THE ELEVENTH WORKSHOP WITH INTERNATIONAL ELECTRONIC PARTICIPATION ON BIOLOGICAL ACTIVITY OF METALS, SYNTHETIC COMPOUNDS AND NATURAL PRODUCTS

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with electronic international participation

14-16 December 2016

Institute of Experimental Morphology, Pathology and Anthropology with Museum

at the Bulgarian Academy of Sciences

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THE ELEVENTH WORKSHOP

"BIOLOGICAL ACTIVITY OF METALS, SYNTHETIC COMPOUNDS AND NATURAL PRODUCTS"

with electronic international participation

IS ORGANIZED BY THE INSTITUTE OF EXPERIMENTAL MORPHOLOGY, PATHOLOGY AND ANTHROPOLOGY WITH MUSEUM (IEMPAM)

UNDER THE AUSPICES OF

THE BULGARIAN ACADEMY OF SCIENCES

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The Program of the Workshop

Wednesday, 14 December 2016

9.00 – 9.30 OPENING CEREMONY

Session A.

Chairpersons:

Prof. Stefka Valcheva-Kuzmanova, MD, PhD, DSc
Medical University, Varna
Assoc. Prof. Radostina Alexandrova, MSc, PhD
Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences
Secretary: Tanya Zhivkova, MSc
Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences

9.30 – 10.00

AO1. HUMAN MICROBIOME AND APPROACHES FOR INFLUENCING

Tsvetelina Velikova

University Hospital St. Ivan Rilski, Laboratory of Clinical Immunology

10.00 – 10.30

AO2. PLANT GENOTYPE DIVERSITY INDUCED BY STRESSED ENVIRONMENT – A WAY TOWARDS ADAPTATION OR EXTINCTION?

Borislava Kukurina and George Miloshev

Laboratory of Molecular Genetics, Institute of Molecular Biology, Bulgarian Academy of Sciences

10.30 – 11.00

AO3. CYTOTOXIC ACTIVITY OF NON-STEROIDAL ANTI-INFLAMMATORY AGENTS, BILE ACIDS AND THEIR METAL COMPLEXES

Lora Dyakova[1], Daniela-Cristina Culita[2], Milena Georgieva[3], Tanya Zhivkova[4], George Miloshev[3], Gabriela Marinescu[2], Marin Alexandrov[4], Luminita Patron[2], Radostina Alexandrova[4]

[1]Institute of Neurobiology, Bulgarian Academy of Sciences, Sofia, Bulgaria
[2]Institute of Physical Chemistry "Ilie Murgulescu", Bucharest, Romania
[3]Institute of Molecular Biology, Bulgarian Academy of Sciences, Sofia, Bulgaria

[4]*Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria*

11.00 – 11.20 Coffee Break

11.20 – 11.35

A04. MACROSCOPIC EVALUATION OF THE PROTECTIVE EFFECT OF ARONIA MELANOCARPA FRUIT JUICE IN A MODEL OF TRINITROBENZENESULFONIC ACID-INDUCED COLITIS IN RATS

A. Kuzmanov[1], V. Kuzmanova[1], S. Valcheva-Kuzmanova[2]

[1]*Student, Medical University Prof. Dr. Paraskev Stoyanov, 9002 Varna, 55 M. Drinov Str., Bulgaria*
[2]*Department of Preclinical and Clinical Pharmacology, Medical University Prof. Dr. Paraskev Stoyanov, 9002 Varna, 55 M. Drinov Str., Bulgaria*

11.35 – 12.05

AO5. BIOCHEMICAL EVALUATION OF THE EFFECT OF *ARONIA MELANOCARPA* FRUIT JUICE IN A MODEL OF TRINITROBENZENESULFONIC ACID-INDUCED COLITIS IN RATS

A. Kuzmanov[1], V. Kuzmanova[1], S. Valcheva-Kuzmanova[2]

[1]*Student, Medical University Prof. Dr. Paraskev Stoyanov, Varna, Bulgaria*
[2]*Department of Preclinical and Clinical Pharmacology, Medical University Prof. Dr. Paraskev Stoyanov, Varna, Bulgaria*

12.05-12.20

AO6. STUDY ON THE EFFECTS OF ALKALINE EARTH METALS ON THE ACTIVITY OF AMINOPEPTIDASE A IN NORMAL AND TUMOR HUMMAN MAMMARY GLAND-DERIVED CELLS

V. Petrova[1], V. Pavlova[1], I. Iliev[1], V. Lozanov[2], I. Ivanov[2], M. Dimitrova[1]

[1]*Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria*
[2]*Department of Chemistry and Biochemistry, Faculty of Medicine, Medical University –Sofia, Bulgaria*

12.20 – 12.35 Discussion

12.35-12.50 Lunch time

Session B.

Chairpersons:

Prof. Ivo Grabchev, MSc, PhD, DSc
Faculty of Medicine, Sofia University "St. Kliment Ohridski"

Assoc. Prof. Radostina Alexandrova, MSc, PhD
Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences

Secretary: Lora Dyakova, MSc
Institute of Neurobiology, Bulgarian Academy of Sciences

13.45 – 14.00

BO1. MACROSCOPIC EVALUATION OF THE EFFECT OF ANETHOLE IN A MODEL OF TRINITROBENZENESULFONIC ACID-INDUCED COLITIS IN RATS

S. Valcheva-Kuzmanova[1], M. Zhelyazkova[1], M. Eftimov[1], V. Marinov[1], M. Tzaneva[2]

[1]Department of Preclinical and Clinical Pharmacology, Medical University Prof. Dr. Paraskev Stoyanov, Varna, Bulgaria
[2]Department of Preclinical and Clinical Sciences, Medical University Prof. Dr. Paraskev Stoyanov, Varna, Bulgaria

14.00 – 14.30

BO2. INVESTIGATION OF BIOCHEMICAL MARKERS IN TWO MODELS OF TRINITROBENZENESULFONIC ACID-INDUCED COLITIS IN RATS

S. Valcheva-Kuzmanova[1] , M. Zhelyazkova[1], M. Eftimov[1], V. Marinov[1], M. Tzaneva[2]

[1]Department of Preclinical and Clinical Pharmacology, Medical University Prof. Dr. Paraskev Stoyanov, Varna, Bulgaria
[2]Department of Preclinical and Clinical Sciences, Medical University Prof. Dr. Paraskev Stoyanov, Varna, Bulgaria

14.30-15.00

BO3. BIOACTIVE COMPOUNDS ISOLATED FROM GARDEN SNAILS

Pavlina Dolashka[1], Aleksander Dolashki[1], Lyudmila Velkova[1], Stefan Stevanovic[2], Laura Molin[3], Pietro Traldi[3] Radostina Velikova[1] and Wolfgang Voelter[4]

[1] Institute of Organic Chemistry; [2] Institute for Cell Biology, Department of Immunology, University of Tübingen, Germany; [3] R-ISTM, Padova, Italy;
[4] Interfacultary Institute of Biochemistry, University of Töbingen, Germany

15.00-15.15

BO4. N- LINKED CARBOHYDRATE STRUCTURES OF MOLLUSCAN HEMOCYANINS FROM SNAILS

Lyudmila Velkova, Aleksandar Dolashki, Pavlina Dolashka

[1]Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria

15.15 – 15.35 Coffee Break

15.35-16.05

B05. MICROBIOLOGICAL ACTIVITY OF Cu(II) AND Zn(II) COMPLEXES OF A NEW BENZANTHRONE TRIPOD

D. Staneva[1], S. Grabchev[2], E. Nikolova[2], E. Vasileva-Tonkova[3], P. Bosch[4], I. Grabchev[5]

[1]University of Chemical Technology and Metallurgy, Sofia, Bulgaria
[2]Sofia University "St. Kliment Ohridski", Faculty of Chemistry and Pharmacy, Sofia, Bulgaria
[3]Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria
[4]Institute of Science and Technology of Polymers, CSIC, Madrid, Spain
[5]Sofia University "St. Kliment Ohridski", Faculty of Medicine, Sofia, Bulgaria

16.05 – 16.20

BO6. ACTIVITY OF WATER EXTRACT FROM *NEPETA NUDA* L. AGAINST ACV-RESISTANT HUMAN HERPES VIRUS TYPE 2

Petia Angelova[1], Anton Hinkov[1], Venelin Tsvetkov[1], Kalina Shishkova[1], Daniela Dragolova[2], Veneta Kapchina-Toteva[2], Stoyan Shishkov[1]

[1]Laboratory of Virology, Faculty of Biology, University of Sofia ''St. Kl. Ohridski'', Sofia, Bulgaria
[2]Department of Plant physiology, Faculty of Biology, University of Sofia "St. Kl. Ohridski", Sofia, Bulgaria

16.20 – 16.35 Discussion

Thirsday, 15 December 2016

Session C.

Chairpersons:

Assoc. Prof. Julia Radenkova-Saeva, MD, PhD
Clinic of Toxicology, Department for Adult, Emergency University Hospital "N.I.Pirogov"
Assoc. Prof. Radostina Alexandrova, MSc, PhD
Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences
Secretary: Boyka Andonova-Lilova, MSc
Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences

9.00-9.30

CO1. TRACKING RESISTANCE OF COMBINATIONS OF PROBIOTIC BACTERIA IN MODEL CONDITIONS OF DIGESTION INCLUDED IN GEL OF CHITOSAN

Iliana Nacheva, Aleksandar Valchkov, Kamelia Loginovska, Daniela Miteva

Institute of Cryobiology and Food Technologies, Sofia, Bulgaria

9.30-10.00

CO2. USAGE EVALUATION OF ZINC INORGANIC COMPOUNDS AND ZINC ORGANIC COMPLEXES (CHELATES) IN REGARD TO THEIR FAVOURABLE PROSPECTS, INVOLVING OVERDOSE BIOASSAYS ON CHICKENS

Sofiya Ivanova[1], Katerina Todorova[2], Margarita Marinova[1], Petar Stamberov[3], Georgi Kalistratov[1], Russy Russev[2]

[1]National Diagnostic and Research Veterinary Medical Institute, Sofia, Bulgaria
[2]Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria

10.00 – 10.30

CO3. LYOPHILISED MEAT FOODS DEVELOPMENT FOR SPECIALIZED NUTRITION HAVING RADIOPROTECTIVE EFFECT

Daniela Miteva[1], Plamen Petrunov[2], Iliana Nacheva[1], K. Dimov[1], A. Valchkov[1]

[1]Institute of Cryobiology and Food Technologies, Sofia

[2]Military Medical Academy, Sofia, Bulgaria

10.30 – 11.00

CO4. "SACRED PLANTS" WITH PSYCHOACTIVE PROPERTIES

Radenkova-Saeva J.

Toxicology Clinic, UMHATEM "N.I.Pirogov", Sofia, Bulgaria

11.00 – 11.20 Coffee Break

11.20 – 11.35

CO5. КОНСУМАЦИЯ НА АЛКОХОЛ И ОКИСЛИТЕЛНО УВРЕЖДАНЕ НА ДНК

Мария Минчева и Катя Попова

Биологически факултет, СУ „Св. Кл. Охридски", София, България

11.35-11.50

CO6. ZINC IN THE ETIOLOGY OF ACRODERMATITIS ENTEROPATHICA

Liliya Lazova, Vasil Boyanov

Medical University of Sofia

11.50 – 12.05

CO7. TOPICAL IMMUNOTHERAPTY TREATING ALOPECIA AREATA

Vasil Boyanov, Liliya Lazova

Medical University of Sofia

12.05 – 12.20

CO8. SILYMARIN AND LIVER

Liliya Lazova, Vasil Boyanov

Medical University of Sofia

12.20 – 12.50 Discussion

Friday, 16 December 2016

Session D.

Chairpersons:

Assoc. Prof. Anna Tolekova, MD, PhD
Medical Faculty, Trakia University, Stara Zagora, Bulgaria

Assoc. Prof. Radostina Alexandrova, MSc. PhD
Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences

Secretary: Desislav Dinev, MSc
Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences

9.00 – 9.30

DO1. КОЛОРЕКТАЛЕН КАРЦИНОМ: ДИАГНОСТИЧЕН И ТЕРАПЕВТИЧЕН АЛГОРИТЪМ И НОВИ ТЕХНИКИ ЗА ЛЕЧЕНИЕ

Бойка Андонова-Лилова, Радостина Александрова

Институт по експериментална морфология, патология и антропология с музей, Българска Академия на Науките, София, България

9.30 – 10.00

DO2. INFLUENCE OF THE REACTION MEDIUM ON THE CHEMICAL, PHASE AND MORPHOLOGICAL CHARACTERISTICS OF DOUBLE-DOPED AMORPHOUS CALCIUM PHOSPHATES

K. Sezanova, R. Gergulova, D. Rabadjieva, S. Tepavitcharova, R. Ilieva

Institute of General and Inorganic Chemistry, Bulgarian Academy of Sciences,
Sofia, Bulgaria

10.00-10.15

DO3. RAT BLOOD BIOCHEMICAL MARKERS TESTED AFTER CALVARIA IMPLANTATION WITH ION-MODIFIED CALCIUM PHOSPHATE BIOMATERIALS

Kostadinka Sazanova[2], Marin Alexandrov[1], Veselin Nanev[1], Neli Tsocheva-Gaytandzhieva[1], Ivelin Vladov[1], Petar Dimitrov[1], Margarita Gabrashanska[1]

[1]Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria
[2]Institute of General and Inorganic Chemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria

10.15 – 10.30

DO4. SYNTESIS AND CHARACTERIZATION OF NEW DOUBLE QUATERNARY POLYMERS AND THEIR HYDROGELS AS BIOMATERIALS WITH POTENTIAL ANTIMICROBIAL APPLICATIONS

Denitsa Nikolova[1], Konstans Ruseva[1], Stephan Metsov[2], Elena Vassileva[1]

[1]Laboratory on Structure and Properties of Polymers; [2]Department of Pharmaceutical and Applied Organic Chemistry, Faculty of Chemistry and Pharmacy, Sofia University "St. Kl. Ohridski, Sofia Bulgaria

10.30 – 11.00

DO5. Ability of polyether ionophore Monensin to bind La(III) metal ions

Veneta Ivanova[1], Ahmed Nedzhib[2], Ivayla Pantcheva[1]

[1]Laboratory of Biocoordination and Bioanalytical Chemistry, Faculty of Chemistry and Pharmacy, "St. Kl. Ohridski" University of Sofia, Bulgaria
[2]Research Laboratory of Military Toxicology, Department of Disaster Medicine and Toxicology, Military Medical Academy, Sofia, Bulgaria

11.00 – 11.20 Coffee Break

11.20 – 11.50

DO6. PCR DETECTION OF SIX TRICHINELLA SPECIES

V. Dilcheva, I. Vladov, S.Petkova

Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria

11.50 -12.05

DO7. ANALYSIS OF SOME LIVER BIOCHEMICAL INDICES AND PARAMETERS OF THE STATE OF HEALTH IN *ASCARIDIA GALLI* INFECTED AND BASIC ZINC-COPPER SALT TREATED CHICKS

Veselin Nanev, Ivelin Vladov, Margarita Gabrashanska, Neli Tsocheva-Gaytandzhieva

Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria

12.05 – 12.20

DO8. КРАТЪК ПРЕГЛЕД НА ОПИТА В ПРОТИВОПАРАЗИТНОТО ТРЕТИРАНЕ НА ДИВИ ПРАСЕТА

Василена Дакова, Мариана Панайотова-Пенчева, Делка Салкова

Институт по експериментална морфология, патология и антропология с музей, Българска Академия на Науките, София, България

12.20 – 12.35

DO9. ФОЛИЕВА КИСЕЛИНА

Надежда Стоянова[1], Стефани Димитрова[1], Емил Белински[2]

[1]*Медицински университет София, Медицински факултет*
[2]*Клиника по съдова хирургия, Токуда Болница, София*

12.35 – 13.45 Lunch time

Session E.

Chairpersons:

Prof. Anna Tolekova, MD, PhD
Medical Faculty, Trakia University, Stara Zagora, Bulgaria
Assoc. Prof. Radostina Alexandrova, MSc, PhD
Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences

Secretary: Abedulkadir Abudalleh, MSc, PhD
Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences

13.45 – 14.00

EO1. BEIGE CELLS AND DO THEY HAVE A POSSIBLE ROLE IN THE TREATMENT OF METABOLIC SYNDROME

Angel Todev, Alexander Brunkov, Viktoriya Trendafilova

Medical Faculty, Trakia University, Stara Zagora, Bulgaria

14.00-14.15

EO2. ENDOTELIAL RECEPTORS ANTAGONISTS IN PULMONARY HYPERTENSION

Yuksel Mekov, Georgi Madjarov

Medical Faculty, Trakia University, Stara Zagora, Bulgaria

14.15 – 14.30

EO3. EXPERIMENTAL MODEL FOR DIET- INDUCED DIABETES TYPE 2

Petya Hristova, Vencislava Dimitrova, Daniel Addai, Jacqueline Zarkos

Medical Faculty, Trakia University, Stara Zagora, Bulgaria

14.30 – 15.00

EO4. PODOPLANINS AS MARKERS FOR ANGIOSARCOMA

Georgi Madjarov, Yuksel Mekov

Medical Faculty, Trakia University, Stara Zagora, Bulgaria

15.00 – 15.20 Coffee Break

15.20-15.50

EO5. COMPLECATED FACE OF MULTIPLE SCLEROSIS

Sonya Ivanova[1], Vera Kolyovska2

[1]*University Hospital for Active Treatment in Neurology and Psychiatry; "St. Naum", Medical University of Sofia, Bulgaria*
[2]*Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences*

15.50 -16.20

EO6. One in six people is at risk for stroke. It could be prevented. Act now.

Vera Kolyovska[1], Desislava Drenska[2], Dimitar Maslarov[2]

[1]*Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Sofia, Bulgaria,*
[2]*Neurology Clinic, First MHAT-Sofia*

16.20-16.35 Discussion

16.35-16.50 Closing Remarks

Posters

AP1. TARGETED THERAPY OF BREAST CANCER: THE MAGIC BULLETS OF MODERN ONCOLOGY

Radostina Alexandrova[1], Katerina Petrova[2], Tim Vladimirov[2], Osama Azmi[3]

[1]Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria
[2]Faculty of Medicine, Sofia Univesrity "St. Kl. Ohridski", Sofia, Bulgaria
[3]Medical Research Division, Institutes of National Research Centres, Gyza, Egypt

AP2. NANOPARTICLES IN TARGETED CANCER THERAPY

Radostina Alexandrova[1], Abedulkadir Abudalleh[1], Orlin Alexandrov[2], Tanya Zhivkova[1], Lora Dyakova[3], Boyka Andonova-Lilova[1], Desislav Dinev[1], Milena Glavcheva[1], Vladimir Kulchitsky[4]

[1]Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria
[2]Health Service, Gorna Malina, Bulgaria,
[3]Institute of Neurobiology, Bulgarian Academy of Sciences,
[4]Institute of Physiologuy, National emy of Sciences of Belarus, Minsk, Belarus

BP1. SYNTHESIS, CHARACTERIZATION AND ANTIMICROBIAL ACTIVITY OF 4F COMPLEXES CONTAINING THE ANTI-INFLAMMATORY DRUGS ISOXICAM AND TENOXICAM

Daniela C. Culita[1], Gabriela Marinescu[1], Luminita Patron[1], Nicolae Stanica[1], Sultana Nita[2], Mariana C. Chifiriuc[3]

[1]"Ilie Murgulescu" Institute of Physical Chemistry, Bucharest, Romania
[2]National Institute of Chemical-Pharmaceutical Research and Development, Bucharest, Romania
[3]University of Bucharest, Faculty of Biology, Department of Microbiology, Bucharest, Romania

BP2. THE MORPHOLOGY AND ANTIBACTERIAL PROPERTIES OF ELECTROSPUN NONWOVEN MATERIALS WITH SILVER NANOPARTICLES

Solveiga Pupkevičiūtė, Erika Adomavičiūtė, Sigitas Stanys

Kaunas University of Technology, Faculty of Mechanical Engineering and Design, Department of Materials, Kaunas, Lithuania

DP1. INFLUENCE OF BARON DOPPED HYDROXYAPATITE ON THE STRUCTURE OF ELECTROSPUN FIBRES FOR BIODEGRADABLE SCAFFOLDS

E. Bolskis[1], E. Adomavičiūtė[1], S. Stanys[1], V. Jankauskaitė[1], O. Albayrak[2]

[1] Kaunas University of Technology, Kaunas, Lithuania
[2] Mersin University, Mersin, Turkey

DP2. CELL CULTURES AS MODEL SYSTEMS IN BIOCOMPATIBILITY EVALUATION OF NEW MATERIALS FOR BONE IMPLANTS

Radostina Alexandrova[1], Orlin Alexandrov[2], Abdulkadir Abudalleh[1], Virginija Jankauskaitė[3], Nabanita Saha[4], Milena Fini[5], Olafur Sigurjonsson[6]

[1]Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences,
[2]Health Service, Gorna Malina, Bulgaria,
[3]Kaunas University of Technology, Kaunas, Lithuania
[4]University „Tomas Bata", Zlin, Czech Republic
[5]Instituto Ortopedico Rizzoli, Bilogna, Italy
[6]Department of Science and Engineering, Reykjavik, University, Oslo, Iceland

Session A.

Chairpersons:

Prof. Stefka Valcheva-Kuzmanova, MD, PhD, DSc
Medical University, Varna

Assoc. Prof. Radostina Alexandrova, MSc, PhD
Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences

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AO1. HUMAN MICROBIOME AND APPROACHES FOR INFLUENCING

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Abstract

The human microbiota is an aggregate of microorganisms that reside on the skin and mucosa. These different colonizing bacteria act as a protective factor against pathogens from adhering by competition for substrates and places of adhesion, and they simultaneously produce antibacterial substances and stimulate the production of specific antibodies and mucus. A dominant flora, representing the 90% of the population, is composed of Bifidobacteria and Lactobacilli, whereas the residual or fluctuating flora (less than 0.01%) of the population is more diversified and contains the potentially pathogenic species. Microbiota changes over time: the gastrointestinal tract of a foetus is usually sterile, then breastfeeding directs microbiome towards Bifidobacteria whereas bottle-feeding – towards more to Bacteroidetes and less to Bifidobacteria. In adults, dominant phyla are Firmicutes, Bacteroidetes and Actinobacteria, and less dominant: Proteobacteria and Verrucomicrobia. Microbiota also stimulates the development of the immune system by many mechanisms. Furthermore, gut microbiome may regulate the Treg/Th17 axis in the intestinal mucosa. Studies of the gut microbiota include traditional techniques for microbial identification based on morphological and biochemical characterization and the molecular techniques using real-time polymerase chain reaction, metagenomics, and proteomics approach, which evaluate the expression of genes of interest or the changes in the host due to the microorganisms impact. Many factors can influence the human microbiome such as the use of antibiotics, probiotics, and fecal microbiome transplantation. Application of probiotics can influence the microflora composition by increasing the number of lactobacilli and other beneficial anaerobes. In conclusion, the human microbiome is a universe within the human body which requires further investigations and the influence of microbiome offers many opportunities in healthcare by providing new approaches for management of numerous diseases.

1. Introduction

Mucous membranes of the body are in direct contact with the antigens from the environment. Early colonisation of the gut with living micro-organisms, previously named as microflora, is important for the development of the gut protection barrier and immune system function driving postnatal maturation of immune regulation (6).
Human microbiota comprises of over 100 trillion microbial cells harbored by each person, primarily in the gut (13). Furthermore, this gut microbiota of higher vertebrates is host-specific (11).

2. The human microbiome

The human microbiota is an aggregate of microorganisms that reside on the skin, in the saliva and oral mucosa, in the conjunctiva, the urogenital mucosa, to some extent the respiratory and almost all the gastrointestinal tract (14). Mucous membranes are the unique environment where different bacterial species are able to survive and to express their properties. About 1014 bacteria of more than 200 species, 40–50 genera live on these surfaces. Approximately it is 7 m long, comprising a 300 m^2 surface area in adults (19). 99% of the whole bacterial population on mucous membranes occurs in the distal segment of the small intestine and in the proximal part of the colon (8). Interestingly, the intestinal tract of adult carries 1–2 kg of microbes (9).

In addition, there is a great diversity of species, some of which have not yet been identified or cultured, and understanding the dynamics of this population is a challenge due to its complex ecosystem comprising nearly two million genes. Indeed, the number of bacteria within the gut is about 10 times that of all of the cells in the human body. At birth, the entire intestinal tract is sterile; bacteria enter the gut with the first feed. Following infancy, the composition of the intestinal microbiota remains relatively constant thereafter (19). This will be covered in the next section.

Microbiota is affected by several factors; some are determined by the interactions between genetic, environmental or disease factors to which the individual is exposed, the diet, the secretion of mucus, digestive enzymes, and intestinal peristalsis. As a result, each individual has a unique characteristic microbiota (3).

Human microbiota includes oral (Streptococcal anaerobes - Streptococcus mutans and Streptococcus sanguinis, Lactobacilli, Staphylococci, Corynebacteria and Bacteroides, respiratory (α-and β-hemolytic streptococci, anaerobes, Staphylococci, Neisseriae and Diphtheroids, Haemophilus, Mycoplasmas and Pneumococci, Bordetella pertussis), conjunctival (Haemophilus and Staphylococcus, urogenital (S. epidermidis, enterococci, diphtheroids, E. coli, Proteus, and Neisseria, Lactobacillus spp. - predominantly L. acidophilus, Corynebacteria, Peptostreptococci, Staphylococci, Streptococci and Bacteroides) and intestinal microbiota (comprised mainly of anaerobes such as Bacteroides, Porphyromonas, Eubacterium, Butyrivibrio, Bifidobacterium, Lactobacillus, Fusobacterium and Clostridium) (14). Enteroccocus and Escherichia coli constitute less than 1% of all intestine micro-organisms. Anaerobes dominate upon facultative anaerobes and microaerophiles at the ratio of 1000:1. A dominant flora, representing the 90% of the population, is composed of Bifidobacteria and Lactobacilli, whereas the residual or fluctuating flora (less than 0.01%) of the population is more diversified and contains the potentially pathogenic species (8).

The largest and earliest source of microbial exposure in human subjects comes from the intestinal tract. The gut contains a large and diverse population of microbes which stimulate the immune system (10). When disturbed, the microbiota has a remarkable capacity

to restore itself and to return to exactly the same state as it was before (10). Because of the normal motility of the intestine (peristalsis) and the anti-microbial effects of gastric acid, the stomach and proximal small intestine contain relatively small numbers of bacteria in healthy subjects. Bacterial colony counts may be as high as 109 CFU/mL in the terminal ileum immediately proximal to the ileocecal valve, with a predominance of Gram-negative organisms and anaerobes (19). However, the lower portion of the GI tract, comprising the lower duodenum and small and large intestines, contains a complex and dynamic microbial ecosystem, with a high density of live bacteria (3). The predominance of anaerobes in the colon reflects the fact that oxygen concentrations in the colon are very low. The explanation for that is: the microbiota has adapted to survive in the hostile environment (19).

The normal enteric bacterial microbiota influences a variety of intestinal functions and plays a key role in nutrition, in maintaining the integrity of the epithelial barrier and in the development of mucosal immunity. The microflora in our digestive system takes part in food digestion, killing of pathogens, and secreting vitamins (e.g. vitamin B) and some essential amino acids, enzymes help in digesting complicated fibers in the food, acid (e.g. lactic acid) helps to prevent pathogenic microflora from exceeding their number limit, and to perform many other vital activities (1). Unabsorbed dietary sugars (lactose), and alcohols are salvaged by bacterial disaccharidases, converted into short-chain fatty acids and used as an energy source by the colonic mucosa. Nutrients and vitamins, such as folate and vitamin K, are produced by enteric bacteria. The relationship between the host's immune system and nonpathogenic microbiota is important in protecting the host from colonization by pathogenic species. For this intestinal bacteria produce a variety of substances, ranging from relatively nonspecific fatty acids and peroxides to highly specific bacteriocins, which can inhibit or kill other, potentially pathogenic bacteria. Furthermore, bacterial metabolism of some medications (such as sulfasalazine) within the intestinal lumen is essential for the release of their active moieties (19).

Microflora of the gastrointestinal tract plays a crucial role in the anatomical, physiological, and immunological development of the host. It stimulates the immune system to respond rapidly to infection with pathogens and through bacterial antagonism it inhibits the colonisation of the gut by harmful or pathogenic bacteria (8).

Bacterial colonisation of the intestine undergoes changes depending on age. It is influenced by local immunity, bacterial fixation factors, and the phenomenon of colonisation resistance. Bacterial strains from the neonatal period are replaced during the life by other bacterial strains characteristic of particular specimens and hosts (8). This will be covered in the following section. Conditions such as stress, excessive alcohol use, high fat diets, meat, sugar, genetic disorders, chlorine and fluoride in drinking water, antibiotics, inadequate food, exposure to environmental toxins and many others factors could change the balance of our intestinal flora (1).

3. Development of human microbiome

In human subjects, the gastrointestinal tract is sterile at birth. Multiple factors determine gut colonization, including bacterial characteristics, mucosal cell characteristics, mode of delivery, and type of diet (10). The initial neonatal gut colonization is determined either by maternal flora or bacteria from the immediate environment (i.e., hospital and health care workers), depending on the mode of delivery. Neonates born by means of vaginal delivery are exposed to the mother's vaginal and intestinal flora as they pass through the birth canal and typically microbiome of newborn resembles those of the mother's flora (10). Compared with vaginal delivery, cesarean section is associated with early gut colonization with Klebsiella species, Clostridium species, and Enterobacteriaceae other than Escherichia

coli. Moreover, children born by means of cesarean section are colonized later and less frequently by Bacteroides species and E. coli (10) and this composition lasts up to at least 6 months of age. Clinically cesarean section results in increased risk for atopy, asthma, and allergic rhinitis perhaps because of a lack of exposure to the maternal vaginal flora, gut flora, or both during normal delivery.

The type of feeding early in life also influences the human microbiome. Hospitalizations and premature birth were also associated with a high prevalence of C. difficile counts similar to those seen after cesarean delivery, which might be related to hospital environmental exposure (10). Although bacterial colonization of the gut is completed approximately 1 week after birth, the numbers and species of bacteria fluctuate markedly during the first few months of life. During the first days of life, the microorganism population is unstable and tends to stabilize with breastfeeding or the intake of breast milk substitutes. The greatest change in this composition, however, occurs through the introduction of solid foods (3). Significant differences between the gut flora of children in industrialized and developing nations suggest that the high prevalence of allergic diseases (e.g., atopic asthma) and obesity in affluent nations might be due to changes in the intestinal flora of young infants (10).

The maternal microbial environment could possibly influence infant immune maturation and T effector and T regulatory immunity. Th1, Th2, and Th17 differentiation is controlled epigenetically, and human T regulatory cell differentiation needs demethylation of the FOXP3 promoter (6).

After adulthood, the intestinal microorganisms change significantly. The gut microbiota of aging people is likely to be influenced by close co-habitation making them more susceptible to microbial imbalance and ultimately infection with pathogens such as C. difficile (4). The healthiest elderly live in a community setting, have a high-quality diet and subsequently possess a distinct gut microbiota from those in long-term residential care. There are a large number of factors that contribute to health decline and composition of the gut microbiota.

In conclusion, gastrointestinal tract of foetus is usually sterile, then breastfeeding directs microbiome towards Bifidobacteria whereas bottle-feeding – towards more to Bacteroidetes and less to Bifidobacteria. During childhood, there is an increase in microbial diversity following intake of solids. In adults, dominant phyla are Firmicutes, Bacteroidetes and Actinobacteria, and less dominant: Proteobacteria and Verrucomicrobia. In elderly persons, compared to healthy adults, there is a reduction in Firmicutes and Bifidobacteria and an increase in Bacteroidetes and Proteobacteria (13).

4. Interactions of microbiome with the immune system

Commensals are not ignored by the immune system, rather they are tolerated via a concerted action of epithelial cells and immune cells (12). In some circumstances, the oral tolerance can be abrogated and an immune response arises. This immune response is mainly humoral mediated by IgA plasmocytes and secretory IgA, which constitute almost 80% of all antibodies produced in mucosal associated tissue. These antibodies inhibit the microbial adherence and also prevent absorption of antigens from mucosal surfaces (18). Certain lactic acid bacteria are able to induce specific secretory immunity, and others can enhance the gut inflammatory immune response (18). Microbiota stimulates the proliferation of epithelial cells and increases the whole intestinal surface and the colonisation of the gut with commensal microflora affects the development of the immune system (8).

Most recently, gut microbiota has been linked to low-grade inflammation through activation of innate immunity through the LPS–Toll-like receptor 4 axis (10). Except for

stimulation of innate immune system, microbiota can sense the adaptive immune system through transportation via M cells. The viability of the probiotic cells may be crucial for the uptake of the probiotic antigens through the Peyer's patches, which could be due to the greater ability of the viable in comparison with non-viable microorganisms to bind to M-cells. Attachment of a strain to the intestinal mucosa is one of the main selection criteria for probiotic microorganisms (16). The importance of the gut microbiome in regulating the Treg/Th17 axis became widely appreciated when different groups reported that germ-free mice demonstrate a decreased frequency of colonic Th17 cells and Tregs. One of the most widely investigated commensal bacteria in the context of Th17 immunity is segmented filamentous bacteria (15). When the intestinal barrier is disrupted, systemic dissemination of microbial products occurs, which invokes the IL-23 pathway and initiates barrier repair, as well as Th17 responses aimed to neutralize invading commensal microbes (15). This circuit promotes IL-17 expression in RORγt+ T cells, especially in the terminal ileum, which is the site of attachment of the segmented filamentous bacteria to the epithelium, the essential condition for Th17 induction (15). In fact, high-fat diet-derived microbiota decreases Th17 cell frequency and the ability of intestinal antigen-presenting cells to generate Th17 cells *in vitro*, thus contributing to low-grade inflammation.

There are some studies exploring the association between microbiome disturbances and many autoimmune diseases, such as diabetes type I, Inflammatory Bowel disease, systemic sclerosis, lupus, asthma, atopic dermatitis, and some other disorders like Irritable bowel disease, obesity, constipation, colorectal cancer (1, 10).

5. Investigation of human microbiome

Studies of the gut microbiota, that use traditional techniques for microbial cultivation, are supported by phenotypic analysis based on morphological and biochemical characterization. These techniques are laborious, time consuming, subject to misinterpretation and identify only approximately 40% of the microbiota (3). Thus, for more precise information on the gut microbial population, appropriate samples should be collected during endoscopies or surgical procedures and then tested by novel identification techniques (3).

The number of species detected molecularly has exceeded on a large scale the number of species accessible by cultivation-dependent methods. The molecular techniques ranging from the identification of intestinal microbiota, particularly probiotic microorganism in different environments, detection of pathogenicity genes in foods, identification and quantification using real-time polymerase chain reaction (PCR), also proteomics approach, which evaluate the expression of genes of interest or the changes in the host due to the microorganisms impact. These novel methods have provided new perspectives in the investigation of diversity, abundance and dynamics of the intestinal ecosystem (3). When we talk about "omics", we have to focus on molecular characterization of specific environments such as GIT, as well as their interactions with probiotic bacteria (21). In line with this, metagenomics is the study of genetic material retrieved directly from environmental samples including the gut, soil, and water. Typically, human gut microbiota behaves like a multicellular organ, which consists of nearly 200 prevalent bacterial species and approximately 1000 uncommon species. All their genetic material presented in an environmental sample, consisting of the genomes of many individual organisms, is called metagenome. Metagenome could be investigated by metagenomic sequencing: the high-throughput sequencing of metagenome using next-generation sequencing technology and descriptive metagenomics is an estimation of microbial relative abundance based on different physiological and environmental conditions to reveal community structure and variation of the microbiome. There is also functional metagenomics which is the study of host–microbe

and microbe–microbe interactions toward a predictive dynamic ecosystem model to reflect a connection between the identity of a microbe or a community (11). All mentioned methods could be employed with a benefit for identification of human microbiome, especially in clinical context.

6. Ways of influencing the human microbiome
    a. Antibiotics

Antibiotics can largely influence the human microbiome. Antibiotic use in the first month of life was associated with reduced numbers of anaerobes, such as Bifidobacteria and Bacteroides species. Many studies confirmed that antibiotic use in early life might lead to alterations in gut microbiota and, ultimately, abnormal development of the immune system (10).

    b. Pro-, pre- and synbiotics

Probiotics, derived from the Greek and meaning "for life", are defined as live organisms that, when ingested in adequate amounts, exert a health benefit to the host (19). Application of probiotics can influence the microflora composition by increasing the number of lactobacilli and other beneficial anaerobes (8). According to World Health Organisation 'there is good evidence that specific strains of probiotics are safe for human use and able to confer some health benefits on the host, but these benefits cannot be extrapolated to other strains without experimentation' (12). A probiotic is classically defined by its "local" beneficial effects in the intestinal tract. For human nutrition, the following definition has been proposed: "a live microbial food ingredient that is beneficial to health". Salminen gave this definition in 1998, and it is still used (7). First reported use of probiotics was in 76 BC when the Roman historian Plinius recommended the administration of fermented dairy products for the treatment of gastroenteritis (3).

Ilya Metchnikoff, the Nobel Prize winner in Medicine in 1908, at the Pasteur Institute, was the first who spotted the effect of what is called now Probiotic. In 1907, he postulated that bacteria were involved in yogurt fermentation. Lactobacillus bulgaricus and Streptococcus thermophilus suppress the putrefactive-type fermentations of the intestinal flora and consumption of yogurt was important in maintaining health. He correlated the long life of Bulgarian peasants and their good health to yogurt intake which contained the Lactobacillus species. In Japan, in the early 1930s, Shirota succeeded in isolating strains existing in healthy individuals' intestinal bacteria. Such strains are able to survive and to passage through the gut. He has used such strains to develop fermented milk and test such milk effects on patients (1).

Selection criteria for probiotic microorganism are the following: human origin, if intended for human use, acid and bile stability, adhesion to mucosal surfaces, safe for food and clinical use, clinically validated and documented health effects, good technological properties. The mechanisms of action of probiotics can be quite disparate. Most probably they are multi-factorial, involving a variety of effector signals, cell types, and receptors, and strains may differ in their respective ability to trigger these signals considering both immunocompetent and intestinal epithelial cells (5). The most common bacteria strains used in probiotic drugs are shown on table 1.

Table 1. The most common bacteria strains used in probiocs/synbiotics production (18)

Lactobacillus species	Bifidobacterium species	Others
L. acidophilus	*B. bifidum*	*Bacillus cereus*
L. casei	*B. longum*	*Eschericia Coli*
L. rhamnosus	*B. breve*	*Saccharomyces cerevisiae*
L. reuteri	*B. infantis*	*Saccharomyces boulardii*
L. bulgaricus	*B. lactis*	*Enterococcus faecalis*
L. plantarum	*B. adolescentis*	*Streptococcus thermophilus*
L. johnsonii		
L. lactis		
L. gasseri		

Probiotic bacteria have been shown to modulate the permeability of epithelial barriers, alter the inflammatory potential of epithelial cells, compete with pathogens for mucosal colonization, or directly modify the activity of immune cells (12). One of the most important hypotheses regarding the effect of probiotics on diarrhea might be the competition for binding sites on the intestinal epithelium. When *Lactobacilli* are ingested, they will compete for binding sites, leaving less binding sites open for pathogens. Pathogens will pass through the gut and leave the body sooner if no binding site is available. Another mechanism concerns competition for nutrients. This has been discussed shortly in antibiotic-associated diarrhea. When many harmless bacteria are present in the gut, they take many nutrients, leaving fewer nutrients for pathogenic bacteria, which may not be able to survive because of starvation. In addition, the entrance of probiotics in the gut may also stimulate the production of secretory IgA. In the absence of intestinal microflora, the intestinal immune system is underdeveloped and intestinal morphology is disrupted. The mice who were kept germ-free after birth have underdeveloped Peyer's patches, decreased macrophage chemotaxis, and a lower capacity for intracellular killing of pathogens compared with macrophages from conventionalised animals. The number of lymphocytes in germ-free mice in the intestine is also greatly reduced. In most cases, they have a predominant production of IgM, little IgG, and no IgA at all. When these mice are given probiotics or are transferred to a conventional area, the immune system will develop into a normal regular immune system. They begin to produce a greater diversity of antibody isotypes, including antibodies specific for resident intestinal bacteria (7).

Probiotics may also produce other chemicals, including neurotransmitters that are normally found in the bowel that can modify other gut functions, such as motility or sensation. Other probiotics have been shown to enhance epithelial barrier function through direct effects on mucin expression, proteins of the cytoskeleton and intercellular tight junctions and indirect effects emanating from interactions between the bacterium, the mucosa and the mucosa-associated lymphoid tissue (19). The facilitation of oral tolerance and innocent bystander suppression by probiotic bacteria support the fact that particular probiotics not only drive protection against infection throughout the mucosal immune system but also regulate the effector response (14).

In conclusion, beneficial effects of probiotics include effects on lactose malabsorption, diarrhea, hypersensitivity reactions, candida-induced vaginitis, cancer, high blood cholesterol, hypertension, and immunity. Benefits of probiotic use have been reported especially for certain high-risk groups such as premature infants, travelers, and people receiving antibiotics (7).

By the same token that probiotics may enhance health due to immunologic mechanisms, influences on the immune system may also have deleterious effects on health. Alterations in specific immune parameters cannot be interpreted unidirectionally as beneficial or deleterious. For instance: an increased Th1 response may enhance resistance to an

infection, yet also increase the expression of autoimmunity. Increased natural killer activity may enhance the resistance to viral infections, yet through enhanced interferon production also enhance the severity of inflammatory responses to bacterial infections. Hence, immune alterations may be indicative of biological effects of exposure to probiotics, they are not necessarily indicative of the direction of the health effect (7) although probiotics have an excellent overall safety record, they should be used with caution in certain patient groups—particularly neonates born prematurely or with immune deficiency. They reviewed case reports of instances of abscesses and endocarditis in relation to probiotic use (19). Thus, probiotics could be used with cautious depending on the case.

Probiotics have some common characteristics:

1 – They are useful and friendly microbes.

2 – They are able to compete with the pathogens and colonize our digestive system.

3 – They are able to ferment our food to simpler byproducts and could promote our health by many different mechanisms.

4 – Their amount could be deteriorated due to many factors, such as incorrect diet, alcohol, age etc. This is why they should be taken through our regular diet.

5 – In particular cases such as after antibiotic treatments, where they are expected to be affected severely, they should be taken orally in considerable amounts or with food.

6 – Probiotics promote health while they:

a. Remove the side effect of the pathogens or the harmful microbes.

b. Supply the body with useful byproducts.

c. Reduce the jobs of our digestive system.

d. Reduce the effect of the first attack of harmful compounds, instead of our cells, by their biofilm, which protects our digestive system.

e. Reduce the amount of food needed by our bodies due to the correct digestion and metabolism of any amount of food.

f. Probiotics in some cases could complement the deficiency in our genetic materials by helping us to borrow the products of their genes (such as in the case of the lactose fermentation deficiency) (1).

In probiotics production, 5 crucial technological and clinical properties must exert:

- Origin: bacteria descending from the human gastrointestinal tract (preferably);
- Safety: probiotic bacteria should be non-pathogenic and sensitive to the most commonly used antibiotics;
- Resistance: the bacterial strains should be able to survive the action of the stomach acid, the bile acids, and the protease enzymes;
- Viability: these bacteria must survive the production process, proliferate in the small and/or large intestine, adhere to the gut epithelium and even colonize the small intestine and/or the colon for a finite time;
- Positive effect: their intake should be beneficial for the health of the human macroorganism (14).

Synbiotics, defined as a combination of a probiotic and a prebiotic, aim to increase the survival and activity of proven probiotics in vivo, as well as stimulating indigenous bifidobacteria and lactobacilli (19). Prebiotics are food components which encourage the growth of beneficial bacteria. We have own results regarding the use of probiotics in healthy persons. Twenty healthy persons were enrolled in our study and they were administered to a novel synbiotic containing five Lactobacillus strains, arabinogalactan and colostrums. After 21 days we observed that the percentage of activated NK cells was higher than the percentage

before ($p = 0.03$), although we did not find significant alteration in total NK cells number. Two of the investigated cytokines in stool samples – IL-6 and IFNγ, showed a significant decrease after synbiotic application ($p < 0.001$). Taken together our results demonstrated an immunomodulating effect of oral administration of synbiotic in direction of increased percentage of activated NK cells in the peripheral blood, as well as decreased levels of IL-6 and IFNγ in stool samples. Thus, the synbiotic could be implemented with beneficial effect in prophylaxis with its system anti-viral effect but could induce local immune tolerance in the gut where it is most needed (20).

c. Fecal microbiota transplantation

More recently, transplantation of a human microbiota from a lean donor to obese subjects induced an improvement of insulin-resistance confirming that microbial imbalance is not solely a secondary consequence, but can contribute to the aetiology of certain diseases (2). Fecal microbiota transplantation (FMT) is another approach that can be used in diseases linked to gut dysbiosis. FMT is a novel technique in which the gut microbiota is transferred from a healthy donor to the patient with the overall goal to introduce a stable microbial community in the gut. Until recently, FMT has primarily been utilized to successfully treat recurrent antibiotic-resistant *C. difficile* infection. The clinical efficacy of FMT in ulcerative colitis is promising as FMT has been shown to induce remission in a greater percentage of patients than placebo and no difference in adverse events were reported. FMT has been investigated in few other diseases including Crohn`s disease, albeit data are too limited to determine clinical usefulness. More randomized controlled trials are warranted to evaluate donor selection and the frequency of FMT administration (2).

7. Conclusion

Investigation of the human microbiome and relevant mechanisms poses a significant challenge because of the complexity of the relationships between the microbiota with host genetics and environmental factors. Future microbiome studies should accurately examine how particular microbial groups are altered in different health conditions. There is an urgent need for novel approaches toward the gut ecosystem dynamics. Such models may then, predict the outcome of the influences in the gut microbiota and eventually aid in therapeutic intervention.

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AO2. PLANT GENOTYPE DIVERSITY INDUCED BY STRESSED ENVIRONMENT – A WAY TOWARDS ADAPTATION OR EXTINCTION?

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Technical progress and global industrialization have had a profoundly adverse impact on the environment. Along with the agricultural practices, species introduction and deforestation pollution takes a considerable part of the human intervention in the ecosystems. Heavy metal pollution in particular drives substantial attention. The major harmful effects are executed through its influence on plants' fitness and populations' survival.

As sessile organisms plants are unable to escape unfavorable conditions, therefore to survive in heavy metal polluted environment plants face a great challenge to keep their cellular metabolism and functions in norm [4].

Heavy metals are known to trigger changes in plant genomes, and thus can act as mutagens or genotoxins and directly to damage DNA molecules. Generally, this can lead to two completely different responses of plant population. On the one hand, heavy metal genotoxicity raises mutational rates and can decrease the population's size and threaten the impacted populations of extinction [1, 3]. On the other hand, being under stress plants can activate genetic mechanisms which lead to genetic rearrangements. This creates genotype variability and is seen as a strategy for adaptation [5]. The preservation and the spread of *de novo* arisen genotypes in populations under stress are believed to be a step towards speciation and evolution [2]. Which way will be chosen by a population - towards adaptation or extinction, is a cross point for the fields of molecular genetics and ecology. It is of utmost interest to examine the potentiality of a population to restore its original genotype variability after removing the source of contamination. Our results revealed dramatic changes in the population genotype diversity due to heavy metal pollution. More intriguing, we did not detect any return to the normal variability after several years of natural recovery in absence of source of pollution. Latest understanding of the long-term effects of heavy metal pollution on populations' genotype diversity will be presented in order to discuss its role in species' evolution and extinction.

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AO3. CYTOTOXIC ACTIVITY OF NON-STEROIDAL ANTI-INFLAMMATORY AGENTS, BILE ACIDS AND THEIR METAL COMPLEXES

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AP1. TARGETED THERAPY OF BREAST CANCER: THE MAGIC BULLETS OF MODERN ONCOLOGY

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AP2. NANOPARTICLES IN TARGETED CANCER THERAPY

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A04. MACROSCOPIC EVALUATION OF THE PROTECTIVE EFFECT OF ARONIA MELANOCARPA FRUIT JUICE IN A MODEL OF TRINITROBENZENESULFONIC ACID-INDUCED COLITIS IN RATS

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Trinitrobenzensulfonic acid (TNBS) is an agent commonly used to induce experimental colitis in animals.

The aim of this study was to evaluate the effect of *Aronia melanocarpa* fruit juice (AMFJ) in a rat colitis model using criteria for macroscopic scoring of colonic damage and to compare AMFJ effect with that of sulfasalazine.

Male Wistar rats (200-250 g) were divided into 6 experimental groups, each of 12 rats: Control, TNBS, TNBS+$AMFJ_{2.5}$, TNBS+$AMFJ_5$, TNBS+$AMFJ_{10}$ and TNBS+S. Colitis was induced by TNBS (10 mg dissolved in 0.25 ml of 50% ethanol) applied in the colon by a soft cannula at a depth of 10 cm from the anus. Control rats received 0.25 ml of 50% ethanol. The oral treatment of the animals began on the 2nd day (24 hours after the induction of colitis) and lasted till the 14th day of the experiment. The animals were treated orally using an orogastric cannula with distilled water (groups Control and TNBS), AMFJ at doses of 2.5 ml/kg, 5 ml/kg and 10 ml/kg (groups TNBS+$AMFJ_{2.5}$, TNBS+$AMFJ_5$, TNBS+$AMFJ_{10}$) or sulfasalazine at a dose of 400 mg/kg (group TNBS+S). On the 15th experimental day the severity of colitis was evaluated using macroscopic criteria: colon length, colon weight, colon weight/length ratio, wall thickening, area of necrosis and adhesions.

The results showed that TNBS caused a significant shortening of the colon ($p<0.05$), a tendency to increase colon weight, a significant increase of the weight/length ratio ($p<0.05$), a significant thickening of the colon wall ($p<0.05$), adhesions of the colon to the other organs ($p<0.05$) and a significant area of necrosis ($p<0.05$). AMFJ at all the doses improved the macroscopic signs of colitis to such an extent that the measured indices did not significantly differ from those of the controls with the exception of the wall thickness of TNBS+$AMFJ_{2.5}$ group which was significantly higher than the control one ($p<0.05$). In sulfasalazine treated rats all macroscopic indices of colon damage were not significantly different from those of the control animals.

In conclusion, AMFJ decreased the TNBS-induced damage in the experimental model of colitis. The effect of AMFJ was comparable to that of sulfasalazine. The effect of AMFJ in this experiment might be the result of its potent antioxidant and anti-inflammatory properties.

Key words: TNBS, colitis, *Aronia melanocarpa* fruit juice, sulfasalazine, rats

A05. BIOCHEMICAL EVALUATION OF THE EFFECT OF *ARONIA MELANOCARPA* FRUIT JUICE IN A MODEL OF TRINITROBENZENESULFONIC ACID-INDUCED COLITIS IN RATS

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In inflammatory bowel disease (IBD), experimental models have proven to be important tools for detecting potential therapeutic agents. Trinitrobenzensulfonic acid (TNBS) is an agent commonly used to induce experimental colitis in animals. Oxidative stress has been proposed as a mechanism underlying the pathophysiology of IBD. The condition is associated with the generation of reactive oxygen species that might be capable of contributing to the inflammatory response.

The aim of this study was to evaluate the effect of *Aronia melanocarpa* fruit juice (AMFJ) in a rat colitis model using biochemical markers and to compare AMFJ effect with that of sulfasalazine.

Male Wistar rats (200-250 g) were divided into 6 experimental groups, each of 12 rats: Control, TNBS, TNBS+$AMFJ_{2.5}$, TNBS+$AMFJ_5$, TNBS+$AMFJ_{10}$ and TNBS+S. Colitis was induced by TNBS (10 mg dissolved in 0.25 ml of 50% ethanol) applied in the colon by a soft cannula at a depth of 10 cm from the anus. Control rats received 0.25 ml of 50% ethanol. The oral treatment of the animals began on the 2nd day (24 hours after the induction of colitis) and lasted till the 14th day of the experiment. The animals were treated orally using an orogastric cannula with distilled water (groups Control and TNBS), AMFJ at doses of 2.5 ml/kg, 5 ml/kg and 10 ml/kg (groups TNBS+$AMFJ_{2.5}$, TNBS+$AMFJ_5$, TNBS+$AMFJ_{10}$) or sulfasalazine at a dose of 400 mg/kg (group TNBS+S). On the 15th experimental day serum, colon, pancreas and liver were taken for biochemical investigation. Thiobarbituric acid reactive substances (TBARS) in colon, liver, pancreas and serum were measured as markers of lipid peroxidation. Serum liver enzymes were used as markers of liver function, creatinine and urea were measured as markers of kidney function.

The results showed that the concentrations of TBARS in colons of rats belonging to group TNBS were significantly higher ($p<0.05$) in comparison with the control level. Colon TBARS concentration of rats belonging to groups TNBS+$AMFJ_{2.5}$, TNBS+$AMFJ_5$, TNBS+$AMFJ_{10}$ and TNBS+S did not differ significantly from the control level. The concentration of TBARS in serum, liver and pancreas were not significantly different in all experimental groups. There were no statistically significant differences in the liver enzyme activities as well as creatinine and urea concentrations of rats belonging to the control group, TNBS group and the groups treated respectively with the three AMFJ doses and sulfasalazine.

In conclusion, TNBS created oxidative stress in colon tissue resulting in a higher level of lipid peroxidation. AMFJ at the three used doses as well as sulfasalazine prevented the increase of TBARS in rat colons. That effect might be the result of the pronounced antioxidant properties of AMFJ and could contribute to its protective effect in the rat colitis model.

Key words: TNBS, colitis, *Aronia melanocarpa* fruit juice, biochemical markers, rats

AO6. STUDY ON THE EFFECTS OF ALKALINE EARTH METALS ON THE ACTIVITY OF AMINOPEPTIDASE A IN NORMAL AND TUMOR HUMMAN MAMMARY GLAND-DERIVED CELLS

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Aminopeptidase A (APA, EC 3.4.11.7) or glutamyl aminopeptidase is a membrane-associated Zn-dependant enzyme of M1 family, catalyzing the hydrolysis of aspartate and glutamate from the N-terminal of peptide substrates. The enzyme is activated by Ca^{2+}-ions [5]. APA is a part of the central and local renin-angiotensin systems (RASs) where it hydrolyses angiotensin II (AngII) to AngIII thus controlling the tissue levels of these effector molecules. Additionally, it is well known that AngII participates in the mechanisms of tumor genesis and progression [3] whereas AngIII is recently recognized as the central effector peptide in the brain RAS for the control of blood pressure [6]. Nowadays, the participation of APA in the pathogenic mechanisms of tumor diseases is a subject of increasing scientific interest [reviewed in 5]. Different studies show that the enzyme participates in the angiogenesis [4] and growth of solid tumors [2, 4]. In breast cancer, APA activity has been shown to decrease in comparison to the adjacent healthy tissues [1]. However, the mechanisms of APA involvement in breast cancer are largely unknown.

The aim of the present study is to follow up the changes in APA activity in three human cell lines – MCF-10A (non-malignant mammary gland epithelial cells), MCF-7 (low invasive mammary gland carcinoma) and MDA-MB-231 (highly invasive mammary gland carcinoma) using the newly developed specific enzyme substrate α-Glu-2-aminoacridone (Glu-AMAC) in the presence of alkaline earth metal ions – Ca^{2+}, Sr^{2+} or Ba^{2+} as enzyme activators.

Using the above substrate in the presence of calcium ions, the specificity constant of APA (V_{max}/K_m) increased in the order MCF-10 < MCF-7 < MDA-MB-231 to show that the catalytic efficiency of the enzyme increased with increasing the invasiveness of the tumor cell line. Similar relationship was observed in the presence of barium but not strontium ions. This result indicates possible changes in the enzyme molecule in breast cancer cells, favoring its catalytic efficiency. It might represent a cell response to the reduced amount of enzyme molecules in pathologically altered tissue.

The Michaelis-Menten constant (K_m) is lowest when using Ca^{2+} as APA activator in all the three cell lines. The activation of APA in MDA-MB-231 cells by Sr^{2+} or Ba^{2+} leads to an order higher K_m than the one obtained when using Ca^{2+}. These results give reason to conclude that barium and strontium ions deteriorate the binding of Glu-AMAC in the active center of APA. The values of Km in the cell line MCF-10 are linearly dependent (r = 0.9994) on the ionic radius of alkaline earth metals whereas this is not the case in tumor cell lines. This result indicates once again that the enzyme molecule is changed in tumor cells.

Our results show that the observed lower APA activity in mammary gland carcinoma is not due to a decreased ability of the enzyme to hydrolyze the substrates but to a reduced synthesis and expression of the enzyme molecule.

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Session B.

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B01. MACROSCOPIC EVALUATION OF THE EFFECT OF ANETHOLE IN A MODEL OF TRINITROBENZENESULFONIC ACID-INDUCED COLITIS IN RATS

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Trinitrobenzensulfonic acid (TNBS)-induced experimental colitis is animals is a commonly used model to investigate the pathogenesis of inflammatory bowel disease.

The aim of this study was to evaluate the effect of anethole (AN) in a TNBS-induced rat colitis model using criteria for macroscopic scoring of colonic damage and to compare the effect of AN with that of sulfasalazine.

Male Wistar rats (200-250 g) were divided into 6 experimental groups, each of 10 rats: Control, TNBS, TNBS+$AN_{62.5}$, TNBS+AN_{125}, TNBS+AN_{250} and TNBS+S. Colitis was induced by TNBS (10 mg dissolved in 0.25 ml of 50% ethanol) applied in the colon by a soft cannula at a depth of 10 cm from the anus. Control rats received 0.25 ml of 50% ethanol. The oral treatment of the animals began on the 2nd day (24 hours after the induction of colitis) and lasted till the 6th day of the experiment. The animals were treated orally using an orogastric cannula. Groups Control and TNBS were treated with sunflower oil (10 ml/kg). Groups TNBS+$AN_{62.5}$, TNBS+AN_{125}, TNBS+AN_{250} were treated with AN at doses of 62.5 mg/kg, 125 mg/kg and 250 mg/kg dissolved in sunflower oil to a total volume of 10 ml/kg. Group TNBS+S was treated with sulfasalazine at a dose of 400 mg/kg dissolved in sunflower oil to a volume of 10 ml/kg. On the 7th experimental day the severity of colitis was evaluated using macroscopic criteria: colon length, weight of rectum plus part of the colon (total length 10 cm from the anus), wall thickening and area of necrosis.

The results showed that TNBS caused a significant shortening of the colon ($p<0.001$ vs. Control), a significant increase ($p<0.001$ vs. Control) of weight of rectum plus part of the colon (10 cm from the anus), a significant thickening of the colon wall ($p<0.001$ vs. Control) and a significant area of necrosis ($p<0.001$ vs. Control). AN at the doses of 125 mg/kg and 250 mg/kg slightly improved the colon shortening and weight increase ($p<0.01$ vs. Control). AN at the dose of 250 mg/kg slightly reduced the wall thickening ($p<0.01$ vs. Control). The

area of necrosis was also reduced, so it was not significantly different from the Control in rats treated with AN 6.25 mg/kg, and was reduced but was still significantly different from the Control in groups TNBS+AN_{125} ($p<0.01$ vs. Control) and TNBS+AN_{250} ($p<0.05$ vs. Control). In sulfasalazine treated rats the colon length and area of necrosis were not significantly different from the control values, the weight and the wall thickening were reduced in comparison with TNBS group but were still significantly higher than that of the Control group, respectively $p<0.01$ vs Control for the weight and $p<0.05$ vs Control for the wall thickening.

In conclusion, AN slightly decreased the TNBS-induced damage in the experimental model of colitis. The effect of AN was lower than that of sulfasalazine. The effect of AN in this experiment might be the result of its antioxidant and anti-inflammatory properties.

Key words: TNBS, colitis, anethole, sulfasalazine, rats

B02. INVESTIGATION OF BIOCHEMICAL MARKERS IN TWO MODELS OF TRINITROBENZENESULFONIC ACID-INDUCED COLITIS IN RATS

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Inflammatory bowel disease (IBD) is characterized by a chronic inflammatory process of the gastrointestinal system. The condition is associated with the generation of reactive oxygen species that may be capable of contributing to or even initiating an inflammatory response. Trinitrobenzensulfonic acid (TNBS) is an agent commonly used to induce experimental colitis.

The aim of this study was to compare some biochemical markers in two colitis models resulting from administration of two different doses of TNBS dissolved in different volumes of two ethanol concentrations.

Male Wistar rats (200-250 g) were divided into 3 experimental groups, each of 10 rats: Control, $TNBS_{20}$ and $TNBS_{10}$. TNBS was applied in the colon by a soft cannula at a depth of 10 cm from the anus. TNBS at a dose of 20 mg dissolved in 0.4 ml of 40% ethanol solution was administered to group $TNBS_{20}$. TNBS at a dose of 10 mg dissolved in 0.25 ml of 50% ethanol solution was administered to group $TNBS_{10}$. Control rats were not treated. Biochemical investigations were made 24 hours after the induction of colitis. Thiobarbituric acid reactive substances (TBARS) in colon, liver and serum were measured as markers of lipid peroxidation. Serum liver enzymes were used as markers of liver function and urea was measured as a marker of kidney function.

The results showed that the concentrations of TBARS in colons of rats belonging to groups $TNBS_{20}$ and $TNBS_{10}$ were significantly higher (respectively $p<0.0001$, $p<0.001$) in comparison with the control level. Colon TBARS concentration of rats belonging to group $TNBS_{20}$ was significantly higher ($p<0.05$) than that of rats from group $TNBS_{10}$. The concentration of TBARS in serum and liver were not significantly different between the two colitis models and the control rats. There were no statistically significant differences in the

liver enzyme activities as well as urea concentrations between the two colitis groups and the control group.

In conclusion, TNBS caused a dose-dependent increase of the concentration of TBARS in rat colons. The higher TNBS dose probably created a higher level of oxidative stress in colon tissue resulting in higher level of lipid peroxidation.

Key words: TNBS, colitis, doses, biochemical markers, rats

BO3. BIOACTIVE COMPOUNDS ISOLATED FROM GARDEN SNAILS

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ABSTRACT

The recent appearance of a growing number of resistant to conventional antibiotics, has become a serious medical problem. To overcome this resistance, the development of new compounds is encouraged. Hemolymph and mucus of *Helix lucorum* and *Helix aspersa* garden snails and *Rapana venosa* marine snail are a complex mixture of biochemically and pharmacologically active components.

Glycoprotein 'hemocyanin' and antimicrobial peptides from the hemolymph and mucus are important components of the innate immunity. Some isoforms and peptides serve as effector molecules of the defense system, providing an efficient initial effect against infectious pathogens.

The *in vitro* antitumor activity of Helix and Rapana hemocyanins and their isoforms with different oligosaccharide structures was established on the bladder carcinoma permanent cell lines T-24. This is probably due to the specific oligosaccharide structures of hemocyanins which are exposed on the surface of the molecule.

Key words: Antibacterial activity, Antitumore activity, Hemocyanins, Helix lucorum, Helix aspersa, peptides,

Introduction

The Phylum Mollusca is probably the third most important animal group after the arthropods and vertebrates, forming a major part of the world fauna. Although most natural medicines are derived from plants, marine invertebrate phyla, including the Mollusca, are of increasing interest as a source of novel bioactive compounds [1,2,6,7]. Molluscs are currently used for a range of therapeutic applications, with purified or synthesised bioactive compounds developed as pharmaceuticals and crude or semi-purified extracts as nutraceuticals (Dwek et al. 2001; Dolashka-Angelova et al. 2008).

Snails belong to the class Gastropoda and land snails are one of the most numerous with almost 35,000 described species of the world. The marine snail *Rapana venosa* and garden snails *Helix aspersa* and *Helix lucorum*, from the family Helicidae are very well known species of gastropod mollusk. The hemolymph from snails contain bioactive compounds as glycans, peptides, glycopeptides, proteins. Many of them have been discovered in recent years [8,9,15,18].

Several glycoproteins as hemocyanins and lectines were isolated from the hemolymph of marine and garden snails and analysed using different methods and techniques. Recently, we identified that two structural subunits, RvH1 and RvH2, with molecular masses of 400 - 450 kDa aggregate into didecamers for *R. venosa* hemocyanin and three subunits (□-HIH, α_D-HIH and α_N-HIH) for *H. lucorum* and *H. aspersa* hemocyanins. Each structural subunit is composed of eight FUs of masses of ~ 50 kDa, which can be isolated from the structural subunits of RvH, HlH and HaH [8,9,12,18].

Moreover, we identified several novel proline-rich antimicrobial peptides with molecular masses between 3000 and 9500 Da from the hemolymph of *R. venosa* snails and garden snails *H. lucorum* showing strong structural subunits antimicrobial activities against the Gram-positive (Gram+) and the Gram-negative bacteria [10,11]. The antibacterial activity of hemolymph from *Galleria mellonella* infected with entomopathogenic strain of *Pseudomonas aeruginosa* and non-pathogenic bacterium *Escherichia coli* was also established [1]. *In vivo*, the antimicrobial activity induced by *E. coli* sustained on the high level until 48 h after infection, while the maximum level for *P. aeruginosa* reached the at 18 h postinjection.

Recently, a series of active peptides and glycoproteins with different physiological functions were also extracted from snail mucus. The amount of mucin isolated from *H.pomatia* species is higher than mucin isolated from *H. aspersa*. However, the amount of mucoproteins isolated from *H. aspersa* species after shaken overnight at 4^0C is higher than mucuproteins isolated from *H. pomatia* [15].

In recent past, people used to eat alive snails to ease heartburn: when snails reach stomach they produce mucus, contrasting acidity. Considering the role of snail mucus in repairing ulcers and thinking about the role of human mucus to prevent or fight acidity, it has been developed syrups against stomach acidity and gastric-esophagus reflux.

Several scientific researches have demonstrated that some of these bioactive compounds-derivated drugs can be used in a large variety of therapies, as in creams to ease skin abrasions and scars, to cure respiratory diseases, heartburn and at last scientists discovered unexpected and previously unknown properties [3,7,16].

The present study dealt with the analysis of the extracts of marine snail *R. venosa* and two garden snail *H. aspersa* and *H. lucorum* and their putative application.

Materials and Methods

Isolation of hemocyanins

Helix and *R. venosa* hemolymphs were isolated from the leg of collected snails, solubilized in 50 mM sodium acetate buffer, pH 5.8, and the hemocyanin was sedimented as described by [9,15,18]. After removal of the blue native hemocyanin pellet, the supernatant was lyophilized.

Isolation of peptides from the mucus

Collected mucus from the snails were lyophilized and separated using Milipore filters (3 and 10 kDa). The lyophilized supernatant from the hemolymph was also separated using the same filters. Three fractions were obtained: Fraction A (masses between 0-3 kDa), Fraction B (masses between 3-10 kDa), and Fraction C (masses above 10 kDa).

Fraction B was lyophilized and then applied on a Nucleosil C18 column, equilibrated with 0.10% trifluoroacetic acid (TFA, v/v) (solution A). Elution was performed with a linear gradient formed by solutions A (0.1% TFA/water) and B (80% acetonitrile in 0.1% TFA (v/v)) at a flow rate of 1.5 ml/min, over 60 min. Ultraviolet absorption was monitored at 214 nm. The eluted fractions were collected and lyophilized. The fractions were reconstituted in Milli Q water containing 0.10% TFA (v/v). The molecular masses of isolated fractions were measured by an Autoflex™III, High-Performance MALDI-TOF & TOF/TOF System (Bruker Daltonics).

Amino acid analisys of mucus

Approx. 1-2 mg of sample B was weighed accurately in a hydrolysis vial, solved in 800µL 6N HCl, closed under vacuum (< 10mbar) and was hydrolysed for 24 hours at 110°C. The HCl was evaporated and the sample was solved in sample dilution buffer to obtain a concentration of approx. 1 mg sample per ml buffer.

Antibacterial assays of the hemocyanins and their isoforms

The antimicrobial activities of the isolated fraction from the mucus of *H. aspersa,* were tested against two Gram+ strains, *Propionibacterium acnes* (strain 266 (IA) and *Propionibacterium acnes* KPA171202) and two Gram- bacteria (*E.coli* NBIMCC and *Helicobacter pylori*). The samples were qualitatively tested according to the growth inhibition assay. Antimicrobial assays of isolated Fractions were obtained on agar plates containing the likewise Gram+ and the Gram- bacteria. Each fraction was spread on agar medium with two different amounts (5 and 20 µl of the sample solutions). The incubation was for 24-36 h at 37°C**.**

Antiproliferative activity of the tested hemocyanins

Experiments were carried out with one commercially available permanent human tumor cell line from different stages of human urinary bladder transitional carcinoma cells (TCC) :

- T-24 cells were established from the primary tumor of an 81-year-old Caucasian woman with urinary bladder cancer (transitional cell carcinoma (TCC), grade III) in 1970 producing a variety of cytokines (e.g. G-CSF, IL-6 and SCF) with a p53 mutation.

The T-24 cells were cultured in Dulbecco Modified Eagle's Medium (DMEM, Lonza, Austria), supplemented with 10% fetal bovine serum (Gibco, Austria), 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 1% non-essential amino acids in 75 cm^3 tissue culture plastic flasks (Falcon).

The T-24 tumor cells were treated for 24,48, and 72h, respectively, with various concentrations (0.25 and 1.0 mg/ml) of the test substances, doxorubicin (DOX, 0.1mg/ml positive control), and cultured medium (negative control). The antiproliferative activity of the tested hemocyanins on T-24 cell line of native molecule of RvH and HlH and their isoforms, subunits and functional units, on cell viability were assessed in 50 mM Tris/HCl buffer, pH 8.0, using the WST-1 and BrdU ELISA assays (Roche Diagnostics, Germany).

Results

In the recent years, the extracts from marine and garden snails were analysed and was found that there are very rich sources of bioactive compounds. Several relatively small antimicrobial peptides and the much larger protein hemocyanins are isolated from the hemolymph of molluscs [6,14,19]. We investigated the structures and properties of hemocyanins isolated from the marine snail *Rapana venosa* (RvH) and the garden snail *H. lucorum* and *H. aspersa*, and studied their structural and functional units (FUs) using different techniques [13-16]. In this study we represent the properties and antitumore, and antimicrobial activities of bioactive compounds isolated from hemolymph and mucus of garden snail *H. aspersa* [9,18].

Purification of bioactive compounds

After collection and purification the mucus from garden snail *H. aspersa* was subdivide into three fractions: Fraction A (masses between 0-3 kDa), Fraction B (masses between 3-10 kDa), and Fraction C (masses above 10 kDa), using Millipore filters with a cut-off of 3 and 10 kDa, respectively.

Upon testing their antimicrobial activity on an agar medium after incubation for 24-36 h at 37°C, only Fraction B (masses between 3-10 kDa), appeared to generate a zone of inhibition of bacterial strains of *Propionibacterium acnes* (strain 266 (IA), and KPA171202), *Helicobacter pylori* and *Echerishie coli* NBIMCC 3486 (not illustrated). Therefore, Fraction B was purified and the structures of some compounds were analyzed.

Table 1. *Amino acid composition of Fraction B from the mucus of garden snail Helix aspersa*

Amino acid	µg/mg	Amino acid	µg/mg
Asp	52.611	**Met**	7.350
Thr	23.271	**Ile + allo-Ile**	21.995
Ser	21.237	**Leu**	36.921
Glu	63.277	**Tyr**	14.372
Pro	25.108	**Phe**	21.396
Gly	38.474	**His**	15.317
Ala	27.497	**Lys**	29.882
Cys(O_3H) + Cys +Cys_2	2.758	**Trp + deg. prod. Trp**	0.000
Val	25.532	**Arg**	21.915
		TOTAL:	**448.913**

High concentrations of Asp, Glu, Gly, Leu, Pro and Lys were calculated by the amino acid analyses of Fraction B (Table 1).

Fraction B was applied on a Nucleosil 7 C18 column (Figure 1) and fourteen fractions were eluted by reversed-phase column chromatoghy.

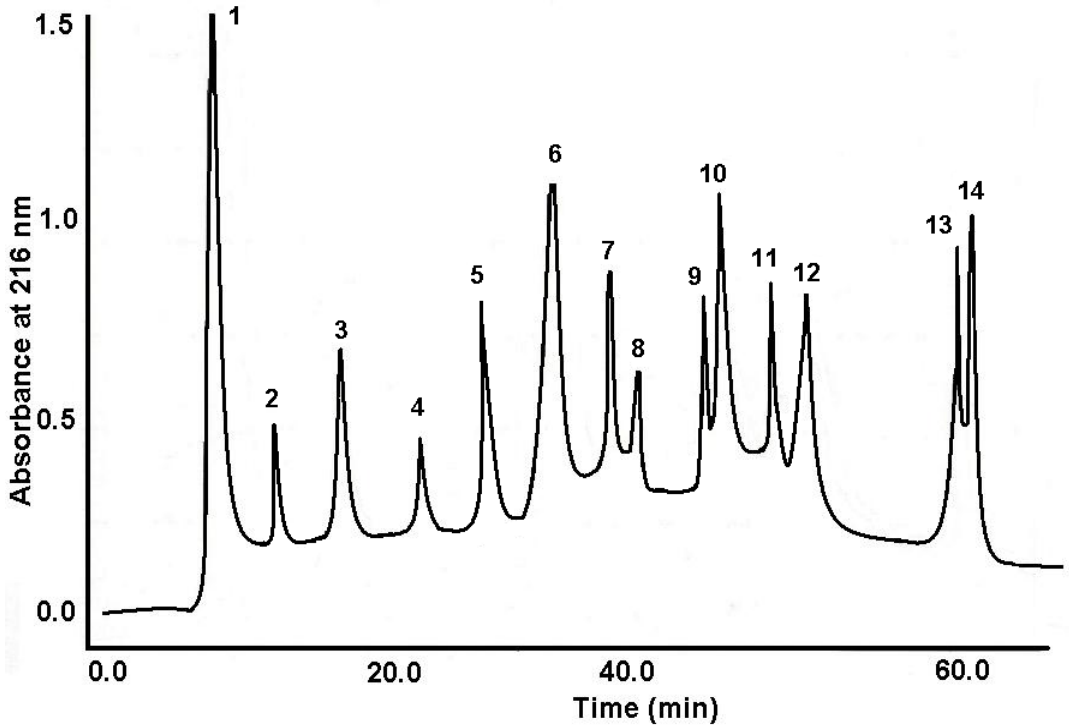

Figure 1. *HPLC purification of peptides fromFraction B of the mucus of garden snail Helix aspersa on a Nucleosil 7 C18 column (250x10 mm; Machery–Nagel, Duren, Germany) using the following conditions: eluent A, 0.1% trifluoroacetic acid; eluent B, 80% acetonitrile in A; gradient program, 15% B for 5, followed by 15–100% B in 55 min at a flow rate of 1 ml/min.*

They were additionally purified on the same column and analysed by orcinol–sulphuric test. As is shown on figure 2 (spot 3) no brown colour was observed for the hemolymph of crab *Eriphia verrucosa*.

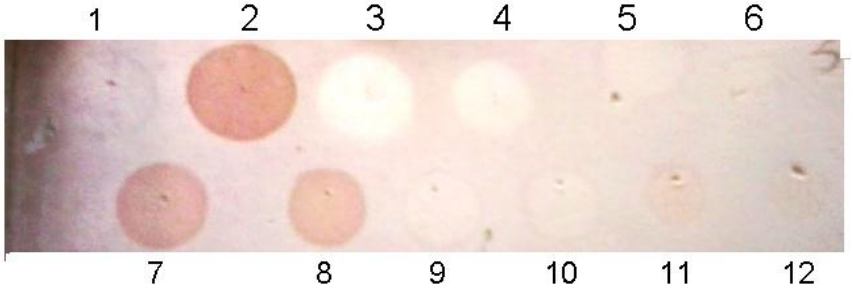

Figure 2. *Orcinol–sulphuric acid test of peptides eluted by HPLC and applied on to a silica-gel plate. The spots on positions: 1). Whater; 2). Glucose 3); Eriphia verrucosa Hc < 10 kDa; 4). Fraction 2 of mucus < 10 kDa; 5). Fraction 3 of mucus < 10 kDa; 6). Fraction 4 < 10 kDa; 7). Fraction 5 < 10 kDa; 8). Fraction 6 < 10 kDa; 9). Fraction 7 < 10 kDa; 10). Fraction 8 < 10 kDa; >10 kDa; 11). Fraction 9 < 10 kDa; 12). Fraction 10 < 10 kDa*

However, the acid test shows that peptides eluted as Fraction 5 (spot 7) and Fraction 6 (spot 8) by HPLC change the colour in brown on spots 7 and 8 on a silica-gel plate. No brown colour was observed for the other fractions isolated by HPLC. Therefore, Fraction 5 was analysed by MALDI-TOF. A mass spectrum of Fraction 5, containing peptides with masses between 2 and 10 kDa, is shown in **Fig. 3.**

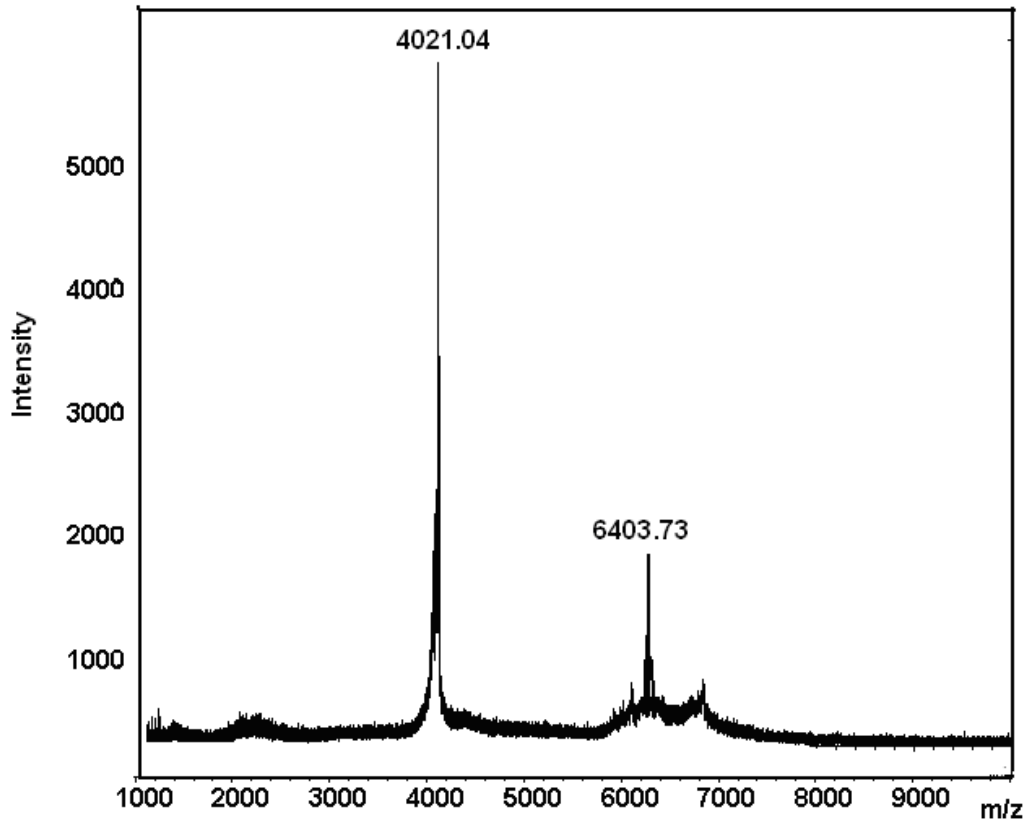

Figure 3. *MALDI spectrum of Fraction 5 from the mucus of garden snail Helix aspersa purification on a Nucleosil RP C18 column (Figure 2). The sample was measured by MALDI-TOF Ultraflex II (Bruker Daltonics, Bremen, Germany).*

The molecular masses of the isolated peptides were determined by MALDI/MS. Two main ions were identified on MS spectrum on Fraction 5, revealed a main ion at m/z 4021.04 $(M+H)^+$ and ion at m/z 6403.73 $(M+H)^+$ **(Fig. 3).**

Antibacterial activity of peptides from the mucus of garden snail Helix aspersa

In vivo, the antimicrobial activity of fractions isolated from the mucus of garden snail *H. aspersa* were tested against different species of Gram+ (*Propionibacterium acnes* strain 266 (IA) and *Propionibacterium acnes* KPA171202) and two Gram- bacterium (*E.coli* NBIMCC and *Helicobacter pylori*). The organisms were chosen because they are human pathogenic bacteria and commonly used in antimicrobial tests. The results show that Fraction 2 exhibited inhibition effect on growth of the bacteria *Propionibacterium acnes* strain 266 (IA) and *Helicobacter pylori* (Fig. 4 A,B).

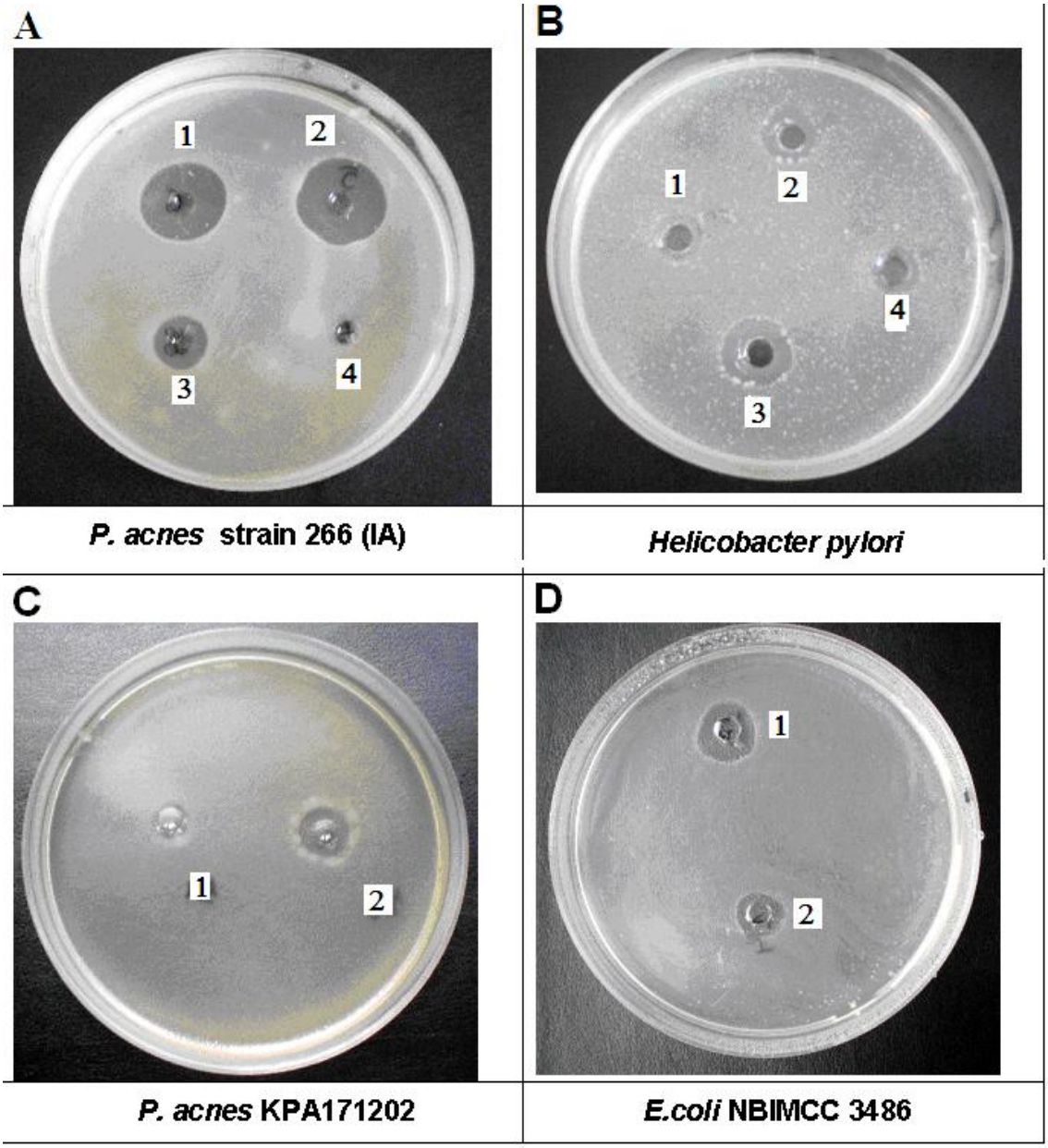

Figure 4. *Antibacterial effect of different fractions: position1)Fraction A; position 2. Fraction B; position 3) Fraction C; position 4) Water) of mucus from the snail Helix aspersa against: A) Propionibacterium acnes (strain 266 (IA) and B) Helicobacter pylori. Antibacterial activity of (1) Fraction 5 and (2) Fraction 6 against C) Propionibacterium acnes KPA171202 and D) E.coli NBIMCC 3486.*

To explain the observed effects of the mucus against various bacteria, the peptides and glycopeptides of mucus were purified by high-performance liquid chromatogram (Fig. 1), and the antibacterial effect of the resulting pure fractions was also tested against *Propionibacterium acnes* KPA171202 and *E.coli* NBIMCC 3486. Fig. 4 C and D shows the antibacterial effect of the two peptides with masses of 4021.04 and 6403.73 Da, isolated from the mucus of the snail, which to varying degrees affect the *Propionibacterium acnes* KPA171202 and *E.coli* NBIMCC 3486.

Antitumore activity of hemocyanins isolated from the hemolymph of snails

After purification of the native hemocyanin from *R. venosa*, *H. aspersa* and *H. lucorum* hemolymphs and dissociation against 0.13 M Glycine buffer, pH 9.0, three isoforms were identified by electrophoresis (data not shown). The direct *in vitro* effect of the isolated

hemocyanins with concentration of 500 μg/ml on bladder cancer cell lines T-24 were evaluated in a number of experiments lasting 24h, 48h and 72h. The effects of the native molecule of molluscan Rapana, Helix and keyhole limpet hemocyanins, structural subunits and functional units on cell line T-24 are presented in Fig. 4.

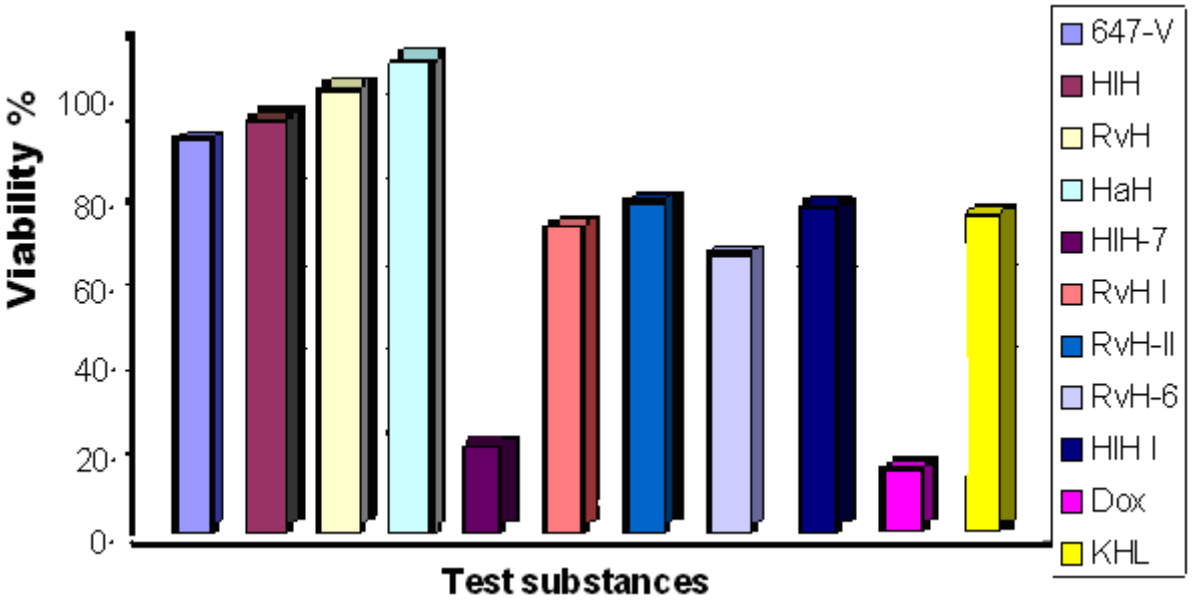

Figure 4. *Effect on the human tumor cell lines T-24 after 72h of incubation with native molecule of RvH, HaH and HlH, structural subunits and functional units with concentration of 500 μg/ml in the presence of negative control and positive controls (Doxorubicin hydrochlorid and KLH).*

From the Fig.4 it is clearly visible that only HlH showed a cytotoxic effect after 72 h of incubation compared to the native molecules of others Hcs. Very slight inhibition effect was observed after 72 h of treatment of T-24 cells with subunits RvH I, RvH II and HlH I. In the opposite, the lack of cytotoxic effect, even stimulation was measured with the native molecule of RvH, HaH and KLH. However, two functional units exhibited very high inhibition effect, FU RvH-6 and HiH-7. A cytotoxic effect after 72 h of incubation of HlH-7 was similar to Doxorubicin hydrochlorid (21%).

We have observed an antiproliferative effect of the functional units isolated from RvH and HlH against bladder cancer cell line T-24. The effect was found to be dose- and time-dependent and similar to the effects of doxirubicin. Therefore, the effect of this FU was studied additional.

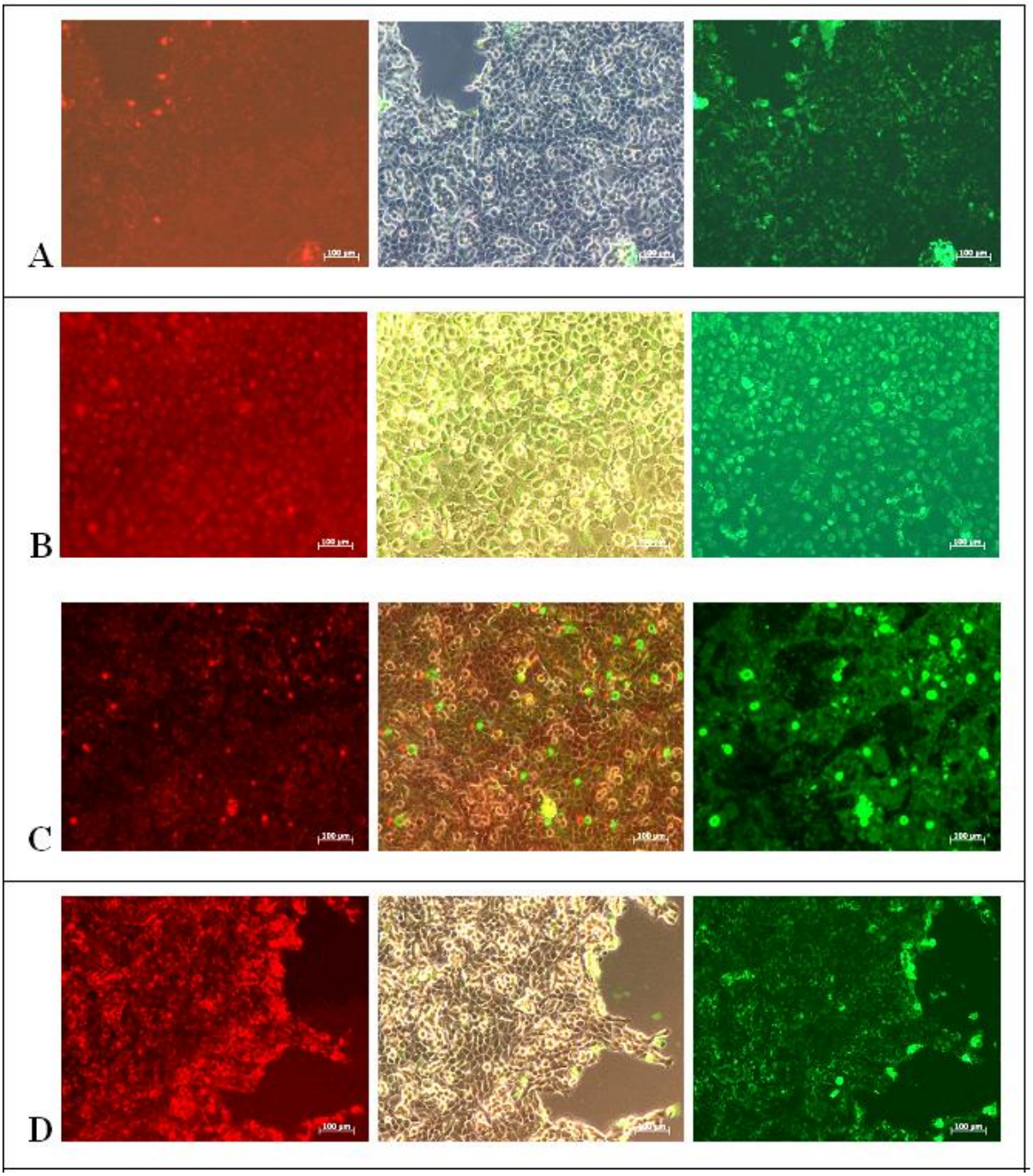

Figure 6. *Fluorescence of T-24 cancer cells treated with Annexin-V-Fluos Kit and PI and incubated for 24 h with 1,5 mg/ml of RvH1 and 1.0 mg/ml of RvH1-c. Cells were cultured in DMEM and maintained at 37o C and 5% CO2: (A) Fluorescence microscope photographs of T-24 control cells without treatment. (B) T-24 cells: treated with mg/ml of doxorubicin, (C) with RvH1, (D) with RvH1-6. Left side micrographs, red filter (PI fluorescence, red); center: merged images of red and green fluorescence and day light; right side micrographs, green filter (Annexin-V-FLUOS fluorescence, green).*

To identify, if the subunit RvH1 and FUs of RvH1 induced reduction in viability of T-24 tumor cells *via* apoptosis, they were incubated with the cells and stained using Annexin-V-Fluos Kit and co-stained with PI. Figure 5 shows fluorescent micrographs of T-24 cells after 24 h incubation with the hemocyanins tested. The left side shows micrographs with necrotic cells (PI fluorescence, red), in the center are merged images of the red and green fluorescence

and day light and the right panel demonstrates apoptotic cells (Annexin-V-FLUOS fluorescence, green). The results was compared with the control (non-treated cells) and doxorubicin-treated cells (Fig. 5A,B).

After treatment of T-24 cells with the structural subunit RvH1 (Fig.5C), both populations were found in the wells – apoptotic cells fluorescing in green only and bright green cells with bright red nuclei. These cells could be late apoptotic or necrotic as well. The same behavior was observed after treatment of the cells only with one FU RvH1-c, identified, as described below (Fig.5D).

Discussion

Recently, a series of active peptides and glycopeptides with different physiological functions were extracted from marine molluscs [6,15,17]. Somel peptides/proteins from the hemolymph of molluscs and arthropods were also found to exhibit a broad-spectrum of microbial activity against Gram-positive (Gram+) and Gram-negative (Gram-) bacteria and yeast.

We have isolated and analysed several bioactive compounds, peptides, glycopeptides, hemocyanins, from marine and garden snails [8,9,12,18]. Biochemically and pharmacologically active peptides in the hemolymph of garden snail *Helix lucorum* and marine snails *R. venosa* were analysed [11,13]. Some of them, rich in Cys, Pro, Ser or Gly residues showed high antimicrobial activity against *S. aureus* and low activity against *Klebsiella pneumoniae* [11].

Here we represent the peptides isolated from the mucus of garden snail *H. aspersa.* It is known that mucus has lots of active compounds, many of them have been discovered even in early history and in recent years scientific researches have demonstrated that mucus-derivated drugs can be used in a large variety of therapies [4,7]**.**

Here we represent two glycosilated peptides with mass of 4021.04 and 6403.73 Da, isolated from Fraction B (2-10 kDa) on a Nucleosil column. Antibacterial test on these peptides and Fraction B against Gram+ (*Propionibacterium acnes* strain 266 (IA) and *Propionibacterium acnes* KPA171202) and two Gram- bacterium (*E.coli* NBIMCC and *Helicobacter pylori*) showed the inhibition activity of Fraction 2 on growth of the bacteria *Propionibacterium acnes* strain 266 (IA) and *Helicobacter pylori* and the activity of two peptides on *Propionibacterium acnes* KPA171202 and *E.coli* NBIMCC 3486.

It is possible that the Gly- and Pro-content in peptides plays a structural role in the activity against this bacteria [16]. Understanding the function and mechanism of action of the new antibacterial peptides from the mucus of *H. laspersa* may contribute to the potential of this compound in anti-infection therapeutics.

Glycoproteins with antitumore activity

Hemocyanins from mollusks are very well known as immunostimulators, and possess an antimicrobial, an antifungal, an antiviral and an antitumore activities [5,6,14,17,19].

In vitro experiments were performed to compare the anti-tumor activities of the native molecules of *R. venosa*, *H. lucorum* and *H. aspersa* and some isoforms, and optimal doses that arrest T-24 acancer cell and benign urothelial cell growth. Alterations in the cell morphology of the treated and untreated cells were observed. Comparison of the actions of the structural and the functional units RvH, HlH and HaH on T-24 tumor lines and benign urothelial cells show that treatment with the functional unit RvH1-c is most effective after the 72 h application on tumor cells without disturbing the metabolism and proliferation of normal cells. The potent inhibiting activity of the functional unit is probably due to its specific oligosaccharide structures. Functional units, RvH1-c, shows the most significant inhibitory effect against T-24 bladder carcinoma cells, which is comparable to the antitumoral activity of doxorubicin without disturbing the metabolic activity or proliferation of normal HL 10/29 urothelial cells. Cells treated with RvH1-c showed mostly apoptotic and less necrotic cell populations, and lots of cells were observed in the medium which lost adherence. These cells could be late apoptotic

or necrotic as well, but there is no accurate fluorescence test which could identify the difference between these two types of cell deaths at this stage.

This exciting efficacy of a natural glycoprotein needs, as next, to be confirmed by animal trials, and experiments to elucidate the mechanism of action which are in our pipeline.

ACKNOWLEDGEMENT

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BO4. N- Linked carbohydrate structures of molluscan hemocyanins from snails

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Abstract

Molluscan hemocyanins (Hcs) have recently particular interest due to their significant immunostimulatory properties. This is mainly related to their high carbohydrate content and specific monosaccharide composition. We present comparative studies in oligosaccharide structures of structural subunits from *Rapana venosa* (RvH), *Haliotis tuberculate* hemocyanin and *Helix lucorum* hemocyanin by mass spectrometry.

Two approaches were applied to analyse the isolated glycans. The first approach included sequencing of the glycans by specific glycosidases and analysis of the fragments via MALDI-TOF-MS before and after treatment with the enzymes giving only preliminary results about the structures of the glycans. Therefore, the second approach, tandem mass spectrometry was applied, and the glycan structure being derived from their MS/MS spectra, obtained by tandem mass spectrometry, on a hybrid quadrupole-linear ion trap mass spectrometer - ESI-Q-Trap system.

The characterization of the N-linked glycans found in HlH, RvH and HtH, in this study, is revealed in part novel structural motifs which might contribute to the pronounced immunogenicity of this gastropod glycoprotein. It is obvious that gastropods have a wide capacity to modify the basic biantennary N-glycan structure with many species-specific peculiar structures. The oligosaccharide moieties found in HlH, RvH and HtH are a potential

source of novel N-glycans that are important for the stimulation of the immune response and/or for the production of antibodies used in diagnosis and therapy.

Keywords: molluscan hemocyanins (Hcs), Rapana venosa (RvH), Haliotis tuberculate hemocyanin (HtH), Helix lucorum hemocyanin (HlH), structural subunit; mass spectrometry; ESI-Q-Trap; N-glycans.

BO5. MICROBIOLOGICAL ACTIVITY OF Cu(II) AND Zn(II) COMPLEXES OF A NEW BENZANTHRONE TRIPOD

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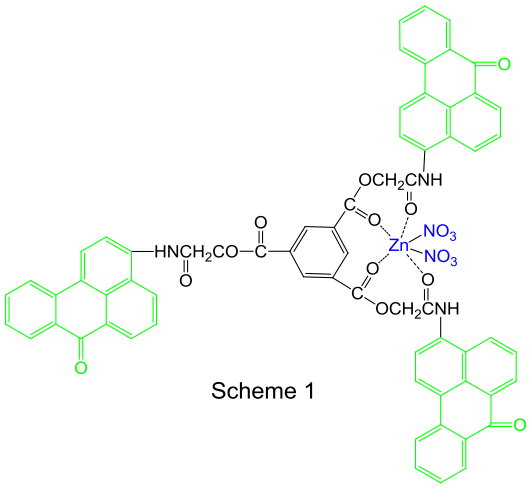
A new green fluorescent tripod has been synthesied by reaction of 2-Chloro-N-(7-oxo-7H-benzo[de]anthracene-3-yl)-acetamide and bnzene-1,3,5,-tricarboxilic acid and its Zn(II) complex has been also synthesized [Zn(BT)$(NO_3)_2$] (Scheme 1). The chemical structures of both compounds were confirmed and analyzed by electronic (UV/Vis and fluorescence), Fourier transform infrared (FTIR), nuclear magnetic resonance (NMR), scanning electron microscopy (SEM) and by ionization-electrospray mass spectrometry (API-ES-MS).



Scheme 1

The inhibitory effect of [Zn(BT)$(NO_3)_2$] was evaluated against model yeasts and Gram (+) and Gram (-) bacterial strains. The compound was found more effective against Gram-positive test cultures in comparison to Gram-negative, with the lowest MIC_{90} of 450 μg/ml determined against *B. cereus* The textile sample impregnated with [Zn(BT)$(NO_3)_2$] complex was investigated for antimicrobial activity against bacterial strains *B. cereus*, *P. aeruginosa* and *E. coli* (Figure 1).

The results demonstrated good antimicrobial effect of the obtained cotton-based material against *B. cereus* (about 64% growth reduction). The cotton sample caused only about 5% reduction of the growth of *E. coli*, and didn't inhibit the growth of *P. aeruginosa*. The antimicrobial effect should be due to the release of $[Zn(BT)(NO_3)_2]$ from the cotton textile by diffusion.

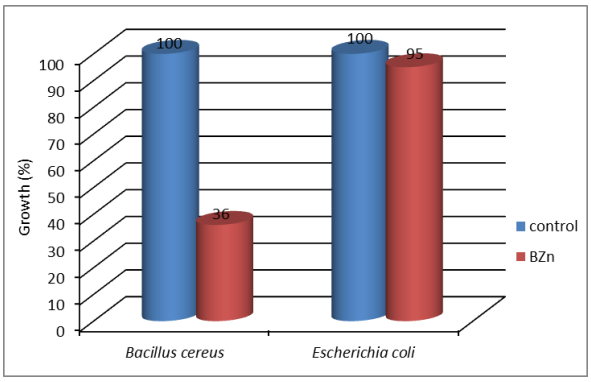


Figure 1

The results suggest their potential application in biomedicine in designing new effective antimicrobial preparations.

Acknowledgment: The authors, acknowledge with thanks to COST-CA15114: Antimicrobial coating innovations to prevent infectious diseases (AMICI)

BO6. ACTIVITY OF WATER EXTRACT FROM *NEPETA NUDA* L. AGAINST ACV-RESISTANT HUMAN HERPES VIRUS TYPE 2

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Herpes simplex virus type 2 (HSV-2), a human pathogen, is a member of the large family of Herpesviridae. HSV-2 infection is usually transmited sexually and can cause recurrent, painful genital ulcer. In neonate the infection is associated with significant morbidity and mortality. Moreover, HSV-2 infection increase the risk of human immunodeficiency virus (HIV) acquisition. For treating of herpesvirus infections there are about 11 licensed antiherpetic drugs. (2). The most commonly used ones are the nucleoside analog acyclovir, its derivatives and cidofovir (3). Unfortunately, continuous therapy leads to a selection of resistant strains (6). Current data indicate the existence of mutant clinical strains, with cross-resistance and double-crossed resistance against these antiviral drugs (7). This requires development of new antivirals, which are pointed to other viral targets. Moreover, the toxicity associated with some antivirals limits their use, and therefore less toxic and more effective drugs are needed. For these reasons a special attention is focused on compounds with natural origin. Plant extracts have complex chemical structure, lower cytotoxicity and due to this the occurrence of resistant strains against their action is delayed.

The genus *Nepeta* (Lamiaceae), comprises about 250 species distributed in the central and southern parts of Europe, Asia and the Middle East. *Nepeta* species are widely used in folk medicine because of their antispasmodic, expectorant, diuretic, antiseptic, antitussive, antiasthmatic and febrifuge activities. (1,5,10).

We are studied the antiviral activity of water extract fom *Nepeta nuda* L. derived from *in vivo* propagated plants. The cytotoxicity was tested on Madin Darby Bovine Kidney (MDBK) cell line. Maximal nontoxic concentration (MNC) and cytotoxic concentration (CC50) of the extract was determined by colorimetric method (MTT assays) (4) The results were measured at 48 hour and 72 hour after adding of the extract. Maximal nontoxic concentration (MNC) of the extract determined at 48 hour is ≈ 4 mg/ml and cytotoxic concentration (CC50) is ≈8 mg/ml. Results obtained for MNC and CC50 at 72 hour after adding of the extract are 2 mg/ml and 4.96 mg/ml, respectively. To determine the antiviral activity of the extract against HSV-2 strain DD-RRR we used modification of MTT assays at low MOI (9) (effect was expressed as % of protection). As a long term experiment (results are measured 5-6 days p.i.) we used the values for MNC measured at 72 hour after adding of the extract. Water extract from Nepeta nuda inhibited significantly the replication of HSV-2, strain DD-RRR. The percentage of protection is up to 65% (IC50 is ≈ 0.919 mg/ml). We conduct also a yield-reduction assay at high MOI (8). As long as this experiment is terminated at the 24th hour this allowed us to use MNC measured at the second day (effect was expressed as % of inhibition). Inhibition yield production reached ≈ 92 % at 4.5 mg/ml. There was almost no activity at 2 mg/ml. Further we tested the direct inactivating effect of the extract against extracellular form of the HSV-2, strain DD-RRR. The extract did not show any change in the virus titer.

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BP1. SYNTHESIS, CHARACTERIZATION AND ANTIMICROBIAL ACTIVITY OF 4F COMPLEXES CONTAINING THE ANTI-INFLAMMATORY DRUGS ISOXICAM AND TENOXICAM

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Six lanthanide(III) complexes with isoxicam and tenoxicam with general formula $[Ln_2(HL)_2(CH_3COO)_4]\cdot xH_2O$ (H_2L = isoxicam, tenoxicam; Ln = Pr, Nd, Gd) have been synthesized and characterized by elemental analysis, IR, UV-Vis-NIR, thermal analysis, magnetic and conductivity measurements. The antimicrobial efficiency of the complexes and the free ligands were examined by in vitro methods against a wide array of planktonic and adherent bacterial and fungal strains, including four ESKAPE pathogens, namely *E. coli, S. aureus, P. aeruginosa* and *E. faecalis*, capable of "escaping" from the biocidal action of antibiotics due to resistance mechanisms. The tested compounds were found to possess a more intensive antimicrobial activity against the Gram-positive bacterial strains as compared to the Gram-negative ones and none of them exhibited antimicrobial activity against the fungal strain. The investigation of the anti-biofilm activity of the complexes revealed a different behaviour as compared to their microbicidal properties, the MBEC values being much higher than the MIC ones, taking into account the increased resistance of biofilm embedded bacteria to antimicrobial and other limitative factors.

Acknowledgements. Financial support of UEFISCDI (PNII–PCCA 126/2012) is gratefully acknowledged.

BP2. THE MORPHOLOGY AND ANTIBACTERIAL PROPERTIES OF ELECTROSPUN NONWOVEN MATERIALS WITH SILVER NANOPARTICLES

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Polymer nanocomposites have attracted a great deal of attention due to their properties such as optical, electrical, catalytic and antimicrobial. These properties allows them to use in biomedical applications such as sensors, catalysts and antibacterial materials. Silver nanoparticles (Ag-NPs) represent one of the most extensively studied nanomaterials, due to their antibacterial activity against bacteria, optical, catalytic and sensing properties. Electrospinning allows us to form nonwoven material from nano/micro fibres. Nonwoven

materials from nano/micro fibres are widely used and studied in biomedicine for tissue engineering scaffolds, wound dressing, and etc.
The aim of this study is to form nonwoven material from nano/micro fibres containing antibacterial silver nanoparticles (Ag-NPs) by roller type "NanospiderTM" electrospinning equipment and to estimate the influence of Ag-NPs on antibacterial properties and structure of electrospun nonwoven material from polyvinyl alcohol (PVA) nano/micro fibres.
Electrospinning solutions of 15 wt% were prepared dissolving PVA polymer in distill water and stirred by magnetic stirring equipment Yellow Line MSH basic (Germany) with heating. Two different concentration of Ag-NPs suspensions were mixed with PVA polymer solution. The viscosity of solutions were estimated by viscometer BROOKFIELD DV-II+Pro Viscometer. Nonwoven materials from PVA polymer with Ag-NPs were formed by electrospinning equipment "NanospiderTM" at applied voltage U=70 kV and distance between electrodes L=13 cm. The structure of nonwoven materials, analysis of Ag-NPs were determined using SEM S-3400N. Diameter of nano/microfibers were evaluated using SEM images and software Lucia Image 5.0. Antimicrobial activity of nonwoven materials from PVA nano/microfibers with Ag-NPs was determined at the Institute of Microbiology and Virulogy of the Lithuanian University of Health Sciences. The antibacterial and antifungal activity was tested in vitro using agar diffusion method in Mueller-Hinton II agar medium (BBL, Cockeysville, USA). Antimicrobial activity of nonwoven materials from PVA nano/microfibers with Ag-NPs was tested in vitro in these standard bacterial cultures: Staphylococcus epidermidis ATCC 12228, Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853.
In this investigation nonwoven materials from PVA polymer with Ag-NPs was successfully formed using roller type electrospinning equipment "NanospiderTM". It was observed that antibacterial Ag-NPs cause the formation of thinner (fibres with diameter up to 200 nm) nano/micro fibres, however the morphology of different electrospun nonwoven materials were quite similar. The antibacterial activity test showed, that electrospun nonwoven materials from PVA nano/micro fibres have antimicrobial activity against bacteria Staphylococcus epidermidis ATCC 12228, Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853. It was also noticed that highest antimicrobial activity was observed for nonwoven materials from PVA nano/micro fibres with higher concentration of Ag-NPs.

Key words: electrospining, nonwoven material, polyvinyl alcohol, silver nanoparticles, antibacterial.

Session C.

Chairpersons:

Assoc. Prof. Julia Radenkova-Saeva, MD, PhD
Clinic of Toxicology, Department for Adult, Emergency University Hospital "N.I.Pirogov"

Assoc. Prof. Radostina Alexandrova, PhD
Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences

Secretary: Boyka Andonova-Lilova, MSc
Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences

CO1. TRACKING RESISTANCE OF COMBINATIONS OF PROBIOTIC BACTERIA IN MODEL CONDITIONS OF DIGESTION INCLUDED IN GEL OF CHITOSAN

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Abstract

The survival rate of three combinations (variants 1, 2 and 3) of probiotic bacteria in model conditions of digestion has been examined. The strains *L.bulgaricus* 1381, *Str.thermophilus* 1374 participate in all three combinations, the ratio being 1: 3, in the capacity of a classic yeast for Bulgarian yoghurt with proven synergy in between. In order to increase the sustainability and probiotic effects of the combinations, *L.acidophilus* 1379, *B.bifidum* 1370 and *L.casei* 1014 have been added into this association. As a result of the experiment it was found that variants 2 and 3 demonstrate resistance and close values of viable cells in model conditions of the gastrointestinal tract, however, they proved to be of a lower survival rate in comparison with the microorganisms in variant 1. The survival of the chosen combination of variant 1 model in terms of gastric and intestinal fluid as a free suspension has been traced and included in polysaccharide gel matrix (chitosan). Studies show that the inclusion of lactic acid bacteria in chitosan gel results in stabilization of their resistance in model digestion conditions and provides for a high concentration of viable cells.

Keywords: survival, probiotic bacteria, chitosan

Introduction

Probiotic bacteria improve the microbial balance in the gastrointestinal tract and are safe to be used in the diet of humans. In the presence of lactic acid bacteria a lactic acid fermentation takes place, which is characterized by the fermentation of sugars and production of lactate [1,5].

L.bulgaricus is not a normal inhabitant in the gastrointestinal tract of mammals. Cells are rod-shaped with dimensions 0,6-0,8 / 4,0-8,0 μm and of round edges. *L.acidophilus* is a natural inhabitant of the intestinal tract of both humans and animals. Its cells are rod-shaped

with round corners, of 0,6-0,9 / 1,5-6,0 μm dimentions. They occur as single cells, in pairs and in the form of short chains. *L.casei* has smaller dimensions than *L.bulgaricus*. Probiotic effects are expressed in modulating the activity of gut microflora and an increase of the amount of immunoglobulin in blood [1,2,3,6].

Str.thermophilus is one of the important bacteria in dairy industry. Along with *L.bulgaricus* it is used as a starter for yogurt, white brine cheese, etc. What distinguishes it from the rest of lactic acid streptococci of the *Lactococcus* genus is its thermal stability. Bifidobacteria are typical lactic acid bacteria which are normal inhabitants of the intestinal tract of humans and animals [1,7].

In recent years, the chitosan polysaccharide provokes scientific interest as a prebiotic as well as in its composition and physiological effect. It stimulates the growth of intestinal bacteria thus having the potential to be used as a prebiotic nutritious additive [4]. So far there exist scant data on the immobilization of lactic acid bacteria in chitosan. Researches in this direction are of scientific interest not only because of the fact that the chitosan polysaccharide use has been allowed for nutritious purposes but also because of the favourable impact it has on human health.

Due to the increased interest worldwide in the production of new probiotic milk-based products, constant development of new combinations of different strains of probiotic bacteria is being observed, including lactobacillus, laktococcus, bifidobacteria as starter cultures as well as different prebiotic supplements to obtain new probiotic nutrients.

The authors pursue the following experimental purposes:

1. Tracing the survival rate of cells of different combinations of probiotic strains both of lactic acid bacteria and bifidobacteria in model conditions of gastric and intestinal fluids to establish the highest level of resistance of cells under these conditions.

2. Tracing the survival rate of the selected combination of probiotic strains in model conditions of gastric and intestinal fluid as a free cellular suspension and included in polysaccharide gel matrix (chitosan).

Materials and Methods

For the purposes of the experiments freeze-dried strains of *L.bulgaricus* 1381, *Str.thermophilus* 1374, *L.acidophilus* 1379, *L.casei* 1014 and *B.bifidum* 1370 were used. They were rehydrated with physiological saline in advance and activated in a thermostat for about 30 min. Their development took place in sterile skimmed milk under conditions consistent with the specifics of cultivation of each strain.

Based on selected lactic acid bacteria strains by mixing in volume ratio of 1:3:1, three probiotic combinations were created:

• Variant 1. *L.bulgaricus* 1381, *Str.thermophilus* 1374, *L.acidophilus* 1379 - at a ratio of 1:3:1;

• Variant 2. *L.bulgaricus* 1381, *Str.thermophilus* 1374, *B.bifidum* 1370 - 1:3:1;

• Variant 3. *L.bulgaricus* 1381, *Str.thermophilus* 1374, *L.casei* 1014 - 1:3:1.

L.bulgaricus 1381, *Str.thermophilus* 1374 strains at a ratio of 1:3 participate in all the three combinations (variants 1, 2 and 3) as a classic yeast for Bulgarian yoghurt with proven synergy in between. The positive effect is expressed in the growth rate, acidifying and flavor formation. The positive effect is expressed in the growth rate, acidifying and flavour formation. Incorporation of one of the other three strains - *L.acidophilus* 1379, *B.bifidum* 1370 and *L.casei* 1014 into this association aims at increasing the sustainability and probiotic effect of combinations.

Survival rate determination of selected strains in model conditions of gastric and intestinal fluid

Combinations of strains were enriched to an initial concentration and it is on this basis that their survival rate in model conditions of gastric and intestinal fluid was determined and compared. Simulated gastric fluid was prepared by dissolving pepsin (3g/l) in a sterile saline solution (0,5%) and adjusting to pH 2,0 by addition of 1M HCl. Simulated intestinal fluid was prepared with pH 7,0 (Phosphate) containing sodium chloride (0,5%) and pancreatin (1g/l). 1ml of the cell suspension underwent incubation with 9 ml of the resulting simulated gastric and enteric fluid, respectively, for 24 hours at a suitable for the strain analyzed temperature. At the end of the 0, 4-th and 24-th hour, aliquots were taken to determine the total number of viable cells (CFU/ml).

Number of viable lactic acid bacteria determination was carried out following a limited dilution method, the latter comprising subsequent calculations in compliance with the table of McCrady. A sample of 1g (1ml) was used to prepare dilutions in physiological saline solution from 10^{-1} to 10^{-10}. The dilution was poured into three (3) tubes of 9 ml of dry, sterile skimmed cow's milk each and inoculations of 1 ml were made. Cultivation was carried out at 40-42^{o}C to result in coagulation. From the tubes with coagulated milk preparations were drawn, and after fixation, coloration and drying, they were observed by means of the immersion system of microscope "Carl Zeiss- Jena".

Incorporation of the chosen combination of probiotic strains in polysaccharide gel matrix (chitosan)

The method of mechanical incorporation in the polymer network of a hydrocolloid gel was applied. For this purpose "Chitosan" (Deacetylated chitin, Poly (D-glucosamine) of "SIGMA" company was used.

Results and Discussion

The results of the experiments with the three variants of probiotic bacteria mentioned above are presented in figures 1 and 2.

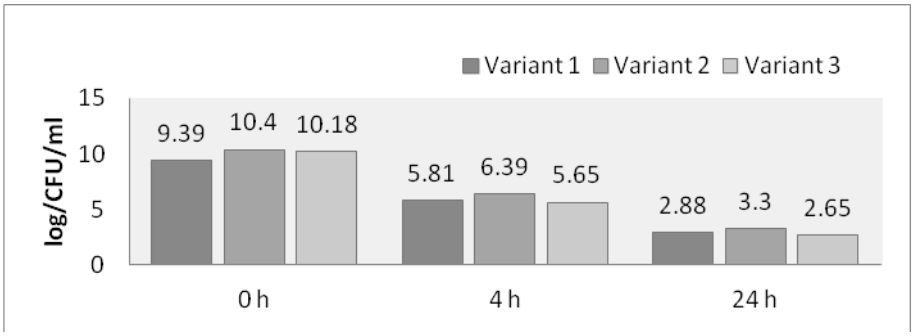

Figure 1. Total number of viable cells (log/CFU/ml) in the studied variants (1, 2 and 3) in simulated gastric fluid

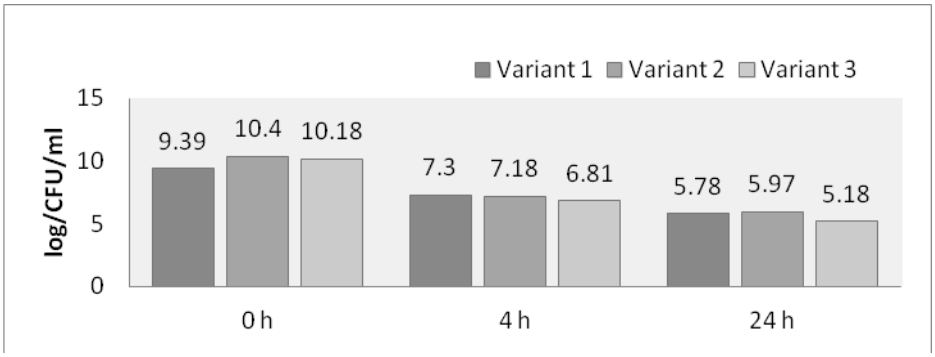

Figure 2. Number of viable cells (log/CFU/ml) in the studied variants (1, 2 and 3) in simulated intestinal fluid

In variant 1 a reduction was observed as to the the number of living cells for a 24 hours period of time in the conditions of gastric and intestinal fluid (Figure 1 and Figure 2), which is less pronounced in comparison with variants 2 and 3. In the model conditions of gastric fluid, during the 0-4 hours period of time the number of cells was reduced by 3,58 log units and during the 24 hours it was reduced to 6,51 log units. In the medium of simulated intestinal fluid, the number of living cells for 4 hours was reduced by 2,09, while for 24 hours - by 3,61 log units.

The cellular number of the **variant 2** strains was affected more significantly by the change of pH of the medium and in the conditions of simulated gastric fluid for the period from 0 to 4 hours it was reduced by 4,01, while for 24 hours it was reduced to 7,1 log units. In the medium of simulated intestinal fluid (Figure 2), the number of viable cells for 4 hours was reduced by 3,22, and for 24 hours - by 4,43 log units.

The combination of strains in **variant 3** is characterized by the highest overall sensitivity to the conditions of simulated gastric and intestinal fluid in comparison with variants 1 and 2.

In the gastric fluid medium, the number of cells in 4 hours was reduced by 4,53, and in 24 hours - by 7,53 log units. In a medium of simulated intestinal fluid, the cellular number at the fourth hour was reduced by 3,37, and at the 24th hour - by 5 log units. The results of the experiment demonstrated variants 2 and 3 resistance as well as close values of viable cells in model conditions of the gastrointestinal tract, but they had a lower survival rate in comparison with the microorganisms in **variant 1.**

The comparative results for the survival rate of experimental strains of variant 1, having been incorporated in a polysaccharide matrix of chitosan in the model conditions of gastric and intestinal fluid are shown in figures 3 and 4.

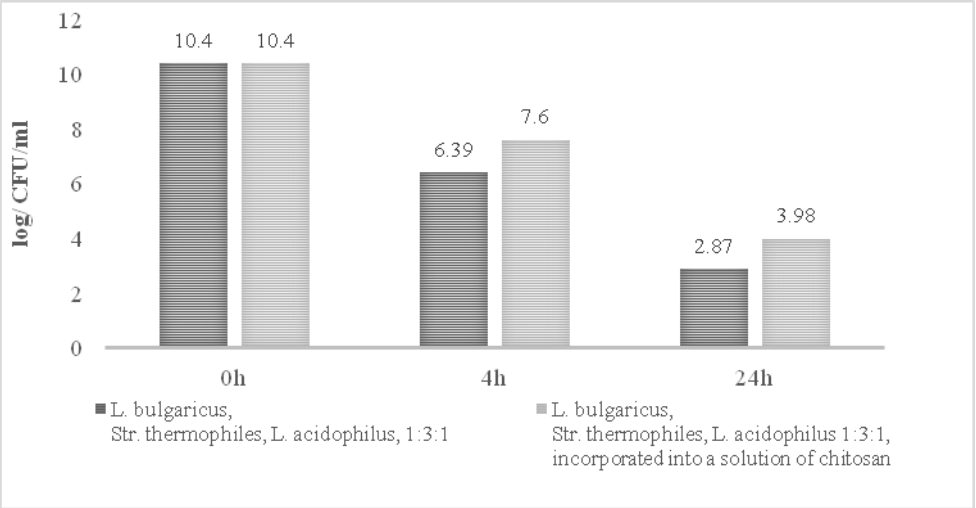

Figure 3. Total number of viable cells (log/CFU/ml) in variant 1 for simulated gastric fluid with and without chitosan presence.

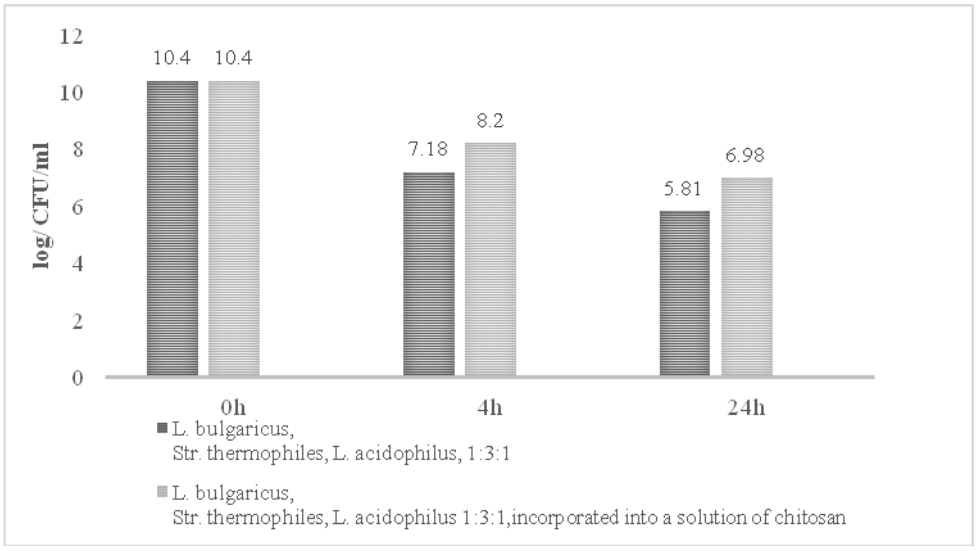

Figure 4. Number of viable cells (log/CFU/ml) in variant 1 in simulated intestinal fluid with and without chitosan presence.

When depositing a pure cell suspension under the conditions of simulated gastric fluid (Figure 3), the number of living cells in 4 hours was reduced with 4,01 log units, and in 24 hours - by 7,53 log units. In the model environment of intestinal fluid (Figure 4) the amount of living cells at the fourth hour decreased by 3,22 log units, and at the 24th hour - by 4,59 log units. Chitosan strains included in the solution demonstrated a higher resistance in a simulated gastrointestinal tract for a period of 24 hours compared to the pure strains. In the medium of gastric fluid for 4 hours, the number of live cells was reduced by only 2,8 log units, and for 24 hours - by 6,42 log units. In the medium of simulated intestinal fluid, the number of cells for 4 hours was reduced by 2,2 log units, and for 24 hours - by 3,42 log units. The experiments which were carried out showed that lactic acid bacteria *L.bulgaricus* 1381, *Str.thermophilus* 1374 and *L.acidophilus* 1379 in chitosan gel incorporation leads to a stabilization of their resistance to model conditions of digestion and an increase of their biological value.

Conclusion

It was established that variant 1 out of the three tested combinations of probiotic strains has the highest survival rate in model conditions of gastric and intestinal fluid. The incorporation of this combination in chitosan gel leads to a stabilization of cells resistance in the model conditions of digestion and an increase of the biological value. Thus, selected strains usage in the composition of probiotic products will provide for a high concentration of viable cells.

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CO2. USAGE EVALUATION OF ZINC INORGANIC COMPOUNDS AND ZINC ORGANIC COMPLEXES (CHELATES) IN REGARD TO THEIR FAVOURABLE PROSPECTS, INVOLVING OVERDOSE BIOASSAYS ON CHICKENS

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Human and animal health and food safety are top priority in EU policy, leading to risk assessment associated with the food chain. In this regard started the development and implementation of organic complexes (chelates) of minerals (zinc, copper, iron, selenium, chromium, etc.) and protein compounds that are reported to be mastered better from animals and stimulate their metabolism, immunity and productivity. Widely used zinc inorganic compounds have been identified as forage component with number of drawbacks, such as poor absorption, poor bioavailability and high environmental contamination by metals.

In the present study have been investigated the toxicity and effects of inorganic and organic zinc compounds and evaluated high doses in experimental *in vivo* conditions, by means of spectro analytical procedure and histopathological studies, on the basis of which we aimed to contribute the assessment and to supplement the existing information on health impacts.

Experimental design:

Products used in the studies:

Zinc bis-DL-methionate (Zn-Met) - synthesized by prof. L. Vezenkov in the laboratory of peptide synthesis of the Department of Organic Chemistry at the University of Chemical Technology and Metallurgy – Sofia, containing 14.6% zinc, 16.6% H_2O and 68.8% methionine.

Reference medicinal product: Zinc sulphate heptahydrate ($ZnSO_4.7H_2O$) - containing 22.7% Zn.

Methods and treatment:

The content of zinc in prepared crop recepies was tested by atomic absorption spectroscopy and atomic absorption spectrophotometer "Perkin Elmer" 3030.

Forty two broiler chickens Cobb-500 were treated once p.o. with zinc sulfate and zinc methionate at doses of 1000 mg / kg m., 1500 mg / kg m. and 2000 mg / kg m and organs were routinely processed and Hematoxylin and Eosin (H&E) stained for morphological examination.

Our results showed that high doses of the inorganic zinc ingredient significantly decreased the general health condition of chickens and led to dose dependent, from mild to severe histopathological alterations of liver, kidneys and heart. In the groups treated with the organic zinc methionate lethal dose was not reached, changes of organ morphology were less pronounced compared to poultry treated with the inorganic zinc sulfate. However histopathology showed dose dependent variations of organ morphology, compared with controls.

As conclusion we admit that the used organic zinc ingredients in chicken forages can be safely used in appropriate doses, which is cost-effective and less harmful to animals and environment, than the inorganic such.

Key words: Zinc bis-DL-methionate, Zinc sulphate heptahydrate, acute toxicity.

CO3. LYOPHILISED MEAT FOODS DEVELOPMENT FOR SPECIALIZED NUTRITION HAVING RADIOPROTECTIVE EFFECT

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Summary

The application of advanced and modern technological approaches with the addition of oils and food of vegetable origin is described, aiming at creation of fortified meat foods to optimize the composition of human nutrition. Freeze-dried foods were developed based on buffalo meat intended for specialized nutrition of people suffering from cancer. The radio-protective effect of freeze-dried foods was studied on test animals (male white mice BDF), subjected to whole body external radiation at relatively low doses of radiation. The effect of biological radio-protection was experimentally proved, as far as nutrition is concerned and its role as a possible factor for reduction of the level of radiation damage. The established positive effect of the tested food components will enable their inclusion in diets for complex treatment.

Keywords: specialized feeding, buffalo meat, biological experiment, lyophilization

Introduction

Dietetic nutrition is a milestone in the complex treatment of patients under stationary conditions. Targeted use of food products and special additions to them provides a number of options for lower radiation damage to the body. One of the alternatives to this is the creation of foods with anti-ray effect. Therefore our efforts were directed towards the study of biological radioprotection, especially nutrition as a possible factor in reducing the incorporation of radionuclides and decreasing the extent of radiation damage as well.

Various teams from Bulgaria have conducted experiments on the impact of nutrition on radiation damage in mammals. It has been found that diets of higher content (up to 60%) of vegetable fats enhance the radioresistance of the organisms. The good deincorporating effect of anthocyanic preparations was proved. Preference to deincorporating components of natural origin was given based on the experiments. Their ability for both multiple application was proved and for long periods without toxic adverse events in a chronic receipt of radionuclides in the body [1,2,3].

Clinical testing was carried out of food products (meat, fish and cereal based) with the addition of anthocyanins, pectins, vitamins A, B1, B6, C and E, Biostar preparation and

lactobacilli in various combinations. Good tolerability of these products was accounted for by patients undergoing radiotherapy and cytotoxic therapy [1,5].

The inevitable conclusion is made that the use of various food products in the diet of radiation affected patients is a complex problem and that the final effect depends on multiple factors. Purposeful experimental studies and clinical trials are required in accordance with the complex pathogenesis of radiation damage [4]. External and internal irradiation performed by radionuclides has damaging effects the basic mechanisms of which have been established long ago in radiology. Vigorous chemical agents have been created to provide for prevention from X-ray damages as well as for disincorporation of radioactive isotopes that have been taken on in various ways. These agents, however, distinguish high degrees of toxicity, which excludes their massive and repeated administration with humans. Therefore our efforts have been directed towards the study of biological radioprotection and nutrition in particular, as a possible factor for reducing incorporation of radionuclides and decreasing the extent of ray damage.

The main objective of the study was to develop a lyophilised meat foods for specialized nutrition having radio-protective effect. An attempt was made to find a modern formula for the prophylaxis of oncological diseases by means of therapeutic feeding and special nutritional meat-based concentrates in particular. The radio-protective effect was traced of the freeze-dried foods on experimental animals (male white mice BDF) subjected to whole body external radiation at relatively low doses of radiation.

Materials and Methods

Prescription formulas have been developed to obtain freeze-dried foods for clinical meat-based nutrition (buffalo meat). The newly created foods contain growth and energetic stimulants, also physiologically active substances of different origin, namely, enzymatic lean buffalo meat, vegetable and cereal components, polysaccharides, ascorbic acid, natural antioxidants, lecithins, etc.

To obtain lean meat enzyme preparations were used such as papain (Merck) and bromelain (Merck). Freeze-drying тоок place in a sublimation installation of the company - "Hochvakuum-TG -16.50".

The test animals used during the experiments were male white mice BDF weighing 18-23g. Whole body irradiation was applied externally from ^{137}Cs source of experimental groups a 2,25 Gy gamma rays dose. Dose rate created by the sources was 1,78 Gy / min.

Five experimental groups were used, of five test animals each:

Group 1: Exposed to radiation control group of animals, fed with vivarium food (tableted yeast and water);

Group 2: Non-irradiated and fed a special food throughout the study period;

Group 3: Irradiated and treated by means of curative nutrition for four days after exposure;

Group 4: Irradiated and treated for preventive and curative reasons for four days before and 12 days after exposure;

Group 5: Non-exposed to radiation control group of mice, fed with vivarium food.

The animals from each group were kept and grown under the same conditions. Access to food and water was available throughout the day. Food was protruded every morning.

Preventive treatment with the additive mentioned continued for 4 consecutive days prior to irradiation at a dose of 2,25 Gy. The curative treatment took place 12 consecutive days post irradiation at a dose of 2,25 Gy. The chosen model of food provision was applied to account for possible prophylactic (prior to irradiation) or medical (after irradiation) effect of tested products. Weight and hematological parameters were investigated. Mice received the supplement together with their usual food and our calculations showed that each mouse had consumed 3 g per day. The investigated parameters included: 1) The concentration of leucocytes in the blood; 2) Weight. Statistical analyzes were performed using Anova program of Excel for Microsoft Office.

Results and Discussion

Experimental data on the concentration of leucocytes in the blood of mice at different feeding regimes are presented in Table 1.

Table 1. Hematological parameters - testing the concentration of leucocytes in the blood of mice at different feeding regimes

№	Control group	Concentration of leucocytes in the blood WBC (pcs./mm^3)			
		4 days before irradiation	Immediately before irradiation	On the 6th day after irradiation	On the 12th day after irradiation
1.	Irradiated mice fed with yeast	10990	11330	9030	8680
2.	Non irradiated mice fed with special food	10490	12760	11972	12730
3.	Mice - irradiated and curatively treated	12725	9630	6675	6700
4.	Mice - irradiated, curative and preventive treated	11840	11130	6860	9630
5.	Non-irradiated mice fed with yeast	12160	11860	11450	11150

Haematological tests included measurement of the factor of leucocytes concentration in the blood of the test specimens. What was special with irradiated objects was a sharp drop in values of the factor immediately after irradiation (Figure 1). The diverse feeding regimes demonstrated pronounced trends. When fed with normal food we ascertained a steady decline in the concentration of leucocytes for the entire studied period while with curative nutrition a slight increase of factor values at the end of the period was found. The effect of special food nutrition was most noticeable in preventive nutrition, for then significant recovery of the concentration of leucocytes (over 20%) was observed at the end of the period. The statistic processing of the results obtained turned out to be statistically insignificant as far as the differences between the variants are concerned (Table 2). This is probably attributed to the small amount of studied objects (5 pcs per variant) and the significant intragroup dispersion between individual specimens in particular.

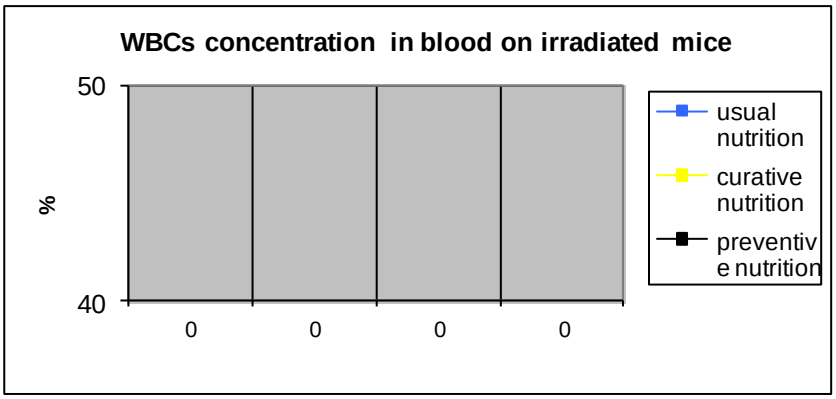

Figure 1. WBCs concentration in irradiet mice blood

Studies of leucocytes concentration in the blood of non-irradiated mice, fed with regular and special food, showed beneficial impact of dietary supplement on the values of this factor in comparison with the control group. Significantly higher values of the factor were registered throughout the period (Figure 2). This was certified by the statistically processed experimental data as well where the beneficial impact of nutrition with special food was manifested with a reliable rating at P = 0,01 (Table 2).

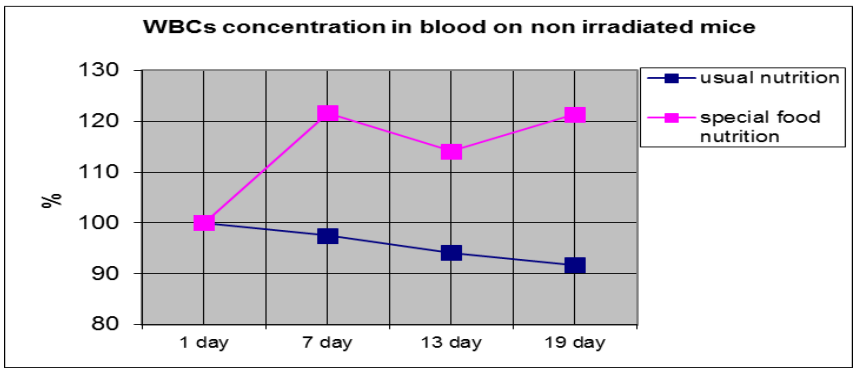

Figure 2. WBCs concentration in blood on non-irradiated mice

Table 2. Statistical parameters of study of the concentration of leucocytes in irradiated mice

Study factor	Rank of proof				
Way of feeding	F op	F_{kp} 0,05	F_{kp} 0,01	F_{kp} 0,001	Rank
Irradiated mice	0,49	3,89	6,93	12,97	-
Non-irradiated mice	12,13	5,32	11,25	25,41	**

P< 0,001 - *** ; P< 0,01 - **; P< 0,05 - *

The impact of different feeding regimes onto post irradiation weight factor of mice were studied with the test animals at a dose of 2,25 Gy (Table 3).

Table 3. Measured values of mice weight at various feeding regimes

№	Control group	Weight in grams by dates of measurement								
		1 day	3 day	5 day (Irradiation)	7 day	9 day	11 day	13 day	15 day	
1.	Irradiated mice fed with yeast	19,90	23,12	24,98	23,20	25,88	25,78	25,72	24,70	25,68
2.	Non-irradiated mice fed with special food	19,66	22,06	21,64	22,46	23,00	23,94	23,72	26,40	28,36
3.	Mice - irradiated and curative treated	20,96	23,66	23,40	25,34	25,06	26,90	26,46	28,90	28,22
4.	Mice -irrad. and curative and prev. treated	20,62	22,96	23,30	24,02	23,76	23,84	25,68	27,36	26,76
5.	Non-irradiated mice fed with yeast	19,42	23,16	23,36	24,48	25,94	25,74	26,38	26,82	25,84

The results of the measurements of the weight factor for irradiated test groups are shown in Figure 3. There is a more rapid increase in weight of the mice fed with customary food before irradiation compared to the other embodiments. However after irradiation (on day 5) a significant decrease in weight was observed with mice fed in this manner, followed by a gradual rise to values higher (5%) than the ones registered before irradiation. The next two variants manifested a significantly higher degree of adaptation of the organism after stress factor application. The increase in weight was respectively 23% for curative nutrition and 17% for preventive nutrition. A despersive analysis of the results obtained was performed. The weight of test animals immediately after irradiation was studied as a basis for comparison as well as the weight at the end of the feeding period with a special diet (Table 4). The beneficial impact of curative and preventive nutrition compared to the control group was demonstrated at reliability rating P = 0,05. Statistically significant differences between curative and preventive nutrition were not substantiated. Figure 4 shows a graph of change in weight of non-irradiated mice fed with customary and special food. Initially we ascertain a faster rise in the weight of mice fed the usual way, after that a gradual levelling of the values of the factor at the end of the period can be observed.

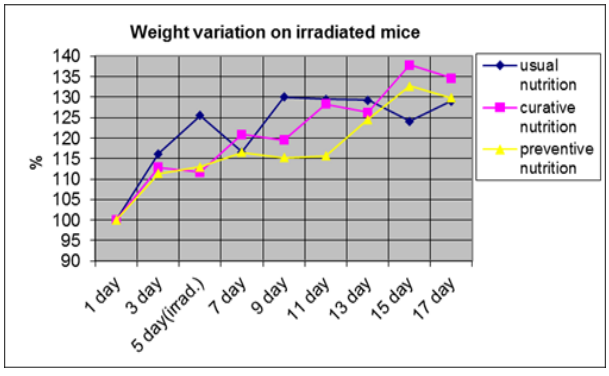

Fig.3. Weight variation on irradiated mice mice

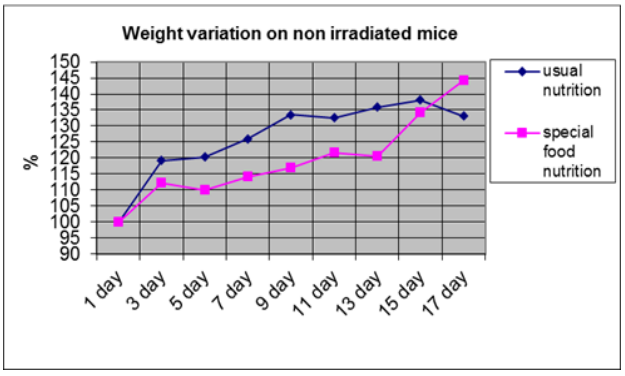

Fig.4. Weight variation on non-irradiated

Table 4. Statistical differences between feeding regimes in relation to weight factor of irradiated mice

Factor study	Rank of proof				
	F op	F_{kp} 0,05	F_{kp} 0,01	F_{kp} 0,001	Rank
Between control and curative nutrition	9,54	5,32	11,26	25,41	*
Between control and preventive nutrition	7,15	5,32	11,26	25,41	*
Between curative and prophylactic nutrition	0,06	5,32	11,26	25,41	-

P< 0,001 - *** ; P< 0,01 - **; P< 0,05 - *

Conclusions

1. A high degree of probability for the beneficial impact of nutrition with special food was demonstrated onto the leucocytes factor in the blood of mice that were not treated with gamma-rays.

2. Statistically significant differences of weight index of mice was registered between the variants depending on whether they had been fed with special food before and after irradiation compared to the control group fed in the usual way, which demonstrate the beneficial impact of the dietary supplement used.

3. The effect of biological radioprotection was proved, namely nutrition as a possible factor for reducing the level of radiation damage.

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CO4. "SACRED PLANTS" WITH PSYCHOACTIVE PROPERTIES

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Alstract.

Some psychoactive substances are used in religious ceremonies, shamanic and spiritual rituals since prehistoric times. There is archaeological evidence for the use of psychoactive substances, dating back at least 10 000 years. Most often these are herbs, plants and fungi, which are representatives of hallucinogenic and other substances with psychoactive properties.

The author presents some of the traditional "sacred plants" and active substances with psychoactive properties: cannabis, coca, kykeon, iboga, soma, peyote, ayahuasca, ergine, psilocybe mushrooms, opium and others.

Key words: psychoactive substances, sacred plants, hallucinogens

Някои психоактивни вещества, особено халюциногени, са били използвани за религиозни цели още от праисторически времена.

Има археологически доказателства за употребата на психоактивни вещества, датиращи от преди 10 000 години.

През 1979 година екип от изследаватели на психоактивните вещества предлага термина **ентеоген** за веществата, които предизвикват промени в съзнанието и мистични преживявания.

Ентеоген в тесния смисъл на думата, е психоактивно вещество, което се използва в религиозни церемонии и спиритуални ритуали. Терминът произлиза от две думи на старогръцки, (entheos) и (genesthai). Буквалното значение на думата ентеоген е преживяване, свързващо човека с вътрешния Бог.

Ентеогените са били използвани в продължение на векове. Има сведения, че те са били известни на почти всички древни цивилизации.

Най-често това са билки, растения и гъби, които са представители предимно на халюциногенните вещества. Примери за традиционните ентеогени включват: кикеон, сома, пейот, аяхуаска, канабис, етанол, алкалоиди на моравото рогче, псилоцибин, опиум (13, 14, 18, 22, 23, 31, 39, 40, 49).

Цикличните култове са характерни за различни общества, като най-ранни свидетелства има за **Елевзинските мистерии**.

В Гърция, в град Елевзина, се е провеждал годишен религиозен празник в чест на Деметра и Персефона. Централно място в тези празненства заема мотива за вечността и безсмъртието. Култът към Деметра бележи завръщане към хтоничното, към богове, естеството на които е свързано с тайните на плодородието, живота и смъртта.

Елевзинските мистерии се празнували всяка година в продължение на близо две хиляди години. Вероятно те възпроизвеждат връщането на Персефона от подземния свят, като символ на възкресението. Съвременните учени смятат, че основната идея на церемониите е проявление на вярата в безсмъртието на човека, който - според идеите на древните гърци, след смъртта се възражда, както семената, засети в почвата поникват и дават нов живот ...

Интересно е, че в град Елевзина е роден първият от гръцките трагици - Есхил.

Някои учени смятат, че „Мистерията" на Елевзина се основава на въздействието върху участниците на халюциногенни вещества, съдържащи се в ***свещената напитка***

кикеон. Кикеон се споменава в текстовете на Омировата "Илиада" и се описва като състояща се от ечемик, вода, билки и козе сирене.

Използването на отвари за магически или религиозни цели е относително често в Елада и древния свят.

Посветените в мистериите, подготвени от предходна церемония, може да са били задвижвани от ефектите на мощна психоактивна отвара с дълбоки духовни и интелектуални последици.

В опозиция на тази идея, други скептични учени отбелязват липсата на солидни доказателства и подчертават колективния, а не индивидуален характер на посвещение в Мистериите.

Много психоактивни вещества са били предложени като значим елемент от кикеон, макар и без консенсус или убедителни доказателства.

Едно от предположенията е, че напитката съдържа алкалоиди на Моравото рогче, с ефекти, подобни на тези на ЛСД.

Моравото рогче /Claviceps purpurea/ е паразитна гъба, която се среща при някои житни и тревисти растения. Алберт Хофман - швейцарски химик, открива психоактивните свойства на алкалоидите на Claviceps purpurea и синтезира ЛСД.
Въпреки това, съвременните опити да се приготви кикеон чрез използване на заразен с Мораво рогче ечемик са дали незадоволителни резултати.

Халюциногенните гъби са друг кандидат за психоактивни вещества в състава на напитката. Терънс Маккена спекулира, че „Тайните“ са фокусирани около магическите гъби Psilocybe. Една съвременна хипотеза предполага, че древните египтяни са култивирали Psilocybe cubensis на ечемик и употребата му е свързана с култа към бог Озирис.

Други ентеогенни гъби, като Червената мухоморка, също са били обсъждани.

Друг кандидат за психоактивно вещество е опиат, получен от *Опиевия мак*.

Според друга теория, психоактивното вещество в кикеон е *диметилтриптамин (ДМТ)*, който се среща в много диви растения от Средиземноморието, включително различни видове акация.

Алберт Хофман и други учени, потвърждават хипотезата, че свещената напитка в древните Елевзинските мистерии е съдържала ечемик или ръж, заразени със склероции от Мораво рогче, причиняващи халюцинации в посветените в свещеното тайнство.

Религиозната и културна стойност на действителната "Тайна" все още е слабо разбрана. „Мистерията" на Елевзина продължава да вълнува въображението на изследователите.

Заслужава да се отбележи, че поклонници в Елевзинския храм са били Платон и Сократ, Софокъл, Омир, Еврипид - най-великите умове на древността (9, 30, 45, 51).

Храмът на Аполон в Делфи, разположен в подножието на планината Парнас е най-популярното и прочуто прорицалище през Античността в цялото Средиземноморие. Това е най-добре документираният религиозен обект на класическия свят. От 1400 пр. н. е. до 381 г. от н. е. хиляди поклонници от всички краища на древния свят са се устремявали в Елада, в знаменития храм на Аполон, за да чуят пророчествата на Делфийския оракул.

Днес науката разполага с много литературни, епиграфски и археологически сведения за Делфийския оракул. Но въпреки това, не всичко е съвсем ясно и не всички тайни са разгадани.

Първоначално Оракулът се е намирал под покровителството на богинята-майка Гея – хтонично божество, което е имало връзка с европейските праисторически, матриархални култури. Вълшебният змей Питон – еманация на божествената енергия е бил пазител и главен жрец на светилището в Делфи. Лавровото дърво, свещеният

извор, триножника, процепа в скалата с опияняващите изпарения – вероятно всички тези, типични за Делфийския Оракул атрибути са били свързани изначално с древния хтоничен култ и ритуалите посветени на Гея. Вероятно още оттогава светилището, освен всичко друго е било важен стратегически център, в който са се концентрирали власт, пари и информация…

Митът за Бог Аполон, който разказва за битката със страшния, свещен змей Питон потвърждава хипотезата за война и победа на новите култове над старите. Аполон убива Питон и взема Оракула под свое попечителство и управление.

Днес особено монументални и внушителни са останките именно от храма на бог Аполон. Обикновените простосмъртни не са имали достъп до вътрешността на храма. Само избрани са можели да чуят думите на пророчицата. Олтарът е представлявал композиция от живи лаврови дървета, статуята на бог Аполон, триного столче, на което е сядала жрицата и омфалоса, символизиращ „пъпа на света“. Пития е името на всяка жрица на бог Аполон в светилището му в Делфи.

Преди всяко пророчество, Пития се измивала в Касталския извор, обличала златотъкана дреха, разпускала косите си, слагала лавров венец на главата си, пиела вода от свещения извор, палела лаврови клончета, дъвчела дафинови листа, сядала на висок триножник и правела своите предсказания.

Има хипотеза, че Питиите са употребявали халюциногенни вещества, най-вероятно растителни, които са предизвиквали видения - *канабис, опиум или блян*.

Според друга хипотеза, под триножника е имало земна пукнатина, от която са излизали *опияняващи газове – етан, етилен*, които са предизвиквали състоянието на транс. Според знаменития Плутарх, който някога също е бил жрец в храма на Аполон, пророческото могъщество на питията се основавало на вдишването на тайнствени изпарения, излъчващи се от недрата на земята. Той отбелязва в съчиненията си, че в пещерата на пророчицата се усещал дъхът на сладък газ и свързва наличието на този газ с постоянния транс, в който се намирала жрицата.

Съвременните проучвания наистина доказват, че храмът се намира върху дълбоки пукнатини. Учените анализирали също така състава на водата в изворите наоколо и установили висока концентрация на метан, етан и етилен.

Сред най-известните антични писатели, които говорят за светилището на Аполон в Делфи са Пиндар, Херодот, Есхил, Софокъл, Еврипид, Платон, Аристотел, Диодор, Страбон, Павзаний, Плутарх, Ливий, Овидий, Лукан, Юлиан, Юстин (24, 35, 18, 21).

Ентеогените са играли ключова роля в духовните практики на **американската култура** в продължение на хилядолетия.

Кактусите на Новия свят имат психоактивни свойства, но може би *пейотът* е най-известният сред тях /Lophophora williamsii/. Този кактус, наричан от местните жители meskal или пейот (peyotl) расте от централната част на Мексико до река Рио Гранде. В Перу свещения кактус е Echinopsis pachanoi - San Pedro.

Пейотът е бил използван от коренното население в северната част на Мексико и от различни индиански племенни групи на територията на Оклахома и Тексас.

Има археологически доказателства, че той е бил известен на местните жители най-малко от преди 8000 г. Заради специфичните му свойства в продължение на векове е играл важна роля в религиозните вярвания и ритуали.

Обикновено се консумира като чай и действието трае между 10 - 12 часа.

Пейотът бързо става известен в Европа и много учени и интелектуалци са пленени от неговата способност да потапя индивида в друга реалност. Пейот отворя вратите на възприятието. "Двери на възприятието" - това е заглавието на книгата на Олдъс Хъксли, посветена на опита му с този ентеоген.

Заедно с психологическите изследвания се търси активното начало на пейот.

През 1900 г. вече е известно, че това е алкалоид, наречен *мескалин* - безцветно масло, разтворимо във вода, алкохол и хлороформ.

Мескалинът предизвиква състояния на дълбока медитация и прозрение от духовно естество. Понякога може да има и изразени визуални и звукови ефекти.

От пейот са изолирани и други алкалоиди, например lofoforin който има стрихинин - подобни свойства. В по-малка степен, те също допринасят за общия ефект на пейота.

Днес мескалин и LSD са стандарт за халюциногенни вещества (6, 11, 12, 37, 53).

Коренното население на Южна Америка използва голямо разнообразие от ентеогени. Забележителен пример е **аяхуаска.** Това е южноамериканска отвара, за която се твърди, че има силен духовен ефект и удивителни физически лечебни свойства. Ритуалът при приемне на на този ентеоген е религиозно тайнство, в съответствие с философията и космологията на южно-американския шаманизъм. Практикува се сред местното население на Перуанска Амазония. Ayahuaska е посочена като нектар на боговете.

Напитката се приготвя като части от лианата *Banisteriopsis caapi* /"Лоза на духовете"/, растяща в амазонската джунгла се сваряват самостоятелно или с листа от други растения, между които *Psychotria viridis* („чакруна" на местния език кечуа) или *Diplopterys cabrerana* (наричано също „чакропанга"). Получената напитка съдържа *МАО-инхибиращи харминови алкалоиди и халюциногенния алкалоид N,N диметилтриптамин (ДМТ)* - психеделик, който действа орално, само когато е съчетан с МАО инхибитор. Харминовите алкалоиди в *Banisteriopsis caapi* служат като МАО инхибитори в аяхуаска.

Свещеното питие се споменава в текстове на някои от най-ранните мисионери в Южна Америка. Тя може да се нарече „специалната шаманска съставка" или дори да се приеме като цяла лечебна традиция на територията на Амазонка.

Аяуаска е известна със способността си да предизвиква силни ефекти, водещи до усещане за промяна на съзнанието и достигане на реално познание природата на душата и околния свят. Ефектът е силно индивидуален, но като цяло се наблюдават изменение на възприятията, изостряне на слуха - повишена чувствителност към звуци, особено чувство за собственото тяло, изменение на мисленето и пр. Пост-ефектът се отличава с изключителна острота на възприятията и интуицията, които се съпровождат с чувство на изтощение, раздразнение, сънливост (1, 3, 7, 20, 48, 49).

Коката /Erythroxylum coca/ е храст, произхождащ от Амазония, обитаващ топли и влажни долини между 1000 и 2000 метра надморска височина. Това растение е фундаментално за индианските култури на Андите и Амазония. От този вид кока се произвежда кокаинът.

Откакто е познат в човешката история, листата на този храст се дъвчат заради техния стимулиращ ефект и облекчаването на дихателните проблеми, дължащи се на живота в толкова големи надморски височини.

Според цитат от първите испански хроники, индианците ценели растението повече от златото и среброто, защото човек не чувства нито глад, нито жажда, когато дъвче листа от кока.

Инките са използвали листата за религиозни цели.

В наши дни чаят от кока е легален в Перу, Боливия, Колумбия, Еквадор. Много от коренните жители на Андите използват чая за медицински цели. Чай от кока често се препоръчва на туристите по високите части на Андите за предотвратяване на височинната болест - сороче. Въпреки това неговата действителна ефективност никога не е изцяло проучвана (10, 38).

„Африканското дърво на познанието" - Tabernanthe iboga е обявено от Министерски съвет на Република Габон за национално богатство през 2000 г.

При по-ниски дози Iboga има стимулиращ ефект и се използва за поддържане на бдителността по време на лов. Най-добре познатият ентеоген, използван в културата на Африка е препарат на основата кори от Tabernanthe iboga (4).

Един от най-широко използваните ентеогени е **канабис**, който се използва в Китай, Европа, Индия, в продължение на хиляди години.

Канабисът има дълга история, като още преди векове е бил използван като средство за предизвикване състояния на транс. Някои историци и етимолози твърдят, че канабисът е бил използван по време на религиозни тайнства от древните евреи, ранните християни и мюсюлманите от ордена Суфи.

Най-често наблюдаваните *ефекти* са: цялостна промяна на възприятието, лека еуфория, чувство на спокойствие и намаляване нивото на стрес, общителност, засилване на епизодичната памет, повишена чувствителност, параноя или тревожност.

Ранно гръцката история и съвременната археология сочат, че народите от Централна Азия са използвали канабис преди 2500 години. Употребата на канабис се явява като част от религиите и културите на индуизма, скитите, исляма. Канабис или ганджа е свързан с почитането на хиндуисткия бог Шива. Херодот пише за канабис в церемониални практики на скитите, за които се счита, че са възникнали от 5-ти до 2-ри век преди новата ера. Ранните християни използват масло от канабис за медицински цели и като част от различни религиозни церемонии. Канабисът се споменава в различни свещени еврейски текстове, Тора, Стария Завет. В древни немски текстове канабисът е свързан с немската богиня на любовта Фрея. Вярвало се е, че Фрея живее като плодородна сила в женските съцветия на растението (2, 23, 27, 28, 34, 41, 43).

Етанолът е мощно психоактивно вещество и едно от най-старите рекреационни средства. Той все още е най-често срещаното вещество, което причинява релаксация, възбуда или гняв.

Археологически доказателства сочат, че лозата, е култивирана от човека около 6000 г. пр.н.е., и че всички цивилизации на древния свят - Египет, Гърция, Рим, са познавали алкохолни напитки - предимно бира и вино. Дионис е богът на виното и сексуалните оргии. Той се изобразява, придружен от менади и сатири - хора с кози крака. Техният лидер е Пан. Северните народи също имали свои алкохолни напитки. Германци и славяни пиели медовина, степните народи - ферментирало мляко - "кумис,, а китайците - напитка от ферментирал ориз. Ислямът строго забранява алкохола, но въпреки негативното отношение към него, именно арабските алхимици са получили спирт в чист вид. Те вярвали, че чрез дестилация се достига „душата“ на упойващите напитки.

Алкохолът придобива популярност сред масите едва през 16-ти век, когато става по-евтин и кръчмите изникват навсякъде (42, 45).

Гъбите имат специална роля в митологията на почти всички народи.

Червената мухоморка се среща в цялото Северно полукълбо, най-вече в близост до брези, борове, смърчове и ели. В западен Сибир шаманите използвали червената мухоморка, за да постигнат състояния на транс.

Качествата на **червената мухоморка** са били известни по целия свят. Гордън Уосън сравнява гъбата с възпятата в древните индийски химни напитка на безсмъртието – сома, като в подкрепа на своята хипотеза привежда няколко аргументи. Сомата е определена като „слънчево растение без листа” в индийските религиозни химни. **Мухоморката**, отличаваща се се със своя яркочервен цвят, се счита за една от най-красивите гъби и напълно се вписва в описанието. Прадедите на индоарийците са дошли от север и вероятно са познали свойствата на тази гъба, играеща съществена роля при сибирските народи и религиите им. Психоактивното вещество в Червената мухоморка е мусцимол.

Много автори смятат, че популярният в средновековна Скандинавия Берсерк дължи славата си на Червената мухоморка. Берсерк (човек-мечка) - така били наричани хората, изпадащи в особено състояние след поемане на питие от мухоморка. В повечето случаи напитката е била използвана от войните преди да влязат в битка. Предизвиквала е възбуда и ярост, и съответно страх и ужас у противника. След битката били необходими няколко дни за възстановяване на бойците. През 1123 г. употребата на мухоморката е била забранена от норвежкия крал (5, 26, 44).

Халюциногенните гъби – Psilocybe са били част от културата на някои цивилизации още от преди векове. За тях се споменава в първите писмени сведения от много ранната история. Древни рисунки на гъбата, датиращи от преди 5000 години пр. Хр. са били открити в пещери в Северен Алжир. В централна и Южна Америка строяли храмове за „Боговете гъби“ още 1000-1500 година пр.Хр.

Халюциногенните гъби били използвани от ацтеките в церемонии и ритуали. Psilocybe е била известна на мексиканците като teonanácatl - "Божествена гъба". Те ги поднасяли заедно с мед или шоколад на някои от най-свещените им събития. Сервирана е и при коронацията на Монтесума II в 1502.

Поглъщането на гъбите засяга зрението и слуха, промяната в настроението може да варира от много приповдигнато до много меланхолично.

След испанското завоевание на Северна и Южна Америка, употребата на халюциногенни растения и гъби, както и други предхристиянски традиции са били насилствено потиснати (15, 16, 19, 25, 29, 32, 33).

Към групата на природните продукти принадлежат и **делириантите,** използвани още от зората на човешката цивилизация като лечебни и "магически" инструменти (**беладона, мандрагора, татул**). По-късно много химични съединения са синтезирани по тяхното подобие, някои от които са сред най-активните известни психоактивни вещества. Лекарства, съдържащи делирианти от този вид имат дълга история на употреба.

На много места в Европа, включително България, Северна Америка и Азия, има растение със свойства на делириант - Datura stramonium - татул. Интоксикация се причинява от съдържащите се в *растението алкалоиди - атропин, хиосциамин, скополамин.* Известни са около 15-20 вида Datura, предизвикващи силни психоактивни ефекти.

Пророчиците в храма на Аполон в Делфи използва малки дози татул, преди да направят своите предсказания. Омир споменава татула в своята епична „Одисея“. Жреците на древните траки по нашите земи, използвани делирианти в церемониите си. Семена и листа от Datura са добавяни към ганджа в Индия, за постигане на допълнителен ефект върху психиката. Шаманите на перуанските индианци използвали свещен татул за въвеждане на младите хора в тайните на духовния свят. Ацтеките са ги използвали за ритуали в храма на Слънцето (7).

Мандрагората се е използвала като силен халюциноген. Корените съдържат психоактивни алкалоиди. Древните гърци смятали мандрагората за растението, от сока на което Цирцея превърнала спътниците на Одисей в свине.

Мандрагората има болкоуспокояващо и сънотворно действие, според начина на приготвянето. Близка е по действие с беладоната. В египетските папируси е описана като средство, възбуждащо чувствителността. От древни времена Мандрагора се смята за афродизиак. От плодовете на мандрагората египтяните приготвят любовен елексир. Асирийците са използвали мандрагората като обезболяващо и сънотворно средство. Хипократ е считал, че в малки дози тя е ефективно средство против страх и депресии. Римският лекар Гален бил почитател на виното от мандрагора, като наблягал на това,

че същото имало сносни увеселителни качества. Мандрагора често се споменава в произведенията на Шекспир, на Николо Макиавели (36, 47).

Отглеждането на **Сънотворен мак** за ритуални цели датира от неолита (новокаменната епоха). Опиумът се споменава в най-важните медицински текстове на древния свят, включително Еберс папирус и трудовете на Диоскорид, Гален и Авицена.

Макове са били намерени в египетски гробници, датиращи от преди 3000 години. Открита е и рецепта, приготвяна от него, с която се спирал плача на децата. Легенда за мака е свързана и с Деметра, която поискала да заспи след загубата на дъщеря си Персефона. Други двама гръцки богове, двамата братя близнаци Хипнос и Танатос, са представени като короновани с макове или с макове в техните ръце.

Очевидно гърците са били наясно с факта, че сънят, предизвикан от опиум може да доведе до смърт. Плиний дава подробно описание как да се събира цветето и предупреждава, че големи количества от него убиват. Древните гърци смятат, че маковете са знак за плодородие (8, 50, 51, 52).

ЗАКЛЮЧЕНИЕ

Ентеогените са били използвани от хората в продължение на векове. За това има потвърждение в цялата писана история на човечеството.

Въпреки, че ентеогените са табу и повечето от тях са забранени в християнските и ислямски общества, тяхното присъствие и значение от гледна точка на духовните традиции на различните култури е безспорна.

В духовните философски и мистични традиции: веди, будизъм, даоизъм, суфизъм, гностицизъм, християнство, мистицизъм, кабала, е описан опит, който човекът не осъзнава във всекидневния си живот. Много от религиозните системи са продукт на вековно изследване на човешката психика и съзнание.

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CO5. КОНСУМАЦИЯ НА АЛКОХОЛ И ОКИСЛИТЕЛНО УВРЕЖДАНЕ НА ДНК.

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CO6. ZINC IN THE ETIOLOGY OF ACRODERMATITIS ENTEROPATHICA

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Acrodermatitis enteropathica is an autosomal recessive metabolic disorder affecting the uptake of zink – an essential trace nutrient required for the proper function of more than 100 enzymes and playing a crucial role in nucleic acid metabolism. The main symptoms of the condition are periorificial and acral dermatitis and diarrhea. Alopecia, nail dystrophy, dry skin cerebral cortical atrophy, short stature are also common. A mutation of the SLC39A4 gene on chromosome 8 q24.3 is responsible for the disorder. The gene encodes a transmembrane protein that is part of the zinc/iron-regulated transporter–like protein (ZIP) family required for zinc uptake. This protein is highly expressed in the enterocytes in the duodenum and jejunum therefore, affected individuals have a decreased ability to absorb zinc from dietary sources. Treatment of acrodermatitis enteropathica requires lifelong zinc supplementation. Typically, 1-3 mg/kg of zinc gluconate or sulfate is administered orally each day.

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CO7. TOPICAL IMMUNOTHERAPTY TREATING ALOPECIA AREATA

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Topical immunotherapy is defined as the induction and periodic elicitation of allergic contact dermatitis by applying a potent contact allergen. Such chemicals are dinitrochlorbenzene, squaric acid dibutyl ester and diphenylcyclopropenone. Contact immunotherapy acts by varied mechanisms. According to the concept of antigen competition it redirects the autoimmune attack on the hair follicles. It is noted a decrease in CD4 to CD8 count, decrease in CD6 lymphocytes and Langerhans cells and increase in suppressor T cells. Another theory is about a cytokine inhibitor. It proposes possible interference of contact allergens with the proinflammatory cytokines, simultaneously synthesizing local cytokines.

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CO8. SILYMARIN AND LIVER

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Silymarin is a flavonoid complex that is delivered from milk thistle seeds. Its major active constituent is silibinin. It also contains isosilibinin, silicristin, silidianin and others. It is hepatoprotector and is available as drug under several trade names. It affects liver in a couple of ways . It shows membrane stabilising activity, interacts with toxin receptors, upregulates glutation thus acting as antioxidant. It prevents cirrhosis, helps regeneration of hepatocytes and improves liver microcirculation. Silymarin is under investigation to see whether it may have a role in cancer treatment.

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Session D.

Chairpersons:

Assoc. Prof. Anna Tolekova, MD, PhD
Medical Faculty, Trakia University, Stara Zagora, Bulgaria

Assoc. Prof. Radostina Alexandrova, MSc. PhD
Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences

Secretary: Desislav Dinev, MSc
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DO1. КОЛОРЕКТАЛЕН КАРЦИНОМ: ДИАГНОСТИЧЕН И ТЕРАПЕВТИЧЕН АЛГОРИТЪМ И НОВИ ТЕХНИКИ ЗА ЛЕЧЕНИЕ

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DO2. INFLUENCE OF THE REACTION MEDIUM ON THE CHEMICAL, PHASE AND MORPHOLOGICAL CHARACTERISTICS OF DOUBLE-DOPED AMORPHOUS CALCIUM PHOSPHATES

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Abstract: Double-doped (with Mg^{2+} and Zn^{2+}) amorphous calcium phosphates (ACP) with molar ratios $(Ca^{2+}+Mg^{2+}+Zn^{2+})/P$ = 1.4 - 1.6, $Mg^{2+}/(Ca^{2+}+Mg^{2+}+Zn^{2+})$ = 0.8 - 0.14 and $Zn^{2+}/(Ca^{2+}+Mg^{2+}+ Zn^{2+})$ = 0.017 – 0.029 were biomimetically prepared in different reaction media with the purpose of studying the influence of the chemical composition and rheology of the reaction medium on the chemical and phase composition, shape, size, pore distribution and specific surface area of the ACP particles. Two buffer media, ammonia and glycine, differing in the type of ligands that could participate in the complex formation with Ca, Mg and Zn ions, were examined. The rheology of the glycine buffer and other initial solutions was modified by a polysaccharide hydrogel (xanthan gum). Chemical, XRD, TEM and BET analyses were applied. It was found that the reaction medium does not influence the phase composition of the precipitate and the specific surface area of the particles, but influences their shape, size and pore distribution. Incorporation of Zn and Mg depends on the reaction medium due to the formation of different chemical species in the solution. The presence of ammonia buffer leads to the formation of spherical particles (sized 50-100 nm), while glycine buffer (pure or in the presence of xanthan gum) - to folded-sheet particles (sized 100-150 nm).

Introduction

The chemical and structural similarity between calcium orthophosphate compounds and "biological apatite" makes the former suitable for developing biomaterials which can be used for bone regeneration and reconstruction [1-3]. Important structural characteristics of biological apatite are the equally oriented uniform nanosized building particles. Therefore, nucleation and crystal growth of the obtained calcium phosphates should be strictly controlled and all factors which may influence their preparation should be taken into account [4]. Precipitation of calcium phosphates in aqueous-salt systems usually leads to aggregation of the precipitated particles and makes impossible the preparation of fine nanosized powdered products. To solve this problem, organic-assisted or polymer-controlled precipitation is proposed in the literature. The influence of asparagine (Asp), glycine (Gly), lysine (Lys), alanine (Ala) and arginine (Arg) on the nucleation and growth of hydroxyapatite (HA) at pH 10 was investigated by Palazzo *et al.* [5] and Yang *et al.* [6]. All amino acids can either chelate the Ca^{2+} and PO_4^{3-} ions in solution or cover the surface of the HA nuclei [7] thus inhibiting their further growth and reducing the size of the particles. Palazzo *et al.* [5] showed that in presence of Asp and Arg needle-shaped crystals are obtained, which are 3 times smaller in diameter and 5 times longer than those obtained in the presence of Ala. Another approach is the use of natural polymers as chitosan, cellulose [8, 9], etc., or synthetic polymers as polyethylene glycol (PEG), polyacrylate (PA) [10], etc., to tailor particle size, reduce agglomeration and add specific functionalities to the calcium phosphate materials.

The aim of this study was to investigate the influence of chemical composition and rheology of the reaction medium on the chemical and phase composition, shape, size, pore distribution and specific surface area of the particles of double-doped (with Mg^{2+} and Zn^{2+}) amorphous calcium phosphates (ACP) precipitated at pH 8. Two buffer media, ammonia and glycine, differing in the type of ligands that could participate in the complex formation with the Ca, Mg and Zn ions, were examined. The rheology of the glycine buffer and other initial solutions was modified by the addition of a polysaccharide hydrogel (xanthan gum).

Materials and methods

Simulated body fluids

The popular conventional simulated body fluid (SBFc) [11] was used to provide an electrolyte medium for the precursor preparations. Modified calcium-free conventional simulated body fluid (SBFc-*Cam*) was used as a solvent for K_2HPO_4; modified phosphorus-free conventional simulated body fluids were used as solvents for $CaCl_2$, $MgCl_2$ (SBFc-*Pm1*) and $ZnCl_2$ (SBFc-*Pm2*) in order to avoid preliminary precipitation [12,13]**.** All simulated body fluids used in the experiments (Table 1) were prepared by successive mixing of previously prepared solutions of KCl, NaCl, $MgCl_2.6H_2O$, $CaCl_2.2H_2O$, $ZnCl_2.1.5H_2O$, $NaHCO_3$, Na_2SO_4, and K_2HPO_4 salts in distilled water. The pH of the solutions was adjusted to 8.2 – 8.4 using 0.1M HCl or 0.05M tris (hydroxymethyl) aminomethane. Analytical grade reagents were used throughout.

Table 1. Composition of blood plasma, SBFc and modified simulated body fluids (*SBFc-Cam, SBFc-Pm1 and SBFc-Pm2*) (mmol.dm^{-3})

Ion content	**Blood plasma**	**SBFc** [11]	***SBFc-Cam***	***SBFc-Pm1*** (Ca^{2+} + Mg^{2+})	***SBFc-Pm2*** (Zn^{2+})
Na^+	142.0	142.0	150.4	133.6	133.6
K^+	5.0	5.0	505.6	3.0	3.0
Mg^{2+}	1.5	1.5	1.5	60.2	1.5
Ca^{2+}	2.5	2.5	-	379.6	2.5
Zn^{2+}			-		14.9
Cl^-	103.0	147.8	142.8	1019.4	172.7

SO_4^{2-}	0.5	0.5	0.5	0.5	0.5
HCO_3^-	27.0	4.2	8.4	-	-
HPO_4^{2-}	1.0	1.0	251.3	-	-

Preparation of double-doped (with Mg^{2+} and Zn^{2+}) amorphous calcium phosphate (ACP)

ACP was prepared by the method of continuous co-precipitation in three different media keeping pH = 8 by 1 M KOH. The precipitates were stirred in the mother liquid for 1 h at room temperature, then washed through decantation and lyophilized.

Procedure SP1: All reagents (solutions *SBFc-Cam, SBFc-Pm1* and *SBFc-Pm2*, Table 1) were added to an ammonia buffer medium ($V_{SBFc\text{-}Cam}=V_{SBFc\text{-}Pm1}=V_{SBFc\text{-}Pm2}=V_{am.b.}$) at a rate of 3 ml/min.

Procedure SP2. All reagents (solutions *SBFc-Cam, SBFc-Pm1* and *SBFc-Pm2*, Table 1) were added to a glycine buffer medium ($V_{SBFc\text{-}Cam}=V_{SBFc\text{-}Pm1}=V_{SBFc\text{-}Pm2}=V_{gl.b.}$) at a rate of 3 ml/min.

Procedure SP3. All reagents (solutions *SBFc-Cam*, *SBFc-Pm1* and *SBFc-Pm2*, Table 1) and glycine buffer were first subjected to gelling with xanthan gum (5 g/l). Then the gelled solutions *SBFc-Cam*, *SBFc-Pm1* and *SBFc-Pm2* were added to the gelled glycine buffer ($V_{SBFc\text{-}Cam}=V_{SBFc\text{-}Pm1}=V_{SBFc\text{-}Pm2}=V_{gl.b.}$) at a rate of 3 ml/min.

Characterization of the precursors

Chemical analysis

The chemical composition of the lyophilized precursors was analyzed as follows: the concentrations of Mg^{2+}, Zn^{2+}, K^+ and Na^+ ions were measured on an inductively coupled plasma optical emission spectrometer (ICP-OES ULTIMA 2 analyzer, Jobin Yvon, France); those of PO_4^{3-} and Cl^- ions - on a NOVA 60 spectrophotometer using Merck and Spectroquant test kits; Ca^{2+} was determined complexometrically with EDTA at pH 11 with murexid as an indicator.

X-ray diffraction analysis

The phase composition of the precursors was determined on a D 500 (Germany) apparatus for XRD analysis, applying CuKα radiation obtained with the monochromator of the secondary beam, within the 2θ range of 10-60°, at a step of 0.02°2θ and counting time of 30s/step.

TEM images

The size and shape of the powder particles were determined by transmission electron microscopy (TEM) on a JEOL JEM-2100 apparatus.

Specific surface area and pore distribution

The specific surface area and pore distribution were determined by low-temperature (77.4 K) nitrogen adsorption on a Quantachrome Instruments NOVA 1200e (USA) apparatus. The specific surface areas (S_{BET}) were determined on the basis of the BET equation and the pore-size distributions were calculated by the BJH method using the desorption branch of the isotherms.

Results and Discussion

Ion-modified calcium phosphates have been developed to simulate the composition of the mineral component of bone tissues and to strengthen some specific biologically important parameters. We chose Mg^{2+} and Zn^{2+} ions as composition modifiers because they are essential for the living organisms. Thus, the biologically active Mg plays an important role in the formation and initial growth of the bone tissue [14], while Zn is an important element for the normal growth and development of the skeletal system [15].

It is well known that the reaction conditions strongly influence the size and morphology of calcium phosphate particles. Three different media were used in our

experiments in order to study their influence on the chemical and phase composition, shape, size, pore distribution and specific surface area of the ACP particles. Two buffer media, ammonia and glycine with different compositions, were examined. Additionally, the rheology of the system was modified by a polysaccharide hydrogel (xanthan gum).

Series of calcium phosphate compounds can precipitate from solutions depending on the pH [12, 13]. That is why, a buffer medium is necessary to maintain a constant pH during the whole precipitation process. The ammonia buffer (NH_4Cl/NH_4OH) is a typical inorganic buffer whose components can be readily removed from the precipitate. We chose the glycine buffer (glycine/NaOH/NaCl) as an alternative because Na^+ and Cl^- ions are present in the SBFs used by us and glycine is an essential amino acid for the biological systems and is also a representative of organic macromolecules in our experiments. Precipitation of calcium phosphates in aqueous salt systems usually leads to aggregation of the precipitated particles and makes impossible the preparation of fine nanosized powdered products. To solve this problem we additionally modified the rheology of all solutions with xanthan gum. The latter is a natural polysaccharide able to enhance viscosity and stabilize, over a wide temperature and pH range, disperse systems with a high salt content.

Our experiments revealed that there are no differences in the phase compositions of the precipitates in the studied systems. In all cases double-doped (with Mg^{2+} and Zn^{2+}) XRD-amorphous (Fig. 1) calcium-deficient (molar ratios $(Ca^{2+}+Mg^{2+}+Zn^{2+})/P = 1.4 – 1.6$, Table 2) phosphates (ACP) were obtained.

The biomimetic approach includes precipitation of a simulated body fluids in a modified electrolyte medium and provides modification of the precipitated ACP with Na^+ (0.03 – 0.04 mmol/g), K^+ (0 - 0.01 mmol/g) and Cl^- (below 0.05 mmol/g). These concentrations are close to those in biological hard tissues [16].

Unlike Zn^{2+} ions, the inclusion of Mg^{2+} ions strongly depends on the reaction medium. In aqueous solutions of ammonia buffer one third of Mg^{2+} ions were included in the precipitates. In the case of glycine buffer only a half of the initial amount of Mg^{2+} ions were included in the precipitates, while the whole amount of Zn^{2+} ions were included (Table 2) in both cases. In hydrogel media all Mg^{2+} ions and 2/3 of the Zn^{2+} ions were included. Unfortunately, our studies did not differentiate between the types of inclusion – incorporation into the crystal lattice or co-precipitation, nor revealed their ratio. The experimentally found differences were related to the different behavior of the two ions, Zn^{2+} and Mg^{2+}, in the solutions under study. According to Pearson concept of “hard” and “soft” Lewis acids and bases [17], as well as the Klopman scale of hardness and softness [18], "hard acids" coordinate to "hard bases" and *vice versa*, "soft acids" – to "soft bases". Mg^{2+} is a "hard acid" and Zn is a "soft acid". The ligands present in the solutions – glycine zwitterions $^+H_3NCH_2COO^-$, HCO_3^-, SO_4^{2-} and Cl^- ions are "soft bases" while H_2O, OH^- and HPO_4^{2-} are "hard bases". In a medium of ammonia buffer at pH 8 the rapid incorporation of Zn^{2+} ions into the calcium phosphate is accompanied by the precipitation of $Zn(OH)_2$ owing to the shortage of "soft acids" which could form stable zinc complexes in the solution. The glycine zwitterions $^+H_3NCH_2COO^-$ present in the system could compensate for the lack of "soft bases" by acting as monodentate or bidentate ligands through their two O atoms and preferentially coordinate to Zn^{2+} ions rather than the hard H_2O molecules. This effect is still more pronounced in the presence of xanthan gum which lowers the activity of water in the solution. As a result, the possible co-precipitation of basic zinc salts significantly decreases. When Mg^{2+} is added to a medium of ammonia buffer at pH 8, the stable $[Mg(H_2O)_6]^{2+}$ complexes dominate. The decrease in water activity after the addition of glycine and xanthan gum to the solution favors the coordination of the hard OH^- ions to the Mg^{2+} ions and the co-precipitation of basic magnesium salts (Table 2).

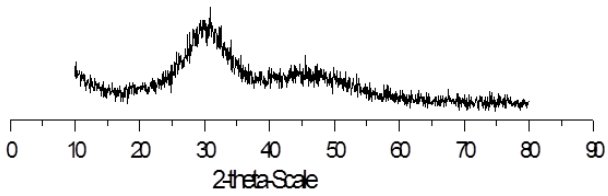
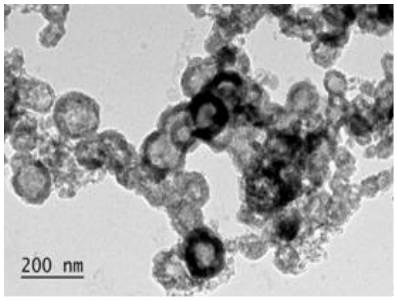


Fig. 1 XRD powder pattern of ACP

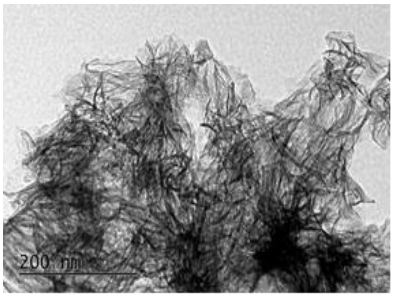
Table 2. Molar ratios and SSA (m^2/g) of the precipitated ACP

Synthesis	**(Ca+Mg+Zn)/P**	**Zn/(Ca+Mg+Zn)**	**Mg/(Ca+Mg+Zn)**	**SSA**
Initial ratio	1.80	3.00	14.00	
Ammonium buffer (SP1)	1.59	2.95	4.65	34
Glycine buffer (SP2)	1.54	2.89	8.50	28
Glycine buffer in the presence of xanthan gum (SP3)	1.40	1.74	13.68	32

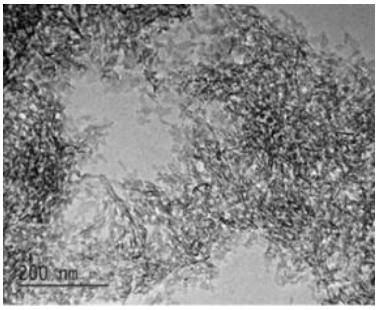
The TEM images of the obtained precipitates (Fig. 2) reveal that the presence of organic molecules has an influence on the morphology of the precipitated precursors. In ammonia buffer medium the obtained particles are spherically shaped while in glycine buffer and hydrogel media the particles are shaped as folded sheets. Spherical Posner clusters with a chemical formula $Ca_9(PO4)_6$ and a size of 0.95 nm are considered to be the energetically most advantageous calcium phosphate species which are the first ones obtained in the precipitation process, thus forming the structure of the amorphous calcium phosphate (ACP) [19]. The chemical formula proposed in the literature presupposes that no other ions but Ca^{2+} and PO_4^{3-} participate in the precipitate. We consider that under the conditions of biomimetic precipitation, other ions like Na^+, K^+, Mg^{2+} and Zn^{2+} are present in the solution, which compete with the Ca^{2+} ions, while CO_3^{2-}, HPO_4^{2-} and OH^- ions compete with the PO_4^{3-} ions for incorporation into the structure. As a result, the spherical clusters obtained have a considerably more complex elemental and stoichiometric composition. In the presence of a glycine buffer the clusters formed are probably restructurized under the effect of the chains of glycine zwitterions and calcium-glycine complexes. In aqueous solution the glycine molecular chains exist as zwitter (bipolar) ions. Well known is their ability to form complexes with a number of inorganic ions by coordinating to the Me^{2+} ion with an O atom from the –COOH group and forming zigzag shaped chains or complexes.



a



b



c

Fig. 2. TEM images of the particles obtained in ammonia buffer (a), glycine buffer (b) and xanthan gum hydrogel (c)

In the xanthan gum hydrogel system the folded-paper shaped particles are with a significantly smaller size than those formed in an aqueous medium. This is due to the higher viscosity of the xanthan gum medium which suppresses the growth of the primary nuclei.

The effect of the medium of synthesis on the texture of the obtained powders is shown on Fig. 3. The adsorption-desorption isotherms are of II-V type according to the IUPAC classification [19], which implies domination of meso and macro pores. The porosity of the powders obtained in glycine buffer (SP2, Fig. 3b) is the lowest one and the pore distribution shows pores with almost equal volumes in the diameter range of 15-60 nm. Powders with spherical particles, obtained in an ammonia buffer, display higher porosity and the maximum of the pore volumes lies in the diameter range of 12 - 22 nm (SP1, Fig. 3b). In xanthan gum hydrogel medium there are two maxima in the ranges 5 - 12 nm and 15 - 30 nm (SP3, Fig. 3b), which point to an increase in the micro and meso pores.

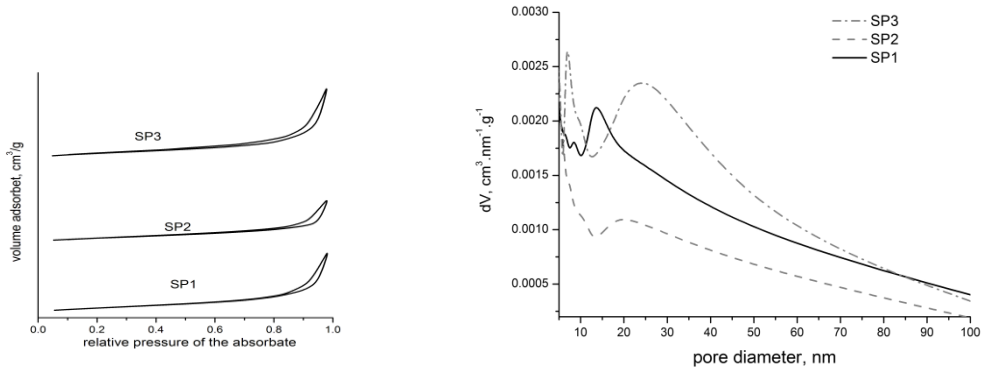


Fig. 3. Adsorption-desorption isotherms (a) and pore size distribution (b) of the obtained powders

ACKNOWLEDGEMENTS: This work was financially supported by the Bulgarian Ministry of Education and Science under Project DFNI T02-5/2014.

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DO3. RAT BLOOD BIOCHEMICAL MARKERS TESTED AFTER CALVARIA IMPLANTATION WITH ION-MODIFIED CALCIUM PHOSPHATE BIOMATERIALS

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Abstract

The present study was carried out for evaluation of some blood biochemical markers in rats with artificially created calvarial defects, implanted with modified calcium phosphates cements. Cements were synthesized on the bases of TTCP/DCPA, carboxylic acids, xanthan gum and glycerin. Rat blood biochemical parameters alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein and total glucose were investigated. No significant differences in their levels were established between the animals from the both groups with implants. The obtained data might be useful in future for *in vivo* experiments with new biomaterials.

Key words: blood biochemical markers, biomaterials, bone implantation, calvaria, rats

Introduction

Biomaterials are used on a daily basis in the field of medicine. The scientific focus in engineered scaffold systems is continuously evolving to create natural, functional bone tissues from synthetic materials. As the biomaterial research is relatively new field of study, animal studies with biomaterial implantations are needed to obtain adequate data for evaluating suitable biological and biochemical parameters.

For replacement of damaged bone, the most promising materials are these based on calcium phosphate (CaP) which are similar in composition to the mineral component of bone tissues. CaP cements (CaP c) have many favorable properties that support their clinical use in the repair of bone defects. CaPc are cementing systems consisting of powder and liquid phases: powders are dicalcium phosphate anhydrous (DCPA) and tetracalcium phosphate (TTCP); liquid phase are carboxylic acids. They are designed for plastic filling of bone defects and joining bone fragments [2]. Further investigations are necessary to take advantage of the excellent biological properties of cements under clinical application. The continuous long-term exposure of blood to materials initiates various cellular reactions and protein conformational changes, depending on the physic-chemical nature of materials [6].

More morphological, biochemical, hematological and molecular information, regarding development of bone implants and their effects in the body, is necessary.

In the present study we have evaluated the *in vivo* biocompatibility of new biomaterials on Wistar rat experimental model. The effects of implanted biomaterials on vital body organs such as liver, kidney, etc. have been studied by the blood biochemical markers – serum enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein and total glucose.

Material and methods

Modified cements preparation. DCPA ($CaHPO_4$, dicalcium phosphate) was prepared from DCPD ($CaHPO_4.2H_2O$, dicalcium phosphate dihydrate) by thermal dehydration at 200^oC. It was signed as a material 1. TTCP ($Ca_4(PO_4)_2O$, tetracalcium phosphate) was prepared by sintering of equimolar mixture of DCPA and $CaCO_3$ at 1500^oC for 5 hours. It was signed as a material 2. The as prepared solid phases were ball milled for 5 hours [7]. Equimolar mixtures of DCPA and TTCP powders with particle size less than 28 μm and activated surfaces (by milling) were continuous mixed with liquid phases in a ratio solid to liquid 2.6 g/ml to form plastic mass. 18 mass % solutions of tartaric or ascorbic acids were used as liquid phases, modified by glycerin (5 mass %). Xanthan gum (2 mass %) was added to the initial solid mixtures to improve their mechanical characteristics [5].

Animal model. Eight-week old male Wistar rats, weighed approximately 350 g, were used in the experiments. The rats were allocated to three experimental groups. Animals in the Group 1 are controls. The control group received a critical size skull defect (CSD) with no scaffold implantation. CSD models are often used to study orthopedic materials. It was been defined as the smallest *in situ* bone defect that could not heal spontaneously by bone formation during the lifespan of the animal. The rat calvarial model offers advantages: the parietal bone is a large plate that facilitates the operation and the analysis, no implant fixation is required, and costs are limited in comparison with large animal model [3]. The rest two groups received implants as follows - material 1 (Group 2) and material 2 (Group 3). General anesthesia was given. To create a CSD in the skull the head was shaved and cleaned with antiseptic. A lateral longitudinal incision over the head was made under aseptic conditions. The skull cortex was drilled and a calvarial bone defect 1.8 mm wide and 6 mm long was created. The biomaterials were implanted into the defect zone and their position was checked. The wound was then closed with continuous subcutaneous stitches. The animals had free access to food and water and were monitored daily in the postoperative period for any complications or abnormal behavior. The experiments lasted 12 weeks. Blood markers were evaluated before operation, 1 week post operation and 12 weeks post operation. The blood biochemical markers (AST, ALT, protein and glucose) were determined by the apparatus Mindray, BC – 88A.

The experiments were conducted in accordance with the requirements of the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes.

Results and Discussion

The results of the activity of the aminotransferases are presented in Figures 1 and 2.

Fig.1. Serum ALT activity in rats with implantations (U/l)

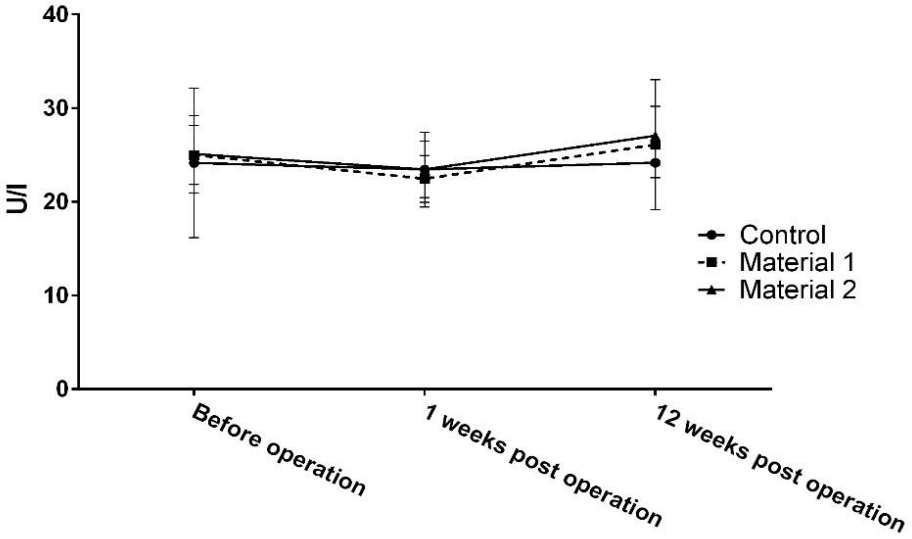
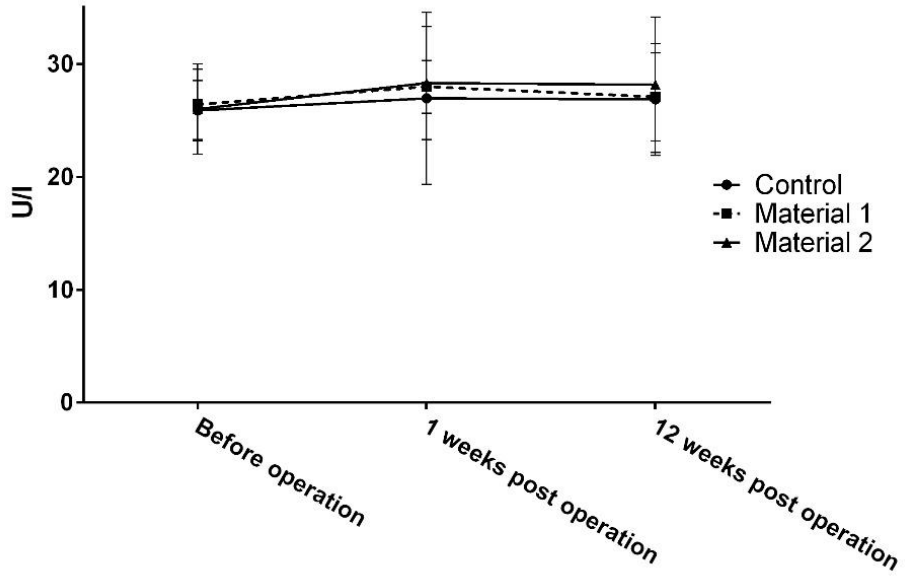


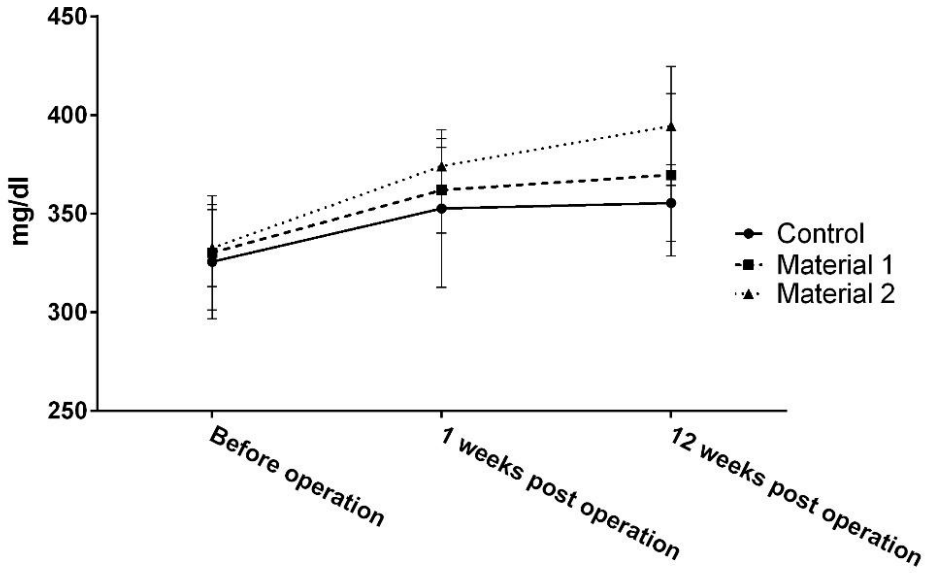
Fig.2. Serum AST activity in rats with implantations (U/l)



The levels of ALT and AST were evaluated as indicators of possible hepatotoxicity by different types of implants. In all groups, the result of ALT activity remained within the limits of the average values and did not differ from preoperative values during the testing period. (Fig.1). AST in all groups was not changed compared to the control values in the studied period (Fig. 2). The quantitative dynamics of these evaluation criteria of the enzymes in both groups with implants during the whole time of the experiment remained similar.

The comparative analysis showed that the next week after the intervention, the glucose concentration in the blood serum in all groups tended to increase - in the bigger extend in the group with material 2 (Fig. 3).

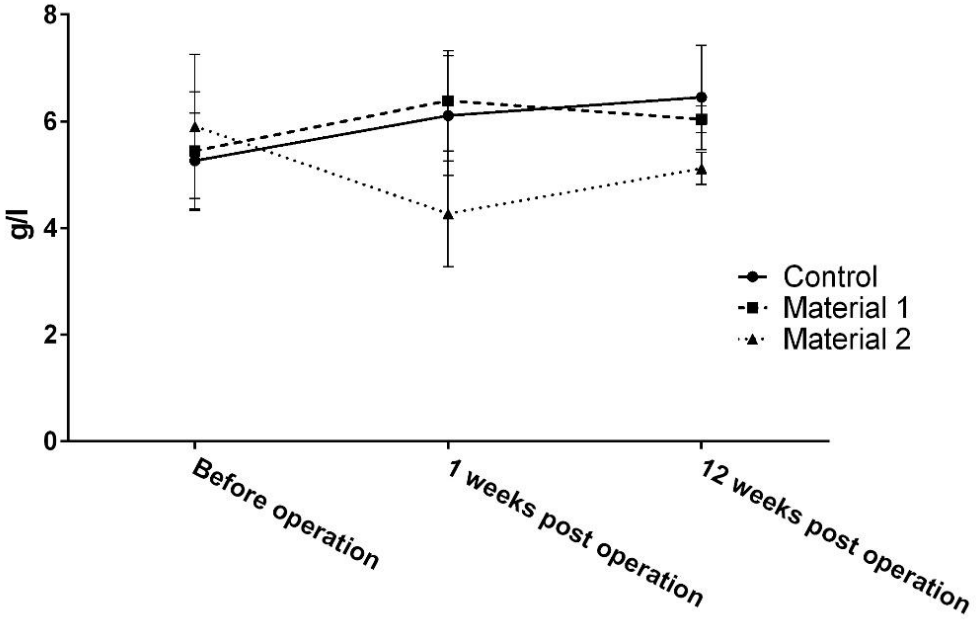
Fig. 3. Serum total glucose content in rats with implantations (mg/dl)



Insignificant changes in glucose level were within physiological range for this type of animals after a week post operation. At the end of the experiment total glucose level was statistically significant higher in the rats with implants from the material 2. May be this hyperglycemia was the consequence of postoperative stress. As a result of an injury, body tissues produce resistance to the insulin which is in part due to the release of anti-insulin hormones and anti-inflammatory cytokines (reaction to stress) [8]. Anti-inflammatory cytokines are of significant importance in the stress induced hyperglycemia after operative intervention. With the mechanical injury the reason explaining the hyperglycemia can also be an increase in the production of glucose in the liver, but not the disturbance of its utilization by tissues.

The absolute values of the total protein during the experiment in the animals from the control group and group 2 did not exceed the physiological limits. Significant differences were observed only in the dynamics of the parameter being investigated in the experimental group 3. The level of total protein from group 3 was significantly lower than that in group 1 and group 2 (Fig. 4).

Fig. 4. Serum total protein content in rats with implantations (g/l)



The levels of ALT and AST were evaluated as indicators of possible hepatotoxicity by different types of implants. In both groups, the result of ALT and ALT remained within the limits of the average values and did not differ from preoperative values. The values obtained for the AST/ ALT activities suggest no post-surgical liver damage in the experimental animals.

During the whole experiment, we did not observe any essential systemic damage, neither because of operative intervention, nor used implantation materials. There was no disruption of glucostatic and protein synthetic function of the liver. The stable level of the routine enzymatic markers (ALT, AST) excluded any sigh of cytolysis [4]. The study showed no sign of hepatotoxic effect or liver cells cytolysis.

No adverse reactions such as necrosis or inflammation were noted, indicating good long term tolerability of the both test compounds. The study showed that no adverse effect they cause when implanted in the calvarial defect site and can be further optimized for bone regeneration and repair.

The continuous long-term exposure of blood to materials initiates various cellular reactions and protein conformational changes, depending on the physic-chemical nature of materials [1].

The surgical procedure is usually connected with several stress symptoms in the animal organism. The level of damage and the rate of tissue regeneration can be monitored by the determination of specific muscle and liver biochemical markers commonly used in clinical practice.

Among the biomarkers, AST and ALT are commonly determined. It is a known fact that that increased levels of ALT and AST are generally a result of liver disease with some degree of hepatic necrosis such as cirrhosis, carcinoma, viral or toxic hepatitis and obstructive

jaundice. Elevated ALT and AST activities have been also found in extensive trauma and muscle disease, hypoxia, and hemolytic disease [4]. In our case the biochemical blood markers were within the normal range after 12 weeks, indicating that the implantation procedure were devoid of any infection.

Conclusions

Although further work is needed to fully understand the mechanism of the action of newly implants tested *in vivo*, this study has opened new avenues of research towards understanding the *in vivo* function of the additives xanthan gum, carboxylic acids and glycerin lengthened the manipulation time due to their hydrophobicity. The study revealed the potential use of these innovative CaPcs in the healing of bone lesions.

Acknowledgements

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DP1. INFLUENCE OF BARON DOPPED HYDROXYAPATITE ON THE STRUCTURE OF ELECTROSPUN FIBRES FOR BIODEGRADABLE SCAFFOLDS

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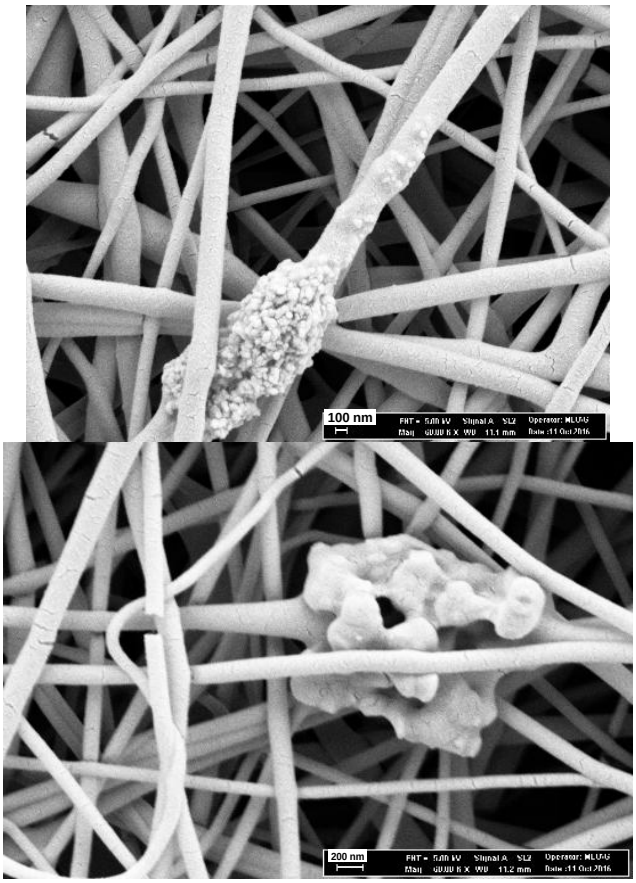
Electrospinning is a process to produce ultrafine fibers with nanometer or submicrometer diameter from various nature polymer solutions. Electrospun fibers exibit several unique characteristics, such as large surface area to mass or volume ratio, small pore size between depositing fibers, surface functionalization flexibility, etc. Besides, electrospun synthetic biodegradable polymers have special attention due to the elimination of a secondary surgery to remove the implanted carrier. Incorporation of hydroxyapatite (HAp) particles can increase such polymeric mat compatibility with bone tissues. HAp is widely used for biomedical applications due to chemical and structural similarity to the mineral phase of bone and tooth. The advantages of HAp can be improved by doping other active elements, such as boron. Baron shows positive effect on bone formation [1] and potentially has antibacterial effect [2].

The goal of this study was to evaluate the influence of baron doped hydroxyapatite particles on the electrospun polyvinyl alcohol fibers geometrical parameters.

In this work boron doped hydroxyapatite (b-HAp) nanoparticles was obtained by acid-base method using 2 wt.% of baron in the synthesis stage of HAp powders. Another group of b-HAp particles after precipitation were sintered consecutively at 1000°C for 2 h. Electrospinning solution (C=12%) was prepared by dissolving of polyvinyl alcohol (PVA, M_w = 72000 g/mol) in distilled water at 80 °C temperature and then 5 wt.% of b-HAp particles were dispersed ultrasonically.

SEM investigations show that b-HAp nanoparticles tend to attach to PVA fibres and form large agglomerates (see Fig.). Baron doped powder sintering at high temperature influences on the formation of more than twice larger b-HAp particles (from *ca*. 80-100 nm up to *ca*. 200-300 nm).



a b

Fig. SEM images of electrospun PVA nano/microfibers with incorporated b-HAp (a) and sintered b-HAp (b) particles

Besides, baron doping in the hydroxyapatite particles influences on the increase of PVA fibres diameter. The incorporation of b-HAp instead of HAp decreases nanofibers (diameter less than 100 nm) amount from 60 % down to 13 %. In this case amount of microfibres with diameter of 100-200 nm arise up to 75 %, while for HAp nanoparticles such dimension fibres reach less than 40 %. However, such fibre dimensions obtained by electrospinning successfully may be used for biomedical application [3].

Thus, electrospun polyvinyl alcohol nano/microfibre mats can be modified with bioactive baron doped hydroxyapatite particles and used as high biocompatibility bone scaffolds materials.

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DP2. CELL CULTURES AS MODEL SYSTEMS IN BIOCOMPATIBILITY EVALUATION OF NEW MATERIALS FOR BONE IMPLANTS

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DO4. SYNTESIS AND CHARACTERIZATION OF NEW DOUBLE QUATERNARY POLYMERS AND THEIR HYDROGELS AS BIOMATERIALS WITH POTENTIAL ANTIMICROBIAL APPLICATIONS

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Polymers, containing quaternary ammonium salts as pending groups, are well known in medicine and find substantial commercial use in the household chemical products because of their antimicrobial and antibacterial properties. Their unique performance is due to and depend on the number and the distribution of ammonium groups in their macromolecules. Adding a second quaternary nitrogen atom in their pending chains is expected to increase their antibacterial properties, as it is known for e.g. surfactants with similar structure. This double quaternary side chains polymers however are never synthesized until now.

In this study, for the first time, we have synthesized a methacrylic monomer, which contains in its side chain two quaternary ammonium groups, namely N^1-(2-(methacryloyloxy)ethyl)-$N^1N^1N^2N^2N^2$-pentamethylethane-1,2-diaminium dichloride. Then, via crosslinking polymerization we have obtained polymer networks from it using two different crosslinking agents poly(ethylene glycol diacrylate) (PEGDA) and N,N'-methylene bisacrylamide (MBAA) with different concentrations. The obtained hydrogels were characterized in terms of their equilibrium swelling ratio, elastic modulus, thermal properties and their biological activity was also tested.

DO5. ABILITY OF POLYETHER IONOPHORE MONENSIN TO BIND LA(III) METAL IONS

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The study on the properties of polyether ionophorous antibiotics and their metal complexes (more specifically Monensin) has become an important reason of research in the biological chemistry field due to diverse activity of ligands against parasites, Gram-positive microorganisms, fungi, tumor cells, etc. Knowledge on coordination chemistry of polyether ionophores and their complexes with monovalent and divalent metal ions was further extended to the evaluation of ability of Monensin to bind trivalent metal ions of lanthanum (La(III)).

The complex formation of Monensin with La^{3+} ions was assessed in methanol solutions for the first time by synchrotron radiation circular dichroism (SRCD spectroscopy). It was found that depending on metal-to-ligand molar ratio a neutral $[La(Mon)_3(H_2O)_3]$ and two positively charged $[La(Mon)_2(H_2O)_2]^+$ / $[La(Mon)(H_2O)]^{2+}$ complexes were formed.

The neutral complex was successfully isolated in solid state. It was characterized by various spectroscopies as IR, ESI-MS, FAB-MS, as well as by elemental analysis. It is assumed that the structure of the complex corresponds to the mononuclear complex species, containing three deprotonated ligands. Each Monensin monoanion is coordinated to the metal ion in bidentate mode *via* carboxylate moiety and hydroxyl function, both located at the opposite ends of the molecule. The cavity of ligands is occupied by water molecule coordinated to the metal center and participating in hydrogen bonds of different origin.

Coordination of La^{3+} ions in the structure of Monensin improves its bactericidal activity against *B. Subtilis, B. Mycoides* and *S. Lutea*, which could be due to the introduction of three moles of the ligand in the composition of the neutral complex.

DO6. PCR DETECTION OF SIX TRICHINELLA SPECIES

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1. Introduction:

Nemathodes of the genus *Trichinella* are the causative agent of trichinellosis. They are zoonotic parasites with a cosmopolitan distribution which infect over 2500 people annually (http://www.who.int). This nematode was discovered 180 years ago [3,4] and the next hundred years, the large number of human infections due to the consumption of domestic pork products focused the attention of the veterinarians, physicians and public health authorities on the important role of some hosts (more than 100 species of mammals, birds and reptiles) in the natural cycle of this nematode. Since it was discovered, this genus was expended from

being monospecific to nine species including three other genotypes of undetermined taxonomic rank. The helminths belonging to the genus are unique among zoonotic nematodes. They are characterized two generations in the same host and by infective first stage larvae (L1) rather than infective third stage larvae (L3) which typify most other nematode species. Inasmuch as discriminating morphological have been scant, our understanding of the genus *Trichinella* has been relegated to a compilation of molecular, biochemical, genetic and biological data. Unfortunately present methods for detection of *Trichinella spp*. (compressorium method, artificial digestion) do not always sufficiently recognize *Trichinella* larvae and these techniques are labor-intensive, time consuming and do not differentiate isolate on the species level since there are no distinguishing morphological features.
The aim of this study is to obtain new data on the taxonomy and genetic characteristics of *Trichinella* isolates collected from different regions of the world and Bulgaria by using PCR assay. This is a novel technique for species detection and identification based on the specific hybridization of a probe designed for a certain species with the DNA in the samples to be analyzed. Only the DNA complementary to the specific probe will hybridize to it (M. Alonso et al. 2011). The chosen method is accurate, rapid and highly suitable for specific identification of the common species of the genus *Trichinella*.

2. Materials and metods:

2.1 Truchinella isolates

Trichinella reference strains (ISS03- *Sus scrofa*, Poland; ISS13-*Nyctereutes procyonoides*, Caucasus, Russia; ISS010- *Ursus maritimus*, Island; ISS02-*Vulpes vulpes*, Italy; ISS029-*Crocuta crocuta*, Kenya, Africa; ISS35- *Ursus americanus*, Pennsylvania, South America) used in this study were kindly provided by prof. Edoardo Pozio (European Union Reference Laboratory for Parasites) and were kept in mice in the vivarium of IEMPAM-BAS. During the experiments were satisfied all the requirements of Regulation No. 20/2012. (Animal Protection Act). Larvae of reference strains were isolated from muscle tissue using artificial digestion at 37 °C and continuous stirring. After several washing steps with distilled H_2O, larvae were stored in 1,5 ml distilled H_2O at -20°C prior DNA extraction.

2.2 DNA extraction from larvae

For the DNA- extraction was used NZY Tissue gDNA Isolation kit. All DNA isolation steps were performed on ice. 180 µl of Digestion solution and 25 µl Proteinase K solution were added to the samples. All the sample were mixed by Vortex. Mixture was incubated at 56 °C for 60 min. and vortexed occasionally during the incubation. According to the protocol larvae were homogenized in 200 µl Lysis solution, mixed by Vortex, washed in 400 µl 50% ethanol and transferred to a column. Each sample was centrifuged for 1 min. at > 11000xg. The flow was discarded through and the columns were placed in new collection tubes. After washing with 500 µl Wash buffer 1 and Wash buffer 2 and subsequent centrifugation respectively 8000x g for 1 min and 12000xg for 3 min, the purified DNA was transferred to a clean tube, washed twice with Elution solution and centrifuged at 8000x g for 1 min. The thus obtained DNA was used for PCR.

2.3 Primer design

Based on described in the literature [6] primers corresponding to the specific gene sequences ITS 1, ITS 2 (Internal Transcribed Spacer) and ESV (expansion segment V), which represent the DNA regions with a relatively high degree of variation even at closely related

species located between highly conserved genes of ribosomal subunits was sought match in the current data in NCBI. The chosen five different PCR primer sets are listed in tabl.1. All sequences were aligned with sequence data analysis software. When used simultaneously, this primer mix generates a specific and unique amplificates for each species and genotype.

Species	Primer 5'-3' (sense/antisense)	Amplicon size
T. spiralis	GTTCCATGTGAACAGCAGT (19bp) CGAAAACATACGACAACTGC(20bp)	173bp
T. britovi	GTTCCATGTGAACAGCAGT (19bp) CGAAAACATACGACAACTGC(20bp) GCTACATCCTTTTGATCTGTT(21bp) AGACACAATATCAACCACAGTACA(24bp)	129bp 253bp
T. pseudospiralis	GTTCCATGTGAACAGCAGT (19bp) CGAAAACATACGACAACTGC(20bp)	295-315bp
T. nelsoni	GTTCCATGTGAACAGCAGT (19bp) CGAAAACATACGACAACTGC(20bp)	155bp
T. nativa	GTTCCATGTGAACAGCAGT (19bp) CGAAAACATACGACAACTGC(20bp)	129bp
T. murrelli	GTTCCATGTGAACAGCAGT (19bp) CGAAAACATACGACAACTGC(20bp) GTGAGCGTAATAAAGGTGCAG(21bp) TTCATCACACATCTTCCACTA(21bp)	127bp 315-317bp

Table 1. The composition of the primers and size of the received non-empirical amplicons.

2.4. Multiplex PCR

The PCR reaction was performed in a volume of 50 µl. We used mix and amplification primers composed of company Nzytech Lda (Portugal). Primers were optimized by dilution according to the manufacturer. The reaction mixture contained 10 µl of sample, 25 µl of amplification mix of 12 µl of the primer mix and 3 µl of nuclease free water. All reactions were carried out in The Applied Biosystems® Veriti® 96-Well Thermal Cycler under the following cycle conditions: (initial denaturation) - 10 min. / 95º C, melting (melting) - 1 min. / 95º C, (annealing) - 1 min. / 55° C and (extension) - 1 min. / 68º C for 40 cycles. The last cycle for a further extension of the DNA strand (final extension) - 10 min. / 68ºC.

2.4 Real-time PCR

The PCR reactions were carried out in total volume of 50 µl containing 10 µl of DNA template, 25 µl qPCR SuperMix SYBR®GreenER TM (Invitogen), 2µl of each primer (concentration 0,2 µM) and 11 µl nuclease free water. The amplification of the desired regions had the following mode: A single initial denaturation step of 5 min. at 95ºC was followed by 60 cycles of 94ºC for 15sec. (denaturation) and 25 sec. at 55 °C- attaching primers to single DNA strands (annealing) and polymerization the DNA chain (elongation).

4. Results and discussion

Primer pairs are connected in three species-specific genetic regions- ESV, ITS1 and ITS2. Universal primer pair, encoding ESV genes, form with all species of the genus *Trichinella* amplified products (amplicons). In those species that have been observed identical weight products, e.g., *T. britovi, T. nativa* and *T. murrelli*, after amplification of the gene were added ESV species specific primer pairs coding ITS1 for *T. britovi* and ITS2 for *T. murrelli.* The aim was to obtain additional bands characterizing each of the two types, and as a final result, species specific diagnostic bands for each of the six species. The results of the multiplex PCR products reported on 1% agarose gel are presented in figure 1, after having the molecular weights of the resulting peaks measured by software Gene Tools Company Cynoptics Ltd.

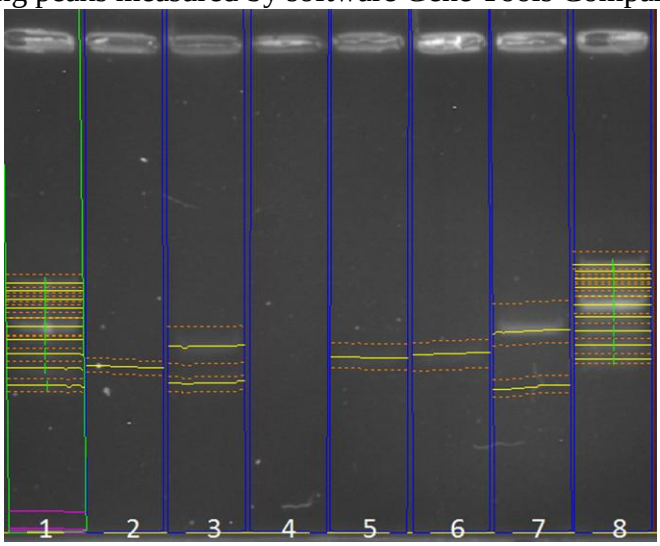

Fig.1 Agarose gel separation of multiplex PCR products from: 1. Molecular weight marker (100-1 000 bp) 2. T. spiralis, 3. T.britovi, 4. T. pseudospiralis, 5. T. nelsoni, 6.T. nativa, 7. T. murrelli, 8. Molecular weight marker (100-1 000 bp).

Results indicates unique and simple banding patterns for each of the genotypes. Electroferograms show obtained amplicons as follows: *T. spiralis* - a band - 173 bp, *T. britovi* - two bands - 129 bp and 253 bp, *T. pseudospiralis*- not noticeable amplification product, *T. nelsoni* - a band - 155 bp, *T. nativa*- a band - 129 bp, *T. murrelli* - two bands - 127 bp and 315 bp. At least one PCR fragment from each genotypic binding pattern was generated from the DNA -derived ESV.

Of great significance to us is that the pair of primers corresponding to the large rDNA ribosomal subunit (ESV) initiates amplification products with different sizes in common sylvatic species in Bulgaria - *T. spiralis, T. pseudospiralis* and *T. britovi.* The specific results we received and the results described in the literature [1,5,6], also the fact that it is a simple, one-step assay for the differentiation of all *Trichinella* genotype define the method as a modern, credible and easily executable.

Since in multiplex PCR we could not get amplification of a specific product of *T. pseudospiralis* (probably due to the insufficient amount of DNA) we decided to examine samples with real-time PCR method, which gives a clear visual representation of each cycle of the reaction from the start-up process of hybridization, in contrast to conventional PCR, which distinguishes products according to their size at the end of the reaction.

Real-time PCR method allows displaying the results at the stage of annealing while it is running, saving time from secondary visualization or identification techniques of the PCR products. For each of the six included *Trichinella* species we were able to establish a species-specific amplification with primers shown in *Table.1*. Using pure *Trichinella* DNA, all

reference strains showed C_T values about 30 threshold cycle (when fluorescence signal increases to a level of reporting), indicating the presence of amplification which allows early detection and differentiation (Fig.2)

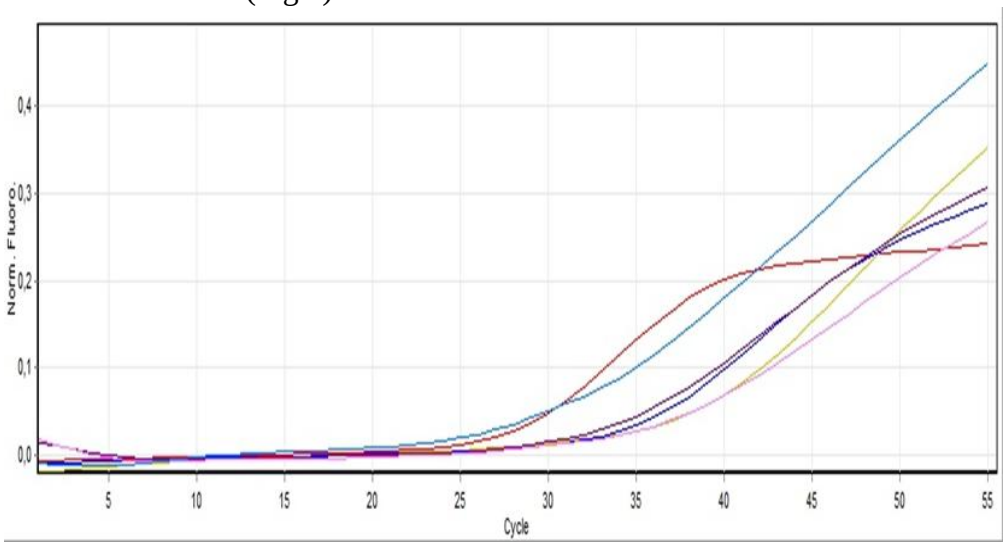

Fig. 1 Amplification curves of the six Trichinella species analyzed by RotorGene 6000 v 1.7.softwear.

In contrast to other typing methods (trichinoscopy, artificial digestion) the PCR is rapid and reliable method enables the accurate identification of *Trichinella* infections. C_T values allow a distinct species identification which was confirmed by the results obtained in Multiplex PCR. Real-time PCR has become important technique in many fields of food industry, it is a powerful tool in the food quality and security control. The developed methodology allows the adequate meat control for prevention of trichinellosis in human, in mode fast and effective, easing the routine inspection by statement agencies, and increasing the confidence of the consumer [2].

It can be concluded that the Multiplex PCR can be routine one-step assay profiling multiple geographical isolates and genotypes. Real-time PCR is acquiring more importance because of its high speed and sensitivity. The results of this study provide an additional information, generate new datasets and re-evaluate the existing data which could help us for developing a new approach for treatment, control and prevention of trichinelosis.

Acknowledgments:
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DO7. ANALYSIS OF SOME LIVER BIOCHEMICAL INDICES AND PARAMETERS OF THE STATE OF HEALTH IN *ASCARIDIA GALLI* INFECTED AND BASIC ZINC-COPPER SALT TREATED CHICKS

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Abstract

Contents of some liver trace elements, antioxidants and quantitative parameters of the state of body health were investigated in chicks after experimental *Ascaridia galli* infection and basic zinc-copper (Zn-Cu) salt application. The levels of trace elements Zn and Cu and antioxidants vitamin C and vitamin E were decreased in the livers of *A. galli* infected chicks. The contents of liver Zn and Cu were significantly increased and the concentrations of vitamins E and C were slightly elevated after the salt application on healthy or *A. galli* infected chicks. The treatment with the basic Zn-Cu salt showed a trend for normalization of the decreased from the parasitic infection chick liver antioxidant and trace element status. Quantitative parameters of the chicks' state of health: parasite burden, body weight and mortality were detected in the experimental birds. The parasite burden was reduced in *A. galli* infected chicks, received the basic Zn-Cu salt. The salt application increased the body weight in the salt treated chicks (healthy or infected). The mortality of the birds from the treated with the basic Zn-Cu salt groups was decreased. The treatment with the basic Zn-Cu salt on experimentally *A. galli* infected chicks had beneficial effect on all investigated body indices.
Key words: Antioxidants, trace elements, liver, parasite burden, body weight, mortality, experimental *Ascaridia galli* infection, basic zinc-copper salt, chicks.

Introduction

Ascaridia galli is a common parasite of poultry and has been reported in chickens, turkeys, guinea fowls, pigeons, ducks and geese. It is the largest internal parasitic nematode causing helminthiasis in poultry. Increased feed intake, blood loss, anaemia, reduced body weight, and increased mortality may occur during the parasitic disease [18]. The most important clinical symptom of natural and experimental *A. galli* infections is loss of body weight, which increases parallelly to worm load. *A. galli* infection is known to alter some biochemical parameters and to cause disturbance in the host mineral balance [11, 12, 22].

In poultry, trace elements copper (Cu), zinc (Zn), manganese (Mn) and cobalt (Co) are essential for the health, growth, production and reproduction of the birds [14]. Salts of these trace elements are used to correct mineral deficiencies arising during different parasite infections. Data exist, that the treatment of mineral disbalance with basic salts of certain transitional elements is better tolerated by the animal organism than the application of normal salts. Benefits, associated with the application of basic salts, persiste even after more prolonged treatment of different hosts. Application of basic salts of different metals leads to

effects of improvement of growth, survival and trace element balance in the infected host organisms in comparison with the normal salts [1, 13]. Examinations also exist on the effects of organically complexed metal chelates of Cu, Mn and Zn on broilers or laying hens' growth performance and body quality in comparison with the respective inorganic trace mineral compounds [5, 24].

Antioxidant defence system of the body is very important in protecting tissues from oxidative destruction during different pathological processes. It has a cellular protective effect against oxidative stress, which resulted in cells, organs and tissues damages during different parasitic infections [13]. Vitamins C and E are very important antioxidants. Vitamin E has been reported as an excellent biological chainbreaking antioxidant that protects cells and tissues from lipoperoxidative damage induced by free radicals [16]. Vitamin C has been demonstrated to enhance antioxidant activity of vitamin E by reducing the tocopheroxyl radicals back to their active form of vitamin E or by sparing available vitamin E [17, 23].

The aim of the present study is to be investigated liver trace element (Zn and Cu) status, antioxidant (vitamins E and C) status and some quantitative parameters of the state of body health (parasite burden, body weight and mortality) in chicks after experimental *A. galli* infection and basic Zn-Cu salt application.

Material and methods

Eighty in number, one-day-old male chicks, belonging to the Hisex breed (a crossbreed of the Dutch Leghorn), were divided in 4 groups of 20 animals: Group 1 - controls (healthy birds); Group 2 – chicks treated with the basic Zn-Cu salt; Group 3 – *A. galli* infected birds and Group 4 - infected with *A. galli* and treated with the basic Zn-Cu salt chicks.

The chicks were placed on pine shavings in 1.2 X 3.6 m pens and were maintained on a 24 h constant light schedule in heated, thermostatically controlled, stainless starter batteries with raised wire floors. Feeders and water cointainers were also of stainless steel construction to minimize environmental metal contamination. All chicks were fed on a corn-soybean meal diet formulated to meet the nutrient requirements of the growing chicks [19]. Chicks' accesses to food and water were ad libitum. The chicks from Groups 3 and 4 were experimentally infected per os with 450 embryonated eggs at 20 days posthatching per chick [21]. The chicks from Groups 2 and 4 were treated with 0.170 g basic Zn-Cu salt per 1 kg food per day. The salt was given orally with the food for twenty days, starting on the 5^{th} day post infection (p.i.).

The experiments were conducted in compliance with the requirements of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Specific Purposes and the current Bulgarian laws and regulations.

The body weights and mortality were studied on the 60^{th} day p.i. in the chicks from all investigated groups.

Chicks were euthanized by CO_2 inhalation on the 60^{th} day p.i. Alimentary tracts of the experimentally infected birds were opened and the intestinal contents were examined for presence of immature and mature parasites *A. galli*, which were counted.

The chick livers were collected and were investigated for contents of vitamins E and C and trace elements Zn and Cu. Vitamin E concentration was determined by modified fluorimetric method [4]. Vitamin C level was determined spectrofotometrically by modified method of Omaye et al. [20]. For detecting the trace elements contents, the liver samples were dried at 100^0C for 24 h, weighted, ground and then burned slowly in muffle furnace at up to 480^0C for 48 h. The ashes obtained were treated with a mixture of concentrated H_2S0_4 and $HN0_3$ (1:5) in a sand bath and the wet residues were dissolved in 1M HCl. The determination of Cu and Zn levels was made using an atomic absorption spectrophotometer PU-900 (Pye Unicam, Madrid) [2].

The received results were statistically processed using variation analysis and Student's t-test. $P < 0.05$ was considered statistically significant.

Results

The results from the investigations of liver trace elements, antioxidant status and quantitative parameters parasite burden, body weight and mortality are presented in Fig. 1 – 7.

The liver contents of Zn and Cu are reduced with 37% and 24% respectively in *A. galli* infected chicks ($P<0.001$). The addition of the basic Zn-Cu salt leads to increasing of the levels of Zn and Cu in the healthy and *A. galli* infected chicks. The increasing of these elements in the only basic salt treated chicks is 22% and 32% respectively compared to the controls ($P<0.001$). The elevation of Zn and Cu levels in *A. galli* infected and the basic Zn-Cu salt treated chicks is 51% and 40% compared to the amounts in only infected chicks ($P<0.001$). The contents of these trace elements in the livers of combined influenced with the *A. galli* infection and salt treatment chicks are similar to the control levels. So the losses of chick liver trace elements Zn and Cu, caused by the parasitic infection are restored by the application of the basic Zn-Cu salt (Fig. 1, 2).

Fig. 1. Zinc (Zn) concentrations in the livers of *Ascaridia galli* infected and basic Zn-Cu salt treated chicks (µg/g)

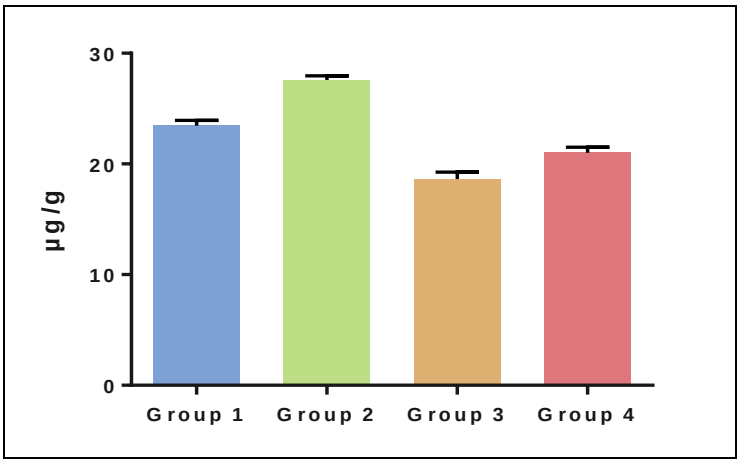
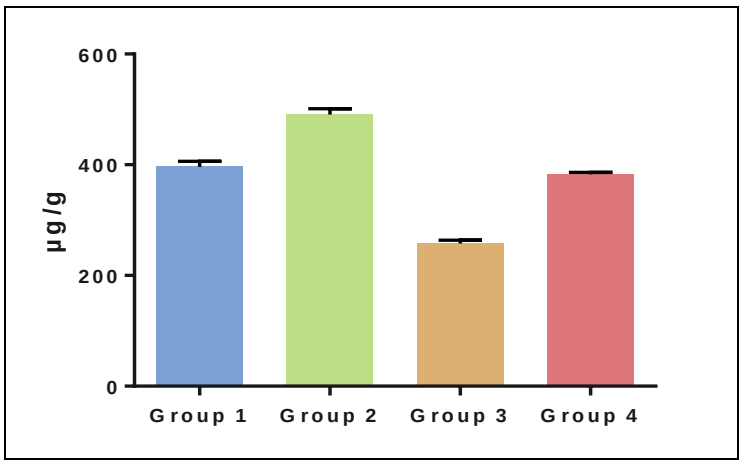


Fig. 2. Copper (Cu) concentrations in the livers of *Ascaridia galli* infected and basic Zn-Cu salt treated chicks (µg/g)



The levels of liver vitamins E and C are reduced in *A. galli* infected chicks with 34% and 15% respectively compared to the healthy chicks (P<0.001). The addition of Zn-Cu salt leads to increasing of the levels of vitamin E in healthy and *A. galli* infected chicks, with 14% and 21 % respectively (P<0.001). The vitamin C amount is increased with 9% in the healthy birds and with 10% in infected chicks after the treatment with the basic Zn-Cu salt (P<0.01) (Fig. 3, 4).

Fig. 3. Vitamin E contents in the livers of *Ascaridia galli* infected and basic Zn-Cu salt treated chicks (mg %)

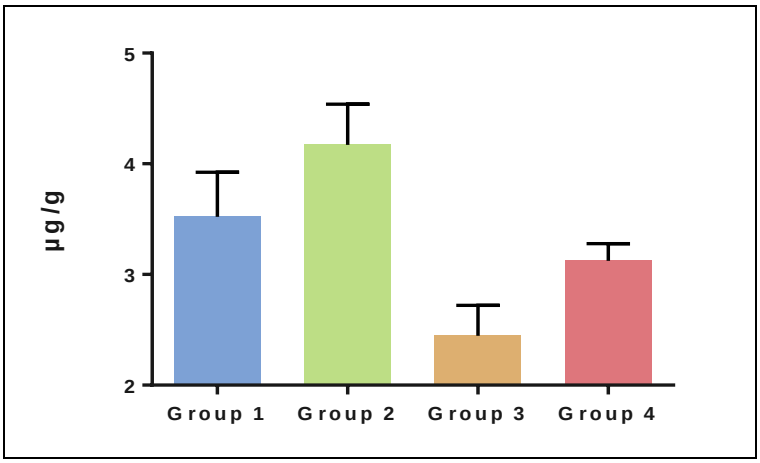
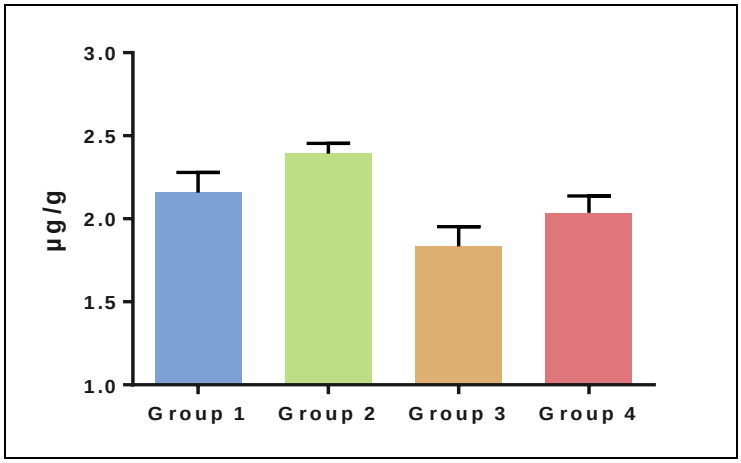
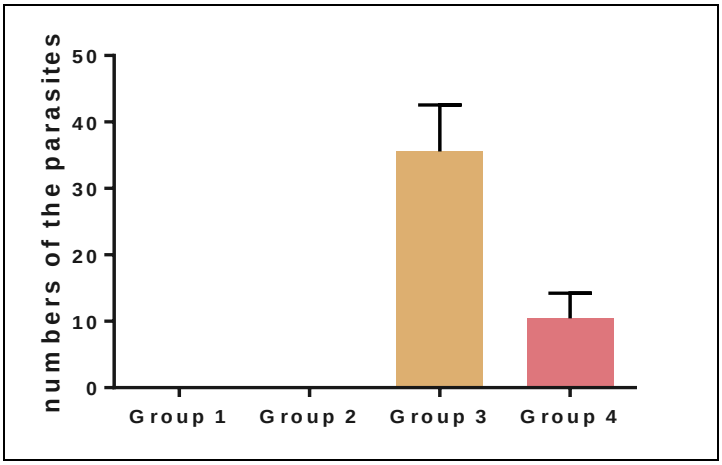


Fig. 4. Vitamin C contents in the livers of *Ascaridia galli* infected and basic Zn-Cu salt treated chicks (mg %)



The body weights of the experimental birds are taken on the 60th day p.i. Reduction of the body weight is detected in *A. galli* infected chicks compared to the controls (P<0.001). The body weight is increased both in the healthy and *A. galli* infected chicks after application of the basic Zn-Cu salt (P<0.001) (Fig. 5). It is established that the applied basic Zn-Cu salt influences positively the decreased from *A. galli* infection chick body weights.

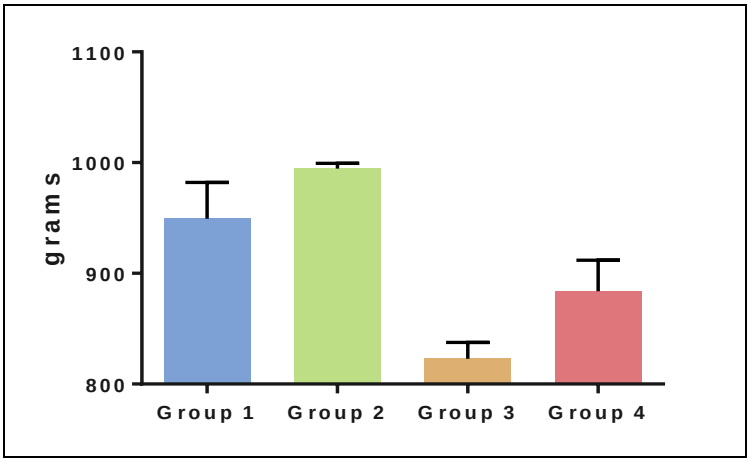
Fig. 5. Parasite burdens in *Ascaridia galli* infected and basic Zn-Cu salt treated chicks (numbers of parasites)



Quantitative analysis of the parasite burdens, body weights and mortality are carried out in the experimental chicks from all investigated groups.

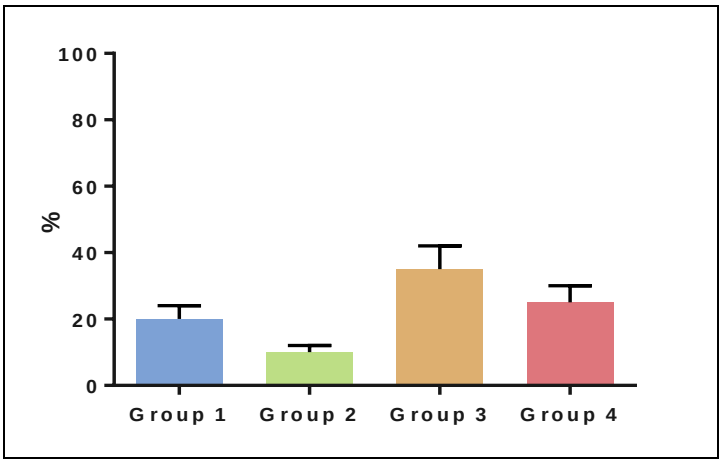
The parasite burden in only infected with *A. galli* chicks is higher than in *A. galli* infected and basic Zn-Cu salt treated experimental birds (Fig. 6). It is shown that the applied basic Zn-Cu salt has an anthelminthic effect on the parasites *A. galli*.

Fig. 6. Body weights of *Ascaridia galli* infected and basic Zn-Cu salt treated chicks (grams)



The mortality of *A. galli* infected chicks, find on the 60th day p.i. is 35%, which is with 10% higher than that in the healthy controls (20%). The chicks' mortality after single basic Zn-Cu salt treatment is 10% and after the combined influence of parasite infection and basic Zn-Cu salt is 25%. It is clear that the basic Zn-Cu salt addition decreases mortality in the groups of the salt treated healthy or *A. galli* infected chicks similarly (Fig. 7).

Fig. 7. Mortality of *Ascaridia galli* infected and basic Zn-Cu salt treated chicks (percents)



Discussion

The parasites *A. galli* cause alterations in various biochemical parameters of the infected birds, including disturbance in the trace element balance. It may be due to the various toxic substances secreting by the parasite via its excretions and secretion into lumen of the gastrointestinal tract of the host. These toxic substances affect the metabolism and all activity of the host. Liver and kidney are the targeting organs which accumulate toxic substance [26]. Trace elements are essential for health, growth, production and reproduction of the organisms. Different metal compounds were applied for treatment of the mineral deficiency of different organisms caused by various pathogenic factors, including helminth infections [12, 14].

In poultry, trace elements Cu, Zn, Mn and Co are essential for optimal bird performance [6]. The ordinary diet in poultry is frequently supplemented with excess of Cu, Zn, Co and Mn in an effort to meet their nutritional requirements [13]. Investigations exist on the effects of single Cu or Zn basic salts or triple mixed basic salts on the liver trace elements balance in chicks with experimental ascaridiasis [10, 12, 14, 15].

In the present study, the levels of liver trace elements Zn and Cu are found to be significantly decreased in *A. galli* infected chicks than in controls. After the basic Zn-Cu salt application, it is established that liver contents of Zn and Cu are increased in the healthy chicks and the losses of these liver trace elements in *A. galli* infected chicks are restored.

In the present study, the liver levels of antioxidants vitamins E and C are found to be significantly lower in *A. galli* infected chicks than in controls. Probably it is consequently to post infection oxidative stress. The oxidative stress is manifested via reduction of some antioxidants such as vitamins C, A and E [9, 13]. It is established that liver vitamin A concentration is also decreased in experimentally *A. galli* infected chicks at the 40^{th} day p.i. [3]. The authors suggest that this finding attributes to the damage of the chick intestinal wall and thus has decreased absorption of vitamins and electrolytes under ascaridiasis.

Dede et al. [8] find out that the concentration of vitamin E is decreased in hosts infected with different parasites in comparison to healthy controls. These data support the findings of the present study.

It has been reported that vitamin C levels decline in sheep infected with *Fasciola hepatica* and *Trichostrongylidae* spp. while infections with *Plasmodium* spp. result in opposite situation. These findings indicate that the concentration of vitamin C is affected by the types of parasites and the hosts they infected [13].

In the present study, it is established that the antioxidant defence system is enhanced after addition of the basic Zn-Cu salt, by increasing of the liver contents of vitamins E and A

and trace elements Zn and Cu both in healthy and as well as in *A. galli* infected chicks. It may be connected to their synergic interactions in the host body [17].

The early stage of *A. galli* infection leads to retarding of the growth and body weight loss. The body weight of infected chick is much lower than that of uninfected ones. *A galli* leads to malnutrition and lowering of products derived from poultry [26]. It is also found that the experimentally *A. galli* infected chicks with different parasite egg doses, show variable decreases in body weight compared to the controls [22]. The authors conclude that the body weight loss is due to reducing or lack of absorption of nutrients, electrolytes and vitamins in damaged intestinal wall in the infected chicks. Considerably decreased body weight and increased mortality in *A. galli* infected chicks in comparison with these of healthy control birds are established in our previous investigations [11].

In the present study it is established that the parasite burdens, body weights and mortality are influenced positively from the application of the basic Zn-Cu salt in experimentally *A. galli* infected chicks. The treatment with the investigated salt increases body weights, decreases mortality in the groups with infected and healthy chicks and reduces parasite burdens in the infected birds. It is in a good agreement with the results of other authors about the beneficial effects of single neutral and basic Cu salts on these parameters [10, 25]. Cu salts show anthelminthic effect on *A. galli* in chicks. The applied in the present study basic Zn-Cu salt also has an anthelminthic effect on *A. galli.*

It is well known that the nutritional status of the infected host exerts influence on both the host susceptibility to parasitic disease, and the severity of the pathological processes. Our investigations are in a good agreement with the results from other studies, where the positive roles of trace element supplementations are proved at the background of different parasitoses [7]. The presently investigated basic salt of combined metals Zn and Cu influences positively the liver trace element status, antioxidant status and some quantitative parameters of the state of health in chicks under experimental ascaridiasis. The antioxidant nutrients Zn and Cu, combined together in a basic compound, can be useful for protecting chick tissues from the oxidative destructions, arising during the parasitic disease ascaridiasis.

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DO8. A SHORT REVIEW ON THE EXPERIENCE OF ANTHELMINTIC TREATMENT IN WILD BOARS

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Abstract

A literary survey has been made about the use of anthelmintics in the European wild boar. The most frequently used anthelmintics have been from the Benzimidazole group (Albendazole, Mebendazole, Febantel, Fenbendazole, Flubendazole). They have been used against gastrointestinal and lung nematodes. Their effect has been strongest against gastrointestinal strongylids, it has been less effective against lungworms and *Capillaria* have been least affected species. Another popular group is that of tetrahydropyrimidine (Pyrantel, Morantel). They have been very efficient against intestinal nematodes. The efficiency against lung nematodes has been also good, with the egg output being stopped, although their adulticid effect has been smaller. Imidazotiazoles (Levamisol, Tetramisol) are a third anthelmintic group that is used in wild boars. The effectiveness of the Imidazotiazole group has been very good against gastrointestinal and lung nematodes, but the risk of drug overdose and intoxication has been higher than the other groups. Studies about the Ivermectin from the Avermectin group have shown that this drug is suitable for treatment of gastrointestinal and lung nematodes and mites, but the effect against *Capillaria* spp. and *Trichuris* spp. has been insufficient.

КРАТЪК ПРЕГЛЕД НА ОПИТА В ПРОТИВОПАРАЗИТНОТО ТРЕТИРАНЕ НА ДИВИ ПРАСЕТА

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Резюме
Извършен е литературен обзор по отношение на използваните при дивата свиня противопаразитни средства. Най-често използваните антихелминтици са били от групата на бензимидазолите (албендазол, мебендазол, фебантел, фенбендазол, флубендазол). Те са използвани срещу стомашночревни и белодробни нематоди. Действието им е било най-силно срещу стомашночревните стронгилиди, по-слабо при белодробните нематоди, а най-слабо са се повлиявали капилариите. Друга група, която е била използвана е на тетрахидропиримидините (пирантел, морантел). Те са били с добър ефект при чревните нематоди. Действали са добре и срещу белодробните нематоди. Макар че адултицидният ефект при тях е бил по-слаб, те са спирали отделянето на яйца. Имидазотиазолите (левамизол и тетрамизол) са трета група противопаразитни средства, изпробвана върху дивите свине. Те са имали много добро действие срещу стомашночревните и белодробните нематоди, но е установено, че опасността от предозиране и отравяне при тях е по-висока. От групата на авермектините са правени опити с ивермектина, който се е оказал подходящ за третиране на белодробните и стомашночревните нематоди и крастните кърлежи, но е бил с по-слабо действие срещу капилариите и трихурисите.

Увод

Дивата свиня е повсеместно разпространен вид и е популярен ловен обект в България. Всяка година от една майка се раждат средно 5-6 малки. Оцеляването на възрастните, броят на приплодите и тяхната преживяемост са свързани с тежестта на паразитното бреме. Паразитите отнемат хранителни вещества и витамини (риск от недоимъчни заболявания), предизвикват възпаления там, където са локализирани, отслабват имунната система и целия организъм, което е предпоставка за инфекциозни заболявания. Паразитите биха могли пряко да доведат до смъртта на своя гостоприемник [12]. За премахване на тези нежелани ефекти се прилагат противопаразитни средства. Тяхното прилагане при дивите животни обаче има своите специфики. Например индивидуалното третиране на дивите животни е невъзможно, използваните лекарствени средства са тествани само при домашни животни, съществува риск от поглъщане на по-голямо количество от лекарството, когато животните се третират групово с храната и т.н. В тази връзка беше поставена целта на настоящата работа, а именно да извършим литературен обзор по отношение на протовопаразитното третиране на диви прасета, като по този начин предоставим на експертите в областта информация, която би ги улеснила при предприемане на действия за контрол и превенция на паразитозите при дивта свиня.

Материали и методи

Настоящата работа представлява литературен обзор по отношение на опита в противопаразитното третиране на диви свине в полеви и експериментални условия. За удобство данните са подредени по хронологичен ред и според фармацевтичната група, към която принадлежат лекарствените средства.

Резултати

Използване на бензимидазоли

Мебендазол даван двукратно орално в доза 15 mg/kg живо тегло е дал добър ефект срещу белодробните инвазии у домашната и дивата свиня [7]. При доза 3 mg/kg за 5 поредни дни мебендазола успешно е намалил *Ascaris suum, Globocephalus urosubulatus* и *Trichuris suis*, но е бил безполезен срещу *Metastrongylus* sp.

Zajícek et al. [15] са провели изследване върху 24 диви свине от двата пола на възраст 1-2 години, намиращи се в резерват и естествено инвазирани с белодробни, стомашночревни нематоди и с кокцидии. Изпитани са били пирантел тартарат + диетилкарбамазин тартарат (в дози от 25 + 50 mg/kg живо тегло), мебендазол 5% премикс и мебендазол 50% премикс (дози от 10 mg/kg и 40 mg/kg живо тегло), като всички са давани в продължение на три дни. Постмортално паразитологично изследване е показало, че най-ефективен е бил мебендазол 50% премикс с 85.4% до 100% освобождаване от паразитите, след това е била комбинацията от пирантел тартарат + диетилкарбамазин тартарат с 58.1%-100% и мебендазол 5% премикс с 47.8%-100% освобождаване от паразитите. Процентът е варирал спрямо вида на нематодите. Изследването на копропроби е показало следните резултати: при пирантел тартарат + диетилкарбамазин тартарат на 6-ия и 15-ия ден след прилагането им намаляването на отделените яйца е било със съответно 99.2% и 70.65%. При мебендазол 5% премикс на 10-ия и 20-ия ден след прилагането му е 94.4% и 79.41%, а при мебендазол 50% премикс е достигнало 96% и 100% на 10-ия и 20-ия ден след прилагането му.

В няколко австрийски резервата заедно с храната на диви свине са давани фенбендазол в доза 30 или 35 mg/kg и флубендазол при лечебен курс за 6-10 дни в доза 150 mg/kg в една трета от дажбата [4]. Прилагането на фенбендазол е намалило отделянето на яйца от *Metastrongylus* spp. с 9,3% до 100%, от *Ascaris suum* с 99%, от *Globocephalus urosubulatus* със 70% до 100% и от *Trichuris suis* с 0 до 100%. Флубендазолът е намалил отделянето на яйца от *Metastrongylus* spp. с 58% до 100%, от *G. urosubulatus* с 67% до 100%, от *T. suis* с 83% до 98% и от *Capillaria* spp. с 25% до 86%. Медикаментите са били приемани добре, без странични ефекти.

Kutzer & Prosl [6] са изследвали влиянието на фенбендазола върху паразитозите при дивата свиня. Авторите са установили, че най-добрата дозировка на препарата е била петкратен прием по 1 mg/kg, която се е оказала 100% ефикасна срещу *Globocephalus urosubulatus, Oesophagostomum dentatum, Ascarops strongylina* и *Physocephalus sexalatus* и от 91.2 до 100% за метастронгилидите. Малкото животни, инвазирани с *Trichuris suis,* също са били напълно обезпаразитени. Лекарството е давано с пелетирана храна и е приемано с охота без странични ефекти, поради което то се препоръчва от авторите за третиране на животните, обект на лов.

Kutzer [5] е изследвал действието на различни дози фебантел срещу различни нематоди (*Metastrongylus, Globocephalus urosubulatus, Oesophagostomum dentatum, Ascaris suum, Trichuris suis* и *Capillaria*). Най-добри резултати срещу метастронгилидите са били получени при 60 ppm/kg храна за 5 дни. Единична доза от 5 mg/kg се е оказала недостатъчна срещу *Metastrongylus* и *T. suis*, а 10 mg/kg е била недостатъчна срещу *T. suis* и *Capilaria* (при 5-дневен курс). Според автора минималните изисквания при групово третиране са 60 ppm за 5 дни, а единичните дози трябва да са 15-20 mg/kg.

Munro et al. [8] са изследвали фекалиите за паразити на 10 мъжки и 8 женски бабирузи (дива свиня в Индонезия). Открити са *Metastrongylus* spp., *Ascarops* sp. и голям брой *Oesophagostomum* sp. Не са били наблюдавани клинични признаци на хелминтоза. Пет животни с телесна маса около 60 kg са били третирани орално с

албендазол в доза 4 mg/kg. Пет дни след това във фекалиите е преустановено откриването на паразитни яйца.

Nesterov & Milla [9] са изследвали екстензивността на инвазия с *Metastrongylus* при диви свине в Румъния. Установили са, че тя е била 89.47% в планински популации и 77.58% при тези от хълмистите области. Лечение с 10% Ринтал (фебантел), даван в доза 500 mg на дива свиня за 3 дни, е постигнало 100% успеваемост.

Използване на имидазотиазоли

Kutzer [4] е третирал диви прасета с левамизол. Препаратът е прилаган заедно с храната в доза 7,5 mg/kg живо тегло, Прилагането му е намалило отделянето на яйца от *Metastrongylus* spp. с 9,3% до 100%, от *Ascaris suum* с 99%, от *Globocephalus urosubulatus* със 70% до 100% и от *Trichuris suis* с 0 до 100%. Авторът посочва, че левамизолът е предизвикал краткотраен пристъп на кашлица при животните, която обаче не е била опасна.

Veselova et al. [13] са третирали млади диви прасета с естествена метастронгилидна инвазия с левамизол, прилаган с храната в доза 15 mg/kg живо тегло. Получените резултати след аутопсия на животните са показали намаляване на паразитното бреме с 99,8%.

Pen'kevich et al. [10] са изследвали екстензивността на метастронгилидната инвазия по диви свине в Беларус в периода 1974-1978 г., при което са установили, че тя се е покачила от 71,4% през 1974 до 100% през 1978 г. Други открити хелминти са били *Trichuris suis, Ascaris suum, Physocephalus sexalatus* и *Heobocephalus urosubulatus*. Проведено е лечение с левамизол и тетрамизол. Левамизолът, даван с храната в доза 10 mg/kg живо тегло за 3 поредни дни, е бил 100% ефикасен срещу нематодите в 938 млади глиганчета. Тетрамизолът, прилаган като 20% гранули в доза 80 mg/kg живо тегло, двукратно през интервал от 3 дни е бил 100% ефикасен. Профилактиката с тетрамизол с ежедневна доза 12 mg/kg за 30 дни се е оказала паразитологично и икономически изгодна.

Goreglyad et al. [2] са установили, че в гората Белавежа, Белоруска ССР, дивите свине са били инвазирани с *Metastrongylus* (38,3%), *Oesophagostomum* (15,4%), *Ascaris* (14,2) и *Trichuris* (11,5%). Профилактичното третиране с тетрамизол-гранули (20%) в доза 12 mg/kg живо тегло, добавян към храната за един месец е било ефикасно.

Използване на тетрахидропиримидини

Kutzer [3] е описал използването на банминт (пирантел) и морантел за контрол над хелминтите (главно на стомашночревните и белодробните нематоди) в дивите преживни и дивата свиня в Австрия. При дивите свине най-задоволителни резултати са получени от 25 mg/kg банминт и 10 mg/kg морантел, като и двата са давани в продължение на два дни. Всички дозировки са били добре понасяни.

Дивите свине в зоологическа градина в Индия са били инвазирани с *Ascaris suum*. Приложен е пирантел памоат в доза 15mg/kg [12]. Той е бил 100% ефективен, което е било доказано от изследване на копропроби. Не е било наблюдавано реинвазиране 55 дни след третирането.

Използване на авермектини

Fernandez-De-Mera et al. [1] са изследвали ефективността на даван с храната ивермектин върху естествено опаразитени с множество хелминти европейски диви свине. Ивермектинът е прилаган в доза 2.4 ppm. Постмортално е установено, че ефективността на ивермектина срещу зрелите форми на *Metastrongylus* sp., *Ascaris suum*, *Ascarops strongylina*, *Physocephalus sexalatus* и *Simondsia paradoxa* е била 100%, а ефикасността срещу *Oesophagostomum dentatum* е била 85,1%. Резултатите при *Globocephalus urosubulatus*, *Trichuris suis* и *Capillaria garfiai* са били незадоволителни. Авторите препоръчват при противопаразитно третиране на дивата свиня, ивермектинът да се

комбинира с втори препарат с по-съществено действие върху трихурисите и други хелминти.

Rajković-Janje [11] е изследвал пет естествено загинали прасенца в ловен район в Хърватска, при което е установил наличието на *Metastrongylus apri* и *Metastrongylus pudendotectus* в белите дробове и *Ascarops strongylina, Physocephalus sexalatus* и *Globocephalus urosubulatusin* в стомашночревният тракт. Открити са също кокцидии и саркоптидни кърлежи. Било е проведено лечението на три различни обекта в резервата с 0,6% ивермектин в храната за 7 дни. Преди третирането са били установени стронгилидни яйца в 70-100% от фекалните проби и яйца на *Strongyloides ransomi, Trichuris suis, Ascaris suum, A. strongylina* и *Ph. sexalatus* в 10-50% от пробите. На 14 ден от началото на лечението са били установени стронгилидни яйца само в един от трите обекта в 10% от фекалните проби, а в нито един обект не са били установени яйца на други видове хелминти.

Обобщените литературни данни показват следното:

- Противопаразитните средства при дивите свине са били прилагани перорално след добавянето им към фураж за примамка.
- Повечето противопаразитни средства са прилагани многократно, в продължение на 2-3 до 5-7 дни. Действието на еднократния прием е изследвано при албендазола, фенбендазола, флубендазола, левамизола и пирантела. Ефектът от еднократното прилагане се е оказал в повечето случаи недостатъчен за пълното елиминиране на метастронгилидни и трихурисни инвазии. Само албендазолът и в по-висока доза левамизолът са показали добри редултати срещу метастронгилидите.
- Най-често използваните противопаразитни средства при дивите свине са били бензимидазолите. Те са били по-ефикасни срещу стомашночревните стронгилиди, отколкото срещу белодробните нематоди, трихурисите и капилариите. За елиминиране на по-устойчивите хелминти обикновено е имало нужда от по-висока дозировка.
- От имидазотриазолите най-ефективни са били левамизолът в доза 15 mg/kg ж.т. и тетрамизолът, прилаган двукратно през три дни в доза 80 mg/kg ж.т. Тетрамизолът е използван и като профилактично средство, като в продължение на месец е прилаган в доза 12 mg/kg ж.т.. Двукратното приложение на пирантел и морантел е показало добри резултати срещу стомашночревните и белодробните нематоди.
- По-рядко при дивите свине е прилаган ивермектин. При това по-добър резултат, достигащ до 100% ефективност срещу хелминти и крастни кърлежи, е постигнат при използване на по-висока дозировка за период от 7 дни.
- При сравняване дозировките на лекарствените средства, прилагани при дивите прасета, спрямо тези, прилагани при домашните се установява следното: албендазолът и мебендазолът са прилагани в по-ниски дози, пирантелът е прилаган при дивите прасета в доза, препоръчвана и за домашните, но с двукратен прием, останалите лекарствени средства са прилагани в по-високи дози или са били с по-дълъг лечебен курс.

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DO9. ФОЛИЕВА КИСЕЛИНА

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Фолиевата киселина е водоразтворим витамин, който не се натрупва и синтезира от организма. Затова е много важно да си я набавяме ежедневно чрез храната или под формата на хранителни добавки.

Тя заема централна роля в умножението и растежа на клетките, участва в образуването на плацентата и изграждането на костния мозък на ембриона. Тя е отговорна за производството на ДНК в началния стадий на бременността и участва във формирането на тъканите. Приемът на фолиева киселина по време на бременност намалява риска от

вродени увреждания на бебето до 70% и спомага синтеза на ДНК и РНК. Витаминът влияе положително върху организма на бъдещата майка – като потиска образуването на стресови хормони. Фолиевата киселина се съдържа в повечето тъмнозелени зеленчуци, в някои меса, черния дроб и зърнените продукти.
Организмът е особено уязвим при недостиг на фолиева киселина и витамини B1, B6 и B12. По време на бременност недостигът на фолиева киселина може да доведе до дефекти на невралната тръба или т. нар. Spina bifida.
Spina bifida е вроден дефект, който се проявява през първите четири седмици на бременността. Спина бифида представлява вродена аномалия в развитието на гръбначния стълб и гръбначния мозък, при която има отвор в гръбначния канал, през който е възможно да се подава част от гръбначния мозък.
Има различни типове спина бифида най-чест, от които е менингоцелът. При него между прешлените се формира достатъчно голям отвор, така че гръбначният мозък и гръбначномозъчните обвивки се подават извън гръбначния стълб, формирайки своеобразна „торба“ под кожата на гърба на новороденото. Това прави гръбначния стълб силно уязвим на механични въздействия и инфекции. Макар че дефектът може да бъде коригиран по хирургичен път, нервната система често понася поражения преди това да се случи. Това може да доведе до пълна или частична парализа на долните крайници, уринна или фекална континенция или загуба на сетивност.
Симптомите и усложненията на спина бифида се разделят в три основни категории – когнитивни, двигателни и симптоматика от страна на вътрешните органи.
Лечението е оперативно, като неговата цел е репонирането във вертебралния канал и покриването с нормална кожа на херниираните нервни тъкани с пластика на вертебралния канал.

Session E.

Chairpersons:

Prof. Anna Tolekova, MD, PhD
Medical Faculty, Trakia University, Stara Zagora, Bulgaria

Assoc. Prof. Radostina Alexandrova, MSc, PhD
Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences

Secretary: Abedulkadir Abudalleh, MSc, PhD
Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences

EO1. BEIGE CELLS AND DO THEY HAVE A POSSIBLE ROLE IN THE TREATMENT OF METABOLIC SYNDROME

Angel Todev, Alexander Brunkov, Viktoriya Trendafilova

Medical Faculty, Trakia University, Stara Zagora, Bulgaria

EO2. ENDOTELIAL RECEPTORS ANTAGONISTS IN PULMONARY HYPERTENSION

Yuksel Mekov, Georgi Madjarov

Medical Faculty, Trakia University, Stara Zagora, Bulgaria

EO3. EXPERIMENTAL MODEL FOR DIET- INDUCED DIABETES TYPE 2

Petya Hristova, Vencislava Dimitrova, Daniel Addai, Jacqueline Zarkos

Medical Faculty, Trakia University, Stara Zagora, Bulgaria

EO4. PODOPLANINS AS MARKERS FOR ANGIOSARCOMA

Georgi Madjarov, Yuksel Mekov

Medical Faculty, Trakia University, Stara Zagora, Bulgaria

EO5. COMPLECATED FACE OF MULTIPLE SCLEROSIS

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Abstract: Multiple sclerosis is an increasingly complex disease in terms of its pathogenesis, comorbidities and prognosis. It is progressive neurodegenerative disease typically affect young adults. There are different pathways to tissue injury in multiple sclerosis. Inflammation, demyelination, and axonal degeneration are the major pathologic mechanisms that cause the clinical manifestations. However, the cause of multiple sclerosis remains unknown. Although traditionally considered a disease of focal white matter lesions, the spectrum of multiple sclerosis pathology is now understood to encompass a broader array of abnormalities, including diffuse damage of so-called normal-appearing white matter and normal-appearing grey matter on magnetic resonance imaging, both of which are associated with a progressive loss of brain volume. Multiple sclerosis affects more women than men. There are no clinical findings that are unique to multiple sclerosis, but some are highly characteristic. The core multiple sclerosis phenotypes are those of relapsing and progressive disease. In addition, these phenotypes are modified by assessments of disease activity and disease progression. Recognition of an inflammatory and neurodegenerative phase of multiple sclerosis has allowed the targeting of therapies for various phase of disease. The treatment consists of acute relapse management, symptomatic treatment, rehabilitation and disease modifying therapy. Nowadays clear treatment goals are defined: maximize neurological reserve, cognitive function and physical function by reducing disease activity. Preventing disability for patients with progressive disease is still unmet need. That is why truly achieving new concept of "no evidence of disease activity" will require the development of new agents that directly target mechanisms of disease progression.

Key words: multiple sclerosis, multiple sclerosis treatment, disease-modifying therapies, relapsing-remitting multiple sclerosis, magnetic resonance imaging

Multiple sclerosis (MS) is an increasingly complex disease in terms of its pathogenesis, comorbidities and prognosis. It is progressive neurodegenerative disease that typically affects young adults, causing irreversible physical and mental disability. During its course, a range of problems, impacting on activities of daily living and on social and/or occupational functioning can occur. The disease thus negatively affects the lives of people with multiple sclerosis and their families, and lead to large, long-term health and economic burdens.

There are different pathways to tissue injury in multiple sclerosis [30]. Inflammation, demyelination, and axonal degeneration are the major pathologic mechanisms that cause the clinical manifestations [5, 6]. However, the cause of multiple sclerosis remains unknown [13, 20]. The most widely accepted theory is that multiple sclerosis begins as an inflammatory immune-mediated disorder characterized by autoreactive lymphocytes [27, 30]. Later, the disease is dominated by microglial activation and chronic neurodegeneration [5]. Typical neuropathologic feature of multiple sclerosis is the presence of focal demyelinated plaques within the central nervous system, accompanied by variable degrees of inflammation and gliosis, with partial preservation of axons [12, 24]. These lesions tend to be located in the optic nerves, spinal cord, brainstem, cerebellum, and the juxtacortical and periventricular white matter [23]. In addition, demyelinated lesions can also be found in the corpus callosum and cortical grey matter [4, 19]. Axonal injury can be a prominent pathologic feature of the MS plaque, though not in the acute phase [3, 29]. Although traditionally considered a disease of focal white matter lesions, the spectrum of MS pathology is now understood to encompass

a broader array of abnormalities, including diffuse damage of so-called normal-appearing white matter (NAWM) and normal-appearing gray matter (NAGM) on magnetic resonance imaging (MRI), both of which are associated with a progressive loss of brain volume [17].

Multiple sclerosis affects more women than men; the estimated female-to-male ratio of MS incidence is approximately 2:1, with some data suggesting the ratio is even higher. The median and mean ages of MS onset are 23.5 and 30 years of age, respectively [25]. The peak age of onset is about five years earlier for women than for men. Onset of MS can rarely occur as late as the seventh decade. Genetic factors appear to contribute to the risk of MS, especially variation involving the HLA-DRB1 locus. Although many viruses, and particularly the Epstein-Barr virus, have been associated with MS, there is no specific evidence linking viruses directly to the development of MS. The incidence and prevalence of MS vary geographically. There is an inverse relationship between sun exposure, ultraviolet radiation exposure, or serum vitamin D levels, and the risk or prevalence of MS [8].

There are no clinical findings that are unique to MS, but some are highly characteristic of the disease. Common symptoms and signs of MS include sensory symptoms in limbs or face, unilateral visual loss, acute or subacute motor weakness, diplopia, gait disturbance and balance problems, Lhermitte sign (electric shock-like sensations that run down the back and/or limbs upon flexion of the neck), vertigo, bladder problems, limb ataxia, acute transverse myelitis, and pain. The onset is often polysymptomatic. The typical patient presents as a young adult with two or more clinically distinct episodes of central nervous system dysfunction with at least partial resolution. The first presentation of MS is often a clinically isolated syndrome (CIS).

The core MS phenotypes are those of relapsing and progressive disease [18]. The pattern and course of MS is further categorized into several clinical subtypes as follows: Clinically isolated syndromes; Relapsing-remitting MS; Secondary progressive MS; Primary progressive MS. In addition, these phenotypes are modified by assessments of disease activity and disease progression [18]. Disease activity is determined by clinical relapses or magnetic resonance imaging (MRI) evidence of inflammation- contrast enhancing lesions and/or new or enlarging lesions. Disease progression is a process that is independently quantified from relapses, and is characteristic of primary and secondary progressive MS. Secondary progressive MS is preceded by relapsing-remitting MS, hence the designation of "secondary." Worsening of disability due to MS is highly variable. The impact of MS varies according to a number of measures, including severity of signs and symptoms, frequency of relapses, rate of worsening, and residual disability. Accumulating evidence suggests that, in most patients, worsening is slow. At the extreme ends of the severity spectrum, there are benign and malignant forms of MS, but the determination of these is always retrospective and must be made cautiously [2]. There are a variety of possible prognostic indicators in MS [7]. With better prognosis are associated relapsing form of MS (not progressive disease); early symptoms as sensory compliance and optic neuritis (not bowel-bladder and brainstem syndromes); monosymptomatic onset of MS (not polysymptomatic); less extent of MRI abnormalities as well as demographics, cigarette smoking, vitamin D intake and other characteristics. However, none are established as reliable. Ability to predict individual patient’s outcomes with MS precisely is quite limited. The development of a progressive course of MS may be the single most adverse factor influencing prognosis.

Multiple sclerosis is primarily diagnosed clinically. The diagnosis is relatively straightforward for patients who present with symptoms and MRI findings that are typical for MS and have a relapsing-remitting course [15]. The typical patient presents as a young adult with one or more clinically distinct episodes of central nervous system dysfunction such as optic neuritis, long tract symptoms/signs, a brainstem syndrome, or a spinal cord syndrome, followed by at least partial resolution. The core requirement for the diagnosis is the

demonstration of central nervous system lesion dissemination in time and space, based upon either clinical findings alone or a combination of clinical and MRI findings. The history and physical examination are most important for diagnostic purposes. Nowadays MRI is the test of choice to support the clinical diagnosis of MS [9]. The recent McDonald diagnostic criteria include specific MRI criteria for the demonstration of lesions dissemination in time and space [22]. Future revisions of diagnostic criteria are likely [10]. Diagnostic difficulties arise in patients who have atypical presentations, monophasic episodes, or progressive illness from onset.

Recognition of an inflammatory and neurodegenerative phase of MS has allowed the targeting of therapies for various phase of disease. The treatment of MS consists of acute relapse management, symptomatic treatment, rehabilitation and disease modifying therapy. Nowadays clear treatment goals are defined as maximize neurological reserve, cognitive function and physical function by reducing disease activity.

Although nowadays have seen significant advances in the treatment of multiple sclerosis, with an increasing number of disease-modifying therapies (DMTs) becoming available, it remains a potentially serious and debilitating condition as none of the current treatments halts or cures the disease. A number of immunomodulatory agents have important beneficial effects for patients with relapsing-remitting multiple sclerosis (RRMS) - decreased relapse rate and slower accumulation of brain lesions on MRI. At present, it is common practice for patients to receive several first-line therapies, such as interferon (IFN)-β, glatiramer acetate, teriflunomide or dimethyl fumarate (DMF), before therapies with greater efficacy, such as fingolimod, natalizumab or alemtuzumab, are tried [26]. It is recommended both early treatment rapidly after diagnosis, and early treatment changes in the event of insufficient response to initial treatment choice [31]. Monitoring MS disease activity is a key to achieving optimal outcomes. Initiation of new agent should be done as quickly as deemed safely possible to minimize the period of time in which a patient is not receiving the personal potential benefit of disease – modifying therapy. However, the heterogeneity of the disease, and the complexity of the underlying biological mechanisms, can render this challenging. The two pathology hallmarks - inflammation and progressive neuroaxonal damage [14] may be suspected from clinical point of view. Inflammation is infrequently associated with the subacute onset of clinical signs and symptoms and focal lesions on magnetic resonance imaging (MRI) that usually show temporary permeability of the blood–brain barrier, reflected by contrast enhancement at sites of acute inflammation. By contrast, axonal degeneration and loss of neurons are associated with sustained disability and evidence of brain or spinal cord atrophy on MRI over time. Axonal transection is a consistent pathological feature of acute MS lesions, and the incidence of neuronal damage correlates with the extent of inflammation within the lesion [29]. Importantly, such damage may be present in the early stages of MS [16]. In early stage it can be masked by mechanisms such as recruitment of other neuronal pathway. Such brain plasticity compensates functional loss for some period of time. That is why progressive damage may go disingenuous and unrecognized until it is too late for update the treatment as to be beneficial [28]. Clinical disease monitoring in MS should consist of measuring disease activity as manifested in relapses (reflecting inflammation), disability (reflecting neuroaxonal loss) and functionality (reflecting the degree of compensation or cerebral reserve). The grow body of evidences that there is limited window of opportunity for effective intervention in MS with currently available drugs. Prompt interventions in cases of suboptimal response are essential to prevent long-term disability. Nowadays potential strategies for the management of MS can be defined, depending on the level of disease activity. In patients with little evidence of disease activity at baseline, treatment can be started with conventional first-line therapies such as IFN-β, glatiramer acetate, DMF or teriflunomide. Treatment should be monitored at 6 month intervals. If signs of disease activity

such as frequent relapses, increasing disability, or worsening MRI lesion burden are observed treatment switch to more affective agent as fingolimod, natalizumab or alemtuzumab should be recommended. For patients with highly active disease at baseline or rapidly evolving severe disease, newer agents can be used as first-line therapy. In recent years understanding of treatment goas has been changed. The concept of "no evidence of disease activity" (NEDA) has become attractive, not only in the assessment of clinical trial data, but also as a treatment target in clinical practice. This concept focus on clinical and MRI measures of disease activity but also on patient-reported outcomes - progression of symptoms, adverse effects of treatment, and an inability to tolerate injections may also constitute grounds for switching treatments. The increasing number of highly active treatments becoming available raises the possibility of treatment election when necessary [32].

The specific treatment of the symptoms is an essential component of the overall management of multiple sclerosis. Treatment of mobility impairment is essential and including weakness, spasticity, ambulatory imbalance. Fatigue is a characteristic finding and primary fatigue felt to be part of the disease process itself. Possible pathophysiologic mechanisms include brain inflammation, cytokine effects, neuroendocrine abnormalities, or autonomic dysfunction [1]. Cognitive decline may be caused by the lesions, but may also be secondary to other factors. When evaluated with neuropsychological tests, up to 70 percent of patients have some cognitive impairment. There are no proven therapies for the treatment of cognitive impairment related to multiple sclerosis, although some disease-modifying agents have a beneficial impact [21]. Cross-sectional studies have shown some degree of affective disturbance in up to two-thirds of patients with multiple sclerosis. Depression is the most common manifestation. Paroxysmal attacks of motor or sensory phenomena can occur also. They are likely caused by ephaptic transmission of nerve impulses at sites of previous disease activity. Although troublesome to the patient, these symptoms do not indicate a true exacerbation of the disease. Seizures associated with multiple sclerosis are generally benign and transient and respond well to antiepileptic drugs or require no therapy. Several different pain conditions can be expected-central neuropathic pain, pain associated with multiple sclerosis complications or with injectable therapies. Sexual dysfunction is common in men and women and can be the result of multiple problems, including the direct effects of lesions in spinal cord, psychological factors, mechanical problems created by spasticity, paraparesis. The prevalence of lower urinary tract dysfunction is 32-97 percent. Bowel issues commonly coexist with urinary symptoms in multiple sclerosis patients and can be extremely distressing [11]. Physical activity in multiple sclerosis may be of value in alleviating some symptoms, preventing complications and possibly being neuroprotective. One possible mechanism of neuroprotective effect is by reducing obesty, as adipose tissue has been shown to be a source of inflammatory cytokines and adipokines. Recent data suggest that exercises can promote increase in neurotrophins and anti-inflammatory cytokines and increase synaptic density. Symptomatic treatment is aimed at the elimination or reduction of symptoms impairing the functional abilities and quality of life of the affected patients.

Management of multiple sclerosis has improved in the present days. Although the past decade has numerous successful clinical trials yielding the approval of new agents and the diagnostic criteria have been honed, MS remains a heterogeneous and unpredictable disease. New conception of disease course has delineated disease activity and progression as parallel processes to better individuate disease description. In light of enhanced vigilance for disease activity, the treatment approach to relapsing disease is much more proactive. The extent to which success in preventing relapses and the accumulation of lesions preserves of long term progression relapsing-remitting MS remains uncertain. Preventing disability for patients with progressive disease is still unmet need. That is why truly achieving NEDA will require the development of agents that directly target mechanisms of disease progression. Furthermore,

the next revolution in MS therapeutics is remyelination. Such remyelination strategies will likely warrant rationally designed combination therapy approaches to both prevent further disease activity and push central nervous system repair. The application of new prognostic biomarkers will provide new progress of multiple sclerosis management.

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EO6. ONE IN SIX PEOPLE IS AT RISK FOR STROKE. IT COULD BE PREVENTED. ACT NOW

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"The big question is how much we can modify the risk factors and, by doing this, how much of the stroke burden can we eliminate?"

Dr Martin O'Donnell

World stroke campaign: "1 in 6". Not many people are aware of that fact how widespread stroke is. The lifetime risk of stroke is 1 in 5 for women, 1 in 6 for men and women have a higher risk because of longer life expectancy. Cardiovascular disease (CVD) includes coronary heart disease, stroke, and other diseases of the circulatory system. CVD is linked to risk factors associated with the metabolic system.

Stroke is defined as an acute onset focal neurological deficit of vascular etiology, persisting for > 24 hours. Stroke is a leading cause of death and disability, especially in low-income and middle-income countries. The potentially modifiable risk factors for stroke in different regions of the world are different and in key populations and primary pathological subtypes of stroke.

Ten potentially modifiable risk factors are collectively associated with about 90% of the population associated risk of stroke in each major region of the world, among ethnic

groups, in men and women, and in all ages. Previous history of hypertension or blood pressure of 140/90 mm Hg or higher, regular physical activity, apolipoprotein ApoB/ApoA1 ratio, diet, waist-to-hip ratio, psychosocial factors, current smoking, cardiac causes, alcohol consumption, diabetes mellitus were associated with all stroke subtypes. Collectively, these risk factors accounted for 90,7% of the population associated risk for all stroke worldwide (91,5% for ischaemic stroke, 87,1% for intracerebral haemorrhage), and were consistent across regions, sex, diet, and age groups. Hypertension was more associated with intracerebral haemorrhage than with ischaemic stroke, whereas current smoking, diabetes, apolipoproteins, and cardiac diseases were more associated with ischaemic stroke.

Atrial fibrillation (AF) is the most prevalent sustained arrhythmia, impacting 30 million people worldwide, with 3 million people in the United States alone. Notably, this number is expected to increase to >10 million Americans by 2050. AF results in an increased risk for morbidity, as well as mortality, with patients having a 5-fold increase in risk of stroke and a 1.5- to 1.9-fold overall increase risk of mortality, after adjusting for other risk factors.

AF risk is multifactorial, with a common risk factor for AF being increased age. For example, the prevalence of AF increases from 4% in individuals > 60 years of age to 10% in individuals over 80 years. In addition to age, other risk factors for AF include cardiac (sinus node dysfunction, valvular heart disease, cardiomyopathy) and noncardiac (diabetes) phenotypes, as well as environmental factors. Furthermore, while not generally considered an inherited arrhythmia, there are now significant data to support the role of genetics in AF. Considering the broad interplay between various risk factors and genetic tendencies that contribute to AF, as well as heterogeneity of disease pathways (electrical, inflammation, and fibrosis), it is not surprising that the efficacy of AF treatment strategies is variable.

Stroke Riskometer™ is a unique and easy to use mobile app tool for assessing your individual risk of a stroke in the next five or ten years and what you can do to reduce the risk.

Telestroke is one of the most frequently used and rapidly expanding applications of telemedicine, delivering much-needed stroke expertise to hospitals and patients. This document reviews the current status of telestroke and suggests measures for ongoing quality and outcome monitoring to improve performance and to enhance delivery of care.

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