hard-passing illnesses with non-favourable prognosis;
- Low level of immune status of the human population;
- An opportunity with transforming of anyone species of rickettsia to impart resistance to tetracycline and chloramphenicol exists nowadays, using resistant genes and selecting of transformants under antibiotical pressure;
- Possible way of appliance is scattering of some kinds of vectors and reservoirs, like ticks, louses, fleas. The diseasing potential of the rickettsioses increases from the fact that they survive in faeces of fleas and lous es and in ticks for period of months. That way is less possible, but it is a possible way.

Mediterranean spotted fever (MSF, Marseille fever) is found for its first time in Bulgaria by professor I. Vaptsarov in 1948. A. Mitov reported for Q-fever cases one year later - 1949. Many natural and agricultural foci of MSF, other tick-borne spotted fevers (SFG) and Q-fever were found and explored in our country. They have different stage of dynamics and activity. Many epidemics activated by different local and global factors have been reported.

The risk of high increasing of morbidity and creating epidemic situation goes very high in conditions of emergency situations. A possible terrorist act will cause a very similar result.

Effective counteractions, avoiding the consequences of possible bioterrorist attacks or other emergency situation are strongly connected with preliminary preparation and organised work between different departments and structures. It is very important to find the agent early in the area of defeat and the disaster to be reacted adequately. It is important also to know the NF and the AF of the area and their condition and circulating of rickettsiae and Coxiellae. That means presence of teams for observation, laboratories and tools for quick diagnosis. Developing of new methods for diagnose in very
A little time period is needed, also improving the old ones. Medical teams and the population need to be educated for that kind of situations for better results when it is needed.

The extreme situations with different kinds, the preventing therapy and the urgent prophylactics, as well as the quick diagnosis, take a leading place among the complex of actions for effective fight with the rickettsiae infections, independently of the originating way – natural or artificial. The question for etiological treatment of rickettsioses was successfully solved with leading in the antibiotics from the tetracycline and chloramphenicol groups. 100% favourable exit was achieved in hard-passing rickettsioses like epidemical spotted typhus, SFRM, fever tsutsugamushi, Q-fever. It was realised shortness of the sick-process, lighter passing of reconvalescent period and preserving from chronifisizing. The question for the prophylaxis, the preventive therapy with antibiotics, has always been a topical, because in some cases that kind of proceeding is rational and warrant. The selection of antimicrobial drugs, the dosage the scheme of interaction, the purpose which is given. The tetracycline from the second generation fit good to that therapy. Fluoroquinolones from second and third generation and the new macrolides are alternative, which is used to little children and pregnant women. If it is necessary Doxycycline is used as a treatment of choice during the therapy of ehrlichioses/anaplasmoses, hard-passing rickettsioses, and a short course of treatment is recommended.

In this connection when developing regimens and schemes for the administration of anti-rickettsial agents in conformity with the above mentioned requirements, it is necessary to keep in mind the some general principles of applicability: selection of effective agent, with possible the vastest spectrum, studying the pathogenesis of the rickettsial infections and immunogenesis under the condition of anti-rickettsial treatment, duration of the therapeutic and preventive treatment and the optimal dose.

Rickettsial researches at the Scientific Laboratory (SRL) of virology at Military Medical Academy (MMA) – Sofia in collaboration with specialists from the Faculty of Military Medicine – Hradetz Kralove, The Czech Republic have been constructed universal two-cyclic scheme of preventive therapy and treatment of rickettsioses, which is based on the specific features of the pathogenesis and immunogenesis of rickettsioses. Treatment of choice is the Doxycycline, which has inhibiting effect on the model rickettsiae and coxielae (R.conorii, R.rickettsii, C.burnetii).

Thick-borne rickettsioses and the Q-fever were reproduced by the principle of the guinea-pigs and white mice, which were infected intraperitoneal or by aerosol way with different infectious doses from the agents. Doxycycline had appliance per oral in combination with vitamine from the B group according to the following three schemas: conventional – 5 days with an interval between cycles 3 or 10 days. The prophylaxis had become at 12 or 24 hour after the infection and the treatment at the third day.

The valuation of the effect was accomplished on the basis of complex of clinical and laboratory indexes. They were traced at: 1-4-10-24-96-120 hour and 7-14-21-28 and 60 day.

The overall results of the study have established the following:
- In a single antibiotic course is observed moving of the maximum of infectious process toward the later terms: 14-21 day, and the peak at the non prophylaxed (no treated) animals is at 5-7 day. Doxycycline retarded the reproduction of the microorganisms (rickettsiotostatic effect). It is not observed maximum inhibiting of the growth of coxiellae and rickettsiae SFG. Electro-microscopic examinations deter-
mine changes in the wall of the cell of coxiellae in animals which were prophylaxed with doxycyclin at 5-7 day after a stop of the treatment with antibiotic.
- the administration of the second cycle – it leads to strongly expressed inhibitory effect over the reproducing of the micro organisms after a ten-days interval, detention of constantly low level in the 7-21st day interval. That resembles infecting with very low doze, stimulating of the immunogenesis, gradually increasing of the titre values of the specific antibodies, which reaches 1:320-1:640 titres at the 30th day, a total releasing (rickettsial clearens) of the macroorganizm from the agents (absence in spleen and other organs) at about 28-30-60 day. In fig. No 1 are presented the results from the preventive therapy of the Q-fever in white mousse by the two-cyclic scheme. In fig. No 2,3 is presented aerosol chamber of the dynamic flow type.
- instigation with small infectious dozes of 21-28th day leads to next increasing of antibodies, with no development of the clinic picture.

Researches over pathogenesis of the Q-fever and of the rickettsioses from SF group gave an opportunity of clarifying a big number of special features in the passing and the dynamic of the rickettsiae infectious process in the progress of naturally passing infection and in the influence of Doxycycline and to construct an original two cyclic scheme for preventing therapy.

The scheme expects treatment with Doxycycline – 100 mg/24h. one time p.o. in 2 - 5-day cycles, with 10-day interval between them.

This scheme passed successfully a clinical trial in humans (n=500), participants who had come in contact or were exposed to agents of Q-fever and TSF. None of the individuals, receiving the preventive therapy developed a disease. The specific features of the used model rickettsiae and coxiella gave us a reason to extrapolate these methods to the spotted typhus group (epidemic and endemic).

We have to take an account of that the preventive therapy/urgent prophylactic is applied only in strictly recommended testifications and estimation.

The specialists from MMA (SRL of virology and NRLR) have a long year experience (over 45 years) in the area of creating and leading in new methods for diagnosis of the rickettsioses. Complex researches over the diffusion of these health and social taking place infectious deceases in the territory of the country are accomplished and are doing together with the specialist from NCIPD and NDSRVMI and also the circulating agents, condition of NF and AF, studying of the factors, which determine the cycles and the dynamic of the rickettsioses.
Figure 1. 2-cycle scheme for preventive therapy and treatment of rickettsial diseases

Figure 2. Natural and agricultural foci of Tick Borne Spotted Fever and Q-fever in Bulgaria 1986-2008
References

Chapter 46

Effective Real-Time PCR SYBR Green System for Early and Rapid Detection of Coxiella Burnetii

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Abstract - Coxiella burnetii is the cause for Q-fever that is spread all over the world and affects both, animals and humans. Q fever is a disease of great significance which may take a chronic or even lethal course. Coxiella burnetii is a potential biological agent that is possible to be used as a biological weapon. Prompt and specific diagnostic tools as well as elaborating new detection methods are needed for an exact diagnosis and a correct etiological treatment. One of the most contemporary monocular – biological methods for a prompt and specific detection at genetic level is the real-time Polymerase Chain Reaction (PCR) with conventional primers CB1/CB2 and SYBR green dye.

We successfully tested its specificity, sensitivity and effectiveness in sequence detecting of Coxiella burnetii genome which is strongly peculiar to that kind of microorganism.

Keywords - Real-time PCR (Polymerase Chain Reaction), SYBR Green dye, Coxiella burnetii

Introduction

Coxiella burnetii is an obligatory intracellular gram-negative bacterium which develops in cell culture, hen embryo and guinea pigs, but cannot be grown in axenic medium. It belongs to a gamma–partition of Proteobacteria, Legionellales order, Coxiiellaceae family, and Coxiiella genus. The size of the genome varies between different Coxiella burnetii strains, from 1,5 to 2,4 Mb. It is considered to have a circular BI-chained DNA and there are 4-plasmide types depending on the different kind of strains: QpH1, QpRS, QpDG,QpDV (18). Coxiella manifests a high level of steadiness in external environment. A lot of mammal, bird and tick species can be a reservoir for nurturing of that kind of microorganism. The infection caused by Coxiella
*Coxiella burnetii* most often takes a latent course in animals constantly eliminating the bacterium in the surrounding area.

*Coxiella burnetii* can enter the human organism through the aerosol (the most important way); through the alimentary tract by absorption of an overdose of the microorganism with the food and water; the transmission vector way (tick); congenital infection via placenta; blood transfusion; sexual contact; skin and mucosa are of least significance (via contact). Q fever is an infection that shows a varied clinical picture and the lack of specific features hampers its diagnosis, prevention and correct etiological treatment. *Coxiella burnetii* has two manifestations – acute (atypical pneumonia and hepatitis) or chronic (endocarditis). Nearly 60% of the cases take an asymptomatic course. During pregnancy the disease can lead to abortion or prematurely born fetus.

For the *Coxiella burnetii* detection a complex of methods for lab diagnosis, including: isolation (most often of cell cultures, hen embryos), serology (the immune – fluorescent method, as a golden standard, ELISA and etc.), molecular-biological methods (conventional PCR, real-time PCR) are used. *Coxiella burnetii* isolation must be performed only in the set of high bio-security laboratories, level 3. The diagnosis of *Coxiella burnetii* via direct and indirect immune-fluorescent method is proved to be referent and has been used most often.

Our attention is drawn at the perspective molecular – biological methods that are currently going through a rapid development and their significance in detection of that microorganism is increasing. These complement the complex of methods by their features: promptness, sensitivity, and specificity.

One of these molecular – biological methods for a rapid and specific detection at genetic level is the real-time PCR with conventional primers CB1/CB2 and SYBR green dye. A pair of 20- and 19-residue oligonucleotide primers (primer C.B.-1 [5’-ACT CAA CGC ACT GGA ACC GC-3’] and primer C.B.-2 [5’-TAG CTG AAG CCA ATT CGC C-3’]) were synthesized (Invitrogen, USA) according to the published DNA sequence of the gene encoding the superoxide dismutase enzyme of C. bumetii. SYBR Green is an asymmetrical cyanine dye used as a nucleic acid stain in molecular biology. SYBR Green binds to double-stranded DNA. The resulting DNA-dye-complex absorbs blue light and emits green light. From a military medical point of view it is important to be mentioned that *Coxiella burnetii* is a potential biological agent possible to be used as a biological weapon. It belongs to B category (Center for Disease Control and Prevention) as a bio-agent with less dissemination, causing infections of medium severity with less mortality, but requiring an enforced diagnosis capacity from CDCP and undivided attention. Prompt and specific diagnostic tools as well as elaborating one detection method are needed for an exact diagnosis and a correct etiological treatment. If the disease is not treated correctly, it may take a severe, chronic and even lethal course.

**Materials and Methods**

oligonucleotide primers (primer C.B.-1 [5'-ACT CAA CGC ACT GGA ACC GC-3'] and primer C.B.-2 [5'-TAG CTG AAG CCA ATT CGC C-3']) were synthesized (Invitrogen, USA) according to the published (A. STEIN 1 AND D. RAOULT 11992). That primer pair was analyzed for specificity by NCBI Blast Software (http://www.ncbi.nlm.nih.gov/BLAST) and no significant similarities to other sequences were identified. We have studied a purified corpuscular antigen derived from yolk sacs infected with *Coxiella burnetii*, strain Heinzerling, 0.025% phenol-treated and ether-purified. DNA extraction was carried out from purified corpuscular antigen of *Coxiella burnetii* by standard phenol/chloroform method after incubation with proteinase K within 2 hours. Precipitation was accomplished with absolute ethyl alcohol and following centrifugation at 13400 rpm for 30 min. Derived pellets have been dehydrated in 50 μl ddH2O. DNA concentration was measured spectrophotometrically (Eppendorf, Bio Photometer). Qualitative values of DNA were obtained automatically or calculated by the following formula: Xμg/μl = A260.50 μg/μl. D, where X is DNA concentration in μg/μl; A260 is DNA extinction, wavelength 260nm and D is a diluting factor. The obtained DNA preparations were led to work concentration 0.5-2.0 μg/μl, distributed on aliquots and kept at –700C against some contamination.

SYBR Green-real-time PCR was carried out by thermocycler Opticon 2 (MJ Research, USA). The reaction compound of primer pair CB1/CB2 consisted of 1 ready buffer system SYBR® Green qPCR Super Mix UDG (Invitogen,), carried all necessary nucleotides (220 μM each one), 22 mM Tris-HCl (pH 8.4); 1.65 mM MgCl2; 55 mM KCl, Taq-polymerase (2U) and SYBR Green dye in volume 25 μl; 2 μl CB1 and 2 μl CB2 were added which is 30 pmol from each primer, 10 μl DNA, 11 μl H2O in 50 μl total volume.

Determination of primers specificity was done by real-time PCR with control genomes of both phylogenetically close and distant microorganisms - *Escherichia coli* (ATCC® 25922), *Legionella pneumophyla* (ATCC® 33152), *Chlamydia psittaci* (ATCC VR 125), *Rickettsia rickettsii* (ATCC VR 891), *Rickettsia prowaceksi* (ATCC VR 142) and *Rickettsia siberica* (ATCC VR-1526T).

Sensitivity estimation of primers was performed by SYBR Green real-time PCR with different DNA concentrations extracted from purified *Coxiella burnetii* corpuscular antigen, strain Heinzerling. All SYBR Green real-time PCR were performed at least three times for reliability.

**Results and Discussion**

The work protocol for these primers was optimized using positive *Coxiella burnetii* DNA samples.

The work protocol includes Initial denaturation - 95°C / 5 min; Terminal extension - 72°C /10 min and 30 Cycles (Denaturation - 95°C / 30 sec.; Annealing - 52°C / 60 sec; Extension - 72°C / 60 sec.).

Primer system CB1/CB2 shows high sensitivity and specificity during the trials.
**Primer Specificity:** This was tested by SYBR Green real-time PCR with genome control samples of both: phylogenetically close and distant microorganisms - *Escherichia coli* (ATCC® 25922), *Legionella pneumophila* (ATCC® 33152), *Chlamydia psittaci* (ATCC VR 125), *Rickettsia rickettii* (ATCC VR 891), *Rickettsia prowacekes* (ATCC VR 142) and *Rickettsia sibirica* (ATCC VR-1526T). As a result of the study (Fig. 1) no specific amplification except for the positive control sample, containing *Coxiella burnetii* DNA (line 1) was shown, which proves the unique value of primer system CB1/CB2.

![Fig. 1. Determination specificity SYBRGreen real-time PCR with primer pair CB1/CB2](image)

**Primers Sensitivity:** The sensitivity estimation of CB1/CB2 primers was accomplished through SYBR Green real-time PCR with different DNA concentrations (Fig. 2) extracted from purified *Coxiella burnetii* corpuscular antigen, strain Heinzerling. DNA concentration was measured by Bio Photometer (Eppendorf), and an extraction of 58.7 ng/μl was reported. Concentrations of DNA were used as follows: 5.87 ng/μl – diluted 1:10 (line 1); 0.587 ng/μl - diluted 1:100 (line 2); 0.0587 ng/μl – diluted 1:1000 (line 3). The high primers sensitivity is evident as it detects DNA concentration in 5.87 ng/μl – diluted 1:10.
Conclusions.

The SYBR Green real-time PCR with primer pair CB1/CB2 is specific and sensitive and can be successfully used together with the complex of diagnostic methods for rapid detection of *Coxiella burnetii*.

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