

# PROCEEDINGS

## OF THE EIGHTH WORKSHOP ON BIOLOGICAL ACTIVITY OF METALS, SYNTHETIC COMPOUNDS AND NATURAL PRODUCTS



**NOVEMBER 27 – 29, 2013**  
**SOFIA, BULGARIA**  
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# PROCEEDINGS

OF THE EIGHT WORKSHOP ON BIOLOGICAL ACTIVITY OF METALS, SYNTHETIC  
COMPOUNDS AND NATURAL PRODUCTS

November 27 – 29, 2013

Institute of Experimental Morphology, Pathology and Anthropology with Museum  
at the Bulgarian Academy of Sciences

Edited by: Dimitar Kadiysky and Radostina Alexandrova



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- **Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences**

THE EIGHT WORKSHOP  
“Biological activity of Metals, Synthetic Compounds  
and Natural Products”

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, 27 November 2013**

**9.00 – 9.30 REGISTRATION**  
**9.30 – 9.45 OPENING CEREMONY**

Session A.

## Chairpersons:

**Assoc. Prof. Stefka Valcheva-Kuzmanova, MD, PhD**

*Faculty of Medicine, Medical University - Varna*

**Assoc. Prof. Pavlina Dolashka, PhD**

*Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences*

**Secretary: Pavel Mitrenga**

*Institute of Neurobiology, Bulgarian Academy of Sciences*

**9.45 – 10.15**

### **AO1. БИОЛОГИЧНО-АКТИВНИ МЕД-СЪДЪРЖАЩИ ГЛИКОПРОТЕИНИ**

Павлина Александрова Долашка

*Институт по органична химия с център по фитохимия, Българска академия на науките*

**10.15 – 10.30**

### **AO2. ANTI-CANCER PROPERTIES OF GASTROPODAN HEMOCYANINS ON MURINE MODEL OF COLON ADENOCARCINOMA**

Vera Gesheva<sup>a</sup>, Stela Chausheva<sup>a</sup>, Nikolina Mihaylova<sup>a</sup>, Iliyan Manoylov<sup>a</sup>, Lyuba Doumanova<sup>a</sup>, Krassimira Idakieva<sup>b</sup>, Andrey Tchorbanov<sup>a,\*</sup>

<sup>a</sup>*The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences*

<sup>b</sup>*Institute of Organic Chemistry, Bulgarian Academy of Sciences, Bulgaria*

**10.30 – 10.45**

### **AO3. HELIX POMATIA HC (HPH) HEMOCYANIN AS PROTEIN-CARRIER AND BIO-ADJUVANT OF BACTERIAL AND VIRAL PROTEINS**

Nadia Stefanova<sup>a</sup>, Stela Chausheva<sup>a</sup>, Vera Gesheva<sup>a</sup>, Nikolina Mihaylova<sup>a</sup>, Krassimira Idakieva<sup>b</sup>, Lyuba Doumanova<sup>a</sup>, Andrey Tchorbanov<sup>a</sup>

<sup>a</sup>*Institute of Microbiology, Bulgarian Academy of Sciences,*

<sup>b</sup>*Institute of Organic Chemistry, Bulgarian Academy of Sciences, Bulgaria*

**10.45 – 11.05 Coffee Break**

**11.05 - 11.20**

**AO4. COMPARATIVE STUDY OF THE PROTECTIVE EFFECT OF ARONIA  
MELANOCARPA FRUIT JUICE AND QUERCETIN IN A MODEL OF  
PARACETAMOL-INDUCED HEPATOTOXICITY IN RATS**

Stefka V. Valcheva-Kuzmanova<sup>1\*</sup>, Krasimir A. Kuzmanov<sup>2</sup>

<sup>1</sup>Department of Preclinical and Clinical Pharmacology, Faculty of Medicine, Medical  
University – Varna, <sup>2</sup>Vivarium, Medical University – Varna, Bulgaria

**11.20 – 11.35**

**AO5. ATTENUATION OF CELLULAR OXIDATIVE STRESS BY ARONIA  
MELANOCARPA TOTAL EXTRACT AFTER DOXORUBICIN EXPOSURE**

I. V. Sainova, V. G. Pavlova, I. P. Valkova, B. L. Alexieva, E. B. Nikolova

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

**11.35 – 11.50**

**AO6. РАСТЕНИЯ ПРИТЕЖАВАЩИ АНТИВИРУСНА ЕФЕКТИВНОСТ СРЕЩУ  
ХЕРПЕС СИМПЛЕКС ВИРУС 1 и 2**

Ваня Младенова

*Institute of Biology and Immunology of Reproduction, Bulgarian Academy of Sciences*

**11.50 – 12.20**

**AO7. CYTOTOXIC AND ANTIHERPES VIRUS EFFECTS OF ORIGINAL  
BULGARIAN STRAINS LACTOBACILLUS**

Petya Genova<sup>1</sup>, Petia Genova-Kalou<sup>2</sup>, Mira Yordanova<sup>2</sup>, Rositsa Tropcheva<sup>3</sup>,  
Svetla Danova<sup>3</sup>

<sup>1</sup>University of Chemical Technology and Metallurgy, Department of Biotechnology, Sofia;

<sup>2</sup>National Centre of Infectious and Parasitic Diseases, Department of Virology, Sofia;

<sup>3</sup>Bulgarian Academy of Sciences, The Stephan Angeloff Institute of Microbiology, Department  
of General Microbiology, Sofia

**12.20 – 12.35**

**AO8. КАКВО (НЕ) ЗНАЕМ ЗА СРЕБРОТО?**

Радостина Александрова<sup>1</sup>, Абдулкадир Абудалех<sup>1,2</sup>, Таня Живкова<sup>1</sup>, Павел  
Митренга<sup>1,2,3</sup>, Лора Дякова<sup>3</sup>, Бойка-Андонова-Лилова, Катя Попова<sup>1,2</sup>, Лилия  
Николова<sup>1,2</sup>, Мария Минчева<sup>1,2</sup>

<sup>1</sup>Институт по експериментална морфология, патология и антропология с музей –  
БАН; <sup>2</sup>Биологически факултет, СУ “Св. Климент Охридски”; <sup>3</sup>Институт по  
невробиология □ БАН

**12.35 – 12.50**

**AO9. ПЪРВИ СРЕЩИ С ФАРМАКОГЕНЕТИКАТА**

Георги Семовски<sup>1</sup>, Пламена Димова<sup>2</sup>

<sup>1</sup>Медицински факултет, СУ “Св. Климент Охридски”; <sup>2</sup>Езикова гимназия, Пловдив

**AP1. ДИХАТЕЛНИ ПИГМЕНТИ**

Милен Лазов

Медицински факултет, СУ “Св. Климент Охридски”

**Thursday, 28 November 2013**

Session B.

**Chairpersons:**

**Assoc. Prof. Julia Radenkova – Saeva, MD, PhD**

*Toxicology Clinic, UMHATEM “N. I. Pirogov”*

**Assoc. Prof. Radostina Alexandrova, PhD**

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

**Secretary: Abdulkadir Mahadi Abudalech**

*Faculty of Biology, Sofia University “St. Kliment Ohridski”*

**9.30 – 10.00**

**BO1. ВЛИЯНИЕ НА МЕТАЛНИ СЪЕДИНЕНИЯ ВЪРХУ ПРЕЖИВЯЕМОСТТА  
И ПРОЛИФЕРАТИВНАТА АКТИВНОСТ НА ТРАНСФОРМИРАНИ С ВИРУСИ  
ТУМОРНИ КЛЕТКИ И РЕПЛИКАЦИЯТА НА ЧОВЕШКИ И ГОВЕЖДИ  
ХЕРПЕСНИ ВИРУСИ**

Абдулкадир Махади Абудалех<sup>1,2</sup>, Стоян Шишков<sup>1</sup>, Радостина Александрова<sup>2</sup>  
<sup>1</sup>Биологически факултет, СУ „Св. Кл. Охридски“; <sup>2</sup>Институт по експериментална  
морфология, патология и антропология с музей - БАН

**10.00 - 10.05**

**BO2. EFFECT OF Zn/Au AND Zn/Ag COMPLEXES WITH SALEM ON VIABILITY  
AND PROLIFERATION OF VIRUS-TRANSFORMED ANIMAL AND HUMAN  
CELLS**

Tanya Zhivkova<sup>1</sup>, Pavel Mitrenga<sup>1,2,3</sup>, Lora Dyakova<sup>3</sup>, Milena Georgieva<sup>3</sup>, George Miloshev<sup>3</sup>,  
Daniela-Cristina Culita<sup>4</sup>, Gabriela Marinescu<sup>4</sup>, Luminita Patron<sup>4</sup>, Radostina Alexandrova<sup>1</sup>  
<sup>1</sup>Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian  
Academy of Sciences; <sup>2</sup>Faculty of Biology, Sofia University “St. Kliment Ohridski”; <sup>3</sup>Institute  
of Neurobiology, Bulgarian Academy of Sciences; <sup>4</sup>Institute of Molecular Biology, Bulgarian  
Academy of Sciences; <sup>5</sup>Institute of Physical Chemistry “Ilie Murgulescu”, Romanian  
Academy, Bucharest, Romania

**10.05 - 10.20**

**BO3. CLUSTERING OF DETONATION NANODIAMOND PARTICLES IN  
PHYSIOLOGICAL SOLUTIONS**

M. Keremidarska<sup>1</sup>, A. Ganeva<sup>1</sup>, D. Mitev<sup>2</sup>, T. Hikov<sup>3</sup>, I. Tsvetanov<sup>3</sup>, L. Pramatarova<sup>3</sup> and N.  
Krasteva<sup>1</sup>

<sup>1</sup>Institute of Biophysics, Bulgarian Academy of Sciences,

<sup>2</sup>ACROSS, School of Chemistry, University of Tasmania, Australia

<sup>3</sup>Georgi Nadjakov, Institute of Solid State Physics, Bulgarian Academy of Sciences

**10.20 – 10.35**

**BO4. ZnO NANOSTRUCTURES AND THEIR POTENTIAL APPLICATION IN BIOMEDICINE**

A. Og. Dikovska<sup>1</sup>, N. N. Nedyalkov<sup>1</sup>, R. A. Toshkova<sup>2</sup>, E. G. Gardeva<sup>2</sup>, L. S. Yossifova<sup>2</sup>, P. A. Atanasov<sup>1</sup>

<sup>1</sup>*Emil Djakov Institute of Electronics, Bulgarian Academy of Sciences;*

<sup>2</sup>*Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences*

**10.35 – 10.55 Coffee Break**

**10.55 – 11.25**

**BO5. PERSISTENT ORGANIC POLLUTANTS - EFFECTS ON HUMAN HEALTH**

Julia Radenkova – Saeva

*Toxicology Clinic, UMHATEM „N.I. Pirogov“, Sofia, Bulgaria*

**11.25 – 11.40**

**BO6. EFFECT OF HEAVY METALS ON SOME PHYSIOLOGICAL PARAMETERS IN TRITICALE LINES**

Irina Moskova, Nina Georgieva, Vera Alexieva, Iskren Sergiev

*Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences*

**11.40 – 12.10**

**BO7. INFLUENCE OF POPOLIS ON SOME PROTOZOAN PARASITES**

Delka Salkova

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

**12.10 – 12.25**

**BO8. CONCENTRATIONS OF SELENIUM AND SIALIC ACIDS IN SERUM FROM RATS INFECTED WITH *TRICHINELLA SPIRALIS***

Dimitar Ivanov, Rositsa Milcheva, Svetlozara Petkova, Margarita Gabrashanska

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

**12.25 – 12.40**

**BO9. STUDY ON THE EFFECT OF DIVALENT METAL IONS ON THE ACTIVITY OF AMINOPEPTIDASE A**

V. Petrova<sup>1</sup>, I. Ivanov<sup>2</sup>, D. Tasheva<sup>3</sup>, M. Dimitrova<sup>1</sup>

<sup>1</sup>*Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria*

<sup>2</sup>*Faculty of Biology, Sofia University “St. Kl. Ohridsky”*

<sup>3</sup>*Faculty of Chemistry and Pharmacy, University of Sofia “St. Kl. Ohridsky”*

**12.40 – 12.55**

**BO10. НОВИ ПОДХОДИ В ТЕРАПИЯТА НА НЯКОИ ПАРАЗИТНИ ЗАБОЛЯВАНИЯ С МЕТАЛ-СЪДЪРЖАЩИ ПРЕПАРАТИ**

Кристияна Добрикова<sup>1</sup>, Петя Генова-Калу<sup>2</sup>

<sup>1</sup>*Софийски Университет “Св. Кл. Охридски”, Биологически факултет;* <sup>2</sup>*Национален*

Център по Заразни и Паразитни Болести, Отдел „Вирусология“, Лаборатория „Рикетсии и тъканни култури“

**BP1. НАКРАТКО ЗА ДЕНДРИМЕРИТЕ И НАНОМЕДИЦИНАТА**

Людмила Стоева<sup>1,2</sup>, Надежда Йорданова<sup>1,2</sup>, Павел Митренга<sup>1,2,3</sup>,  
Радостина Александрова<sup>1</sup>

<sup>1</sup>Институт по експериментална морфология, патология и антропология с музей – БАН; <sup>2</sup>Биологически факултет, СУ “Св. Климент Охридски”; <sup>3</sup>Институт по невробиология - БАН

**BP2. HEAVY METALS IN THE PARASITE-HOST SYSTEM IN TERRESTRIAL ANIMALS**

M. Gabrashanska<sup>1</sup>, V. Ermakov<sup>2</sup>

<sup>1</sup>Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences

<sup>2</sup>Vernadsky Institute of Geochemistry and Analytical chemistry, Russian Academy of Sciences

**BP3. ТИТАНЪТ В ОРТОПЕДИЯТА**

Кристина Генкова, Симона Спасова

Медицински факултет, СУ “Св. Кл. Охридски”

**BP4. CALCIUM PHOSPHATES AS BIOMATERIALS FOR BONE IMPLANTS**

R. Alexandrova<sup>1</sup>, B. Andonova-Lilova<sup>1</sup>, P. Mitrenga<sup>2,3</sup>, T. Zhivkova<sup>1</sup>, L. Dyakova<sup>3</sup>, D. Rabadzhieva<sup>4</sup>, S. Tepavitcharova<sup>4</sup>

<sup>1</sup>Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences; <sup>2</sup>Faculty of Biology, Sofia University “St. Kliment Ohridski”;

<sup>3</sup>Institute of Neurobiology, Bulgarian Academy of Sciences; <sup>4</sup>Institute of general and Inorganic Chemistry, Bulgarian Academy of Sciences

Session C.

**Chairpersons:**

**Prof. Margarita Gabrashanska, DVM, PhD**

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

**Assoc. Prof. Anna Tolekova, MD, PhD**

*Faculty of Medicine, Trakya University, Stara Zagora*

**Secretary: Lora Dyakova**

*Institute of Neurobiology, Bulgarian Academy of Sciences*

**14.00 – 14.30**

**CO1. COMPLEX ASSESSMENT OF GRAIN QUALITY FUSING DATA FROM IMAGE AND SPECTRA ANALYSES**

Mirolyub I. Mladenov<sup>1</sup>, Martin P. Deyanov<sup>2\*</sup>, Roumiana Tsenkova<sup>3</sup>

<sup>1</sup>Department of Automatics and Mechatronics, University of Rousse, Ruse, Bulgaria;

<sup>2</sup>Department of Automatics and Mechatronics, University of Rousse, Ruse, Bulgaria;

<sup>3</sup>Biomeasurement Technology Laboratory, Kobe University, Japan

**14.30 - 14.45**

**CO2. ДМСО – ПРИЛОЖЕНИЕ И РОЛЯ В МЕДИЦИНАТА И КРИОБИОЛОГИЯТА**

Кирил Лазов

*Institute of Biology and Immunology of Reproduction "Acad. Kiril Bratanov", Bulgarian Academy of Sciences*

**14.45 - 15.00**

**CO3. SELECTIVE ANGIOTENSIN II BLOCKERS - EFFECT ON CONTRACTILE ACTIVITY OF UTERUS AND URINARY BLADDER**

P. Hadzhibozheva, A. Tolekova, Ts. Georgiev

*Dept. of Physiology, Pathophysiology and Pharmacology, Medical Faculty, Trakia University, Stara Zagora, Bulgaria*

**15.00 – 15.15**

**CO4. AGE DEPENDANT DISTRIBUTION OF CARBOHYDRATES IN MURINE INTESTINAL GLYCOCALYX**

V. Pavlova<sup>1</sup>, Ts. Paunova<sup>2</sup>, S. Stoitsova<sup>2</sup>, E. Nikolova<sup>1</sup>

<sup>1</sup>*Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Science;*

<sup>2</sup>*The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences*

**15.15 – 15.35 Coffee Break**

**15.35 – 15.50**

**CO5. ОРИГИНАЛНИ ВЪГЛЕХИДРАТНИ И СОЛЕВИ НАПИТКИ ЗА ПРИЛОЖЕНИЕ В ХИРУРГИЯТА И МЕДИЦИНАТА - ПЪРВОНАЧАЛНИ КЛИНИЧНИ РЕЗУЛТАТИ**

Д. Дарданов<sup>1</sup>, В. Стоянов<sup>2</sup>, П. Недков<sup>3</sup>, П. Христов<sup>3</sup>

<sup>1</sup>5 МБАЛ, СУ - МФ, <sup>2</sup>Александровска Болница, МУ - София, <sup>3</sup>ИОХЦФ - БАН

**15.50 – 16.05**

**CO6. MORPHOLOGICAL AND IMMUNOLOGICAL ASSESSMENT OF THE INDIVIDUAL AND COMBINED EFFECTS OF TWO MYCOTOXINS-FUMONISIN B1 AND DEOXYNIVALENOL IN VIVO**

K. Todorova, P. Dimitrov, R. Toshkova, S. Lazarova, E. Gardeva, L. Yossifova, B. Andonova-Lilova, R. Milcheva, R. Russev

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

**16.05 – 16.20**

**CO7. MEDICAL MARIJUANA AND MULTIPLE SCLEROSIS**

Vera Kolyovska<sup>1</sup>, Dimitar Maslarov<sup>2</sup>

<sup>1</sup>*Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences*

<sup>2</sup>Medical University of Sofia, Neurology Clinic, First MHAT - Sofia

## 16.20 – 17.00 Poster Presentation

### CP1. HORMONE REPLACEMENT THERAPY AND BREAST CANCER

Federica Salvoldi<sup>1,2</sup>

<sup>1</sup>University of Studies of Brescia, Italy;

<sup>2</sup>Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences

### CP2. ЦИНКЪТ, БЕЗ КОЙТО НЕ МОЖЕМ

Радостина Димитрова<sup>1,2</sup>, Мелинда Здравкова<sup>1,2</sup>, Димитър Тенев<sup>1,2</sup>, Абдулкадир Абудалех<sup>1,2</sup>, Радостина Александрова<sup>1</sup>

<sup>1</sup>Институт по експериментална морфология, патология и антропология с музей – БАН; <sup>2</sup>Биологически факултет, СУ “Св. Климент Охридски”

### CP3. ВАНАДИЯТ – ПОЗНАТ И НЕПОЗНАТ

Галина Димитрова<sup>1,2</sup>, Анна Джисова<sup>1,2</sup>, Лилия Донова<sup>1,2</sup>, Абдулкадир Абудалех<sup>2,3</sup>, Радостина Александрова<sup>2</sup>

<sup>1</sup>Медицински факултет, СУ “Св. Климент Охридски”; <sup>2</sup>Институт по експериментална морфология, патология и антропология с музей – БАН;

<sup>3</sup>Биологически факултет, СУ “Св. Климент Охридски”

### CP4. ОЩЕ НЕЩО ЗА ЗЛАТОТО

Мария Минчева<sup>1,2</sup>, Лилия Николова<sup>1,2</sup>, Катя Попова<sup>1,2</sup>, Абдулкадир Абудалех<sup>1,2</sup>, Радостина Александрова<sup>1</sup>

<sup>1</sup>Биологически факултет, СУ “Св. Климент Охридски”; <sup>2</sup>Институт по експериментална морфология, патология и антропология с музей – БАН

### CP5. THE GOLDEN STORY OF GOLD IN MEDICINE

Radostina Alexandrova<sup>1</sup>, Abdulkadir Abudalech<sup>1,2</sup>, Tanya Zhivkova<sup>1</sup>, Pavel Mitrenga<sup>1,2,3</sup>, Reni Kalfin<sup>3</sup>, Daniela Culita<sup>4</sup>, Gabriela Marinescu<sup>4</sup>

<sup>1</sup>Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences; <sup>2</sup>Faculty of Biology, Sofia University “St. Kliment Ohridski”; <sup>3</sup>Institute of Neurobiology, Bulgarian Academy of Sciences; <sup>4</sup>Institute of Physical Chemistry “Ilie Murgulescu”, Romanian Academy, Bucharest, Romania

### CP6. ХИСТОНОВИ БЕЛТЪЦИ

Никола Симеонов, Методи Янев, Константин Илиев, Кристина Радилова  
Медицински факултет, СУ “Св. Климент Охридски”

### CP7. НЕХИСТОНОВИ БЕЛТЪЦИ

Методи Янев, Никола Симеонов, Кристина Радилова, Константин Илиев  
Медицински факултет, СУ “Св. Климент Охридски”

### CP8. ПРОПОЛИС

Дарина Кадийска, Дарко Стаменов  
Медицински факултет, СУ “Св. Климент Охридски”

### **CP9. ЗА ШОКОЛАДА – С ЛЮБОВ!**

Драгомира Угринова, Ели Трифонова, Васил Бранков, Юлиана Иванова  
*Медицински факултет, СУ “Св. Климент Охридски”*

### **CP10. С ДЪХ НА КАНЕЛА**

Каролин Мишел Шоайб<sup>1</sup>, Александра Петрова<sup>1</sup>, Константин Проевски<sup>1</sup>, Емил Чиков<sup>2</sup>  
<sup>1</sup>*Медицински факултет, СУ “Св. Климент Охридски”*; <sup>2</sup>*Физически факултет “Св. Климент Охридски”*

### **CP11. НА ЧАША ВИНО**

Елмер Кехайов, Ива Янкулова, Кристиан Кирилов  
*Медицински факултет, СУ “Св. Климент Охридски”*

### **CP12. ПОЛЕЗНА ЛИ Е БИРАТА?**

Шенол Чакър, Ардит Каси  
*Медицински факултет, СУ “Св. Климент Охридски”*

### **CP13. HELICOBACTER PYLORI – БИОМАРКЕР И ЗА ДИАБЕТ ТИП 2?**

Даниела Иванова, Теодора Йорданова, Виктор Стойков, Лина Абужамус, Станислава Благоева  
*Медицински факултет, СУ “Св. Климент Охридски”*

**Friday, 29 November 2013**

Session D.

#### **Chairpersons:**

**Prof. Ilza Pajeva, PhD, DCs**

*Institute of Biophysics and Biomedical Engineering, Bulgarian Academy of Sciences*

**Assoc. Prof. Anna Damianova, MD, PhD**

*Institute for Nuclear Research and Nuclear Energy,  
Bulgarian Academy of Sciences*

**Secretary: Tanya Zhivkova**

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

**9.30 – 10.00**

#### **D01. MODES-OF-ACTION RELATED TO REPEATED DOSE TOXICITY: FROM PPAR $\gamma$ LIGAND-DEPENDENT DYSREGULATION TO NON-ALCOHOLIC FATTY LIVER DISEASE**

M. Al Sharif<sup>1</sup>, I. Tsakovska<sup>1</sup>, P. Alov<sup>1</sup>, V. Vitcheva<sup>1,2</sup>, I. Pajeva<sup>1</sup>

<sup>1</sup>*Institute of Biophysics and Biomedical Engineering, Bulgarian Academy of Sciences,*

<sup>2</sup>*US FDA CFSAN, College Park, MD, USA*

**10.00 - 10.15**

#### **DO2. ANALYSIS OF BOAR SEMINAL PLASMA PROTEINS WITH PROTECTIVE EFFECT DURING LOW TEMPERATURE STORAGE**

Denica Daskalova, Alexander Kukov, Irina Kirilova, Maria Ivanova-Kicheva

*Institute of biology and immunology of reproduction “Acad. Kiril Bratanov”, Bulgarian Academy of Sciences*

**10.15 - 10.30**

**DO3. SMALL MAMMALS AS INDICATORS OF STATUS OF THE ENVIRONMENT NEAR RESEARCH REACTOR**

S. Dimitrov<sup>1</sup>, E. Geleva<sup>2</sup>, A. Damianova<sup>2</sup>, M. Iovtchev<sup>2</sup>

<sup>1</sup>*Permanent Repository for Radioactive Waste – Novi Han, State Enterprise Radioactive Waste, “G.M.Dimitrov” Blvd., 52A, 1797 Sofia, Bulgaria*

<sup>2</sup>*Institute for Nuclear Research and Nuclear Energy, Bulgarian Academy of Sciences*

**10.30 - 10.45**

**DO4. STUDY ON MINERAL WATER IN NORTHWEST BULGARIA**

Anna Damianova, Ilia Penev, Zornitsa Tsoleva

*Institute for Nuclear Research and Nuclear Energy,  
Bulgarian Academy of Sciences*

**10.45 – 11.05 Coffee Break**

**11.05 – 11.20**

**DO5. METAL IONS IN HUMAN SEMEN**

Galina Nenkova, Almira Georgieva, Elina Tzvetanova, Albena Alexandrova

*Institute of Neurobiology, Bulgarian Academy of Sciences*

**11.20 - 11.35**

**DO6. THE ANTITUMOR POWER OF PLATINUM COMPOUNDS**

Radostina Alexandrova<sup>1</sup>, Metin Mazgaldzhi<sup>1,2</sup>, Iva Gavrilova<sup>3</sup>, Konstanta Timcheva<sup>3</sup>

<sup>1</sup>*Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences;*

<sup>2</sup>*Medical Faculty, Medical University, Sofia;*

<sup>3</sup>*National Specialised Hospital for Active Treatment in Oncology*

**11.35 – 12.30 Poster Session**

**DP1. CONTROL OF CHRONIC MICROELEMENTOSES IN FARM ANIMALS FROM EAST SIBERIA BASED ON THE CHEMICAL ELEMENTAL COMPOSITION OF THEIR HAIR (CECH)**

Vadim Ermakov, Valentina Danilova, Anastasia Danilogorskaya, Sergey Tjutikov, Margarita Gabrashanska\*, Milena Anisimova\*

*Vernadsky Institute of Geochemistry and Analytical Chemistry of RAS.*

*Moscow, Russia; \*Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences*

**DP2. ACCUMULATION OF METALS BY MILK XANTHINE OXIDASE**

Vadim Ermakov, Sergey Tjutikov, Valentina Danilova, Sabzbakhor Khushvaktova

*Vernadsky Institute of Geochemistry and Analytical Chemistry of RAS.*

*Moscow, Russia*

**DP3. БИОЛОГИЧНИ И СИНТЕТИЧНИ ЕКЗОГЕННИ МУТАГЕНИ**

Емилиян Ицков Джустов, Ефросини Захариас Таскуди

*Биологически факултет, СУ “Св. Климент Охридски”*

**DP4. КОНСЕРВАНТИТЕ И ХРАНАТА, КОЯТО ОБИЧАМЕ**

Александра Петрова, Даниела Гулева, Николай Димитров  
*Биологически факултет, СУ "Св. Климент Охридски"*

**DP5. ЗМИЙСКА ОТРОВА – ПЪТ КЪМ СМЪРТТА ИЛИ ПЪТ КЪМ ЖИВОТА**

Мария Младенова, Симона Такова, Ирена Михайлова, Елвира Никова  
*Медицински факултет, СУ "Св. Климент Охридски"*

**DP6. КОЙ СВИРИ ХЕВИ МЕТЪЛ В НАШИЯ ОРГАНИЗЪМ?**

Даниела Цветкова, Мария Кръстева, Камелия Лазарова, Йоанна Маламуси  
*Медицински факултет, СУ "Св. Климент Охридски"*

**12.30 – 12.45 Closing Remarks**

**Бел. ред. Всеки автор носи отговорност за представения от него материал**

# АО1. БИОЛОГИЧНО АКТИВНИ МЕДНИ ГЛИКОПРОТЕИНИ

Павлина Александрова Долашка

*Институт по органична химия с център по фитохимия, Българска академия на науките*

## Abstract

Copper ions are considered as multifunctional participating in a broad spectrum of intracellular processes under normal and pathologic conditions. The role of copper and its complexes in medicine is a challenging task that awaits further exploration.

An interesting copper binding protein found in some of fungal strain and yeast (superoxide dismutases) as well in the lower eukaryotes (hemocyanins).

Hemocyaninins are found in a majority of arthropods and mollusks, and they are called "Blue Blooded Organisms" contain 320 and 96 copper atoms, respectively. They turns blue in color upon oxygenation and serves as primary carrier of oxygen.

Phenoloxidase is another such copper binding protein that binds to dioxygen with a different physiological function. Three copper ions are involved in the molecule but two of them are in the active site.

Hemocyanins and superoxide dismutases are copper-containing glycoproteins with a presence of carbohydrate component. Combining in vitro and in vivo methods, the antiviral, antitumor and antibacterial properties of several hemocyanins was suggested that carbohydrate moieties play a basic role for the potential therapeutic antitumor potential of different hemocyanins

## УВОД

Металите, както в свободно състояние, така и свързани в сложни комплекси, играят важна роля за протичането на редица процеси. Едни от най-често срещаните в природата са мед-съдържащите гликопротеини, в активния център на които участват медни йони. Гликопротеини са едни от най-разпространените протеини в живата природа. Те се срещат в гръбначни и безгръбначни организми, микроорганизми, растения, вируси и др. Гликопротеините участват в почти всички биологични процеси, поради което предизвикват голям научен интерес. Едни от най-често срещаните гликопротеини, които изпълняват 'транспортна' или 'защитна' функция след взаимодействие с молекула кислород или кислородни радикали, са супероксид дисмутази (СОД) и хемоцианини (Хц) (1-4). Те притежават разнообразни въглехидратни структури и включват един или два медни йона в активния център, които взаимодействат по различен начин с кислорода.

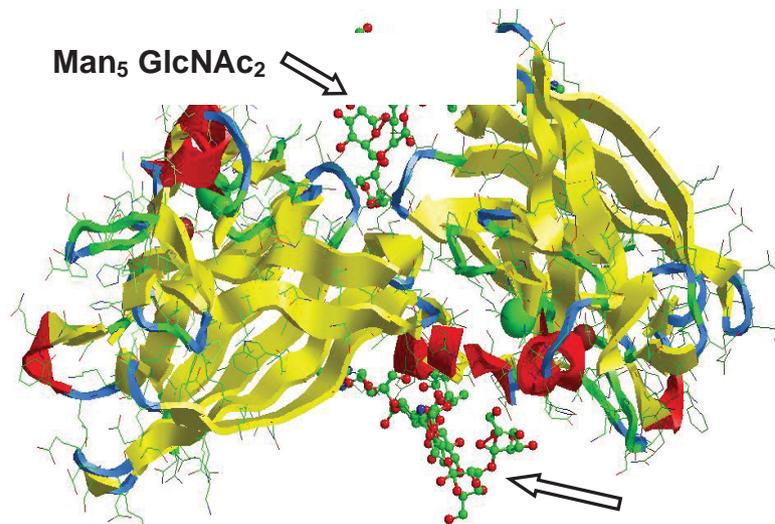
Известна е двойствената роля на кислорода: едновременно с безспорното му предимство като източник на енергия, той е свързан и с вредното въздействие на неговите реакционноспособни метаболити или така нар. свободни окси-радикали.

Ензимът Cu/Zn-супероксид дисмутаза, който включва един меден йон в активния си център, играе ключова роля в антиоксидантната защита, като елиминира  $\cdot\text{O}_2$  радикали и ограничава количеството на  $\text{H}_2\text{O}_2$ . Докато двата медни йона в активния център на хемоцианините изпълняват друга изключително важна роля, а именно, пренос на молекула кислород до тъканите в респираторните организми.

Голяма е групата на мед-съдържащите гликопротеини от типа 3, които са свързани с еволюцията на двуядрения процесор при хемоцианините (Хц), тирозинази (Ти) (7) и фенолоксидази (Фо). Те притежават подобен активен център, но доста голямо различие в размера, първичната и четвъртична структури на молекулата, както и във физиологичната функция, която изпълняват (2-7). Известно е, че гликопротеините включват различен брой медни йони в активния център, като два медни йони участват в активния център при хемоцианините, докато в молекулата на тирозинази/фенолоксидази са разположени 3 медни йона. Ензимът тирозиназа се нуждае от 3 медни йона, два разположени в активния център и един в страничния домен, за да може да осъществи своята функция (7).

## МЕД-ЦИНКОВИ СУПЕРОКСИД ДИСМУТАЗИ

Супероксид дисмутазата (СОД) включва само един меден йон в активния център и е основен ензим, който участва в защитата на клетката срещу увреждания, предизвикани от свободни радикали. Ето защо е открито голямо разнообразие от супероксид дисмутази във всички организми/СОД-зи/. Изследванията върху гъбичните щамове *Humicola lutea* 110, *Humicola lutea* 103, *Aspergillus niger* 26 и дрожди *Kluyveromyces marxianus* NBIMCC са показали, че те са добри продуценти на двата вида СОД: Mn-СОД и Cu/Zn-СОД, а от определените аминокиселинни последователности на изолираните Cu/Zn-СОД е установено наличие на вероятно гликозилирани центрове (1,8). Позицията 32-35 (-Asn-Ile-Thr-) на свързване на този гликан към полипептидната верига на Cu/Zn-КмСОД беше определена, анализирайки изолираните пептиди, след хидролиза на ензима с трипсин и секвениране с MALDI, както е описано на фигура 1 (8).

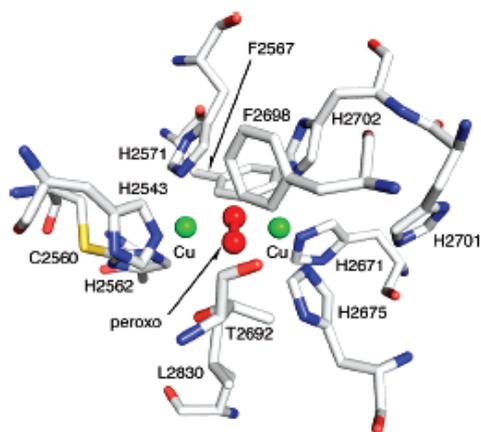


**Фигура 1.** 3D-модел на Cu/ZnСОД, изолирана от дрождев щам *K. marxianus*, и получен чрез програмата MODELLER, и е отбелязана позицията на въглехидратната структура, прикрепена към полипептидната верига.

Позицията на гликаните в двете субединици на Cu/Zn-КмСОД беше определена от 3D модела на молекулата, като се вижда, че двете въглехидратни вериги в димерната молекула на Cu/Zn-КмСОД са разположени на повърхността и не влияят на свързването на субединиците в нативната молекула.

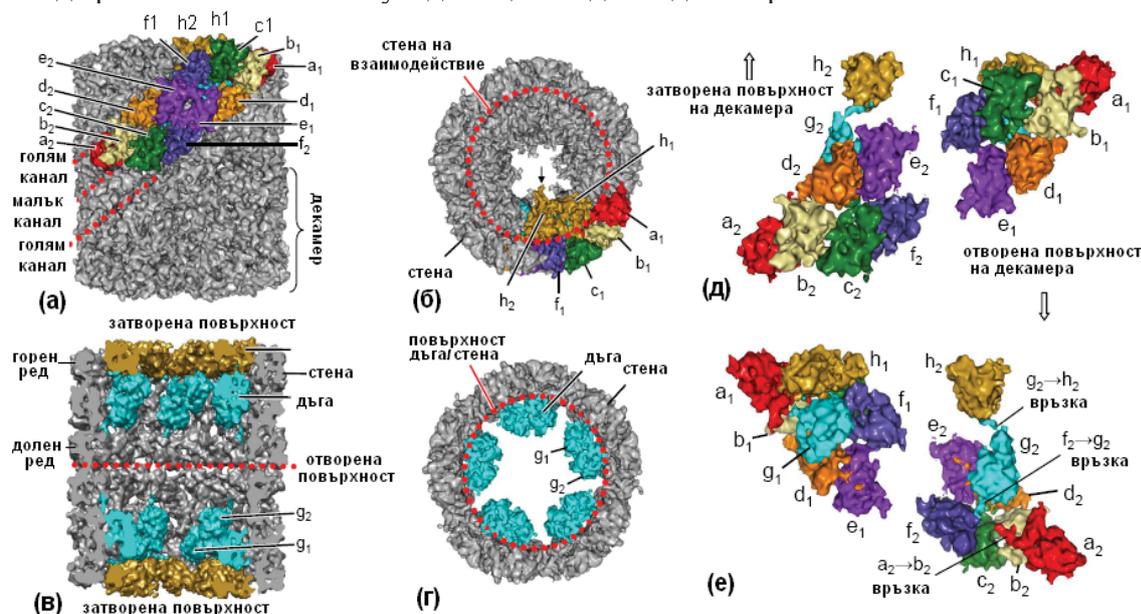
## КИСЛОРОД-СВЪРЗАЩИ МЕДНИ ГЛИКОПРОТЕИНИ

От няколко десетилетия огромните протеини (хемоцианини), чиято молекулна маса може да достигне до няколко милиона Далтона, привличат вниманието на учените, поради интересната им структура, свойства и организация. Хемоцианините са представени в два основни класа: молюски и артроподи. Те се срещат единствено в циркулиращата течност, хемолимфата, изпомпвана от сърцето на някои артроподни (раци, омари, скорпиони, паяци и др.) и молюскови (охлюви, сепии, октоподи, миди и др.) организми. Те имат активен център с приблизително еднакво съдържание на медни йони (молюскови - 0,25% Cu, артроподни – 0,17% Cu), изпълняват еднаква функция, но съществено се различават в структурната организация и въглехидратното съдържание (3-6). Биологичната функция на тези гликопротеини е чрез двата медни йона в активния си център да свързват обратимо молекула кислород (Фиг. 2).



**Фигура 2.** Активен център на хемоцианин от молюски

Хемоцианините от артроподи са изградени от 4,6 или 8 хексамери, като всеки хексамер включва по 6 структурни субединици с маса около 75 кДа. Много по-сложна е структурата на хемоцианини от молюски, където масата на нативната молекула достига до 9 000 кДа и тя може да бъде образувана от димери или мономери. От предложения модел за четвъртичната структура на молекулата на KLN, показан на фигура 3 се вижда разположението на субединиците в двата декамера.



**Фигура 3.** Модел на четвъртичната структура на молекулата на KLH: а) и в) страничен поглед върху дидекамера; б) и г) поглед от горе и от долу на декамера; д) и е) разположението на ФЕ в двете субединици

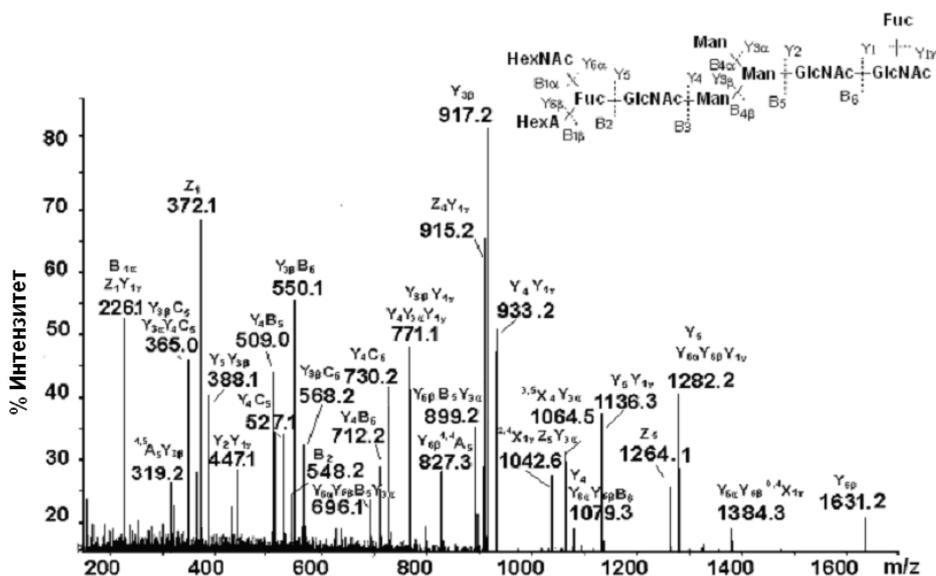
Една, две или три субединици изграждат тези огромни молекули, но основните градивни елементи са седем или осем функционални единици, с маса около 50 кДа (6).

Повечето хемоцианини от молюски са гликопротеини с голяма разнообразие на въглехидратното им съдържание и структурата. Гликозилирането на хемоцианините от молюски предизвиква особен интерес, поради връзката му с имуностимулиращ ефект, проявен от тези медсъдържащи гликопротеини. Чрез прилагане на различни съвременни методи и техники да се анализират въглехидратните структури на хемоцианини от различни молюсковидни организми, обитаващи различни условия на живот, като хемоцианинът изолиран от черноморския охлюв *Rapana venosa* (RvH) и градински охлюв *Helix vulgaris* (HvH) и сравнени с хемоцианина от гигантската мида *keyhole limpet* (KLH) обитаващ северното крайбрежие на Америка (9,10).

Използваната стратегия за характеризиране на въглехидратната структура на хемоцианин *Rapana venosa*, включва два основни подхода:

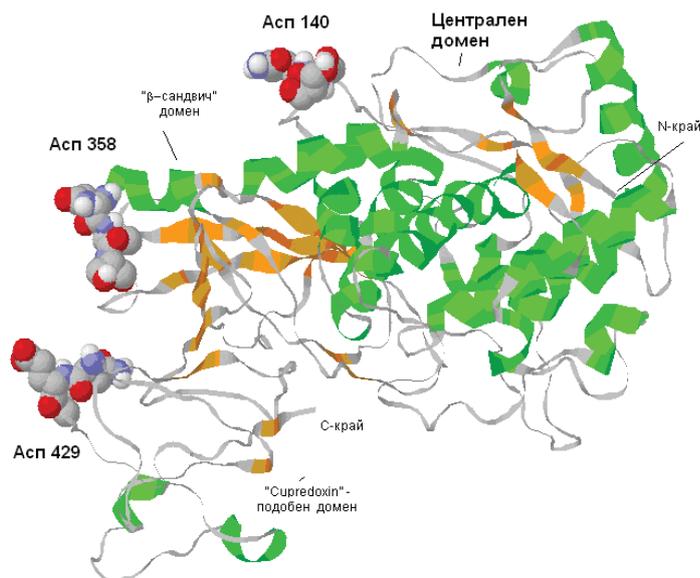
**Първият подход** включва характеризиране на гликани, изолирани след хидролиза на структурните субединици на хемоцианините със специфична гликозидаза PNGase F. Така получените гликани са анализирани чрез капилярна електрофореза, MALDI-TOF и Q-Тгар системи.

**Вторият подход** е свързан с характеризиране на гликопептиди, изолирани от функционалните единици и структурните субединици RvH1 и RvH2. Получените гликопептиди след ензимна хидролиза с трипсин са охарактеризирани с LC/ESI-MS, Q-Тгар-LC/MS/MS или нано-електроспрей йонизационна маспектрометрия.



**Фигура 4.** MS/MS спектър и структури с фрагментационна номенклатура на двойно заредения йон  $[M+2Na]^{2+}$  на гликана при  $m/z$  915.2, изолиран от RvH2.

От получените MS/MS спектри, както е показано на фигура 4, за редица гликани и гликопептиди, изолирани от хемоцианините, са определени въглехидратните им структури (9,10). Установено е, че едната фукоза е локализирана към крайния GlcNAc, докато другата е разположена в средата на въглехидратната верига и е свързана с GlcNAc ( $m/z$  1631,2). За първи път беше определено наличието на хексурониева киселина в хемоцианини (Фиг.4).

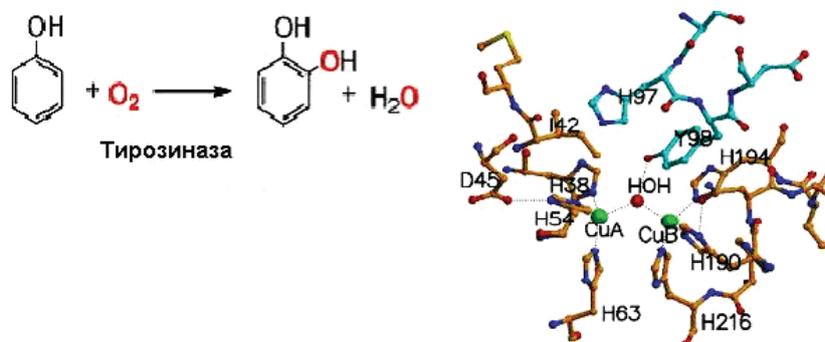


**Фигура 5.** 3-Д модел на функционална единица НН-н.

От построения модел на една функционална единица от хемоцианин от *Helix lucorum* се наблюдава разположението на гликаните на повърхността на молекулата (Фиг. 5). Разположението на въглехидратните вериги на повърхността на молекулата играе важна функция за структурата и свойствата на хемоцианините.

## ТИРОЗИНАЗИ

Тирозиназите са мембранни гликопротеини, изградени от около 330 аминокиселинни остатъка, които са групирани в два богати на цистеини (17 Cys) домени и включват 4 *N*-гликозилирани центъра. За разлика от хемоцианините, тирозиназите съдържат три медни йона, като два са разположени в активния център (Cu A и CuB) и един на повърхността на молекулата. Всеки меден йон е свързан с три хистидинови остатъка. Активирането на ензима се осъществява *in vivo* след лимитирана протеолиза, което увеличава достъпа на субстрата до медните йони в активния център. Същите свойства са наблюдавани и при тирозиназа от бактериалния щам *Laceyella sacchari* (7). Окси-формата на тирозиназата катализира превръщането на пирокатехола до о-хинон, както е показано на фигура 6.



**Фигура 6.** Активен център на ензима тирозиназа

## ПОТЕНЦИАЛЕН ТЕРАПЕВТИЧЕН ЕФЕКТ НА МЕДНИТЕ ГЛИКОПРОТЕИНИ

През последните години гликопротеините представляват интересни молекули, поради наличието на въглехидратни вериги с разклонената природа и различни типове свързвания. Предполага се, че тези въглехидратни вериги са отговорни за структурата и терепевтичните свойства на тези гликопротеини. Също така гликопротеините са отговорни за редица заболявания или се включват, като важна съставка в лекарствени препарати.

#### Антитуморен и антивирусен ефект на супероксид дисмутази

Така например потенциален терапевтичен ефект на ензима Cu/Zn-SOD е потвърден след третирането на белите мишки с 500 Е Cu/Zn-SOD от *H. lutea* 103, което води до увеличаване на преживяемостта им до 86% при грипна инфекция от вирус А/Aichi (H3N2) Прилагането на 250 Е също намалява смъртността, макар и в по-слаба степен, като протективният ефект на препарата се определя от неговата доза (11).

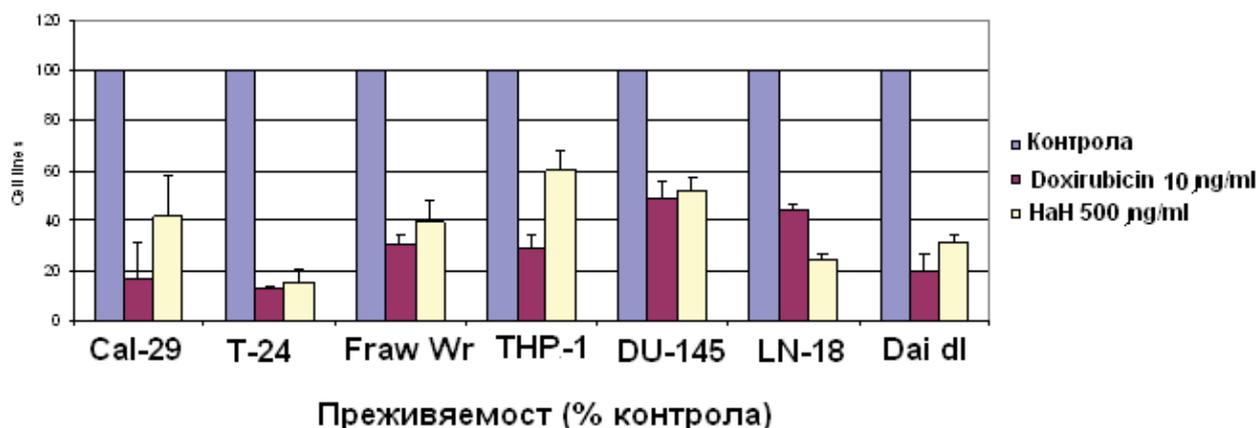
Също така, е установено, че третирането на хамстери с трансплантиран тумор на Графи с Cu/Zn-SOD води до забавяне на туморния растеж, особено значимо в ранните стадии на туморната прогресия. На 10-ия ден след трансплантацията се отчита 73-75% намаление в размерите на тумора, като антиоксидантната активност в чернодробна тъкан от тумор-носител хамстер (ТНХ) се променя значително в процеса на туморната прогресия (12,13). С времето, както SOD активността така и нивото на този ензим намалява непрекъснато.

Както е известно, появата на туморите се съпътства с повишаване нивото на свободните окси-радикали и едновременно с това, с намаляване нивото на антиоксидантните ензими в клетките, като супероксид дисмутазите (СОД) и др. Ето защо след инжектиране на СОД в организма води до редукция на потока от радикали и предпазва клетката от изменения. Терапевтичният ефект на СОД, вероятно е свързан със способността му да обезврежда  $O_2^-$ , а не с директно антивирусно действие.

#### Антивирусен, антитуморен и антибактериален ефект на хемоцианини

Друг меден гликопротеин (хемоцианин) също показва антивирусна, антитуморна и антибактериална активност. След третиране на различни вируси с нативните молекули на хемоцианини *Rapana venosa* (RvH), *Helix lucorum* (HhH), *Helix aspersa* (HaH) и KLH, както и с тяхните изоформи, беше установено, че една функционална единица от RvH инхибира развитието на вируса (10). Тък като структурата на 9 от 11 протеини от обвивката на HSV-1 са добре изучени, това допринася за предсказване на взаимодействията между въглехидратната верига от протеините от вирусната обвивка и хемоцианините.

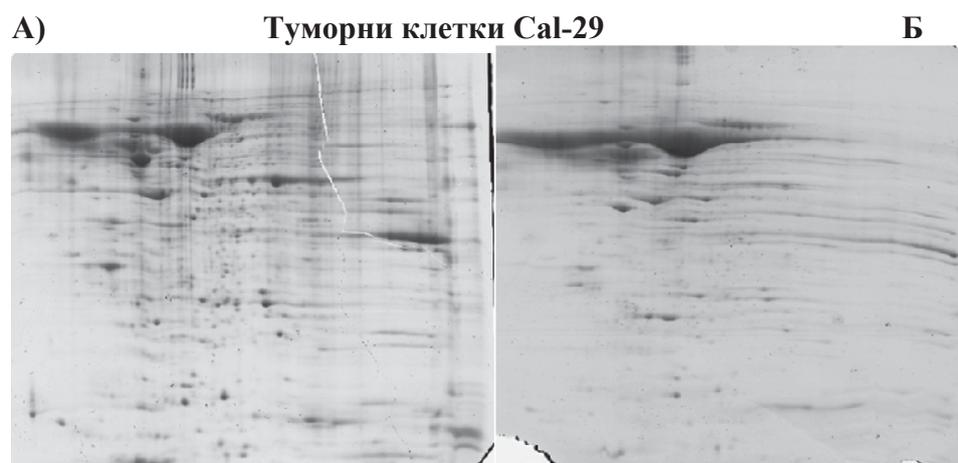
Известно е, че KLH се прилага в имунотерапията на карцином на пикочния мехур, поради антигенно родство с епитопи на туморни антигени от този вид тумор. Доказани са и предимствата на хемоцианин от *Carcinus* и *Helix* като носител на висок антигенен потенциал в сравнение с KLH (13) и антитуморния ефект на *Helix* хемоцианин срещу *Guerin ascites* тумор (14). След определяне на специфичната активност на Hc и на изоформите, е установен антитуморен ефект на хемоцианина от *Helix aspersa* (HaH) и изоформите срещу туморни клетъчни линии T-24 и Cal-29 (тумор на пикочния мехур) (фиг. 7) (15).



**Фигура 7.** Антитуморен ефект на хемоцианин от *H. aspersa* срещу туморни клетъчни линии T-24 и Cal-29.

Хемоцианинът от градински охлюв от *H. aspersa* не само стимулира имунната система на организма, но и пряко атакува туморните клетки. За изясняване на механизма на разграждане на туморните клетки от хемоцианин *H. aspersa* е използван протеомен анализ, като са проследени промените в експресията на протеините в туморните клетки преди и след третиране с нативната молекула на НН, както и изоформите ѝ.

Промяната на експресията на някои протеини в човешки туморни клетъчни линии T-24 и Cal-29, третирани с нативната молекула на НН след 24 часа, са проследени чрез 2Д-електрофорези (Фиг. 8А и Б). След третиране на HL 10/29 уроепителни клетки с хемоцианини не се наблюдава промяна в морфологията на клетките.



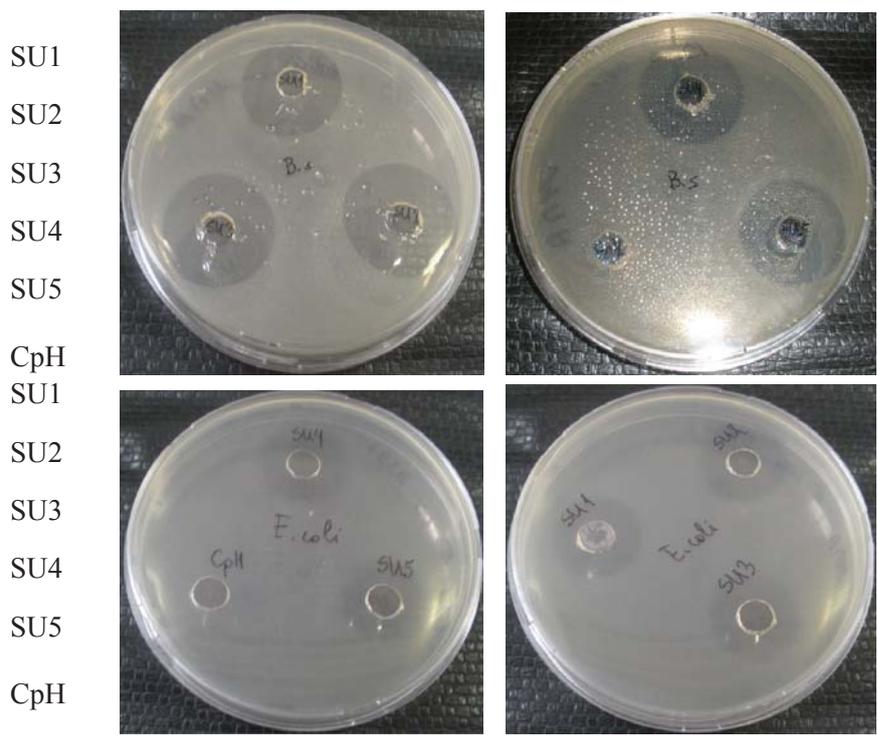
**Фигура 8.** А) 2Д-електрофорези след 24 часа от третирането с нативната молекула на НН на човешки туморна клетъчна линия Cal-29.

Антибактериален ефект на хемоцианините

Проведени са редица изследвания за определяне на ефекта на хемоцианини от артроподи и молюски срещу различни бактерии. Тестът за антибактериална активност включва инокулирането на хранителната среда със суспензия от бактериални клетки, накапване на съответните проби и тяхното дифундиране в средата и отчитане на диаметъра на стерилна зона, образувана след 24 h култивиране. Проведените анализи показват промяна в зоната за стерилност (17 мм) при третиране на бактерията *Bacillus subtilis* с функционална единица 6 от хемоцианин от градински охлюв *Helix aspersa* (Фиг. 9).



**Фигура 9.** Третиране на бактерията *Bacillus subtilis* с функционални единици от хемоцианин от градински охлюв *Helix aspersa*  
 Докато само една функционална единица от HaH проявява инхибиращ ефект, то почти всички структурни субединици от хемоцианин от *Cancer pagurus* потискат растежа на бактериите *Bacillus subtilis* и *Escherichia coli* 3397 (Фиг. 10 А,Б).



**Фигура 10.** А) Третиране на бактериалните щамове *Bacillus subtilis* и Б) *Escherichia coli* 3397 със структурни субединици от хемоцианин от *Cancer pagurus*.

## ЗАКЛЮЧЕНИЕ

Тъй като пазарът се нуждае от нови активни средства, това налага необходимостта от търсене на други гликопротеини, проявяващи подобни свойства. Получените резултати от проведените изследвания върху антивирусния, антитуморен и антибактериален ефект на медните гликопротеини, като супероксид дисмутази, хемоцианини от молюски и артроподи, както и изграждащите ги изоформи, потвърждават, че те са потенциални терапевтични компоненти, най-вероятно поради високото им въглехидратно съдържание и специфична монозахаридна композиция. Ето затова определянето на първичната и въглехидратни структури на супероксид дисмутазите от *Humicola lutea* 103 и дрожди *Kluyveromyces marxianus* NBIMCC, както и на хемоцианините от *Rapana venosa* и *Helix vulgaris*, сравнени с тази на хемоцианина от keyhole limpet, е от значение за определяне на свойствата и механизма на действие на тези протеини.

Експериментални данни ни дават основание да продължим изследванията върху медните гликопротеини, супероксид дисмутази и хемоцианини от молюски и артроподи, с цел приложението им като лечебно средство в имунотерапията, бактериални и вирусни инфекции, и най-вече при туморна терапия.

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## AO2. ANTI-CANCER PROPERTIES OF GASTROPODAN HEMOCYANINS ON MURINE MODEL OF COLON ADENOCARCINOMA

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### Abstract

Various immunotherapeutic approaches have been used for treatment of cancer. A number of natural compounds are designed to repair, stimulate, or enhance the immune system's response. Among them are the hemocyanins (Hcs) - extracellular copper proteins isolated from different arthropod and mollusc species. Hcs are oxygen transporter molecules and normally are freely dissolved in the hemolymph of these animals. Because of their immunostimulatory properties as well as an absence of toxicity or side effects hemocyanins are very promising class of an anti-cancer therapeutics. KLH (*Megathura crenulata* hemocyanin) is the most studied molecule of this group setting a standard for natural carrier protein for small molecules and has been used in anti-tumor clinical trials.

Hcs isolated from marine snail *Rapana thomasiana* (RtH) and the terrestrial snail *Helix pomatia* (HpH) perform strong *in vitro* and *in vivo* anticancer and antiproliferative effects in murine model of colon adenocarcinoma. Immunization of the developed model with RtH and HpH prolonged survival of the treated animals, improve humoral anti-cancer effect, and moderate the typical manifestation of C-26 carcinoma model, like tumor growth, splenomegaly and appearance of lung metastasis compared to untreated ones.

**Keywords:** C-26 carcinoma model, hemocyanins; anti-cancer activity;

### **AO3. HELIX POMATIA HC (HPH) HEMOCYANIN AS PROTEIN-CARRIER AND BIO-ADJUVANT OF BACTERIAL AND VIRAL PROTEINS**

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#### **Abstract**

Killed viral vaccines and bacterial toxoids are weakly immunogenic. The hemocyanins are widely used as immune modulators. In the present study we promote the hemocyanin, isolated from the terrestrial gastropod *Helix pomatia* (HpH) as protein-carrier as well as bio-adjuvant. The purified HpH was combined with standard antigens and a construct of HpH with influenza virus hemagglutinin intersubunit peptide (IP) or HpH – tetanus toxoid were used for immunization.

An immunization of mice with Influenza peptide conjugated to *Helix pomatia* hemocyanin induced a strong anti-influenza cytotoxic response. The IgG antibody response to the TT combined with HpH was comparable to the response of the toxoid in CFA. Immunization of experimental animals with HpH combined with IP or TT led to generation of enhanced levels of key cytokines for development of Th1 or Th2 - related immune response.

The vaccination of mice demonstrates that the HpH is acceptable as a potential bio-adjuvant for subunit vaccines and it could be used as natural adjuvant or protein-carrier.

# AO4. COMPARATIVE STUDY OF THE PROTECTIVE EFFECT OF *ARONIA MELANOCARPA* FRUIT JUICE AND QUERCETIN IN A MODEL OF PARACETAMOL-INDUCED HEPATOTOXICITY IN RATS

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## Abstract

*Aronia melanocarpa* fruits are extremely rich in polyphenolic substances (procyanidins, flavonoids and phenolic acids) with pronounced antioxidant and anti-inflammatory activities. Quercetin is a naturally occurring polyphenolic compound, one of the flavonoids in aronia fruits, which has already been demonstrated to ameliorate paracetamol-induced liver damage.

The aim of the present study was to investigate the effect of *Aronia melanocarpa* fruit juice (AMFJ) in comparison with quercetin in a model of paracetamol-induced hepatotoxicity in rats.

AMFJ was applied daily orally at doses of 2.5 and 5.0 ml/kg from day 1 to day 7 of the experiment. Quercetin was administered also orally from day 1 to day 7 to other groups of rats at doses of 50 and 100 mg/kg. Paracetamol was applied intraperitoneally (1.0 g/kg) on day 5. Blood and liver were taken for biochemical investigations on day 7 (48 hours after paracetamol administration). Liver toxicity was estimated by the activities of serum enzymes – aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP). Oxidative stress was estimated by the levels of thiobarbituric acid reactive substances (TBARS) in serum and liver homogenate.

The administration of paracetamol caused a significant elevation of serum AST ( $p < 0.01$  vs. control) and ALT ( $p < 0.05$  vs. control), and induced lipid peroxidation as measured by the significant increase of TBARS in serum ( $p < 0.01$  vs. control) and liver ( $p < 0.05$  vs. control). AMFJ at the two tested doses prevented the increase of liver enzyme activities. The effect of AMFJ was comparable to that of quercetin. Thus, in animals pretreated either with AMFJ or quercetin liver enzyme activities did not differ significantly from the control levels. AMFJ prevented the elevation of TBARS in the liver at the two applied doses and in the serum at the dose of 5.0 ml/kg. Similar were the effects of quercetin: it prevented the elevation of TBARS in the liver at the two applied doses and in the serum at the dose of 100 mg/kg.

The present study demonstrated the protective effect of both AMFJ and quercetin in a model of paracetamol-induced liver toxicity. This hepatoprotection is probably due to the antioxidant activities of the tested substances.

Key words: *Aronia melanocarpa* fruit juice, quercetin, paracetamol, hepatotoxicity, rats

## **AO5. ATTENUATION OF CELLULAR OXIDATIVE STRESS BY ARONIA MELANOCARPA TOTAL EXTRACT AFTER DOXORUBICIN EXPOSURE**

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### **Introduction:**

Cellular oxidative stress *in vitro* and *in vivo* after Doxorubicin treatment was investigated by measurement of the intracellular reduced Glutathione (GSH) levels after application of total extract from the medical plant *Aronia melanocarpa*.

### **Materials and Methods:**

Normal 3T3 fibroblasts from Balb/c experimental mice, mouse myeloma cells and mixed cell cultures, were cultivated in the presence of *Aronia*-extract alone; of 0.05M Doxorubicin alone; of both substances, as well as untreated controls. Analogically, laboratory mice from the same line were divided into four groups: treated by *Aronia*-extract alone; by Doxorubicin alone; by both substances and untreated controls.

### **Results:**

In all cases, despite of the established decreased levels of GSH in the presence of Doxorubicin, certain restoration in them was noticed in the presence of *Aronia*-extract, but, on the other hand, significant differences in these levels were not observed. Similar tendency was noted in *in vivo*-tests, where the highest GSH amounts were detected in the livers of the animals from the four groups tested.

### **Discussion:**

The results obtained were in agreement with literature findings about the antioxidant and detoxification role of GSH [1-3]. On the other hand, those data supported the cited literature about a certain depletion of GSH after Doxorubicin exposure [4, 5].

### **Conclusion:**

Further studies, directed to investigation on the influence of the separate antioxidant components (polyphenols and anthocyanins) from the *Aronia melanocarpa* total extract on the levels of intracellular GSH should be necessary, including determination of the levels of enzymes, participating in its metabolism.

**Keywords:** GSH-levels, Doxorubicin, *Aronia melanocarpa* total extract.

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## АО6. РАСТЕНИЯ ПРИТЕЖАВАЩИ АНТИВИРУСНА ЕФЕКТИВНОСТ СРЕЩУ ХЕРПЕС СИМПЛЕКС ВИРУС 1 И 2

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Едни от най – разпространените полово предавани заболявания в световен мащаб са тези предизвикани от херпес симплекс вирусите (8, 22). Основният причинител на гениталният херпес при хората е херпес симплекс вирус – 2(HSV-2), който се пренася почти изцяло по полов път(4). Заразяването с херпес симплекс вирус 1(HSV-1)от своя страна, обикновено става през ранна детска възраст, не по полов път. В някои развиващи се страни обаче, HSV-1 е основен причинител на гениталният херпес (9,16,22). И двата вируса могат да преминат през клетките на епителната мукоза, както и през кожа със наранена повърхност, след което да мигрират до нервната тъкан, където перзистират в латентната си фаза. HSV-1 предизвиква основно орофациални лезии, като преобладаващо се локализира в тригеминални ганглии, докато HSV-2 се установява предимно в лумбосакралния ганглий. Не на последно място трябва да се отбележи, че и двата вируса могат да заразят както орофациалната област, така и гениталния тракт (11).

По данни на Chauvichitsilp et al. (7) между 60 и 90% от възрастните индивиди в света са заразени със HSV-1.

Херпес симплекс вирусите принадлежат сем. Herpesviridae, към което се отнасят херпес симплекс вирус 1(HSV-1), херпес симплекс вирус 2 (HSV-2), варицела зостер вирус,

цитомегаловирус, вирусът на Епщайн – Бар, човешките херпесвируси 6 и 7, както и вирусът причиняващ Саркома на Капоши (10, 18, 21).

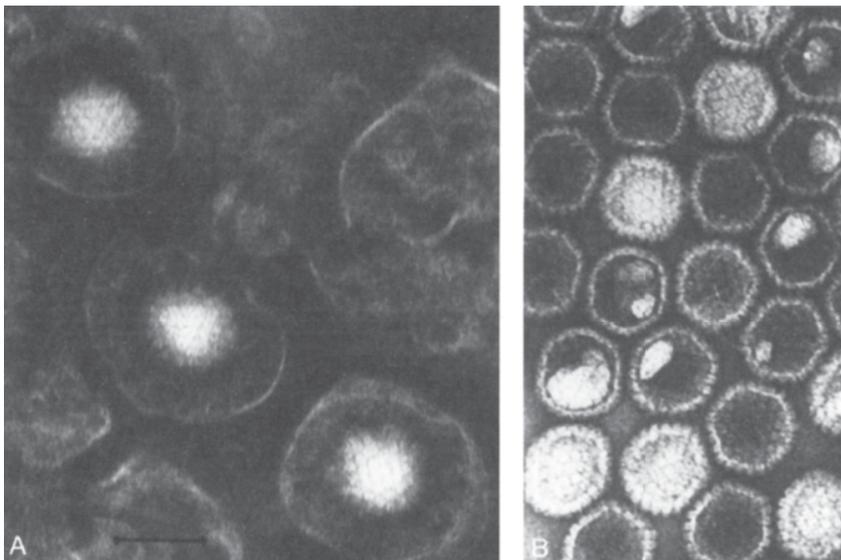
HSV-1 и HSV-2 принадлежат към подсемейство Alphaherpesvirinae, род Simplexvirus (15)

Вирионът (фиг. 1) се състои от двойно верижна ДНК, геномът е около 150 kbp, заобиколен от капсид, тегумент и външна обвивка (енвелоп) (15).

Поради факта, че инфекциите предизвикани от HSV-1 и HSV-2, представляват голям проблем за човешкото здраве в световен мащаб, множество проучвания са извършени за намиране на ефективни методи за отстраняване на етиологичните агенти.

Лечението на орофациалните и гениталните херпесни инфекции включва преди всичко симптоматична терапия, целяща облекчаване на клиничните проявления на болестта. Най – широко приложение за етиологична терапия срещу херпес симплекс вируси имат антивирусните препарати: aciclovir (acyclovir), valaciclovir (valacyclovir), famciclovir и penciclovir. За съжаление нито един от тях не води до пълно отстраняване на вируса от заразеният организъм.

В търсене на други антивирусни агенти, ефективни срещу херпес симплексните инфекции са били изследвани голям брой растения и екстракти от тях.



Фиг.1 Херпес симплекс вирус тип 1(негативно оцветяване, електронен микроскоп). (Ляво) Вириони със обвивка. (Дясно) Капсиди без обвивка. Вар: 100 nm. (15).

*M. officinalis* (lemon balm) е многогодишно тревисто растение от сем.Lamiaceae, характерно за западна Азия и източното Средиземноморие, като е култивирано в централна, източна и западна Европа и САЩ (6, 23). То има доказан вируциден ефект върху Herpes simplex type 1 и Herpes simplex type 2, при изследвания ин витро (2, 5, 13, 17).

Като основна съставка с антивирусно действие в екстрактите от *M. officinalis* се посочва розмариновата киселина (5).

Abad et al. (1) провеждат изследване за определяне антивирусната активност на растения растящи на Иберийският полуостров срещу HSV-1. Установено било, че растенията *Nepeta nepetella* (150–500 mg/mL), *Nepeta coerulea* (150–500 mg/mL), *Nepeta*

tuberosa (150–500 mg/mL), *Dittrichia viscosa* (50–125 mg/mL) и *Sanguisorba minor magnolii* (50–125 mg/mL), проявяват антивирусна активност срещу HSV-1.

При изследване на антивирусната активност на метаноловите екстракти от различни растения от държавата Того/Африка/ срещу херпес симплекс вирус, Sindbis вирус и pollovirus, Ananil et al. (3), установяват, че видовете *Conyza aegyptiaca* и *Palisota hirsuta* и особено при *Adansonia digitata*, тя е най силно изразена. Освен тях, ефективни срещу херпес вирус били и растенията *Ficus ovaia*, *Mitracarpus villosus* и *Paullinia pinnata*.

Sydiskis et al. (19) приготвили горещи глицеринови екстракти от растенията *Rheum officinale*, *Aloe barbadensis*, *Rhamnus frangula*, *Rhamnus purshianus* и *Cassia angustifolia* и тествали ефективността им срещу HSV-1. Всички екстракти инактивирали вирусът. След извършване на сепариране върху тънкослойна хроматография, било установено, че активните им компоненти са антраквинони. Авторите приготвили пречистена проба от антраквинона *aloe emodin*, и третирали с него HSV-1 и HSV-2. С помоща на електронния микроскоп било установено, че третираните с антраквинони вируси, били с частично разрушена външна обвивка. На тази база е направен извода, че антраквиноновите екстракти от тестваните растения имат директен вируциден ефект срещу вируси с обвивка.

Müller et al.(14), проучили антивирусният ефект срещу HSV-1, шамове KOS и 29-R на 18 екстракта и фракции от различни Южно Американски растения. При 6 екстракта и 4 фракции бил установен антивирусен ефект. *Plex paraguariensis*, *Lafoensia racari*, *Passiflora edulis*, *Rubus imperialis* и *Slonea guianensis* показали индекс на селективност (SI) > 7 срещу двата използвани щама на HSV-1.

При изследване на антивирусният ефект на 17 растения, произхождащи от Северна Нигерия, било установено, че 4 от тях - *Bauhinia thonningii*, *Anacardium occidentale*, *Dichrostachys glomerata* и *Sterculia setigera* са ефективни ин витро срещу HSV-1 (12).

Както е видно има голям брой растения по цял свят, притежаващи антивирусна активност срещу HSV-1 и HSV-2. За съжаление, повечето данни се отнасят за ин витро изследвания, проведени на клетъчни култури в контролирана лабораторна среда.

Един от редките случаи, в които е установена, както ин витро, така и ин vivo антивирусна активност срещу HSV е водният екстракт от корени на растението *Carissa edulis* (20). Това е лечебно растение, отглеждано в Кения. При изследване на антивирусните му свойства срещу диви шамове на HSV (7401H HSV-1 и Ito-1262 HSV-2), както и срещу резистентни към конвенционалната антихерпесна терапия шамове на HSV (TK(-) 7401H HSV-1 и AP(r) 7401H HSV-1), било установено, че екстрактът инхибира значително формирането на плаки върху клетъчна линия Vero E6, а при прилагането му на заразени мишки, забавил проявлението на заболяването с над 50 %, като същевременно намалили смъртността сред заразените животни от 70 до 90 %.

От представените данни до тук е видно, че се работи усилено върху търсенето на решение на проблема с лечението на херпес симплекс вирусните инфекции при хората. Проблемът е особено актуален, поради наличието на резистентни шамове на HSV срещу регулярно използваните антивирусни препарати, като ацикловир.

Много от учените по цел свят са се насочили към откриване на натурални продукти, които да бъдат използвани в борбата с това заболяване. Наред с другите си предимства пред синтетичните препарати, екстрактите от растения се използват в традиционната медицина на много страни за лечение на голям брой заболявания. Едва през последните 60 – 70 години, науката е напреднала достатъчно, за да може да разбере механизмите, чрез които тези растения помагат при едно или друго заболяване. Нашите надежди са, че в скоро време ще бъдат идентифицирани и приложени за лечение на инфекции предизвикани от HSV-1 и HSV-2, вещества от естествен произход, които да имат

достатъчно добре изразени антивирусни свойства, без да оказват негативно влияние на човешкият организъм.

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## **AO7. CYTOTOXIC AND ANTIHERPES VIRUS EFFECTS OF ORIGINAL BULGARIAN STRAINS LACTOBACILLUS**

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Infections with human herpes virus type 1 and 2 (HSV-1 and HSV-2) are widespread and result in persistent and latent infection.

The lactic acid bacteria (LAB) play an important role in healthy balance in the ecosystems, such as gastrointestinal and urogenital tract. A new medical concern for a *Lactobacillus* application in therapy and prophylaxis of vaginal disorders become more acceptable. However, there is not presently a work discussing the possible role of non-vaginal LAB.

The aim of study was to evaluate *in vitro* the cytotoxic and anti-herpes virus type 1 and type 2 activities of selected original Bulgarian strains. The *Lactobacillus* strains (*LB13*, *LB131*, *LB51*, *LBC1*, *LB3*, *LB4* and *LB11*) from the collection of the Institute of Microbiology were included in the present study. They were isolated from artisanal dairy products, identified to the species level and pre-selected as bio-protective cultures. The MTT colorimetric assay was performed to detect the effect of tested strains on growth kinetics, cell viability, maximal nontoxic concentration (MNC) and the IC<sub>50</sub> after incubation in continuous MDBK cells at 48 h. The cytotoxicity of strains *LB3* and *LB11* were more cytotoxic than the strains *LB13*, *LB131*, *LB51*, *LBC-1* and *LB4*. The influence-of the *Lactobacillus* strains on HSV replication

was evaluated on the basis of their effects on the infectious titers on two *wt* HSV clinical isolates - TM (HSV-1) and Bja (HSV-2). The most effective was *L. bulgaricus* LB13. Furthermore, it was the only strain, able to affect the HSV-1 and HSV-2 replication. To our knowledge these is the first result for antiviral activity of *L. delbrueckii* subsp. *bulgaricus*.

**Key words:** *Lactobacillus* strains, HSV-1, HSV-2, antiviral effect

## **АО8. КАКВО (НЕ) ЗНАЕМ ЗА СРЕБРОТО?**

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## **АО9. ПЪРВИ СРЕЩИ С ФАРМАКОГЕНЕТИКАТА**

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**BO1. ВЛИЯНИЕ НА МЕТАЛНИ СЪЕДИНЕНИЯ ВЪРХУ  
ПРЕЖИВЯЕМОСТТА И ПРОЛИФЕРАТИВНАТА АКТИВНОСТ НА  
ТРАНСФОРМИРАНИ С ВИРУСИ ТУМОРНИ КЛЕТКИ И  
РЕПЛИКАЦИЯТА НА ЧОВЕШКИ И ГОВЕЖДИ ХЕРПЕСНИ  
ВИРУСИ**

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**BO2. EFFECT OF Zn/Au AND Zn/Ag COMPLEXES WITH SALEM ON  
VIABILITY AND PROLIFERATION OF VIRUS-TRANSFORMED  
ANIMAL AND HUMAN CELLS**

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### **BO3. CLUSTERING OF DETONATION NANODIAMOND PARTICLES IN PHYSIOLOGICAL SOLUTIONS**

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Recently nanodiamond (ND) particles are gaining much interest in biomedical applications due to their unique properties such as chemical stability, high hardness, small size, large surface area and high adsorption capacity [1]. One of the problems, however in application of diamond nanoparticles and of nanoparticles (NPs) in general in the human body and in *in vitro* studies is their trend to aggregate in solutions with physiological salt concentration and pH values [2]. In order to stabilize particles and to prevent their clustering different stabilizers have been used, including Tween, pulmonary surfactant, albumin or serum as well as treatments with high ultrasound energy. Unlike albumin and serum coatings sonication does not change chemically NPs surface and does not provoke immune reaction in the organism after introduction of sonicated NPs in the body. Therefore, in our experiments we have chosen sonication to disperse NDs before to incubate them with cells. It was important to know also how long our particles will be stable in different physiological solutions after treatment with ultrasonic energy. For this goal we have incubated for three days four types of detonation nanodiamond (DND) particles (named as NASFPA, NASHCl, YTM and DND-30) in different physiological solutions - phosphate buffered saline (PBS), Dulbecco's Modified Eagle Medium (DMEM) with and without Fetal Bovine Serum (FBS) and blood plasma, after treatment of the NPs with ultrasound energy. The results showed that in PBS NASFPA particles are stable for 2 days, DND-30 - for 3 days while the other two types, NASHCl and YTM formed clusters on the first day; in serum-free medium all types of DND particles formed clusters while in DMEM with FBS only insignificant clustering was observed e.g. FBS seems to suppress aggregation. Surprisingly, in blood plasma we have also observed aggregation of DND particles although based on the literature data we expected that plasma would stabilize the particles.

In general we can conclude that the method of sonication used by us needs to be optimized in order to stabilize DND particles for a longer time.

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## ZnO NANOSTRUCTURES AND THEIR POTENTIAL APPLICATION IN BIOMEDICINE

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## Abstract

In the last decade, nanoscale materials have become an area of extensive research due to their potential application in wide areas of science, technology and medicine. In what concerns medicine, inorganic metal oxides have attracted scientific interest as antimicrobial agents because of their safety and stability. Among these, zinc oxide nano objects are at the forefront of research due to their unique properties and widespread applications.

In this study, we synthesized different types of ZnO samples (thin and nanostructured films) and investigated the effect of substrate morphology on the cell viability. Samples were prepared by pulsed laser deposition (PLD), applied at different experimental conditions, which allows excellent control over stoichiometry and surface morphology. Three permanent cell lines (HeLa and MCF-7 human tumor cells and 3T3 mouse fibroblast cells) were cultured on the tested samples at the same condition and concentration. It was found that cells respond differently, in a cell-type dependent manner, to smooth surfaces compared to nanoscale roughness. Nano surfaces of the samples maintains HeLa cell viability, while only a small amount of MCF-7 cells survives on them, established by double staining assay with acridine orange and ethidium bromide. The ability to control cell adhesion and response is desirable in cell engineering and suggests that nanotopography will have an important role in the development of next-generation biomaterials and biosensor devices.

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## **BO5. PERSISTENT ORGANIC POLLUTANTS - EFFECTS ON HUMAN HEALTH**

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### **Abstract**

Persistent Organic Pollutants (POPs) are toxic chemicals that adversely affect human health and the environment around the world. Because they can be transported by wind and water, most POPs generated in one country can and do affect people and wildlife far from where they are used and released. They persist for long periods of time in the environment and can accumulate and pass from one species to the next through the food chain.

The studies have shown that chronic exposure to low doses of certain POPs can result in reproductive and immune system deficits. Exposure to high levels of certain POPs chemicals - higher than normally encountered by humans and wildlife - can cause serious damage or death. Epidemiological studies of exposed human populations and studies of wildlife might provide more information on health impacts.

The Stockholm Convention on Persistent Organic Pollutants, which was adopted in 2001 and entered into force in 2004, is a global treaty whose purpose is to safeguard human health and the environment from highly harmful chemicals that persist in the environment and affect the well-being of humans as well as wildlife. The Convention requires parties to eliminate and/or reduce POPs, which have a potential of causing devastating effects such as cancer and diminished intelligence and have the ability to travel over great distances.

**Keywords:** Persistent Organic Pollutants, human health, environment

**Persistent Organic Pollutants**, known as POPs, are toxic substances - carbon-containing compounds, released into the environment through a variety of human activities. They have adverse effects on the health of ecosystems, wildlife and people.

POPs have a number of common properties:

POPs are persistent in the environment. They resist degradation or breakdown through physical, chemical, or biological processes;

- POPs generally are semi-volatile. They evaporate relatively slowly but when they enter the air, they travel long distances on air currents. They return to earth in rain and snow in the colder areas of the globe, resulting in their accumulation in regions such as the Arctic, thousands of kilometres away from their original sources;
- POPs generally have low water solubility (they do not dissolve readily in water) and high lipid (fat) solubility (they do dissolve easily in fats and oils). Persistent substances with these properties bioaccumulate in fatty tissues of living organisms. In the environment, concentrations of these substances can increase by factors of many thousands or millions as they move up the food chain; and
- POPs have the potential to injure humans and other organisms even at the very low concentrations at which they are now found in the environment, wildlife and humans. Some POPs in extraordinarily small amounts can disrupt normal biological functions, including the activity of natural hormones and other chemical messengers, triggering a cascade of potentially harmful effects (6, 13).

The Stockholm Convention on Persistent Organic Pollutants is a legally binding international agreement to protect human health and the environment from some of the most dangerous chemicals on earth. POPs are defined by their persistence in the environment, their bioaccumulation in nature and in people, and the harm they pose often far from the source. Countries that ratify the treaty are known as Parties commit to abide by its provisions. The POPs treaty calls on Parties to take action to eliminate the production of POPs, minimize unintentional sources, and clean-up and safely manage remaining stockpiles and wastes (8, 12).

The Stockholm POPs treaty names twelve POPs chemicals (the "Dirty Dozen"), which are listed under three Annexes. The nine POPs listed on Annex A are destined for elimination with specific, time-limited exemptions. This includes the agricultural chemicals aldrin, chlordane, dieldrin, endrin, heptachlor, mirex, and toxaphene, as well as the industrial chemicals hexachlorobenzene (HCB), and polychlorinated biphenyls (PCBs). In contrast, POPs listed on Annex B are subject to restrictions on production and use, but eligible for specific exemptions for acceptable purposes. Annex B contains just one substance, the pesticide DDT. Annex C contains POPs that are unintentionally produced, for example as industrial byproducts and combustion processes. Four of the Dirty Dozen POPs are listed under Annex C: polychlorinated dioxins, polychlorinated furans, PCBs, and HCB (8, 13, 19).

The POPs treaty was completed and opened for signature in May 2001 and entered into force on May 17, 2004 following ratification by the 50th nation. As of January 1, 2011, a total of 172 countries, including 25 Member States of the EU have ratified the Convention.

Stockholm Convention was signed by Bulgaria on 23 May 2001 and has been ratified by an Act of the National Assembly on 30 September 2004 (promulgated in State Gazette № 89/12.10.2004 years). Bulgaria is a party to the Convention of March 20, 2005.

**Polychlorinated dibenzodioxins (PCDDs)**, or simply **dioxins**, are a group of halogenated organic compounds. They are commonly referred to as dioxins for simplicity in scientific publications because every PCDD molecule contains a dioxin skeletal structure. Members of the PCDD family have been shown to bioaccumulate in humans and wildlife due to their lipophilic properties, and are known teratogens, mutagens, and suspected human carcinogens. Dioxins are produced in small concentrations when organic material is burned in the presence of chlorine, whether the chlorine is present as chloride ions or as organochlorine compounds so they are widely produced in many contexts. According to the US EPA data, the major sources of dioxins are: coal fired utilities; municipal waste incinerators; metal smelting; diesel trucks; land

application of sewage sludge; burning treated wood; trash burn barrels. These sources together account for nearly 80% of dioxin emissions (4).

There have been many incidents of dioxin pollution resulting from industrial emissions and accidents; the earliest such incidents were in the mid 18th century during the Industrial Revolution (31).

*Toxicity:* Dioxins are absorbed primarily through dietary intake of fat, as this is where they accumulate in animals and humans. In humans, the highly chlorinated dioxins are stored in fatty tissues and are neither readily metabolized nor excreted. The estimated elimination half-life for highly chlorinated dioxins (4-8 chlorine atoms) in humans ranges from 7.8 to 132 years (16).

*Health effects in humans:* Exposure to high levels of dioxins in humans causes a severe form of persistent acne, known as chloracne (15). A case-control study has shown an elevated risk of sarcoma associated with low-level exposure ( $4.2 \text{ fg/ m}^3$ ) to dioxins from incineration plants (35). High levels of exposures to dioxins have been shown by epidemiological studies to lead to an increased risk of tumours at all sites (35). Other effects in humans may include: Developmental abnormalities in the enamel of children's teeth (1, 24), central and peripheral nervous system pathology (23), thyroid disorders (22), damage to the immune systems (3), endometriosis (14), diabetes (2).

Dioxins accumulate in food chains in a fashion similar to other chlorinated compounds (bioaccumulation). This means that even small concentrations in contaminated water can be concentrated up a food chain to dangerous levels due to the long biological half life and low water solubility of dioxins.

*Studies of dioxins' effects in Vietnam.* US veterans' groups and Vietnamese groups, including the Vietnamese government, have convened scientific studies to explore their belief that dioxins were responsible for a host of disorders, including tens of thousands of birth defects in children, that have affected Vietnam veterans as well as an estimated one million Vietnamese, due to their exposure during the Vietnam War to Agent Orange, a defoliant chemical which was widely sprayed over Vietnamese land and which was found to be highly contaminated with TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin - the most toxic dioxin). Several exposure studies showed that some US Vietnam Veterans who were exposed to Agent Orange had serum TCDD levels up to 600 ppt (parts per trillion) many years after they left Vietnam, compared to general population levels of approximately 1 to 2 ppt of TCDD. In Vietnam, TCDD levels up to 1,000,000 ppt have been found in soil and sediments from Agent Orange contaminated areas, three to four decades after spraying. In addition, elevated levels have been measured in food and wildlife in Vietnam (25).

*Dioxin - potent Immune System Poison.* U.S. Environmental Protection Agency's (EPA's) 1994 draft reassessment of dioxin emphasized that dioxin damages the immune system directly and indirectly. From studies of rats, mice, guinea pigs, rabbits, cattle, marmosets, monkeys, and humans, EPA concludes that even low doses of dioxin attack the immune system. Dioxin directly reduces the number of B cells (immune cells that develop in the bone marrow, then circulate throughout the blood and lymph, fighting off invaders). And it reduces the number of T cells (immune cells that develop in the thymus, then circulate throughout the body, attacking invaders), but dioxin's attack on T cells seems to be indirect. One potentially important indirect mechanism is via effects on the endocrine system. Several endocrine hormones have been shown to regulate immune responses, including glucocorticoids, sex steroids, thyroxine, growth hormone, and prolactin. Importantly, TCDD [dioxin] and other related compounds have been shown to alter the activity of these hormones (30).

It is important to consider that if an acute exposure to TCDD even temporarily raises the TCDD body burden at the time when an immune response was initiated, there may be a risk of adverse impacts even though the total body burden may indicate a relatively low average TCDD level. In other words, a single dose of dioxin at the wrong time may damage immune system's ability to protect people. Furthermore, because TCDD alters the normal differentiation of immune system cells, the human embryo may be very susceptible to long-term impairment of immune function from in utero effects of TCDD on developing immune tissue. In other words, dioxin can prevent the immune system from developing properly in an unborn child, with lifelong consequences. Animal studies suggest that some immunotoxic responses may be evoked at very low levels of dioxin exposure (30).

Dioxin appears to be a carcinogen in fish, rodents, and other mammals, including humans. But dioxin can also modulate (modify) the immune system resulting in an inability to fight disease. It is a very powerful immunosuppressant. But it can also upregulate (excite) the immune system so that the people start becoming hypersensitive, developing autoimmunity and allergies. Depending upon the stage (of growth) of the animal and the species, sometimes it could be observed immunosuppression and in other cases it could be observed upregulation (5).

Birnbaum goes on to describe Taiwanese children, exposed to dioxin-like chemicals, who had unusually frequent respiratory infections and ear infections (otitis). Further, she described an Inuit population in Quebec with elevated levels of dioxin in their bodies from eating the fat of marine mammals (seals); their children have very high incidences of respiratory infections and otitis [ear infections], and also a very decreased take of vaccinations (5). In other words, vaccinations don't work well in these children, perhaps because their immune systems have been damaged.

There is no threshold for immunotoxic responses to dioxin - there is no level of dioxin below which the immune system is not affected (5). Put another way: any amount of dioxin seems to do some damage to the immune system, at least in animals; there is no "safe" dose.

In laboratory mice, a single tiny dose of dioxin causes increased deaths when the mice are challenged with an influenza virus (24)- 32. It is worth emphasizing that the effective dose of dioxin is very small.

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

#### *Dioxin exposure incidents*

- In 1949, in a herbicide production plant for 2, 4, 5-T in Nitro, West Virginia, 240 people were affected when a relief valve opened (10).
- In 1963, a dioxin cloud escapes after an explosion in a Philipsa-Duphar plant (now Solvay Group) near Amsterdam. In the 1960s, Philips-Duphar produced 2250 tonnes of "Agent Orange" for the US Army (31).
- In 1968, an explosion of a reactor with 2,4,5-trichlorophenol in Spolana Neratovice plant in Czechoslovakia seriously poisoned about 60 workers with dioxins; after the incident Spolana stopped manufacture of 2,4,5-T (most of which was supplied to the US military in Vietnam). Major parts of the Spolana chemical plant were heavily contaminated by dioxins. A large amount of dioxins were flushed into the Elbe and Mulde rivers during the 2002 European flood, contaminating the soils. The consumption of local fish, eggs, poultry and some produce was prohibited because of the post-flood contamination (9).
- In 1976, large amounts of dioxins were released in an industrial accident at Seveso, Italy. A cloud of toxic chemicals, including 2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin, or TCDD, was

released into the air and eventually contaminated an area of 15 square kilometres where 37 000 people lived. Extensive studies in the affected population are continuing to determine the long-term human health effects from this incident. These investigations, however, are hampered by the lack of appropriate exposure assessments. A minor increase in certain cancers and effects on reproduction have been detected and are being further investigated. Possible effects on the children of exposed people are currently being studied (14).

- In 1978, dioxins were some of the contaminants that forced the evacuation of the Love Canal neighborhood of Niagara Falls, New York. Dioxins also caused the 1983 evacuation of Times Beach, Missouri. From 1982 through to 1985, Times Beach, Missouri was bought out and evacuated under order of the US EPA due to high levels of dioxins in the soil. The town eventually disincorporated.
- In December 1991, an electrical explosion caused dioxins (created from the oxidation of polychlorinated biphenyl) to spread through four residence halls and two other buildings on the college campus of SUNY New Paltz.
- In May 1999, there was a dioxin crisis in Belgium: quantities of dioxins had entered the food chain through contaminated animal feed. 7,000,000 chickens and 60,000 pigs had to be slaughtered. This scandal was followed by a landslide change in government in the elections one month later.
- Explosions resulting from the terrorist attacks on the US on September 11, 2001 released massive amounts of dust into the air. The air was measured for dioxins from September 23, 2001, to November 21, 2001, and reported to be "likely the highest ambient concentration that have ever been reported (in history)". The US EPA report dated October 2002 and released in December of 2002 titled "Exposure and Human Health Evaluation of Airborne Pollution from the World Trade Center Disaster" authored by the EPA Office of Research and Development in Washington states that dioxin levels recorded at a monitoring station on Park Row near City Hall Park in New York between October 12 and 29, 2001, averaged 5.6 parts per trillion, or nearly six times the highest dioxin level ever recorded in the U.S. Dioxin levels in the rubble of the World Trade Centers were much higher with concentrations ranging from 10 to 170 parts per trillion. The report did no measuring of the toxicity of indoor air.
- A few cases of intentional human poisoning have also been reported. The most notable incident is the 2004 case of Viktor Yushchenko, President of the Ukraine, whose face was disfigured by chloracne.
- In 2007 in Italy thousands of tonnes of foul-smelling refuse are piled up in Naples and its surrounding villages, defacing entire neighbourhoods. Polychlorinated dibenzodioxins are found in animals and humans over lethal dose. Sources of Polychlorinated dibenzodioxins was identified in refuse and PVC combustion and industrial refuse disposal in uncontrolled industrial waste disposal.

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

The following reviews show a strong link between PCBs and a weakened immune system:

Immunologic effects of pre- and postnatal polychlorinated biphenyl (PCB)/dioxin exposure in Dutch infants from birth to 18 mo of age are explored. The study show: background levels of PCB/dioxin exposure influences the human fetal and neonatal immune system; increase in the total number of T cells; increase in the number of TcR gamma delta+ T cells; increased in the number of CD8+ (cytotoxic), TcR alpha beta+, and TcR gamma delta+ T cells; lower monocyte and granulocyte counts; no relationship between pre- and postnatal PCB/dioxin exposure and upper or lower respiratory tract symptoms or humoral antibody production; study examined only 4 types of PCBs (118,138,153 and 180) for prenatal tests; study examined only 8 types of dioxin-like PCBs along with 17 types of dioxin for tests after birth (33).

Tsuji, 2000 evaluate chronic immune effects of endocrine disruptors. Serum autoantibodies and immunoglobulin concentrations were studied in patients with Yusho disease. PCBs are linked to long-term immune effects (decades after poisoning); autoantibodies in patients with Yusho PCB poisoning are frequent (which may make them vulnerable to autoimmune disorders); 45.6% for antinuclear antibody, 12.7% for rheumatoid (arthritis) factor and 11.1% for thyroglobulin antibody; serum levels of immunoglobulin A(IgA), immunoglobulin G(IgG) and immunoglobulin M(IgM) were elevated in 12.7%, 24.1% and 8.9% ; thyroglobulinantibody was detected in 19.5% of people with higher PCB exposures; mean absolute density of CD4 positive lymphocytes was higher; exposure to background levels of chlorinated dioxins and related chemicals through breast milk may cause some immunologic disorder; chlorinated dioxins and related chemicals from the breast milk correlated negatively with the percentages of CD8 positive lymphocytes (29).

Increased levels of dioxins and related compounds (PCBs) correlate with negative changes in lymphocyte subpopulations and CD4+/CD8+ biomarkers; dioxin and related compounds may be related to pathology of the immune system, such as atopic dermatitis; exposures occurred at background levels (18).

The immunologic effects of in utero exposure to polychlorinated biphenyls (PCBs)/polychlorinated dibenzofurans (PCDFs) were evaluated in the Yucheng children. The study subjects consisted of 105 Yucheng children and 101 control children. The Yucheng children were born, between July 1978 and June 1987, to women who had exposed to high dose of PCBs/PCDFs through consumption of contaminated rice bran oil in 1978-1979. These children had been reported to have higher frequencies of bronchitis than their controls in the first six months of life, and higher frequencies of respiratory tract and ear infection in a 6-year follow-up. The low resistance of the Yucheng children to infection suggested that their immune function was suppressed by the PCBs/PCDFs they had exposed to in utero. In the summer and fall of 1995, a thorough physical examination and blood draw were performed on the study children. The Yucheng children were reported by their parents to have higher frequencies of influenza attacks than the control children during the six months prior to the examination (34).

Non-Hodgkin's lymphoma risk increases in humans with immune deficiencies or autoimmune disorders; non-Hodgkin's lymphoma has been associated with PCB exposure; non-Hodgkin's lymphoma may result from a combination of chemical immunosuppression and virus infection (17).

Organochlorines have been reported to adversely affect the human immune system, reducing defenses against cancer; organochlorine exposure has been shown to increase risk of non-Hodgkin's lymphoma; immune damage combined with estrogenic qualities (as with PCBs) have been linked to breast cancer. Prenatal organochlorine exposure could be a risk factor for increased ear infections in infants (11).

Polychlorinated biphenyls (PCBs) are among the most widespread environmental pollutants and a prominent contaminant of the Great Lakes basin. Higher incidence of bacterial infections was reported for breast-fed infants born to mothers who consumed large amounts of Great Lakes fish compared to the incidence in control infants whose mothers ingested low amounts of fish (28).

Respiratory involvement and immune status was studied and followed up for 14 years in 401 patients with polychlorinated-biphenyl (PCB) poisoning caused by ingestion of contaminated edible rice-bran-oil. Respiratory symptoms correlated with PCB levels in blood and were often exacerbated by viral or bacterial infection. Immunoglobulin-A and immunoglobulin-M levels were decreased, while immunoglobulin-G level was increased.

Pathologic changes in lungs and thymus were observed, including necrosis of Clara cells, mild pulmonary edema, and thymic atrophy, with similar but milder effects noted for PCBs. The authors conclude that respiratory involvement in patients with PCB and PCDF poisoning is located mainly in small airways and is due primarily to PCDFs. [Note: PCBs and furans are generally found together] (21).

The concentration of serum Ig and the distribution of different lymphocyte subpopulations were studied in peripheral blood samples obtained from 30 human subjects exposed to PCB (polychlorinated biphenyl) and from 23 normal healthy subjects. PCB caused decreased concentrations of IgA and IgM but not that of IgG. By using different rosette techniques to enumerate the percentages of lymphocyte subpopulations, the percentages of total T cells, active T cells and T-mu cells decreased, while the percentages of B cells and T-gamma cells were not affected. Changes of lymphocyte subpopulations may be responsible for the reported immune deficiency associated with PCB exposure (6). Effects of postnatal exposure to polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and coplanar polychlorinated biphenyls (Co-PCBs) on lymphocyte subpopulations were investigated in the peripheral blood of 36 breast-fed Japanese babies. Ratios of CD4+ to CD8+ T cells showed significant increasing tendency correlated with organochlorine exposure (20).

Consumption of fatty fish species, like salmon and herring, from the Baltic Sea is an important source of human exposure to persistent organochlorine compounds, e.g. polychlorinated dioxins (PCDDs), dibenzofurans (PCDFs) and biphenyls (PCBs). The high consumers had lower proportions and numbers of natural killer (NK) cells, identified by the CD 56 marker, in peripheral blood than the non-consumers (27).

*In conclusion* dioxins are widespread environmental pollutants and they have been reported to adversely affect the human immune system. Immunotoxicity is one of the most significant health effects of dioxins.

Reducing dioxin exposure is an important public health goal for disease reduction, also with respect to sustainable development. Good controls and practices during primary production, processing, distribution and sale are all essential to the production of safe food.

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## **BO6. EFFECT OF HEAVY METALS ON SOME PHYSIOLOGICAL PARAMETERS IN TRITICALE LINES**

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### **Aim**

Investigation of different triticale lines to identify their adaptability to copper and identification of copper tolerant genotypes.

Triticale is a hybrid between wheat and rye. It combines the high yield potential and bread production qualities of wheat with the rye's flexibility to climatic conditions and lower requirements for soil type. The efforts of geneticists focus on creating valuable varieties, especially for regions where conditions are not favorable for wheat growing (1).

Copper, as a component of several enzymes involved in catalyzing the oxidation-reduction reactions in the mitochondria and chloroplasts (2) is an essential microelement for plants. However, at concentrations slightly above the optimal levels it causes changes in the physiological processes which result in inhibition of growth (3). An important feature of copper toxicity is the initiation of oxidative stress - formation of reactive oxygen species such as singlet oxygen, hydrogen peroxide and hydroxyl radicals that cause damages in the membranes, the protein molecules and DNA (4).

In our study, the influence of  $\text{Cu}^{2+}$  on three triticale lines, characterized by high productivity - CCO-1, CCO-3 и CCO-PB, and their parent varieties wheat "Mersia-2" and rye "Lozen-14" was investigated. The measurements were carried out on 18-day-old plants grown as water cultures. Copper is applied in two concentrations (5mg/l and 15mg/l) to the nutrient medium. To assess the physiological status of the plants treated with the heavy metal, we investigated the changes of the growth of roots and above ground part, fresh and dry weight, content of free proline, malondialdehyde (MDA), and free thiol groups.

It was found that  $\text{Cu}^{2+}$  caused inhibition of growth of the above ground part and roots most significantly in CCO-1 line, while CCO-PB was less affected. A similar trend was established in respect of the fresh and dry weight. The high concentration of copper led to increase of the proline content in the whole plants, most significantly in wheat, rye, and CCO-1. It was found that copper induced an increase in the content of MDA primarily in the roots of lines CCO-3 and CCO-PB, while in CCO-1 MDA was significantly increased in the aboveground part also. Regarding the content of thiol groups, certain increase due to the treatments was found in CCO-PB line.

The analyzed lines of triticale showed a higher tolerance to copper than the parent varieties. They are suitable for more extensive studies on the mechanisms involved in the heavy metal resistance and for development of new varieties of triticale.

**Key words:** triticale, copper toxicity, oxidative damage

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## BO7. INFLUENCE OF POPOLIS ON SOME PROTOZOAN PARASITES

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### Abstract

Although honey is perhaps the most famous bee product of interest to human beings, bees also make propolis, another substance that humans have used for thousands of years. Propolis (from the Greek "προ" + "πολις" - "to the city", "protection of the City"), also known as clay is a mixture of resin, wax and pollen from flowers and buds of plants enriched with enzymes and subjected to lactic acid fermentation in the digestive system of bees. Propolis contains vitamins, essential oils, mineral salts, trace elements, hormones, and enzymes. Bees gather and bring to the hive and perform with him disinfection activities. It is their "chemical weapons" against infection. Bees coat the hive with propolis in much the same way we use paint and caulking on our homes. Propolis has been used as a popular remedy for several centuries for a wide array of ailments. Its antimicrobial properties, present in propolis from different origins, have been extensively studied. But, more recently, antiparasitic, anti-viral/immune stimulating, healing, anti-tumor, anti-inflammatory, antioxidant and analgesic activities of diverse types of propolis have been evaluated. The color of propolis is yellow, yellow-green, dark green or gray. Was dissolved in water slightly, most preferably dissolved in alcohol, ether, chloroform, acetone.

**Key words:** propolis, immunomodulatory activity, anti-protozoan properties

## **Introduction:**

Propolis is a bee product, composed mainly of plant resins and beeswax, therefore its chemical composition varies due to the geographic and plant origins of these resins, as well as the species of bee [26]. Propolis is a mixture of balsams and resins, waxes, essential oils, pollen, and other substances which is used by bees in the construction, repair and protection of their hives, mainly due to its mechanical properties and antimicrobial activity [27]. The flavonoids in propolis may be responsible for its antimicrobial effects as well as other alleged health benefits. Propolis has been used in folk medicine due to its many biological properties, such as antimicrobial, antiinflammatory, antioxidant, immunomodulatory activities, among others [19]. People began using propolis more than 2,300 years ago for many purposes, the foremost of which was applying it to wounds to fight infection. Nowadays it is still used in traditional and alternative medicine, but also in the modern biocosmetic industry and in health foods [11]. Pesticides, hormones and antibiotics in food are becoming a problem; consumers in different countries demand more and more natural food of better quality. This tendency is worldwide and can be recognized in different productive chains, such as meat, milk, horticulture, fruticulture, etc [22]. Propolis is not toxic when administered to animals and most of the flavonoids are innocuous when added to the human diet; tolerance and absence of toxicity give propolis its medicinal value. Propolis also shows immunomodulatory and antiparasite activities, and researchers have been attracted to its potential for the development of new drugs [1].

## **Propolis and protozoan parasites**

Current therapy of protozoan infections is not effective for the vast majority of animals with relapsing parasitemia and clinical signs. Recently, attention is being focused on the antiparasitic activity of propolis.

Higashi and De Castro [15], have been studied the ethanolic (EEP) and dimethylsulphoxide extracts (DEP) of propolis, for their anti-protozoan properties and found that they have been active against the different forms of the parasite studied. Total lysis of blood stream trypomastigotes has been observed after 24 hr in the presence of EEP at a concentration of 100 µg/ml. The effect has been found to be temperature-dependent. Treatment of infected peritoneal macrophages and heart muscle cells with EEP strongly have been inhibited infection levels. The anti-protozoan properties of different propolis extracts have been studied regarding *Trypanosoma cruzi* and its interaction with host cells. Ethanolic and dimethylsulphoxide extracts have been both active against the three forms of the parasite, compared with the former compounds [14].

The effect of Bulgarian propolis against *Trypanozoma cruzi* has been studied from Dantas et al. [4]. They established that the treatment of *Trypanosoma cruzi*-infected skeletal muscle cells with ethanol (Et-Blg) extract with known chemical compositions led to a decrease of infection and of the intracellular proliferation of amastigotes, while damage to the host cell has been observed only at concentration 12.5 times higher than those affecting the parasite. Ultrastructural analysis of the effect of both extracts in epimastigotes have been revealed that the main targets have been the mitochondrion and reservosomes. Et-Blg also have been affected the mitochondrion-kinetoplast complex in trypomastigotes, has offered a potential target for chemotherapeutic agents. They have established also that the treatment of experimental infected Swiss mice with *Trypanozoma cruzi* with 50 mg Et-Blg/kg body weight/ day has been led to a decrease in parasitemia and showed no hepatic or renal toxic effect. Treatment with Et-Blg has been led to a decrease in the spleen mass and has been modulated the initial inflammatory reaction as have been demonstrated by analysis of the

leukocyte profile in peripheral blood, quantification of T cells subsets, and phenotypic markers in the spleen [5].

Marcucci et al. [17] have been studied the effect of four compounds, which have been isolated from Brazilian propolis against protozoan *Trypanosoma cruzi* and the bacteria *Escherihia colli*, *Pseudomonas aeroginoza*, *Staphilococcus aureus* and *Streptococcus faecalis*. All four compounds, identified as (1) 3-prenyl-4-hydroxycinnamic acid (PHCA), (2) 2,2-dimethyl-6-carboxyethenyl-2H-1-benzopyrane (DCBEN), (3) 3,5-diprenyl-4-hydroxycinnamic acid (DHCA), and (4) 2,2-dimethyl-6-carboxyethenyl-8-prenyl-2H-1-benzopyran (DPB) have been active against *Trypanozoma cruzi*.

The composition of Brazilian green propolis ethanolic extract (Et-Bra) and its effect on *Trypanosoma cruzi* trypomastigotes and other pathogenic microorganisms have been reported from Salomão et al. [25]. They said that in trypomastigotes, Et-Bra has been led to the loss of plasma membrane integrity. The *in vitro* studies indicate that Et-Bra interferes in the functionality of the plasma membrane in trypomastigotes and of reservosomes and mitochondrion in epimastigotes. Acutely infected mice have been treated orally with Et-Bra and the parasitemia, mortality and GPT, GOT, CK and urea levels have been monitored. According to Salomão et al. [25] the extract (25–300 mg kg<sup>-1</sup> body weight/day for 10 days) reduced the parasitemia, although not at significant levels; increased the survival of the animals and did not induce any hepatic, muscular lesion or renal toxicity and Et-Bra could be a potential metacyclogenesis blocker, considering its effect on reservosomes, which are an important energy source during parasite differentiation.

Salomão et al. [24] have compared the activity between the composition of Bulgarian (Et-Blg) and Brazilian (Et-Bra) ethanolic extracts and have established that in spite of the striking differences, both of them have been active against trypomastigotes of *T. cruzi* and induced in epimastigotes intracellular disorganization.

Da Silva et al. [8] have been investigated the activity of the 15 extracts from different samples of Brazilian propolis against bloodstream trypomastigotes of *Trypanosoma cruzi*, the etiologic agent of Chagas' disease, which is endemic in Latin America with about 16 million of people infected [32]. They concluded that the biological activity of the sample of the extract depends on the extraction method employed. Falcão et al. [10] have been studied that the propolis extracts presenting the relatively highest inhibitory effect against *Trypanosoma brucei*.

Gressler et al., [13] have been evaluated the susceptibility of *T. evansi* to propolis extract *in vitro* and *in vivo*. A dose-dependent trypanocidal activity of propolis extract has been observed *in vitro*. All trypomastigotes have been killed 1 h after incubation with 10 µg mL<sup>-1</sup> of the extract. *In vivo*, the concentrations of 100, 200, 300 and 400 mg kg<sup>-1</sup> administered orally for 10 consecutive days have been showed no curative effect, and the rats have been died from the disease. However, rats treated with the two highest concentrations of propolis extract have been showed higher longevity than the other groups. Based on these data, they have been concluded that *T. evansi* is susceptible to propolis *in vitro*. Despite the lack of curative efficacy observed *in vivo* at the concentrations tested, the propolis extract can prolong life in rats infected with the protozoan.

Leishmania is an intracellular tissue parasite which belongs to the order Kinetoplastida and the family *Trypanosomatidae*. Leishmanias leads to clinical views in visceral, cutaneous and mucocutaneous forms [21].

Ayres et. al. [3] have been investigated the effect of four ethanolic extracts of typified propolis collected in different Brazilian states, on *Leishmania amazonensis* performing assays with promastigote forms, extracellular amastigotes, and on infected peritoneal macrophages. Ethanolic extracts of all propolis samples (BRG, BRPG, BRP-1, and BRV) have been capable to reduce parasite load as monitored by the percentage of infected macrophages and the

number of intracellular parasites. BRV sample which is named *red propolis*, collected in the state of Alagoas, and containing high concentration of prenylated and benzophenones compounds, has been the most active extract against *L. amazonensis*. The anti-Leishmania effect of BRV sample have been increased in a concentration and time dependent manner. They said that the BRV treatment have been non-toxic to macrophage cultures. BRV extract at the concentration of 25 µg/ml has been reduced the parasite load of macrophages while the direct toxicity to promastigotes and extracellular amastigotes has not been found. It has been suggested that constituents of propolis effect on the mechanism of macrophage leads to killing of *L. amazonensis*. Their results have been demonstrated, that the ethanolic extracts of Brazilian propolis reduce *L. amazonensis* infection in macrophages, and encourage further studies of this natural compound in animal models of leishmaniasis.

Extracts of propolis samples collected in Brazil and Bulgaria have been assayed against four Leishmania species - *Leishmania amazonensis*, *L. braziliensis*, *L. chagasi* and *L. major* [16]. They concluded that the overall analysis have been shown that for all the species evaluated, Bulgarian extracts has been more active than the ethanol Brazilian extract. As the assayed propolis extracts with their chemical composition determined further investigations are necessary to clarify the effect of individual components or their combinations on each *Leishmania* species.

Duran et al., [7] have been investigated the *in vitro* activity of Adana propolis sample on *Leishmania tropica*. They have been treated parasite cells with five concentrations of the propolis and they have been found that the concentrations up to 100 µg/ml of the propolis did not exhibit antileishmanial activity against the parasites cells. However, in culture media containing the propolis samples at 250, 500, and 750-µg/ml concentrations, statistically significant differences in cell counts have been observed, compared with the control group ( $p < 0.05$ ). Their results demonstrate that ethanolic extracts of Adana propolis samples reduced the proliferation of *L. tropica* parasites significantly.

In the other study Ozbilge et al. [20] have been investigated the activity of ethanolic extract of propolis from Kayseri on *Leishmania tropica* in concentrations varying from 0.0625 µg/ml to 1024 µg/ml. According to their study the inhibited effect of propolis on the parasites begins at 32 µg/ml concentration and has been increased with increasing concentration and incubation period.

The antileishmanial and immunomodulatory effects of propolis collected in Botucatu, Sao Paulo State, Brazil, have been evaluated in *Leishmania (Viannia) braziliensis* experimental infection from da Silva et al. [9]. They have said that the Brazilian propolis has direct action on the parasite and displayed immunomodulatory effects on murine macrophages. The effects observed could be associated with the presence of phenolic compounds (flavonoids, aromatic acids, and benzopyranes), di- and triterpenes, and essential oils found in propolis sample. Pontin et al. [23] have reported for first time about the *in vivo* antileishmanial activity for Brazilian green propolis and their results indicate that BPE shows both *in vitro* and *in vivo* antileishmanial activity against *L. braziliensis*.

Monzote et al. [18] have been analyzed the antiprotozoal effects of eighteen Cuban propolis extracts (brown, red and yellow type) collected in different geographic areas, using *Leishmania amazonensis* (as a model of intracellular protozoa) and *Trichomonas vaginalis* (as a model of extracellular protozoa). All evaluated propolis extracts have been caused inhibitory effect on intracellular amastigotes of *L. amazonensis*. However, cytotoxicity on peritoneal macrophages from BALB/c mice has been observed. Only five samples have been decreased the viability of *T. vaginalis* trophozoites at concentrations lower than 10 microg/mL. No correlation between the type of propolis and antiprotozoal activity has not been found. Cuban propolis extracts have demonstrated activity against both intracellular and extracellular protozoa model.

The propolis as a natural source of compounds against protozoan investigated has potentiality to obtain new antiprotozoal agents. According to Xu et al. [33] propolis possesses clear *in vitro* anti-trichomonas activity which is relevant to the duration of culture and the concentration of the agent. Starzyk et al. [28] have been found that the propolis extract at certain concentrations has been lethal to *Trichomonas vaginalis*, when applied clinically. It has been killed all active forms of the parasite within 24 hrs.

Syamsudin and Kusmardi [29,30] have been investigated the immunomodulatory and *in vivo* antiplasmodial activities of Indonesian propolis extracts. Their research results have been revealed that Propolis Hydroalcoholic Solution (PHS) has a strong immunomodulatory activity but weak antiplasmodial activity and they have been concluded that PHS have been shown more immunostimulant activity than antiplasmodial activity, proved by the increase of IgG and the macrophage phagocytosis activity and capacity. The antiplasmodial activity of PHS is due to increasing of the mice immunity so that they lived longer.

The effect of an alcoholic extract of propolis (56.15 mg/ml) on the *in vitro* growth of the protozoan parasite *Giardia lamblia* has been evaluated from Torres [31]. The results have been showed growth inhibition of more than 40% with all 3 concentrations tested (3.0, 5.8 and 11.6 µg/ml). The highest concentration leads to 98% inhibition. Propolis extract at 5.8 µg/ml has been destroyed the outer layer of the parasites and rounded forms appeared in the culture. Freitas et al. [12] have been investigated the *in vitro* effects of propolis on *Giardia duodenalis* trophozoites and have been found that the propolis inhibits the growth of trophozoites and the level of inhibition varies according to the extract concentration and incubation times. This dependence has been observed [2] in studying the effect of propolis on *Entamoeba histolytica* (trophozoites) *in vitro*. More comprehensive studies of the effects of propolis on *Giardia duodenalis* have been found necessary [6].

Yin et al. [34] have been investigated the cellular immune responses induced by intranasal immunization with soluble antigen tachyzoites of *Toxoplasma gondii* plus IFN- $\gamma$  and propolis in mice. They have been found that the propolis can be applied to mucosal adjuvant for intranasal immunization against *Toxoplasma gondii*.

## Conclusions:

Propolis is a resinous compound made primarily from tree sap, and contains biologically active compounds called flavonoids, which come from its plant source. Propolis has antiseptic properties. Propolis is a wax-like resin produced by honeybees from substances collected from plants, which are mixed with beeswax and other compounds of bee metabolism. Its chemical composition depends on the specific local flora at the site of collection and also of climatic characteristics, resulting in a striking diversity of constituents. Honey bees harvest resins with antimicrobial properties from various plant species and bring them back to the colony where they are then mixed with varying amounts of wax and utilized as propolis. It shows much attractive substance applied in medicine due to its pharmacological activities. Recently numerous investigations prove that propolis is a promising source of natural compounds for the development of new chemotherapeutic agents for treatment of protozoan diseases. Moreover, further biological and phytochemical investigations have to be pointed to the identification of the active compounds of propolis against protozoan parasites.

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## **BO8. CONCENTRATIONS OF SELENIUM AND SIALIC ACIDS IN SERUM FROM RATS INFECTED WITH *TRICHINELLA SPIRALIS***

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Sialic acid is a general term for a family of unique 9-carbon monosaccharides. In eucaryotic cells, Neu5Ac (sialic acid) occurs essentially as terminal sugar in  $\alpha$  2.3 and  $\alpha$  2.6 linkages onto terminal Gal residues of N-glycans of the N-acetyllactosamine type. As a result of their location, and their negative carboxylate functionality, the sialic acids play important roles in a variety of biological processes like mediating cellular recognition, cell-substrate interaction, maintenance of serum glycoproteins in circulation and protein targeting. More than 40 different sialic acid compounds have been identified with various biological functions. Sialic acids are found in many body fluids, such as serum, urine, breast milk, saliva, semen, cerebrospinal fluid and pleural effusion. Their concentrations usually change during diseases such as neoplastic tumors, myocardial infarction, inflammatory disorders and diabetes mellitus.

Selenium is an essential trace element for the living organism. It is incorporated as selenocysteine at the active site of a wide range of proteins to make selenoproteins which are important antioxidant enzymes such as glutathione peroxidase, gastrointestinal glutathione peroxidase and other peroxidases. They act as a major antioxidant to mitigate the cytotoxic effects of reactive oxygen species. Thioredoxin reductase is a recently identified as a selenocysteine containing enzyme which catalyzes the NADPH dependent reduction of thioredoxin and therefore plays a regulatory role in its metabolic activity. About 20 – 25 different selenoproteins are identified and characterized. Selenium has been suggested to play a role in the prevention of certain forms of cancer, viral infection, and immune system function. Only few data exist about the effect of selenium on helminthic diseases.

Trichinellosis is a disease, caused by parasitic nematodes, which belong to the *Trichinella* genus. They infest a broad range of mammals, birds and reptiles. The life cycle of *Trichinella* comprises an enteric and skeletal muscle stage within the host. The muscle phase is associated with disruption of skeletal muscle fibers, enlargement and centralization of host muscle nuclei and inflammation around occupied sarcoplasm areas.

We studied the content of free, lipid, and protein bond sialic acid in serum of rats infested with *Trichinella spiralis* and after treatment with a commercial preparation containing selenium. Our results showed that the serum sialic acid concentration in the *T. spiralis* infected rats were significantly higher (at week 8) compared to healthy animals. A good correlation was found between the sialic acid level and selenium concentration. Treatment of animals with Sel-plex significantly reduced the levels of serum sialic acid. These results are in good agreement with the data of other authors who have found that the level of serum sialic acid is significant lower in lambs with white muscle disease, after treatment with selenium.

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## **BO9. STUDY ON THE EFFECT OF DIVALENT METAL IONS ON THE ACTIVITY OF AMINOPEPTIDASE A**

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### **Abstract**

Aminopeptidase A (APA) is a zinc-dependent metalloproteinase of M1 family. It catalyzes the cleavage of glutamic and aspartic amino acids from the N-terminus of polypeptides. The enzyme is activated by Ca<sup>2+</sup> and inhibited by chelating agents and heavy metals. In the present study, the effects of alkaline earth metals and lead on the APA activity is evaluated in cryostat sections of mouse kidney and small intestine using the fluorogenic

histochemical substrate 4-( $\alpha$ -glutamylhydrazido)-N-hexyl-1,8-naphthalimide. The results show that all the studied alkaline earth metals activate mouse APA. The enzyme activity decreases in the order  $\text{Ca}^{2+} \gg \text{Ba}^{2+} \geq \text{Sr}^{2+}$  to show that the activation potential of alkaline earth metals does not correlate with the ionic radius. The lack of such correlation is illustrated also by the fact that  $\text{Sr}^{2+}$  is enzyme activator whereas  $\text{Pb}^{2+}$ , which has the same ionic radius, is a powerful inhibitor of APA.

## Introduction

Aminopeptidase A (Glutamyl aminopeptidase, APA; EC 3.4.11.7) is a zinc-dependent membrane-bound aminopeptidase (family M1) that catalyzes the cleavage of glutamic and aspartic amino acid residues from the N-terminus of polypeptides. The enzyme degrades vasoconstricting angiotensin II into angiotensin III and therefore helps to regulate blood pressure [4]. Moreover, APA was shown to be over-expressed in several types of malignant tumours, probably by inducing the formation of as yet unidentified cell proliferating active peptides [1]. The enzyme also is a regulator of blood vessel formation, and is a putative target for angiogenesis in cancer. In mammals and humans high enzyme activity was detected in kidneys, pancreas, small intestine, male and female reproductive organs as well as in the capillaries [2,6]. The enzyme is activated by  $\text{Ca}^{2+}$ . It is inhibited by chelating agents such as EDTA, EGTA and 1,10-phenanthroline [5] and completely inhibited by transitional metal ions -  $\text{Zn}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Hg}^{2+}$  and  $\text{Cd}^{2+}$  at 1 mM [3]. The hydrolysis of  $\alpha$ -L-glutamyl- $\beta$ -naphthylamide from rat APA was markedly enhanced in the presence of  $\text{Ba}^{2+}$ ,  $\text{Ca}^{2+}$  and  $\text{Sr}^{2+}$  [7]. The effects of alkaline earth metals on the APA activity were studied using the enzyme purified from different sources. However, under these conditions, the enzyme is not associated with the cell membrane, i.e. it is not in its natural environment.

The aim of the present study is to evaluate the effects of alkaline earth metals and lead on the APA activity in cryostat sections of mouse kidney and small intestine using the enzyme histochemistry.

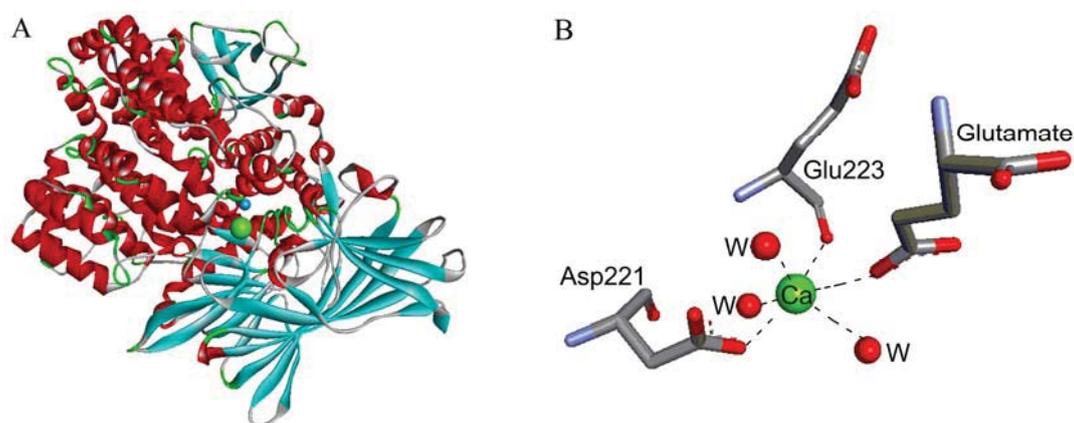
## Materials and methods

Mature BalbC mice of both sexes were decapitated in deep anesthesia. Pieces of kidney and jejunum were extracted and frozen in liquid nitrogen. Ten  $\mu\text{m}$  thin sections were cut on cryotome Reichert-Jung (Germany) at  $-26^\circ\text{C}$ . Before the enzyme reaction all the sections were covered by celloidin (1 % celloidin in absolute ethanol/diethyl ether/acetone 3:3:4) for a minute at room temperature. The sections were incubated in a substrate medium consisting of 0.5 mM substrate 4-( $\alpha$ -glutamylhydrazido)-N-hexyl-1,8-naphthalimide ( $\alpha$ -Glu-HHNI) and 0.5 mg/ml piperonal in 0.1 M cacodylate/HCl buffer, pH 7.4 for 120 min at  $37^\circ\text{C}$ . The effects of metals on APA activity were studied in the same substrate media but supplied with 1mM of the respective metal ion. Then, the sections were post-fixed in neutral formaline for 15 min at room temperature, stained with haematoxyline consistent with classical methods of histology and embedded in glycerol/jelly. All the sections were studied under the microscope Leica DM5000B (New York, USA).

## Results and discussion

APA is a zinc-dependent aminopeptidase of M1 family, the only member of the family, which activity is modulated by calcium ions. The crystal structure of the human enzyme (Fig. 1A) shows that the Ca-binding site is in the S1 pocket and  $\text{Ca}^{2+}$  is situated in the immediate vicinity to the catalytic  $\text{Zn}^{2+}$  [8]. The calcium ion coordinates with Asp221, Glu223,

glutamate and three molecules of water (Fig. 1B). The binding of calcium leads to a change in substrate specificity of APA. In the presence of calcium, the binding of acidic residues is enhanced as they ligate with the cation, whereas the binding of basic residues is no longer favorable [8]. The enzyme isolated from rat prostate hydrolyses  $\alpha$ -L-glutamyl- $\beta$ -naphthylamide more effectively in the presence of  $\text{Ca}^{2+}$ ,  $\text{Sr}^{2+}$  and  $\text{Ba}^{2+}$  [7].



**Figure 1.** Structure of Human Aminopeptidase A (Protein Data Bank code 4FKE). **A.** Enzyme shown with solid ribbon,  $\text{Zn}^{2+}$  as a blue sphere and  $\text{Ca}^{2+}$  as a green sphere. **B.** Calcium-binding site in the S1 pocket of glutamate-bound APA. The calcium ion coordinates with Asp221, Glu223, glutamate and three molecules of water.

We evaluated the effects of alkaline earth metals on the APA activity in cryostat sections of mouse kidney and small intestine using the enzyme histochemistry. Lead was chosen to assess the effects of heavy metals on the activity of the enzyme. No data were found about its effect on APA activity. Moreover, the charge of the lead's ion is the same as that of the alkaline earth metals, its ionic radius is equal to that of  $\text{Sr}^{2+}$  (Table 1). Lead ions have a higher preference to form complexes with oxygen donor ligands than with nitrogen donor ligands, such as alkaline earth metals.

**Table 1.** Ionic radii for coordination number 6 ( $\text{\AA}$ )

Metal ion			
$\text{Ca}^{2+}$	$\text{Sr}^{2+}$	$\text{Ba}^{2+}$	$\text{Pb}^{2+}$
1.00	1.18	1.35	1.19

In the present study, the effects of metal ions on APA activity were evaluated using the histochemical substrate  $\alpha$ -Glu-HHNI in cryostat sections of mouse kidney (Fig. 2) and small intestine (Fig. 3). In the lack of metal ions, the enzyme activity proved to be low in the kidney and almost missing in the jejunum. In both organs,  $\text{Ca}^{2+}$  strongly activated the enzyme whereas  $\text{Pb}^{2+}$  completely inhibited it. The other alkaline earth metals' ions also activated APA but to a lesser extent in comparison to the calcium ions. In the presence of  $\text{Ba}^{2+}$  the enzyme reaction in the small intestine was stronger than in the presence of  $\text{Sr}^{2+}$  (Fig. 2). Such differences were not detected in the kidney (Fig. 3).

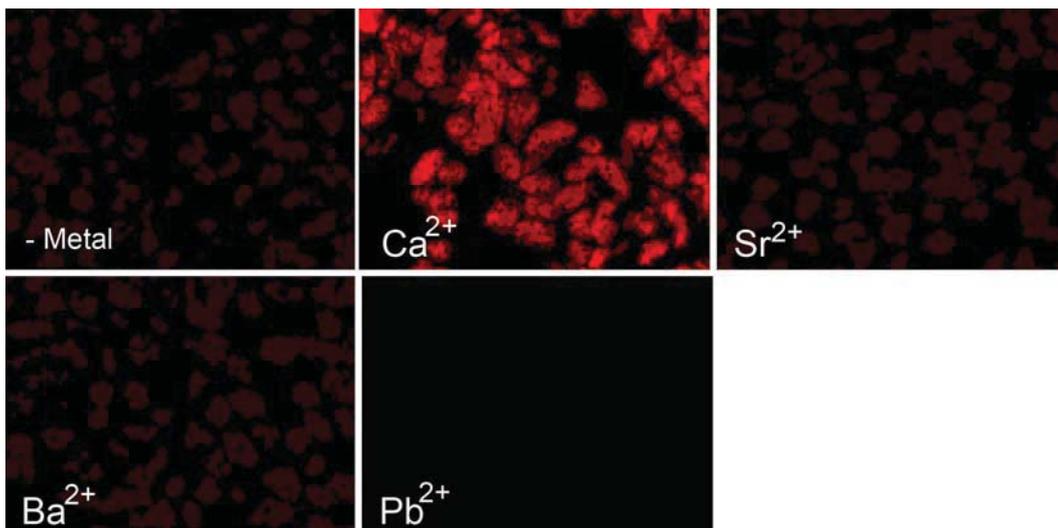


Fig. 2. APA activity in mouse kidney. Magn. 200x

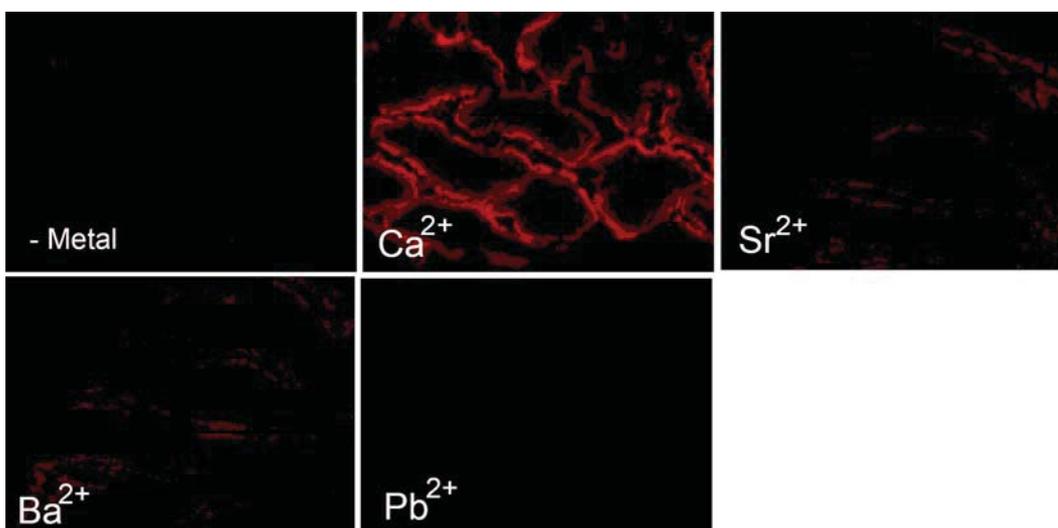


Fig. 3. APA activity in mouse jejunum. Magn. 200x

In conclusion, our results show that all the studied alkaline earth metals activate mouse APA from kidney and small intestine. The enzyme activity decreases in the order  $\text{Ca}^{2+} \gg \text{Ba}^{2+} \geq \text{Sr}^{2+}$  to show that the activation potential of alkaline earth metals does not correlate with the ionic radius. The lack of such correlation is illustrated also by the fact that  $\text{Sr}^{2+}$  is enzyme activator whereas  $\text{Pb}^{2+}$ , which has the same ionic radius, is a powerful inhibitor of APA.

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## **ВО10. НОВИ ПОДХОДИ В ТЕРАПИЯТА НА НЯКОИ ПАРАЗИТНИ ЗАБОЛЯВАНИЯ С МЕТАЛ-СЪДЪРЖАЩИ ПРЕПАРАТИ**

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Паразитните заболявания представляват сериозен здравен проблем в целия свят. По данни на СЗО около 4,5 милиарда души в света са носители на поне един паразит. За съжаление и в наши дни голяма част от страдащите от тези болести умират. Поради ограничения брой ефективни антипаразитни средства и честата поява на химиорезистентност се налага спешна необходимост от разработване на нови лекарства срещу тези заболявания. Напоследък се наблюдава подчертан интерес към използването на метални комплекси в лечението на тропически болести като трипанозомоза, малария и лайшманиоза, въпреки факта, че те са считани за едни от шестте заболявания, определени като основни световни здравни проблеми. Два са благоприятните ефекта при свързването на металния йон с органичните лекарства. На първо място повишаване на биологичната активност на органичните лекарства, причинени от комплексообразуване с метален йон, което вероятно се дължи на по-

дългото време на пребиваване на лекарството в организма. Вторият ефект е намаляване на токсичността на металения йон, което се дължи на факта, че комплексообразуването с органични лекарства прави металения йон по-малко на разположение за нежелана реакция като инхибиране на ензими. Свързването на металния йон с органичните лекарства води и до други биологични ефекти. Чрез вмъкването на метал в структурата на установени антипаразитни лекарства води до засилване на фармакологичното му действие. Металните комплекси в класическите антипаразитни лекарства променят техните фармакокинетични параметри.

**Ключови думи:** метални комплекси, терапия, *Trypanosoma*, *Plasmodium*, *Leishmania*

## **ВО10. НОВИ ПОДХОДИ В ТЕРАПИЯТА НА НЯКОИ ПАРАЗИТНИ ЗАБОЛЯВАНИЯ С МЕТАЛ-СЪДЪРЖАЩИ ПРЕПАРАТИ**

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## **ВР1. НАКРАТКО ЗА ДЕНДРИМЕРИТЕ И НАНОМЕДИЦИНАТА**

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## **BP2. HEAVY METALS IN THE PARASITE-HOST SYSTEM IN TERRESTRIAL ANIMALS**

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### **Abstract**

Metals are natural constituents of the abiotic or biotic components of all ecosystems, and under natural conditions they are cycled within and between the geochemical spheres. Human activities have altered natural concentrations of many substances in the environment and added numerous new chemicals. In general there is a relationship between metal concentrations among several species. Bioaccumulation capacity has been well documented for some groups of organisms, as well as for some metals. Parasites are ubiquitous. There is a relationship between concentrations of heavy metals in the parasite and those in the target tissues of their host. Some helminthes can selectively accumulate certain metals, and others – cannot, unlike their host. Several studies were done in the host (terrestrial animals) - parasite systems and demonstrated their possibility to act as bioindicators for heavy metal pollution in the environment. Analysis of such situations can lead to the use of the species to provide evidence of the burden of pollution of ecosystems. As cestodes and nematodes are more abundant in terrestrial mammals than acanthocephalans and thus potentially more useful in attempts toward passive as well as active biomonitoring several host-parasite systems were studied in mammalians. Cestodes and acanthocephalans are able to effectively accumulate heavy metals and are considered as the most promising groups of potential indicators of heavy metal pollution in terrestrial habitats.

**Key words:** host-parasite system, heavy metals.

### **Introduction**

Metals are natural constituents of the abiotic or biotic components of all ecosystems, and under natural conditions they are cycled within and between the geochemical spheres. Human activities have altered natural concentrations of many substances in the environment and added numerous new chemicals. In general there are was a relationship between metal concentrations among several species. Food chain effects, or bioaccumulation within successive trophic levels, have been well documented for some groups of organisms, as well as for some metals. By following the cycles of trace elements in the environment, it is possible to demonstrate the existence of species particularly sensitive to certain elements.

Analysis of such situations can lead to the use of the species to provide evidence of the burden of pollution of ecosystems.

There is information on metal concentrations in various animals as indicators of environmental pollution [10]. Parasites are ubiquitous. They occur in virtually all food webs

at all trophic levels. In general there is a relationship between concentrations of heavy metals in the parasite and those in the target tissues of their host. This relationship was most obvious for the nonessential heavy metals (Cd and Pb). The results indicated that there do not exist qualitative differences in the trace element composition in the studied host-parasite systems, while their quantity varies within one and the same limits, with a certain shifts due to the parasite species [11]. Obviously the biogenic trace elements take part in the regulation of the helminth metabolism as in the case with the mammals.

Certain organisms are able to provide information about the chemical state of their environment through their presence or absence. Others are less affected by toxic substances but show an ability to concentrate environmental pollutants inside their tissues. Thus far, studies have mainly focused on the use of parasites as accumulation indicators of heavy metals, less frequently, investigations have dealt with organic pollutants. This is obviously related to different accumulation patterns of hydrophilic and lipophilic substances. Lipophilic chemicals mainly accumulate in fats and therefore become biomagnified along food webs, whereas hydrophilic substances are distributed more evenly among tissues. Parasites having a low percentage of fats, are not able to bioconcentrate lipophilic substances above the levels of the host tissues. Even if parasites do not accumulate organic pollutants, they are able to alter the uptake of chemicals of their hosts, including metals.

Some helminths can selectively accumulate certain metals, and others – cannot, unlike their host. It is possible that the host organism performs a barrier function to the access of some elements to the parasites when there is an increased concentration of that in the environment. The other possibility is that the helminths are adapted to significant deviations of metal levels in the hosts animals. Some helminth species possess a negative gradient of accumulation to some metals, and a positive gradient to others.

The place of the parasites in the food chain is not studied enough, either. It recently became clear that acanthocephalans parasitizing fishes can bioconcentrate several metals to conspicuously higher concentrations than the tissues of their definitive hosts [12]. Information on parasites of terrestrial vertebrates as sentinels for heavy metal environmental pollution and the benefit to their host is scarce. As cestodes and nematodes are more abundant in terrestrial mammals than acanthocephalans and thus potentially more useful in attempts toward passive as well as active biomonitoring several host-parasite systems were studied in mammalians. Little is known about the interrelation between different elements in the parasitized host. Cu, Zn, Mn are elements of physiological importance for most animals, it is possible that competition among parasites for these elements may lead to increased absorption of other, non-essential or toxic elements, such as Cd and Pb, and the host can be detoxified.

### **Interactions between metals and helminths.**

Sures et al. proposed a cestode/rodent model successfully evaluated for urban areas (*Hymenolepis diminuta*/*Ratus norvegicus*) [11, 12]. Concentration of Pb was determined in *Hymenolepis diminuta* and in its host *Ratus norvegicus* in comparison. Rats were sampled in 2 sites with different pollution. The host-parasite system rat-*H. diminuta* appears to be a promising and a useful bioindicator at least for Pb in urban ecosystems as rats as well as the tapeworm are globally and easily accessible.

Torres et al. studied the systems *Gallegoides arfaai*/*Apodemus sylvaticus* and *Skrjabinotaenia lobata* / *Apodemus sylvaticus* [14, 15]. Torres et al. evaluated the parasite model constituted by the wood mouse *Apodemus sylvaticus* and an intestinal cestode *Skrjabinotaenia lobata* as a potential bioindicator of Pb and Cd in the urban dumping site and in the references[15]. The proposed model seems to be promising bioindication to evaluate environmental Pb exposure in terrestrial habitats. Torres et al. assessed the concentration of

Pb and Cd in the model *Apodemus sylvaticus* and *Gallegoides arfaai* [14]. They proposed the model as a promising bioindication system to evaluate Pb exposure in terrestrial habitat especially for non-urban areas.

Eira et al. verified the inadequacy of cestode/lagomorph model (*Mosgovoyja ctenoides* (Cestoda) /*Oryctolagus cunicula*) as a bioindicator system [3]. They determined some toxic metals (Cd, Pb, Hg and As). The most significant relationship for Pb and As were detected between elements concentration in the cestode and in host muscle. The results did not confirm the role of the model (*Mosgovoyja ctenoides* (Cestoda) /*Oryctolagus cunicula*) as a promising bioindicator system.

Jankovska et al. studied the heavy metals in tissues in small terrestrial rodents [6]. The systems of field voles (*Microtus agrestis*) and field mice (*Apodemus flavicolis*) and their helminthes *Mastophorus muris* (Nematoda) and *Paranoplocephala* (Cestoda) were investigated for heavy metals. The content of heavy metals in the hosts was decreased with the increasing abundance of cestodes [6].

Species response models were demonstrated that sheep tapeworm *Moniezia expansa* accumulate much higher lead levels than in the host tissues [8]. Thus *M. expansa* might be used as an accumulation indicator for heavy metals in terrestrial biotopes especially as it is a very abundant parasite of sheep and cattle.

Influence of parasitism on trace element contents in tissues of red fox (*Vulpes vulpes*) and its parasites *Mesocestoides spp.* (Cestoda) and *Toxascaris leonine* (Nematoda) was studied by [7] in fox and its parasite (*Vulpes vulpes*) /*Mesocestoides spp.*. They could be a promising bioindication system serving as a complement to the rodent models. The herbivorous species may be more susceptible to Pb and other heavy metals pollution than their predators. High metals accumulation in worms affected the metal levels in the tissues of a definite host. The pollutants negatively affected chemical conditions in pedosphere. The exposed ecosystems are also affected by other pollution components. There are two points of view according host-parasite systems: 1. Using of parasites of carnivore as sentinels in the monitoring of environment pollution and 2. Using of some hosts tissues as biomonitors [8]

Anisimova et al. studied the trace elements levels in the system mouflon-endohelminths from two regions with different pollution [1]. The detected elements were Co, Cu, Mn, Fe, Zn, Mo, Se, Cd, Pb. Bioaccumulation of Zn, Cd and Pb was established in the systems from the both regions. The higher concentration of those elements was shown in the system from the more polluted region. It reflected the anthropogenic pollution.

Gabrashanska et al. assessed the heavy metal accumulation in the host-parasite system of hare (*Lepus europeaus*) - *Passalurus ambiguus* (Nematoda) in two sites with different pollution in Bulgaria [4]. Zinc, iron, copper, manganese, lead and cadmium were determined in kidney and in liver as well as in *P. ambiguus*. Hares and their helminths showed similar qualitative but different quantitative metal status. There were some differences in the studied systems from both sites. Zn, Fe, Mn, Cd and Pb levels in the liver and kidney of parasite-infected hares were lower in comparison to uninfected ones mainly captured in polluted site. Zn concentration was similar in the liver tissues and in the nematode in the system from less polluted site. Fe concentration was lower in the infected host v/s uninfected ones and v/s the nematode from the both sites. Cu level was higher in the nematode than in the host tissues of hares from the same site. Mn concentration was lower in the parasite than in the host tissues from both sites and was similar in the infected and uninfected hares. The level of Pb was similar in the host tissues and in the parasite. The level of Cd was higher in the nematode than in the host from the less polluted site. Cd level in the kidney of infected hares from the same site was lower v/s uninfected. Zn level was reduced in the tissues of infected hares v/s uninfected ones from polluted site. It was lower in the nematode than in the host. Fe concentration was lower in the infected host as well as in *P. ambiguus* in comparison to

uninfected hosts. Cu was higher in the nematode than in the host. The concentration of Mn was two times higher in the nematodes v/s that in the kidney of hares found in polluted site. Cd level was reduced in liver from the infected in comparison with uninfected hares in the same system. Mn level was higher in the nematode than that in the infected host. According to our results, the nematode *P. ambiguous* presented more Mn, Cu, Pb and Cd compared to those detected in the kidney and liver of the hares from polluted region. The host-parasite system from this industrial region reflects the higher concentrations of some metals.

The impact of the helminth burden on bioaccumulation of heavy metals in the livers and kidneys as the target organs of hares was expressed in our study concerning mainly polluted region. The tissues of hares captured in industrial emission affects area showed higher levels of Mn, Cd and Pb in comparison from less polluted region (reference site). Comparison of heavy metals content in the organs of hares uninfected and infected with nematodes, respectively, showed lower concentration of Fe, Mn, Cd and Pb in hares with nematodes from polluted region..

Gabrashanska et al. assessed the accumulation of heavy metals (Cd, Pb,Co, Zn,Hg andCu) in the same host -hares (*Lepus europeaus*) and their helminths (*Mosgovayia pectinata*, Cestoda) and *Trichostrngylus retortaeformis* , Nematoda) from industrial polluted field conditions [5]. Host infected with cestodes showed lower Pb and Cd concentrations than hosts infected with nematodes. Levels of Co, Zn and Cu were similar in the hosts infected with cestodes or nematodes. The content of heavy metals in hosts was decreasing with increasing of cestode burden.

Lafferty suggested that if the use of parasites as indicators of pollution can be justified the host species must be abundant and easily accessible, and the species of parasites, despite their over dispersed distribution, must show a high prevalence and abundance in the host population [9]. Parasites should be also easily removed and counted, and details of the ecology and biology of both host and parasites should be available.

The results demonstrate the parasite suitability as bioindicators of environmental pollution and suggest that they are sharing more burden in their soft tissues as well as persistent in contaminated environment therefore act as a bioremediator for host (removing heavy metals) and help in the survival of host with toxins. Parasites reduce environmental stress in host through bioremediation by concentrating the metals in their soft body tissues and minimizing the site disturbances within the host body is comparable to conventional clean up technologies [2].

In most cases, when monitoring large scale environmental pollution the intestinal tracts of collected animals are usually not investigated parasitologically. However, parasites can interfere with established bioindication procedures owing to their effects on the physiology and behavior of the host. This is the reason why it is necessary to select as a biomarker tissue from animals that do not have parasitic helminthes in their intestines. It is clear that we are still lacking considerable amounts of information on the effects of parasite on common bioindication, procedures such as the analysis of biomarkers, and the use of parasites as indicators. We need much more knowledge about the basic physiological effects that parasites have on their hosts at the time of infection, development, and reproduction. More information is required to establish certain host-parasite assemblages, as effect indication system. The need for sentinels in terrestrial habitats should encourage scientists to intensify research in this interdisciplinary area.

## **Conclusion.**

The influence anthropogenic and migration process expressed in the wildlife, makes it possible for some metals to be accumulated in the parasites and in their hosts. The

investigation showed that not only the influence of the environment on the metals migration in the chain environment-host (animals)-parasite can be seen but also their relative stability in the mineral status of host-parasite system. It be concluded that metals levels in the host (animal) – endohelminth system is a direct reflection of that in the geochemical medium and this system is a sensitive and specific zoomonitor for metal pollution. The migration of elements in the host-parasite system showed their place in the biogeochemical chain, The data indicated the influence of the geochemical medium on one side and the other – the relative stability in the mineral status, which is formed as a result of relationship parasite-host (medium from the first order) and host (medium from second). The similar mineral status of the parasites and their hosts show that they are an integrated, dynamic system, whose evaluation is headed towards establishment of more narrow relations between them. This proximity arises due to the mutual adaptation of parasites and their hosts on one side and on the other – due to adaptation to the external geochemical medium. The system a host-parasite system could be considered an integral part of the biosphere and presents an united ecosystem.

### **Future research.**

There are promising three directions for future research in the field of parasitology and ecotoxicology [13].

Parasite as sinks for pollutants within their hosts: Some parasites are able to reduce pollutant levels in the tissues of their hosts.

Parasites as diagnostic tools to test bioavailability of substances.

Changes of biomarker responses of the host against pollutants. Parasites can alter physiological reactions of their hosts against pollutants in different ways

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## **BP3. ТИТАНЪТ В ОРТОПЕДИЯТА**

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## **BP4. CALCIUM PHOSPHATES AS BIOMATERIALS FOR BONE IMPLANTS**

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**CO1. COMPLEX ASSESSMENT OF GRAIN QUALITY FUSING  
DATA FROM IMAGE AND SPECTRA ANALYSES**

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**Abstract:**

The paper presents the approaches, methods and tools for assessment of main quality features of grain samples which are based on color image and spectra analyses. Visible features like grain color, shape, and dimensions are extracted from the object images. Information about object color and surface texture is obtained from the object spectral

characteristics. The categorization of the grain sample elements in three quality groups is accomplished using two data fusion approaches. The first approach is based on the fusion of the results about object color and shape characteristics obtained using image analysis only. The second approach fuses the shape data obtained by image analysis and the color and surface texture data obtained by spectra analysis. The results obtained by the two data fusion approaches are compared.

**Keywords:** grain sample quality assessment, color image analysis, spectra analysis, classification, data fusion

## INTRODUCTION

One of the main factors of human life quality is the food quality and safety. The food provides the energy, needed for the human body for movement, physical and intellectual activity. It is a source of proteins, fats, carbohydrates, vitamins and minerals, due to them the cells and tissues are renovated. As a result of the feeding the human organism produces hormones, enzymes and other regulators of the metabolic processes.

The assessment of food quality and safety is an important part of food production chain. The grain is a main part of the human and animal food. The higher food quality requirements demand development of new, objective, intelligent technologies, methods and tools for assessment of main food quality and safety features.

The grains and the cereals are an essential part of the human food. The cereals assure the half of the daily energy ration of the people in the developed countries and 80% in the developing countries. The grains of the wheat, maize, rice, barley, oats and millet contain about 60-80% carbohydrates, 8-15% proteins and 1.5-2% fats [8].

The problem for rapid, objective, automated, express and nondestructive grain quality assessment is a complex and multilevel task, related to the analysis of the appearance, the visible features as well as the grain contents, smell, flavor, moisture content, infections, non-grain impurities, etc. of the grain sample elements.

This investigation is focused on the assessment of main corn grain quality features. There are proposed and investigated methods and tools for feature extraction and data dimensionality reduction, analysis and identification of the grain sample elements. They are based on the analysis of color images and spectral characteristics of the investigated objects, as well as the fusion of the results of the two kinds of analyses. This approach is realized in the frame of INTECHN project “Development of Intelligent Technologies for Assessment of Quality and Safety of Food Agricultural Products” founded by Bulgarian National Science Fund [7].

According to the Bulgarian national standards the main quality features are as follow: the appearance, shape, color, smell, taste, moisture and impurities typical for the variety. Some of these features of a corn grain sample are presented in Table 1.

Table 1. Corn grain quality groups

Grain quality groups	Grain quality features
First group - standard kernel	Whole grains and broken grains bigger than the half of the whole grain, with appearance, shape and color typical for the variety
Second group -grain impurities	Broken grains smaller than the half of the whole grain, heat-damaged grains, small grains, shriveled grains, green grains, sprouted grains, infected (with Fusarium) grains, smutty grains.
Third group – non-grain impurities	Corn-cob particles, leaf and stem fractions, pebbles, soil and sand, as well as harmful elements

The assessment of grain quality features presented in Table 1 is mainly related to the visible features of the grain sample elements and features related to the grain content, dry matter content, moisture content, starch, protein, glutenin, vitamins, toxins and mineral content [3].

It is obvious that all of the features mentioned above cannot be evaluated using the information extracted from one sensor source only. A huge part of these features (for example grain appearance, shape and color) are evaluated by an expert on the base of visual assessment only. That's why such features can be efficiently evaluated using a Computer Vision System (CVS). A review of the progress of computer vision in the agricultural and food industry is given in [2].

Some features like grain composition, content, infections etc. cannot be evaluated by means of CVS. Spectra analysis is mainly used for assessment of such features. Other features like moisture content, specific weight etc., are evaluated by other standard physicochemical methods. To obtain a complex assessment of the grain quality using data about color, shape and dimensions of the grain sample elements is a complicated and multilevel task [25]. This is because the color, the shape and the dimensions of the elements in a sample vary within a wide range.

There are many publications related to the assessment of some particular quality features using color image analysis. A digital image analysis algorithm [18] based on the textural features is developed for classification of individual kernels of cereal grains. Color analyses are used to assess variety [18,19], infections [28,23], germination [22], weed identification [1], etc.

The grain variety is usually assessed by means of different morphological features related to the shape and geometrical parameters. A set of eight morphological features namely area, perimeter, length of major axis, length of minor axis, elongation, roundness, Feret diameter and compactness are used to recognize five different kinds of cereal grains [31]. A broader investigation about classification of barley, Canada Western Amber Durum wheat, Canada Western Red Spring wheat, oats, and rye is presented in [29]. It is based on a total of 230 features (51 morphological, 123 color, and 56 textural). A profile analysis through one-dimensional digital signals [12], by modeling the shape by means of a set of morphological features [30] and by shape curvature analysis [25] is performed for assessment of grain purity. Computer vision methods are also used to determine kernel mechanical damage, mold damage [27], broken kernels in threshing process [37], etc.

A preliminary investigation [23] shows that we can't get a precise assessment of some of the grain sample elements like smutty grains, infected grains and non-grain impurities, using an image analysis. It is difficult to detect the small changes of surface texture through a CVS. That's why we expect a more accurate assessment of such features to be gotten by spectra analysis. Unfortunately information about shape and dimensions cannot be extracted from spectra.

Visible (VIS) and Near Infrared (NIR) spectra analyses are applied in the assessment different food products [38, 26, 4, 25], as well as of grain quality features like grain composition, dry matter content, moisture content, starch, protein, glutenin, vitamins, toxins, mineral content, etc [10, 25, 10, 5, 9]. Different calibration models are developed for predicting grain composition and content [34, 32, 33, 10, 40, 20].

Modified partial least squares models on NIR spectra (850–1048.2 nm) are developed to predict grain quality features [32]. The best models are obtained for protein, moisture, wet gluten, and dry gluten with  $r^2 = 0.99, 0.99, 0.95, \text{ and } 0.96$ , respectively.

The spectra analysis is also used for detection of different grain infections. Determination and prediction of the content of ergosterols and different kinds of mycotoxins like aflatoxin, fumonisin and others are very important tasks because mycotoxins are toxic for animals and humans. Reflectance and transmittance VIS and NIR spectroscopy are applied to detect fumonisin in single corn kernels infected with *Fusarium verticillioides* [6]. A method for determination of *Fusarium graminearum* infection is proposed in [30]. The classification accuracy reaches to 100% for individual samples. Transmittance spectra (500 to 950 nm) and reflectance spectra (550 to 1700 nm) are suggested as tools for aflatoxin determination in single whole corn kernels [34]. The authors use discriminant analysis and partial least squares regression for spectral data processing. The best results are obtained using two feature discriminant analyses of the transmittance data. A NIR spectroscopy (NIRS) method for estimation of sound kernels and *Fusarium*-damaged kernels proportions in grain and for estimation of deoxynivalenol levels is proposed in [35]. The method classifies *Fusarium* damaged kernels with an accuracy of 99.9%. A neural network based method is developed for deoxynivalenol levels determination in barley using NIRS from 400 to 2400 nm [36]. Fourier transform NIRS is applied for rapid and non-invasive analysis of deoxynivalenol in durum and common wheat [9]. A qualitative model for discrimination of blank and naturally contaminated wheat samples is developed. Classification accuracy of the model is 69% of the 65 validation samples.

A comparatively new approach for grain quality assessment is based on the Hyperspectral Imaging System (HIS). A HIS get data about object spectra at some regions (pixels) of the object area. Every pixel contains spectral reflection data for many narrow situated spectral bands usually in VIS and NIR spectrum. Spectral data is normally presented as a hyperspectral cube. HIS could be considered as a variant of a color image analysis, where the object image is divided into pixels, every pixel is analyzed using multiband spectral analysis instead of three band analysis (R, G, B).

The hyperspectral analysis is applied for assessment of different grain features. For example, Mahesh et al. [17] use HIS for developing class models of different wheat varieties in Western Canada. The grain samples are scanned in NIR spectrum (960–1700 nm) at an interval of 10 nm. 75 different values of the intensity of the reflection are obtained from hyperspectral images and they are used for class model development. These models assure about 90% classification accuracy.

NIR spectroscopy is applied for assessment of grain moisture level too [14, 15, 16,]. The authors present a new method using NIR hyperspectral imaging system (960–1,700 nm) to identify five western Canadian wheat classes at different moisture levels. They are found that the linear discriminant analysis (LDA) and quadratic discriminant analysis (QDA) could classify moisture contents with classification accuracies of 89–91% and 91–99% respectively, independent of wheat classes. Once wheat classes are identified, classification accuracies of 90–100% and 72–99% are observed using LDA and QDA, respectively, when identifying specific moisture levels.

The HIS (350–2500 nm) is used for assessment of protein content in wheat grains [39] too.

## **MATERIALS AND METHODS**

### **Color image analysis. Grain groups and subgroups.**

Some features of grain sample elements, which are in principle evaluated by an expert on the basis of visual estimation, are assessed using CVS within the framework of this investigation. These features are related to the appearance, the color, the shape and the dimensions of the grain sample elements.

Groups (classes) and subgroups (subclasses) in which the corn grain sample elements are distributed are presented in Table 2. The tree normative quality classes are based on the corresponding color and shape subclasses presented in the same row of the table.

Table 2. Corn grain sample classes and subclasses

Normative (quality) classes	Color classes	Shape classes
1cst - standard kernel (whole grains and broken grains bigger than the half of the whole grain,) with appearance, shape and color typical of the variety	1cc- grains with color typical of the variety, back side	1csh- with typical of the whole grains variety shape
	2cc- grains with color typical of the variety, germ side	2csh- broken grains bigger than the half of the whole grain
2cst-grain impurities: broken grains smaller than the half of the whole grain, heat-damaged grains, small grains, shrivelled grains, green grains, sprouted grains, infected (with Fusarium) grains, smutty grains.	3cc- heat-damaged grains	3csh- broken grains smaller than the half of the whole grain and small and shrivelled grains
	4cc- green grains	
	5cc- mouldy grains	
	6cc- smutty grains	
	7cc- infected (with Fusarium) grains	
	8cc- sprouted grains	
3cst-non-grain impurities: corn-cob particles, leaf and stem fractions, pebbles, soil and sand, as well as harmful elements	9cc- non – grain impurities	4csh- non – grain impurities

Because the color and shape features are extracted and represented in a different manner, it is expedient their assessment to be made separately. After that the results from the two assessments have to be fused to obtain the final classification to one of the normative classes. Color and shape groups are divided in several subgroups in order to simplify the classification procedure. Color features are divided in 8 basic classes corresponding to the typical of different sample elements color zones and one additional class that corresponds to the non-grain impurities (it is impossible to define a compact class for non-grain impurities). The sample elements are divided in 3 basic shape classes corresponding to the whole grains, broken grains bigger than the half of whole grain and broken grains smaller than the half of the whole grain, and one additional class that corresponds to the non-grain impurities. Each of the three basic shape classes is divided in 6 shape subclasses.

### Features extraction from images

RGB, HSV, XYZ, NTSC and YCbCr color models are used for extracting the object area from background and for different color zones extraction in the frame of object area. These zones are typical of the standard grains, heat-damaged grains, green grains, smutty grains, infected (with Fusarium) grains, bunt and non-grain impurities. Furthermore four color texture models [24] are development for this purpose. It is expected they will better underline the difference between the color zones in the input RGB image. The texture models can be presented by the following equations:

First texture model. It is constructed on the basis of RGB model. Its components are the normalized differences between the R, G and B components:

$$d_1 = \frac{R-G}{R+G+B}; d_2 = \frac{B-R}{R+G+B}; d_3 = \frac{G-B}{R+G+B}; d_1 + d_2 + d_3 = 0 \quad (1)$$

Second texture model. This model includes non-linear transformation onto R, G and B components as follows:

$$O_1 = \frac{R}{G}; O_2 = \frac{B}{R}; O_3 = \frac{G}{B}; O_1 O_2 O_3 = 1 \quad (2)$$

Third texture model. It is similar to the second model. The pixel intensity is added as fourth coordinate:

$$O_1 = \frac{R}{G}; O_2 = \frac{B}{R}; O_3 = \frac{G}{B}; I = R + G + B; O_1 O_2 O_3 = 1 \quad (3)$$

Fourth texture model. This model converts input RGB space into one-dimensional texture feature  $T_k$ :

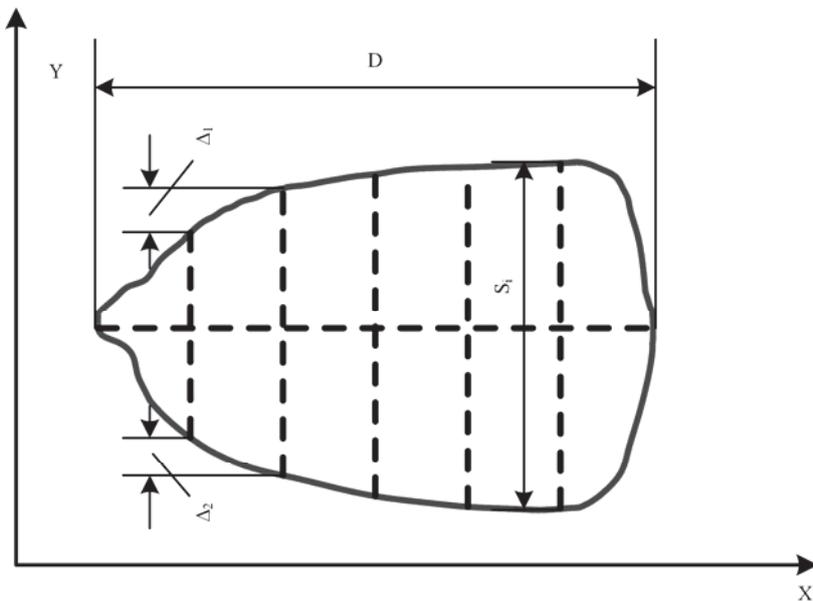
$$T_k = \frac{3(R + mG + nB)}{(R + G + B)(1 + m + n)}; m = \frac{G}{R}; n = \frac{B}{R} \quad (4)$$

The best results related to the extraction of the object area from background are obtained using the second texture model. When extracting different color zones within the object region, the best performance have the HSV and RGB color models. Therefore, the operator, who performs the training procedure, can choose the appropriate color or texture model based on the results obtained during the training procedure.

Ten-dimensional descriptions are applied to represent the shape of the grain sample elements [25, 21]. The following procedure is realized to obtain the shape description. First, the binary image of the object area is created. After that the object's peripheral contour is extracted and the bisection line of the object contour is found. An odd number of cross-sections perpendicular to the bisection line are built (Fig. 1).

The relative length  $h_i = s_i/D$  of the cross-sections, as well as the size and the sign of the difference between two neighbor cross-sections  $\Delta_i = \Delta_1 - \Delta_2$  are calculated. Finally, the object shape description is presented in the following form:

$$X_{sh} = (h_1, h_2, \dots, h_n, \Delta_1, \Delta_2, \dots, \Delta_n) \quad (5)$$



**Fig.1.** Object shape description:  $D$  – length of the bisection line;  $h_i = s_i/D$  – length of a cross-section;  $\Delta_i = \Delta_1 - \Delta_2$  - difference between neighbor cross-sections.

The contour line of the corn kernels has a huge asymmetry along to the bisection line. It is easy to locate the germ in the whole grain and to build contour descriptions and models with proper orientation. For broken grain, depending on what part of the whole grain is remained

(with the germ or without it) the contour descriptions could be sufficiently different. It is necessary to define two types of descriptions and models for 2csh and 3csh classes (for its corresponding subclasses): for broken grain where the germ exists in the remaining part of the grain and where it does not exist. The all shape groups are divided into 18 subgroups (subclasses).

### **Spectra analysis. Grain groups and subgroups.**

The spectra analysis is used to evaluate the color and texture features of grain groups like Fusarium infected grains, shriveled grains, sprouted grains, smutty grains and non grain impurities. The color image analyses doesn't enable to obtained sufficiently precise assessment of such features.

The groups and subgroups in which the corn grain sample elements are distributed based on the spectra analysis are shown in Table 2.

### **Features extraction from spectra and data dimensionality reduction**

Different methods like Principal Component Regression, Partial Least Squares Regression, Principal Component Analysis, Hierarchical Cluster Analysis and other methods are applied for developing a model to predict a property of interest, as well as for feature extraction and large and complex spectra data reduction. Methods like K-Nearest Neighbors (KNN), Linear Discriminant Analysis (LDA), Quadratic Discriminant Analysis (QDA), Cluster Analysis (CA), Support Vector Machines (SVM), Neural Networks (ANN), and Soft Independent Modeling of Class Analogy (SIMCA) are mainly used for assessment of different grain features using data from grain spectra.

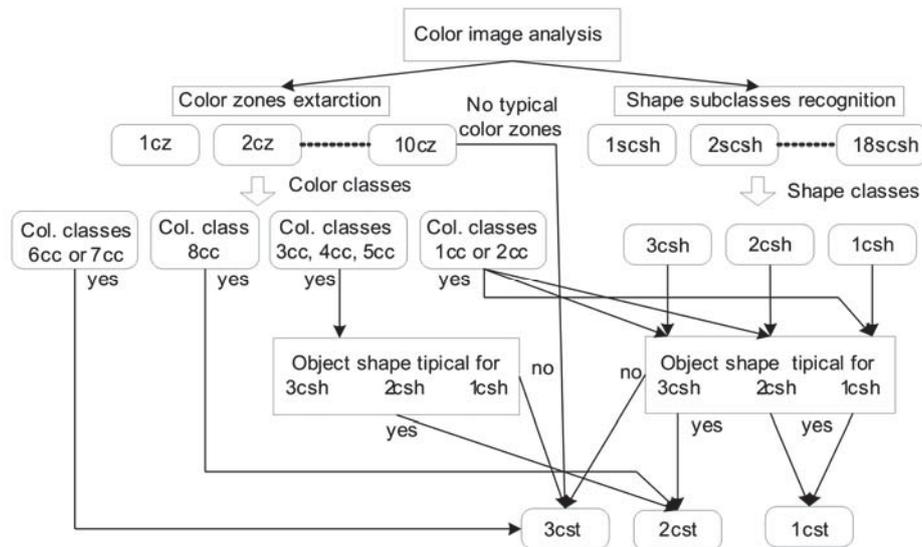
The spectral characteristics are obtained using QE65000 spectrophotometer. Each characteristic is a vector with about 1500 components. Principle Component Analysis (PCA) and combination of Wavelet descriptions and PCA are applied for extracting typical features from object spectra and for spectral data dimensionality reduction. The Wavelet1(detail coefficients) and Wavelet2 (approximation coefficients) and the Haar, Daubechies2, Coiflet2, Symlet2 wavelet functions are used in this investigation. The level of decomposition is varied from  $m=1$  to  $m=4$ . The most informative wavelet coefficients are chosen using PCA method.

### **Grain quality assessment fusing data from image and spectra analyses**

Because the color and shape features are extracted and described in a different manner, the assessment of these characteristics is separately done. After that the results from the two assessments are fused in order to obtain the object's final categorization to one of the normative classes.

Different variants of data fusion schemes are developed at different stages of the study [25]. The schemes developed could be associated with hierarchical clustering algorithms. Their typical feature is that different criteria for class merging are used at different levels of data fusion.

**Variant 1.** The First scheme uses a simplified fusion scheme. It is presented in Fig. 2.



**Fig. 2** Data fusion of the color and shape characteristics based on an image analysis only.

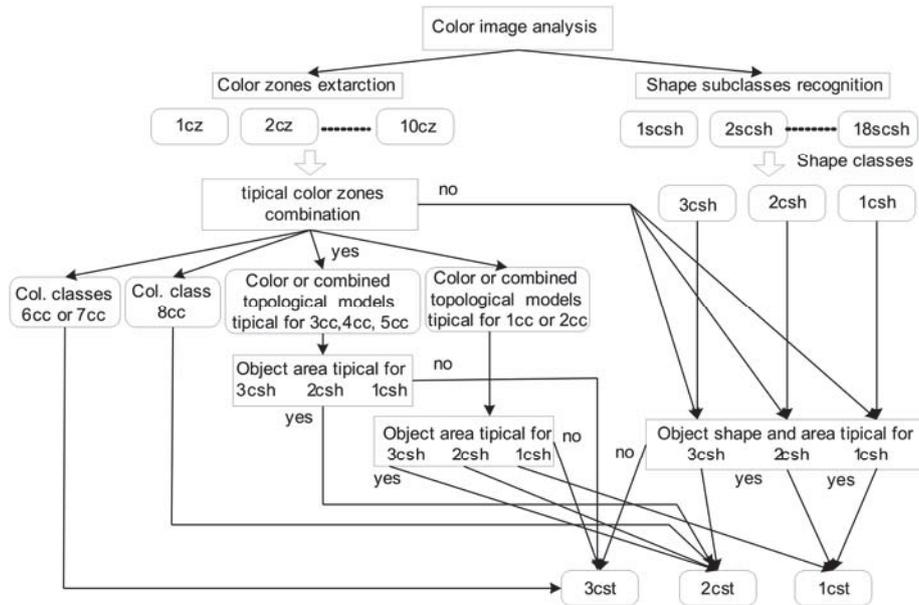
The input data (input classes) are separated in two groups – data about object color characteristics and object shape data. The first group consist of 10 color zones typical of the corn grains. These are regions of pixels in the grain image with similar color characteristics. The color zones were extracted from kernel images within the framework of a preliminary investigation.

The second group consists of 18 shape subclasses (1scsh, 2scsh, ..., 18scsh). The first six of them correspond to different shape models of whole grains, the next six – to models of broken grains bigger than the half of whole grain and the last six – to models of broken grains smaller than the half of whole grain.

The color class (1cc, 2cc, ..., 8cc) is determined on the basis of preliminary defined combinations of color zones at the first stage of fusing the results from the color analysis. The shape subclasses are merged into one of the three main shape classes (1csh, 2csh and 3csh).

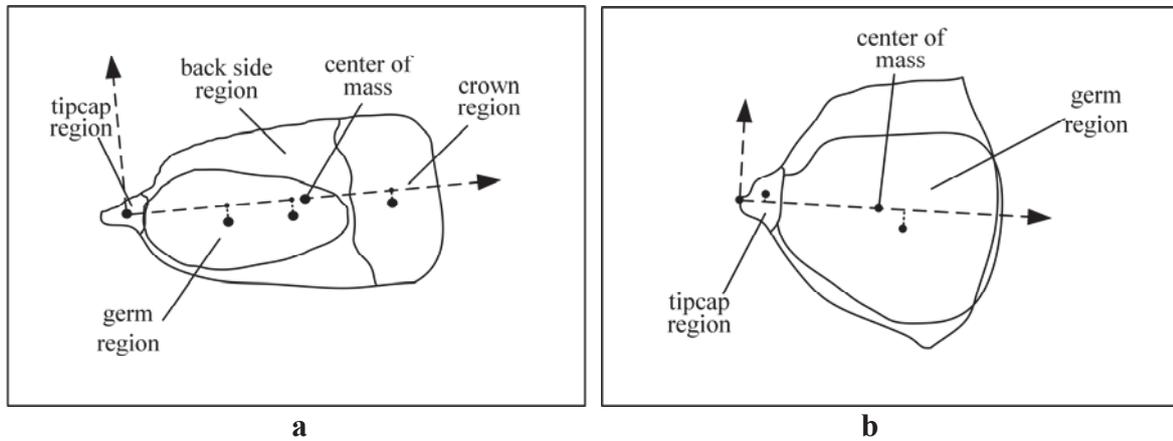
At the second stage of the analysis the fusion of color and shape classes is made in order to form the final decision of object classification in one of the three normative classes (1cst, 2cst and 3cst). The assessment whether the shape of the object is typical of one of the three classes or not, is used as a fusion criterion for color classes 1cc to 5cc. For 6cc, 7cc and 8cc classes the shape is not important at all.

**Variant 2.** The second scheme (Fig. 3) uses color and combined topological models of typical color zones. The topological models represent the plane distribution of the color zones



**Fig. 3** Data fusion of color and shape characteristics based on an image analysis and grain topological models.

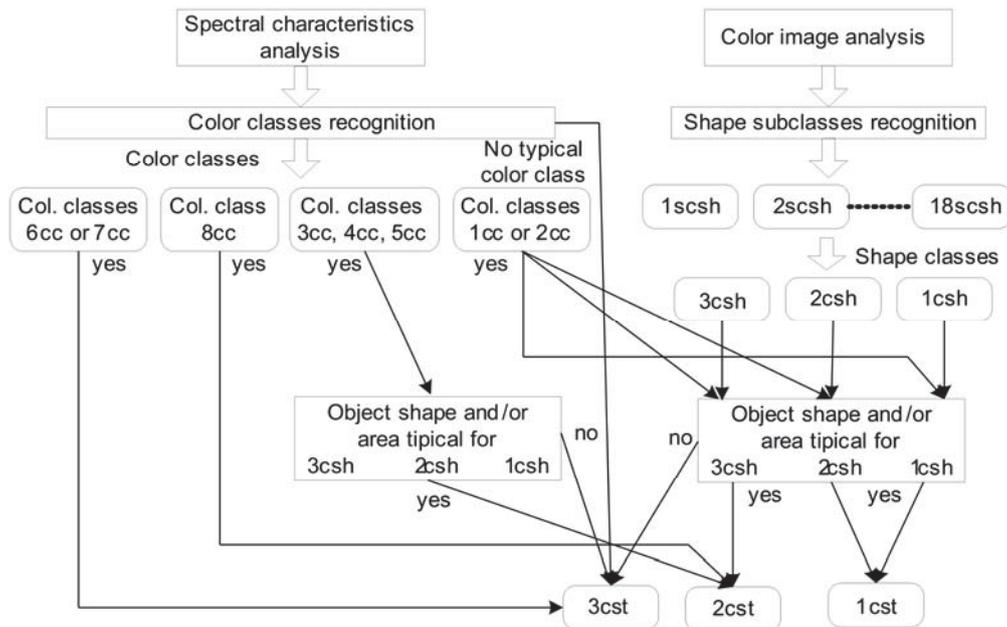
within the object area. A set of color topology models (when 3 or more typical for the kernels color zones are found fig. 4a) and combined topology models (when only 2 typical color zones are found fig. 4b) is preliminary defined. The combined topological models represent the plane distribution of some shape element (kernel tipcap or crown region) and the color zones found.



**Fig. 4** Color (a) and combined (b) topology

The final categorization when such topology is found is performed on the base of the object area only. The object shape and the object area are important for the final categorization, when one typical color zone is found. For 6cc, 7cc and 8cc classes the shape is not important at all.

**Variant 3.** The third scheme (Fig. 5) fuses color characteristics extracted from spectra and shape characteristics extracted from images. This is because the color class recognition is more precise when we use spectra analysis instead of image analysis.



**Fig. 5** Data fusion of color characteristics extracted from spectra and shape characteristics extracted from images

The main criterion for final categorization is the object color class. The correspondence of the object shape and/or the object area to the typical for the grain sample elements shapes is an additional criterion.

The variants 1 and 2 of color and shape data fusion could be associated with the first level of multisensory data fusion (Direct fusion of sensor data [13]). We can consider the results from color and shape analyses as signals obtained from two different sensors (the shape and the color are extracted and presented in a different way).

The third variant of data fusion is a typical example of third level of multisensory data fusion (Decision level fusion). The color and shape data are obtained from two different sensors – spectrophotometer and RGB camera. This data is separately processed and after that the results are combined to get the final decision.

It is expected that the data fusion procedures can improve the final classification in normative classes in comparison with the variants, when the categorization is made using object color data or object shape data only. This expectation is based on the assumption, that the fusion procedure can ignore or decrease some of the factors, which determine big errors of the final classification, when we use object color or object shape data only. The effectiveness of the proposed methods for data fusion is confirmed with the results presented in table 5 and table 6.

### Classification of the grain sample elements

Specific classification strategy, classifiers, and validation approach [25, 21] are applied for the categorization of the grain sample elements, which are conditioned by the specificity of the classification tasks.

**Classification approach.** *If the classes (related with the color, shape, PCA and Wavelet+PCA descriptions) are presented in the feature space, a part of them (1cc, 2cc, ...8cc) will form comparatively compact class regions. The sets of descriptions extracted from the grain sample training sets are used for developing the models of these grain sample groups. Each class model is presented by the class centre (the average value of the class training data) and the class boundary surface. The boundary surface is determined through a threshold value*

of the covariance of the class training data. A correct model for the 9cc class (non-grain impurities) could not be created because the characteristics of the elements of this class could be sufficiently different in each subsequent grain sample.

As a correct model for the 9-th grain group could not be created, a part of the descriptions of such objects of the testing set could get into the boundaries of the other eight classes. A big part of them would get outside the class regions and could be located in a random place in the feature space. These descriptions could be considered as noisy vectors. It could be assumed that the comparatively compact class regions of the objects from the first eight groups are submerged in a noisy environment. Therefore the task for categorization of the grain sample elements can be interpreted as a task for classification in classes, whose boundaries have definite shapes, dimensions and location in the feature space, and they are situated in a noisy environment [21].

Under this formulation, the use of popular strategies like LDA, CA, SVM, KNN and some other methods, which build boundaries between class regions, is obviously not a good choice. This is due to the fact that for the class 9cc a correct model cannot be created.

Furthermore if there are too big deviations of the actual values of the object characteristics and intensive measurement noise, the class areas can be overlapped. Very often correct information about prior probabilities of the classes is missing. This makes the classification problem more complex. If we use a classifier, which demands the prior probabilities to be known (for example Bayesian classifier), the training procedure has to be implemented using the prior probabilities obtained from the number of elements in training sets. When we assess quality of an unknown sample, the ratio of the number of elements from different classes could be sufficiently different from this ratio in the training sets. The classifier decision can be sufficiently different from the optimal decision under these circumstances. In this case the classification task is reduced to a task for approximation of overlapping class areas when the classes are situated in noisy environment and correct information for class a priori probabilities is missing.

**Classifiers.** The task for grain class modelling is reduced to a task for approximation of the boundaries of the grain class regions. For this purpose classifiers based on Radial Basis Elements (RBEs) could be used. Such classifiers will easily perform the approximation of the class regions and will simplify the classification procedure.

*The following classifiers [25] are used for class area approximation: CSRBE, CDRBE and CRBEP.*

### Classification accuracy

The accuracy of classification procedure is evaluated on the bases of the following classification errors:

$$e_i = \frac{FN_i}{(TP_i + FN_i)} \quad (9)$$

$e_i$  gives the relative part of objects from some class  $i$ , which are assigned incorrectly to other classes  $k=1..N$ , where  $FN_i$  is the number of elements from the  $i$ -th class classified incorrectly to other classes,  $TP_i$  is the number of correctly classified elements from the  $i$ -th class;

$$g_i = \frac{FP_i}{(TP_i + FP_i)} \quad (10)$$

$g_i$  gives the relative part of objects from other classes, which are assigned to class  $i$ , where  $FP_i$  is the number of elements from other classes assigned to the  $i$ -th class;

$$e_o = \frac{\sum_{i=1}^N FN_i}{\left( \sum_{i=1}^N TP_i + \sum_{i=1}^N FN_i \right)} \quad (11)$$

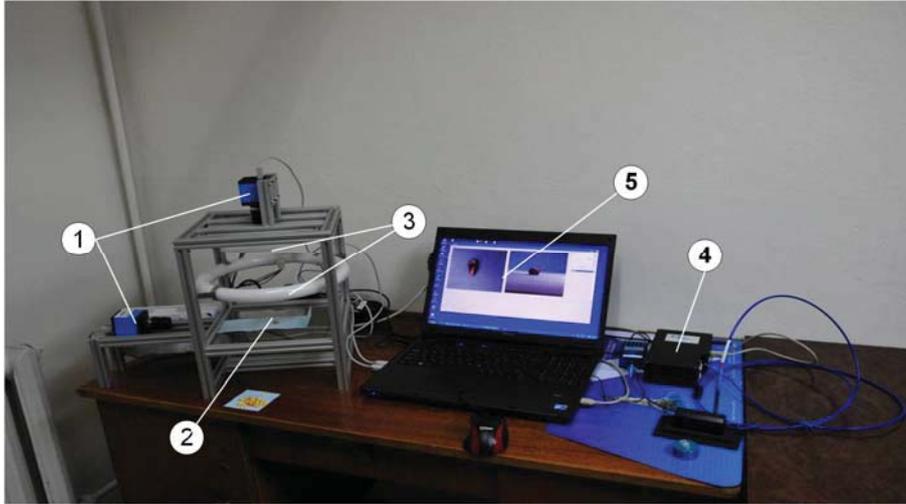
$e_o$  (classification error rate) gives the relative part of all incorrectly classified objects, where  $N$  is the number of classes.

### Test Setup

The hardware system consists of the following main components: computer vision system (CVS) (5) and spectrophotometer (4) (Fig.7). The CVS includes two color CCD cameras (1) which give a possibility to obtain color images of investigated object (2) in two planes (horizontal and vertical). The illuminant system (3) is used for direct object illumination. The reflectance spectral characteristics are obtained using spectrophotometer type QE65000 (4). The specifications of the camera, lenses, spectrophotometer and computer are presented in table 3.

Table 3 Technical specifications of camera, lens, spectrometer, computer and lighting system.

Name	Information
<u>Color camera</u> DFK 31AU03	Color digital video camera with USB interface, 1/3" Sony CCD sensor with progressive scanning, resolution – 1024x768 pixels;
<u>Camera lens</u> T2Z 3514 CS	Lens with variable focal length – 3.5 to 8 mm, diaphragm – from 1.4 to infinity, CS – assembly, MOD 0.3m;
<u>Computer system</u> Dell Vostro 1720	CPU - Intel Core 2 Duo P8700 (2.53 GHz, 3MB L2 Cache, 1066 MHz FSB) RAM - 4 GB (2x2048 GB) DDR2, 800 MHz Video card - NVIDIA GeForce 9600M GS 512MB
<u>Spectrometer</u> QE65000	Detector: Hamamatsu S7031-1006; Range: 350-1000 nm; Resolution: 1024 x 58 pixels; Optical resolution: ~0.14-7.7 nm FWHM; S/N ratio: 1000:1; ADC: 16 bits; Dynamic range: 7.5 x 10 <sup>9</sup> , 25000:1 for single measurement; Integral time: 8 ms to 15 min ; Adjusted linearity: >99.8%;
<u>Lighting system</u> Fluorescent lamps	The lighting system is compound of two ring-shape fluorescence sources with different diameters. It is used for direct illumination of investigated objects. They are placed so that the light can uniformly illuminate the object.



**Fig. 7** Test Setup. 1- color CCD camera; 2- investigated object; 3- illuminant system; 4- spectrophotometer; 5- computer vision system (CVS)

All investigations are carried out in laboratory conditions with a constant artificial lighting. Investigations in industrial environments are not made.

The object color images are obtained in a dark room. The objects are separately placed on a color pad with a color different from the grain sample elements. It allows the object zone to be accurate extracted from the background.

## RESULTS AND DISCUSSIONS

### Training and testing sets.

The developed procedures for grain sample quality assessment are trained, validated and tested with sets presented in Table 4. The training and testing sets include a number of different elements (whole grains, broken grains, etc.).

Table 4. Training and testing sets

Color classes recognition using CVS							
Classes	1cc	2cc	3cc	5cc	7cc	8cc	9cc
Training sets	10	10	12	15	18	19	
Testing sets	47	81	44	24	74	39	168
Object shape recognition							
Classes	1csh	2csh	3csh	4csh			
Training sets	120	135	135				
Testing sets	122	63	11	256			
Color classes recognition using spectra analysis							
Classes	1cc	2cc	3cc	5cc	7cc	8cc	9cc
Training sets	120	120	80	53	19 2	42	536
Testing sets	30	30	20	13	48	11	134

The results from objects classification in shape and color classes are presented in Table 5. All classifiers described in the section “*Classifiers*” are used in the investigation. The “selected classifier” is the classifier with the best performance.

Table 5. Classification errors in shape and color classes recognition

Color and shape class recognition						
	Shape class recognition		Color class recognition using CVS		Color class recognition using spectra analysis	
Selected classifiers	CRBEP		CDRBE		CDRBE Wavelet1+PCA model	
Errors	Test. errors		Test. errors		Test. Errors	
Class	$g_i, \%$	$e_i, \%$	$g_i, \%$	$e_i, \%$	$g_i, \%$	$e_i, \%$
1	39.5	5.7	8.9	8	0	15.2
2	62.0	69.8	8.3	4.9	0	5.1
3	87.5	77.8	0	15.9	0	0
4	21.9	43.1				
5			79.4	8.3	4.5	12.5
6						
7			35	13.5	19.5	5.4
8			0	5.3	0	10.3
9			23.2	68.4	12.0	6.6
	$e_0=35.6\%$		$e_0=30.6\%$		$e_0=7.3 \%$	

The results from objects classification in normative classes using the three variants of data fusion are presented in Table 6. Two classifiers are placed in the field “Selected classifiers”. The first classifier is applied in color class recognition and the second classifier is used in shape class recognition.

Table 6. Classification errors in normative classes recognition

Color and shape data fusion						
Fusion variant	Variant 1		Variant 2		Variant 3	
Selected classifiers	CDRBE-CRBEP		CDRBE-CRBEP		CRBEP-CDRBE Wavelet1+PCA model	
Errors	Test. errors		Test. errors		Test. Errors	
Class	$g_i, \%$	$e_i, \%$	$g_i, \%$	$e_i, \%$	$g_i, \%$	$e_i, \%$
1cst	7.7	28.2	4.2	1.7	2.7	6.8
2cst	27.9	32.2	17.2	14.4	0.8	12.7
3cst	12.7	0.8	6.2	9.1	8.4	0.4
	$e_0=15.3\%$		$e_0=8.6\%$		$e_0=5.3 \%$	

1. The test results in shape class recognition show that the rate of objects from class 1csh assigned to other classes is comparatively small (4.9%). On the other hand, the rate of objects assigned to this class which actually belong to other classes is sufficiently bigger (35.2%). The rate of objects from class 3csh assigned to 2csh and 4csh is big too.

2. The classification error rate of objects from class 3csh (parts of kernels) is large. This is an expected result because it is impossible to define some standard shape for objects from this class. In many cases even a qualified expert will not recognize such objects if no color characteristics but only shape is taken into consideration. During the classifiers training models of broken kernels are created on the basis of whole kernels models and that is why the training sample classification error rates for classes 2csh and 3csh are small. This explains the big difference between training and testing classification results for these two classes.

3. The testing error in color class recognition using spectra analysis (7.3%) is acceptable bearing in mind the specific investigation conditions and the diversity of grain sample elements.

4. The comparative analysis of the results obtained using different variants of classifier validation, training and testing confirms the effectiveness of the classification strategy, classifiers, validation approach and data models. For example, if we use the three data models: PCA, Wavelet1+PCA and Wavelet2+PCA the training errors are 6.8%, 6.3% and 10.3% respectively using the CDRBE classifier. The validation approach (when the non – grain impurities are included in validation procedure, but are excluded from training sets) decreases the testing error 3.8 times (from 27.6% to 7.3%) in comparison with the traditional validation approach (when the non – grain impurities are simultaneously excluded or included in validation and training sets). The choice of an appropriate classifier for specific classification task has an influence over the classification accuracy too. For example, the training errors obtained using the CDRBE, CSRBE and CRBEP classifiers and PCA data model are 6.8%, 72%, and 7.3% respectively.

5. The classification errors in color class recognition using spectra analysis are sufficiently smaller than the errors using image analysis. For example, the testing errors are 1.3% and 10% respectively using the two approaches when the non grain impurities are excluded from the validation and testing sets. When we include the non grain impurities in validation and testing sets these two errors are 7.3% and 42%. The big difference between the two errors can be explained by the fact that the object spectral characteristics contain not only information for objects color characteristics, but for their surface texture too. Although typical for some grain groups color zones are found in a big part of non – grain impurities, the surface texture of these elements is sufficiently different from the typical for the grains.

6. Object classification in normative classes (1cst, 2cst and 3cst) includes complex assessment of color and shape characteristics of the investigated objects. For this purpose color data and shape data are fused. The data fusion procedure improves sufficiently the final classification results. The classification error rate  $e_0$  in normative classes using CVS (Selected variant CDRBE–CRBEP) is 15.3% when data fusion Variant 1 is used and 8.6% when Variant 2 is used, while the errors of object color zones extraction and object shape recognition are 30.6% and 35.6% respectively. When we use Variant 3 for classes' recognition the classification error rate  $e_0$  decreases about 1.6 times in comparison with the better result obtained using CVS. This is due to the fact that the spectra analysis gives the best result in color classes' recognition.

## CONCLUSIONS

The results from the investigation at this stage of the INTECHN project implementation concerning grain sample quality assessment using complex assessment on the basis of color image and spectra analyses can be summarized as follow:

1. The developed approaches, methods and tools for grain samples quality assessment based on the complex analysis of object color, object surface texture and object shape give an

acceptable accuracy under specific experimental circumstances. The error rate  $e_0=5.3\%$  of the final categorization in the normative classes can be accepted as a good result at this stage of project implementation.

2. The data fusion procedure improves sufficiently the final classification results. The classification error rate  $e_0$  using CVS is 15.3% when Variant 1 is used and 8.6% when Variant 2 is used, while the errors of object color zones extraction and object shape recognition are 30.6% and 35.6% respectively.

According to the minimal quality requirements of grain samples defined in Bulgarian Standard Regulation 1272/2009 the impurities (grain class 2cs and non-grain 3cs) in a grain sample cannot be more than 12%. About 28% of standard grains are recognized as impurities using Variant 1. This means that the error is bigger than the permissible percentage of the impurities and this variant is not applicable for the analyses of real grain samples. The errors of the Variant 2 and 3 are permissible from the point of view of the Regulation 1272/2009. Variant 3 is preferred because the error (these are objects from third and second class recognized as objects from first class) is about 2 times smaller than the same error of the Variant 2.

3. The results obtained show that the choice of an appropriate procedure for fusion the results from color characteristics and objects shape analysis has a significant influence over the final classification accuracy. When we use the second algorithm (Variant 2) which is based on color or combined topology assessment the classification error rate decreases about 1.8 times compared to the first algorithm (Variant 1) in which color class assessment is based on the registration of the typical color zones combinations only. When we fuse the results from color classes recognition obtained on the basis of spectra analysis and shape classes recognition obtained on the basis of image analysis (Variant 3) the final classification accuracy is increased 2.9 and 1.6 times in comparison with Variant 1 and Variant 2 respectively.

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## **CO<sub>2</sub>. DMSO-APPLICATION AND ROLE IN THE FIELDS OF MEDICINE AND CRYOBIOLOGY**

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### **Abstract**

The usage of cryo protectors is a problem representing big interests in the field of medicine and cryo medicine, because of the fact that they provide conditions for optimal biological storage of the cells and tissues. One of the largely used cryo protectors is dimethyl sulfoxide (DMSO). DMSO is used for cryo conservation of gametes, cells and tissues. In addition, its application is related to the cryo conservation of blood plasma rich in trombocytes. DMSO is in a leader position as a cryo protector in the technology for long term storage of trombocytes. DMSO has growing role in conservation of leucocytes. It's technology in which mainly cryo protectors on the base of glycerin are used.

It is clear that after the thaw of frozen trombocytes and their infusion, DMSO enters into the organism. The procedure of DMSO removal is needed to avoid its toxic effects. Different procedures are applied to avoid DMSO. The literature data shows that 95% of DMSO content in an organism can be removed after such procedures. This percent of the removed DMSO is too high but in living organisms in this case 5% of avoided DMSO remains after transfusion. The doses of the rested DMSO in living organisms are not toxic but the remaining quantity of DMSO has a noxious effect on organisms. By this point of view, the studies related to the DMSO action in nontoxic doses on the organisms are of interest for our laboratory. The question related to the study of other DMSO effects is interesting for us and the subject of our studies will be to clarify if there are large spectral therapeutic effects or other effects on the organisms.

The implementation of DMSO in medicine is related to the name of the scientist Jacob who described the extraordinary features of this chemical substance (Jacob et al, 1964). The medical application is described for the first time in the USA in 1966. DMSO is currently applied in medicine and in therapy for different diseases.

The action of DMSO on organisms in therapeutic doses.

DMSO and micro circulation DMSO possesses a vasodilating action and decreases the resistance of peripheral blood vessels. It improves the micro circulation in brain tissues. It decreases the possibility of red blood cells to aggregate. DMSO increases the permeability of the histo-hematological barriers of the lung and the liver, but it does not influence them in the kidneys.(1)

### Anti-inflammatory effect of DMSO

Anti-inflammation effects of DMSO are comparable with the anti-inflammation effects of non-steroid anti-inflammation substances. It increases the secretion of cortisol while also stabilizing lysosomal membranes. It possesses anti-exudative effect, it decreases the cellular infiltration in the damaged section, it accelerates the development of granulose tissue, it interacts with the metabolic products of the protein degradation forming intermolecular bonds, in this way making their diversion from the organism possible. We can say that DMSO plays the role of a chemical nurse of places of inflammation.

DMSO decreases the level of prostaglandins and acts like an anti-pyretic. DMSO does not have allergic effects in cases of contacts with the skin, but it possesses anti-sensibility effect. The base of its anti-allergenic activity is its ability to suppress B-lymphocytes and plasma cells and to inactivate histamines. It possesses painkiller effect. (1)

The usage of DMSO for blockage of nerves in combination with local anesthetics

It is considered that DMSO potentiates the effects of antibiotics. The cooperative application of DMSO and antibiotics increases the antibiotic concentration 2,3 to 3 times in the damaged locations. We can conclude that DMSO disposes of the antibiotic to the damaged locations. (1).

### Anti-mutagenic activity of DMSO

It was proved that DMSO has anti-oxidant effects. It prevents the  $H_2O_2$  lipid oxidation and captures free radicals like OH. DMSO has antiseptic effect because it is a good oxidizer.(1)

#### Clinical application of DMSO

DMSO has application in psychiatry. It possesses sedative and tranquilizing effects.

#### Application of DMSO in the neurology

DMSO is used in cases of hemorrhagic insults and traumatological damages of the brain and the spinal brain because it has anti-swollen effects as a result of its osmotic functions. (1)

#### DMSO application in the ophthalmology and otorhinolaryngology

DMSO is applied as treatment for the conjunctivitis and blepharitis in the form of eye drops. In case of treatment of purulent otitis and purulent sinusitis it is used in the form of washings.(18)

#### Application of DMSO in gastroenterology

DMSO can prevent from ulcer. It decreases the secretion of the stomach glands as well as acidity in the stomach. At the same time it increases the exocrine function of the liver increasing the secretion of the gall.

#### DMSO application in rheumatology

DMSO is applied as a treatment for rheumatologic arthritis, bursitis, tendovaginitis. This is a result of the possibility of the substance to modulate the connective tissue metabolism of the collagen in the cells.(19,20),

#### Application of DMSO in dermatology and in cosmetics

The application of DMSO in cases of closing wound. The result of the treatment is that there is a better cicatrix formed. DMSO is used for the treatment of different skin diseases like

dermatitis with Streptococcus origin, infections of different origin. In these cases for treatment its iodine solution is used. (1,2).

### DMSO in urology

The substance is used as a treatment for cystitis.(3)

#### Application of DMSO in surgery

It is effective in cases of treatment of purulent ulcer, in the form of a solution in concentration 30-50% in combination with antibiotics. It decreases the pain as well as the possibility of an inflammation. (6,7,8)

#### DMSO and oncology, other diseases and applications

Currently there is not data for the antitumor effect of the DMSO. The substance provokes the effect of storage of antitumor drugs in the neoplastic formations.(5) The local application of DMSO solution of the antitumor drugs of a patient with melanoma basal cellular cancer and Bowen disease gives positive result. The clinic trials of 30-50% fluoracil solution in DMSO give positive results in the case of vulva cancer. The solution of radiosensibilizer (5FX-metranidazol) increases the sensibility of skin tumors to radiotherapy. (5) Some of the clinic trials show that it can be used in cases of amiloidosis. The ability of the DMSO to be absorbed in the skin, creates a possibility for DMSO to be used as a transport molecule for others chemical substances in the skin. These abilities of DMSO make the use of DMSO and iodoxirine in the local treatment of Herpes zoster possible in the UK.

One of the DMSO characteristics is that it has the ability to increase a transdermal transport.(3) This fact makes DMSO a possible candidate for the group of the chemical arms. The mixture of DMSO and chemical arm substance leads to faster penetration of the arm through the skin.

In conclusion, it is clear that the DMSO is used to cure a large spectrum of different diseases. The Food and Drug Administration (FDA) has decided that the DMSO can be used while conveying treatment for interstitial cystitis. The DMSO substance is used in the Russian Federation as an anti-inflammatory drug for treatment of diseases of the osteo system and as part of the therapy of Alkalizing spohdolit , rheumatoid arthritis, osteoporosis, , in the case of erithema nadosus, in combination with heparine in the treatment of thromboflebitis . The market name of DMSO using in the therapy of different diseases is Dimexid.

At the same time, it is clear that the DMSO has negative effects on the living organisms and it is dangerous in cases of disfunction of the liver, stenocarditis, pregnancy, cataracts.

#### Toxic effects of DMSO

The experiments with animals have established that the DMSO in the air is not highly toxic. The animals(guinea pigs) are alive after the exposure with DMSO in the form of gas in concentrations 1048, 1600, 2000, 2900 mg/m<sup>3</sup> and exposition 4, 24 and 40 h. The concentrations are too high and lung swelling was observed. The threshold concentration is 258 mg/m<sup>3</sup> for rats and 285 mg/m<sup>3</sup> for mice. Decrease of the muscle vitality has been observed. DMSO applied in a dose of 606 mg/kg. , in humans has effects like nausea, vomiting, and fibril cancellation of skeletal muscles. The dose 1800mg/kg in human skin provoked hemolytic anemia cyanosis.

It is observed that doses 45mg/m<sup>3</sup> applied on the animals, like rat hemodynamic disturbances and dystrophy of the inner organs and in the brain. A month later the animals are relaxed and patho-anatomic disturbances are not observed.

The absorption and excretion of DMSO were studied in Rhesus monkeys (Macaca mulatta) given daily oral doses of 3 gms DMSO/kg B.W. for 14 days. DMSO and its major metabolite, dimethyl sulfone (DMSO<sub>2</sub>), were measured in serum, urine and feces by gas-liquid chromatography. DMSO was absorbed rapidly, reached a steady state blood level after 1 day

and then was cleared from blood within 72 hrs after ending treatment. Serum DMSO declined in a linear fashion on semilogarithmic coordinates as described by second order kinetics. It had a half-life of 16 hrs. DMSO<sub>2</sub> appeared in blood within 2 hrs and reached a steady state concentration after 4 days of treatment. DMSO<sub>2</sub> was cleared from blood about 120 hrs after DMSO administration was stopped. Its half-life in blood was calculated to be 38 hrs. Urinary excretion of unmetabolized DMSO and DMSO<sub>2</sub> accounted for about 60% and 16%, respectively, of the total ingested dose. Neither DMSO nor DMSO<sub>2</sub> was detected in fecal samples. However, when added to fecal samples, DMSO was degraded rapidly. Although dimethyl sulfide (DMS) was not measured, some DMSO was metabolized to this compound because of the particular sweetness of breath of the monkeys. The absorption of DMSO by monkeys is similar to that for humans, but that its conversion to DMSO<sub>2</sub> and urinary elimination are more rapid in monkeys.

The literature reference for DMSO as a drug done in this work has shown that DMSO has a large spectrum of influences on the organism on the level of cells and this fact gives possibility for its application in medicine. These characteristics as well as the possibilities to use DMSO like cryo protector of cells and tissues give us the basis to conclude that the future studies related with DMSO as a drug are a real perspective, and would be interesting in the field of cryobiology and medicine.

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### **CO3. SELECTIVE ANGIOTENSIN II BLOCKERS - EFFECT ON CONTRACTILE ACTIVITY OF UTERUS AND URINARY BLADDER**

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The significance of Angiotensin (Ang II) as a regulatory peptide has been expanded considerably, concerning many systems outside the cardio-vascular and renal ones. Moreover, the heterogeneity of Ang II receptors – the existence of AT1 and AT2 subtypes, further complicates the possible role of this octapeptide with respect to the various tissues. Since it is not still well studied, the AT2 receptor remains a mystery. This receptor binds Ang II with a high affinity but is not responsible for mediating the classical physiological actions of AngII, all of which involve the AT1 receptor. During the past years, much information has been gathered about the possible role of AT2 receptor, and the increasing number of publications indicates a growing interest in this new and interesting area of research. A number of studies suggest a participation of AT2 receptors in brain, renal, and cardiovascular functions and in the processes of apoptosis and tissue regeneration.

The better study of the effects mediated by the different Ang II receptor subtypes requires specific blockers to be used. Losartan is the first approved non-peptide AT1 blocker in the

medical practice. Except for the treatment of hypertension, losartan has additional beneficial effects such as regression of myocardial fibrosis and microcirculation flow improvement. The selective AT<sub>2</sub> - blocker PD 123319 is still under testing, but AT<sub>2</sub> receptor blockade with this highly selective substance reveals that these receptor subtypes largely modulate or even counteract the effects, mediated by activation of AT<sub>1</sub> receptors.

The effects of Ang II on the smooth muscle activity of the urogenital tract are of particular interest, regarding the pathogenesis and treatment of many micturition disturbances and uterine disorders. It has been established that Ang II causes a potent contraction in urogenital tract, mainly via AT<sub>1</sub> receptors. However, both AT<sub>1</sub> and AT<sub>2</sub> receptors have been demonstrated to exist in urinary bladder and uterus, suggesting a possible role for AT<sub>2</sub> receptors in Ang II – mediated processes in these organs. The application of PD 123319 on isolated smooth muscle strips from rat uterus revealed significantly reduced parameters of AngII-mediated contraction, compared to the classical response of AngII. Obviously AT<sub>2</sub> receptors are of great importance for the development of uterine contraction. Regarding urinary bladder, the opposite tendency was observed: the AT<sub>2</sub> blockade with PD 123319 led to increased response to AngII. In previously conducted experiments we have described the effect of PD 123319 on Ang II-mediated gastro-intestinal contractions. Our experience with the study of Losartan and PD 123319 on smooth muscles showed that these blockers affect the motility of the gastro-intestinal system as well as urogenital tract.

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## CO4. AGE DEPENDANT DISTRIBUTION OF CARBOHYDRATES IN MURINE INTESTINAL GLYCOCAL

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The enterocyte is a unique cell governing an array of processes. It is covered by a specific coat of carbohydrates responsible for gut colonization - the gut glycocalyx. This "sugar-rich" covering that lines plasma membranes, is an extraneous coat and an integral part of the plasma membrane as well. The intestinal glycocalyx and secreted mucins constitute a glycosylated milieu which has a number of physiological and protective functions. The epithelial mucins are a heterogenous family of glycoproteins, containing an array of O-linked carbohydrates. These glycans are rich in the amino-acids serine and threonine. The latter are favorable for O-linked glycosylation of glycans in the Golgi apparatus. The glycocalyx provides a matrix for concentrating intestinal enzymes and their substrates and products in a close proximity to enterocyte membranes thus facilitating the digestive-absorptive function. It also prevents enzymatic self-digestion of cell membranes. Another important function of intestinal epithelial cell glycocalyx is its barrier function against microbial adherence to different membrane glycolipids. Thus, the glycocalyx is an important part of the mucosal immune system in newborn animals.

The aim of the present study was to identify the carbohydrates in the small bowel glycocalyx of Balb/c mice at different ages. We used plant lectins with different sugar specificities. Fluorescein labeled lectins binding different carbohydrate moieties were detected using confocal laser scanning microscopy technique. Biotinilated lectins were used for the transmission electron microscopy observations of the constituents of the gut glycocalyx at different periods of postnatal development in mice.

Concanavalin A (Con A), that is specifically binding mannose was evenly distributed on tip of microvilli. Wheat Germ agglutinin (WGA) is specifically binding N-acetyl- $\beta$ -glucosamine oligomers. We detected that colloidal-gold staining of WGA was abundant on the surface of whole villi, covering duodenum, jejunum and ileum of murine small bowel. Age related expression patterns of the latter carbohydrates were being discussed. Confocal laser scanning microscopy (CLSM) observations were performed to trace the binding of fluorescein labeled Con A and WGA lectins. Confocal microscope micrographs revealed age dependent distribution patterns of carbohydrate moieties in the murine intestinal glycocalyx of developing animals. These differences could be important determinants in cell-cell and cell-matrix interactions.

Different carbohydrate moieties were identified in the glycocalyx of murine small intestine. They followed different distribution patterns and characteristics. Since carbohydrates present on mucus surface depend on tissue localization, cell type, and on stage of development, the distribution and mucins glycosylation could be of interest in analyzing the response of mucosal barrier to intestinal pathogens, causing infection or inflammation.

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## **CO5. ОРИГИНАЛНИ ВЪГЛЕХИДРАТНИ И СОЛЕВИ НАПИТКИ ЗА ПРИЛОЖЕНИЕ В ХИРУРГИЯТА И МЕДИЦИНАТА - ПЪРВОНАЧАЛНИ КЛИНИЧНИ РЕЗУЛТАТИ**

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## **CO6. MORPHOLOGICAL AND IMMUNOLOGICAL ASSESSMENT OF THE INDIVIDUAL AND COMBINED EFFECTS OF TWO MYCOTOXINS-FUMONISIN B1 AND DEOXYNIVALENOL IN VIVO**

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Mycotoxins are health problem as they are natural contaminants of corn, wheat, oats, barley, rice, ect. and their by-products used in food and feed industry. Two of them-fumonisin B<sub>1</sub> (FB<sub>1</sub>) and deoxynivalenol (DON) are the predominant representatives detected in samples of grains from different regions of Bulgaria. They lead to acute and chronic intoxications, and different pathological alterations in humans and animals. Chickens are potential target, because their feed is based on wheat and corn. More investigations are needed to evaluate the impact of FB<sub>1</sub> and DON toxicity on the profitability of the poultry industry.

The objective of our study was to investigate the morphological and immunological effects of fumonisin B<sub>1</sub> and deoxynivalenol, and their combination in chickens, used in concentrations found in grains from Bulgaria.

Materials and methods: FB<sub>1</sub>- 10 mg/kg and DON-1,15 mg/kg forage were applied, either separately or in combination, in the diet of 30 days old Lohmann brown female chickens for a period of two weeks. Histological and ultrastructural examinations were performed. Thiazolyl blue tetrazolium bromide test (MTT), trypan blue assays, tests for proliferation and mitogenic response of blood and spleen lymphocytes and spreading and phagocytosis of macrophages were carried out.

The results showed reduced viability, proliferative activity and mitogenic response of the blood lymphocytes. The spleen lymphocytes proliferation was diminished too. The functions-spreading and phagocytosis of peritoneal macrophages were decreased significantly

after the treatment. The histological and ultrastructural findings revealed alterations in the lymphoid organs, esophagus and duodenum that were highly expressed in the FB<sub>1</sub> and DON consuming group. We concluded that the applied FB<sub>1</sub> and DON concentrations and particularly their combination can compromise the health of chickens.

*Key words:* fumonisin B<sub>1</sub>, deoxynivalenol, morphological and immunological effects, chickens

## Introduction

Mycotoxins are metabolites of microscopic fungi that grow on various plants including maize, wheat, oats, barley, rice, ect. The moulds of the genus *Fusarium* are spread worldwide and are common contaminants of crops. They synthesize in the field or during storage three main groups of metabolites with toxic action: trichothecenes, fumonisins and zearalenone. These mycotoxins can prevalent in grains used for preparation of different types of food for human consumption [1, 2]. Fumonisins belong to the recently (1988) discovered toxins produced by *F.verticillioides (moniliforme)*, *F.nygamai*, *F. anthophilum* and *F. proliferatum* [3]. Fumonisin B<sub>1</sub> (FB<sub>1</sub>) is the predominant representative. It disturbs cell growth and differentiation, and causes apoptosis in various organs [4]. DON was first obtained in Japan from contaminated barley infected with *Fusarium* species [5]. As trichothecene, it inhibits DNA, RNA and protein biosynthesis, leads to apoptosis in tissues and has a haemolytic effect [6]. Both toxins lead to acute and chronic intoxications and various pathological effects in humans and animals. They were detected in samples of grains from different regions of our country, which proves their natural occurrence in crops in Bulgaria and justifies their studying [7, 8]. Chickens are potential target of mycotoxic action, because of their feed based on wheat and corn. The adverse effects on birds consist of: acute and chronic intoxications, poor absorption of feed, reduced body weight, decreased productivity and reproduction and immunosuppression, which severely compromise the profitability of the poultry industry. The mechanisms injuring cardiovascular system, reproduction and immunity are still not well known. There are data about the influence of extracts from *F. verticilloides*, containing fumonisins and other mycotoxins [9] and DON [10, 11, 12] that showed altered small intestinal morphology in broilers. But few data are available concerning only fumonisin B<sub>1</sub> effects and the impact of the interaction of FB<sub>1</sub> and DON on the gastrointestinal morphology in chickens.

We aimed to investigate the morphological and immunological effects on chickens, of fumonisin B<sub>1</sub> and deoxynivalenol, and their combination, used in concentrations found in grains from Bulgaria.

## Materials and methods

*Animals and treatment.* 30 days old female Lohmann brown-classic chickens were separated into four groups (10 chickens each). The toxins used in fodder mixtures were FB<sub>1</sub> (Genaxxon bioscience GmbH, Germany) and DON (Sigma- Aldrich Chemie GmbH, Germany). The fodders were prepared as follows: free of mycotoxins-for the control group of chickens, 10 mg FB<sub>1</sub>/kg forage for the second group; 10 mg FB<sub>1</sub>/kg and 1.15 mg DON /kg forage for the third group and 1.15 mg DON /kg forage for the fourth group. The used concentrations were verified by ELISA (Ridascreen, R-Biopharm AG, Darmstadt, Germany) and applied for a period of two weeks. The experimental animals were treated according to guidelines of the Local Committee for Experimental Use of Animals.

*Gross pathology and histology.* Gross pathology examinations were performed on whole organs. Tissue samples from thymus, spleen, bursa Fabricii, oesophagus, duodenum, ovaries and heart were routinely processed, H&E stained and examined under light

microscope (Leica DM 5000B, Wetzlar, Germany). Transmission electron microscopy (JEOL 1200 EX) was performed for examination of ultrastructural alterations.

**Hematology and immunology.** Trypan blue viability tests (Countess TM automated cell counter, Invitrogen Life Technologies Corporation Carlsbad, California) were carried out for isolated blood and spleen lymphocytes, and peritoneal macrophages. Colorimetric MTT (Sigma-Aldrich) assays were performed to estimate viability, proliferation and mitogenic response to phytohemagglutinin (PHA) and lipopolysaccharide (LPS) (Sigma-Aldrich) of blood and spleen lymphocytes. The macrophage functional activity was estimated by *in vitro* tests for spreading and phagocytosis according to Rabinovich and De Stefano [13], with modification of Passeti [14].

**Statistical analysis.** The statistical significance was evaluated by one-way ANOVA and with Bonferroni's post hoc test using GraphPad InStat Software (San Diego, CA, USA). Results were expressed as mean  $\pm$  SEM.

## Results

No clinical signs were shown during the experiment. The gross pathology revealed few haemorrhages in thymus and bloated intestines with enlarged and visible blood vessels in the FB<sub>1</sub> and DON, and only DON consuming groups.

The combination of FB<sub>1</sub> and DON decreased significantly the cell viability and cell proliferation of blood lymphocytes presented by Trypan blue and MTT test results. The viability and proliferation activity of isolated spleen lymphocytes and the viability of peritoneal macrophages were affected but not significantly decreased.

The mitogenic response of blood lymphocytes toward PHA (T-cell) and LPS (B cell) mitogenes was decreased in all treated experimental groups (Fig.1-b, c, d), while in the controls the used mitogenes had normally stimulated cells (a). The mitogenic response to B-cell mitogenes was strongly affected as it is shown (Fig.1-b, c, d: green columns). The mitogenic response of spleen lymphocytes toward both mitogenes remained unchanged as in the control group.

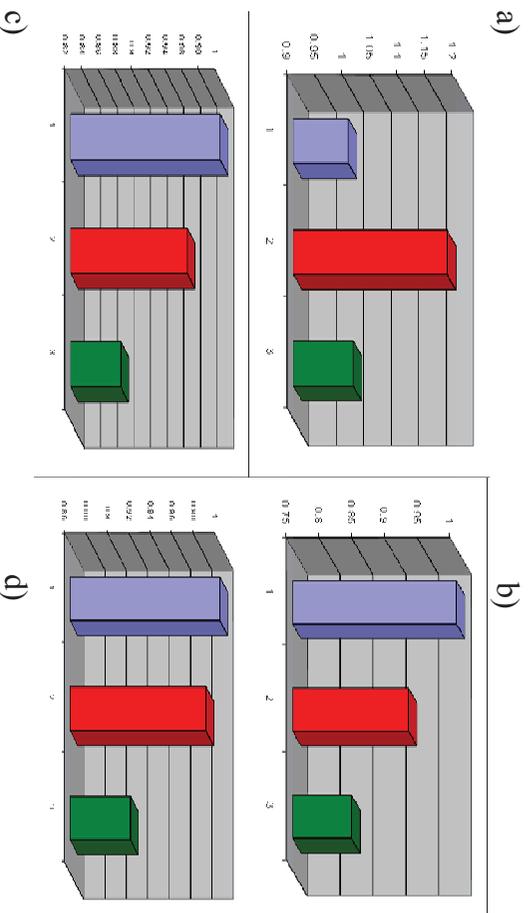
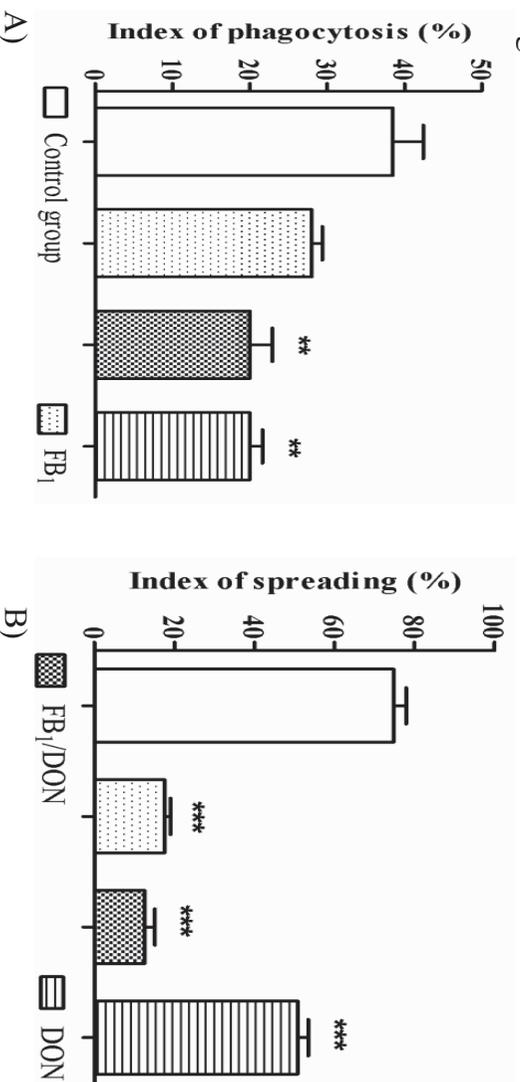


Fig. 1 Mitogenic response of blood lymphocytes towards PHA and LPS.

blue color - not treated lymphocytes, red color - treated with PHA (T-cell), green color - LPS (B-cell) mitogenes.

The used concentrations of FB<sub>1</sub> and DON decreased the functional activity of peritoneal macrophages illustrated by the lowered phagocytosis (Fig. 2 (A)) and spreading (Fig. 2 (B)) indexes, calculated as a percentage of total number of macrophages.

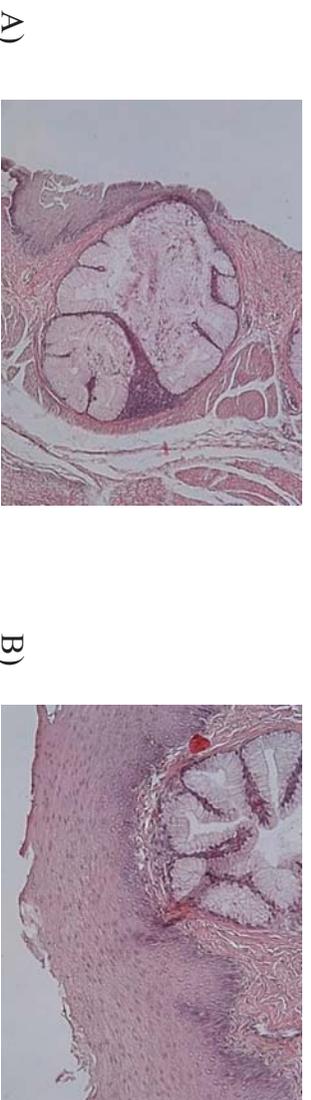
Fig.2



The error bars represent ±SEM; the stars indicate statistically significant difference between the treated birds versus the control group: \*\*P<0.01; \*\*\*P<0.001

The results from our histological examinations demonstrated changed morphostructural characteristics in the thymus, spleen and bursa Fabricii. They were expressed mainly in reduction of lymphoid cells, focal cell necroses and morphological signs of apoptosis - pycnotic nuclei and apoptotic bodies, especially in the groups consuming only FB<sub>1</sub> and both toxins. The white pulp was decreased and replaced by red pulp in the spleens of chickens consuming FB<sub>1</sub> and the combination of both. The histopathological alterations in esophagus consisted of thinning of the mucosal stratified squamous epithelium and degeneration of epithelial cells of mucous (mixed) glands of chickens from the three experimental groups, especially in the group consuming FB<sub>1</sub> and DON (Fig.3 A)). For comparison the esophagus of a control chicken is presented (Fig. 3 B)).

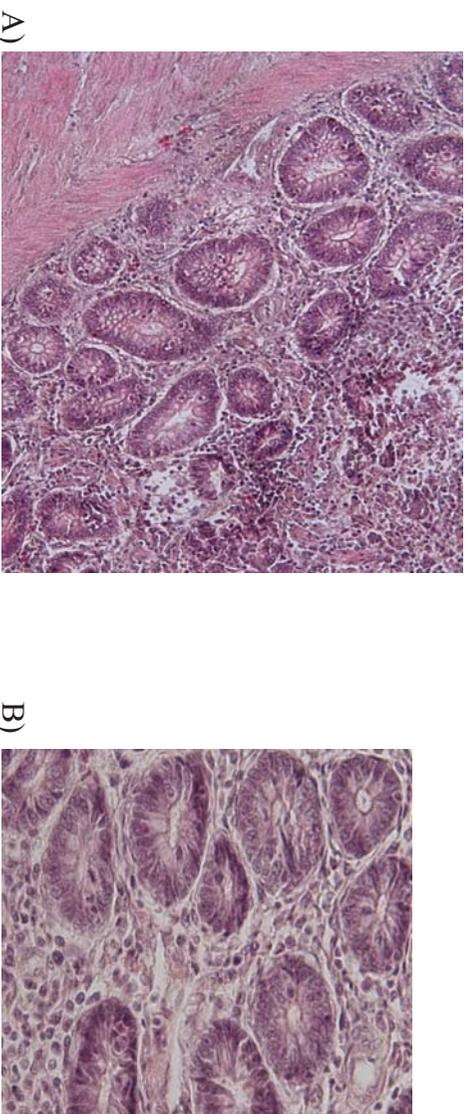
Fig. 3 Esophagus- A) denudation of the esophageal mucosa in FB<sub>1</sub> and DON consuming chicken; B) control chicken: note the thick, nonkeratinized squamous epithelium on the mucosal surface and the well arranged epithelium in the mixed glands; H&E, x 40



The crypts of Lieberkuhn and the intestinal villi in the duodenum were affected. More observable in the groups treated with both mycotoxins and FB<sub>1</sub> only and less in the group

treated with DON only. They were covered by epithelium where rounded cells with pyknotic nuclei and pale cytoplasm or highly vacuolated ones were seen near the columnar cells (Fig 4 A)). The epithelium of the duodenum of the control chickens remained intact (B)).

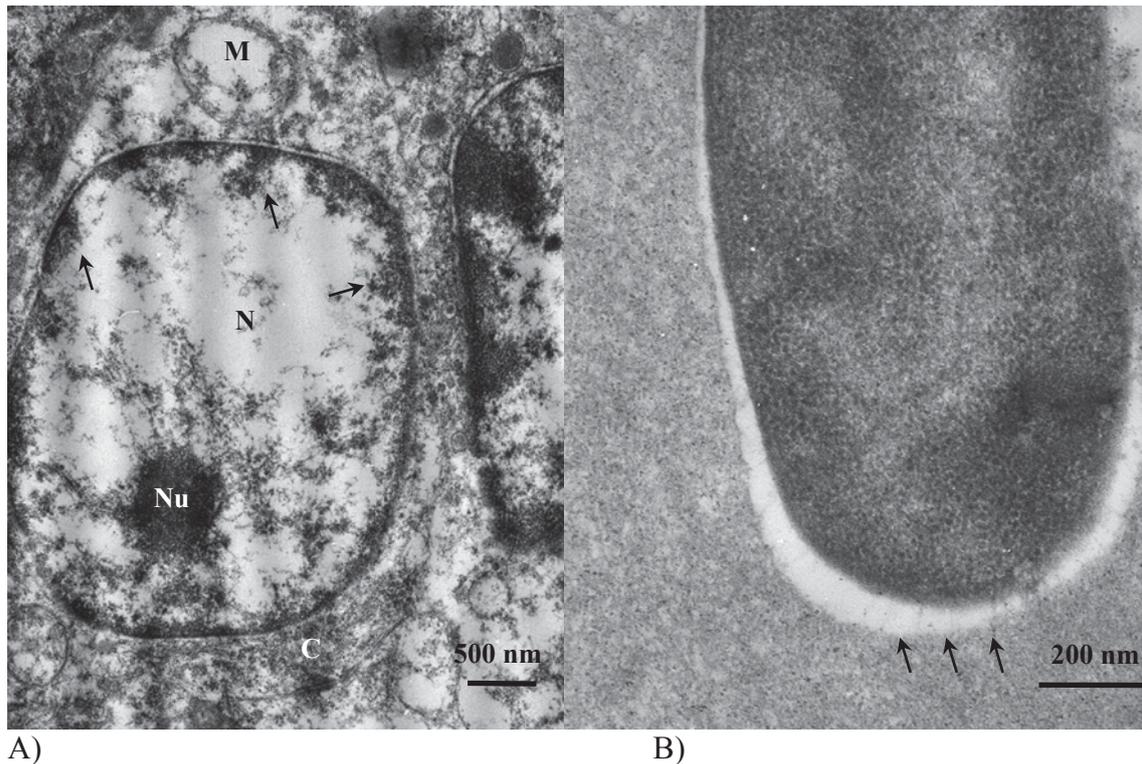
Fig.4 Crypts of Lieberkuhn A) degenerating enterocytes in FB1 and DON consuming chicken, B) control chicken; H&E, x 40



The examination of the heart and ovaries did not show any alterations for that period of the treatment.

The main ultrastructural characteristics (Fig. 5) of the effects of FB<sub>1</sub> and the combination of both consisted of chromatin margination, undistinguishable RNP structures, condensation of nucleolus with indefinite fibrillar and granular components, swollen mitochondria with lysed cristae and vacuolated cytoplasmic organelles of lymphocytes and epithelial cells in lymphoid organs, esophagus and duodenum. Enlarged perinuclear spaces were seen in some erythrocytes in spleen. The group consuming only DON demonstrated less intensive, as frequency, alterations mainly chromatin margination and vacuolated cell organelles mainly in the esophagus and duodenum.

Fig. 5. Electron micrographs of spleen from FB1 and DON treated chicken. A – lymphocyte with clear nucleus (N) with undistinguishable RNP structures and margination of chromatin (arrows); condensation of nucleolus (Nu) with indefinite fibrillar and granular components; swollen mitochondria (M) with lysed cristae; cytoplasm (C) shows vacuolated organelles. B – erythrocyte with enlarged perinuclear space (arrow).



## Discussion

The results obtained by this experiment evaluate fumonisin B<sub>1</sub> and deoxynivalenol as important pathogens in fodders. Despite the fact that poultry are considered to be more resistant to mycotoxic pollutants than mammals, it is evident, that growing birds can be sensitive. It was shown that the combination of FB<sub>1</sub> and DON decreased significantly the viability and functional activity of highly differentiated blood lymphocytes which are one of the first defending barriers in organism. In some experiments estimating FB<sub>1</sub> toxicity only, a reduced rate of proliferation of blood lymphocytes [15] and increased apoptosis [16] were observed too. The decreased mitogenic response of peripheral blood lymphocytes to PHA and especially to the B-cell mitogen-LPS presented in our results, can explain the mild to severe (depending on dose and time) immunosuppression accompanying these mycotoxicoses. Our histological and ultrastructural analyses revealed morphological signs of apoptosis and lack of normal organelles in many developing B-cells in bursal follicles and those in spleen that participate in the protein synthesis including antibodies. But the difference in the exhibited above MTT results, for the unchanged mitogenic response of spleen lymphocytes, can be interpreted with the possibility, that some cells isolated from the whole organ remained untouched for that short treatment period, and still processes of compensation and preservation were active. Reduction of macrophage functions was observed after FB<sub>1</sub> exposure [17] but the data concerning the influence of DON and the combined effect of both mycotoxins on macrophages were insufficient. Our results demonstrated that low doses of deoxynivalenol and its combination with fumonisin B<sub>1</sub> suppress significantly macrophage activity for the period of 14 days.

Our histological and ultrastructural studies of ovaries and heart indicated that the used concentration of both toxins did not affect organs for that period of time. In our opinion, further investigations of chronic exposure to these concentrations are needed. Morphological features of cell death in lymphoid organs at light and electron microscopic levels after FB<sub>1</sub> treatment were described already [18]. As it was demonstrated by our examinations,

gastrointestinal tract appears to be a target for fumonisin and deoxynivalenol action. The results indicated that both toxins in combination have more strong effects than alone. We assume that participation in the processes of feed refusal, low nutrient absorption and weight loss observed in chickens fed FB<sub>1</sub> and DON contaminated forages could play the diminished epithelial cell elements of mucosa in esophagus and injured epithelium in duodenum incapable to produce digestive enzymes or absorb nutrient. In conclusion, we observed stronger pathomorphological effects in lymphoid organs in FB<sub>1</sub> and DON treated group mainly due to the fumonisin B<sub>1</sub> toxicity, enhanced by deoxynivalenol. While epithelium in esohpagus and duodenum were susceptible to all treatments, including the used low dosage of deoxynivalenol, again presented more acute in birds consuming both mycotoxins.

According to the results of this study 10 mg FB<sub>1</sub>/kg fodder and 1,15 mg DON/kg fodder could affect the structure and function of organs. These investigations demonstrated that fumonisin B<sub>1</sub> and deoxynivalenol, especially their combination, applied in naturally occurring concentrations influence poultry health status with possible unfavourable economic outcome.

### **Acknowledgments:**

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## **CO7. MEDICAL MARIJUANA AND MULTIPLE SCLEROSIS**

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Cannabis is normally a dioecious plant with male and female flowers developing on separate plants. Contemporary uses of cannabis are as a recreational or medicinal drug, and as part of religious or spiritual rites, the earliest recorded uses date from the 3rd millennium BC [2]. Marijuana is a colloquial name for dried leaves and flowers of cannabis varieties rich in 1

to 20 percent  $\Delta$ -9-tetrahydrocannabinol (THC) [10]. It is one of 483 known compounds in the plant, including at least 84 other cannabinoids, such as cannabidiol (CBD), cannabinol (CBN), tetrahydrocannabivarin (THCV) and cannabigerol (CBG) [4]. Cannabis – based medicines have been used for thousands of years in Asia and were popular for 100 years in Western medicine after their introduction in the mid-nineteenth century. One of the first extensive descriptions concerning the application of Indian hemp was provided in 1830 by the chemist and botanist Theodor Friedrich Ludwig Nees from Esenbeck [10]. In 1899 was published “The pharmacology of Cannabis indica” in British Medical Journal by W.E.Dixon [3].

In 1964 the Israeli biochemist Rafael Meshulam (with Bulgarian lineage) was isolated the basic component of marijuana  $\Delta$ -9-tetrahydrocannabinol and he created the synthetic drug “marinol” used to prevent vomiting after chemotherapy.

The 1990s witnessed major advances in our scientific understanding of how THC and other cannabinoids act on the central nervous system. The discovery that the brain and some other organs contain specific protein receptors that recognize the drug and trigger cell responses is analogous to the discovery in the early 1970s of opiate receptors in the brain that bind morphine and other opiates. As with the opiates, the discovery of specific cannabinoid receptors prompted the search for putative naturally occurring chemicals that interact with the receptors [10]. Cannabis is often consumed for its psychoactive and physiological effects, which can include heightened mood or euphoria, relaxation, and increase in appetite. Unwanted side-effects can sometimes include a decrease in short-term memory, dry mouth, impaired motor skills, reddening of the eyes, and feelings of paranoia or anxiety [2].

Since the beginning of the 20th century, most countries have enacted laws against the cultivation, possession or transfer of cannabis. Since the early 20th century cannabis has been subject to legal restrictions with the possession, use, and sale of cannabis preparations containing psychoactive cannabinoids currently illegal in most countries of the world; the United Nations has said that cannabis is the most-used illicit drug in the world [8].

The high lipid-solubility of cannabinoids results in their persisting in the body for long periods of time. Even after a single administration of THC, detectable levels of THC can be found in the body for weeks or longer (depending on the amount administered and the sensitivity of the assessment method). A number of investigators have suggested that this is an important factor in marijuana's effects, perhaps because cannabinoids may accumulate in the body, particularly in the lipid membranes of neurons [5].

More than 100 controlled clinical trials of cannabinoids or whole-plant preparations for various indications at the neuronal level studied have been conducted since 1975. The findings of these trials have led to the approval of cannabis-based medicines (dronabinol, nabilone, and a cannabis extract [THC: CBD=1:1]) in several countries [4]. Researchers have subsequently confirmed that THC exerts its most prominent effects via its actions on two types of cannabinoid receptors, the CB<sub>1</sub> receptor and the CB<sub>2</sub> receptor, both of which are G-Protein coupled receptors. The CB<sub>1</sub> receptor is found primarily in the brain as well as in some peripheral tissues, and the CB<sub>2</sub> receptor is found primarily in peripheral tissues, but is also expressed in neuralgia cells as well. THC appears to alter mood and cognition through its agonist actions on the CB<sub>1</sub> receptors, which inhibit a secondary messenger system (adenylate cyclase) in a dose dependent manner. These actions can be blocked by the selective CB<sub>1</sub> receptor antagonist SR141716A (rimonabant), which has been shown in clinical trials to be an effective treatment for smoking cessation, weight loss, and as a means of controlling or reducing metabolic syndrome risk factors. However, due to the dysphoric effect of CB<sub>1</sub> antagonists, this drug is often discontinued due to these side effects [5].

Spasticity (an increase in muscle tone) is a common symptom of multiple sclerosis (MS), resulting in muscle spasms, immobility, disturbed sleep and pain. Complex drug

combinations are sometimes necessary to manage symptoms of MS, but these are often only partially effective and associated with unacceptable side effects [11].

A large proportion of patients with MS have spasticity, which has a marked impact on their quality of life. Anecdotal evidence suggests a beneficial effect of cannabis on spasticity as well as pain. Recently, randomized, double-blind, placebo-controlled studies have confirmed the clinical efficacy of cannabinoids for the treatment of spasticity in patients with MS. Based on these data, nabiximols (Sativex - GW Pharma Ltd), a 1:1 mix of  $\Delta$ -9-tetrahydrocannabinol and cannabidiol extracted from cloned Cannabis sativa chemovars, received approval for treating MS-related spasticity in various countries around the globe [7]. Sativex is the first cannabinoid preparation to be approved as a symptomatic treatment option. Sativex has been licensed "for symptom improvement in adult patients with moderate to severe spasticity due to MS who have not responded adequately to other anti-spasticity medication and who demonstrate clinically significant improvement in spasticity related symptoms during an initial trial of therapy" [11].

In Germany, a cannabis extract was approved in 2011 for the treatment of moderate to severe refractory spasticity in multiple sclerosis. It is commonly used off label for the treatment of anorexia, nausea, and neuropathic pain. Patients can also apply for government permission to buy medicinal cannabis flowers for self-treatment under medical supervision. The most common side effects of cannabinoids are tiredness and dizziness (in more than 10% of patients), psychological effects, and dry mouth. Tolerance to these side effects nearly always develops within a short time. Withdrawal symptoms are hardly ever a problem in the therapeutic setting [4].

Now in Bulgaria there is a license laboratory which produce cannabis for scientific and police needs in the town of Silistra.

A review of six randomized controlled trials of a combination of THC and CBD extracts for the treatment of MS related muscle spasticity reported, "Although there was variation in the outcome measures reported in these studies, a trend of reduced spasticity in treated patients was noted." The authors postulated that "cannabinoids may provide neuroprotective and anti-inflammatory benefits in MS." A small study done on whether or not cannabis could be used to control tremors of MS patients was conducted. The study found that there was no noticeable difference between of the tremors in the patients. Although there was no difference in the tremors, the patients felt as if their symptoms had lessened and their quality of life had improved. The researchers concluded that the mood enhancing or cognitive effects that cannabis has on the brain could have given the patients the effect that their tremors were getting better [6].

Compared with those taking a placebo, almost twice as many patients with MS prescribed an oral cannabis extract reported relief from muscle stiffness, a new study has found. The cannabis agent also proved better at relieving symptoms of body pain and muscle spasm, and sleep disturbances.

Results of the phase 3 Multiple Sclerosis and Extract of Cannabis (MUSEC) study confirm the patient-rated benefits of cannabis on MS symptoms that were found in the earlier Cannabinoids in Multiple Sclerosis (CAMS) study, researchers say.

"The evidence behind using cannabinoids for symptom relief is pretty strong now, and the MUSEC study is another piece of evidence to support that view; in fact, it's probably the strongest evidence so far," said study lead author John Peter Zajicek, PhD, professor and chair of clinical neurology at the University of Plymouth, United Kingdom [1].

With the increasingly abundant anecdotal but very high profile reports, neurologists can likely expect a surge in interest from parents, particularly those frustrated by intractable epilepsy in their children, who are logically asking "why not?"

"When patients, children or otherwise, are faced with bad situations and no good treatment they, or their parents, look 'out of the box' to find one," said David M. Labiner, MD, a neurologist with the University of Arizona and director of the Arizona Comprehensive Epilepsy Program, in Tucson.

"Medical marijuana is one of those things being utilized now" he told *Medscape Medical News* [9].

Largely as a response to political pressure and the limited availability of high quality commercial cannabis, the home growing of this crop, whether for medical or recreational use, is a trend rapidly spreading across North America and Europe. Cannabis smoking and cultivation for personal medical use will eventually be legalized or tolerated in many places, if not by the public openly favoring marijuana legalization, then by increasing awareness of the advantages of this potentially useful medicine.

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## **CP1. HORMONE REPLACEMENT THERAPY AND BREAST CANCER**

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## **CP2. ЦИНКЪТ, БЕЗ КОЙТО НЕ МОЖЕМ**

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## **CP3. ВАНАДИЯТ – ПОЗНАТ И НЕПОЗНАТ**

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## **CP4. ОЩЕ НЕЩО ЗА ЗЛАТОТО**

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## **CP5. THE GOLDEN STORY OF GOLD IN MEDICINE**

Radostina Alexandrova<sup>1</sup>, Abdulkadir Abudalech<sup>1,2</sup>, Tanya Zhivkova<sup>1</sup>, Pavel Mitrenga<sup>1,2,3</sup>,  
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## **CP6. ХИСТОНОВИ И НЕХИСТОНОВИ БЕЛТЪЦИ**

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## **CP7. ПРОПОЛИС**

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## **CP8. ЗА ШОКОЛАДА – С ЛЮБОВ!**

Драгомира Угринова, Ели Трифонова, Васил Бранков, Юлиана Иванова

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## **CP9. С ДЪХ НА КАНЕЛА**

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## **CP10. НА ЧАША ВИНО**

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## **CP11. ПОЛЕЗНА ЛИ Е БИРАТА?**

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## **CP12. HELICOBACTER PYLORI – БИОМАРКЕР И ЗА ДИАБЕТ ТИП 2?**

Даниела Иванова, Теодора Йорданова, Виктор Стойков, Лина Абужамус, Станислава Благоева

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**Friday, 29 November 2013**

Session D.

**Chairpersons:**

**Prof. Ilza Pajeva, PhD, DCs**

*Institute of Biophysics and Biomedical Engineering, Bulgarian Academy of Sciences*

**Assoc. Prof. Anna Damianova, MD, PhD**

*Institute for Nuclear Research and Nuclear Energy,  
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**Secretary: Tanya Zhivkova**

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**D01. MODES-OF-ACTION RELATED TO REPEATED DOSE  
TOXICITY: FROM PPAR $\gamma$**

**LIGAND-DEPENDENT DYSREGULATION TO NON-ALCOHOLIC  
FATTY LIVER DISEASE**

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**Abstract**

The Adverse Outcome Pathway (AOP) methodology has been shown to support the regulatory risk assessment based on the comprehensive understanding of toxicological mode-of-action (MoA). The MoA description of key events at different levels - from molecular to organism's individual response - that are both measurable and necessary for the adverse toxicity effect, has been considered a key step toward superseding the current repeated dose toxicity methodology with new generation predictive toxicology tools [1]. Since the chronic hepatotoxicity is of particular interest, we focused on the description of toxicological MoAs of non-alcoholic fatty liver disease (NAFLD).

Peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ) is well known for its role in a wide spectrum of biological pathways, notably lipid homeostasis and immune response. Because of its importance for glucose metabolism PPAR $\gamma$  has long been considered an attractive therapeutic target for synthetic anti-diabetic compounds with agonistic activity. However there are growing concerns for the safety profile of full exogenic PPAR $\gamma$  agonists in relation to their adverse obesogenic effect. At the same time novel cosmetics based on PPAR $\gamma$  antagonism are developed without enough safety data. These facts underline the demand of critical evaluation of the role of the receptor in the etiology of many human diseases. PPAR $\gamma$  has been recently proposed as one of the nuclear receptors involved in the molecular initiating event (MIE) for NAFLD [2, 3]. In this study we performed a detailed analysis of research and review papers with significant data regarding the possible role of ligand-dependent PPAR $\gamma$  dysregulation in disruption of lipid homeostasis. We conclude that either inappropriate activation (by receptor agonist) or inhibition (by receptor antagonist) of PPAR $\gamma$  with emphasis of the site of action (hepatic or adipose tissue), could be a MIE for ectopic lipid deposition in the liver and, eventually, for NAFLD.

### **Acknowledgements:**

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## DO2. ANALYSIS OF BOAR SEMINAL PLASMA PROTEINS WITH PROTECTIVE EFFECT DURING LOW TEMPERATURE STORAGE

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### Abstract

The study aimed to investigate the effect of different seminal plasma proteins (SPPs) on boar spermatozoa functional characteristics. We investigated the putative protective effect of SSPs on sperm cells motility and velocity during low temperature storage at 4°C. Nine fractions of SPPs were obtained by Gel Permeation Chromatography (GPC) and further characterized by 12% SDS PAGE. Sperm Computer Analysis (SCA) after incubation of spermatozoa with separated proteins revealed that low molecular weight (MW) proteins could preserve spermatozoa motility and velocity better when compared to those with higher MW.

### Introduction

The protein composition of mammalian SP varies among species and could affect spermatozoa functions (8, 8). Several SPPs have been reported to influence motility, survival rate and fertility potential of spermatozoa (9). Also a supposed role of SPPs in prevention of sperm cells from the effect of low temperature has been suggested (2). It is well known that boar spermatozoa are more sensitive to cold shock stress than other species such as human and bulls (8). It should be noted that the fertilizing ability of boar sperm cells after cold-shock is unsatisfactory (4). One promising field of investigations in this direction is the evaluation of boar SPPs as natural protectors which might play a crucial role in spermatozoa viability after cooling. The majority of boar SP proteins belong to the group of spermadhesins, which have important physiological role and participate in sperm cells' development, including capacitation, interaction of gametes, preparation of the uterus for conception (1, 2, 10). It has been shown that ram SPPs with molecular weight of 14 and 20 kDa could have a protective effect on sperm function after storage at low temperatures (2, 2). The biological effects of SPPs on spermatozoa, functions are still not fully understood (5, 6, 10). This prompted us for further evaluation of the role of boar SPPs on sperm cells motility and viability after cooling.

The aim of the study was to investigate the effect of SPPs with different molecular weights on the motility and velocity parameters of boar spermatozoa after *in vitro* storage at low temperatures (4°C).

### Materials and methods

Preparation of samples: The ejaculates were collected from healthy Large White boars. Immediately after collection of semen, sperm motility and concentration, number of live and dead sperm cells, semen pH, survival and morphological analysis were assessed. SP was yielded by centrifugation of semen at 2000 rpm, at 4°C for 5 min. Afterwards supernatant was collected and again centrifuged at 12 000 rpm for 5 min, than filtered through a 0.22 µm membrane (Millipore) and kept on -80°C until assay.

The semen samples used in the study were odorless, without agglutination, with concentration of 200-400 x 10<sup>6</sup> sperm cells/ml and with semen pH between 7.3 - 7.8.

Chromatography separation of seminal plasma: For Gel Permeation Chromatography 1 ml of the SP was loaded onto semipreparative column TSK gel G3000SW, (TOSOH BIOSCIENCE) at a flow rate of 6ml/min. After GPC nine SPPs fractions were obtained. Protein content in the collected samples was determined and visualized by 12% SDS PAGE after silver staining (Garl Roth).

Evaluation of seminal plasma proteins effect: To evaluate the effect of SPPs on spermatozoa eight ejaculates from Large White boars were used. After quantification of semen parameters samples were divided into two equal parts, each one was diluted 1:2 with extender. Controls were included as follows: C+ (with whole SP) and C- (without SP), instead SPPs to C-only extender was added. Semen samples were centrifuged at 2000 rpm for 5 min at room temperature, to exclude the SP, treated sperm cells were resuspended with extender at concentration of 1:4. Resuspended sample were aliquated into equal volumes and 500 µl of SPPs from fractions 1, 2, 3, 4, 5, 6 and 7 were added to the appropriate sample, to a final volume of 1 ml. The samples were incubated at 4<sup>0</sup>C for 24h.

Evaluation of boar sperm motility by sperm computer analyzer: Motility of sperm cells was monitored prior and after incubation at 4<sup>0</sup>C for 24h, the assessment was done by Sperm Class Analyzer (Micropticum, Spain). Measurements were conducted using the Motility&Concentration Software and for each sample following parameters were recorded: progressive motility (an actual space-gain motility); non progressive motility, static, curvilinear velocity (VCL; shows the rate of sperm for the actual time elapsed from point to point, or distance traveled over a given period of time); straight line velocity (VSL; the straight line distance from beginning to end of a sperm track divided by the time taken) and average path velocity (VAP; the speed of sperm motion for the average distance traveled in space time), Velocity data were in µm/sec.

## Results

### *Result after chromatography separation of SPPs.*

After GPC nine major SPPs fractions was obtained. Fraction 1, 2 and 3 contained SPPs with high MW from 55 to 200 kDa, fraction 4 and 5 contained proteins with MW from 20 to 97 kDa, and low MW proteins (up to 30 kDa) were found in samples 6 and 7.

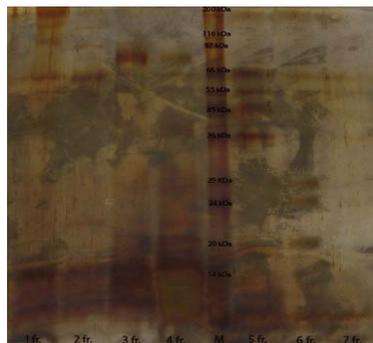


Figure 1. SDS PAGE of separated seminal plasma proteins.

### *Evaluation on sperm velocity parameters*

Sperm motility was influenced in the samples after incubation of boar spermatozoa at 4<sup>0</sup>C for 24h with separated SPPs. It was shown at Table1, that cooling affected in a significant reduction of total motility of the spermtozoa.

The percentage of static cells was significantly higher in samples that contained SPPs from fractions 2, 3, 4 and C- than in the samples C+ and 6. The percentage of progressive motile spermatozoa in samples 6 and C+ was 51.00±5.4 and 43.70±9.49, while in samples C,

1 and 3 it was  $14.63 \pm 2.06$ ,  $31.60 \pm 7.18$  and  $30.32 \pm 5.53$ , the difference between samples 6 and 3 was statistically significant ( $p < 0.05$ ).

Table 1. Sperm motility parameters (%) after 24 h incubation at 4°C (n=8)

Samples	Progressive motile	Non progressive motile	Static
C+	$43.70 \pm 9.49^a$	$40.75 \pm 5.70^a$	$15.55 \pm 4.05^c$
C-	$14.63 \pm 2.06^c$	$26.31 \pm 4.29^c$	$59.08 \pm 2.32^a$
1 fr.	$31.60 \pm 7.18^b$	$42.63 \pm 4.01^c$	$25.77 \pm 4.09^b$
2 fr.	$28.76 \pm 8.51^b$	$39.01 \pm 3.06^c$	$32.23 \pm 5.04$
3 fr.	$30.32 \pm 5.53^b$	$39.20 \pm 8.28$	$30.48 \pm 10.49$
4 fr.	$33.28 \pm 5.62$	$32.1 \pm 5.77$	$34.65 \pm 11.09$
5 fr.	$39.73 \pm 11.72$	$40.54 \pm 6.17$	$19.73 \pm 4.10^c$
6 fr.	$51.00 \pm 5.41^a$	$32.05 \pm 2.11^a$	$17.95 \pm 3.64^c$
7 fr.	$37.25 \pm 6.92$	$41.18 \pm 6.70$	$21.57 \pm 1.34^b$

Data are presented as mean  $\pm$  SD, Significant difference shown between <sup>a</sup> and <sup>b</sup>  $p < 0.05$ ; <sup>a</sup> and <sup>c</sup> at  $p < 0.001$ .

The velocity parameters after incubation at 4°C for 24h are shown on Table 2. In all samples the mean values decreased significantly during low temperature storage. The higher values of VCL were in samples C+ and in samples incubated with fraction 6 in contrast to parameters found in samples C-, 1, 2, 3 and 7. Differences between samples C+, 6 and C- were statistically significant ( $p < 0.001$ ). VAP was lower in samples without SP and in samples with spermatozoa that were incubated with high MW seminal plasma proteins VSL parameters were lower in sample C- compared to fraction 6.

Table 2. Sperm velocity parameters - VCL, VSL and VAP after 24 hours incubation the boar sperm of 4°C (n=8).

Samples	VCL ( $\mu\text{m}/\text{sec}$ )	VSL ( $\mu\text{m}/\text{sec}$ )	VAP ( $\mu\text{m}/\text{sec}$ )
C+	$46.56 \pm 5.39^a$	$19.10 \pm 8.24$	$31.65 \pm 9.48^a$
C-	$14.63 \pm 2.84^b$	$8.63 \pm 1.29^b$	$13.63 \pm 1.69^b$
1 fr.	$39.96 \pm 12.64$	$12.73 \pm 3.35$	$18.60 \pm 3.78$
2 fr.	$34.90 \pm 2.25$	$18.61 \pm 4.57$	$25.69 \pm 4.97$
3 fr.	$36.87 \pm 11.18$	$12.30 \pm 2.11$	$21.85 \pm 5.42$
4 fr.	$39.96 \pm 6.90$	$15.25 \pm 2.47$	$26.08 \pm 4.77$
5 fr.	$33.93 \pm 3.55$	$11.70 \pm 3.01^b$	$19.40 \pm 3.11$
6 fr.	$47.25 \pm 4.64^a$	$26.75 \pm 2.16^a$	$23.85 \pm 3.10$
7 fr.	$27.35 \pm 6.33$	$13.00 \pm 1.90^b$	$21.88 \pm 2.40$

Data are presented as mean  $\pm$  SD. Significant difference shown between <sup>a</sup> and <sup>b</sup> at  $p < 0.05$ ; between <sup>a</sup> and <sup>c</sup> at  $p < 0.001$

## Discussion

In the study the ability of SPPs to improve viability and velocity parameters during low temperature storage of boar spermatozoa was tested. It was established that storage of spermatozoa at low temperature resulted in a significant reduction of the progressive motile sperm cells. It has been already shown that the addition of definite SPPs may protect sperm cells (7). Recently it has been demonstrated that proteins from ram SP with low molecular weight may protect the sperm PM and revert the damage of spermatozoa caused by cold

shock (2). Our results coincide with the findings of other authors which address the relevance of specific seminal plasma proteins for the function and viability of sperm cells (2, 3, 9). We found that boar spermatozoa incubated in extenders which contained low molecular weight SPPs from 16kDa to 29 kDa (contained in fraction 6) retain better progressive motility, VCL, VSL and VAP parameters. These proteins may be candidates' for protective agents during low temperature storage of boar semen. We suppose that the effect of these proteins might be exerted by adhesion in specific regions of the plasma membrane. By means of adhesion the proteins may stabilize the membrane and most probably might prevent damages caused by low temperature storage. Spermatozoa with stabilized PM showed better viability and motility of boar spermatozoa and most probably have retained fertilizing ability. Our results indicated that isolated seminal plasma proteins, contained in sample 6 retained the greatest number of spermatozoa with well-preserved velocity parameters in comparison with other samples after storage at 4°C for 24 hours. Consequently the presence of proteins with MW lower than 30 kDa may protect spermatozoa through preservation of PM integrity and sperm velocity parameters.

In conclusion, our results indicated that the incubation of boar spermatozoa with separated low molecular weight seminal plasma proteins (16-29 kDa), preserved better the motility, velocity parameters (VCL, VSL, VAL) of the germ cells. This fraction (fr.6) contained low molecular weight SPPs, which might have a protective action on boar spermatozoa, function and prove candidate for a protective agent in media designed for storage of boar semen.

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### **DO3. SMALL MAMMALS AS INDICATORS OF STATUS OF THE ENVIRONMENT NEAR RESEARCH REACTOR**

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The biomonitoring is a component of the radiation monitoring for assessment of the impact of the nuclear facilities on the living organisms.

The biomonitors are the key indicators for the contaminant distribution and effect upon biota. Small rodents belong to the most abundant vertebrates living in the forest ecosystems. For this reason, they became popular as biomonitors in studies on the effect of the pollutants on living organisms. Small rodents as biomonitors satisfy the conditions - to be small, easy to catch, they should inhabit a small area. The rodents *Microtus arvalis* (Field vole), *Apodemus flavicollis* (yellow-necked wood mouse), *Clethrionomys glareolus* (bank vole) were used to estimate the environmental status.

Seven sampling control points were selected in the site of IRT-2000 research reactor in Sofia. The global positioning system device is used for easy access to the sampling points.

Sampling is performed during the period 2010 – 2011 in monitoring network points around the reactor and reference point „Valchata scala”, Vitosha mountain. The rodents were studied for their  $\beta$  and  $\gamma$  radionuclides.

For  $\gamma$  measurement the whole body of the animals were dried at 60<sup>0</sup> C to obtain constant weight and then were homogenized

The radionuclide content in the samples from investigated area has been performed by low level gamma spectrometry. All results show content of natural and artificial radionuclide under the lowest limit of detection (LLD) for the analytical procedure applied, except the natural radionuclides <sup>228</sup>Ra, <sup>226</sup>Ra, <sup>228</sup>Th and <sup>40</sup>K and the artificial <sup>137</sup>Cs.

The results obtained are compared with these from the referent point at Vitosha Mountain.

For gross beta activity (beta-emitting isotopes  $^{90}\text{Sr}$ ,  $^{40}\text{K}$  and etc.) the method includes preliminary preparation of samples, ashing at a temperature of  $450^\circ\text{C}$  for 4 hours and measurement with a LAS 3A low level activity system (30 % efficiency on  $^{40}\text{K}$  and background 1 cpm-1). The results for gross beta activity varies between 163 and 242 Bq/kg.

## **DO4. STUDY ON MINERAL WATER IN NORTHWEST BULGARIA**

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### **Abstract**

The paper deals with the determination of radioactivity of mineral water from northwest Bulgaria. Different parameters in mineral water were analyzed: physicochemical indices and radionuclides.

For the determination of physical and chemical indices (pH, temperature, conductivity and dissolved oxygen) a portable pH meter "SENSION 156" was used.

The gamma emitters (Cs-137 and K-40) in mineral water were determined by two High Purity Germanium (HPGe) detectors and program ANGES.

### **INTRODUCTION**

The small territory of Bulgaria is among the richest in mineral water regions of the world [2]. Nowadays, there are more than 550 mineral water deposits found in Bulgaria [2], with about 1600 springs, having different temperature, mineral composition and radioactivity.

The specific chemistry and purity of the natural mineral water are preconditions for the satisfaction of the human needs for water, mineral salts and trace elements. This main purpose of use requires periodic updating, refining and adding information for the mineral waters. The study of the properties of the mineral waters in Bulgaria allows optimization of their use in a range from spa treatments to drinking and household needs [1].

Natural waters contain a number of alpha ( $^{238}\text{U}$ ,  $^{230}\text{Th}$ ,  $^{226}\text{Ra}$ ,  $^{210}\text{Po}$ ) and beta emitters from the natural decay series of uranium, thorium and actinium, the primordial isotope,  $^{40}\text{K}$  and artificial isotopes, such as  $^{137}\text{Cs}$ .

The aim of the study is parallelly with the parameters already investigated (as pH, mineral content etc.) some new characteristics (as gamma emitters) to be measured, not only in the well known mineral sources (Gorna Bania, Devin, Bankia etc.) but in some not investigated until now mineral sources in North-West Bulgaria to be studied.

### **MATERIAL AND METHODS**

**Sampling.** Subject of research is the mineral water from different springs near the town of Berkovitsa (Lakatnik, Varshets, Spanchevtsi, Slatina, Burzia) and Vratsa (Liliache and Banitsa) (Figure 1).

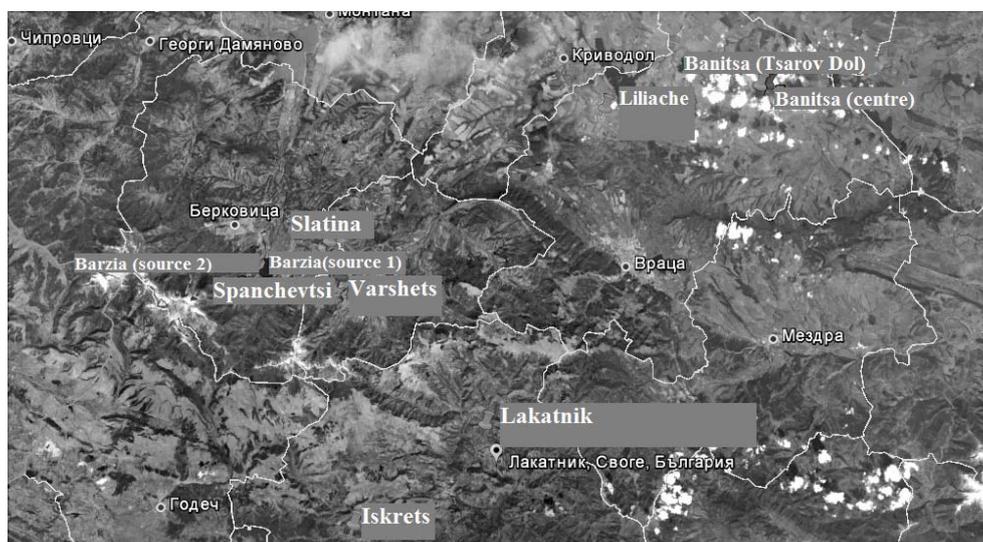


Fig.1 Localization of samples of mineral water from Berkovitsa and Vratsa region

Geographical parameters of each sampling point were measured by GPS (Table 1).

Table 1. Geographical characteristics of mineral water

№	Sampling point	GPS - data	Altitude, m	Gamma-background $\mu\text{Sv/h}$	pH	Temp., $^{\circ}\text{C}$	Dissolved Oxygen, mg/L	Conductivity, $\mu\text{S/cm}$
01	Iskrents	N 42° 59' 13.4'' E 023° 15' 0.05''	546,5	0,13	7,7	20.0	6.3	287.0
02	Lakatnik	N 43° 04' 58.3'' E 023° 23' 32.1''	491,6	0,12	8,0	25.0	5.8	466.0
03	Varshets	N 43° 11' 31.0'' E 023° 17' 13.6''	396,6	0,14	9,6	30.8	3.0	171.4
04	Spanchevtsi	N 43° 11' 13.0'' E 023° 14' 09.6''	523,0	0,08	9,4	26.4	5.4	159.0
05	Slatina	N 43° 12' 53.5'' E 023° 13' 25.1''	369,5	0,13	9,6	25.1	3.1	304.0
06	Barzia (source 1)	N 43° 11' 25.2'' E 023° 09' 16.7''	574,3	0,09	8,2	25.0	6.3	94.8
07	Barzia (source 2) „Kom”	N 43° 11' 24.2'' E 023° 09' 31.3''	520,8	0,11	10,0	30.7	3.6	198.9
08	Liliache	N 43° 19' 21.2'' E 023° 31' 21.2''	204,0	0,10	7,7	18.9	7.3	207.0
09	Banitsa /center/	N 43° 20' 27.1'' E 023° 41' 30.5''	264,2	0,09	7,4	16.0	5.4	686.0
10	Banitsa /Tsarov Dol/	N 43° 20' 28.8'' E 023° 42' 03.8''	254,0	0,09	7,5	17.9	4.7	883.0

Mineral water was taken directly from the springs. For sampling standard plastic bottles prepared by a standard procedure: washing three times with distilled water, followed by 48 hours standing with distilled water, were used.

**Physicochemical indices.** The temperature and the physicochemical indices (dissolved oxygen content, pH and conductivity) were measured by a portable pH meter SENSION 156.

**Gamma activity.** As a pretreatment step for gamma measurements, water samples were evaporated from 1 L to 40 ml at 80-100 °C to increase the concentration of radionuclide. Gamma analysis was carried out with two HPGe detectors [3], with 33% relative effectiveness and 2.0 keV energy resolution, and with 50% relative effectiveness and 2.5 keV energy resolution, respectively. Background spectrum was measured several times for 600 000 sec. All samples were measured for 86400 sec (24 hours). The spectra are processed by ANGES program [4]. Assigned were parameters for energy and effectiveness calibration. The program calculated automatically peak position, area, FWHM, with their uncertainties. ANGES identified the radionuclide and calculated its activity in Bq.l<sup>-1</sup>.

## RESULTS AND DISCUSSION

**Chemical Reaction pH.** The results obtained show that the mineral water under investigation have alkaline reaction – pH between 7,4 to 10,0 (Table 1).

**Temperature.** The temperature of the measured water varies from 16,0 °C to 30,8 °C (see Table 1).

**Dissolved Oxygen.** Dissolved oxygen analysis measure the amount of gaseous oxygen (O<sub>2</sub>) dissolved in an aqueous solution. Oxygen gets into water by diffusion from the surrounding air, by aeration (rapid movement), and as a waste product of photosynthesis. In our measurements the values for dissolved oxygen are from 3,0 mg/l to 7,3 mg/l.

**Electrical conductivity.** The electrical conductivity of water estimates the total amount of solids dissolved in it – Total Dissolved Solids (TDS). The conductivity varies from 94,8 µS/cm to 883,0 µS/cm (See Table 1).

**Gamma Analysis.** Gamma analysis is carried out with two HPGe detectors with different effectiveness. Measurement time (24 hours) is maximum acceptable for ecological samples. It ensures enough count statistics for the range of interest (0 to 3000 keV). Under these conditions we didn't detect any gamma peaks over the background. Nevertheless, we processed two areas: 662 keV – the position of the peak of Cs-137, the most detected radionuclide from human activity; 1460 keV – the peak of K-40, one of the most widespread natural radionuclides. In accordance to the expectation, the definite values for specific activity are under the Minimum Detection Limit (MDA), which are respectively 0.04 Bq/ l<sup>-1</sup> for Cs-137 and 0.50 Bq/ l<sup>-1</sup> for K-40 (Table 2).

Table 2. Results from gamma-spectrometric analysis for <sup>137</sup>Cs and <sup>40</sup>K

Sample №	Position	A <sub>0</sub> <sup>137</sup> Cs, Bq.l <sup>-1</sup>	A <sub>0</sub> <sup>40</sup> K, Bq.l <sup>-1</sup>
01	Iskrets	≤ 0,04	0,60
02	Lakatnik	≤ 0,04	≤0,5
03	Varshets	≤ 0,04	≤0,5
04	Spanchevtsi	≤ 0,04	≤0,5
05	Slatina	≤ 0,04	≤0,5
06	Barzia (source 1)	≤ 0,04	≤0,5
07	Barzia (source 2)	≤ 0,04	≤0,5
08	Liliache	≤ 0,04	≤0,5
09	Banitsa /Center/	≤ 0,04	≤0,5
10	Banitsa /Tsarov Dol/	≤ 0,04	≤0,5

**Note: MDA** =0,04 for Cs-137 and 0,5 Bq.l<sup>-1</sup> for K-40

The results prove the absence of gamma emitters in investigated mineral water samples.

## CONCLUSION

Mineral waters from 10 springs in Northwest Bulgaria (Berkovitsa and Vratsa deposits) have been investigated. Physicochemical investigation and gamma spectroscopic analysis were used. We established the absence of pollution by gamma emitting radionuclides, including human done Cs-137 and natural K-40.

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## DO5. METAL IONS IN HUMAN SEMEN

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Human semen contains high concentrations of trace elements including iron and copper, in bound and free (ionic) form. These trace elements play an essential role in affecting various parameters of semen. On one hand these elements could play a beneficial role, because they are co-factors of antioxidant enzymes, determining their activity. On the other hand they could act as prooxidants (via Fenton/Fenton-like reaction) when they are present in excess. Thus, iron is an essential constituent of catalase enzymes, and copper is a component of Cu,Zn-superoxide dismutase, but each of them could initiate oxidative stress outcomes. Human spermatozoa are abundant of polyunsaturated fatty acids and this fact determines their high

sensitivity to lipid peroxidation, which in turn leads to decreased motility and difficult fusion between sperm and oocyte. The analysis of the seminal trace element concentration and their correlation with various sperm parameters indicated that in the astheno-group the concentrations of iron and copper are usually low and they are accompanied with decreased glutathione level and increased level of malondialdehyde (an index of the lipid peroxidation). Along with these observations there were some cases with much higher (doubled) concentration of the iron and motility less than 10%, comparing to the normo-group. Therefore it could be concluded that an optimal physiological level of the trace metals is necessary for proper sperm functioning and any alteration on either side of this range is likely to induce a negative effect.

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## DO6. THE ANTITUMOR POWER OF PLATINUM COMPOUNDS

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#### Abstract

The review summarizes data about biological activity of platinum anticancer agents. Mechanism(s) of action, therapeutic ability, advantages and disadvantages of the leading antitumor drug cisplatin as well as of the other two worldwide available second and third generation drugs carboplatin and oxaliplatin are presented. Information about locally available drugs Lobaplatin (China), Nedaplatin (Japan), Heptaplatin (South Korea) and about the investigational orally available lipophilic platinum anticancer agent Satraplatin is also discussed

## 1. Cisplatin

### 1.1. Discovery

The square planar platinum (II) complex,  $\text{cis-}[\text{PtCl}_2(\text{NH}_3)_2]$  (cisplatin, cis-DDP, or CDDP), was first synthesized in 1844 by Michael Peyrone in Turin and is historically known as Peyron's chloride. The structure of cisplatin was unknown until the work of Alfred Werner in 1893 who developed the theory of coordination chemistry and showed that ammonia can bond to a metal ion like platinum +2 by donating its lone pair of electrons in a dative or coordinate bond. Thus, in the end of 19<sup>th</sup> century Werner correctly assigned to this compound a cis-geometry, which was confirmed in an X-ray crystal-structure determination reported in 1966.

The biological activity of cisplatin was accidentally discovered in 1965 by Barnet Rosenberg, a biophysicist at the University of Michigan, and his coworkers who reported first the inhibitory activity of this compound on *Escherichia coli* division. It was noticed that when treated with cisplatin *E. coli* bacteria grow to 300 times their normal size but do not divide. Rosenberg hypothesized that if cisplatin could inhibit bacterial cell division it could also stop tumor cell growth. A little bit later these scientists performed experiments with sarcoma 180 and leukemia L1210 bearing mice and the promising results obtained led to cisplatin entering phase I clinical trials in 1971. In 1978, the U.S.A. Food and Drug Administration (FDA) approved cisplatin under the name of Platinol® (Bristol-Myers Squibb) for treating patients with metastatic testicular or ovarian cancer in combination with other drugs but also for treating bladder cancer [1, 2, 21, 43, 53, 58, 62].

### 1.2. Therapeutic ability

Evidence for antitumour activity in humans was first reported against testicular and ovarian cancer, in terms of both objective response and of prolonged survival. Because of its marked renal toxicity, cisplatin was almost withdrawn from clinical trials. Interest was rekindled when hyperhydration with isotonic saline circumvented this problem. One of the advantages of cisplatin, besides its efficacy, is that its toxicity is different from that of other anticancer drugs, thus making it attractive for combination regimens.

Today cisplatin is the world's leading anti-tumour drug for the chemotherapy of human cancer. This platinum compound continues to be used in 50-70% of all cancer patients especially against testicular and ovarian cancer, and it is also used in case of head and neck cancer, cervical and bladder carcinomas, bronchogenic carcinoma, small-cell and non-small cell lung cancers, lymphoma, osteosarcoma, melanoma and neuroblastoma [1, 30, 60, 85,]

In particular, cisplatin-based chemotherapy is curative for the majority of patients with advanced testicular germ cell cancer, which was almost uniformly fatal in the pre-cisplatin era. Prior to the advent of cisplatin combination chemotherapy, the older standard regimens achieved a 50% response rate and a 10% to 20% complete remission rate; however, the cure rate was only 5% to 10%. Currently, the 10-year survival rates of testicular cancer exceeds 95% and the treatment of this disease with cisplatin-based chemotherapy has become a model for a curable neoplasm [10, 26, 56, 72]. It has to be emphasized here that although testicular germ cell tumors are relatively uncommon, they are particularly important as they tend to affect children and young men, representing the most frequently occurring form of cancer in male aged from 15-20 to 35-40 years [15, 57].

### 1.3. Challenges

Although cisplatin is highly effective anticancer agent, it is not ideal because of: 1) low or no activity against some of the most common and socially important malignant neoplasms such as breast, gastrointestinal and renal cancers, leukemia; 2) the development of

drug resistance during treatment is not rare; 3) undesired side effects such as nephrotoxicity, nausea and vomiting, peripheral neuropathy, myelotoxicity, ototoxicity [1, 23, 30] Cardio toxicity (prolongation of QT-interval) has also been associated to CDDP treatment [27].

Nephrotoxicity is a major and dose-limiting side effect of cisplatin, with an incidence reported as 6-13% [24]. Recent studies have shed new lights on the mechanism of cisplatin nephrotoxicity, especially on the signaling pathways leading to tubular cell death and inflammation. It has been suggested that nephrotoxicity is mediated through drug transport into renal epithelial cells, which subsequently causes injury to nuclear and mitochondrial DNA, activation of cell apoptosis and necrosis, and stimulation of inflammatory responses (with T lymphocytes as direct mediators of experimental CDDP nephrotoxicity). Cisplatin induced production of reactive oxygen species (ROS) has also been implicated in its nephrotoxicity [71, 20, 45]. Renoprotective approaches are being discovered and future studies including clinical trials will clarify their effectiveness in the prevention of this problem [19, 55, 61].

CDDP is used to treat *pediatric solid tumors*. A major dose-limiting toxicity in children is hearing loss. A large individual variation exists, since it does not occur in all patients even if they are similarly treated [39]. There are data that up to 93% of patients receiving cisplatin chemotherapy develop progressive and irreversible sensorineural hearing loss which leads to a decreased quality of life in cancer survivors. No treatment is currently available for cisplatin-induced ototoxicity. There is a need for otoprotective agents that could be administered without compromising cancer treatment. Various studies have aimed to assess the potential protective effects of compounds such as antioxidants, anti-inflammatories, caspase inhibitors, anti-apoptotic agents and calcium channel blockers against the toxicity caused by cisplatin in the inner ear with variable degrees of protection. Nevertheless, the pathophysiology of cisplatin-induced ototoxicity remains unclear [27, 28].

The major barrier to cisplatin efficacy is perhaps the drug resistance, which can be either intrinsic or acquired. Even if an initial response occurs, acquired resistance due to mutations and epigenetic events limits efficacy. For cisplatin, which is routinely used in dosages at the limit of its systemic toxicity, the level of resistance can completely eliminate clinical effectiveness. Resistance to cisplatin has been studied extensively and multiple potential mechanisms of resistance have been identified at the cellular and molecular level. The process of cisplatin resistance appears to be multifactorial and includes changes in drug transport leading to decreased drug accumulation, increased drug detoxification, changes in DNA repair and damage bypass and/or alterations in the apoptotic cell death pathways. Reduced platinum accumulation and increased cytoplasmic detoxification by glutathione and/or metallothioneins represent the major causes of inadequate drug concentrations reaching DNA. The mechanism by which platinum drugs enter cells has traditionally been attributed to simple passive diffusion. However, recent evidence suggests a number of active uptake and efflux mechanisms are at play, and altered regulation of these transporters is responsible for the reduced accumulation of drug to the resistant cells. Once DNA binding has occurred, resistance mechanisms include increased DNA repair of adducts, and an ability to tolerate greater levels of DNA damage with concomitant failure to engage programmed cell death (apoptotic) pathways. Decreased DNA binding (e.g. due to high intracellular pH) was described. Alterations in proteins that recognize cisplatin-DNA damage such as mismatch repair and high-mobility group family proteins, alter the cellular response to induce damage and may contribute to damage tolerance. It is generally accepted that during treatment with cisplatin the formation of cisplatin-DNA-adducts is the main lesion leading to cytotoxicity. The fate of cells following cisplatin treatment depends both on the extent of damage induced and on the cellular response to damage. Cisplatin was shown to kill cells by apoptosis. There are data indicating a general association between susceptibility to apoptosis and response to

chemotherapy. Alterations in proteins that determine susceptibility to apoptosis induction such as p53 and the pro- and anti-apoptotic members of the Bcl2 family proteins may also contribute to damage tolerance and therefore to resistance. It is known that the tumor-suppressor proteins p53 and p73, and the oncoprotein c-Myc, which function as transcription factors, influence cellular sensitivity to cisplatin. Several transcription factors involved in cisplatin resistance, including Y-box binding protein-1 (YB-1), CCAAT-binding transcription factor 2 (CTF2), activating transcription factor 4 (ATF4), zinc-finger factor 143 (ZNF143) and mitochondrial transcription factor A (mtTFA) have been identified. The importance of several signaling pathways as well as of the presence of quiescent non-cycling cells for cisplatin resistance phenomenon was also discussed in the literature [9, 11, 34, 40, 48, 56, 64, 66, 68, 74, 77]. Elucidation of these mechanisms of resistance has been essential in involving a basis for the development of Pt-based complexes capable of circumventing cisplatin resistance [1]. Different strategies for overcoming cisplatin resistance have been proposed such as application of copper-lowering agents [14], DNA repair inhibitors [6].

Cisplatin resistant cell lines are often more sensitive to another chemotherapeutic drug paclitaxel, or are able to be sensitized to cisplatin with paclitaxel pre-treatment. The understanding of this sensitization by paclitaxel using cell models of cisplatin resistance will lead to improvements in the clinical treatment of cisplatin resistant tumours [75].

#### **1.4. Mechanism of action**

Platinum chemotherapy exerts its cytotoxic effect by forming DNA adducts and subsequently inhibiting DNA replication. After injection (oral administration is not possible due to highly gastric acidity) cisplatin binds to plasma proteins and is renally excreted (30-70%). The remaining fraction is transported by the blood in an unaltered form. After passive transport of neutral cisplatin through cell membranes, it is rapidly hydrolyzed due to the markedly lower chloride concentration in intracellular regions. The hydrolysis reaction is the rate-determining step for DNA binding. Within cells, about 40% of the platinum is present as  $[\text{cis-Pt}(\text{NH}_3)_2\text{Cl}(\text{H}_2\text{O})]^+$  which is assumed to be the active form of the antitumor agent. Furthermore, the cationic species would be likely to approach and coordinate to the negatively charged DNA [3, 38]

Depending on cell type and concentration, cisplatin induces cytotoxicity, e.g., by interference with transcription and/or DNA replication mechanisms. Additionally, cisplatin damages tumors via induction of apoptosis, mediated by the activation of various signal transduction pathways, including calcium signaling, death receptor signaling, and the activation of mitochondrial pathways. The role of early plasma membrane events in cisplatin apoptosis such as changes in plasma membrane fluidity, inhibition of NHE1 exchanger, activation of acid sphingomyelinase and their consequences on the Fas death pathway in response to cisplatin have been reported. CDDP is primarily considered as a DNA-damaging anticancer drug, forming different types of bifunctional adducts with cellular DNA [27, 59].

#### **2. Other platinum compounds**

The limitations of cisplatin have stimulated research in the field of platinum antitumour chemistry by giving specific goals. These include reduction in toxicity of cisplatin; circumvention of the acquired drug resistance in certain tumors; increased spectrum of activity and oral administration for the new anticancer drugs [1].

Since the introduction of cisplatin around three thousand platinum derivatives have been synthesized and tested against tumour cells; but only thirty compounds have reached clinical trials and more than half of those have been rejected. The main aim of these intensive investigations was to obtain drugs with at least an equal activity but reduced toxicity

compared to cisplatin. Today, six are used clinically: three of them – cisplatin, carboplatin and oxaliplatin, gaining international marketing approval, whereas the other three are applied in a few countries - nedaplatin in Japan, lobaplatin in China, and heptaplatin – in South Korea. Currently there are four drugs in the various phases of clinical trial (satraplatin, picoplatin, Lipoplatin and ProLindac). No new small molecule platinum drug has entered clinical trials since 1999 which is representative of a shift in focus away from drug design and towards drug delivery in the last decade [1, 22, 84].

Cisplatin is not only the first platinum complex which exhibited antitumour activity, but it is also the complex with the simplest structure. Modifications to the leaving groups or the two amine ligands of cisplatin resulted in the development of carboplatin and oxaliplatin. Carboplatin was developed to lower the toxicity profile of cisplatin by replacing the dichloride-leaving groups with 1,1-cyclobutanedicarboxylate. Oxaliplatin was designed with a 1,2-diaminocyclohexane group in place of the two amine ligands based on the prediction that a bulkier platinum-DNA adduct would interfere with DNA repair and overcome cisplatin resistance. Satraplatin (JM216) was developed to circumvent acquired resistance by replacing one of the amine ligands of cisplatin with a bulkier cyclohexylamine group. More recently, a strategy to overcome resistance due to interaction with thiol-containing molecules led to the synthesis of picoplatin (AMD473) in which one of the amines linked to Pt was replaced by a bulky methyl substituted pyridine allowing the drug more time to reach its target, DNA, and preventing the platinum center from being inactivated by glutathione [1, 53, 67].

As for the mechanism of action, it is now accepted that the primary target of platinum drugs is DNA, to which they bind covalently, most frequently to neighbouring guanine N7 sites. Chelation of platinum induces distortion of the double helix that affect both replication and transcription and ultimately lead to cell death [65, 76].

## 2.1. Carboplatin

The strategy to reduce toxicity involved increasing the solubility in water and stability of the complexes. This has been generally achieved by replacing the chloro ligands either with chelating carboxylate, oxalate, sulfate or glycolate. This kind of leaving group is the main feature of the second generation platinum drugs. The most successful of them is carboplatin  $[(\text{NH}_3)_2\text{Pt}(\text{C}_4\text{H}_6)_4]$ . Carboplatin entered the U.S.A. market as Paraplatin(®) in 1989 for initial treatment of advanced ovarian cancer in established combination with other approved chemotherapeutic agents [53].

Carboplatin is better tolerable due to a reduced toxicological profile while having an essentially identical spectrum of activity as cisplatin. In practice carboplatin has replaced cisplatin in a number of indications. Besides its efficacy and lower toxicity, the single intermittent bolus or shorter infusion schedule is more practical than the protracted infusion of cisplatin. Also, although carboplatin is more expensive than cisplatin, the complete cost of treatment is cheaper. Cisplatin and carboplatin have substantial activity in sensitizing tumour cells to radiotherapy in head and neck cancer, lung, oesophagus, cervix, bladder and rectal cancer. Unfortunately, cross-resistance occurs to both platinum agents. The dose-limiting toxicity of carboplatin is myelosuppression, mainly thrombocytopenia [1, 21, 30, 74].

## 2.2. Oxaliplatin

Oxaliplatin, a third-generation platinum drug with a 1,2-diaminocyclohexane (DACH) carrier ligand, was approved in 1994 under the name of Eloxatin(®). This drug has a higher efficacy and a lower toxicity than cisplatin in *in vivo* preclinical studies. Oxaliplatin gave interesting results in ovarian, breast, head and neck cancer, in most non-Hodgkin's lymphoma, malignant melanoma, glioblastoma and non small cell lung cancer (NSCLC). The best results to date have been obtained in the treatment of (metastatic) colorectal cancer - it is

the first platinum-based drug found to be active against metastatic colorectal cancer in combination with 5-fluorouracil and folinic acid [13, 30, 53].

Various mechanisms of action are ascribed to oxaliplatin. Like other platinum-based compounds, oxaliplatin exerts its cytotoxic effect mostly through DNA damage. Apoptosis of cancer cells can be caused by formation of DNA lesions, arrest of DNA replication, inhibition of RNA synthesis, and triggering of immunologic reactions [2, 47]. Although analogous mechanism of action cisplatin and oxaliplatin might activate different signal transduction pathways, ultimately leading to apoptotic DNA fragmentation and cell death [78].

It has to be emphasized that oxaliplatin expresses no cross resistance with cisplatin nor carboplatin [2].

Oxaliplatin has a unique pattern of side effects unrelated to those observed with other therapeutic platinum derivatives. During the course of oxaliplatin clinical trials, the adverse effects more often cited were myelosuppression, gastrointestinal tract toxicity, and a neuropathy. These side reactions have been easy to manage with appropriate awareness from patients and care providers [4, 13]. Oxaliplatin produces a symmetric, axonal, sensory distal primary neuropathy without motor involvement that has been found to be dose-limiting [4]. The neuropathy appears to be cumulative with successive cycle, but is rapidly reversible after drug cessation [30].

To date, several neuroprotective agents including thiols, neurotrophic factors, anticonvulsants and antioxidants have been tested for their ability to prevent neuropathy but the clinical data are still controversial [2, 4]. The ability of calcium gluconate and magnesium sulphate (when infused before and after oxaliplatin administration) to significantly reduce the incidence of chronic peripheral neuropathy symptoms secondary to oxaliplatin has been recently reported. There is no evidence to suggest a decrease in the anticancer effects of oxaliplatin when calcium and magnesium infusions are administered [2, 31, 33]. Oxaliplatin also exhibits synergism with other cytotoxic drugs, but the underlying mechanisms of those effects are not well clarified. Synergy was observed with leucovorin and 5-fluorouracil (5-FU) even in 5-FU-resistant tumours [18, 22]

It has recently been reported that Dasatinib (oral multi- BCR/Abl and Src family tyrosine kinase inhibitor) plays synergistic role with oxaliplatin in inhibiting gastric cancer cell growth both *in vitro* and *in vivo*, via inhibiting Src activity stimulated by oxaliplatin [69]. Many studies revealed the efficacy of oxaliplatin in combination with other anticancer agents (Bevacizumab - monoclonal antibody blocking VEGF, Cetuximab – EGF receptor monoclonal antibody, Irinotecan, Raltitrexed, 5-fluorouracil and oral fluoropyrimidines such as Capecitabine) in the treatment of metastatic colorectal cancer [7, 12, 36 49, 50, 54].

### **2.3. Lobaplatin**

Lobaplatin was introduced into clinical trials in 1992 for cisplatin-resistant ovarian cancer, head and neck malignant neoplasms, and small-cell lung cancer. This third-generation platinum antineoplastic agent has been approved in China for the treatment of breast cancer and has been used also to treat patients with small-cell lung cancer, and chronic myelogenous leukemia [1, 86].

Intrapleural or intraperitoneal infusion of lobaplatin (20-30 mg/m<sup>2</sup>) has been reported to be a safe treatment with encouraging efficacy for patients with malignant pleural effusion or ascites [37]. It has been demonstrated that the use of lobaplatin alone or in combination with antitubulin agents might be a rational and novel therapeutic strategy for human NSCLC [86].

The results obtained by Wang et al., indicate that Lobaplatin could be an effective chemotherapeutic agent in human cholangiocarcinoma treatment through induction apoptosis and cell cycle arrest [81].

## 2.4. Nedaplatin

Nedaplatin (cis-diammine-glycolatoplatinum) was developed in 1983 in Japan to provide a treatment with effectiveness similar of that of cisplatin but with decreased renal and gastrointestinal toxicities. This compound received approval for clinical use in Japan in 1998, the official indications are head and neck, testicular, lung (NSCLC and SCLC), oesophageal, ovarian and uterine cervical cancer. Unfortunately, nedaplatin (which is a cisplatin analogue with two ammine ligands, like carboplatin) is cross resistant with cisplatin. The dose-limiting toxicity of nedaplatin is myelosuppression, including leucopenia, anemia, and primarily thrombocytopenia. Nedaplatin-induced nephrotoxicity can be also observed [22, 70].

High anticancer activity has been reported for nedaplatin in Phase II studies of patients with non-small cell lung cancer (NSCLC), esophageal cancer, uterine cervical cancer, or head and neck cancer [70]. Combination of nedaplatin and paclitaxel achieved an encouraging clinical outcome, with relatively minimal toxicities for patients with metastatic esophageal carcinoma [32]. The combination of nedaplatin and docetaxel was found to be effective for recurrent esophageal cancer [28] as well as for recurrent squamous cell carcinoma of the cervix [81]. Nedaplatin with concurrent radiotherapy is an effective and well-tolerated regimen for advanced squamous cell carcinoma of the uterine cervix [87].

## 2.5. Heptaplatin

Heptaplatin, cis-malonato [(4R,5R)-4,5-bis(amino-methyl)-2-isopropyl-1,3-dioxolane] platinum (SKI-2053R, Sunpla) is a new platinum derivative with anti-tumour activity comparable to cisplatin on various cell lines. Preclinical studies suggest that it is less nephrotoxic than cisplatin [63].

Heptaplatin has been approved for the treatment of gastric cancer in South Korea. There are data that low dose paclitaxel may sensitize tumour cells to heptaplatin. This compound also shows greater penetration through multilayers of tumour cells compared to cisplatin and oxaliplatin, which may be an important benefit for solid tumour treatment. Overall, there are data supporting the clinical development of heptaplatin in combination with low-dose paclitaxel against cultured head and neck squamous cancer cells [41].

An additive cytotoxic effect on NCI-H520 human cell line (squamous carcinoma cell line of the lung) was observed when low dose heptaplatin was combined with high dose ionizing radiation. A moderate dose of heptaplatin and a low dose of ionizing radiation were found to express an additive cytotoxic effect on the growth of SQ20B human squamous carcinoma cell line of the larynx. The effects of heptaplatin and ionizing radiation were additive rather than synergistic because both cell lines are radioresistant [63]. Heptaplatin showed similar effects to cisplatin when combined with 5-FU in advanced gastric cancer patients with tolerable toxicities [42].

A novel water-soluble heptaplatin analogue, cis-[(4R,5R)-4,5-bis-(aminomethyl)-2-isopropyl-1,3-dioxolane](3-hydroxy-1,1-cyclobutanedicarboxylato)platinum(II), has been synthesized. The complex has been found to be more active and less toxic than its parent drug heptaplatin [46].

## 2.6. Satraplatin

Satraplatin (JM-216) is an investigational third-generation orally available lipophilic platinum anticancer agent, which has demonstrated safety and antitumor activity in vitro, in vivo and in clinical settings. It is active in lung, breast, ovarian and prostate cancer, and appears to have good efficacy in combination with radiation for lung and head and neck cancer. Preclinical data suggest it may also be effective for the treatment of certain cisplatin-refractory tumours. Satraplatin is under evaluation for the treatment of patients with

hormone-refractory prostate cancer whose disease has progressed following prior systemic therapy (Choy, 2006; Bhargava, Vaishampayan, 2009; Sonpavde, Sternberg, 2009; Doshi et al., 2012).

Satraplatin has preclinical antitumour activity comparable with that of cisplatin and, clinically, has a more manageable and milder side-effect profile. Satraplatin was associated with dose-limiting myelosuppression, but no significant ototoxicity, neurotoxicity or nephrotoxicity [5, 25].

Use of satraplatin as an alternative platinum cytotoxic agent is particularly attractive because of: relative ease of administration; potential lack of cross-resistance with other platinum agents; milder toxicity profile; theoretical advantage as a radiosensitizer, and activity in cancers historically nonresponsive to platinum drugs. All these advantages of satraplatin indicate that it could be useful in settings that preclude cisplatin, for example, underlying renal dysfunction, elderly age and poor performance [5, 25].

### 3.

Second and third generation platinum drugs, which are in clinical use, even though they have some improved characteristics compared to the parental compound in terms of reduced toxicity, do not seem able to broaden the spectrum of action of cisplatin significantly [65, 76]. In order to overcome the severe side-effects of a platinum-based chemotherapy, to improve clinical effectiveness and to broaden the spectrum of activity is still a need for novel, innovative platinum antitumour agents. Currently, different concepts are used in the design of new platinum-based antitumour drugs which can be grouped in three categories [30, 83, 88]. It has been suggested that the next big advance in platinum-based chemotherapy is not likely to come from the development of new drugs, but from the controlled and targeted delivery of already approved drugs or those in late stage clinical trials. Encapsulation of platinum drugs inside macromolecules has already demonstrated promise, and encapsulation within cucurbit[n]urils has shown particular potential. Partial or full encapsulation within cucurbit[n]urils provides steric hindrance to drug degradation by peptides and proteins, and the use of different sized cucurbit[n]urils allows for the tuning of drug release rates, cytotoxicity and toxicity [35, 82]. Reformulating platinum drugs using liposomes has resulted in the development of L-NDPP (Aroplatin™), SPI-77, Lipoplatin™, Lipoxal™, and LiPlaCis®. Liposomes possess several attractive biological activities, including biocompatibility, high drug loading, and improved pharmacokinetics, that are well suited for platinum drug delivery [44, 51]. Liposomal cis-platin or lipoplatin is under a phase III randomized clinical trial for patients suffering from small cell lung cancer whereas polymer-based drug, Prolindac™ is currently under investigation for pretreated ovarian cancers in up to eight European centers [53].

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## **DP1. CONTROL OF CHRONIC MICROELEMENTOSES IN FARM ANIMALS FROM EAST SIBERIA BASED ON THE CHEMICAL ELEMENTAL COMPOSITION OF THEIR HAIR (CECH)**

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### **Abstract**

For the case of 6 test sites in Moscow, Voronezh and Chita regions where some 500 cattle hair samples were taken, the chemical element composition of their hair (CECH) was found to have regional features and to show slight dependence on the physiological condition and age of animals. For a number of chemical elements, CECH of cattle shows strong differences depending on the coat color. CECH is rather informative in revealing natural-man-made biogeochemical provinces with imbalance in food chains of iodine, arsenic, selenium, lithium, mercury, cadmium, lead, molybdenum and copper. CECH shows moderate indication capacity with respect to cobalt, nickel, strontium, zinc, manganese and iron, and weak with respect to calcium, magnesium, and probably potassium. CECH is not informative with respect to sodium.

**Key words:** analysis, biogeochemistry, hair, diagnosis, animals, microelementoses, provinces, ecology

### **Introduction**

The topicality of this investigation is related to the biogeochemical heterogeneity of the environment, existence of local and zonal biogeochemical provinces with certain ecological and national agricultural problems, in particular those related to agricultural development and provision of high-quality foodstuffs, in the territory of various states. At that, today's active economic activity of humans contributes to the heterogeneity in the environmental chemical composition. The imbalance of biologically active chemical elements

in the environments leads to the enrichment or depletion of plants, forage and animal organisms in individual chemical elements, which reduce livestock yield and result in diseases, the so called microelementoses [1-3].

Diagnosing microelementoses on the basis of chemical elemental composition of hair (CECH) seeks not only to settle ecological problems and prevent microelementoses, but also to develop a new painless up-to-date technique to reveal mineral dysbolism in animals and assess the evolution of the biosphere's taxons. Therefore, this article is devoted to the assessment of the CECH of cattle as an ecological and biogeochemical marker.

## Methods

The biogeochemical anomaly indication method was tested in specific territories with different ecological status. As experimental sites were chosen Moscow and Voronezh regions (background), Eastern Transbaikalia (areas with selenium deficiency and strontium excess in soil and plant. For the case of 6 test sites in Moscow (Lenin Farm), Voronezh (Razdol'e farm) and Chita regions (Beklemishchevo, Trubachevsk, Nerchinskiy Zavod, Unda farms) 500 cattle hair samples were taken. A sample of hair was taken along the full length of the tail bunch. A section of the bunch is sheared at a distance of 1-2 cm from the skin by means of scissors (preferably with titanium coating). The sample with a label is placed into a paper or plastic bag and delivered to the laboratory. Preparation of cattle hair samples (tail bunch) for determination of some macro and trace elements was carried out according to the procedure [7]. In order to validate the technique, two reference samples of human hair CRM NCS DC 73347 Hair (China National Analysis Center for Iron & Steel 1997) and CRM 397 (Commission of the European Communities, BCR, CRM No 397, Human hair) were analyzed (Table 1). The mineral material of the standard sample CRM NCS DC 73347 Hair left white sediment. By AES, this sediment was found to contain aluminum in the basis. All measurements were performed by the method of standard makeweights.

Table 1 - Results of analysis of standard hair samples (in mg/kg)

Element	CRM NCS DS 73347		Element	CRM 397	
	Certified value	Obtained value		Certified value	Obtained value
Ca	2900±200	2930±500	Fe*	580±10	525±25
Mg	360±30	400±150	Mg*	200±5	193±10
Na	152±10	160±20	Zn	199±5	203±20
K	20±5	23±5	Mn*	11.2±0.3	9.1±1.2
Zn	190±5	210±50	Cd	0.521±0.024	0.55±0.06
Mn	6.3±0.5	5.9±2.2	Mo*	6.6±0.2	7.5±1.6
Sr	24±1	21.5±4.5	Pb	33.0±1.3	29.5±3.5
Cd	0.11±0.02	0.10±0.02	Ni**	39.0±1.4	46±5
Ni	0.83±0.15	1.1±0.4	Co*	0.55±0.03	0.40±0.18

\* - Informative values; \*\* - Indicative values.

In the analysis of the standard sample of hair CRM NCS DS 73347 for lead, cobalt, phosphorus, and selenium were obtained the following data (in mg/kg): lead -  $8.8 \pm 0.9$  (certificate) and  $8.6 \pm 1.1$  (obtained value); cobalt -  $0.071 \pm 0.008$  (certificate) and  $0.055 \pm 0.025$  (obtained value); phosphorus -  $170 \pm 7$  (certificate) and  $161 \pm 6$  (obtained value); selenium -  $0.60 \pm 0.03$  (certificate) and  $0.61 \pm 0.02$  (obtained value).

## Results

CECH was found to have regional features and to show slight dependence on the physiological condition and age of animals (Table 2). Two groups were compared using the F-distribution to assess the effect of physiological condition of cows (dairy cows, incalvers) on the chemical composition of hair.

Table 2 - Statistical parameters determined based on CECH of tail bunch hair samples of cows with different physiological status (Moscow district)

Group	Parameter	Content, mg/kg										
		K	Ca	Mg	Sr	Zn	Mn	Cu	Fe	Mo	Co	Ni
Non-lactating incalvers (n=15)	M <sub>1</sub>	1,790	1,384	977	4.1	115	18.0	7.5	33	0.41	0.046	0.93
	S <sup>2</sup> <sub>1</sub>	2,457,039	513,154	74,910	4.2	343	204	2.9	1,009	0.17	0.0026	0.32
Dairy (n = 13)	M <sub>2</sub>	1517	1,315	1,117	3.8	117	20.8	8.4	70.5	0.43	0.049	1.18
	S <sup>2</sup> <sub>2</sub>	1,018,166	254,710	230,556	1.2	462	116	0.96	5779	0.19	0.0016	0.144
F <sub>calc.</sub>		2.41	2.01	3.08	3.46	1.35	1.76	3.02	5.73	1.12	1.63	2.22

Note: M<sub>1</sub>, M<sub>2</sub> – average values, S<sup>2</sup><sub>1</sub>, S<sup>2</sup><sub>2</sub> – sample variance, F<sub>calc.</sub> - Fisher criterion

At a given probability of P = 0.99, the calculated values of F<sub>calc.</sub> were found to be not higher than the tabular value (F<sub>tab.</sub> = 7.72 at f<sub>1</sub> = 1, f<sub>2</sub> = 26) for all the elements. Therefore, CECH data of the cow groups of different physiological status belong to the same universal set. This means that the physiological condition of cows does not affect the CECH.

For a number of chemical elements, CECH of cattle shows strong differences depending on the coat color. The comparison of the composition of light-brown and brown hair samples from the Trubachevsk Farm revealed no significant difference in CECH with respect to potassium, calcium, magnesium, strontium, zinc and other trace elements. In darker hair samples, the range of variation was generally wider as against light color samples (Table 3).

Table 3 Levels of chemical elements in cow tail bunch hair samples of different color

Color	Parameter	Level of element, mg/kg										
		K	Ca	Mg	Sr	Zn	Mn	Cu	Fe	Mo	Co	Ni
Light-brown (n = 20)	M	110	646	2,370	20.1	107	20.0	7.1	210	0.06	0.056	1.3
	±s	17	34	93	1.6	3	1.3	0.2	35	0.01	0.006	0.15
Brown (n = 10)	M	93	784	2,890	19.8	101	25	6.6	270	0.04	0.078	1.2
	±s	20	44	140	1.2	4	5	0.2	61	0.01	0.016	0.3

A detailed analysis for the hair samples taken on the Beklemishchevo Farm has shown that the accumulation of potassium and magnesium was typical of dark-yellow hair samples (Table 4).

Table 4 - Levels of chemical elements in different-color tail bunch hair samples

Color	Parameter	Level of element, mg/kg										
		K	Ca	Mg	Sr	Zn	Mn	Cu	Fe	Mo	Co	Ni
Dark-yellow (n = 15)	M	336	840	1740	9.4	110	17.0	7.0	69	0.17	0.045	0.76
	±s	74	22	95	0.5	3	1.3	0.3	4	0.02	0.004	0.20
Yellow (n = 36)	M	314	685	1460	9.5	111	15.2	7.1	54	0.14	0.044	0.58
	±s	47	21	54	0.6	2	1.5	0.1	4	0.01	0.006	0.03
Yellowish-gray (n = 8)	M	188	1840	1120	14.2	144	22.9	7.9	85	0.14	0.059	0.47
	±s	74	51	63	0.8	9	2.9	0.6	14	0.03	0.011	0.17
Light-yellow (n = 22)	M	150	1780	996	12.9	127	21.2	7.0	56	0.06	0.066	0.25
	±s	26	53	55	0.8	3	0.9	0.2	3	0.01	0.011	0.02
White (n = 20)	M	122	1340	650	8.9	145	18.3	7.4	60	0.03	0.077	0.32
	±s	12	83	37	1.0	10	1.2	0.2	3	0.01	0.017	0.07

Calcium, strontium, zinc, manganese, copper, iron and molybdenum prevailed in yellowish-gray hair samples. At that, the color gradation was as follows: dark-yellow, yellow, yellowish-gray, light-yellow and white. As a result of statistical data processing, however, the differences proved to be insignificant for Beklemishchevo cows.

Analysis of the body of data obtained testifies to considerable differences in the chemical elemental composition of cattle hair from different regions in terms of K, Na, Ca, Sr, Fe, Mn, Cu, Co and Se levels. For example, cow hair samples from the Trubachevsk farm, Chita region according to the investigation in 2003 year contain small quantities of potassium (23-255 mg/kg), sodium (121-430 mg/kg) and greater quantities of strontium (9.6-41.4 mg/kg), iron (64-700 mg/kg) and manganese (13.6-64.8 mg/kg). However, hair of cows from the Lenin farm, Lenin district, Moscow region, was found to have higher concentrations of strontium (13.6-102 mg/kg). Hair samples from this farm contained unusually high copper levels: 18-196 mg/kg (as against the background – around 7 mg/kg), which might be attributed to the extensive use of fodder enriched in copper. Hair samples from this farm also contained smaller quantities of calcium (660-1950 mg/kg), iron (13.5-34.0 mg/kg), cobalt (0.003-0.007 mg/kg), but greater quantities of potassium (230-3,100 mg/kg) and copper (16.4-160.5 mg/kg) as against the biomaterial sampled in Chita region. In the region of the Urov Kashin-Beck disease, in addition to the low level of selenium, on a number of farms one can also observe increased levels of strontium, which correlates with the levels of these elements in soils and plants (Table 5).

Table 5 - Levels of chemical elements in tail bunch hair of cows

Region	P*	Controlled element, mg/kg										
		K	Ca	Mg	Sr	Zn	Mn	Cu	Fe	Mo	Co	Ni
Moscow district (n=147)	R	1600-2300	1200-1600	900-1100	7-11	110-130	10-20	7-8	20-40	0.14-0.26	0.02-0.04	0.6-1.1
Voronez district (n=133)	R	570-1090	1180-1580	540-680	11-15	110-120	6-8	8-10	50-90	0.02-0.08	0.02-0.04	-
Chita district, Beklemishevo (n=33)	R	160-300	1020-1320	1070-1350	9-12	120-130	16-20	7-8	55-70	0.08-0.14	0.04-0.08	0.2-1.3

Chita region, Unda (n=12)	$\bar{X}$	3728	2288	1425	33	148	22	6.9	160	0.037	0.10	1.09
	$\pm s$	1132	179	117	8,9	13	5	0.3	37	0.007	0.02	0.39
Chita region, Nerchinsk factory (n=13)	$\bar{X}$	575	1244	581	11.2	116	4,4	6.0	44	0.024	0.013	0.28
	$\pm s$	106	174	85	3.0	5.4	0.4	0.5	6	0.004	0.007	0.07
Chita region, Trubachevskoe (n=60)	$\bar{X}$	405	1548	1799	24.7	110	20.4	7.0	359	0.043	0.088	0.80
	$\pm s$	75	118	108	1.2	2	1.0	0.1	36	0.005	0.006	0.1

P\* - parameter or range

For a number of chemical elements, CECH of cattle shows strong differences depending on the coat color. CECH is rather informative in revealing natural-man-made biogeochemical provinces with imbalance in food chains of iodine, arsenic, selenium, lithium, mercury, cadmium, lead, molybdenum and copper. CECH shows moderate indication capacity with respect to cobalt, nickel, strontium, zinc, manganese and iron, and weak with respect to calcium, magnesium, and probably potassium. CECH is not informative with respect to sodium [5, 6].

## Conclusion

The research shows that for most of macro and trace elements, their levels in hair samples taken from the tail bunch are reliably higher than in the samples taken from the withers and back of animals. Taking into account the convenience and simplicity of cattle hair sampling from the tail bunch and experimental data, it was found advisable to take hair samples for chemical analysis from the tail bunch.

The use of CECH of animals in biogeochemical indication of trace elements and their compounds allows researchers to achieve a number of valuable results. The major of these include representativeness related to the ecological connection between these animals and human and the possibility of comprehensive territory assessment with the help of ruminants.

It is of practical interest to use the method in the most ecologically unfavorable azonal and subregional biogeochemical provinces both in Russia and in Bulgaria [3-5]. In Russia these primarily include polymetallic anomalies with predominant associations of Cu-Zn, Cu-Ni, Pb-Zn, Cu-Ni-Co (Southern Ural Mountains, Bashkortostan, Chara, Norilsk, Mednogorsk), nickel provinces (Norilsk, Monchegorsk, Nikel, Polyarnyi, Zapolyarie, Tuva); lead provinces (Altai, Caucasus, Transbaikalia); mercury provinces (Altai, Sakha, Kemerovo region); provinces enriched in fluorine (Kirovsk, Eastern Transbaikalia, Krasnoyarsk, Bratsk); subregional provinces enriched in boron and beryllium. Potentially hazardous are technogenic biogeochemical provinces with unpredictable ecological consequences. Such territories need to be monitored on a regular basis, and in this case CECH as a biomarker could be rather useful.

The method is particularly promising as applied to agrobiogeocenoses. It was established that when cattle is rather closely connected with local conditions of feeding with natural forage planted at forage lands of a farm during the pasture season, enrichment or depletion in available forms of chemical elements in soils results in their deficiency or excess in the animal organism, which leaves its imprint on the chemical elemental composition of cattle hair. Therefore, chemical analysis of cattle hair with respect to a wide range of both

essential elements and xenobiotics can serve as an express method for assessing the elemental status of agricultural territories in terms of sustainable development. The most reasonable way to replenish deficient essential macro and trace elements in the *soil-plant-animal* system is to add them to the soil of forage lands.

On the whole, the problem of human and animal CECH application for diagnosing microelementoses is at the stage of keen interest of scientists and active search for optimal solutions [1, 2]. There are considerable analytical difficulties in choosing biomaterial, its preparation for analysis and interpretation of data obtained. Notable spread of data on the levels of macro elements (Ca, Mg, K, Na) makes it more difficult to diagnose disorders and changes in their metabolism in animals at different physiological conditions and pathologies. On the one hand, this is related to the high degree of tail bunch hair pollution. On the other hand, of high significance is the type of chemical bond between macro and trace elements and keratin and melanin of hair. It is typical of macro elements to form salts, coming from feces and with suint, which readily diffuse into solutions, when hair is cleaned with aqueous solutions. In this respect, there arises the problem of studying the nature of chemical bond between macro and trace elements with the keratin hair matrix, and of more careful analysis of hair biochemistry, including polymerization of keratin structures and intake of trace elements.

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## DP2. ACCUMULATION OF METALS BY MILK XANTHINE OXIDASE

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### **Abstract**

In the course of biogeochemical studies of W-Mo ore landscapes of the North Caucasus (Tyrnyauz) and background areas tungsten and rhenium were found to accumulate not only in plants or soil, but also the element incorporation into an enzyme xanthine oxidase in animals was first determined. In case of increase in molybdenum and/or tungsten (rhenium levels in the environment, the migration of the latter metals enhances significantly in ore zones.

Keywords: biogeochemistry, tungsten, molybdenum, copper, rhenium, xanthine oxidase

### **Introduction**

Molybdenum is a trace element virtually essential for any form of life. Some biogeochemical provinces with excessive molybdenum are identified in the environment where a specific form of endemic gout is spread among the human population or a “molybdenosis”, i.e., a chronic molybdenum toxicosis in animals is diagnosed [5].

At a molecular level, molybdenum and copper compete for the active centre in xanthine oxidase (XO) - EC 1.17.3.2 [1, 7, 10]. There are also some data suggestive of an antagonism of molybdenum and tungsten at their effecting XO activity [9, 11]. It is believed that tungsten was widely used by microorganisms in the period of oxygen deficiency in the primary biosphere, though later on a wide range of organisms lost their potential of using tungsten in catalytic processes [6]. Nevertheless, there is a group of thermophilic bacteria wherein tungsten-containing enzymes play an important role in their vital activity [1, 2, 8].

We identified a range of trace elements in soils, and animals (in blood and/or hair) in a series of open fields and background areas of the North Caucasus and Transbaikalia. The purpose of this study was to determine the specific features of copper and/or molybdenum accumulation rates in animals against the background of various tungsten and rhenium content levels in the environment and animal feeds, and the possible metals incorporation into XO of cow milk.

### **Methods**

The field biogeochemical studies were carried out in the summer seasons of 2009 to 2012 in ore landscapes of W-Mo deposits and background areas of the North Caucasus (Tyrnyauz). Milk, cream and buttermilk samples were kindly submitted to us by the residents of Kudinovo Place (Moscow region, a background level area), Zayukovo Place and Prokhladny (Kabardino-Balkaria, background), as well as of Bylym village and Tyrnyauz (areas with higher content levels of molybdenum and tungsten both in the environment and feeds).

In the elemental analyses of soils, plants or animal fluids, and also fractions of milk XO we used mass spectrometer with inductively coupled plasma (model 7500 CE manufactured by "Aglient Technologis", USA). The measurements were carried out in a scanning regime and quantified using standard Cu, Mo, W, Re or Fe solutions.

Some experiments were carried out to assess the metals effect XO activity of milk. We used an enzyme isolated from cream (buttermilk). In this case, a common method [7] modified by G.G. Tsoy [10] The subsequent XO purification was performed with a preparative column (2.5 cm x 30 cm with TSKGEL HW-65F), and a semipreparative column TSKGEL G3000SW (0.78h30 cm, the sorbent graining 10 µm). The fractions were analyzed with a gel-exclusion HPLC using a column BioSep-SEC-2000 (5 µm, 145 A, 0.6 x 30 cm, OOH-2145-EO) with UV detection (212, 220, 260 and 280 nm). A phosphate buffer (0.1 M) was used as an eluent (pH = 6.8), flow rate - 0.35 ml / min. A part of the column fractions was analyzed using AAS and ICP-mass spectrometry.

We determined that the soils of the Baksan river valley were also significantly enriched with molybdenum (2-60 mg/kg), tungsten (10-102 mg/kg) and rhenium (0.002-0.054 mg/kg) which is associated with ore mineralization process The lateral migration of molybdenum (and, possibly, tungsten or rhenium) is due to some increase in water-soluble forms of the trace elements and acetate buffer-extracted compounds [3, 4].

The biogeochemical investigations of tungsten & molybdenum ore landscapes in the North Caucasus (Tyrnyauz) and background areas (Moscow region) revealed that the metals were accumulated not only with soils, but with pasture plants, too (see Table 1).

Iron concentration levels in plants of certain territories do not show any sharp differences, varying within 200 to 500 mg/kg, nor any significant differences in copper levels among the areas under study are observed. Nevertheless, for molybdenum and tungsten concentration levels the differences are quite significant. Tungsten and/or molybdenum accumulation is most intensive in ore anomalies (Tyrnyauz and Bylym), with Bylym village representing a secondary biogeochemical province as a result of removing large amounts of terrigenous materials from Tyrnyauz ore mines and loci. It should be noted that the hay crops and pasture plants also contained trace amounts of rhenium.

Table 1 - Levels of the metals in plant mowing samples taken in various areas (in mg/kg of dry matter). In brackets - number of samples

Location selection	Fe	Mo	Cu	W	Re
Kudinovo (hay)	390	0.32	3.3	0.01	0.0016
Zayukovo (5)	234-392	0.5-2.2	3.0-13.4	0.03-0.22	0.0010-0.0018
Bylym (12)	242-832	0,7-4,2	3.0-12.1	0.19-1.20	0.011-0.130
Tyrnyauz (16)	228-500	1.2-31.0	2.3-13.4	0.1-5.40	0.050-0.540

The above metals have been found not only in whole cow milk, but in buttermilk, either, with their concentrations in buttermilk raising 5 to 10 times, both in the ore and the control areas. Thus, in the buttermilk collected in a control area (Kudinovo) the following concentrations were determined (µg/l): Cu - 130, Mo - 93, W - 0.4, Re – 0.03 and buttermilk samples taken from cows in Tyrnyauz gave the following indices: Cu - 684, Mo - 556, W - 4.3, Re- 0,86 µg/l (see Table 2).

Table 2. Iron, copper, molybdenum and tungsten in the milk and buttermilk of livestock from anomalous (Tyrnyauz, Bylym) and background areas (Kudinovo, Zayukovo) (µg/l)

Place selection	Buttermilk				
	Fe	Cu	Mo	W	Re
Kudinovo	84	130	93	0,4	0.03
Zayukovo	102	148	189	0,1	0.02

Bylym	68	110	174	2,0	0.32
Tyrnyauz	112	684	556	4,3	0,86

Taking into account the known relationship of molybdenum and copper with the enzyme xanthine oxidase in milk, this enzyme was isolated from cattle buttermilk both in the control areas (Kudinovo and Zayukovo) and the molybdenum- & tungsten-anomalous grounds (Tyrnyauz and Bylym). For the enzyme isolation and purification, both a well-known method [7, 10] and a new one that we modified, were used. In the latter case, for the enzyme purification we used a sequential chromatography of the extract on the preparative and semi-preparative columns TSKGEL HW 65F and TSKGEL G3000SW delivered by TOSOH BIOSCIENCE (Japan). This sorbent was more effective when used for the separation of proteins, as compared with Sephadex G-200, as the number of theoretical plates increased by several times. Several fractions of the enzyme were purified with a column chromatography using a sorbent TSKGEL, and analyzed for protein, XO enzyme activity and metal content.

The purified enzyme exhibited parameters characteristic of XO in milk (see Table 3).

Table 3. Comparative XO chemical composition as observed in milk samples from various areas ( $\mu\text{g}/\text{mg}$  of protein)

The habitat	FAD	Fe	Mo	Cu	W	Re
Sibai *	0.70	0.26	0.12	0.02	-	-
Armenia *	1.20	0.40	0.40	<0.01	-	-
Kudinovo	1.15	0.44	0.31	0.03	0.002	0,0004
Zayukovo	1,50	0.64	0.47	0.06	0.005	0,0004
Bylym	0.80	0.60	0.44	0.01	0.010	0,0005
Tyrnyauz	0.80	0.56	0.60	0.03	0.080	0,0006

\* According to G.G. Tsoy [13]; FAD - flavine adenine dinucleotide

The table presents comparative data on the characteristics of XO samples collected by our group or other researchers [10]. In the latter case, the results of the enzyme analyses for tungsten content indices are not available. The authors used a photometric method for molybdenum identification using zinc dithiol, which did not allow molybdenum and tungsten differentiation, as far as both of the metals reacted with zinc dithiol similarly.

It should be noted that the tungsten and rhenium content in the enzyme is relatively lower than the molybdenum one. The tungsten quantity as observed in the purified fraction of the enzyme is reduced compared to the metal content in the substrate (e.g., in buttermilk). If we consider the total content of tungsten in the buttermilk, and in the whole fraction of the purified enzyme, there is a suggestion that not all the metal bulk is incorporated into milk XO. It is also possible that some part the tungsten is lost at the enzyme purification. In addition, in the process of the isolated enzyme storage, its portion is hydrolyzed to its subunits, since when carrying out HPLC of the enzyme some peaks corresponding to the molecular weights of 130 and 40 kDa appear on the chromatograms, which is consistent with the existing data [9].

Multiple forms of xanthine oxidase is an important mechanism regulating body processes and adaptations. Apparently, xanthine oxidase, by dithio-bond, as metallothioneins, forms the numerous isoforms with a number of metals (molybdenum, copper, tungsten, and rhenium). Centers that are undergoing deep changes of the isoenzymes serve areas with extreme geochemical conditions of molybdenum and copper sub-regions of the biosphere and biogeochemical provinces.

In addition, the gene responsible for the formation of W or Re-containing XO or some other metal-containing proteins is possibly preserved in the process of evolution of organisms, and it is present not only in thermophilic bacteria, but in mammals, too. Yet, the incorporation of tungsten and rhenium in milk XO, along with molybdenum and copper, is proved. The

further analyses of XO would determine the enzyme differentiation into its isoforms and prove the existence of W-containing XO.

### Conclusion

Thus, in the course of carrying out biogeochemical studies of tungsten & molybdenum ore landscape of the North Caucasus (Tyrnyauz) and background areas, a phenomenon of tungsten and rhenium accumulation not only in plants, but also the metal incorporation into a mammalian enzyme XO were determined for the first time. At increasing molybdenum and tungsten levels in the environment, migration of the W and Re increase sharply in ore districts. These data substantially change the current ideas regarding the biological role of tungsten and rhenium in mammals. Nevertheless, deeper investigations into the mechanism of W and Re incorporation into XO lie ahead.

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### **DP3. БИОЛОГИЧНИ И СИНТЕТИЧНИ ЕКЗОГЕННИ МУТАГЕНИ**

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### **DP4. КОНСЕРВАНТИТЕ И ХРАНАТА, КОЯТО ОБИЧАМЕ**

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### **DP5. ЗМИЙСКА ОТРОВА – ПЪТ КЪМ СМЪРТТА ИЛИ ПЪТ КЪМ ЖИВОТА**

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### **DP6. КОЙ СВИРИ ХЕВИ МЕТЪЛ В НАШИЯ ОРГАНИЗЪМ?**

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