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PROCEEDINGS

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

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PROCEEDINGS

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

November 26 - 28, 2014

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 NINTH 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, 26 November 2014

9.30 – 9.50 REGISTRATION
9.50 – 10.00 OPENING CEREMONY

Session A.

Chairpersons:

Prof. Marin Alexandrov, DVM, PhD
Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences

Assoc. Prof. Stefka Tepavitcharova, MSc, PhD
Institute of General and Inorganic Chemistry, Bulgarian Academy of Sciences

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

10.00 – 10.30

AO1. In the world of zinc

Radostina Alexandrova[1], Tanya Zhivkova[1], Abdulkadir Abudalleh[1], Lora Dyakova[2], Pavel Mitrenga[1], Ivo Grabchev[3], Ogo A. Ogo[4], Daniela-Cristina Culita[5], Gabriela Marinescu[5]

[1]Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences; [2]Institute of Neurobiology, Bulgarian Academy of Sciences; [3]Medical Faculty, Sofia University "St. Kliment Ohridski"; [4]Newcastle University, UK; [5]Institute of Physical Chemistry "Ilie Murgulescu", Romanian Academy, Bucharedt, Romania

10.30 – 10.35

AO2. Zinc fingers

Pavel Mitrenga[1], Lora Dyakova[2], Tanya Zhivkova[1], Boyka Andonova-Lilova[1], Ogo A. Ogo[3], Radostina Alexandrova[1]

[1]Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences; [2]Institute of Neurobiology, Bulgarian Academy of Sciences; [3]Newcastle University, UK

10.35 – 10.45

AO3. Anticancer properties of zinc compounds

Radostina Alexandrova[1], Lora Dyakova[2], Tanya Zhivkova[1], Pavel Mitrenga[1], Marin Alexandrov[1], Reni Kalfin[2], Daniela-Cristina Culita[3], Gabriela Marinescu[3], Luminita Patron[3]

[1]Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences; [2]Institute of Neurobiology, Bulgarian Academy of Sciences; [3]Institute of Physical Chemistry "Ilie Murgulescu", Romanian Academy, Bucharedt, Romania

10.45 – 11.00

AO4. Metal complexes and drug resistant cancer cells

Radostina Alexandrova[1], Lora Dyakova[2], Tanya Zhivkova[1], Pavel Mitrenga[1], Marin Alexandrov[1], Reni Kalfin[2], Ramona Tudose[3], Elena-Maria Mosoarca[3], Otilia Costisor[3], Daniela-Cristina Culita[4], Gabriela Marinescu[4], Luminita Patron[4], Katalin Nemet[5], Jan Stenvang[6]

[1]Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences; [2]Institute of Neurobiology, Bulgarian Academy of Sciences; [3]Institute of Chemistry, Timisoara, Romania; [4]Institute of Physical Chemistry "Ilie Murgulescu", Romanian Academy, Bucharest, Romania; [5]Institute of Haematolohy and Immunology, National Medical Centre, Budapest, Hungary; [6]University of Kopenchagen, Denmark

11.00 – 11.20 Coffee Break

11.20 – 11.35

AO5. "pH" In cell biology, microbiology and biotechnology

Daniela Pencheva[1*], Mina Rumenkina[1], Alexandrina Al-Djasem[1], Martin Iliev[1], Velko Karamihov[2], Petia Genova-Kalou[3], Todor Kantardjiev[3]

[1]Bul Bio-National Centre of Infectious and Parasitic Diseases, Sofia, Bulgaria;
[2] Metrohm Bulgaria, Sofia, Bulgaria;
[3]National Centre of Infectious and Parasitic Diseases, Sofia, Bulgaria.

11.35-11.50

AO6. The role of glutathione in the pathogenesis of influenza virus infection

A. Dimitrova[1], M. Mileva[1], D. Krustev[2], Gegova, G.[1], Todorova, K.,[1] A.S. Galabov[1]

[1]The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences;
[2]Medical University of Sofia, Medical Colleague "Jordanka Filaretova

11.50 – 12.05

AO7. Ефект на екстракти от Teucrium chamaedrys спрямо Herpes simplex virus тип 2

В. Цветков, Е. Иванова, Д. Тодоров, Д. Драголова, А. Хинков, Д. Павлова, С. Шишков

Биологически факултет, Софийски университет "Климент Охридски"

12.05 – 12.20

AO8. QSAR models for predicting transcellular permeability of bioactive compounds

A. Diukendjieva, P. Alov, I. Tsakovska, I. Pajeva

Institute of Biophysics and Biomedical Engineering, Bulgarian Academy of Sciences, Sofia, Bulgaria

AP1. The unknown face of Disulfiram

Tanya Zhivkova[1], Lora Dyakova[2], Pavel Mitrenga[1], Desislav Dinev[1,3], Simona Spasova[1,4], Jan Stenvang[5], Radostina Alexandrova[1]

[1]Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences; [2]Institute of Neurobiology, Bulgarian Academy of Sciences;
[3]Faculty of Biology, Sofia University "St. Kl. Ohridski, [4]Faculty of Medicine, Sofia University "St. Kl. Ohridski", Bulgaria; [5]University of Kopenchagen, Denmark

AP2. Effect of Zn/Au and Zn/Ag with Saldmen and Salampy on viability and proliferation of human tumor cell lines

Tanya Zhivkova[1], Desislav Dinev[1,2], Pavel Mitrenga[1], Abdulkadir Abudalleh[1], Lora Dyakova[3], Marin Alexandrov[1], Daniela-Cristina Culita[4], Gabriela Marinescu[4], Luminita Patron[4], Radostina Alexandrova[1]

[1]Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences; [2]Faculty of Biology, Sofia University "St. Kl. Ohridski"; [3]Institute of Neurobiology, Bulgarian Academy of Sciences; [4]Institute of Physical Chemistry "Ilie Murgulescu", Romanian Academy, Bucharedt, Romania

12.20 – 12.45

Poster presentation and Discussion

12.45-13.39 Lunch time

13.30 – 14.00

AO9. Properties and possibilities for application of the hybrid material with silver nanoparticles (PVA / AgNps)

Daniela Pencheva[1*], Rayna Bryaskova[2], Petia Genova-Kalou[3]

[1]"Bul Bio - NCIPD" Ltd., Sofia, Bulgaria

[2] University of Chemical Technology and Metallurgy, Department "Polymer Engineering", Sofia, Bulgaria

[3]National Centre of Infectious and Parasitic Diseases, Sofia, Bulgaria

AP3. Antimicrobial properties of silver

Ivaylo Dankov

Medical Faculty, Sofia University"St. Kliment Ohridski"

AP4. Фаготерапия

Даниела Гулева, Александра Борисова, Адриан Жозе Кардозо, Цветозара Дамянова

Биологически факултет, СУ „Св. Климент Охридски"

AP5. „Избухващият вирус" - Ебола

Фаузия Салах Ел Рантиси, Мохамед Мохедин, Ая Хасанова, Исра Ал Хусейн, Джуди Джабулие, Дания Ясърджи

Палестинско училище, София

AP6. Ебола – новото предизвикателство на 21-ия век

Георги Тошев

Биологически факултет, СУ „Св. Кл. Охридски"

AP7. Прионите и заболяванията, които те предизвикват

Георги Тошев

Биологически факултет, СУ „Св. Кл. Охридски"

Session B.

Chairpersons:

Prof. Mashenka Dimitrova, MSc, PhD
Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences

Assist. Prof. Delka Salkova, DVM, PhD
Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences

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

14.00-14.15

BO1. Trace metals polluted soils in Kardjali region, Bulgaria

S. Tepavitcharova[1], A. Kovacheva[1], D. Rabadjieva[1], J. Stajkova[2], R. Tchilingirova[3]
[1]*Institute of General and Inorganic Chemistry, Bulgarian Academy of Sciences;*
[2]*National Center of Public Health and Analysis, Sofia, Bulgaria;*
[3]*Regional Health Inspectorate, Kardzhali, Bulgaria*

14.15 – 14.45

BO2. Effects of exogenous Abscisic acid on the photosynthetic activity of ferns (*Nephrolepis sp.)*

S. Dimitrova[1], A. Tanev[3], K. Dankov[1], M. Dimitrova[2], V. Goltsev[1], E. Ananiev[3]
[1]*Faculty of Biology, Sofia University "St. Kliment Ohridski";*
[2] *Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences*

14.45 – 15.05 Coffee Break

15.05-15.35

BO3. Honeybee pollen as bioindicator for environmental pollution

Delka Salkova Salkova, Mariana Panayotova-Pencheva
Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia

15.35 – 15.50

BO4. Alterations of antioxidant trace elements and related metalloenzymes in rabbits with eimeriosis

I. Vladov[1], M. Gabrashanska[1], V. Nanev[1], V. Ermakov[2], S. Tyutikov[2]
[1]*Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia*
[2]*V. I. Vernadsky Institute of Geochemistry and Analytical Chemistry, Russian Academy of Sciences, Moscow, Russia*

15.50 – 16.05

BO5. Some aspects of selenium biological activity

Delka Salkova Salkova[1], Zhana ViktorovnaUdalova[2], Svetlana Vasilievna Zinovieva[2]
[1]*Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia;*
[2]*Center of Parasitology, A.N. Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences, Moscow, Russia*

16.05 – 16.20

BO6. Макроелементи

Симона Спасова, Кристина Генкова
Медицински факултет, СУ „Св. Кл. Охридски”

16.20 – 16.35

BO7. Незаконна търговия и бизнес със застрашени животни

Мадлена Младенова, Станислава Тренчева
Биологически факултет, СУ „Св. Кл. Охридски”

BP1. Polymetallic Biogeochemical Province of North Ossetia

S.F. Tyutikov[1], V.V. Ermakov[1], M. Gabrashanska[2], M. Anissimova[2]
[1]*V. I. Vernadsky Institute of Geochemistry and Analytical Chemistry, Russian Academy of Sciences, Moscow, Russia*
[2]*Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia*

BP2. Recent data about biological activity of Monensin

Delka Salkova[1], Tanya Zhivkova[1], Desislav Dinev[1,2], Simona Spasova[1,3], Radostina Alexandrova[1]
[1]*Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia;*
[2]*Faculty of Biology, Sofia University “St. Kl. Ohridski”;*
[3]*Faculty of Medicine, Sofia University “St. Kl. Ohridski”*

BP3. Effect of cobaltbisdicarbolides on viability of cultured tumor permanent cell lines

Radostina Alexandrova[1], Nikola Simeonov[2], Tanya Zhivkova[1], Lora Dyakova[3], Francesc Teixidor Bombardo[4], Clara Viñas Teixidor[4]

[1]*Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences;*
[2]*Medical Faculty, Sofia University "St. Kliment Ohridski";*
[3]*Institute of Neurobiology, Bulgarian Academy of Sciences;*
[4]*Institut de Ciència de Materials de Barcelona (CSIC), Campus de la U.A.B., Bellaterra, Spain*

BP4. Cobaltabisdicarbollides affect viability and proliferation of mouse and hamster tumor and non-tumor cells

Radostina Alexandrova[1], Reneta Toshkova[1], Tanya Zhivkova[1], Lora Dyakova[2], Nikola Simeonov[3], Ivaylo Dankov[3], Francesc Teixidor Bombardo[4], Clara Viñas Teixidor[4]

[1]*Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences;*
[2]*Institute of Neurobiology, Bulgarian Academy of Sciences;*
[3]*Medical Faculty, Sofia University "St. Kliment Ohridski";*
[4]*Institut de Ciència de Materials de Barcelona (CSIC), Campus de la U.A.B., Bellaterra, Spain*

16.35 – 17.00
Poster presentation and Discussion

Thursday, 27 November 2014

Session C.

Chairpersons:

Prof. Lujbomir Angelov, PhD, DSc
Institute of Cryobiology and Food Technology, Agricultural Academy, 53 Cherni vrah, 1407 Sofia, Bulgaria

Assist. Prof. Rumiana Hristova, MSc, PhD
National Centre of Infectious and Parasitic Diseases, Sofia

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

9.30 – 10.00

CO1. Food allergens and food allergy

R. Hristova[1], J. Radenkova - Saeva[2]
[1]Laboratory of Allergy, National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria;
[2]Clinic of Toxicology, Department for Adult, Emergency University Hospital "N.I.Pirogov"

10.00 – 10.30

CO2. Biological active components of ewe's milk and white brine cheese

Silviya Ivanova, Lujbomir Angelov
Institute of Cryobiology and Food Technology, Agricultural Academy, Sofia, Bulgaria

10.30 – 10.45

CO3. The role of hepcidin in regulation of iron homeostasis

Tsvetelina Petkova-Marinova, Boryana Ruseva
Department of Physiology, Medical University – Pleven

10.45 – 11.00

CO4. Advantages of the application of iron methionate compared to iron sulphate in fodders for broiler chickens

A. Arnaudova-Matey [1], K. Todorova[3], T. Todorov[1], T. Yankovska [2], Tsv. Kirilova[2], T. Mehmedov [1], S. Ivanova[4], P. Dimitrov[3], S. Lazarova[3], P. Dilov[1] and G. Angelov[1]

[1]University of Forestry, Faculty of Veterinary Medicine,Sofia, Bulgaria;
[2]Central Laboratory of Veterinary Control and Ecology, Sofia, Bulgaria;
[3] Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Scienc, Sofia, Bulgaria;
[4]National Diagnostic and Research Veterinary Medical Institute, Sofia, Bulgaria

11.00 – 11.20 Coffee break

11.20 – 11.35

CO5. Хемолитична анемия

Никола Симеонов
Медицински факултет, СУ „Св. Климент Охридски"

11.35 – 11.50

CO6. In search of potentially new therapeutic application of coumarins beside as anticoagulants

Syiana Georgieva
Medical Faculty, Sofia University "St. Kliment Ohridski"

11.50 – 12.20

CO7. Национални особености при храненето и заболяванията (на примера на Китай)

П. Джуров
Медицински факултет, Медицински университет, София

CP1. Чили – За лютото с любов!
Елена Манлиева
Биологически факултет, СУ „Св. Климент Охридски”

CP2. Амброзия за здраве и дълголетие (Мед)
Елена Манлиева
Биологически факултет, СУ „Св. Климент Охридски”

CP3. Medical uses of plant extracts from Sideritis scardica
Angelina Bankovska
Medical Faculty, Sofia University “St. Kliment Ohridski”

CP4. Истината е във виното!?
Людмила Стоева, Надежда Йорданова
Биологически факултет, СУ „Св. Климент Охридски”

12.20 – 12.45
Poster Presentation and Discussion

Session D.

Chairpersons:

Assoc. Prof. Mariana Panayotova-Pencheva, 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 Universiyt, Stara Zagora

Secretary: Katerina Todorova, DVM
Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences

13.30 – 14.00
DO1. Angiotensin peptides – the new players in the team of rennin-angiotensin system
P. Hadzhibozheva, A. Tolekova, Ts. Georgiev
Medical Faculty, Trakia University, Stara Zagora, Bulgaria

14.00 – 14.15
DO2. Arginine-vasopressin – beyond the visible
Ts. Georgiev, A. Tolekova, P. Hadzhibozheva
Medical Faculty, Trakia University, Stara Zagora, Bulgaria

14.15 – 14.45
DO3. Moxa- prevention, healing and longevity, part of the alternative/complementary medicine
Y. Staykova-Pirovska
Department of Family medicine, Trakia University, Stara Zagora, Bulgaria

14.45 – 15.05 Coffee Break

15.05 – 15.35

DO4. Възможности на китайската медицина за лечение на депресия

П. Джуров

Медицински факултет, Медицински университет, София

15.35 – 15.50

DO5. Туморсупресорни гени (p53, PTEN)

Людмила Стоева, Надежда Йорданова

Биологически факултет, СУ „Св. Климент Охридски"

15.50 – 16.05

DO6. Повратни точки в канцерогенезата

Георги Семовски

Медицински факултет, СУ „Св. Кл. Охридски"

16.05 – 16.20

DO7. Elizabeth Holmes – founder of "Theranos"

V. Kolyovska, S. Todorov

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

16.20 – 16.45

Poster Presentation and Discussion

Friday, 28 November 2014

Session E.

Chairpersons:

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

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

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

9.30 – 10.00

EO1. Hallucinogenic plants poisoning – case series

Radenkova – Saeva J, Stoyanova R
Clinic of Toxicology, Department for Adult, Emergency University Hospital "N.I.Pirogov", Sofia, Bulgaria

10.00 – 10.15

EO2. Treatment for Cyanide Poisoning – Classical and Developing Methods

Veneta V. Dimitrova, Vladimir P. Milov
Faculty of Medicine, Sofia University "St. Kl. Ohridski"

10.15 – 10.45

EO3. Low salt diet is associated with the prevention of exacerbation in patients with relapsing remitting MS

V. Kolyovska[1], V. Pavlova[1], S. Todorov[1], D. Maslarov[2]
[1]Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences;
[2]Medical University of Sofia, Neurology Clinic, First MHAT-Sofia

10.45 – 11.00

EO4. Болест на Паркинсон и ролята на допамина в нейната етиология

Ирена Михайлова
Медицински факултет, СУ „Св. Климент Охридски"

11.00 – 11.20 Coffee Break

EP1. The potential role of aluminium in Alzheimer's disease

Ivaylo Dankov
Faculty of Medicine, Sofia University "St. Kl. Ohridski"

11.20 – 11.35

EO5. Биохимични механизми на пристрастяването

Мария Валериева Младенова
Медицински факултет, СУ „Св. Климент Охридски"

11.35 – 11.50

EO6. Горенето на живота в пламъка на цигарата

Симона Красимирова Такова
Медицински факултет, СУ „Св. Климент Охридски"

11.50 – 12.05

EO7. Серотонинът – хормонът на щастието и болката

Милен Лазов
Медицински факултет, СУ „Св. Климент Охридски"

EP2. Vanadium and nervous system

Radostina Alexandrova[1], Abdulkadir Abudalleh[1], Delka Salkova[1], Lora Dyakova[2]

[1]Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences;[2]Institute of Neurobiology, Bulgarian Academy of Sciences

EP3. Базални ганглии

Елена Младенова, Ангелина Пискова, Деяна Жекова

Медицински факултет, СУ „Св. Климент Охридски"

EP4. Остър хирургичен корем

Деяна Жекова, Ангелина Пискова, Елена Младенова

Медицински факултет, СУ „Св. Климент Охридски"

12.05 – 12.30

Poster presentation and Discussion

Session F.

Chairpersons:

Prof. Reni Kalfin, MSc, PhD
Institute of Neurobiology, Bulgarian Academy of Sciences

Assoc. Prof. Diana Rabadzhieva, MSc, PhD
Institute of General and Inorganic Chemistry, Bulgarian Academy of Sciences

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

13.30 – 13.45

FO1. Calcium – a vital chemical element for the human body

Aleksey Mitev, Galya Tomova

Medical faculty of Medical University, Sofia

13.45 – 14.15

FO2. Calcium phosphate cements derived from anhydrous dicalcium phosphate and tetracalcium phosphate

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

Institute of General and Inorganic Chemistry, Bulgarian Academy of Sciences

14.15 – 14.45

FO3. Приложение на нанотехнологиите в тъканното инженерство и регенерация

Пламен Славов
Медицински факултет, СУ „Св. Климент Охридски”

14.45 – 15.05 Coffee Break

15.05 – 15.35

FO4. Brief journey in the world of tissue engineering

Radostina Alexandrova[1], Boyka Andonova-Lilova[1], Abdulkadir Abudalleh[1], Tanya Zhivkova[1], Pavel Mitrenga[1], Lora Dyakova[2], Orlin Alexandrov[3]
[1]Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences; [2]Institute of Neurobiology, Bulgarian Academy of Sciences; [3]Health Service, Gorna Malina, Bulgaria;

15.35 – 15.50

FO5. Bone tissue engineering

Radostina Alexandrova[1], Abdulkadir Abudalleh[1], Boyka Andonova-Lilova[1], Tanya Zhivkova[1], Pavel Mitrenga[1], Lora Dyakova[2], Orlin Alexandrov[3], Desislav Dinev[1.4], Simona Spasova[1,5], Olafur Sigurjonsson[6]
[1]Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences; [2]Institute of Neurobiology, Bulgarian Academy of Sciences; [3]Health Service, Gorna Malina, Bulgaria; [4]Faculty of Biology, Sofia University “St. Kl. Ohridski”, Bulgaria; [5]Faculty of Biology, Sofia University “St. Kl. Ohridski”, Bulgaria; [6]The Blood Bank, Landspitali- University Hospital/Reykjavik University, Reykjavik, Iceland

FP1. Развитието на терапевтичните ваксини в борбата с различни заболявания

Ангелина Пискова, Екатерина Петрова, Деяна Жекова
Медицински факултет, СУ „Св. Кл. Охридски”

FP2. Карцином на млечната жлеза – Her2 рецептор

Екатерина Петрова, Ангелина Пискова, Елена Младенова
Медицински факултет, СУ „Св. Кл. Охридски”

15.50 – 16.20

General Discussion and Closing Remarks

Session A.

Chairpersons:

Prof. Marin Alexandrov, DVM, PhD
Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences

Assoc. Prof. Stefka Tepavitcharova, MSc, PhD
Institute of General and Inorganic Chemistry, Bulgarian Academy of Sciences

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

AO1. In the world of zinc

Radostina Alexandrova[1], Tanya Zhivkova[1], Abdulkadir Abudalleh[1], Lora Dyakova[2], Pavel Mitrenga[1], Ivo Grabchev[3], Ogo A. Ogo[4], Daniela-Cristina Culita[5], Gabriela Marinescu[5]

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Abstract
Zinc (atomic number 30, atomic weight 65.37) is the second most common trace element in the human body. Iron, the most abundant metal in humans, occurs primarily in the blood, making zinc the most prevalent metal in human tissue other than the blood. The body of a 70-kg man contains approximately 2.3 g of zinc. About 64% of body's zinc are found in muscle, and about 28% are in the bones. The highest concentrations of zinc are found in tissues of the reproductive organs, most notably in the prostate gland, which contains zinc at a concentration of 87 μg/g wet weight (whole-body average = 33 μg/g wet weight). The relative concentration of free zinc ions in biological system ranges from 10^{-9}M in the cytoplasm of many cells to 10^{-3} M in some organelles.

Zinc is an essential element required for the normal function of more than 300 enzymes (representing more than 50 different classes) and > 1000 transcription factors and is known to take part in many biochemical processes supporting life. The human genome has been found to encode ~3000 zinc proteins. The element is involved in protein, nucleic acid, carbohydrate, and lipid metabolism, as well as in the control of gene transcripotion, cell differentiation, development, and growth. The important role of zinc for the development and maintenance of the immune and nervous systems is well known.

The nutritional essentiality of zinc for the growth of living organisms had been recognized long before zinc biochemistry began with the discovery of zinc in carbonic anhydrase in 1939.

The Recommended Daily Allowance (RDA) for zinc is 12-15 mg/kg, in balanced diets this amount is obtained mainly by eating meat and other sources of animal products. National Academy of Sciences of the United States defines admissible limit the ontake of 40 mg Zn / day in individuals 19 years and older. Zinc deficiency can promote different pathological

states including retardation of growth development in children, hypoginadism, dermatitis and delayed wound healing, alopecia, poor pregnancy outcomes and teratogeny, increased susceptibility to infections, etc. The prevalence of zinc deficiency is estimated to be high, with two billions of people affected, in particular in the developing world. In industrialized countries, elderly people are a high risk group for zinc deficiency. Zinc supplementation may not readily remedy zinc deficiency if other factors limit the capability of a cell to control zinc.

Acknowledgements
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AO2. Zinc fingers

Pavel Mitrenga[1], Lora Dyakova[2], Tanya Zhivkova[1], Boyka Andonova-Lilova[1], Ogo A. Ogo[3], Radostina Alexandrova[1]

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Abstract
Many eukaryotic proteins contain a region folded around a central Zn^{2+} ion resulting in a compact structural motif termed Zn finger. The Zn finger motif was first proposed for DNA binding domains of transcription factor IIIA by Miller et al. in 1985. This motifs can be different classes according to the groups that are in contact to the Zn^{2+} ion (Cys-Cys-His-His; Cys-Cys-Cys-Cys; Cys-Cys-Cys-His). The most common DNA binding motif encoded in the human genome is Cys-Cys-His-His. The 3D structure of Zn fingers is composed of two or three β-layers and one α-helix. The binding of the Zn^{2+} ion by Cys and His residues leads to the exposure of the α helix and it's interaction with DNA's major groove. Zinc fingers can

also provide Protein-protein interactions (Bactreriophage T4 Gene 32) and RNA-protein interactions (S. cerevisiae Nab2). As a DNA binding proteins, many zinc finger proteins are related to cancer, such as ZEB2 (Zinc finger E-box-binding homeobox 2) involved drug resistance in small cell lung cancer.

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AO3. Anticancer properties of zinc compounds

Radostina Alexandrova[1], Lora Dyakova[2], Tanya Zhivkova[1], Pavel Mitrenga[1], Desislav Dinev[1,3], Marin Alexandrov[1], Reni Kalfin[2], Greta Patroniou, Daniela-Cristina Culita[4], Gabriela Marinescu[4], Luminita Patron[4]

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Abstract
The aim of our study was to evaluate the influence of 11 Zn(II) complexes with various ligands (non-steroidal anti-inflammatory drugs, bile acids, etc.) on viability and proliferation of cultured human and animal cancer cells comparing their activity with those of 19 other complexes of Cu(II), Co(II), Ni(II) and La(III) with the same ligands. The experiments were performed using methods with different mechanisms of action and cellular targets such as thiazolyl blue tetrazolium bromide test (MTT) test, neutral red uptake cytotoxicity assay, crystal violet staining, trypan blue dye exclusion technique, double staining with acridine orange and propidium iodide, colony-forming method. Commercially available antitumor agent cisplatin served as a positive control.
Keywords: zinc, metal complexes, cancer cell lines, cytotoxic/cytostatic activity

Introduction
Zinc is an essential element, which plays a crucial role in a variety of cellular processes including DNA synthesis, behavioral responses, reproduction, bone formation, growth, and wound healing. There are data that zinc deficiency leads to various pathologies including growth retardation, delayed sexual maturation and hypogonadism, impaired immune function and increased susceptibility to infections, hair loss, diarrhea, etc. [2-4; 7, 8]. There are data that some zinc compounds exhibit antitumor properties in cultured cells and/or animal model *in vivo* [1, 2].
The aim of our study was to evaluate the influence of eleven Zn(II) complexes with various ligands on viability and proliferation of cultured tumor cells.

Materials and Methods

Compounds

The tested complexes – ligands and their metal complexes are presented in Table 1. Commercially available antitumor agent cisplatin served as a positive control.

Table 1. Metal complexes of Zinc (II) with various ligands

Ligand	Complexes	Number
Cholic acid	Cu(II), **Zn(II),** La(III),Co(II)	**4**
Dexidrocholic acid	**Zn(II),** Cu(II)	**2**
Litocholic acid	Co(II), **Zn(II),** Na(II)	**3**
Ursodeoxy cholic acid	Cu (II), **Zn(II)**, Ni(II)	**3**
Deoxycholic acid	Cu (II), **Zn(II),** Ni(II)	**3**
Meloxicam	Cu (II), **Zn(II),** Ni(II), Co(II)	**4**
Piroxicam	Cu (II), **Zn(II),** Ni(II), Co(II)	**4**
Isoxicam	Cu (II), **Zn(II),** Ni(II), Co(II)	**4**
Morpholine biguanide (MorphBig)	**Zn (II)**	**3**

The compounds (metal complexes and theis ligands) were dissolved in Dimethylsulfoxide (DMSO) and then diluted in culture medium. The final concentration of DMSO in stock solutions (where the concentration of the tested compound was 1 mg/mL) was 2%.

Cell cultures and cultivation

Permanent cell lines established from tumors of various origin (human, rat, chicken), etiology and histological type were used as model systems in our study (Table 2). The cells were grown as monolayer cultures in DMEM medium supplemented with 5-10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. They were kept in a humidified incubator (Thermo Scientific, HEPA Class 100) at 37ºC under 5% CO_2 in air. For routine passages adherent cells were detached using a mixture of 0.05% trypsin and 0.02 % EDTA. The cell lines were passaged 2-3 times per week (1:2 to 1:3 split). The experiments were performed during the exponential phase of cell growth.

Table 2. Permenent cell lines used as experimental models

Origin	Cell line
Human	HeLa – Cervical carcinoma
Rat	LSR-SF(SR) – Transplantable sarcoma induced by *Rous sarcoma virus*, strain *Shmidt-Ruppin*
Chicken	LSCC-SF(Mc29) - Transplantable liver cancer induced by the myelocytomatosis virus Mc29

Experimental disain
The influence of the compounds on cell viability and proliferation was determined in short- (24-48 h, with monolayer cultures) and long-term (16-18 days, with 3D colonies) experiments using methods with different molecular/cellular targets and mechanism(s) of action such as: thiazolyl blue tetrazolium bromide (MTT) test [6], neutral red uptake cytotoxicity assay (NR) [5], crystal violet staining (CVS) [9], trypan blue dye exclusion technique (TB), double staining with acridine orange and propidium iodide [10], colony-forming method [11].

Statistical analysis
Statistical differences between control and treated cells were assessed by unpaired Student *t*-test and calculated by Graph-Pad Prism 4.0 software package. Effective concentrations of the compounds – CC_{50} and/or CC_{90} were estimated by Origin 6.1™ .

Results

The MTT test, neutral red uptake cytotoxicity assay, crystal violet staining and/or trypan blue dye exclusion method were performed after 24h, 48h and 72h incubation periods. Relative cell viability, expressed as a percentage of the untreated control (100% viability), was calculated for each concentration. Concentration–response curves were prepared and the effective concentration of the compounds – CC_{50} (causing a 50 % reduction of cell viability) and/or CC_{90} (causing a 90 % reduction of cell viability) was estimated. Hierarchical orders were prepared according to the cytotoxic/cytostatic activities of the compounds. Some of them are presented in Table 3.

A positive correlation between the cytotoxicity assays – MTT test (reflects damage to mitochondria), Neutral red uptake cytotoxycity test (indicates damage to lysosomes and Golgi apparatus), Crystal violet staining (nuclear staining) and Trypan blue dye exclusion technique (shows damage to plasma membrane) as well as between short term – (MTT, NR, CVS, TB with monolayer cultures) and long term (colony forming method with 3D colonies) experiments was observed. Cytopathologycal changes were visualized by double staining with acridine otange and propidium iodide in the cells treated by effective concentrations of the compounds.

The results obtained revealed that:

i) Zn(II) compounds significantly decrease viability and proliferation of the treated cells in a time- and concentration-dependent manner;

ii) Zn(II) complexes show more pronounced cytotoxic and antiproliferative activities than ligands alone;

iii) The virus-transformed LSCC-SF-Mc29 chicken liver cancer tumor cells that express v-myc oncogene has been found to be the most sensitive to the cytotoxic and cytostatic activities of the compounds;

iv) Zn(II) and Cu(II) complexes are more pronounced cytotoxic/cytostatic agents as compared to Co(II) and Ni(II) complexes with the same ligands;

v) The examined metal complexes are less effective antitumor agents than cisplatin.

Table 4. Hierarchic orders of the compounds investigated according to their cytotoxic and/or antiproliferative activities

According CC_{50} (μM)			
Cell line	**Method**	**Hour**	**Hierarchic order**
LSCC-SF-Mc29	**MTT**	**72h**	**Zn-Meloxicam > Zn-UDCA > Zn-DCA > Zn1 > Zn2 > Zn3 > Zn-Isoxicam > Zn-Piroxicam**
LSR-SF-SR	**MTT**	**72h**	**Zn-Meloxicam > Zn-DCA > Zn-UDCA > Zn3 > Zn1> Zn-Isoxicam > Zn2 = Zn-Piroxicam**
HeLa	**MTT**	**72h**	**Zn-DCA > Zn-UDCA > Zn3 >Zn1 > Zn-Meloxicam > Zn-Isoxicam > Zn2 = Zn-Piroxicam**
According CC_{90} (μM)			
Cell line	**Method**	**Hour**	**Hierarchic order**
LSCC-SF-Mc29	**MTT**	**72h**	**Zn-DCA >Zn-UDCA > Zn2 > Zn1 > Zn3 > Zn-Isoxicam > Zn-Meloxicam = Zn-Piroxicam**
LSR-SF-SR	**MTT**	**72h**	**Zn-DCA = Zn-UDCA> Zn1> Zn-Isoxicam > Zn2 = Zn3 = Zn-Meloxicam = Zn-Piroxicam**
HeLa	**MTT**	**72h**	**Zn-DCA > Zn-UDCA > Zn-Isoxicam > Zn-Meloxicam = Zn2 = Zn-Piroxicam = Zn1 = Zn3**

MTT= thiazolyl blue tetrazolium bromide test

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AO4. Metal complexes and drug resistant cancer cells

Radostina Alexandrova[1], Lora Dyakova[2], Tanya Zhivkova[1], Pavel Mitrenga[1], Marin Alexandrov[1], Reni Kalfin[2], Ramona Tudose[3], Elena-Maria Mosoarca[3], Otilia Costisor[3], Daniela-Cristina Culita[4], Gabriela Marinescu[4], Luminita Patron[4], Katalin Nemet[5], Jan Stenvang[6]

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Introduction. The acquisition of drug resistance in tumor cells is the leading factor responsible for the failure of cancer chemotherapy. Resistance has been documented against every effective anticancer drug and can develop by numerous mechanisms including decreased drug uptake, activation of detoxifying systems, activation of DNA repair mechanisms, evasion of drug-induced apoptosis, etc. A common mechanism of multidrug resistance is the overexpression of adenine triphosphate -binding cassette (ABC) efflux transporters in cancer cells, such as ABCB1/P-glycoprotein (P-gp), ABCC1/multidrug-resistance-associated protein (MRP) and ABCG2/breast cancer resistance protein (BCRP), that pump various structurally unrelated compounds out of cells.

Aim. The aim of our study presented was to evaluate the effect of Zn(II), Cu(I, II), Co(II), Ni(II), Fe(II, III) complexes with various ligands (bile acids, non-steroidal anti-inflammatory drugs, Mannich bases) in multidrug resistant cells.

Materials and methods. The following human cell cultures were used as experimental models – A431 (squamous cell carcinoma) and its clones expressing MDR, MRP or ABCG2 multidrug resistance genes; NCI-H1650 (non small cell lung cancer) and its clones G7 and C11 that are resistant to tyrosin kinase inhibitor Gefitinib. The investigations were performed by MTT test and/or neutral red uptake cytotoxicity assay, double staining with acridine orange and propidium iodide and colony-forming method.

Results. The compounds examined suppress to a different extent the in vitro growth of the treated cells in a time- and concentration- dependent manner.

Conclusion. Searching for new cytotoxic agents in multidrug resistant cells is of particular importance. Experiments are underway to clarify the structure-activity relationship as well as the mechanisms of action of metal complexes in multidrug resistant cells.

Key words: metal complexes, tumor cell lines, drug resistance, cytotoxic/cytostatic activity

Acknowledgement
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AO5 "pH" In cell biology, microbiology and biotechnology

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Abstract
The article shows the importance of pH as a key indicator which is monitored strictly in different types of laboratories associated with the production, quality control and reference activities. It was found direct dependence on the index for growth promotion and differentiating properties of different culture media in microbiology and yield in bioreactor cultivation.
Keywords: pH; bacteria; viruses; culture media; cell culture; bioreactor cultivation.

Introduction
Some microorganisms survive in extreme pH conditions, such as the increasing acidity in volcanic areas. *Helicobacter spp.,* which are associated with ulceration in gastritis, can proliferate in the gastric pH (1-3). *Vibrio Cholerae*, which is the causative agent of cholera, can propagate at pH 8-9. However, most pathogens grow at varying pH values of about 3 units and prefer neutral pH values, because they are close to pH 7.0 in the body fluids. Fungi typically prefer or are tolerant to more acidic pH than bacteria. Both bacteria' and viruses' prevalence vary accordingly to changes of the pH. Most human pathogenic viruses inactivate by acid and the exposure to pH 4 for 6 hours kills the more sensitive of them. An exposure time for several days is sufficient to kill and more resistant. The Enteroviruses that cause

gastrointestinal infections are transmitted by the faecal-oral route, so that they survive during the gastric transit exposure to gastric acid at pH 1-3 [5].
With the advent of the Good Manufacturing Practice and Good Laboratory Practice in the daily lives of many laboratories and companies, the registration of basic microbiological and physico-chemical indicators that provide information on quality of the culture media and changes that occur in the bioreactor cultivation became compulsory. Optimization in terms of pH is absolutely necessary even when working with cell cultures.
The most important indicator of the quality of culture media in microbiology and biotechnology is their growth capacity. This basic indicator is influenced not only by the composition of the medium and the quality of the ingredients, but by the optimal conditions for the cultivation of microorganisms which are established. Incubation temperature, humidity, pH and sterility are indicators, without whom good results cannot be achieved and therefore for all types of culture media are fixed certain standards for deviation of these parameters.
The industrial production of penicillin takes place in fermentation containers with volume of 180,000 liters. The fermentor is inoculated from a seed reservoir with a capacity of 500 liters. After completion of the fermentation, the penicillin is contained in an acellular environment. This means that the culture can be filtered so as to remove the cells and the supernatant is processed so as to extract the antibiotic. In order to remove the antibiotic in the solvent, an acidification is required such as amyl acetate or butyl acetate). This step is performed quickly and at low temperature, as penicillin is unstable in acidic solutions. The addition of a phosphate buffer results in a crystallization of the penicillin, from where it may be washed and dried [5]. Quoted technological process is indicative for the connection between the tolerance to variations in pH and the need to resort to extreme values (within the permissible limitations) of the concerned product.

Methods and equipment
For measuring the pH of agar and liquid culture media ("Bull Bio-NCIPD") was used a pH meter "Methrom 744". To measure the pH of the agar media was used an electrode for surface and for the liquid - for fluids.
The requirement is respected about the measured temperature of the culture medium and the environment temperature, that have to be 24 ± 1 ° C, because the pH is a temperature dependent indicator.
For optimal growth of different types of cell lines is required pH in the range of 6.9-7.4. In most cases, the medium is buffered via CO_2/HCO_3. For maintenance of suitable pH, the bicarbonate concentration in the medium should be in equilibrium with the CO_2 in the atmosphere. Therefore, most cultures are cultivated in a medium containing 23 mM bicarbonate and 5% CO_2 in the atmosphere. The presence of CO_2 is obligatory, because without it the medium quickly alkalinizes. When a CO_2 incubator is missing, the cells are grown in a tightly closed vials, buffered with an organic buffer - mostly Hepes (10-25 mM), which provides a pH in the range of 6.8 to 7.2. Isotonicity of the medium, which is provided by the salts dissolved therein, also plays a role in regulating the pH.

Principle of the method
pH means "pondus hydrogenii“ (potential for hydrogen). A measurement of hydrogen potential is a value for the measurement of the concentration of H+. The pH range goes from acid to basic. The mathematical definition of the pH value is minus log of the hydrogen concentration: pH = - log c (H+). Very important is that it is defined in water as the ion product of water: the concentration of hydrogen ions multiplied by the concentration of hydroxide ions is equal to ten power minus 14: c(H+) x c(OH-) = 10-14. From this

definition one can calculate the expected pH value: c(HCl) = 0.1 mol/L, pH =1; c (NaOH) = 0.1 mol/L, pH = 13. A pH sensor must only respond to changes of the hydrogen ion concentration. Actually measured is „voltage“, the difference of potential between the two electrodes (Figures 1, 2). For every potentiometric measurement 2 electrodes (ME and RE) are needed. The difference is the entrance signal on the pH-meter [3; 4]. The pH meter is a millivoltmeter with a special high impedance input electric circuit and an electric circuit to convert the mV of the electrode in pH output units.

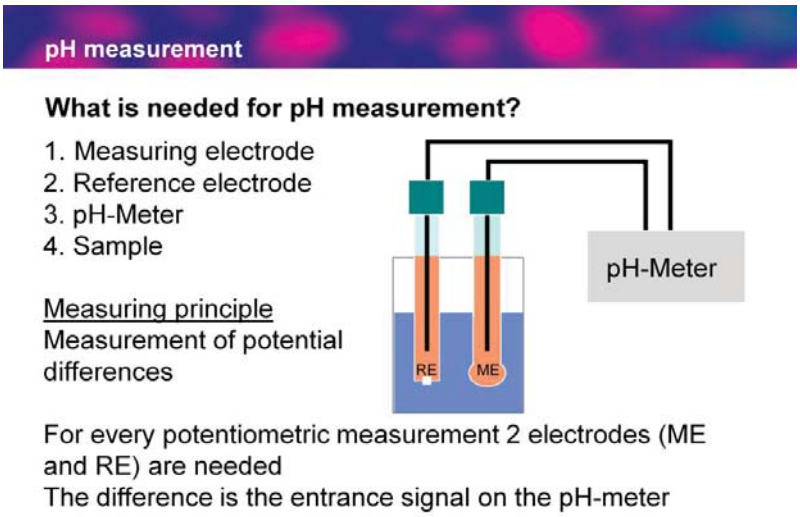

Figure 1: Facilities and principle of pH measurement.

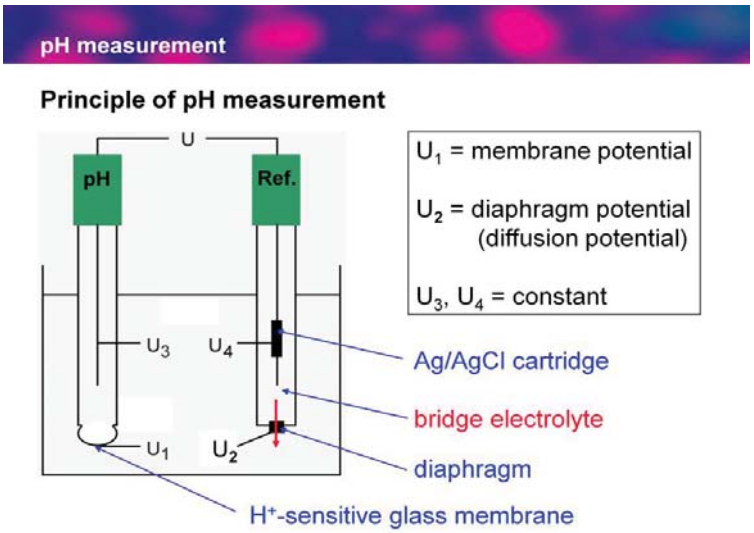

Figure 2: Principe of pH measurement by surface electrode.

Calibration

It is recommended to be made 2- or more point calibration as the pH value of sample must be in within calibration range. The 2 buffers give 2 potentials at 2 pH values which are used to determine the slope. The theoretical value is 59.16 mV for one pH unit at 25°C. Always have to be used fresh buffers. The temperature behavior of the buffers should be similar like the temperature of the real samples [3; 4].

Results and Discussion

The influence of the pH indicator is monitored throughout a continuous and diverse manufacturing of culture media like the one used in "Bull Bio-NCIPD" LTD. All the agar and liquid culture media are followed by daily quality control of this indicator. Both the pH of water for injection, which is used for dissolution of the ingredients and the process of sterilization could have impact on pH of the routinely produced culture media. Empirically, it has been found that once the culture media, which is prepared and then sterilized by autoclaving at 121 ° C after sterilization will be with modified values of this parameter. When pH of the culture media is adjusted without buffers before sterilization, we can observe a change in pH in the opposite direction of the adjustments. For example, if the pH has been increased by sodium hydroxide, after sterilization is observed decrease of pH. Therefore, the pH of the culture media is always measured on the ready for use media.

Produced batch of **Mannitol Salt Agar (MSA)** with abnormal pH affects the growth capacity of *S.aureus* ATCC25923. Taking into account that the control of the growth capacity uses a procedure that guarantees seeding to 100 CFU of the corresponding microorganism, the change in physico-chemical parameters are easily detectable by parallel seeding of microorganism on the reference culture medium. In the case of calculation of the index of productivity - P_R [7] for batch produced of MSA by the formula:

$$PR = N_S / N_O$$

N_O - total number of colonies on standard medium

N_S - total number of colonies on the test medium

It is found that the value of the index is 0, when the standard value ≈ 0,7. The test batch was not released for sale.

Uri test is a product that contains two culture media - MacConkey agar and Cystine Lactose Electrolyte Deficient (CLED) spilled respectively on both sides of the ribbed plate attached to the cap in a plastic container. It is used for quantitative detection of the pathogens presence in the microbial testing of aseptically taken urine. It was found that the abnormal pH of CLED agar leads to reduced growth capacity of *Staphylococcus aureus* ATCC25923.

At **Kligler agar,** which is polytropic culture medium, the abnormal pH of the medium leads to a deviation in its selective identification properties (Figure 3). The degradation of sugars and acids formed during the process affects the phenol-rot, which is an indicator of the pH of the medium, and the change of the color from orange-red to yellow and in an alkaline medium to dark red. Some bacteria convert the thiosulphate to hydrogen sulfide, which reacts with iron ions to form iron sulfide and change the medium color in black.

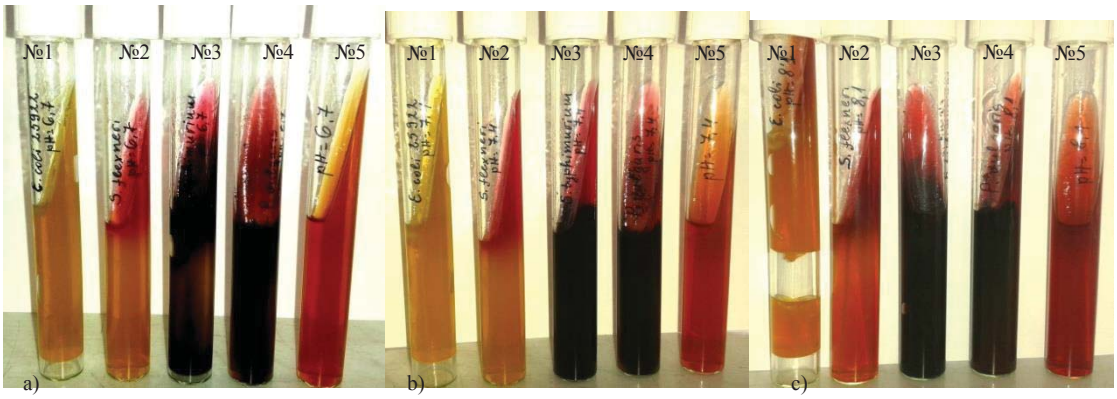

Figure 3: Effect of pH on the differentiating features of Kligler agar.

The pH standard value for Kligler agar is from 7.2 to 7.6. For the purposes of the experiment, two of the three samples have been produced at abnormal pH. The first tube of each sample

with Kligler agar is inoculated with the control strain *Escherichia coli ATCC 25922*. A higher pH than the observed norm leads to increased gas formation and delayed absorption of the lactose (the slope is not completely yellow). The second tube was inoculated with the control strain *Shigella flexneri NCTC 9950.* At a pH above the norm, the yellowing of the stub is not as expressed, which is associated with the assimilation of the glucose. In the tubes №3 and 4, respectively seeded with *Salmonella typhimurium ATCC 14028* and *Proteus vulgaris ATCC 13315*, there are no differences of variation in the pH indicator.

Mueller Hinton Agar(MHA) is a specific medium which is used only for antibiotic sensitivity testing by disk diffusion method of Kirby-Bauer [2]. Due to its specificity, the medium requires strict compliance with the rules in CLSI / NCCLS [1].

The variation in 4 key indicators is monitored through an indirect method by setting antimicrobial disks from different classes of antibiotics.

The first is the thickness of the agar layer, which must be 4 mm, the other three indicators are physicochemical and include: content of thymine and thymidine, content of Ca^{2+} and Mg^{2+} ions and the pH of the ready-to-use medium. The variation of pH is allowed in a narrow range from 7.2 to 7.4. It was found experimentally that due to this narrow range of tolerance, there is an influence over the pH caused by the keeping of the Petri dishes for 24 hours in a refrigerator at 2-8 ° C and subsequent tempering to ≈24 ° C. Because of this, although in the standard CLSI M2-A9 there is not a preliminary condition to leave the Petri dish of MHA in a refrigerator for measurement of the pH, it is accepted as a practice in The Laboratory of Control of In Vitro Diagnostic Devices ("BB-NCIPD"). Deviations in these four indicators lead to a wrong reporting of the zones of suppression. This requires a specifying of the manufacturing process and full compliance with the requirements of Good Manufacturing Practice. Indirect microbiological method developed in The Laboratory of Control of In Vitro Diagnostic Devices for detection of deviations in the qualitative composition of Mueller Hinton agar refers to the observed dissimilarities in the eligible zones of suppression by disk diffusion method of unfastidious referent strains with defined antimicrobial disks or groups of disks on the Petri dish of MHA without blood.

Interpretation of results is performed by using the theoretical data from CLSI / NCCLS M100-S17 Performance standards for antimicrobial susceptibility testing, Table 3C. Disk Diffusion QC Troubleshooting Guide, which are presented in tables with chosen by us groups of antimicrobial disks (Table 1).

Table 1: Interpretation of the deviations in the pH according to the established standards by the CLSI / NCCLS (7.2 to 7.4)

<table>
<tr><td colspan="3">Indicator: pH</td></tr>
<tr><td>Antimicrobial disks</td><td>Zone larger than the upper limit</td><td>Zone smaller than the lower limit</td></tr>
<tr><td>Ciprofloxacin 5µg</td><td rowspan="4">pH is higher than 7.4</td><td rowspan="4">pH is lower than the 7.2</td></tr>
<tr><td>Clindamycin 2µg</td></tr>
<tr><td>Erythromycin 15µg</td></tr>
<tr><td>Gentamicin10µg, Tobramycin 10µg</td></tr>
<tr><td>Tetracycline 30µg, Doxycycline 30µg</td><td>pH is lower than the 7.2</td><td>pH is higher than 7.4</td></tr>
</table>

- At pH lower than 7.2 become inactivate antimicrobial substances from the group of Aminoglycosides, Quinolones and Macrolides, while other agents (tetracycline) are more active and have larger zones of suppression.
- At pH higher than 7.4 it can be expected the opposite effect.

In testing of the quality of batches MHA with 5% blood are applied the rules of zones of suppression of *Streptococcus pneumoniae* ATCC 49619 according to CLSI. M100-S21 (Table 3B. Disk Diffusion: Quality Control Ranges for Fastidious Organisms). Three types of antimicrobial disk in shelf life are selected and the zones of suppression are interpreted according to their norms of *Streptococcus pneumoniae* ATCC 49619 in CLSI M100-S21. According to the requirement of CLSI M6-A2 for testing new reference culture medium of *Streptococcus pneumoniae* ATCC 49619 are used the following antimicrobial disks: Table 2.

Table 2: Antimicrobial Disks control of MHA with 5% blood.

Antimicrobial agent	Antimicrobial disk loading (μg)	Zone of inhibition by CLSI M100-S21 (mm)
Clindamycin	2	19–25
Erythromycin	15	25–30
Levofloxacin	5	20–25
Trimethoprim-sulfamethoxazole	1.25/ 23.75	20–28
Vancomycin	30	20–27

In testing the quality of batches of **MHA with 5% defibrinated horse blood and 20 mg / L ß-NAD** shall be set up three antimicrobial disks with antimicrobial content according to the requirements of the EUCAST on two Petri dishes. The third plate is used for verifying the sterility and for measuring the pH. In literature [6] human blood pH is 7.35. In healthy animals the blood pH is also approximately around the normal. Experimentally has been found that in a batch of MHA with 5% blood, despite the small amount of blood in it, if it is out of normal range of pH, this should affect the pH of the final product.

Experimentally has been found that the temperature and the pH are inversely related, i.e. if the temperature of the test culture medium is higher than normal, it is reported less than the actual value of the pH. If the deviation of the temperature is in the range of 1 to 2 ° C, the difference in the variation of the pH value is very small and is not relevant for the quality of the culture media.

The most significant for registration of deviations in the quality of the culture media and to monitor changes in the process of bioreactor cultivation is the physiochemical parameter "pH" of the environment. The change in pH was monitored hourly during bioreactor cultivation of the microorganisms used in the production of the native tetanus toxoid (Table 3). In the first 72 hours is observed decreasing of the pH and then from 72-th to 144 hour the pH begins to increase. In the first 72 hours there is an increase in the biomass and then begins the process of dying of the bacteria and the releasing of a tetanus toxin, which could be detected in an increased pH value (Table 3).

Table 3:

	0 hours	24 hours	48 hours	72 hours	96 hours	120 hours	144 hours
pH-1	6,61	6,23	6,2	6,1	6,5	6,9	6,91
pH-2	6,82	6,47	6,4	6,3	6,62	7,02	7,03
pH-3	6,78	6,43	6,4	6,3	6,68	6,93	6,94
pH-4	6,6	6,15	6,1	6,1	6,45	6,92	6,93
pH-5	6,22	5,78	5,62	5,7	6,09	6,05	6,71
pH-6	6,42	5,99	5,85	5,9	6,34	6,8	6,83
pH-7	6,64	6,3	6,3	6,2	6,7	7,21	7,22
pH-8	6,97	6,58	6,5	6,5	6,67	6,88	6,96
pH-9	6,81	6,48	6,4	6,4	6,55	6,88	6,9
pH-10	6,74	6,5	6,4	6,3	6,5	6,88	6,89
pH-11	6,75	6,43	6,4	6,3	6,62	7,08	7,12
pH-12	6,81	6,49	6,4	6,4	6,57	7,04	7,08
t C	34	34	34	34	34	34	34
quantity of air in l/min	6	20	20	20	40	40	40

Empirically it has been shown that if the pH does not start to increase after 72 hours, but on the contrary - to decreas, there is a contamination in the process of cultivation. Over the entire period of reactor biocultivation is observed a decreasing of the amount of dissolved oxygen in the reactor.

Conclusion

Although some microorganisms have a wider range of tolerance to changes in the pH of the culture medium, the importance of the pH indicator to the cell cultures and the culture media is essential. Deviations of the growth capacity indicator of culture media is a result of reported pH out of the norm for the given growth medium, which is seen as a priority in certain strains. The pH indicator can affect both the differentiation in the identification of viruses, bacteria and fungy and the yield in bioreactor cultivation.

Acknowledgments

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AO6. The role of glutathione in the pathogenesis of influenza virus infection

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Abstract:

Glutathione (γ-glutamylcysteinylglycine, GSH) is an ubiquitous sulfhydryl-containing tripeptide produced by most mammalian cells. It is often called "the mother of all antioxidants" because it plays significant role in the intracellular redox balance in different ways including mechanism of elimination of reactive oxygen species (ROS). Oxygen-free radicals, more generally known as reactive oxygen species along with reactive nitrogen species (RNS) are well recognised for playing a dual role as both deleterious and beneficial species. The cumulative production of ROS/RNS through either endogenous or exogenous conditions is involved as element of oxidative stress. Many findings have demonstrated that infection by RNA virus, including influenza, induces oxidative stress in host cells.

Introduction:

Reactive Oxygen Species (ROS) are a necessary evil of aerobic life, being generated continuously during the process of respiration, but with the potential to cause oxidative deterioration of protein, lipid and DNA. A range of different stress conditions elevates ROS generation. ROS damage is linked to serious degenerative conditions in humans [2]. The cumulative production of ROS/RNS through either endogenous or exogenous ways is termed oxidative stress [16]. Oxidative stress corresponds to an imbalance between the rate of oxidant production and that of their degradation [15]. It is implicated as a pathogenic factor in a number of viral infections [3] including influenza viral infection. Protective effects of oxidative damages have specific inhibitors of viral replication rimantadin and oseltamivir [10]. Taking of antioxidant as vit. E, vit. C, flavonoids quercetin and rutin on prophylaxis scheme before virus inoculation showed a protective effect on oxidative damages and viral toxicity. The purpose of this study is to elucidate the role of glutathione as a preventive agent in the pathogenesis of influenza viral infection.

What is Glutathione?

Glutathione (γ-glutamylcysteinylglycine, GSH), an ubiquitous sulfhydryl-containing tripeptide produced by most mammalian cells, is the cells principle mechanism of eliminating ROS [12]. GSH is the most abundant low-molecular-weight thiol, and GSH/ glutathione disulfide is the major redox couple in animal cells. The synthesis of GSH from glutamate, cysteine, and glycine is catalyzed sequentially by two cytosolic enzymes, -glutamylcysteine synthetase and GSH synthetase. Compelling evidence shows that GSH synthesis is regulated primarily by γ-glutamylcysteine synthetase activity, cysteine availability, and GSH feedback inhibition. [19]

The definition of antioxidants as compounds that inhibit or delay the oxidation of substrates even if the compound is present in a significantly lower concentration than the oxidised substrate [8] indicates that almost everything found in foods and in living tissues including proteins, lipids, carbohydrates and DNA is able to act as an antioxidant. GSH serves as an important intracellular water-soluble antioxidant and detoxifying agent [2]. It performs various functions ranging from cellular metabolism to transport as well as protection against free radicals and reactive oxygen species. The antioxidant function of the tripeptide is related to oxidation of the thiol group of its cysteine residue with formation of a disulfide (GSSG), which is, in turn, catalytically reduced back to the thiol form (GSH) by glutathione reductase [5].

GSH tripeptide is the most important antioxidant defense of eukaryotic cells. Its antioxidant activity is due to the interconversion of GSH and its oxidized form, glutathione disulfide (GSSG), and under physiological conditions, a GSH/GSSG ratio >1 is maintained [13]. Reduced glutathione (GSH) is the most prevalent non-protein thiol in animal cells [6].

GSH is synthesized de novo in a two-step enzymatic process in which glutamine and cysteine are covalently linked by the heterodimeric enzyme γ-glutamylcysteine synthetase or glutamatecysteine ligase (GCL) to form the product γ-glutamylcysteine. This is the rate limiting step in the synthesis of GSH, and cysteine is both the rate limiting reactant and the component that provides GSH with antioxidant activity, as cysteine's sulfhydryl bond is oxidized during the reduction of ROS. In the second step of the reaction, γ-glutamylcysteine is bonded to glycine to form a complete GSH molecule [12].

It is typically present in high (0.1 - 10 mM) levels and is thus both the most prevalent cellular thiol and the most abundant low molecular weight peptide. In many cells GSH accounts for more than 90% of the total nonprotein sulfur. GSH was recognized 100 years ago, and its structure was established in 1935 (figure 1) (11).

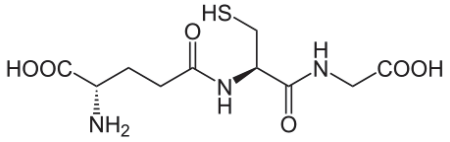
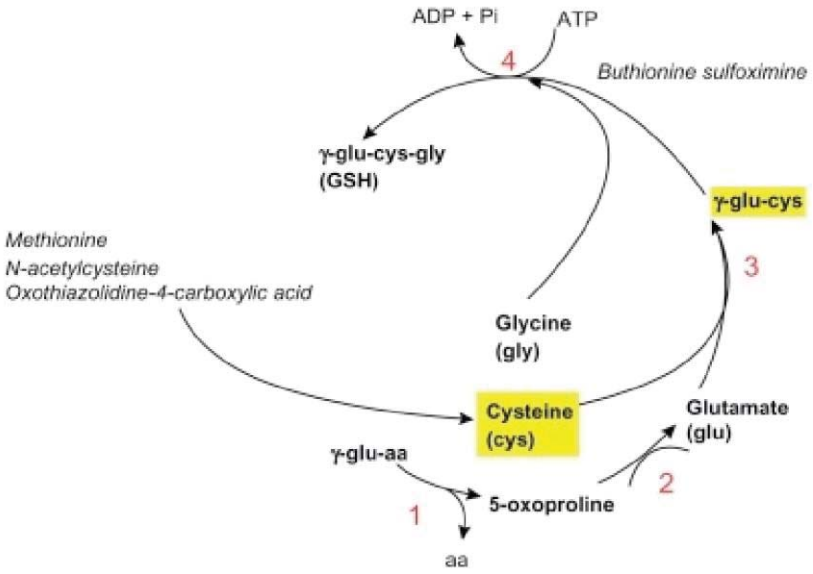


Figure 1. Chemical structure of glutathione.

Efforts to increase cellular GSH levels by the direct administration of reduced glutathione face limitations by solubility, absorption and stability. This has resulted in a significant effort to identify GSH analogues or precursors, or to generate mimic molecules with the capacity to reduce oxygen radical and peroxidation related effects on cells.

Glutathione is an important intracellular antioxidant and redox potential regulator that plays a vital role in drug detoxification and elimination and in cellular protection from damage by free radicals, peroxides, and toxins [18].



(modified by Meister A, 1983)

Figure 2. Glutathione (GSH) biosynthesis. 1) gamma-glutamyl cyclotransferase; 2) 5-oxoprolinase; 3) gammaglutamylcysteine synthetase; 4) Glutathione synthetase. Outlined in yellow are the limiting factors in GSH biosynthesis.

Influenza virus infection

Influenza is a seasonal viral infection associated with significant morbidity and mortality.[14]. The clinical spectrum of pandemic influenza A (H1N1) virus infection was broad, ranging from mild upper respiratory tract illness with or without fever and occasional gastrointestinal symptoms such as vomiting or diarrhea and exacerbation of underlying conditions, to severe complications such as pneumonia resulting in respiratory failure, acute respiratory distress syndrome, multi-organ failure and even death [17]. Most complications have occurred among previously healthy individuals, with obesity and respiratory disease as the strongest risk factors. Pulmonary complications are common. Primary influenza pneumonia occurs most commonly in adults and may progress rapidly to acute lung injury requiring mechanical ventilation. [14]

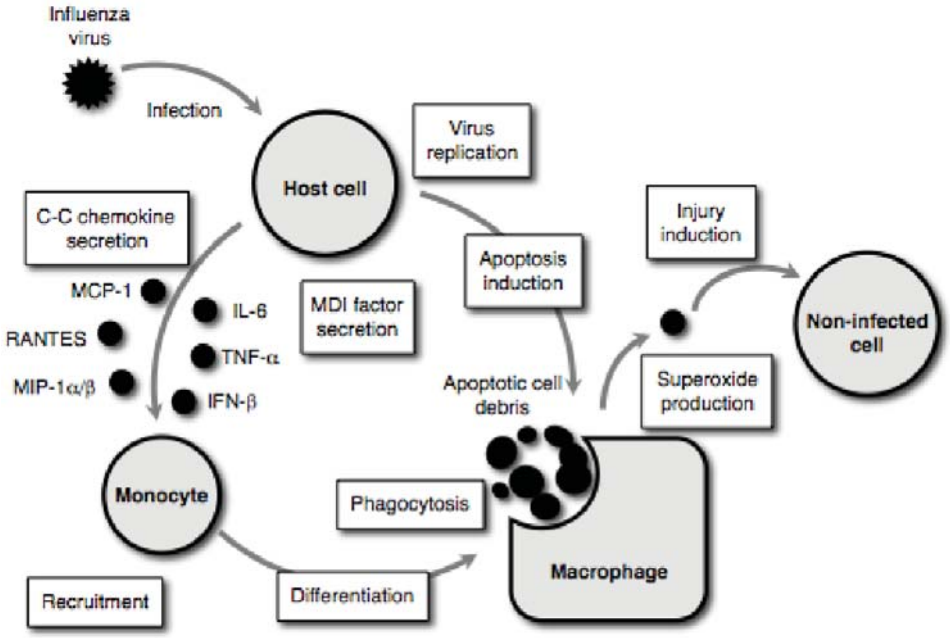


Figure 3. Tissue injury model during influenza virus infection. (Uchide, N. et al.)

Why is GSH important for IAV?

Infection by RNA virus induces oxidative stress in host cells [4]. Influenza virus(IAV) inhibits cellular respiration (mitochondrial O2 consumption),diminishes cellular glutathione(GSH) and triggers apoptosis [1]. Viral infection is often associated with redox changes characteristic of oxidative stress that can be caused by several factors, among which the decrease in intracellular glutathione levels [7].

The mechanisms by which pro-inflammatory cytokines decrease intracellular GSH may be in response to increased levels of free radicals. A study found that increases in pro-inflammatory cytokines resulted in an increase of free radicals which is targeted by free GSH in host cells [12]**.** Cells of different origins display differential permissivity for influenza A virus replication, depending on their intracellular redox power [13].

Accumulating evidence suggests that cellular redox status plays an important role in regulating viral replication and infectivity [4]. Many studies have shown that superoxide anion produced by macrophages infiltrated into the virus-infected organs is implicated in the development of severe influenza-associated complications. Selected antioxidants, such as pyrrolidine dithiocabamate, *N*-acetyl-L-cysteine, glutathione, nordihydroguaiaretic acid, thujaplicin, resveratrol, (+)-vitisin A, ambroxol, ascorbic acid, 5,7,4-trihydroxy-8-methoxyflavone, catechins, quercetin 3-rhamnoside, isoquercetin and oligonol, inhibit the proliferation of influenza virus [17].

Reduced glutathione displays anti-influenza activity *in vitro* and *in vivo.* The addition of reduced glutathione into culture medium exogenously blocked the induction of apoptosis through the inhibition of viral macromolecule synthesis in Madin-Darby canine kidney (MDCK) cells after influenza virus infection. In BALB/c mice, inclusion of reduced glutathione in the drinking water decreased viral titer in both lung and trachea homogenates at 4 days after intranasal inoculation with a mouse-adopted influenza strain A/X-31. Moreover, both the levels of Bcl-2 expression and the content of intracellular reduced glutathione contribute to the ability of host cells for down-regulating influenza virus replication, although their effects are exerted at different stages of the viral life-cycle [17].

Infection in mice reduces lung tissue GSH and inhibition of GSH impairs viral replication.

Exogenous administration of several molecules (i.e. GSH, GSH derivatives, GSH precursor such as glutamine or cysteine, α-lipoic acid) able to increase cellular GSH concentration inhibits the replication of several viruses, including the influenza virus, through different mechanisms. Cai *et al*. (2003) demonstrated that, when added extracellularly, GSH had dose-dependent anti-influenza effect in cultured cells. They suggested that such an effect was probably due to an inhibition of apoptosis in host cells and subsequent release of the active virus from dead cells, but it is likely that other mechanisms are also involved. The anti-influenza activity of GSH has also been demonstrated in an *in vivo* experimental model. In particular, the addition of GSH to the drinking water of influenza infected mice inhibited viral titer in the trachea and lungs [4]. These effects were the result of GSH-C4's interference with maturation of the viral glycoprotein hemagglutinin (HA), a process that is largely mediated by the redox-sensitive activities of host-cell oxidoreductase-protein disulfide isomerase [2].

Conclusions

Growing evidence indicates that viral replication is regulated by the redox state of the host cells. Data prove that the thiol antioxidant GSH has an anti-influenza activity *in vitro* and *in vivo*. In theory, co-medication of specific inhibitors of influenza virus replication with antioxidants which are responsible for the integrity of cell membrane could improve conventional chemotherapy for severe influenza-associated complications.

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АО7. Ефект на екстракти от Teucrium chamaedrys спрямо Herpes simplex virus тип 2

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Резюме

Растенията от род *Teucrium* (*Lamiaceae*) са богати на вещества от групата на флавоноидите, холините и танините, както и етерични масла. Много видове са познати с техните диуретични и антисептични свойства. От популацията на *Teucrium chamaedrys*, около село Фотиново са приготвени три екстракта – хлороформен и два метанолови.

Установено е въздействието им върху клетъчна линия MDBK, като е определена максималната поносима концентрация и концентрацията, при която се наблюдава 50% преживяемост в клетъчната линия. Изследвано е въздействието спрямо на репликацията на HSV-2 като най-голям ефект беше отчетен при хлороформения екстракт.

Определено е също така влиянието на трите екстракта спрямо извънклетъчната форма на вируса. Като най-силно въздействие показва хлороформения екстракт с инактивация на HSV-2 вирион от 99,99% до 100%.

Представените данни са получени при изследвани в рамките на проект от 2014 към ФНИ на СУ.

AO8. QSAR MODELS FOR PREDICTING TRANSCELLULAR PERMEABILITY OF BIOACTIVE COMPOUNDS

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Abstract

The parallel artificial membrane permeation assay (PAMPA) is a high throughput *in vitro* assay system that evaluates transcellular permeation of small drug-like molecules [3]. PAMPA is intensively used in the pharmaceutical research to screen for human intestinal absorption because PAMPA permeability has been shown to correlate with both Caco-2 cell permeability and human intestinal absorption. Previous studies have reported QSAR (quantitative structure-activity relationship) models that relate PAMPA permeability with either descriptors based on free energy of solvation of the compounds in hydrophobic and hydrophylic media [4], or structural descriptors like pKa, logP, surface area of hydrogen bond donors and acceptors, etc [2]. In the present study we report a classical QSAR analysis on a data set of nearly 300 diverse drugs with PAMPA permeability coefficients measured at pH 6.5 and 7.4 [1]. The structures of the compounds were obtained from the NCI's Chemical Identifier Resolver service, and the descriptors were calculated by the software tools ACD/Labs' PhysChem Suite and ChemAxon's Marvin. The best QSAR models included the apparent partition coefficient, the topological polar surface area, and the molecular weight of the compounds. The models were subjected to external validation and proved to have high predictivity. As a future prospect the models will be implemented in the open source knowledge-mining platform KNIME and can be applied for selecting compounds with suitable permeability from chemical libraries.

Acknowledgment. Acknowledgment: The funding from the European Community's 7th Framework Program COSMOS Project (grant n°266835) and from the Ministry of Education, Youth and Science, Bulgaria (grant n°D01-169/14.07.2014) is gratefully acknowledged.

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AP1. The unknown face of Disulfiram

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Abstract

Disulfiram (DSF) is a member of the dithiocarbamate family and has been approved by the Food and Drug Administration (FDA) for the treatment of alcoholism. It blocks the processing of alcohol in the body by inhibiting acetaldehyde dehydrogenase and causes an intolerance to alcohol because of increasing levels of acetaldehyde in blood.

Beyond treatment of alcoholism, disulfiram was found to express anti-neoplastic activity both in vitro and in vivio against different type of cancer (including cancers of the breast, prostate, colon; glioblastoma; melanoma). It is also the subject of ongoing clinical trials for lung and liver cancers. The divalent metal ions (especially Cu^{2+} and Zn^{2+}) have been proved to enhance its antitumor potential.

Recent studies reveal that disulfiram inhibits the activity of ATP-binding cassette (ABC) transporters, which are responsible for the development of multi drug resistance in cancer and fungal cells. It has been suggested that this drug may play an important role as an adjuvant in the chemotherapy of neoplasis as well as in the treatment of drug – resistant fungal infections. Disulfiram is also being studied as a treatment for cocaine dependence.

Acknowledgment

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AP2. Effect of Zn/Au and Zn/Ag with Saldmen and Salampy on viability and proliferation of human tumor cell lines

Tanya Zhivkova[1], Desislav Dinev[1,2], Pavel Mitrenga[1], Abdulkadir Abudalleh[1], Lora Dyakova[3], Marin Alexandrov[1], Daniela-Cristina Culita[4], Gabriela Marinescu[4], Luminita Patron[4], Radostina Alexandrova[1]

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AO9. Properties and possibilities for application of the hybrid material with silver nanoparticles (PVA/AgNps)

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Abstract
PVA/AgNps is a hybrid material with thermally reduced silver nanoparticles, stabilised in polyvinyl alcohol. It is well characterised with experimental results from physicochemical, microbiological and cytological tests. In vivo experiments, in dermal cytotoxicity test and subcutaneous injections on white mouse showed PVA/AgNps is a non-toxic in the enclosed silver concentration. An experiment was conducted to implement it as an inactivator of a bacterial strain *E. coli* O 104 for the preparation of antigen for immunisation of rabbits. It has been used also for preservative of the obtained in consequence of immunisation, hyperimmune *E. coli* O104 rabbit antiserum. It has been successfully used in clinical trials as a treatment agent for cough and recurrent otitis in dogs. The activity of PVA/AgNps was tested on nearly 150 bacterial and fungal strains. The MBC of synthesized samples of the hybrid material are determined also for *E. coli* O 149, *E. coli* O 157 H7 and *S. Typhimurium*, which are established as common pathogens in farm animals with huge losses for animal farming.
Keywords: ghost bacterial cells, silver nanoparticles, vaccine, *E. coli, S. Typhimurium,* cytotoxicicty.

The link between good health of livestock and people's health is indisputable. Zoonoses have been recognized for many centuries, and over 200 have been described. They are caused by all types of pathogenic agents, including bacteria, parasites, fungi and viruses [53]. For animals they subjected to animal husbandry carry the risk of accidents in the process of

applying live (attenuated) vaccines by veterinary professionals, involved with livestock - breeders, farmers, veterinarians and zoo technicians, from diseases affecting people caught through consumption of contaminated products to an economic loss for farms and the holdings [2]. Reducing public health risk from these factors, requiring communication and collaboration among the sectors responsible for human health, animal health, and the environment.

The use of "bacterial ghosts" as a candidate vaccine is a new and progressive approach for the introduction of safe, non-living, active vaccines for the prevention of a wide range of infectious veterinary diseases [49], which can be applied for the development of either prophylactic or therapeutic vaccines. Killed, containing whole cells vaccines have the advantage of representing the full range of antigenic determinants of the immune system. They can also be exploited as a delivery system for proteins, which are expressed on the envelopes before lysis or subsequently loaded [15].

"Ghost" vaccines also retain its natural outer membrane with strong immunostimulatory lipopolysaccharide structure.

There are publications for lethal action of silver nanoparticles against bacteria, fungi, viruses and parasites [7, 46, 16, 42, 13, 41]. They impact on the target object by multiple mechanisms, but a way of killing cells is the formation of 'pores' on their membranes [47, 23] Due to the high internal osmotic pressure, the cytoplasm content is expelled through the tunnel, resulting in an empty bacterial cell envelope [52]. This is what gives a reason to assume that in the treatment of bacterial cells with well-studied physico-chemical, microbiological and cytological hybrid material containing silver nanoparticles, such as PVA/AgNps [32, 36, 38] will produce "ghost" candidate vaccine cells. This is the theoretical basis for fundamental scientific research in this direction.

The improvement of the properties by decreasing the size of the particles is attributed to the increase in the number of active sites on the surface of the material. Nanomaterial with a radius of 10 nm has a higher percentage of active sites on the surface of the particles, rather than a material having a radius of 10μm. Larger particles have more inner active sites, which remain actually intact under chemical reactions, or external forces. Compared with a suspension of colloidal silver, the smaller particles have more surface active sites for interaction of liquid-solid phase and therefore are more likely to remain dispersed [16].

Nanotechnology has emerged as a rapidly growing field with applications in science and technology for the production of new materials with dimensions from the nanoscale. The word "nano" is used to refer to one billionth of a meter or 10^{-9}. The term "nanotechnology" was introduced by Professor Norio Taniguchi from Tokyo Science University in 1974., to be able accurately to describe the production of materials with dimensions in the order of nanometer. The nanoparticles are clusters of atoms in the size range 1-100 nm. "Nano" is a Greek word, a synonym of small, in the sense of extremely small. The use of nanoparticles is stimulated in our century, once were detected owned by them chemical, optical and mechanical properties.

Bionanotechnology occurred as integration between biotechnology and nanotechnology to explore the biosynthetic and environmentally friendly technologies for the synthesis of nanomaterials. Nanotechnology has an especially pressing and beneficial use in the field of medicine. It may be used to provide advanced biomedical research tools [17].

The metal nanoparticles are the most promising, because they showed among others good biomedical properties corresponding to the large surface area relative to their volume. It is known that they have an antiviral activities against various viruses. Viral infections poses significant global health challenges, especially in view of the fact that the emergence of resistant viral strains and the adverse side effects associated with prolonged use of antiviral drugs. Since metals may attack a broad range of targets in the virus there is a lower possibility

to develop resistance as compared to conventional antiviral therapeutics. It is established that there is also an antiviral activity of various metal nanoparticles to the human immunodeficiency virus (HIV-1), herpes virus, respiratory syncytial virus, influenza virus, hepatitis virus, and other [11, 1,13].
Incorporation of silver nanoparticles in different polymer structures allows control over their size and shape, as well as appropriate stabilization due to the possibility for rapid oxidation and aggregation in solution.
Nano-silver serves as a reservoir for supplying dissolved silver ions, which has a strong bactericidal effect. Ionic silver is toxic to bacteria and somewhat for fungi and viruses, making it a very effective biocide. Regardless of the shape of the silver essential characteristic is the concentration of released silver ions.
Silver nanoparticles are produced by chemical or physical methods, and because of their small size can potentially pass through biological membranes and reach more and different organs and tissues in the body. It can be summarized that the toxicity of nanosilver is greater than silver as a whole [30,11]. The silver is significantly more toxic than other heavy metals when they are in the form of nanoparticles [4, 30, 11], and the silver is significantly more toxic than other heavy metals when they are in the form of nanoparticles [4, 45]. Determining the toxicity of nanosilver factors are particle size, shape and concentration [30]. Silver nanoparticles with sizes <10 nm can pass through the cell wall [26]. Numerous researchers believe that silver particles with different sizes have a different toxicity [6, 26, 11].
The mechanism of nano-silver toxicity is still unknown. However, all forms of silver can release silver ions. Silver cations interact with multiple target cell mechanism [22]. On the one hand the positively charged silver cations associated with negatively charged components of the bacterial cell - cell wall and membrane, which induce structural changes and cell lysis. Another mechanism of action is penetration of the silver cations inside of the bacterial cell binding to the negatively charged proteins, enzymes, DNA or RNA, to interfere with electron transport, cell division and cell replication. Mechanisms of toxicity to bacteria cited to in the literature are:

- DNA loses its ability to replicate [44, 3].
- Deactivation of proteins essential for ATP [50].
- Deactivation of membrane bound proteins, leading to structural changes and cell death [47].
- Inhibition of respiratory enzymes to accelerate propagation of the oxygen species, and thus damage or kill cells [30].

- Molecular mechanism: the increase of silver ions (even at very low concentrations), which can pass the cell membrane, deplete the cell wall proteins and already penetrated into the cell results in a loss of energy and cell death [10].

Nano-silver demonstrates activity against fungi by attaching in an analogous mechanism with the negatively charged parts [47]. Dimorphic transition of C. albicans from yeasts to the micellar form is considered to be responsible for pathogenicity. Silver nanoparticles inhibit the extension and the formation of mycelium. They can damage the yeast cells by attacking their membranes, and thus distort the membrane potential.
The silver nanoparticles stirred membrane lipid bilayer, causing outpouring of ions and other materials, and also formation of pores and distribution of the electric potential of the membrane.
At TEM is found that they cause the formation of holes in the cell walls and pores in the cytoplasmic membrane [23].

Ag- nanoparticles cause disturbances in the normal process of budding, which correlates with damage to the membrane [23].
Thus, following attachment of the silver nanoparticles (AgNps) to the cell membrane, they enter in the fungi, forming space with a small molecular weight in the center of the fungus attached to the respiratory chain and eventually stop the cell division, which results in cell death [29].
PVA/AgNps hybrid materials was preparaed by adding a silver salt ($AgNO_3$), the precursor for silver ions, to the PVA solution thus leading to coordination of silver ions with hydroxyl groups (-OH) from PVA. Boiling the PVA solution at 100°C for 60 min in the presence of $AgNO_3$, results to formation of silver nanoparticles stabilized in PVA, which protects the silver nanoparticles from agglomeration and ensure the homogeniuos distribution of silver nanoparticles. The formation of silver nanoparticles was proven by UV-Vis spectroscopy and transmission electron microscopy (TEM) [5].
The formation of silver nanoparticles is evidenced from UV-Vis spectroscopy by the appearance of strong absorption bands at 420 nm (Figure 1) which indicates the formation of AgNPs.

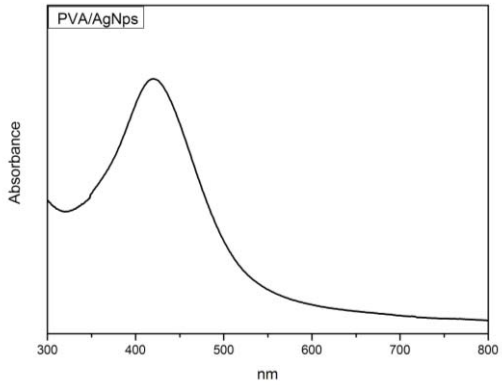


Figure 1. UV-vis spectrum of silver nanoparticles.

Figure 2a shows TEM images of initially produced silver nanoparticles by boiling the PVA/$AgNO_3$ mixture. Spherical silver nanoparticles with an average diameter of 5.0 ± 1.0 nm has been measured by TEM (Figure 2a) and their formation is confirmed using EDX analysis with a peak exhibited at approximately 3 KeV characteristics for the elemental silver (Figure 2b).

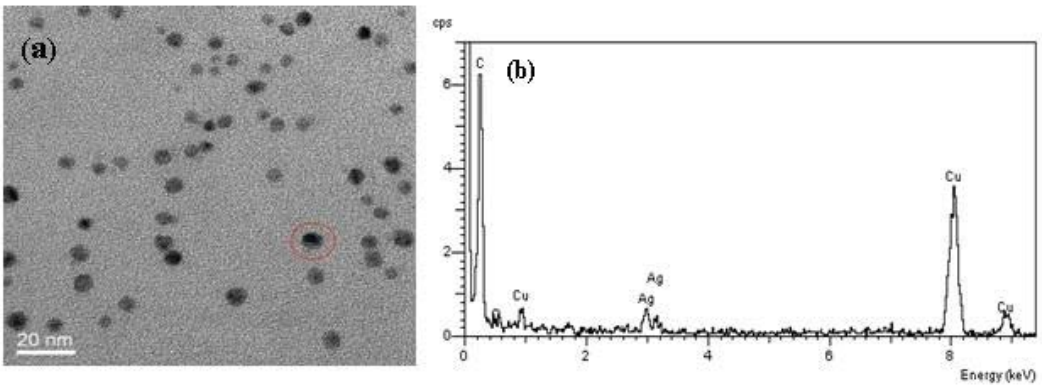


Figure 2.
a) TEM image of PVA/AgNps; b) EDX analysis of the PVA/AgNps.

The sinthjesized solution of hybrid material was diluted with sterile injection water to concentration 30 mg/l. This concentration of silver was chosen on the basis of performed in advance tests onto more than 100 clinical antimicrobial resistant isolates which showed strong bactericidal and fungicidal activity using this silver content. It was taken into account the threshold value for cytotoxity for silver nanoparticles cited in the literature [31]. After that we hold our experiments for determining the anti-bacterial activity of the PVA/AgNps hybrid material were preceded by determination of its cytotoxic effect *in vitro* of different cell lines. Cell viability was estimated by a modification of the MTT assay [27], which determines the metabolically active mitochondria of cells. For this purpose we used three monolayer cell lines of mammalian origin: two kidney lines - Madin-Darby canine kidney (MDCK) and monkey kidney (GMK) and one mouse fibroblasts cell line L20B. Using dose-response curves on the 24h and 48h we calculated maximal nontoxic concentration (MNC) and concentration required to inhibit cell viability by 50% (CD_{50}). The maximal concentration, which altered neither the morphology of monolayer nor the cell survival rate, was recognized as MNC. The CD_{50} of PVA/AgNps hybrid material for three cell lines were calculated from the dose-response curves. On the basis of the data from cytotoxicity experiments, we calculated the CC_{50} (Cytotoxic Concentration 50) to MNC ratio – therapeutic efficacy (TE). The ratio characterizes the tolerable concentration range in which the particular compound could be applied avoiding significant cell alterations.

The growth of all tested cell lines was suppressed in a dose-dependent manner. The hybrid material with included silver nanoparticles was not cytotoxic on the three cell lines tested at concentrations ranging from 0.001 to 1 mg/L. Tested for cytotoxicity hybrid material PVA/AgNp on MDCK, GMK and L20B cell lines showed very good therapeutic effect.

Specified minimum bactericidal concentration and some evidence of therapeutically efficiency (TE) can determine the nearest appropriate range of their application at given silver concentration. For testing the antimicrobial properties of the synthesized hybrid PVA/AgNps material following methods are used [8, 9, 32, 36, 38]:

- DDM (Disk Diffusion Method);
- MIC (Minimal Inhibitory Concentration) by the agar dilution method;
- Method with the macro dilutions;
- Chess method for testing the presence of synergism of PVA/AgNps hybrid material and Pi or Ce;
- Modified method for testing the presence of synergism of the material to antimycotics;
- In vivo tests: dermal test; test for bio toxicity; application as an aseptic agent in the treatment of skin and wound infections in animals and humans.

The influence of the stabilizer (disk impregnated with PVA) compared to the impact of the entire hybrid material, was evaluated in the absence of zone of inhibition on the control bacterial and yeast strains (Figure 3).

Figure 3. Testing the influence of PVA stabilizer on bacteria *E.coli ATCC25922, S.aureus ATCC25923, P.aeruginosa ATCC27853*

Bactericidal properties of hybrid PVA/AgNps materials was initially established through DDM (Figure 4). Calculations of the silver concentration in the synthesized hybrid sample were in this case against the inputted starting concentration of silver nitrate. A sample of hybrid PVA/AgNps material with a concentration of silver precursor of 3.9 mg/mL (3900 μg/mL) was used.

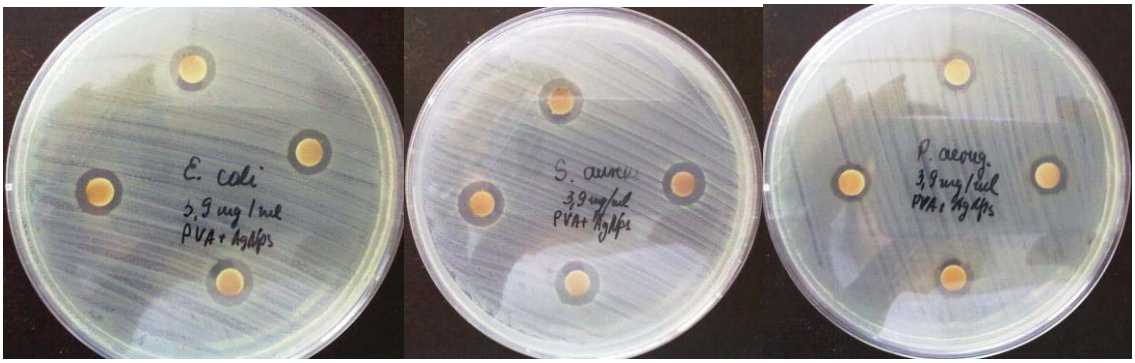
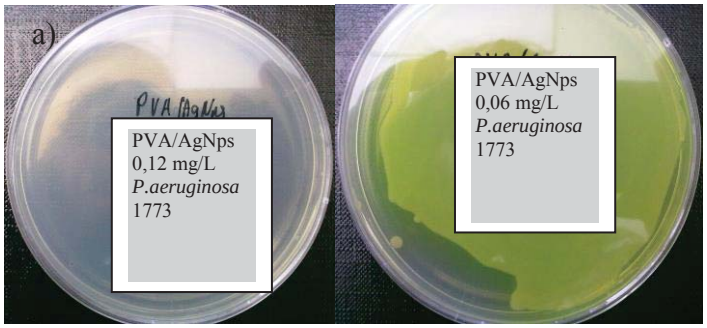


Figure 4. Testing the bactericidal activity of PVA/AgNps hybrid material to control bacterial strains by DDM.

The Agar Dilution Method is very convenient for the simultaneous determination of the Minimum Inhibitory Concentration (MIC) of a large number of strains. The same PVA/AgNps sample (concentration of silver precursor 3.9 mg/mL) against 21 clinical isolates from *Staphylococcus sp*., 24 clinical strains *E.coli* and 26 *P.aeruginosa* was tested.
The tested clinical strains of *S.aureus* and *S. saprophyticus* have demonstrated resistance to six antimicrobial substances. MIC for all staphylococci was ≥24.4 μg/mL with the exception of four strains, wherein it is lower. In two of the tested strains *S.aureus* MIC was 12.2 μg/mL, while in the other two the MIC was 6.1 μg/mL.
The tested clinical strains of *E. coli* have established resistance to 11 antibiotics, and the explored clinical *P.aeruginosa* strains were resistant to eight antibiotics. MIC for all tested Gram negative bacteria was ≥ 24.4 μg/mL.
For the determination of the MBC according to the method of macro dilution PVA/AgNps sample with determined by ICP silver concentration 156, 902 mg/L was used (Figure 5). Four multiresistant clinical isolates were selected - two strains *P.aeruginosa*, one strain *E.coli*, isolated from humans and one strain *A.baumanii* isolated from an ear infection in a dog.



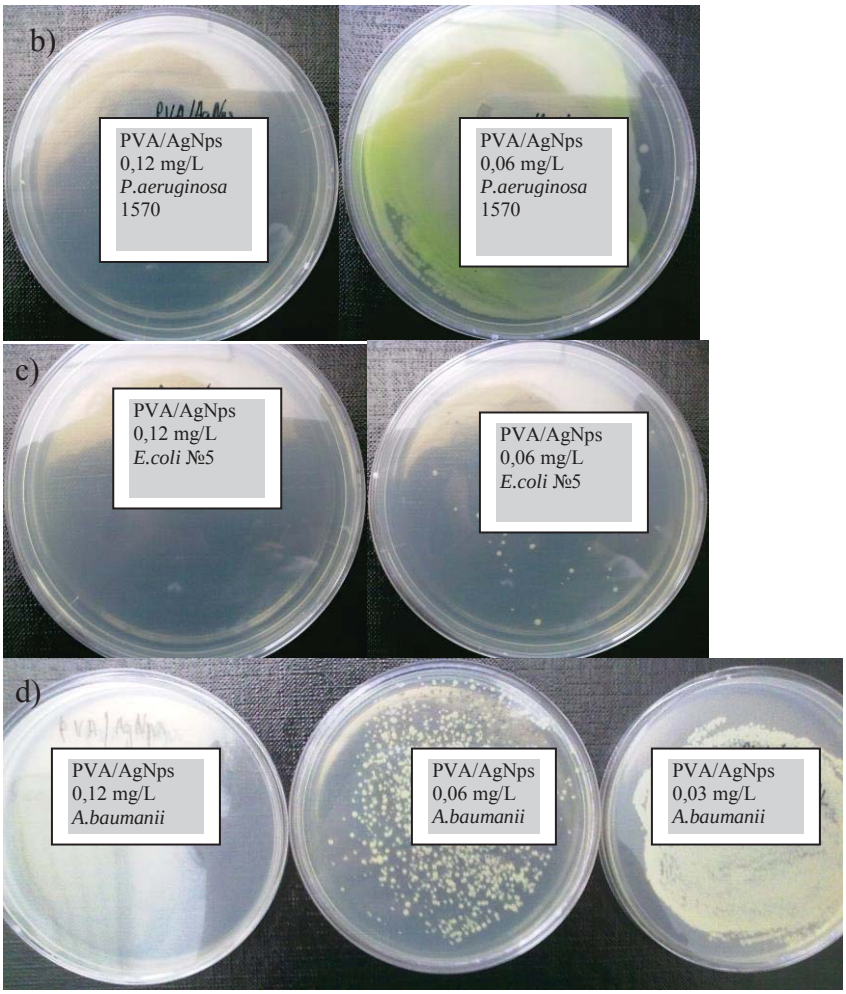


Figure 5. MBC determination of multidrug-resistant clinical strains: a) *P.aeruginosa* 1773, resistant to Pi, Cz, Ct, Ce, Azt, I, G,Cp; b) *P.aeruginosa* 1570, resistant to Pi, Ct, Cz, ,Ce, Azt,G,Cp; c) *E.coli* № 5, resistant to A, A/S, AmC, Cx, Cz, Ct, Cm, Cft,Ce, Azt, G,Cp; d) *A.baumanii*, resistant to Pi, A/S, Cz, Ct, Cft, Ce, I, G, Tb, Am, T, D, Cp, S/T.
Legend: Piperacillin (Pi) , Ampicillin/sulbactam (A/S), Ceftazidime (Cz), Cefuroxime (Cx), Cefotaksime (Ct), Ceftriaxone (Cft), Cefepime (Ce), Imipenem (I), Gentamicine (G), Tobramicine (Tb), Amikacine (Am), Cephamandole (Cm), Tetracycline (T), Doxycycline (D), Ciprofloxacin (Cp), Sulfamethoxazole/ Trimethoprim (S/T), Aztreonam (Azt), Amoxicilin/Clavulanic acid (AmC)

The control strain *E. coli* O104 Kopenhagen was tested as well (Figure 6).

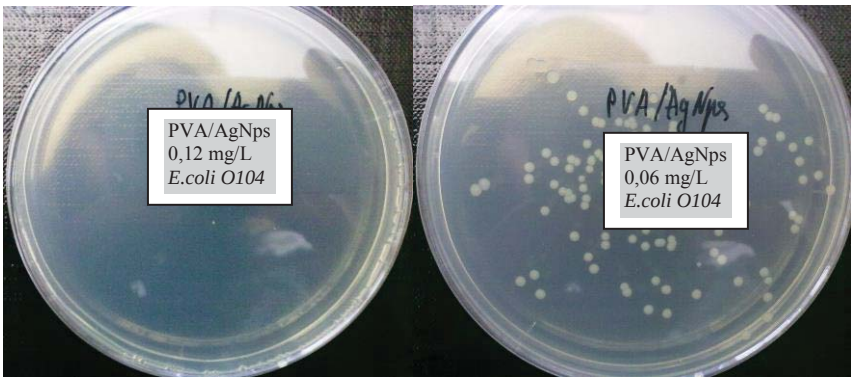


Figure 6. Determination of the MBC of the control strains *E. coli O104* Kopenhagen.
In all of the tested bacterial strains MBC ≥ 0,12 mg/L was determined.

Determination of Minimal Fungicidal Concentration (MFC) of the tested yeasts.
The hybrid materials (PVA/AgNps) were investigated using serial macro-dilutions method. To determine the Minimal Fungicidal Concentration (MFC) of the control and the first five clinical strains, standards of CLSI M26-A and CLSI M27-A2 were applied in modified variant.
It was established that the MFC for the control strains *Candida albicans* and *Candida krusei* was lower than 14.5 mg/L, for *Candida tropicalis* MFC was 28.99 mg/L, for *Candida glabrata* – 115.98 mg/L and for *Aspergillus brasiliensis* MFC was 927.81 mg/L [38].
In order to establish the fungicidal activity of PVA/AgNps against clinical strains with proven resistance to one or more antimycotics, five clinical yeast strains were used and their MFCs were determined. The established values for MFC differ from the results obtained for the control strains. The MFC for *Candida albicans* 8-127 and *Candida glabrata* 8-122 was 463.91 mg/L, for *Candida albicans* 8-137 - 231.95 mg/L, for *Candida krusei* 8-126 MFC was lower than 14.5 mg/L. The results demonstrate a pronounced fungicidal activity against all tested control and clinical strains with MFC in the range of 980 to 30 mg/L. An exception was *C. krusei* 8-112, where no fungicidal activity of the tested PVA/AgNps was observed [33].
The results indicate that the most sensitive control strains were *C. tropicalis* and *C. krusei* with MFC <30 mg/L akin to PVA/AgNps. Further, *C. krusei* 8-112 strain was found to be resistant to silver in PVA/AgNps respectively at as high as 1960 mg/L Ag concentration. This concentration (1960 mg/L) was more than 5 times greater than those established for the other types of clinical strains, which is indicative for the presence of silver resistance strain.
Another four clinical Candida strains (*C. krusei* 8-48, *C. parapsilosis* 0-115, *C. glabrata* 0-73, *C. nivariensis* 383) were tested with the second sample using another validated method, where the initial fungal suspension was standardized using densitometer of 24 - hr culture with water for injection and by adding such a quantity of the suspension to each tube of the reaction system, which guarantees the submission of 10^5-10^6 CFU/mL. MFC of the PVA/AgNps sample (silver concentration determined by ICP: 140 mg/L) for all of them was defined as less than 0.27 mg/L. The growth of all tested with this sample cell lines was suppressed in a dose-dependent manner. These sample hybrid PVA/AgNps material was established as not cytotoxic, on the two monolayer cell lines from animal origin: Madin-Darby canine kidney cell line (MDCK) and embryonic bovine tracheal cells (EBTr) tested at concentrations ranging from 0.0005 mg/L to 1 mg/L and TE = 90 x 10^3 [40]. MTT assay was performed to determine the effect of the nanoparticles on the cell viability and to calculate maximal non-toxic concentration (MNC) and therapeutically efficiency (TE).
In 1968 silver nitrate is combined with sulfonamides and included in the contents of the silver sulfadiazine cream, which was employed as an antimicrobial agent with a broad spectrum for wound treatments. The silver sulfadiazine is effective against pathogen bacteria as *E.coli,*

S.aureus, Klebsiella sp., Pseudomonas sp. and demonstrates particularly antifungal and antiviral properties. Recently as a result of more often appearance of antimicrobial resistant bacteria and the restricted choice of antimicrobials, the clinicians pay attention to the silver wound bandages with different amount of silver [25]. The presence of strong bactericidal activities of synthesized hybrid materials with included silver nanoparticles gives hope for possible solution of this situation. There are published data about established synergistic effect by combination of silver nanoparticles alone or included in hybrid materials with: amoxicillin, [24], rifampicin [21], ampicillin, kanamycin, erythromycin, chloramphenicol, polymyxin B [43]. The problem of this kind of materials is that they are broad-spectrum but not selective. Their combination with antimicrobials could make amends of these disadvantages.

The diameter of the zone is significant not only for bactericidal action but also for the amount of the released in the agar media silver ions. But the absence of any inhibition zone is not indicative for the lack of activity of the silver ion. It is probably a result of impossibility of the functional ion to diffuse in the nutrient media. All of this could be taken in attention analyzing the results of Disk diffusion testing for synergism. There are data in the literature about investigation of the effect by combination of silver nanoparticles and different antibiotics (penicillin G, amoxicillin, carbenicillin, cephalexin, cefixime, erythromycin, gentamicin, amikacin, tetracycline, Co-trimoxasole, clindamycin, nitrofurantoin, nalidix acid and vancomycin) toward *S. aureus* and *E. coli* using DDM with disks, impregnated with solution of both of the tested substances. The increased activity was established by their combination with penicillin G, amoxicillin, erythromycin, clindamycin and vancomycin.

A checkerboard testing method of the synergism of clinical isolates resistant to antibiotics, in combining antibiotics with PVA / AgNps.

1. Resistant to Pi and Ce strains when tested by DDM on CLSI [36]**.**
    - When hybrid material (157 mg/L silver concentration determined by ICP-OES) was tested, the MBCs for both strains were determined as 0.12 mg/L.
    - *P. aeruginosa* strain did not show sensitivity to piperacillin (Pi) even at 128 mg/L and the strain *E. coli* - is resistant even to 32 mg/L cefepime (Ce).
    - The results indicate that, self-administered, the hybrid material has a higher antibacterial activity than in combination with antibiotics. Thus, the hybrid material with a silver concentration 0.25 mg/L exhibits bactericidal effects on the test strain, while its combination in the same concentration with Pi: in concentration - 32mg/L and 16mg/L and with Ce: in concentration 16 mg /L does not exhibit such action.
        - If the definitions of the terms adopted by EUCAST were applied then this result could be defined as presence of antagonistic activity. At lower concentrations of piperacillin the bactericidal activity of the hybrid material was again exhibited. Calculation of Fractional bactericidal concentration (FBC) [12] with those obtained results are greater than 2, wherein the combined effect is evaluated as antagonism.

$$FBC(Pi) = \frac{MBC(Pi \text{ in the presence of } PVA/AgNps)}{MBC\ (Pi \text{ alone})}$$

FBC (Pi)= 32/256= 0,125

$$FBC(PVA/AgNps) = \frac{MBC(PVA/AgNps \text{ in the presence of } Pi)}{MBC\ (PVA/AgNps \text{ alone})}$$

FBC (PVA/AgNps)= 0,25/0,12= 2,1

ΣFBC= FBC (Pi)+FBC (PVA/AgNps)= 2,225

-

$$FBC(Ce) = \frac{MBC(Ce \text{ in the presence of } PVA/AgNps)}{MBC\ (Ce \text{ alone})}$$

FBC (Ce)= 8/64=0,125

$$FBC(PVA/AgNps) = \frac{MBC(PVA/AgNps \text{ in the presence of } Ce)}{MBC\ (PVA/AgNps \text{ alone})}$$

FBC (PVA/AgNps)= 0,25/0,12=2,1

ΣFBC= 2,225

2. Testing for presence of synergism by combining of PVA/AgNps with ceftazidime on *Klebsiella* 2494 [18].

The calculation of Fractional bactericidal concentration (FBC) [12] on the combined action of ceftazidime and the hybrid material was:

$$FBC(Cz) = \frac{MBC(Cz \text{ in the presence of } {}^{PVA}/_{AgNps})}{MBC\ (Cz\ alone)}$$

FBC(Cz)=0.25/2=0.125

$$FBC({}^{PVA}/_{AgNps}) = \frac{MBC(PVA/AgNps \text{ in the presence of } Cz)}{MBC\ ({}^{PVA}/_{AgNps} \text{ alone})}$$

FBC (PVA/AgNps)=0.234/0.937=0.25

ΣFBC= FBC (Cz)+FBC (PVA/AgNps)= 0.375

The result was lower than 2, and according to EUCAST, the combined effect is reported as sinergism.

3. **Testing for presence of synergism by combining of PVA/AgNps with ceftazidime on *Salmonella* Paratyphi B 176** [19].

The calculation of Fractional bactericidal concentration (FBC) [12] on the combined action of ceftazidime and the hybrid material was:

$$FBC(Cz) = \frac{MBC(Cz \text{ in the presence of } PVA/AgNps)}{MBC\ (Cz \text{ alone})}$$

FBC(Cz)=0,25/8=0,03

$$FBC(PVA/AgNps) = \frac{MBC(PVA/AgNps\ in\ the\ presence\ of\ Cz)}{MBC\ (PVA/AgNps\ alone)}$$

FBC(PVA/AgNps)=0,234/0,468=0,5
ΣFBC= FBC (Cz)+FBC (PVA/AgNps)= 0,53
The result was lower than 2, and according to EUCAST, the combined effect is reported as sinergism.

Further, the presence of synergism was investigated combining PVA/AgNps (initial silver concentration 3900 mg/L) and antifungal agents, using a commercial product ATB™. A synergistic effect was observed when PVA/AgNps material with decreasing concentrations of antifungal agents was used. Similar effect was observed even for *C.krusei* 8-112 strain which was resistant to PVA/AgNps when used singularly. However, not all possible combinations of dilutions for both combined agents have been performed and will be a part of our future research reports [34].
In vivo experiments in dermal cytotoxicity test and subcutaneous injections on white mouse showed PVA/AgNps as a non-toxic in the enclosed silver concentration [35]. The synthesized sample was used in a dermal test conducted on white mice at silver concentration determined by ICP as 156, 902 mg/L. After mechanical working on the test field there were not established skin wounds.

- The used in the test hybrid material silver concentration was 5.2 times higher than the announced in the literature on cytotoxicity when tested on human fibroblasts. However, there is not any redness in the experimental field. After 24 hours, the experiment was repeated and the result was the same.
- Skin is calm and fur has grown by about 0,5 cm on the experimental field, which can be considered as aseptically processed and used as an operational field. Upon subsequent deep scarification is reported the absence of redness and dermal infection. Scarify areas healed without scarring.

The same sample hybrid material, but diluted to a concentration of the silver, 30 mg / l was used in two tests for bio-toxicity. In the first one subcutaneous injections of 2 ml of the hybrid material PVA / AgNps were injected on a white mouse "BALB c" with weight 20 g [35]**.** After the performed test for bio-toxicity the mouse used, stayed alive and in perfect health, traced for about a month after the intervention. In the second experiment the whole cycle of intravenous immunizations of the rabbit was finished successfully. An agglutinating antiserum *E.coli* O104 was produced with determined O-antigen titer 1600. The hybrid material PVA / AgNps has been used also for preservative of the obtained in consequence of the immunization hyper-immune E. coli O104 rabbit antiserum which stores its titer three years after the preservation [51].
In the intravenous immunization scheme, involving the killed via the hybrid material an antigen from control strain E.coli O104 Kopenhagen, at the end of the immunization cycle the hare stayed alive, clinically healthy and with normal vital signs. Previously proven highly bactericidal activity of the tested hybrid material PVA/AgNps, the occurrence of fungicidal activity at a concentration of silver nanoparticles 30 mg/L, and the positive test results for bio-toxicity are sufficient reason to start testing it as a vaccine preservative.
Since the 1930s it is applied in vaccines as an approved preservative thiomersal, which is a organomercuric compound [54]. Raised doubts about its participation in some of the side effects of the vaccines, require comprehensive monitoring of all evidence under the supervision of WHO.
Nano-materials as cyanoacrylates and poly(lactide-co-glycolide) copolymers were used successfully as vaccines adjuvant. The polymers used in these adjuvants also are used as suture material, prostheses, and drug carriers and are thought to be nontoxic [48].

Before in the produced agglutinating rabbit antiserum *E.coli O104* to be added as a preservative the hybrid material PVA/AgNps, it was enrolled in antigenicity studies. In the diffusion on Uhterloni onto 2% agarose plate was pipetted into wells of one row with the thus-obtained experimental *E.coli* O104 antiserum. In the rows above and below it is pipetted pure hybrid material PVA/AgNps with silver concentration 30 mg/L. After 24 hours of diffusion observed weak precipitin strips between the middle row wells and that above and below it (Figure 7). This indicates a certain antigenic activity of the hybrid material, as a result of which they form soluble antibodies against the used for the inactivation of the strain hybrid material PVA/AgNps. Given that it is a complex compound having a molecular weight of polyvinyl alcohol only over 20000 daltons, and in the literature chemicals with a molecular weight above 6000 daltons, for the most part are immunogenic can be questioned whether the hybrid material possesses adjuvant properties. This would strengthen the immune response by connecting with haptens.

Figure 7. Linear immunodiffusion on Uhterloni of *E.coli O104* of an experimental antisera and PVA/AgNps.

It is established that microparticles (1–100 μm) can induce CMI, including CTLs, as well as humoral immunity. They usually are not immunomodulators, but immunomodulators can be incorporated to improve their effectiveness. Microparticle adjuvants can protect incorporated antigens from harsh conditions such as low pH, bile salts, and enzyme activities. For this reason, they may be particularly useful in oral and intranasal vaccines. Technical problems in manufacture can be a disadvantage because the encapsulation process may alter antigens and decrease their ability to stimulate the immune system [48]. Theoretically there is a guideline for future work modifying the bacterial genome in order synthesis of cell surface expressed by bacteria viral antigens. Obtained in this way 'ghost' vaccines can provide protection against a wide range of zoonotic agents.

The hybrid material PVA/AgNps with a concentration of 30 mg/L was administered by a veterinarian for the local treatment of skin and wound infections in pets-dogs [37].

- In the first case of purulent wounds in the leg, caused by awn, they are treated on the course of the wound using a sterile swab soaked in a hybrid material where upon its astringent effect is observed and subsequently confirmed the expected bactericidal action of that preparation, with the result that the wounds are healed uncomplicated.
- In the treatment of wounds in the ears with the hybrid material solution was reused, after heavy irrigation of a sterile swab with a long handle which allows local processing them even when they are located in the depth of the ear canal. There was again found an astringent and healing effect.

There are good results of the clinical application of the hybrid material with silver concentration of approximately 200 mg/L in pets-dog as a therapeutic agent for cough [39] and by silver concentration 600 mg/L for application by recurrent otitis externa in Samoyed dog.

Traditional production of non-living (killed) vaccine by heat treatment, irradiation or chemical treatment of the pathogen often leads to denaturation of significant structural components of the cell wall, changing the antigenic character of the vaccine due to the loss of important immunogenic epitopes cannot create a complete immunity [20]. There is scientific evidence for the preparation of bacterial "ghost cells" by a non-denaturing process through controlled expression plasmid PhiX174 of lysis gene E in gram-negative bacteria. The result is a tunnel formed by specific protein E, which is limited to a small fraction of the total cell surface [49]. Thus were obtained ghost cells by different bacterial pathogens of importance in veterinary medicine - *E. coli, K. pneumoniae, Vibrio cholera, Salmonella typhimurium, Actinobacillus pleuropneumoniae*. Analysis of the immune response of such "ghost" in various animal models was shown to induce humoral and cellular immune response against their cell walls, which provide protective immunity [49]. Killed vaccines induce a strong polyclonal immune response and immunity tense as a result of "build in" adjuvants. Such effects are less purified vaccines. The more purified they are, the weaker the immune response and stimulate greater the number of required immunizations, which raises the cost of the vaccine. In highly purified peptides and carbohydrates is essential to add an adjuvant to fail to immune tolerance to them [48]. Most adjuvants are chemicals, microbial components, or mammalian proteins. In general, most appear to enhance antigen presentation, improve antigen stability, or act as immunomodulators. A single adjuvant may have more than 1 mechanism of action. For example, adjuvants that help preserve the antigen's strucure can improve the effectiveness of the vaccine and also increase its shelf life [48]. Against pathogenic bacteria are included both as the specific antibodies also the cell mediated immunity. The antibodies are more effective against extracellular bacteria and bacteria which cause disease which is a result of the production of exotoxins rather than intracellular bacteria. It has been found that inactivated vaccines are able to induce cell-mediated immunity and depending on the adjuvant in the vaccine [14] have been studied the opportunities to create recombinant bacterial "ghosts" through the introduction of foreign protein inside them [20].

Nano-silver damaging bacteria by making holes in the bacterial cell walls, accumulates there and leads to an increase in its permeability and the final cell death. This gives reason to accept as true that as a result of the complex interaction of silver nanoparticles on bacteria form "ghost cells." In the immune defense against pathogenic bacteria, included both specific antibodies and cell mediated immunity. The antibodies are more effective against extracellular bacteria and bacteria which cause disease as a result of production of exotoxins rather than against intracellular or facultative intracellular bacteria. Inactivated vaccines can also induce a cell-mediated immunity depending on the adjuvant in the vaccine [14].

Animal diseases caused by enterotoxigenic *E.coli* (ETEC) appear as typical water diarrhea during the first days after weaning in pigs. They also cause infections in cattle and small ruminants, rabbits, cats and dogs. ETEC adhere to the micro villas of the small intestine without inducing morphological lesions through production of enterotoxins, acting locally on the enterocytes. Adhesins and toxins are the two most important virulence factors in ETEC [28]. The activity of PVA/AgNps was tested and proven in nearly 150 bacterial and fungal strains [38]. The MBC of synthesized samples of the hybrid material are determined also for *E. coli* O 149, *E. coli* O 157 H7 and *S. Typhimurium*, which are established as common pathogens in farm animals with huge losses for animal farming.

Conclusion

As a result of all the in vitro and in vivo tests, the hybrid material was characterised as a non-toxic product with bactericidal and fungicidal actions to control and clinical strains of bacteria and yeast in the established tests limits. There are excellent prospects for this hybrid material

to be used as a preservative for diagnostic serums and as an inactivator for obtaining of ghost cells with capacity for application as whole cell killed vaccines for prophylactic purposes, and theoretical perspectives in the preparation of recombinant vaccines and as immunostimulator.

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AP3. Antimicrobial properties of silver

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AP4. Фаготерапия

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Биологически факултет, СУ „Св. Климент Охридски"

AP5. „Избухващият вирус" - Ебола

Фаузия Салах Ел Рантиси, Мохамед Мохедин, Ая Хасанова, Исра Ал Хусейн, Джуди Джабулие, Дания Ясърджи

Палестинско училище, София

AP6. Ебола – новото предизвикателство на 21-ия век

Георги Тошев

Биологически факултет, СУ „Св. Кл. Охридски"

AP7. Прионите и заболяванията, които те предизвикват

Георги Тошев

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Session B.

Chairpersons:

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

Assist. Prof. Delka Salkova, DVM, PhD
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BO1. Trace metals polluted soils in Kardjali region, Bulgaria

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Abstract

Ecological assessment of soils in the region of Kardjali city, Bulgaria, where lead-zinc industry is developed, was performed by monitoring and thermodynamic studies. The monitoring studies covered 6 soil sampling stations. Their results were used for thermodynamic modeling of the mobile inorganic and organic chemical species of trace metals in these soils. Two thermodynamic models were applied in this study - a classical ion-association model for calculation of the inorganic chemical species, and the Stockholm Humic Model (SHM) for calculation of the organic metal species. Visual Minteq computer program, version 3, was used.

Soil monitoring studies revealed that the total concentrations of the trace elements Mn, Co, Ni, Cu, Zn, Pb, Cd exceeded the allowed levels for Pb-Zn industrial regions (Bulgarian regulation No 3/2008) in 4 of the 6 sample stations; the humus content of the soils was from poor to medium, and their pH varied from slightly acidic to slightly alkaline. The level of mobile ions was found to be low (varying from 0 to 5 %), which means that these microelements are in a sparingly soluble form in the soils. The thermodynamic modeling of the chemical species in 1N KCl extracts of slightly acidic soils (pH 6.44, station S3) pointed out that mainly Me^{2+} followed by $MeSO_4^0$ were typical for Zn, Cd, Mn, Co and Ni despite the higher total organic carbon content in comparison with other stations. For Pb and particularly for Cu bidentate and monodentate complexes were typical. In the station S1 with slightly basic soils (pH 8.14) domination of organic species, especially for Cu and Pb, followed by Ni,

was calculated. An only exception represented Mn for which Mn^{2+} dominated. Fe and Al were mainly present as $(MeOH)^{2+}$ bidentate complexes independent of the solution acidity.

Introduction

The region of Kardjali city, in the central part of Rhodope Mountains, Bulgaria, is known to be highly polluted with heavy metals from the pyrometallurgical activities of the lead-zinc company [1]. The Kardjali city with population of 51 000 is the largest industrial, administrative and economic center of the region. It is situated on both banks of Arda River between two artificial lakes, Kardjali Lake to the west and Studen Kladenec Lake to the east (Fig.1).

Monitoring studies are usually performed to produce data on the total trace metal content without differentiating between mobile and sparingly soluble metal ions. These studies do not provide data on the content of mobile metals and their chemical species [2-4]. The application of thermodynamic modeling is an appropriate approach to calculate chemical speciation distribution because of its facility and rapidity. The accuracy of the calculations depends on the accuracy of the analytical data, as well as on the availability of appropriate thermodynamic data.

The aim of the present study was to evaluate by monitoring studies the pollution level of the soils in the region of Kardjali city, Bulgaria and to calculate by thermodynamic modeling the chemical species of the mobile ions in these soils that are responsible for metal toxicity and bioavailability.

Experiments and methods

Field study - Sampling stations and sampling

The soils used in this study were collected from 6 sampling stations in Kardjali region, Bulgaria. The sampling stations were selected in areas with different pollution: (i) *reference zone* - station S1 (Kardjali artificial lake); (ii) *affected zones:* city zone, station S2 (city central park) and lake zone, S5 and S6 (Studen kladenec lake - on the bank opposite to the lead-zinc factory); (iii) *polluted zone* - two stations (S3 near to the lead-zinc factory; and S4 – tailing pond of Gorubso Ltd).

The sampling was done in 2012 from the topsoil layer (0-20 cm) according to the requirements of ISO standard 10381.

Laboratory studies - Chemical analysis

Samples preparation

The soil samples were air-dried, hand-ground and sieved with a 2 mm mesh sieve. Then solution samples for the analyses (total metals content; total organic carbon; mobile anions content; mobile metals content) were prepared.

The solution samples for determination of total metals (Fe, Mn, Cu, Zn, Co, Ni, Cd, Pb) content were prepared by extracting 0.5 g soil with 10 ml mixture of concentrated nitric and hydrochloric acids in a ratio of 1:3, according to ISO 11466.

The solution samples for total organic carbon (TOC) determination were prepared by temperature oxidation of soil organic matter (1.0 g soil) with 10 ml 0.4 N oxidizing solution ($K_2Cr_2O_4$:H_2SO_4=1:1) in presence of Ag_2SO_4 as catalyser.

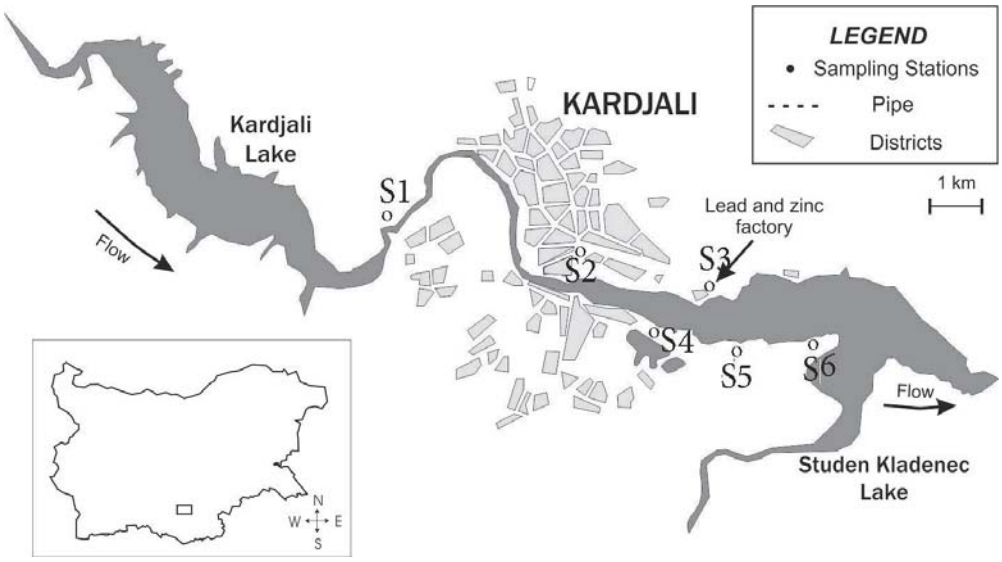

Fig.1. Map of Kardjali region with sampling stations

The solution samples for determination of the content of mobile metals (Al, Mn, Fe, Co, Ni, Cu, Zn, Pb, Cd and As) were prepared by extracting 40 g soil with 100 ml 1N KCl for 1 h.

The solution samples for determination of the content of mobile anions (Cl^-, CO_3^{2-}, PO_4^{2-}, NO_2^-, NO_3^-, SO_4^{2-}) were prepared by extracting 10 g soil with 50 ml distilled water for 30 min.

Chemical methods

Inductively coupled plasma optical emission spectrometry (ICP-OES ULTIMA 2 analyzer) was applied for determination of the concentration of the metals Al, Mn, Fe, Co, Ni, Cu, Zn, Pb, Cd and As.

Spectrophotometry (NOVA 60, Merck apparatus, Spectroquant Test kit) was applied for determination of the concentration of the ions Cl^-, CO_3^{2-}, PO_4^{2-}, NO_2^-, NO_3^-, SO_4^{2-}.

Tjurin method [5] was applied for total organic carbon (TOC) determination.

pH measurements - *acidity/alkalinity* of the soils was measured in 1N KCl and in distilled water according to ISO 10390.

Computational method - Thermodynamic modeling

Two thermodynamic models were applied for calculation of the mobile chemical species. The classical ion-association model [6-7] was applied for the inorganic metal species. The Stockholm Humic Model (SHM) [8] was applied for the organic metal species. Visual Minteq computer program (version 3) was used.

The monitoring data for the mobile metals Al, Mn, Fe, Co, Ni, Cu, Zn, Pb, Cd, As, the anions F^-, Cl^-, CO_3^{2-}, PO_4^{2-}, NO_2^-, NO_3^-, SO_4^{2-} and pH of the soils in distilled water were used in the calculations. As the dissolved organic carbon (DOC) could not be experimentally determined, we accepted it to be 1% of the TOC. Further, we have assigned DOC to fulvic acids because these acids are dominant in maroon forest highly leached soils, to which Kardjali soils belong [9].

All calculations were done with the oxygen couple O^{2-}/O_2, representing the redox conditions in the soil systems, because the studied samples were collected from well oxygenated surface soil layers.

Results and discussion

The monitoring studies (Table 1) revealed that the total concentrations of Zn, Pb and Cd in the soil samples S1, S3 - S5 exceeded the allowed levels for lead-zinc industrial regions [10]. The Cu value for the sample S3 was also above requirements. For samples S2 and S6 only the Cd concentration exceeded the allowed levels. The lowest metal levels were registered for station S2, probably because of the remediation of the soils in the city central park zone (through heaping). It was also found that all soils had a poor humus content (1100 – 15000 mg/kg), and were from slightly acidic (S3) to slightly alkaline (S1, S2, S4, S5 and S6) (Table 1). The soils from the polluted regions S3, S4 и S5 significantly differed by total heavy metal content, the results for S4 and S5 being close to one another. The contents of Cu, Zn, Cd and Pb in the soil of station S3, which is the one closest to the factory, considerably exceeded those of the other two stations (Table 1).

A low level of mobile trace metals (from 0 to 5.65 %) was found in the samples (Fig. 2). Obviously, trace metals are present in the soils as sparingly soluble compounds. The highest mobility of Mn in station S3, Cu in station S2, Co in stations S1 and S5, and Ni in station S2 was registered (Fig. 2). The highest concentrations of mobile ions: Zn (709 mg/kg), Pb (370 mg/kg), Mn (43 mg/kg) and Cd (19 mg/kg), were registered for the S3 sample, which also displayed the highest total metal contents and the highest soil acidity. For Co, Ni and Cu lower mobility was registered (Table 1).

Table 1. Monitoring data for the soil samples

Soil sample	Limit levels [7]	Referent station	Affected stations			Polluted stations	
		S1	S2	S6	S5	S3	S4
Physicochemical characteristics							
pH_{H2O}		8.13	7.88	8.08	7.95	6.44	7.86
pH_{KCl}		6.95	7.04	7.42	6.87	5.68	6.71
Total content							
Fe, mg/kg		11409.47	31700.62	15697.27	16970.36	19392.51	50619.51
Mn, mg/kg		633.686	323.694	1008.491	984.406	1294.206	937.554
Cu, mg/kg	140	32.100	15.409	51.540	29.545	661.738	76.949
Zn, mg/kg	390	444.412	79.947	679.996	246.854	28586.01	468.821
Co, mg/kg		5.872	15.309	10.565	11.034	17.582	28.487
Ni, mg/kg	80	44.334	6.804	33.669	40.215	25.274	106.653
Cd, mg/kg	2.5	7.242	17.210	15.896	9.666	771.928	27.532
Pb, mg/kg	130	203.465	52.631	278.634	103.410	14925.87	846.882
TOC, mg/kg		2600	9200	2100	2000	15000	1100
Water extraction							
F^-, mg/kg		<0.5	<0.5	1.15	<0.5	0.65	<0.5
Cl^-, mg/kg		58	21.5	<12.5	24	24	22.5
CO_3^{2-}, mg/kg		<50	<50	<50	<50	<50	<50
PO_4^{2-}, mg/kg		5.0	11.5	0.5	3.0	0.5	1.5
NO_2^-, mg/kg		4.1	2.75	0.5	0.85	1.85	1.9
NO_3^-, mg/kg		19.5	86	56.5	28.5	176.5	22
SO_4^{2-}, mg/kg		81	15.5	142	146.5	620	61.5
1N KCl extraction							
Al, mg/kg		0.01	0.01	0.01	0.01	0.01	0.01
Fe, mg/kg		0.243	0.039	0.085	0.007	0.183	0.002
As, mg/kg		0.049	<0.013	0.032	<0.013	0.049	0.067
Mn, mg/kg		4.073	2.776	1.791	0.688	43.488	0.553
Cu, mg/kg		0.270	0.319	0.381	0.342	1.821	0.346
Zn, mg/kg		0.434	0.657	0.413	0.609	709.263	0.049
Co, mg/kg		0.332	0.064	0.383	0.049	0.436	0.007
Ni, mg/kg		0.399	0.214	0.325	0.045	0.441	0.180
Cd, mg/kg		0.144	0.401	0.115	0.196	19.152	0.032
Pb, mg/kg		0.446	0.239	<0.013	0.027	370.334	0.007

The concentrations of mobile metals and anions, dissolved organic carbon (assumed as 1% from TOC) and pH (measured in water) were used to simulate the compositions of the soil solutions. Thermodynamic calculations of the chemical species of the metals in these soil solutions were made for the sampling stations S1 and S3, as they are characterized with high metals mobility, different pH (8.13 for S1 and 6.44 for S3) and high TOC (0.26% for S1 and 1.5% for S3). Since the redox processes in the system were estimated using the activity of the redox couple O^o/O^{2-} in the mass-action expressions, the calculated concentrations of Fe^{2+} and Cu^+ were very low so that only the Fe^{3+} and Cu^{2+} states were included in our considerations. Al, Mn and Co in the solutions were estimated as Al^{3+}, Mn^{2+} and Co^{2+} because of the lack of thermodynamic data for other redox states.

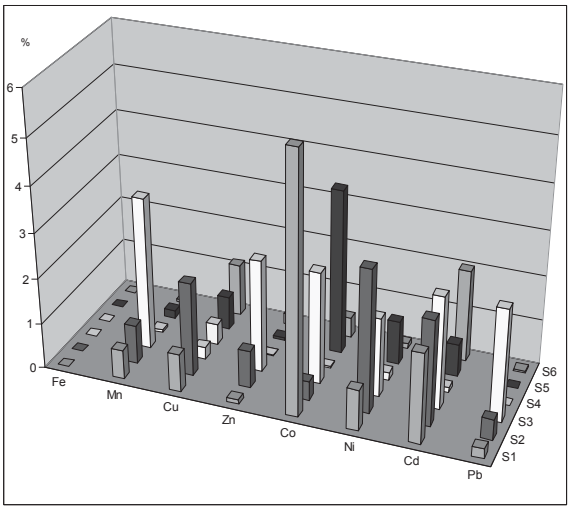

Fig. 2. Percentage of mobile *vs* total concentration of metals

The calculations revealed that inorganic chemical forms (mainly Me^{2+} and $MeSO_4^0$) were dominant for Zn, Cd, Mn, Co and Ni in the soil solution of station S3 (Fig. 3a and b) differently from station S1 (Fig. 3c and d) despite the higher organic content at the former station (Table 1). The lower pH and the higher SO_4^{2-} content are responsible for this phenomenon which is in agreement with the calculations of Cornu *et al.* [11] who reported 48-60% of Cd^{2+} ions in soil solutions with pH 6.2-6.9 at significantly higher DOC. Organic metal species are about 50% for Pb, 65% for Cu and Al and almost 100% for Fe (Fig. 3a and 3b).

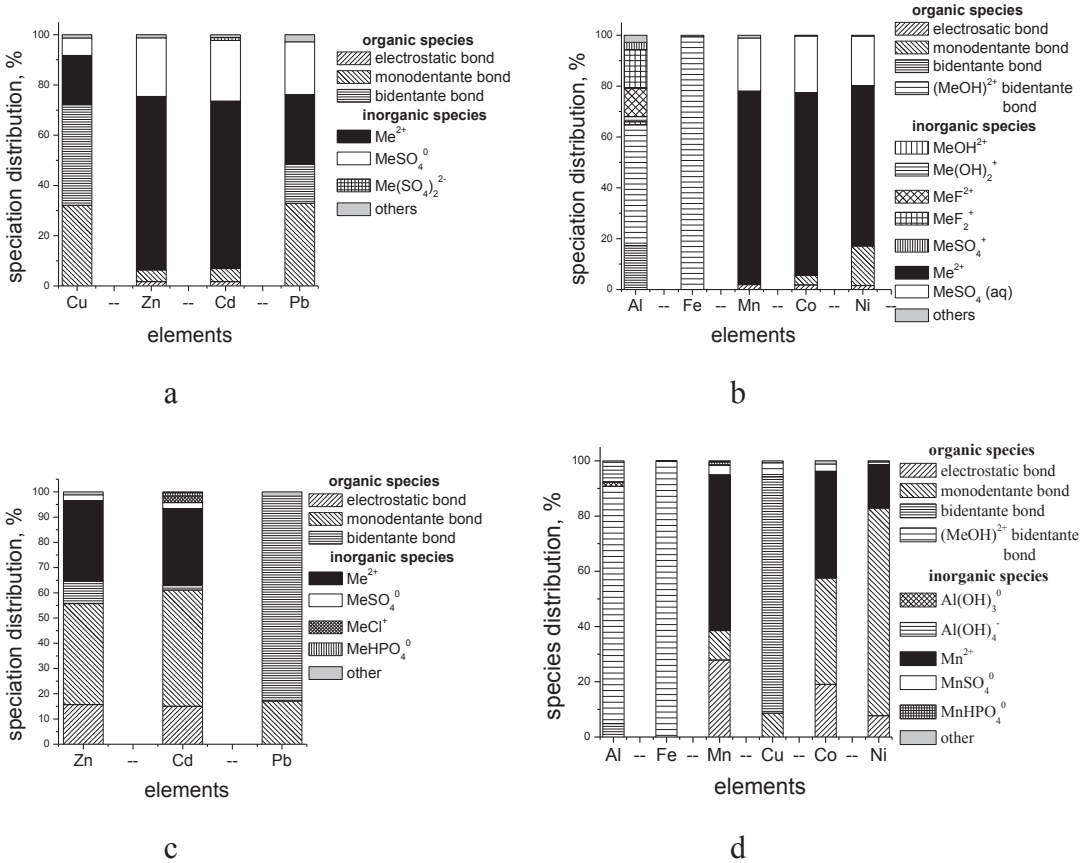

Fig. 3 Distribution of metal species above (a and c) and below (b and d) the limit levels for sample stations S3 (a and b) and S1(c and d)

Organic chemical species are dominant for all studied metals at station S1 with the exception of Mn (Fig.3c and 3d). Here, bidentate complexes are typical for Pb and Cu while monodentate ones were calculated to be dominant for Ni. About 15-20% of electrostatic bonds were calculated for Mn and Co that result from metals interactions with the functional groups of fulvic acids. Fe and Al are present mainly as $(MeOH)^{2+}$ bidentate complexes, independently of the solution acidity.

The results of the thermodynamic modeling of the mobile metals pointed to the advisability of developing thermodynamic models for soil remediation from heavy metals by converting their mobile species into sparingly soluble inorganic compounds.

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BO2. Effects of exogenous Abscisic acid on the photosynthetic activity of ferns (*Nephrolepis sp.)*

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In the light of recent global events such as climate changes, overpopulation and lack of resources, today's agriculture needs to find new ways to sustain healthy, highly productive and stress tolerant plants in order to fulfill the need for plant material [1, 4]. Thus it is very important to develop and test different approaches and methods to establish the best conditions for a given species and to sustain them.

In order to evaluate the influence of environmental factors on plants growth and productivity, we need to be able to test how it reacts to different types of conditions such as different water content of the soil, constitution of inorganic and organic compounds, light intensity, temperature variations, circadian cycle and of course – what is the impact of chemical compounds such as growth regulators, insecticides, herbicides and others, used for various purposes [1,4]. It is important to establish the species limitations and reaction to stress effects, chemical treatment and so on or in other words – the tolerance to different conditions. The functional and structural response of plants to variety of conditions has to be known in order to efficiently improve the selection of quality plants and to sustain healthy, appropriate conditions for their growth and protection from stress.

An investigation with such caliber is very complex [2,5,6]. That is why studies of the photosynthetic apparatus (PSA) of plants are an essential tool in plant physiology. The PSA is very sensitive machinery that senses with great precision every change in the overall physiological state of the plant and thus it is an important indicator about whether the plant is growing and yielding within its best capacity or it experiences some ecological stress.

A lot of experiments clarifying the influence of different biologically active compounds on the PSA of many different species are maintained. Even though nowadays huge amount of information is collected, there is still not enough data about the impact of different compounds to the photosynthetic process of evolutionary lower plants such as lichens, mosses and ferns. Collecting and analyzing data about them could give an answer to variety of questions about the evolution of those plants and of course it could be useful for establishing their potential role in food industries, medicine, cosmetics and many other applications [3].

Abscisic acid (ABA) is a stress hormone in plants. It is synthesized in almost all cells that contain chloroplasts and amyloplasts [3]. It is found in every plant species except in ferns. ABA is overexpressed during water deficiency when it plays a crucial role in plants response and tolerance to dehidratation. When the plant experiences drought stress ABA is transported to the guard cells of the stomata and it serves as a signal for the stomata to close. That way the transpiration is reduced and thus the water loss is reduced as well. Also, ABA inhibits shoots growth while promoting the growth of roots, which adds to the decrease in water loss and its availability [3].

The aim of this study is to trace the dynamics of the photosynthetic process during dark to light transitions of fern plants, treated with the plant hormone ABA in different environmental conditions.

In order to accomplish this we have studied the effects of the hormone in plants in their best condition and in plants, experiencing drought stress.

I. Materials and methods

1. Plant growth and sample preparation

Nephrolepis sp ferns were grown at room temperature in pots with day/night cycle – 16 hours of day and 8 hours of night. They were exposed to 200 μmol photons· m^{-2}· s^{-1} light intensity and watered every 3 days. Four samples were prepared:

Sample	Abbreviation
Control sample, grown in optimal conditions. It is not treated with ABA and it is not subjected to water stress.	Control
A control sample subjected to drought, but without ABA treatment.	(-) ABA (+) drought
Sample, treated with ABA, but grown in optimal conditions.	(+) ABA (-) drought
Sample, subjected both to ABA treatment and drought stress.	(+) ABA (+) drought

ABA treatment is inflicted by spraying the leaves with pulverizer and the plant is watered with the hormone. The concentration of the ABA water solution is 1.10^4 mg/L. Samples are measured 48 hours after treatment.

Drought stress is maintained at 22ºC, ferns are left with no watered for a week.

2. Chlorophyll *a* measurement

Induction kinetics of Prompt chlorophyll a fluorescence (PF) are recorded using fluorometer M-PEA (Multifunctional Plant Efficiency Analyser), developed by Hansatech Instrument Ltd., King's Lynn, Norfolk, UK. Plant samples were kept in the dark during 20 minutes and then fluorescence transients during 120 sec were measured; five repeats are made for bigger statistical accuracy. Three different light intensities were used - 5000, 1000 и 600 μmol photons· m^{-2}· s^{-1}. An OJIP test was performed, comparing 12 chosen photosynthetic parameters accordingly Goltsev et al. [6].

II. Results and Discussion

1. Changes in the kinetics of Prompt Chlorophyll fluorescence

Upon high intensity illumination the kinetics of PF do not show significant difference between the control and the ABA treated plants in the part of the curve which represents the fluorescence rise from F_0 (fluorescence at open reaction centers) to F_M (maximal chlorophyll fluorescence at closed reaction centers). Kinetics of the decay, correlating with the activation of the Rubisco enzyme which pulls reducing equivalents from Photosystem I (PSI) show significant difference between the control sample and the plants, subjected to drought stress and ABA. Plants, treated with both stress factors express a slower decay of the fluorescent signal, thus – a slower activation of Calvin-Benson's cycle, which leads to a longer life time

of the reduced state of the plastoquinone pool. This suggests that during drought stress ABA has a negative effect of carbon dioxide intake trough leaves stomata as it acts as a signal for their closing.

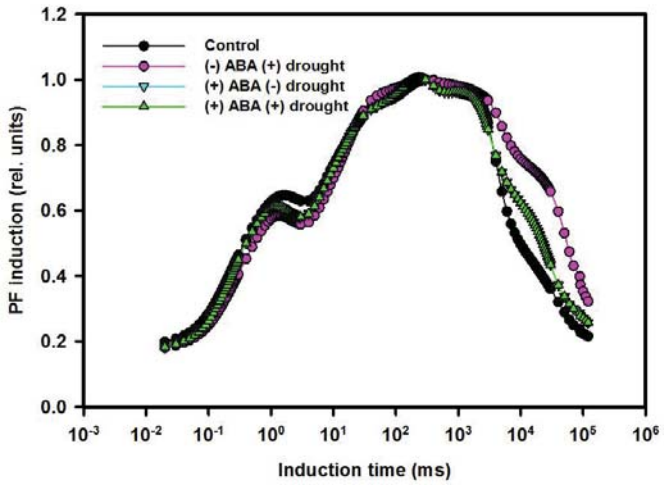

Fig.1. Changes in the PF induction curve in leaves of control fern plants, or treated with ABA during 48 hours and/or dewatered during 7 days. The sample is illuminated with 5000 μmol photons· m^{-2}· s^{-1}.

Upon illumination with lower light intensity (600 μmol photons· m^{-2}· s^{-1}) the delay in Rubisco activation is observed with even better clarity. The slowest decay is observed in plants treated with both ABA and drought stress. The slightest change is recorded in plants, treated only with ABA. This suggests that in lower concentrations and without stress conditions ABA does not affect the PSA of ferns negatively.

When illuminated with light with low intensity there are differences between the control sample and the treated samples in the part of the curve from F_O to F_J, which represents the rate of Q_A reduction to Q_A^-. The samples, treated both with ABA and drought stress show an increase in F_0, which may be due to functionally inactive closed reaction centers in the dark adapted samples or to structural changes in the antennae complexes, interfering with the energy transfer to the reaction center.

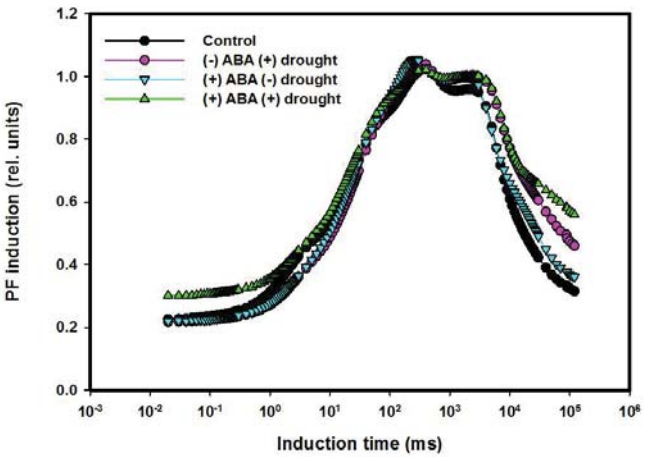

Fig.2. Changes in the PF induction curve in samples (treated as in Figure 1), illuminated with light intensity of 600 μmol photons· m^{-2}· s^{-1}.

The results from the OJIP test show changes in the chosen photosynthetic parameters. The most significant changes are observed in PI_{total}, PI_{abs} and M_0.

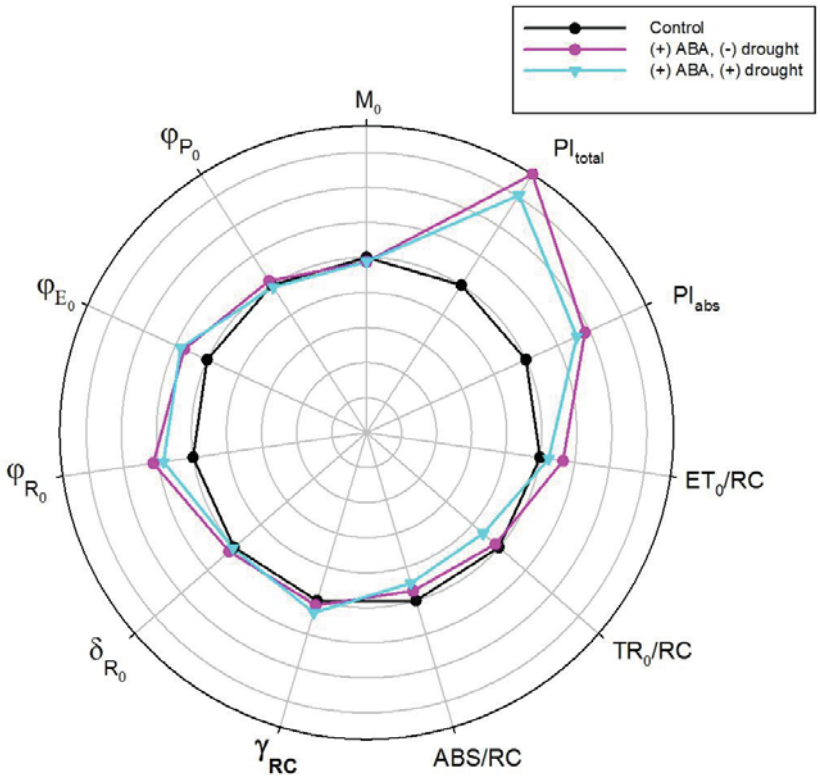

Fig.3. Changes in the parameters of the OJIP test. Control sample values are normalized to 1. Plants are illuminated with 5000 μmol photons· m^{-2}· s^{-1}. Samples are measured 48 hours after treatment with ABA.

PI_{abs} is a parameter, describing the ability of Photosystem II to use the captured energy for reduction of the electron acceptors in the electron transport chain. It gives information about the functional activity of Photosystem II in relation to the absorbed energy.

PI_{total} describes the ability of the PSA to use the captured energy to reduce electron acceptors of Photosystem I.

Plants, treated with ABA show significantly higher PI_{abs} and PI_{total} which suggests that in lower concentrations in plants, which do not synthesize ABA the hormone could have a favorable effect on the PSA as well as on the overall physiological state of the plant. This is also shown by the higher values of φ_{Eo} and φ_{Ro}, which give information about the efficiency of the electron transport of electrons from Q_A to Q_B and to the acceptor side of Photosystem I.

Plants treated with ABA have a smaller initial slope of the curve (M_0), which could be related to the higher rate of the primary photochemical reaction.

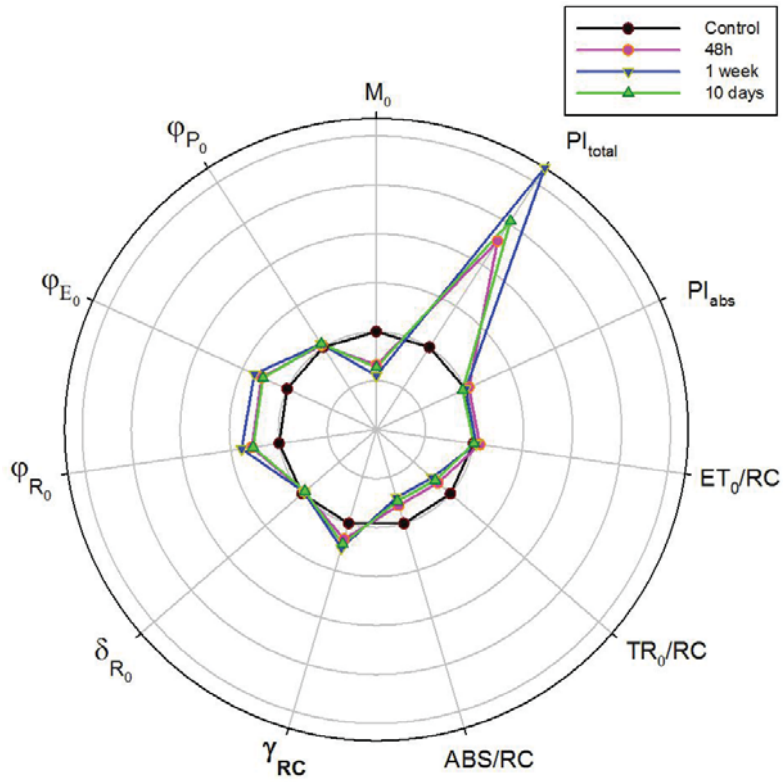

Fig. 4. Dynamics of the changes in the OJIP test parameters in samples, measured 48 hours, 1 week and 10 days after treatment with ABA without drought stress. Samples are illuminated with 5000 μmol photons· m^{-2}· s^{-1}. Control sample values are normalized to 1.

The dynamics of the changes of the parameters of Prompt chlorophyll fluorescence shows an increase in the values of PI_{abs}, PI_{total}, φ_{Eo}, φ_{Ro} during the first 48 hours which continuous to increase until 1 week, where it reaches its peak. After 10 days the values are starting to decrease back to the control sample values. It seems that ferns, subjected to ABA treatment without ecological stress show significantly improved efficiency of the electron flow in the electron transport chain of Photosystem I and II. With time the effect wears off. Simultaneously ABA leads to decrease of the energy, trapped and transformed in the reduction of Q_A for a reaction center (T_{R0}/RC), as well as absorption for an active reaction center (ABS/RC). A higher rate of the primary photochemistry is observed.

Even though ferns don't have endogenous ABA, they successfully include it in their metabolic pathways. In lower concentration ABA has a favorable influence on the PSA of ferns by improving the efficiency of Photosystem II as well as the reduction of the acceptors of Photosystem I.

In stress conditions ABA significantly delays the activation of Rubisco, which leads to blocking of the electron transport chain and slower reopening of the reaction centers which is mostly noticeable when plants are illuminated with higher light intensities. Even though ABA has favorable effects on the activity of both Photosystems, it reduces the energy trapping efficiency. It is possible that the hormone has a destructive influence on the antennae complexes. Since this effect is reversible, there may be some repair mechanisms which bring the control values of the energy trapping back to normal 10 days after ABA treatment.

In certain cases ABA in lower concentrations can positively affect the photosynthetic process of ferns.

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BO3. Honeybee pollen as bioindicator for environmental pollution

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Abstract

Bee products possess therapeutic properties and are the source of many essential trace elements that is way they are regarded as valuable foods. Honeybee pollen is one of the most popular bee products used as a diet complement with immunostimulant effect. On the other hand, bee pollen as a product derived from the pollen of flowers around the hive, gives valuable information about the environmental situation in a given area. As the bees flit about collecting pollen and nectar from flowering plants, they also collect any pollutants that may have settled onto the plants from the air or been drawn up from the ground. The presence of pollutants in these products can lower their quality, which in consequence may endanger human health. In this work some investigations on the use and effectiveness of bee pollen as a bioindicator of environmental pollution with different trace elements, thoroughly heavy metals and pesticides, are presented.

Keywords: honeybee pollen, trace elements, toxic heavy metals, pesticides, environmental pollution

Introduction

Pollen, as a natural plant product, is exposed to different contaminations absorbed by plants from the soil through root system or with water intake, as well as to numerous pollutants of different origin, including anthropogenic ones, deposited directly on pollen [11]. Areas with intensive industry are associated with heavy metal pollution of the environmental, which is a first step of contamination of food sources. On the other hand, some of trace

elements like iron, manganese and zinc are essential elements, very important for biological systems, which can have a harmful effect when their concentration exceeds well known quantities [10]. Many investigators have employed honeybees or honeybee products as tools for assessing environmental pollution in industrial areas.

The honeybees are easy adapted to environment with different ecological characteristics (soil, vegetation, air, and water). Therefore, if they function in a polluted environment, plant products used by them may also be contaminated, and a part of these pollutants can accumulate in the bee products, in particular pollen.

The bees, foragers in particular, are good biological indicators that quickly detect the chemical impairment of the environment by the high mortality and the presence of pollutants in their body or in beehive products [9].

According to Chauzat et al. [4] pollen loads and beeswax have the highest frequency of pesticide occurrence among the apiary matrices, whereas honey samples have the lowest. On the other hand the pollen, which can also serve as a bioindicator of environmental pollution, is one of the sources of honey [3]. Therefore, the aim of this work is to present some of the studies on the role of honeybee pollen as a bio-indicator of environment pollution.

Honeybee pollen as a bioindicator for environmental pollution with heavy metals

Conti and Botrè [5] have measured the concentrations of three representative heavy metals (cadmium, chromium and lead) by atomic absorption spectroscopy in honeybees and in apiary's products (honey, pollen, propolis, and wax). Samples have been collected from five different sampling points: four from areas surrounding the city of Rome, and the fifth in the city center which receives intenseve hicular traffic. All apiaries employed for this study have been specifically constructed without any metal part in order toavoid the risk of contamination of the assayed materials. Sample collection has been conducted over a 3-month period. Experimental data have revealed, in general, statistically significant differences between the background levels of heavy metals recorded from the reference sites and the levels measured in the site located in the center of the city of Rome. The results of the authors indicate that honeybees and, to a lesser extent, some of their products, including pollen, can be considered representative bioindicators of environmental pollution.

Roman [12] has determined the rate of bioaccumulation of chosen heavy metals (As, Pb, Cd, Hg) in fresh pollen obtained in the form of pollen loads. The research material have been samples of fresh pollen obtained in stationary apiaries located in two regions: agricultural woodland and a former military airfield. Samples have been collected in a period of July – August 2005 and 2006. They have been mineralized by microwave method. Quantitative analysis of examined metals (arsenic, lead, cadmium and mercury) has been done using plasma spectrometry (ICP). Research has demonstrated that mean content of elements of toxic properties in pollen from an agricultural woodland region in 2005 and 2006 have been (in mg · kg-1 d.m.) as follows: Pb – 0.804 and 0.491; Cd – 0.234 and 0.272; As – 0.060 and 0.036, and Hg – 0.0038 and 0.0036, respectively. Lead concentration in 20 samples (n=36) in 2005, and in 11 samples (n=36) in 2006 has exceeded acceptable standards (0.50 mg · kg-1 d.m.). However, in pollen from the area of the former military airfield the content of particular elements has been higher for Pb – 0.835 and 0.704, Cd – 0.356 and 0.363, As – 0.093 and 0.099, and for Hg – 0.0066 and 0.0059, respectively.

Lead accumulation has exceeded permissible standards in 33 samples (n=36) from 2005 and in 21 (n=36) from 2006, and cadmium in 31 and 36 samples (n=36), respectively.

Mercury and arsenic have appeared to be metals of toxic properties that do not cause any toxicological problems in pollen from the agricultural woodland and airfield regions since their concentration has been very low. According to author high concentration of cadmium in

pollen from the agricultural woodland deserves attention. Differences in concentrations of analyzed elements between regions may be used as bioindicators of environmental contamination with elements of toxic properties.

A large study on the role of honeybees and their products, including pollen, as bioindicator of environment status has been performed in Bulgaria by Zhelyazkova et al. [14]. The authors have made a comparative analysis of the content of some heavy metals and metalloids (copper Cu, zinc Zn, lead Pb, cadmium Cd, nickel Ni, cobalt Co, manganese Mn, iron Fe) in the body of bees (Apis mellifera L.), fecal mass, bee products (honey, pollen, wax) and sunflower flowers in areas with different degree of anthropogenic impact. Samples have been taken in the active bee season of 2010 (June – July). The study has included two settlements in Stara Zagora region: the town of Gurkovo – an area with low level of anthropogenic impact; the village of Bratya Kunchevi – an area with established anthropogenic impact (working stone quarry on the territory of the village). It has been established that Fe content is of higher values in the bee body, pollen and wax in the station village of Bratya Kunchevi (compared to the samples in the town of Gurkovo). A possible reason for the reported high quantity of iron in the analysed samples of the village of Bratya Kunchevi according to the authors could be the working stone quarry on the territory of the village operating a deposition of andesite tuffs (calcium-magnesium-iron silicates).

The purpose of the study of Popescu et al. [10] has been to evaluate the use of the pollen as bio-indicator of environmental pollution. The content of Fe, Mn, and Zn from pollen samples uniformly distributed in Dambovita region has been determined. The authors have collected samples during April 2009 from 18 bee houses from private farms, kipped in industrial-urban and non industrial-rural areas. The samples have been analyzed with an Elvax ED-XRF spectrometer having a solid state Si-pin-diode detector with thermo-electrical cooling with 165 eV at 5.9 keV (Fe 55 isotope) energy resolution. Differences between the two kinds of samples have been found. The authors have concluded that the pollen can be used for bio-monitoring of environment for Fe, Mn, and Zn.

Morgano et al. [8] have carried out a set of experiments aiming to validate a method for inorganic contaminants in honeybee-collected pollen, consisting of digestion of the samples in a closed microwave-assisted system and quantification of 10 inorganic contaminants by ICP OES. Forty-three samples of Brazilian bee pollen, collected in Southeastern Brazil during one year, have been analyzed. According to the authors determination of these analytes is important both as bioindicators of pollution and to verify the safety of consuming the pollen itself. The method has had satisfactory performance, with good accuracy and precision. The ranges of the mean levels have been 10.4−268.0 mg/kg for Al, <0.01−1.38 mg/kg for As, 2.78−17.63 mg/kg for Ba, 0.003−0.233 mg/kg for Cd, <0.01−1.11 mg/kg for Co, <0.01−2.32 mg/kg for Cr, <0.10−1.13 mg/kg for Ni, <0.01−0.44 mg/kg for Pb, <0.035−1.33 mg/kg for Sb, and <0.0004−0.0068 mg/kg for Hg. Generally higher levels of all studied contaminants have been observed in samples produced in an urban site, compared to those of a rural site. Al, Cd, Co, and Pb have tended to have higher levels during the dry months (July−October). Ingestion estimates have showed that Al and As would have the highest contributions to the adult diet, reaching 27 and 8%, respectively, of the provisional tolerable weekly intake values, considering a daily portion of 25 g.

Some of above mentioned results have been confirmed by other scientists. Apiaries in urban and hedgerow landscapes have appeared more contaminated than apiaries in cultivated and island landscapes. Sampling period has had a significant effect on Pb contamination with higher Pb concentrations determined in dry seasons [7].

Al-Naggar et al. [1] have evaluated the effectiveness of honeybees and their associated products as biological indicators of the presence of lead, cadmium, copper, iron and zinc in the environment of four different Egyptian regions with different anthropogenic activities.

The reported concentrations of heavy metals decreased in the following order: honey bee workers > pollen > honey. The authors have found that the bee gathered pollen heavy metal contents have been higher during spring when compared with those during the summer. These results have indicated that honeybees and, to a lesser extent, some of their products (including pollen), can be considered as bioindicator of environmental pollution with heavy metals.

Honeybee pollen as a bio-indicator for environmental pollution with pesticides and other trace elements

Bernal et al. [2] have performed a study aiming to evaluate the pesticide residues in stored pollen from honey bee colonies and their possible impact on honey bee losses in Spain. In total, 1021 professional apiaries have been randomly selected. All pollen samples have been subjected to multiresidue analysis by gas chromatography-mass spectrometry (MS) and liquid chromatography-MS; moreover, specific methods have been applied for neonicotinoids and fipronil. A palynological analysis also has been carried out to confirm the type of foraging crop. Pesticide residues have been detected in 42% of samples collected in spring, and only in 31% of samples collected in autumn. Fluvalinate and chlorfenvinphos have been the most frequently detected pesticides in the analyzed samples. Fipronil has been detected in 3.7% of all the spring samples but never in autumn samples, and neonicotinoid residues have not been detected. More than 47.8% of stored pollen samples belonged to wild vegetation, and sunflower (*Heliantus* spp.) pollen has been only detected in 10.4% of the samples. A direct relation between pesticide residues found in stored pollen samples and colony losses has not been evident accordingly to the obtained results.

Kasiotis et al. [6] have investigate reported cases of honeybee death incidents with regard to the potential interrelation to the exposure to pesticides. Thus honeybee, bee pollen and honey samples from different areas of Greece have been analyzed for the presence of pesticide residues. In this context an LC–ESI-MS/MS multiresidue method of total 115 analytes of different chemical classes such as neonicotinoids, organophosphates, triazoles, carbamates, dicarboximides and dinitroanilines in honeybee bodies, honey and bee pollen has been developed and validated. LOD and LOQ values have ranged – for honeybees, honey and bee pollen – from 0.03 to 23.3 ng/g matrix weight and 0.1 up to 78 ng/g matrix weight, respectively. Therefore, according to the authors this method is sufficient to act as a monitoring tool for the determination of pesticide residues in cases of suspected honeybee poisoning incidents. From the analysis of the samples the presence of 14 active substances has been observed in all matrices with concentrations ranging for honeybees from 0.3 to 81.5 ng/g, for bee pollen from 6.1 to 1273 ng/g.

Samples of honey, pollen and honey bees have been collected in some regions of Italy after the Chernobyl accident, and have been subjected to gamma spectrometry in order to assess their possible use as markers of the radioactive environmental contamination [13]. The results have shown that bees can be used for the purpose, but their collection is more difficult than pollen and honey. Honey has given only an indication for radioactive pollution. Pollen has been resulted in the best indicator, since according to the authors it reflects exactly the air contamination and therefore it is suitable for obtaining a map of fallout.

Conclusions

Bee pollen is one of the popular bee products, used as a diet supplement with immunostimulatory effect. As the bees flit about collecting pollen and nectar from flowering plants, they also collect any pollutants that may have settled onto the plants from the air or been drawn up from the ground. Determining the quantities of accumulated heavy metals (Fe, Mn, Zn, Al, Cd, Co, Pb As, Hg etc.) and pesticides (fluvalinate, chlorfenvinphos neonicotinoids, fipronil, etc.) in the pollen through specially developed and validated methods

may serve as monitoring of the ecological state of nature. On the other hand, these studies can guarantee the quality of consumed bee products and their safety. The review of the presented scientific experiments shows that the bee pollen can be used as a reliable bioindicator of environmental pollution. Easy way for obtaining of the pollen gives its advantage before other bioindicators (plants, insects, birds, small rodents, fishes) in investigation of environmental status.

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BO4. Alterations of antioxidant trace elements and related metalloenzymes in rabbits with eimeriosis

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Abstract

The aim of this study was to investigate the levels of antioxidant trace elements (zinc, copper, selenium) and the activity of related metalloenzymes (superoxide dismutase: SOD and glutathione peroxidase, GPx) in the blood of rabbits experimentally infected with *Eimeria stiedae.* The results showed that blood Se and serum Zn concentrations, the GPx activity were significantly decreased, serum Cu concentration and blood Cu/ZnSOD activity were increased. As shown in our data the levels of trace elements Zn, Cu and Se and the activity of metallo-enzymes Cu/ZnSOD and GPx were significantly changed Antioxidant trace elements and related metalloenzymes play a vital role in maintaining the antioxidant defense system during eimeriosis.

The importance of the distribution of trace elements used for antioxidant enzyme synthesis should be taken in account in the therapeutic support of the parasitized animals, to adjust the nutritional supplementation to their specific needs and to propose nutritional support recommendation.

Key words: Eimeriosis, SOD, CAT, Se, Zn, Cu

Introduction

Coccidioses caused by protozoans of Eimeria genus (Apicomplexa: Eimeriidae) is a serious problem for the health of rabbits and their production. Of special importance is the hepatic coccidia *Eimeria stiedae*. Hepatic coccidiosis in rabbits is a highly contagious parasitic infection. It infects the bile ducts, leading to severe liver disease and death. Clinical symptoms are characterized by reduced food consumption, diarrhea or constipation, enlargement of liver, ascites, icterus, and finally death [2].

Some investigations have revealed that hepatic coccidiosis leads to increase of serum AST, ALT and GGT activities while albumin level decreases. [7]. several investigations have revealed that some parasitic infections cause changes in trace elements levels and metalloenzymes [6], [11]. Trace elements and related metalloenzymes play an important role in a number of essential metabolic processes [4]. There are no data concerning blood antioxidant trace element levels and an activity of enzymes related with them during the eimeriosis in rabbits.

The aim of this study was to investigate the levels of antioxidant trace elements (zinc, copper, selenium) and the activity of related metalloenzymes (SOD and GPx) in the blood of rabbits experimentally infected with *Eimeria stiedae.*

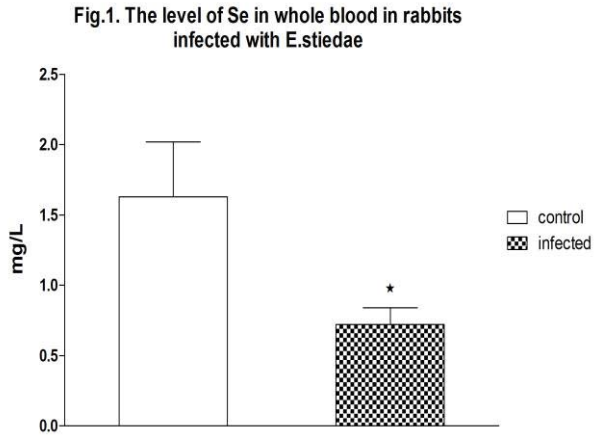
Material and methods

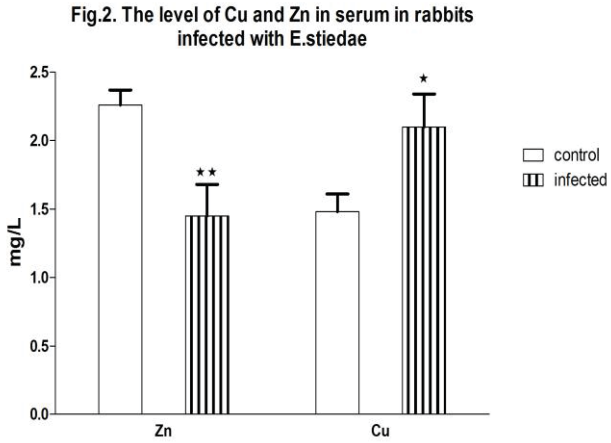
16 healthy New Zealand rabbits, aged 6-7 weeks with a weight 1-1, 5 kg were used. During the experiment the rabbits were individually housed in metal cages. They were fed with commercial pellet food. Food and water were supplied ad libitum. The absence of eimeriae oocysts prior to the experiment was confirmed by fecal examination in rabbits.

The rabbits were divided into two groups each of 8 rabbits- control, healthy and a group infected with sporulated *E. stiedae* oocysts. The inoculum was prepared according to [9] as each ml containing 40000 sporulated *E. stiedae*. At the day 15 post infection blood samples were collected from each rabbit. The level of zinc and copper were determined in the serum using flame atomic absorption spectrophotometry using Varian Techtran, Model AA 220. Se in the whole blood was performed by the fluorometric method of [14]. The blood GPx activity was measured by the method of [1]. Serum Cu/ZnSOD activity was determined according to [10].

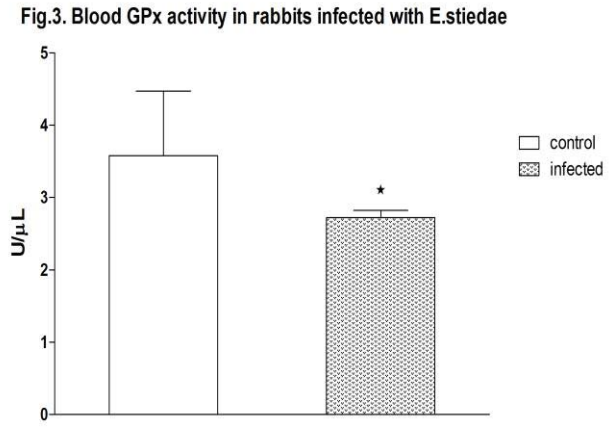
Results and Discussion

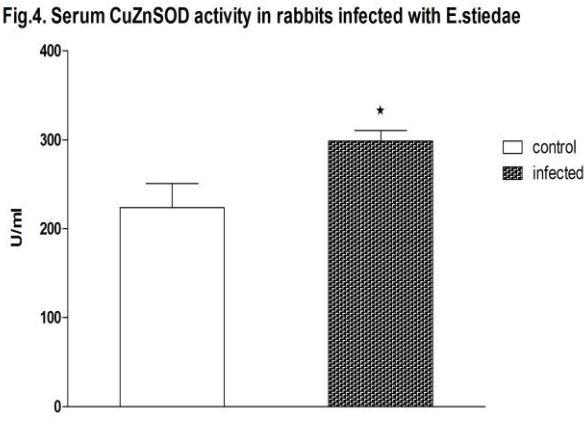
Blood Se was significantly decreased at the day 15 p.i. (Fig. 1). Serum Cu concentration was significantly increased but Zn concentration was significantly decreased (Fig.2).





The activity of the studied enzymes was changed too. Blood GPx activity was decreased and serum Cu/ZnSOD activity was increased at the day 15 p.i. (Fig. 3, 4).





Our data confirmed that parasitoses could cause deviations in the trace element status in the hosts. Parasitic infections induced by *Fasciola hepatica* [6] and *Dicrocoelium dendriticum* [12] cause trace elements imbalance in the infected hosts as well as lipid peroxidation.

Decreased Se level and GPx activity were well expressed in this experiment. Among antioxidant trace elements Se acts in the immune system, antioxidants mechanisms and tissue repair [13]. Se is especially involved in antioxidant enzymatic defenses regarding its structural function in the act site of selenoenzymes and plays a direct role in the regulation in the

inflammatory processes by inhibiting transcription factors of several proinflammatory mediators. Se-intake may be essential in protection against oxidative damage [4]. We observed a blood GPx activity depletion as Se used to maintain GPx synthesis. GPx activity reduction reflects the blood Se decreased. The decreased GPx activity might be associated with excessive free radicals occurring during the infection or a decrease in their production as a result of liver damage [3].

Zn modification could be related to the generalization of the parasites induced inflammatory response [6]. Zn level is associated with impaired endothelial cell barrier function and thus reducing the possibility of entry of ceruloplasmin and consequently decreasing the risk of oxidizing low density lipoprotein (LDL). Zn status may affect LDL oxidisability or SOD activity [4]. Zn is an essential compound of proteins, biomembranes and various enzymes, maintain their normal structure and/or functions. It possess important antioxidant properties that have been linked partially to its role as an integral part of Cu/ZnSOD, as a stabilizer of cell membranes and as a protective factor of the sulfhydryl groups against oxidation of proteins [4].

Cu level was observed to increase in the infected rabbits. In the inflammatory processes a rice of Cu level is observed in our study. It can be postulated that the elevated Cu level is actually inflammatory markers. Increased serum Cu level which might be related to the enhancement of the inflammatory processes. Inflammatory cytokines stimulate hepatic synthesis of ceruloplasmin among other reactants. The increase of ceruloplasmin which is the major transport protein of Cu may explain the enhancement of blood level of Cu [8]. When oxidative stress elevated Zn and Cu mobilization in liver contributes to the expansion of antioxidant organism defense. As Cu/ZnSOD and GPx are joined in cellular defense against oxidants [8]. Decreased GPx activity together with increased Cu/ZnSOD activity revealed unbalanced antioxidant defense capacity. The increased MDA level in serum of rabbits infected with *E. stiedae* and changes in the activity of SOD, CAT and GPx showed a development of oxidative stress during the infection with *E. stiedae* [3].

Copper and zinc are components of numerous enzymes responsible for detoxification and homeostasis protection [8]. Among the major enzymes containing Cu and Zn as cofactors Cu/ZnSOD plays a key role to counteract the oxidative stress induced by eimeriosis in rabbits. The increase of SOD activity are known to limit the diffusion of reactive oxygen species released by injured tissues and therefore to avoid the extension of the oxidative burst. SOD activation should interfere in vivo to limit the inflammatory process [5].

Antioxidant trace elements and related metalloenzymes play a vital role in maintaining the antioxidant defense system during eimeriosis. As shown in our data the level of trace elements Zn, Cu and Se and the activity of metallo-enzymes Cu/ZnSOD and GPx significantly changed.

Decreased blood GPx activity and Se level have suggested that Se deficiency may have a more prominent effect in hepatic coccidiosis in rabbits.

The study demonstrates that eimeriosis induced alterations, mobilization and localization of antioxidant trace elements. It can be postulated that these alterations reflect an adaptive mechanism to counteract the deleterious effect of metabolic disorders and the oxidative stress associated with the parasitosis. The interest of combined antioxidant supplement to restore a balanced antioxidant status is therefore to be considered in the eimeriosis.

The importance of the distribution of trace elements used for antioxidant enzyme synthesis should be taken in account as soon as possible in the therapeutic support of the parasitized animals, to adjust the nutritional supplementation to their specific needs and to propose nutritional support recommendation.

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BO5. Some aspects of selenium biological activity

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Abstract

The biological role of selenium is not enough investigated yet. This element is one of the components of the Earth crust. At high concentrations Se is toxic but in trace amounts Se is an essential micronutrient.This short review summarizies data about some biological functions of Se and its importance in normal metabolic processes of living organisms in respect to human health. Selenium has been found to form part of the important enzyme – glutation peroxidase. Selenium also suppresses the development of carcinoma. Studies connected with Se - its content in the rocks and the soil, the role of microorganisms and plants as mediators for migration of Se from soil to humans are necessary.

Selenium has been discovered in 1817 from Jacob Bercelius. Its atomic weight is 78, 96. Selenium has many similar chemical properties with sulfur (S), because se atom is slightly larger than S atom. Its radius is 0, 5 Å and the S atom radius is 0, 37 Å. They both can exist in five valence status conditions (selenide -2^{-}; elemental Se –0; thyoselenate -2^{+}; selenite – 4^{+}; selenate – 6^{+}) [18,24]. The specific activity of Se depends on redox conditions and pH. Selenate usually is active in aerobic and neutral to alkaline environments. Selenide and elemental Se take part in metabolism under anaerobic environments. Se can exists in volatile forms-dimethyldiselenide and probably dimethyl selenone, dimethylselenylsulfide and methaneselenol [10, 21].

Selenium is toxic at high levels but it is also an essential element for many organisms (bacteria, sertain algae, mammals, including man). Some organisms incorporate selenocystein in so-called selenoproteins which have antioxidant activity. Se deficiency is a problem in Eastern Unated States, Europe, Australia and some regions of China [30].

Selenium is of fundamental importance to human health. Se is an essential element of several metabolic pathways-thyroid hormone metabolism, antioxidant system and immunity. On the other hand Se has been shown as an agent responsible for mortality, developmental defects and reproductive functions in migratory aquatic birds and fish in waters of Kesterson Reservoir in California [19]. Se effect depends on dosages. The range between useful and toxic dosages is very narrow and specific for a given organism. Selenium takes part in formation of important enzymes, like glutation peroxidase [24]. It has been found the suppressive effect of Se on carcinoma and some symptoms of AIDS [14].

The toxicity of Se is due to its ability to replace S in amino acids resulting in incorrect folding of the proteins and consequently nonfunctional proteins and enzymes. Acute professional exposition of SeO_2 leads to bronchitis, irration of respiratory organs, cough, vomitation. Chronic exposition to Se at 1438 μg/day for a man and 1238 μg/day for a women during their life causes lost of hear and nails, rotting of theet, disturbaces of central nervous system [15].

Selenium is incorporated as selenocysteine at the active site of a wide range of selenoproteins. The four glutation peroxidase enzymes (classical GPx1, gastrointestinal GPx2, plasma GPx3, phospholipidhydroperoxide-GPx4). This is the major class of functionally

important selenoproteins, which have to be characterized. It has been shown the higher toxicity of selenites than selenates [22, 24].

Circulation of Se in environment included plants. Some investigations proved that Se may be required for the metabolism of so named plant-accumulators. These higher plants are endemic species to seleniferons soils. Higher plants metabolize Se via the S-assimilation pathway [24, 28]. Higher plants have a main role in Se volatilization (in so-named plant-nonaccumulators). Plants have ability to convert inorganic Se to volatile forms and this process is called phytovolatilization. The translation of Se from soil to the plant roots and shoots depends on the form of Se. Translocation and distribution of Se in plants also depends on the forms and concentration of Se in the roots, as well as on the concentration of other substances [8, 24, 27].

It has been proved that selenate is accumulated in plant cells [3, 24] and in other organisms like Esherichia coli and yeast [6, 24]. Se uptake is mediated by sulfate transporter.

Plants also can absorb volatile se from the atmosphere via the leaf surface [24, 31].

Very probably there is possible in corporation of trace amount of Se into specific selenoproteins and higher plants.

Some group of organisms can volatilize se at high rates compared with higher plants [9, 10, 11, 12, 24]. It has been proved the important role of soil microorganisms in plant volatilization of Se. treated of the roots with penicillin –G and chlortetracycline inhibited almost 95 % of plant Se volatilization [29].

Many environmental factors influenced the process of phytovoltalization of Se. One of important factor is the concentration of sulfates in the substrate. Increasing of sulfate leads to decreasing of the volatilization [24]. On the base of different investigations by means of multiple – regression analysis that water temperature, Se concentration in sediment (or in roots) and the level of microbial biomass (especially in the rhizosphare) have been one of the most important environmental factors influencing phytovolatilization.

Selenium is very important to human health [1]. It takes part as an essential component of major metabolic pathways. At the moment more than 30 different selenoproteins have been investigated. Some of them (about 15) have been purified and characterized in respect to their biological activity. Selenium is incorporated as selencysteine at the active cite of a wide range of selenoproteins [2]. The first of the characterized selenoproteins have been four glutation peroxidase enzymes (classical GPx1; gastrointestinal GPx2; plasma GPx3; phospholipids hydroperoxidase GPx4). They are a major class of functionally important selenoproteins.

Other classes of selenoproteins are absolutely necessary of the prohormone tyrosine (T4) to the active tyroid hormone triodothyronine (T3). Special selenoprotein is responsible for normal formation of sperm. Se is also necessary for muscle metabolism. It has been proved the protective role of Se against some forms of cancer and is of importance for the male fertility and havy cardiovascular disease. Se also has been shown as essential component to human nutrition.

Classical glutation peroxidase (GPx1) has been the first investigated and characterized selenoproteine which is closely connected with Se concentration in erytrocytes.

Gastrointestinal peroxidase (GPx2) is the most important selenoprotein antioxidant in the colon. Oxidative stress is a critical event in tumourogenesis. It is therefore likely that the antioxidant function of GPx2 will provide an early defence against colon cancer [2].

Phospholipids hydroperoxid glutation peroxidase (GPx4) is a monomer and its activity is preserved in preference to GPx1 when Se content is low [2]. It has been found to be responsible as an essential factor to destruction of fatty acid hydroperoxides, which it not reduced to hydroxyl fatty acids and will lead to uncontrolled free radical chain reactions that are obstakle to the integrity of membranes.

The detail mechanism for the activation-inactivation of the enzyme is still unknown but the evidence of high activity in membranes of differentiating spermatogenic cells suggest a possible relationship between cell differentiation and peroxide levels [5].

Extracellular glutation peroxidase (GPx3) poses antioxidant activity. Glutation peroxidase (GPx3) may have a specific antioxidant function in renal tubules. Thioredoxin is possible to act as electron donor and support an antioxidant role for GPx3 in plasma [13].

Thioredoxin also is important antioxidant protein to regulation of cell growth. Thioredoxin reductase plays an important role in the prevention of some forms of cancer.

Another selenoprotein, selenoprotein-P is also present in many tissues and is associated with cell membranes [4].

Iodotbyronine deiodinases are another class of selenoproteins which take part in syntesis of thyroid hormones and have a regulatory role in hepatic enzyme expression and neutrophil function.

Spermatozoa contain the highest concentration of selenium. About 50 % of the capsule materials are GPx4 [25]. Insufficient concentration of Se affects sperm motility and may be induce sterility [26].

Selenoprotein W has been found to be responsible for so named white muscle disease. Recent investigation show role of se to myopathy caused by selenium deficiency [16].

Selenoprotein W is another main selenoprotein.

Investigations of animals metabolism have proved the necessity of Se for their muscle metabolism (skeletal muscle calcification in sheep and cattle-so named white muscle disease). Till now the effect of selenoprotein W on human skeletal muscles is not yet understood in details. Many investigations now are pointed to influence of Se on muscle dystrophies. Se deficiency has been proved as a factor caused myopathy.

The circulation of se from soil to animals and humans is realized through plants.

The level of se indifferent tissues depends on dietary. Dietary traditions are closely connected with geographical conditions and content of Se in the soil. Available Se content is Europe is relatively low. Really, heavy Se deficiency is rare. Such a deficiency has been found to be a cause of fatal cardiomyopathy in China, region Keshan. Comparatively low content of Se has been reported in New Zeland, Finland and some areas of the Eastern United States [2].

Element Se is both essential and highly toxic. Investigations have shown to content of 40 μg Se/day to be sufficient to the Se requirements for men with average body weight of 60 kg [2]. This data is used for recommendation of Se dosage.

Now it is well known that Se is important for a healthy immune system [23]. Se deficiency can cause reduction of T-cells, impaired lymphocyte proliferation and responsivness [17]. A decreasing of se in blood plasma has been reported in respiratory distress syndrome and AIDS. Selenium supplementation reflects the tocoferol status of the blood.

Now there are many data about Se-protective effect against some forms of cancer. Recent studies have proved the positive effect of Se on decreasing of prostate, colon and lung cancers by 63, 58 and 46% respectively [7]. Recently the mechanism of this effect is not known. May be this is due to se depenent redox systems in the cells.

Low concentration of Se in blood has been associated with cardiovascular disease mortality. Very probably this may be a result of sub-optimal GPx4 activity.

Selenium is an integral component of three major metabolic systems essential for normal cell metabolism. Supplementation of Se in prevents development of endemic cardiomyopathy in Keshan (North East China). Blood selenium concentration reflects to synthesis of thyroid hormone metabolism, antioxidant activity and redox system. Clinical studies proved decreasing of infections parallel to increasing of Se concentration.

Individuals have different requirements to Se content in the cells. There are genetic variation among individuals and examination of the nutrient gene interactions are one of the main factors in the future. There are many other factors which influence on Se metabolic pathways. Precise future investigations will provide a real measure of functional necessity of Se.

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ВО6. Макроелементи

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ВО7. Незаконна търговия и бизнес със застрашени животни

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BP1. Polymetallic Biogeochemical Province of North Ossetia

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BP2. Recent data about biological activity of Monensin

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Monensin is the first antibiotic used as anticoccidials. In July 1971, the polyether ionophorous antibiotic monensin was introduced in the United States for the control of coccidiosis in poultry [1]. Monensin is an antibiotic produced as a byproduct of fermentation by *Streptomyces cinnamonensis* and belongs to a family of drugs known as polyether antibiotics or ionophores. Ionophores which are used commercially and monensin is choice of product for broiler chickens mainly because of its broad spectrum activity against majority of pathogenic species of coccidian (*Eimeria spp*.) which are still widely used for this purpose today [4, 9].
Monensin exhibits antibiotic, coccidiostatic, cardiovascular and other important biological and medical properties [3]. Recent experimental data have shown that monensin possesses antiparasitic activity against many kind of parasites such as *Toxoplasma gondii, Neospora caninum, Cryptosporidium parvum, Toxoplasma gondii, Echinococcus granulosus, Plasmodium berghei* and *Pl. yoelii, Fasciola hepatica* [5, 6, 8, 10, 11, 12, 14].

The interest in monensin and its compounds encourages scientists to search and testing of new, more effective ways to use it. Up to now various derivatives of Monensin were synthesized in order to reduce its toxicity and to extend its fields of application [3]. Surolia et al. [13] have been studied the effect of monensin, which had been included in nanoparticles, named PLGA nanoparticles. They have been examined the antimalarial efficacy of monensin-PLGA nanoparticles. Monensin loaded in nanoparticles was 10-fold more effective in inhibiting the growth of P. falciparum in vitro as compared to free monensin. The antimalarial

efficacy of monensin-PLGA nanoparticles was significantly dependent on the molecular weight of the polymer.

The investigations performed in our laboratories indicate that complexes of monensic acid with some biometal (II) ions (Mg, Ca, Co, Mn, Ni, Zn) express significant antineoplastic potential against cell lines obtained from some of the most common and aggressive human cancers (glioblastoma multiforme, cancers of the breast, liver, lung, uterine cervix) being more active as compared to the non-coordinated monensic acid [15].
Due to a broad spectrum of biological activity, monensin derivatives are an important object of research aimed at reducing the toxicity and to obtain new compounds with improved biological properties in terms of further use [7].
Only recently it was shown that Monensin A is also a highly effective ionophore for Li^+, Rb^+ as well as for Pb^{2+} cations [2]. These properties are the basis of many biological and pharmaceutical fields of application of this compound and new ones can be expected in the future.

Keywords: monensin, biological activity, parasites, cancer

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BP3. Effect of cobaltbisdicarbolides on viability of cultured tumor permanent cell lines

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BP4. Cobaltabisdicarbollides affect viability and proliferation of mouse and hamster tumor and non-tumor cells

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Thursday, 27 November 2014

Session C.

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CO1. Food allergens and food allergy

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Abstract

Food allergy is an adverse immune response to certain food proteins or glycoproteins /so called food allergens/ mainly by formation of IgE specific antibodies.

The main factors leading to the onset and development of sensitivity to food are many diseases of the gastrointestinal tract, different intestinal parasites harmful to mucosal tissues and underdeveloped digestive apparatus in children creating conditions for allergic reaction by the enteral route.

Hypersensitivity is depending on the immunogenicity of the organism and is categorized according to the immunological mechanisms which take part in its development. There are four types of Hypersensitivity reaction: Type 1 - Immediate IgE-mediated, Type 2 – Cytotoxic or cytolitic, Type 3 - Immune complex-mediated, and Type 4 - Delayed cell-mediated. The first is an acute response that occurs immediately after exposure to an allergen. This phase can either subside or progress into a "late phase reaction" which can substantially prolong the symptoms of a response, and result in tissue damage.

The food allergens can provoke different type of allergic reactions but the classic immunoglobulin-E (IgE)-mediated food allergies are classified as type-I immediate Hypersensitivity reaction. These allergic reactions have an acute onset (from seconds to one hour) and may include: itching and burning in the mouth, throat, eyes and skin; anaphylaxis - nausea, vomiting, diarrhea, convulsions, stomach and abdominal pain; angioedema - soft tissue swelling, eyelids, face, lips, tongue. It is possible swelling of the larynx and trachea, leading to respiratory obstruction and serious difficulty in breathing or cooking. Most severe

form of allergic reaction is anaphylactic shock affecting the whole body, with the risk of death.

According to a study carried in the Clinic of Toxicology, Department for Adult, Emergency University Hospital "N.I.Pirogov" for a period of one year, the number of patients hospitalized in the Department due to adverse toxoallergic reactions / TAR's / were 749. The patients with adverse toxoallergic reactions, due to medicines were 230 – (30.70%), TAR's due to various protein foods were 519 (69.30%). The anaphylactic triggers, cause the life-threatening anaphylactic and anaphylactoid reactions - anaphylactic shock or variants of shock were the following: drugs – in 29 patients (53.7%), and foods, - in 25 patients (46.3%).

Food allergy is associated with pollinosis. Between 30% and 80% of patients with pollinosis have accompanying food allergy. 50% of patients with a hay fever have oral allergy syndrome - an allergic reaction to fruits and vegetables, occurring in direct contact with the oral mucosa in patients with pollen allergy.

Food allergy often precedes asthma and is a risk factor for its development. Patients with asthma and food allergy may be in greater risk of anaphylactic shock upon contact with the food to which they are allergic.

Key words: food allergy, allergens, hypersensitivity reaction, toxoallergic reactions

CO1. Хранителни алергени и хранителни алергии

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Резюме

Хранителната алергия е състояние на организма, при което той реагира негативно към определена храна, като в резултат се получава отговор на имунната система чрез генериране на IgE-специфични антитела към един или повече протеини или гликопротеини, съдържащи се в нея.

Всички хранителни протеини или гликопротеини независимо от произхода им, предизвикващи реакция на сенсибилизация и имунен отговор, се наричат хранителни алергени. Те са водоразтворими, термостабилни, трудно разграждащи се от киселини и протеази белтъци с молекулно тегло между 10 и 70 kDa.

В зависимост от имуногенността на организма, хранителните алергени могат да предизвикат както реакции от бърз тип (хуморално-антитяло-медиирани), така и забавени клетъчно-медиирани реакции, проявяващи се с различна симптоматика.

Хранителната алергия е свързана и с алергията към полени. Полинозата е предпоставка за развитие на хранителна алергия към плодове и заленчуци, поради наличието на кръстосано реагиращи IgE антитела към общи антигенни епитопи от поленовите алергени и алергените в плодовете и зеленчуците.

В голяма част от случаите хранителната алергия предхожда астмата и създава предпоставка за нейното развитие. В зависимост от чувствителността на организма

някои протеини в храните могат да предизвикат нежелани токсоалергични реакции (ТАР), които налагат спешна хоспитализация и специализиран подход на лечение.

Ключови думи: хранителна алергия, хранителни алергени, реакция на свръхчувствителност, токсоалергични реакции

Хранителна алергия и непоносимост към определени храни са различни понятия. Непоносимостта се изразява в невъзможност на организма да обработва правилно храната, поради недостига или липсата на някои храносмилателни ензими, инфекциозни или механични увреждания на чревната логавица,докато хранителната алергия е отговор на имунната система чрез генериране на специфични антитела към усвоена храна [23].

Свръхчувствителността на организма зависи от неговата генетична предиспозиция и характера на алергена и може да се протече по четири типа алергични реакции [8,16]: тип 1 - незабавни IgE-медиирани реакции, тип 2 - цитотоксични /цитолитични/ реакции, тип 3 - имуно-комплексни и тип 4 - забавени клетъчно-медиирани реакции, появяващи се от няколко часа до няколко дни след приемането на храната.

Реакциите от бърз тип (хуморални-антитяло-медиирани) се появяват от секунди до един час и се проявяват като: сърбеж и парене в устата, гърлото, очите и кожата; анафилаксия - гадене, повръщане, диария, стомашни конвулсии и коремна болка; ангиоедем - подуване на меките тъкани: клепачи, лице, устни, език. Възможно е подуване на ларинкса и трахеята, което да доведе до дихателна обструкция и сериозни затруднения в дишането, дори задушаване; анафилактичен шок - остра алергична реакция, засягаща цялото тяло, която може да доведе до смърт.

Основните фактори водещи до появата и развитието на свръхчувствителност към храна са някои заболявания на стомашно-чревния тракт, създаващи благоприятни възможности за преминаване на цели или не напълно разградени белтъчни молекули през мукозната тъкан [2,11], наличието на различни чревни паразити, които увреждат мукозата и действат чрез промяна на общата реактивност на организма и не на последно място недоразвитият напълно храносмилателен апарат в най-ранна детска възраст, даващ предпоставка за преминаване на цели белтъчни молекули и алергизация по ентерален път. Най-често в тази възраст се стига до сенсибилизация към кравето мляко, а това по-късно може да бъде причина за поява на алергични реакции и към други храни (9,21,25).

Алергията към мляко се подрежда на едно от първите места и се причинява от липса или дефицит на ензима лактаза, който разгражда лактозата до глюкоза и галактоза. Типичните симптоми са подуване, сърбеж, бронхоспазми, треска, падане на кръвното налягане, спазми и разтройство (15). Казеинът е един от основните протеини в млякото, които могат да предизвикат алергична реакция. Той е протеин общ за всички видове мляко и заема около 80% от протеиновото съдържание. В голям процент от случаите алергичните реакции могат да се дължат на *β-лактоглобулина* [27]. Той присъства с около 65% е протеин специфичен за млякото и месото на определен животински вид, а това създава предпоставка за проява на кръстосана сенсибилизация между тях. *α-лакталбуминът* заема около 25% и е протеин, специфичен за всеки отделен вид мляко, различен имунологично от серумния албумин, което изключва проявата на кръстосана сенсибилизация.

В млякото се съдържат също и говежди серумен албумин (8%), имуноглобулини, липаза, трансферази и др. В него са изолирани и антигени, които могат да се получат в стомаха по естествен начин след усвояването/разграждането му.

В киселото мляко протеините са превърнати в пептон и затова алергия към него се наблюдава рядко.

Алергията към млечните произведения - сирене, кашкавал, извара и др., се дължи както на протеините в млякото от което са произведени, така и на микроорганизмите добавени в някои видове, като *Penicillium roqueforti, Penicillium camemberti.*

Яйцата са изключително ценен източник на основните незаменими аминокиселини, кото: левцин, изолевцин, лизин, фенилаланин, треонин, тирозин, триптофан, хистидин, както и витамини: А, Д, Е, В1, В2 и др. В състава им влизат и редица полизахариди и липиди. Белтъчното съдържание в яйцата е около 13% от общата молекулна маса. Те са мощен алерген [9] и често са причина за тежки, стигащи до анафилактичен шок, алергични състояния [12] за възникването на които понякога е необходимо минимално количество от алергена. Може да съществува кръстосана сенсибилизация между яйцата от различни видове птици, но тя зависи главно от сенсибилизиращата молекула, която може да бъде специфична или неспецифична за вида. Сенсибилизация могат да предизвикат както белтъкът, така и жълтъкът, но белтъкът е основния причинител на алергични реакции [5,10].

Екстрактът от яйце съдържа няколко белтъчни фракции, като основната алергизираща съставка е овоалбуминът в белтъка - 60-70% от общата му протеинова маса и молекулно тегло 45k Da. Друга негова съставна част е коналбуминът - 13-16% с молекулно тегло 70 - 80 kDa. Тези два белтъка нямат общи антигени. В състава на яйчния белтък влизат също и глобулин и лизозим. Жълтъкът е изграден от три фракции, които могат да бъдат потенциални алергени, като вителинът (70%) има водеща роля, следван от леветинът (20-25%) и фосфитинът (9%).

Симптомите при сенсибилизация с яйца се проявяват като кожни реакции, бронхиална астма, стомашно-чревни смущения, мигрена, анафилактичен шок.

Рибата е източник намного белтъчини, но въпреки богатото си белтъчно съдържание, с ясно изразени сенсибилизиращи свойства е протеинова фракция с молекулно тегло 12 kDa, намираща се в мускулната тъкан. Сходството в белтъчното съдържание между отделните видове риба е около 35% и е предпоставка за кръстосана сенсибилизация в около 50% от случаите [26]. Алергията към риба се проявява като уртикария, екзема, бронхиална астма, анафилаксия [6].

От морските дарове: членестоноги - раци, скариди, омари и мекотели – миди, стриди, рапани, най-чести алергични реакции причиняват скаридите. Кръстосана реактивност се наблюдава при 40-50% [22].

Антигенните свойства на месото от бозайници се определят от белтъците, които участват в изграждането на мускулната тъкан и които се съдържат в кръвта. С най-силни сенсибилизиращи свойства е свинското месо. Алергия към птиче месо се среща сравнително по-рядко. Не съществува кръстосана сенсибилизация между различните видове птици, тъй като генетично те са твърде отдалечени един от друг. Антигенните свойства на продуктите от птици - месо и яйца, са различни и това обяснява по-рядката едновременна сенсибилизация към тях.

В химически чист вид мазнините не са антигени. Животинските мазнини са много по-слаби антигени в сравнение с животинските продукти, от които са получени. Мазнините от растителен произход - слънчоглед, царевица, маслини, фъстъци, лен, орехи, бадеми и др., са със слаби антигенни свойства.

При храните от растителен произход, най-често употребявана е пшеницата, тъй като влиза в състава на различни видове брашно. Съдържа белтъка глутен който обуславя алергизиращите ú свойства. Ръжта е с по-ниско съдържание на глутен и алергия към нея се среща много по-рядко. Царевицата също е с ниско съдържание на глутен, но в страните, където намира често приложение в храненето (САЩ, Румъния) причинява често алергични реакции. Ечемикът не съдържа глутен и се препоръчва като заместител на пшеницата при хора с доказана алергия към нея. Оризът също не съдържа глутен и алергия към него се проявява изключително рядко.

Хлябът и тестените произведения носят алергизиращите свойства на семената, от които са получени и на продуктите, които се добавят при тяхното производство: брашно, мляко, мая, яйца, подправки. Маята съдържа различни видове захаромицети, които в някои случаи са причина за алергията към хляба.

От семейство „бобови“ често срещана е алергията към фъстъци, особено в детската възраст [14,18,19]. Друг представител на това семейство е соята, която съдържа около 15 протеина, всеки един от които може да провокира алергични реакции. Доказано е, че в семената на боба, грахта, и лещата се съдържа белтъкът легумин, който е с изразени антигенни свойства. От друга страна обаче, всеки отделен вид съдържа и по няколко специфични за него белтъци. Подобно на бобовите растения, ядките - лешници, орехи, кашу, бадеми, шам-фъстък и др. могат да причинят алергични реакции, особено в детска възраст.

Кафето, чаят, както и различните видове подправки причиняват алергични реакции предимно от кожен тип.

Млечният шоколад може да причи алергични реакции дължащи се, както на протеините от млякото, така и на ядките включени в състава му. Чувствителността към какао се проявява с най-различни симптоми от страна на дихателната и храносмилателната система и на кожата. Не са наблюдавани обаче тежки анафилактични реакции.

Медът притежава алергизиращите качества на полена на растенията, от които произхожда, но алергията към него може да е свързана и с някои от алергизиращите съставки на пчелната отрова [13].

Всички плодове могат да предизвикат алергични реакции, като това зависи от генетичното предразположение на организма [19]. Най-често хиперсенсибилизация причиняват ягодите и малините. Алергичните реакции са предимно от кожен и гастроентерален тип.

Зеленчуците дават по-рядко алергични реакции. Симптомите засягат дихателната, храносмилателната система и кожата [19].

В последните години все по-често се наблюдават алергични прояви свързани с т. нар. хистаминови либератори (псевдо-алергени). Тези реакции не са свързани с имунната система. Провокират се от храни, съдържащи хистамин или водещи до освобождаването на хистамин по време на химични реакции в организма. Това са различни видове оцветители: Е100 – Е199, консерванти: Е200 – Е299, антиоксиданти и киселинни регулатори: Е300 – Е399, стабилизатори: Е400 – Е499, киселинни регулатори: Е500 – Е599, аромати: Е 600 – 699, подсладители: Е950 – Е969 и др. които присъстват в голяма част от хранителните продукти.

Хроничните алергични реакции, астмата и хранителната алергия са неопровержимо свързани, като хранителната алергия често предхожда астмата и е рисков фактор за нейното развитие [7,17]. При пациенти с хранителна алергия, астматичният пристъп е една от проявите на алергична реакция към определена храна [4,24]. Пациенти с астма и хранителна алергия са изложени на по-голям риск от анафилактичен шок при контакт с храната, към която са алергични. Парите при готвене

на риба, миди и яйца могат да бъдат причина за проява на алергични и астматични симптоми [3], а инхалирането на пшенично брашно може да причини професионално астматично заболяване при работещите в хлебната промишленост.

В голяма част от случаите хранителната алергия е свързана с алергията към полени (кръстосана реактивност). Доказано е че полинозата е отговорна за развитие на хранителна алергия към плодове и заленчуци. Между 30% и 80% от болните с полиноза, имат придружаваща хранителна алергия [18]. При сенната хрема 50% от болните имат орален алергичен синдром (ОАС) към плодове и зеленчуци. Оралният алергичен синдром е описан за пръв път от Амлот и сътрудниците му през 1987 г. [1] и представлява алергична реакция към плодове и зеленчуци от бърз тип, възникваща при пряк контакт с лигавицата на устата у болни с поленова сенсибилизация. Клиничната картина се проявява със сърбеж по лигавицата на устата, оток на устните, небцето. Симптомите се появяват от минути до един час след консумацията на храните. Причината е кръстосано реагиращи IgE антитела към общи антигенни епитопи от поленовите алергени и алергени в плодовете и зеленчуците. Алергиите към прашеца на брезата се придружава в 80% от хранителни алергии.

Крастосани алергични реакции дава брезовия прашец/полена, който провокира алергични прояви към ябълка, морков, целина, круша, домат, черупкови плодове. Прашецът на дивия пелин реагира кръстосано с моркови, целина, анасон, праскова. Прашецът на все по-широко разпространяващата се амброзия дава кръстосани реакции с пъпеш, краставица, банан, слънчоглед. Повечето хора с сенна хрема реагират кръстосано с две или повече храни.

Друг вид реакции свързани с чувствителността към протеините в храната са токсоалергичните реакции (ТАР).

В Клиниката по Токсикология, към Университетска многопрофилна болница за активно лечение и спешна медицина "Н. И. Пирогов", в отделение "Токсикология - възрастни" е извършено едногодишно епидемиологично, ретроспективно проучване на спешно хоспитализирани пациенти с нежелани токсоалергични реакции за периода от 1-ви януари до 31-ви декември 2012 година. Установено е, че от общия брой 749 хоспитализирани пациенти с ТАР, 519 (69,30%) са постъпили след прием на разнообразна белтъчна храна, останалите 230 (30,70%) са с медикаментозни ТАР. Анафилактични реакции причиняващи животозастрашаващ токсоалергичен шок в резултат от лекарствен прием са наблюдавани при 29 пациенти (53.7%), а в резултат от прием на храна - при 25 пациенти (46.3%).

Лечението на тези пациенти включва: спешна хоспитализация и наблюдение 2-5 дни; адреналин в дози съобразени с тежестта на клиничните прояви; интравенозно вливане на монозахариди и електролитни разтвори; антихистамини /проследяване на кръвното налягане! /; кортикостероиди; кислород; симптоматично лечение.

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CO2. Biological active components of ewe's milk and white brine cheese

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Abstract

Ewe's milk and dairy products are among the main sources of CLA. The essential fatty acids from groups of ω-3 and ω-6 are a vital component of human nutrition and animal health.

The purpose of this study was to provide a biologically and anticancerogenic components in ewe's milk and white brine cheese in three breeds of sheep.

The content of CLA in milk of three different breeds of ewe's reared on the natural pasture range from 2,38 to 3,77 g / 100g fat. A production of white brine cheese doesn't have significant changes on the amount of conjugated linoleic acid. Essential fatty acids in milk from different breeds of sheep are balanced and are maintained during processing technology of white brine cheese.

Key words: ewe's milk, white brine cheese, CLA, omega- 3, omega- 6

Introduction

Ewe's milk and dairy products [15] are among the main sources of CLA. The highest concentrations of CLA -derivatives were detected in the milk and meat in sheep. The content of CLA is very volatile. The concentration of conjugated fatty acids in the raw ewe's milk and dairy products vary depending on several factors: species, season and diet. Jahreis et al., [12,13] found in sheep milk reliably higher levels of CLA compared to other species. Kelly et al., [14], Parodi [22, 23, 24] and Lock et al., [18] found that inhibits CLA carcinogenesis in experimental animals [20] and may reduce the tumours, as well as is given as a cytostatic against existing tumour cells. The studies of Sugano et al., [26] showed that CLA inhibits atherosclerosis and has anti-inflammatory properties. Mihailova et al. [3, 4, 5] found that the concentration of CLA in ewe milk ranges between from 3 to 4 g / 100g milk fat. Sheep and goat milk is superior to cow's content on bioactive substances [2, 8].

Conjugated linoleic acid, a mixture of positional andgeometric isomers of linoleic acid (18:2 *n*–6; LA), is an intermediate in the ruminant biohydrogenation of LA to stearic acid [16]. Conjugated linoleic acid (CLA) has many beneficial effects, including decreased tumour growth in animal cancer models. The enrichment of milk and meat in ruminants CLA required to increase the synthesis of 18: 1 trans-11 in the rumen and increases the activity of the enzyme Δ9-desaturase in tissues [7].

In spite of the high SFA levels in milk fats, sheep milk plays an important role in the human nutrition as it is a source of biologically active substances– linolic acid, conjugated linolic acid (CLA), omega-3 and omega-6 fatty acids. Some of the CLA isomers (cis-9, trans-11 and trans-10, cis-12) are characterized by anticarcinogenic effect, hamper the process of becoming obese, the risk of diabetes, atherosclerosis and act as immunomodulators [25,19].

Ewe's milk is the main source of the conjugated linoleic acid (CLA) and its quantity varies depending on the breed, the season and the feeding regimen [1, 6].

The essential fatty acids from groups of ω-3 and ω-6 are a vital component of human nutrition and animal health. Established a significant imbalance between the two groups of fatty acids, wherein the level of ω-3 fatty acids is very low. Linoleic and linolenic acid are essential for humans, but not synthesized in the body's (essential fatty acids). A balanced intake of ω-6 and ω-3 fatty acids can only be achieved by pre-selection of food and control the composition of the incoming essential fatty acids in the body [17]. The main functions of ω-3 and ω-6 fatty acids are associated with the accumulation of energy in the cell, maintaining body temperature, preventing the skin from drying, reproduction of certain hormones needed for the cells to cellular biochemistry and metabolism of energy maintain cardiovascular and immune systems [9, 10].

The intake of food high content of saturated fatty acids leads to cardiovascular disease, which is one of the most common causes of mortality in Europe, so that interest in science are unsaturated fatty acids and dietary balance between omega-3 fatty acids, the main representative α-linolenic acid and omega-6 fatty acids, the main representative linoleic acid (C18: 2). A high ratio between the two groups of fatty acids is a prerequisite for coronary heart disease and the formation of blood clots leading to stroke, therefore it is advisable not to exceed the ratio of four units [11, 21, 27, 28] .

The purpose of this study was to provide a biologically and anticancerogenic components in ewe's milk and white brine cheese in three breeds of sheep.

Materials and methods

The milk were studied for bioactive components in milk fat from three sheep breeds in second lactation (n= 18)- Middle Rodopean Bread (MRB), Karakachan Bread (KB) and Rhodopean Tsigay Bread (RTB). White brined cheese was manufactured by milk from Middle Rodopean Bread, Karakachan Bread and Rhodopean Tcigay Bread (n = 6). The fatty acid composition of the milk was investigated during the indoor (January-March) and the pasture grass (April-June) rearing periods. The extraction of total lipids was carried out by the method of Roese- Gottlieb, by diethyl and petroleum ether and consequent methylation with the aid of sodium methylate (CH_3ONa, Merck, Darmstadt) and drying with $NaHSO_4.H_2O$. The fatty acids methyl esters /FAME/ was analysed with the aid of gas chromatograph Shimadzu-2010 (Kioto, Japan) equipped with flame-ionizing detector and automatic injection system (AOC-2010i). The analysis was carried out on a capillary column CP 7420 (100m x 0.25mm i.d., 0.2μm film, Varian Inc., Palo Alto, CA). As carrier gas was used hydrogen and as make-up gas- nitrogen. A regime of the furnace was programmed at four steps- the initial temperature of the column- 80ºC/ min., which is maintained for 15 min, after which is increased by 12 ºC/ min up to 170 ºC and is maintained for 20 min, follows new increase by 4ºC/min up to 186 ºC for 19 min and up to 220 ºC c by 4 ºC/min until the end of the process.

The data were processed by the method of variation statistics with the statistical package of EXCEL 2003 software. The validity of the differences between the studied groups was established by the Student's t-test.

Results and Discussion

The milks were studied from three breeds of sheep during the natural pasture period provide information for the synthesis of biologically active components that depend on breed differences. The CLA have a highest value in Karakachan breed - 3.77 and lowest in Middle Rodopean breed sheep - 2.38 g / 100g fat during the dairy period (Table 1).

Table 1. Biological active fatty acids (g/100g fat) in ewe's milk from three breed

FA	MRB	KB	RTB
ΣCLA	3.04±0.18	3.95±1.74	3.59±0.04
CLA	2.38±0.20	3.77±1.59	2.95±0.04
Σ n-3	1.73±0.01	2.03±0.37	1.83±0.00
Σ n-6	3.20±0.01	2.64±0.27	3.42±0.03
Σn-6/Σn-3	1.86±0.01	1.32±0.10	1.88±0.02
C-18:1c9/	15.85±0.11	18.10±5.75	15.10±0.38
C-18:1tr11	4.12±0.39	6.69±2.20	4.18±0.07

The established a low statistical reliability in the total content of CLA and CLA in milk between Middle Rodopean breed and Rhodopean Tsigay. The high content of omega- 3 fatty acids were found in Karakachan breed- 2.03 g / 100g fat, until omega- 6 is well secured a milk from Rhodopean Tsigay. The quantitative proportion between the two groups of essential fatty acids ranged from 1.32 in Karakachan breed to 1.88 in the Rhodopean Tsigay.

Table 2. Statistical reliability of the results for fatty acids in ewe's milk from three breed

FA	MRB	KB	RTB
ΣCLA		*	
CLA		*	
Σ n-3		**	
Σ n-6		*	*
Σn-6/Σn-3	**		**
C-18:1c9/			
C-18:1tr11			

*P<0.05, ** P<0.01, ***P<0.001

Statistical confidence in omega- 3 is found between breeds Middle Rodopean Breed and Rhodopean Tsigay and omega- 6 between Middle Rodopean Breed and Rhodopean Tsigay and between Karakachan Breed and Rhodopean Tsigay. Oleic acid (18: 1cis9) were synthesized in ewe's milk in the highest quantities in Karakachan breed- 18.10 g / 100g fat and lowest in the representatives of the Rhodopean Tsigay-15.10 g / 100g fat. There were not statistically significant differences between the three species in oleic acid and vaccenic acid. The vaccenic acid in the studied breeds was highest in Karakachan breed (6.69 g / 100g fat) and lowest in Middle Rodopean Breed of ewe's (4.12 g / 100g fat).

Production of the white brine cheese leads to minor changes in the fatty acid composition of fat, as is applied a low temperatures to obtain it and not suffer significant changes due to processes of oxidation and isomerization. CLA had the lowest concentration in white brined cheese from the milk of Middle Rodopean Breed- 2.94 g / 100g fat and highest in Karakachan breed- 2.93 g / 100g fat.

The omega- 3 fatty acids are lowest in Karakachan and highest in Middle Rodopean Breed of ewe's (Table 3), while the omega- 6 are in highest concentration in cheese of Karakachan Breed - 3.45 g / 100g fat and lowest from Middle Rodopean Breed - 3.23 g / 100g fat. The ratio between omega- 6 and omega- 3 fatty acids doesn't exceed two, and the lowest is in cheese from Middle Rodopean Breed - 1.73 and highest in cheese from Karakachan Breed - 1.89. Oleic acid in the analysed cheeses range from 14.83 to 14.87 g / 100g of fat, but due to technological process its content decreases as the loss in technological treatments range

from 1 to 3%. The vaccenic acid in white brined cheese have a lowest concentration from Middle Rodopean Breed- 3.88 g / 100g fat and highest in cheese from Karakachan breed 4.16 g / 100g fat.

Table 3. Biological active fatty acids (g/100g fat) in white brine cheese from three breed

FA	MRB	KB	RTB
ΣCLA	2.94±0.02	3.53±0.06	3.56±0.04
CLA	2.36±0.02	2.93±0.03	2.92±0.04
Σ n-3	1.87±0.02	1.80±0.01	1.83±0.02
Σ n-6	3.23±0.12	3.45±0.03	3.40±0.03
Σn-6/Σn-3	1.73±0.09	1.89±0.02	1.86±0.02
C-18:1c9/	14.87±0.35	14,86±0.26	14.83±0.38
C-18:1tr11	3.88±0.10	4.16±0.05	4.13±0.07

Conclusions

The investigation allows us to make the following conclusions:

The content of CLA in milk of three different breeds of ewe's reared on the natural pasture range from 2.38 to 3.77 g / 100g fat. A production of white brine cheese doesn't have significant changes on the amount of conjugated linoleic acid. Essential fatty acids in milk from different breeds of sheep are balanced and are maintained during processing technology of white brine cheese.

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CO3. The role of hepcidin in regulation of iron homeostasis

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Abstract

It is known fundamental importance of iron to humans for the synthesis of haemoglobin and normal erythropoiesis. Occurrence of iron-deficiency anaemia (IDA) is a widespread health problem, regarding not only developing countries, but also advanced nations. In the recent 10 years, there was a new insight in the iron homeostasis following the discovery of hormone hepcidin. This small peptide has a central role in regulation of iron homeostasis. Genetic defects associated with elevated hepcidin levels lead to development of IDA which is refractory to treatment with oral iron preparations.

Hepcidin is synthesized by the hepatocytes in response to changes in saturation of plasma transferrin with iron. At mollecular level, there is an interaction between hepcidin and ferroportin, a transmembrane exporter of iron from the cells into the blood. Formation of hepcidin-ferroportin complex is followed by internalization and lysosomal degradation of both proteins. Inactivation of ferroportin leads to diminished release of iron into plasma from the duodenal enterocytes, tissue macrophages in the process of iron recycling, and iron-storing hepatocytes.

Synthesis of hepcidin is up-regulated by increased plasma iron-transferrin levels, in iron overload, and inflammatory processes. Under conditions of inflammation, elevated hepcidin levels lead to decreased serum concentrations of iron and limited supply of iron for erythropoiesis – a state of iron-deficient erythropoiesis. The so-called functional iron deficiency develops – it is related to impaired mechanisms for iron mobilization in the presence of adequate iron stores. Prolonged suppression of iron absorption induced by increased hepcidin levels may lead to absolute iron deficiency with depleted iron stores.

Hepcidin synthesis is down-regulated in conditions of iron deficiency (ID), hypoxia, anaemia, and hyperactive or ineffective erythropoiesis, which allows increased iron absorption and enhanced release of iron from reticuloendothelial cells.

As it was recently described, transmembrane serine protease S6 (TMPRSS6), also known as matriptase-2, has an essential role for suppressing hepcidin production in ID. Mutations of gene TMPRSS6 encoding this enzyme with autosomal-recessive inheritance are

identified. Deficiency of the enzyme leads to elevated hepcidin levels and causes anaemia which is resistant to oral iron therapy – iron-refractory iron-deficiency anaemia (IRIDA).

Key words: *iron, hepcidin, iron-deficiency anaemia*

CO4. Advantages of the application of iron methionate compared to iron sulphate in fodders for broiler chickens

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Abstract

The test involved 55 broiler chickens aged 10 days, divided into 5 groups of 11 chickens, which were fed fodder supplemented with Bulgarian iron methionate (Fe methionate) administered or iron-sulphate (Fe-sulphate, heptahydrate) in doses of 60 ppm and 300 ppm. The tests continued 35 days. The basic mixed feed was prepared by using a recipe for growing broiler chickens and an average content of 85.6 ± 2.4 mg Fe/kg. The appetite, health status (clinical one) and individual weight of the chickens were controlled. On the 15th and 35 th days, samples of the liver from euthanized chickens from each group, were taken for histological and chemical studies. The liver samples intended for chemical analysis were frozen at-18oC and after 22 days were thawed out and tested for iron content by optical emission spectrophotometer ICP-OES 715-S. Samples of the cloacal content were taken and analysed for iron content by using atomic absorption spectrophotometer equipped with graphic cuvette, model Spectra AA 800. The statistical results were processed by parametric Anova one-way method. During the test period no clinical symptoms and signs of disease or mortality were found in all treated chickens; there were no pathomorphological changes in the organs of treated chickens and livers and spleens were Fe^{3+} negative, revealed by Perls histochemical assay. In general, the utilisation was more favourable for the iron methionate compared to the iron sulphate. It was better expressed in the low concentration (60 ppm) - a steady growth, trend for better deposition in the liver and significantly smaller amount (up to two times) of iron in the cloacal content (beneficial for the environment). The iron deposited in the liver of the treated chickens was from 40 to 60% more than that in the control ones. The LD_0 for Fe-methionate was estimated at 1500 mg/kg bw and for Fe-sulphate - 1000 mg/kg bw.; LD_{05} for Fe-methionate was 2000 mg/kg bw., while for Fe-sulphate it was 1500 mg/kg bw.

Key words: iron methionate, iron sulphate, chickens, utilisation

CO5. Хемолитична анемия

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CO6. In search of potentially new therapeutic application of coumarins beside as anticoagulants

Syiana Georgieva

Medical Faculty, Sofia University “St. Kliment Ohridski”

CO7. Национални особености при храненето и заболяванията (на примера на Китай)

П. Джуров

Медицински факултет, Медицински университет, София

CP1. Чили – За лютото с любов!

Елена Манлиева

Биологически факултет, СУ „Св. Климент Охридски”

CP2. Амброзия за здраве и дълголетие (Мед)

Елена Манлиева

Биологически факултет, СУ „Св. Климент Охридски”

CP3. Medical uses of plant extracts from Sideritis scardica

Angelina Bankovska

Medical Faculty, Sofia University “St. Kliment Ohridski”

CP4. Истината е във виното!?

Людмила Стоева, Надежда Йорданова

Биологически факултет, СУ „Св. Климент Охридски”

Session D.

Chairpersons:

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

Assoc. Prof. Anna Tolekova, MD, PhD
Faculty of Medicine, Trakya Universiyt, Stara Zagora

Secretary: Katerina Todorova, DVM
Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences

DO1. Angiotensin peptides - the new players in the team of rennin-angiotensin system

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Abstract
The renin-angiotensin system (RAS) is in the focus of many studies in the recent decades. It is no longer a mystery that the primary actions of RAS and its main effector - Angiotensin II (Ang II), are the maintenance of the hydro – electrolytic balance and blood pressure control. This conception of RAS has been significantly extended by recent scientific discoveries, which revealed the complexity of the system. Nowadays, RAS is already very far away from the idea of a simple, linear-cascade system. It was discovered that Ang II is not the only biologically active "player" of RAS. Ang II also functions as a precursor for other active fragments, represented by Angiotensin III (Ang III), Angiotensin IV (Ang IV), Angiotensin 1-7(Ang 1-7) and the newly described peptide Alamandine. All these angiotensins exert their own biological actions via classical receptors for Ang II(AT1 or AT2) or specific ones. Ang III has pressor, dipsogenic and vasopressin-releasing effects similar to Ang II, contributing to the regulation of cardiovascular homeostasis and drinking behavior. Ang IV is a vasodilator and is involved in the exploratory behavior, by stimulating the learning, memory and neuronal development (via AT4 receptors). Ang 1-7 produces blood pressure fall, diuresis and antiproliferative reactions through a specific receptor (Mas), unlike Ang II and Ang III, which act mainly via the AT1-receptors. This is why Ang 1-7 could be considered as a possible counter-regulatory factor for the vasoconstrictor effects of Ang II. The biological activities of the newly identified RAS hormone – Alamandine, resemble those of Ang 1–7, although each of these peptides acts through different receptors.
The identification of angiotensin peptides and their role might contribute to a new understanding of the RAS physiology and pathophysiology. It will also set a development of novel clinical opportunities by considering the application of various RAS-affecting drugs.

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DO2. Arginine-vasopressin – beyond the visible

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Abstract

Arginine Vasopressin (AVP) is a hormone secreted by the posterior pituitary gland. In the target cells AVP act via V1 and V2 receptors. The known effects of AVP are primarily associated with the maintenance of water balance in the body, cardiovascular homeostasis, affecting of social behavior and emotions. New studies indicate that AVP is synthesized in many other organs such as the ovaries, testes, uterus, thymus, pancreas, adrenals, etc. Newly discoveries associated AVP with effects on group inclusion, the preference for pair-bonding and parental behavior. Deviations of AVP levels are associated with aggression, obsessive-compulsive disorder and schizophrenia. AVP affects the consumption of food, alcohol and metabolism. The analogues and antagonists of AVP are with increasing clinical use in many cases. Analogues: Desmopressin is used in the treatment of diabetes insipidus; Terlipresin is used in treatment of bleeding esophageal varices, and septic shock. Antagonists: Nelivaptan, is used in the treatment of experimental models of psychiatric disorders; Atosiban, is used for the treatment of premature labor; Relkovaptan, relieves the symptoms of dysmenorrhea, affects the processes of tocolysis. Regarding the non-pregnant uterus AVP is much more potent agonist than oxytocin. Therefore the studies on AVP in recent years are directed towards applications in obstetrics.

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DO3. Moxa- prevention, healing and longevity, part of the alternative/complementary medicine

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Abstract
The moxa is main tool in moxibustion, which is one of the branches of Traditional Chinese Medicine. Moxibustion is preventive and healing method used in China since 3000 years. It is sad that regular applying lead people to longevity. This is method of burning of moxa- a downy substance obtained from the dried leaves of Artemisia vulgaris. This mixture is made like cone or cigar, which is burning on or close to the skin. The purpose is to warm, tonify and stimulate the channels, acupuncture points and organs, by absorbing the heat and smoke from moxa. The leaves contain viscous oil, the borneol is main ingredient with antiseptic and analgesic effect. Other volatile components such as tannin have astringent and hemostatic effect.

Goal
The purpose of this report is to examine and present a natural product- moxa, used as a method of healing in alternative/complementary medicine.

Methods and materials
Bibliographic study of scientific reports and literature materials from different countries.
Results and conclusions
Collected literature and researches, shows that the moxa has a wide range of therapeutic response and long-term impact. Burning moxa close to the skin results first in forming local hyperemia, sense of heat and strange filling called „da qi“. This therapy increase levels of Er, Lue, Hb, stimulate regenerative process of tissues, also and the immune system. Moxa is used more often for wind-cold syndromes, different pain syndromes like abdominal, menstrual, muscle-joints, also sexual weakness, infertility and bedwetting in children. Moxibustion with moxa is friendly, ease and pleasant kind of alternative/complementary method suitable for kids and adults, applying regularly and properly leads to health and longevity.

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DO4. Възможности на китайската медицина за лечение на депресия

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DO5. Туморсупресорни гени (p53, PTEN)

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DO6. Повратни точки в канцерогенезата

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DO7. Elizabeth Holmes – founder of “Theranos”

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Abstract

Theranos is a privately held health technology and medical laboratory services company based in Palo Alto, California that provides blood tests. The company's blood testing platform uses a few drops of blood obtained via a fingerstick rather than vials of blood obtained via traditional venipuncture, using microfluidics technology.

Elizabeth Holmes (born 1984) is an American chemical and electrical engineer and entrepreneur.

Theranos (a health technology and medical laboratory services company) was founded in 2003 by Elizabeth Holmes with the goal of streamlining and standardizing blood tests by creating a handheld device. The company's name is Theranos - an amalgam of "therapy" and "diagnosis". Holmes, then a sophomore majoring in chemical engineering at Stanford University, left the university early, at age 19, to start the company with a bridge loan from a venture capitalist. Before leaving Stanford, Holmes had founded a software company and

worked on a protein microarray for the detection of SARS in Singapore. Holmes' Stanford professor Channing Robertson encouraged her to start the company and was a director.

The company has been secretive about its plans and operations in order to maintain confidentiality. An exclusive interview with Holmes in the Wall Street Journal in September 2013 marked a shift to going more public.

Theranos uses a fingerstick to draw a micro sample of blood into a cartridge, which is loaded into a local reader for analysis. The results are sent wirelessly from the reader to a secure database. The company says that the results are received faster than the usual three-day delay for centralised laboratory testing, up to 30 blood tests can be performed on a single blood sample, and its method ensures accuracy by eliminating or reducing most human handling and delays associated with traditional blood tests. Theranos initially targeted its blood testing services at clinical trials for new drugs, because frequent testing can provide early indication of whether a drug therapy is working or causing adverse reactions.

Theranos began to offer services directly to consumers via Theranos Wellness Centers located inside Walgreens stores in 2013. Theranos offers blood testing services in pharmacies in California and Arizona. Theranos' blood tests mainly cost under $10, e.g. blood cholesterol at $2.99; the company claims low-cost blood tests could save US Medicare and Medicaid around $200 billion a decade. Theranos holds more than 10 patents, including patents on wearable blood monitors and influenza virus detection.

By 2014, the company offered 200 tests and was licensed to run in every state of the US. It had 500 employees and was valued at more than $9 billion. Holmes retained control of more than 50% of the company's equity.

As of 2014, Holmes has 18 US patents and 66 non-US patents in her name and is listed as a co-inventor on over a hundred patent applications. Holmes is the youngest self-made woman billionaire on the Forbes 400 list, where she is №111; her net worth is an estimated $4.5 billion.

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Friday, 28 November 2013

Session E.

Chairpersons:

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

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

Secretary: Abdulkadir Abudalleh, MSc, PhD
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EO1. Hallucinogenic plants poisoning – case series

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Abstract
Objective: To analyze a case series of hallucinogenic plants poisoning in patients hospitalized in Toxicology Clinic, Emergency University Hospital "N.I.Pirogov. Datura stramonium and Atropa belladonna are hallucinogenic plants that cause serious poisoning. The foliage and berries are extremely toxic, containing tropane alkaloids - atropine, scopolamine and hyoscyamine. *Case series:* We report 4 cases of acute poisoning in young men 18-20 years old, due to drinking tea, made from Datura stramonium for recreational use. A 52-year-old man had ingested 5-6 "blueberries" found in the forest. Based on the patient's description and clinical presentation, the "blueberries" were believed to be Atropa belladonna. All 5 patients were hospitalized in the Clinic and presented with a typical anticholinergic syndrome: agitation, confusion, combative behaviour; all of them had mydriasis, dry mouth and tachycardia. The patients presented with visual hallucinations, disorientation, incomprehensible and nonsensical speech. Hallucinations and symptoms resolved within 36-48 hours after hospitalization. The patients were favourably managed with symptomatic treatment and gastrointestinal decontamination with activated charcoal. *Conclusion:* Ingestion of the hallucinogenic plants can result in serious intoxication, requiring hospitalization. It is important to educate health care providers about the hazards and symptoms cause a these plants.

Key words: hallucinogenic plants, anticholinergic syndrome, Datura stramonium, Atropa belladonna

Цел: Да се анализират серия случаи на халюциногенни растения отравяне при пациенти, хоспитализирани в Клиниката по токсикология, Болница Спешна

университет "Н.И.Пирогов. Datura татул и Atropa беладона са халюциногенни растения, които да причинят сериозно отравяне. Листата и плодовете са изключително токсични, съдържащ тропанови алкалоиди - атропин, скополамин и Хиосциамин. Case серия: Ние докладва четири случаи на остро отравяне при млади мъже 18-20 години, в резултат на пиене на чай, изработени от Datura татул за отдих употреба. А 52-годишният мъж е погълнат 5.6 "боровинки", намерени в гората. Въз основа на описанието на пациента и клиничното представяне, "боровинки" се смята, че са Atropa беладона. Всички 5 пациенти са били хоспитализирани в Клиниката и представени с типичен антихолинергичен синдром: възбуда, объркване, войнствено поведение; всички от тях са мидриаза, сухота в устата и тахикардия. Пациентите, представени със зрителни халюцинации, дезориентация, неразбираем и безсмислен реч. Халюцинации и симптомите преминават в рамките на 36-48 часа след хоспитализацията. Пациентите са благоприятно управлявани със симптоматично лечение и стомашно деконтаминация с активен въглен. Заключение: При поглъщане на халюциногенни растения може да доведе до сериозна интоксикация, изискващи хоспитализация. Важно е да се образоват доставчиците на здравни грижи за опасностите и симптоми предизвика тези растения.

EO2. Treatment for Cyanide Poisoning – Classical and Developing Methods

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Abstract

Cyanide poisoning poses a great threat in contemporary industrialized countries. Numerous sources of potentially dangerous cyanide compounds exist: among them industrial and home fires, terroristic attacks and hazardous waste plants leakages. In the 'light' of these constant threats, the problem of cyanide poisoning treatment is highly pressing.

Treatment for cyanide poisoning exists. A classical approach is the administration of sodium nitrite in combination with thiosulfate, although this treatment is highly controversial because of the serious side effects and the slow action. Cyanide poisoning is always an emergency case in clinical practice worldwide. Cyanide compounds are a powerful and extremely fast-acting poison, which stops cellular respiration. Hence, medical workers need an efficacious antidote with the least possible side effects.

Such antidote may be hydroxocobalamine or vitamin B12, which has a great affinity to bind cyanide ions and is relatively harmless in the usual antidotal concentrations. This method has been widely used for many years now and has shown satisfactory results in the long-term period.

However, other antidotal substances have recently been developed such as 4-dimethylaminophenol, 3-mercaptopyruvate prodrugs, dicobalt edetate and vitamin B12 synthetic analogues. It is of interest whether any of these may be a better choice for treating cyanide poisoning than the classical approach. Here, we present a study for the combined use of cobinamide and sulfanegen in cyanide poisoning research with mice.

Cobinamide and sulfanegen are two recently developed antidotes for cyanide poisoning. The first is an analogue to vitamin B12 and the second is a 3-mercaptopyruvate prodrug. In this presentation we outline the detrimental physiological action of cyanide compounds, then we briefly consider the mechanisms of action of the various existing

antidotes and we finally focus on the combined use of cobinamide and sulfanegen as treatment agents.

Although there are several methods for cyanide poisoning treatment, they are not irreproachable. Future research may be directed to the combined usage of two or more alternative methods for the accomplishment of better and faster treating.

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EO3. Low salt diet is associated with the prevention of exacerbation in patients with relapsing remitting multiple sclerosis

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In September at "MS Boston 2014", the 2014 Joint Americas and European Committees for Treatment and Research in Multiple Sclerosis (ACTRIMS/ECTRIMS) meeting, there were few publications about "Sodium intake and correlation with increased disease activity in multiple sclerosis (MS) and in other autoimmune diseases".

Humans with MS having higher sodium intake showed an approximately 3 times higher risk for exacerbations in symptoms as well as disease activity, compared to those with lower sodium intake, a new study showed [3]. The Argentinean authors were cautious about drawing conclusions, however, with various limitations, the study falls short of proving a causal relationship. "This small observational study suggests that there may be a positive correlation between sodium intake and MS disease activity," lead author Mauricio Farez, MD, from the Raúl Carrea Institute for Neurological Research, Buenos Aires, Argentina, told *Medscape Medical News* [1].

"However, we are just starting to address the effect of sodium intake in MS patients, and further and larger studies are required to establish whether sodium restriction is a potential add-on therapy to MS patients." [1].

There has been a marked increase in the incidence of autoimmune diseases in the past half-a-century. Although the underlying genetic basis of this class of diseases has recently been elucidated, implicating predominantly immune-response genes, changes in environmental factors must ultimately be driving this increase [2].

Recently, salt has been shown to modulate the differentiation of human and mouse Th17 cells. Mice that were fed on a high-sodium diet were described to develop more aggressive courses of experimental autoimmune encephalomyelitis. However, the role of sodium intake in multiple sclerosis (MS) has not been addressed. We aimed to investigate the relationship between salt consumption and clinical and radiological disease activity in MS [1].

The study involved 2 separate groups of patients with MS. One group included 70 patients with frequently relapsing MS were followed longitudinally for 2 years. The patients' sodium intake was estimated from urine and serum samples taken on 3 separate occasions over 9 months, and regression analysis was used to determine the effect of the intake on their disease activity [3].

Fares et al. conducted an observational study in which sodium intake was estimated from sodium excretion in urine samples from a cohort of 70 relapsing-remitting patients with MS who were followed for 2 years. The effect of sodium intake in MS disease activity was estimated using regression analysis. The findings were then replicated in a separate group of 52 patients with MS [1].

TH17 cells (interleukin-17 (IL-17)-producing helper T cells) are highly proinflammatory cells, critical for clearing extracellular pathogens and for inducing multiple autoimmune diseases. IL-23 has a critical role in stabilizing and reinforcing the TH17 phenotype by increasing expression of IL-23 receptor (IL-23R) and endowing TH17 cells with pathogenic effector functions. However, the precise molecular mechanism by which IL-23 sustains the TH17 response and induces pathogenic effector functions has not been elucidated. Transcriptional profiling was used to construct a model of developing TH17 cells of their signalling network and nominate major nodes that regulate TH17 development. The authors identified serum glucocorticoid kinase 1 (SGK1), a serine/threonine kinase, as an essential node downstream of IL-23 signalling. SGK1 is critical for regulating IL-23R expression and stabilizing the TH17 cell phenotype by deactivation of mouse Foxo1, a direct repressor of IL-23R expression. SGK1 has been shown to govern $Na^{(+)}$ transport and salt (NaCl) homeostasis in other cells. It was shown that a modest increase in salt concentration induces SGK1 expression, promotes IL-23R expression and enhances TH17 cell differentiation in vitro and in vivo, accelerating the development of autoimmunity. Loss of SGK1 abrogated $Na^{(+)}$-mediated TH17 differentiation in an IL-23-dependent manner.

These data demonstrate that SGK1 has a critical role in the induction of pathogenic TH17 cells and provide a molecular insight into a mechanism by which an environmental factor such as a high salt diet triggers TH17 development and promotes tissue inflammation [4].

The newly identified population of interleukin (IL)-17-producing CD4 (+) helper T cells (TH17 cells) has a pivotal role in autoimmune diseases. Pathogenic IL-23-dependent TH17 cells have been shown to be critical for the development of experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis, and genetic risk factors associated with multiple sclerosis are related to the IL-23-TH17 pathway. However, little is known about the environmental factors that directly influence TH17 cells. Increased salt (sodium chloride, NaCl) concentrations found locally under physiological conditions *in vivo* markedly boost the induction of murine and human TH17 cells. High-salt conditions activate the p38/MAPK pathway involving nuclear factor of activated T cells 5 (NFAT5; also called TONEBP) and serum/glucocorticoid-regulated kinase 1 (SGK1) during cytokine-induced TH17 polarization. Gene silencing or chemical inhibition of p38/MAPK, NFAT5 or SGK1 abrogates the high-salt-induced TH17 cell development [2].

High-salt diet induces Th17 cells *in vivo* and exacerbates experimental autoimmune encephalomyelitis**;** Naïve murine CD4 cells were stimulated with radiated APC, anti-CD3, IL-6 and TGF-β1 in the presence (NaCl) or absence of additional 40mM NaCl and were

analysed by FACS (n=3); IL-17A secretion (ELISA) of primary splenocytes, stimulated by anti-CD3 in the presence or absence of NaCl (n=6); Mean clinical scores of EAE in HSD animals or controls; Spinal cord from EAE animals was analysed by qRT-PCR (n=5–6); Splenocytes from EAE animals were analysed by qRT-PCR (n=4–7); Splenocytes from EAE animals were re-stimulated with MOG for 2 days and supernatants were analysed for IL-17A and IFN-γ by ELISA (n=7–8) or cells were analysed for IL-17A by FACS (n=4). qRT–PCR data are depicted as relative expression [2].

The autors found a positive correlation between exacerbation rates and sodium intake in a multivariate model adjusted for age, gender, disease duration, smoking status, vitamin D levels, body mass index and treatment. They found an exacerbation rate that was 2.75-fold (95% CI 1.3 to 5.8) or 3.95-fold (95% CI 1.4 to 11.2) higher in patients with medium or high sodium intakes compared to the low-intake group. Additionally, individuals with high-sodium intake had a 3.4-fold greater chance of developing a new lesion on the MRI and on average had eight more T2 lesions on MRI. A similar relationship was found in the independent replication group [1].

The TH17 cells generated under high-salt conditions display a highly pathogenic and stable phenotype characterized by the upregulation of the pro-inflammatory cytokines GM-CSF, TNF-α and IL-2.

Moreover, mice fed with a high-salt diet develop a more severe form of EAE, in line with augmented central nervous system infiltrating and peripherally induced antigen-specific TH17 cells. Thus, increased dietary salt intake might represent an environmental risk factor for the development of autoimmune diseases through the induction of pathogenic TH17 cells [2].

These results suggest that a higher sodium intake is associated with increased clinical and radiological disease activity in patients with MS.

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EO4. Болест на Паркинсон и ролята на допамина в нейната етиология

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EP1. The potential role of aluminium in Alzheimer's disease

Ivaylo Dankov

Faculty of Medicine, Sofia University "St. Kl. Ohridski"

EO5. Биохимични механизми на пристрастяването

Мария Валериева Младенова

Медицински факултет, СУ „Св. Климент Охридски”

EO6. Горенето на живота в пламъка на цигарата

Симона Красимирова Такова

Медицински факултет, СУ „Св. Климент Охридски”

EO7. Серотонинът – хормонът на щастието и болката

Милен Лазов

Медицински факултет, СУ „Св. Климент Охридски”

EP2. Vanadium and nervous system

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EP3. Базални ганглии

Елена Младенова, Ангелина Пискова, Деяна Жекова

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EP4. Остър хирургичен корем

Деяна Жекова, Ангелина Пискова, Елена Младенова

Медицински факултет, СУ „Св. Климент Охридски”

Session F.

Chairpersons:

Prof. Reni Kalfin, MSc, PhD
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Assoc. Prof. Diana Rabadzhieva, MSc, PhD
Institute of General and Inorganic Chemistry, Bulgarian Academy of Sciences

Secretary: Boyka Andonova-Lilova, MSc
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FO1. Calcium - a vital chemical element for the human body

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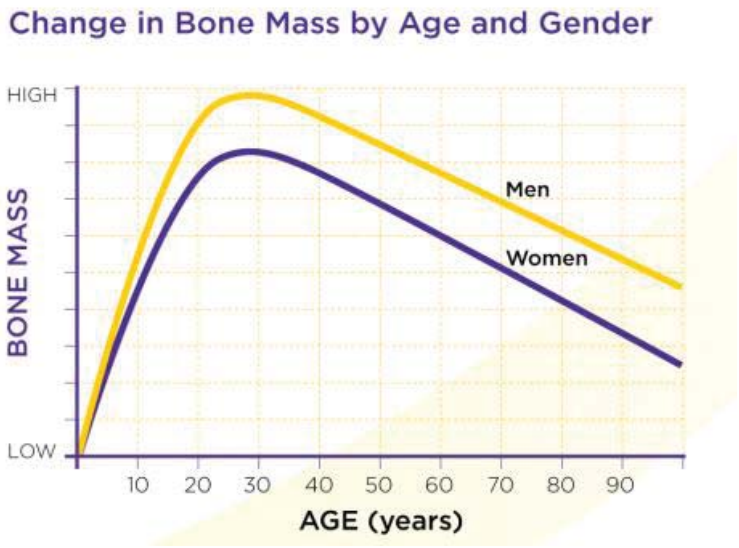
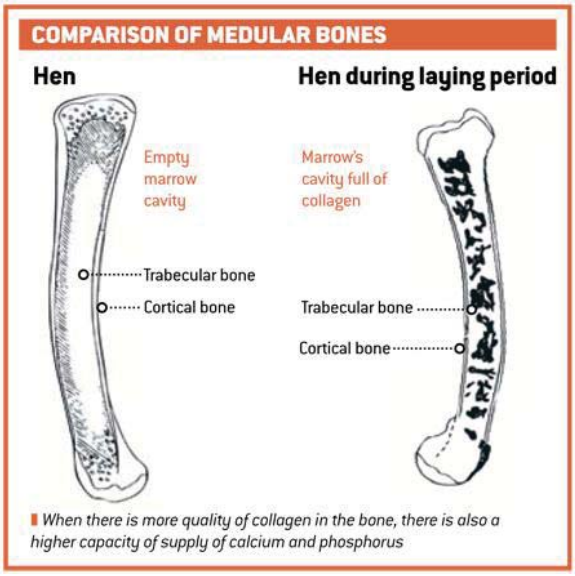
Calcium (Ca) is a chemical element which is typical with its metallic characteristics and soft gray colour. It is widely found in the nature and also in the human body. In the last one there is a plenty of Ca ions which have vital functions for the development and the optimal states of existence for the living organisms. Building of the hardest structures of the body – the bones, important element of the hormones, regulation of normal conditions, contraction of muscle tissue, stabilizing the blood pressure – these are only a few components of the enormously wide possibilities and importance of Ca^{2+}.

Resorption of old bone and formation of new bone are processes that continuously overlap. The importance of these processes varies at different times throughout the life cycle. In general, from birth until about age 20, the bones are in a phase of active growth. This stage is characterized by an increase in bone length and bone width. Shaping of the growing bones, called modeling, also occurs at this time. Between the ages of 12 and 30, the rapid phase of bone dimensional growth tapers off and consolidation occurs with the attainment of peak bone mass. Although dimensional bone growth ceases at maturity, adult bone is constantly being remodeled. It is generally accepted that peak bone mass or maximum bone density and strength occurs by age 30. Studies indicate that peak bone mass at several skeletal sites (especially the proximal femur and vertebrae) may be reached as early as late adolescence. Peak rate of calcium accretion occurs at about age 12.5 years for girls and 14 years for boys. Beginning in the 40s or later, resorption of existing bone starts to exceed formation of new bone, resulting in a net loss. Age-related bone loss is influenced by both genetic and environmental factors. This also occurs at different times in the two different types of bone — trabecular and cortical. Trabecular bone, which is spongy in appearance, forms the internal support network for the cortical shell, vertebrae, and other bones. Cortical or compact bone forms the outer shell of almost all bones and is predominant in shafts of long bones such as those in the arms, legs, hands, and feet.

Overview

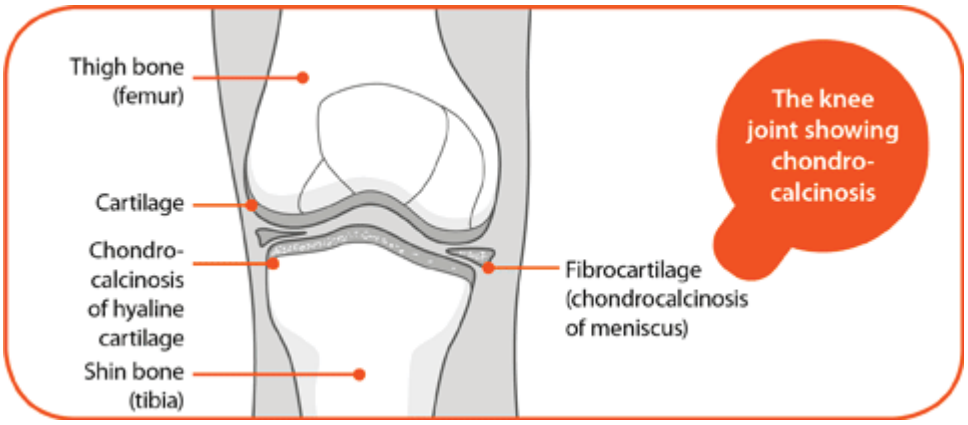
Teeth, like bones, are calcified tissues. The teeth begin to form in the first few months of fetal life and the mineralization process continues into late adolescence. An adequate intake of calcium, in addition to phosphorus, protein, fluoride, and vitamins A, C, and D, is needed for proper development of tooth structures. Calcium and calcium-rich dairy products such as milk and cheese may help protect against dental caries and periodontal diseases. Although the mature tooth is metabolically active, the fully eruptive adult tooth is not significantly subject to resorption. Unlike bone, teeth do not readily release their calcium when the body's needs for this mineral are not met by the diet. Outside of bones and teeth, the level of ionized calcium in the blood must be maintained within a narrow range to perform calcium's regulatory functions . When the diet is low in calcium, the bones release enough calcium into the bloodstream to meet the body's needs.





Although the amount of calcium outside bones and teeth is relatively small, it is required for a number of basic regulatory functions including contraction and relaxation of muscle, coagulation of blood, transmission of nerve impulses, activation of enzyme reactions, stimulation of hormone secretions, integrity of intracellular cement substances.

Bones serve as a basic support system protecting vital organs and as a reservoir for calcium — the most abundant mineral in the body. In fact, 99% of the body's calcium is found in bones and teeth (the other 1% is found in cells, blood, and other body fluids).



Despite its static appearance, bone is constantly being formed and broken down. This process, called remodeling, is the resorption (breaking down) of existing bone and deposition of new bone to replace that which has been broken down. At any one time, about 5% of bone surfaces in adults are undergoing remodeling.

Major functions of calcium in the body

The body regulates blood calcium in three ways. First, the kidneys can decrease urinary calcium excretion. Second, the body can adjust the efficiency of dietary calcium absorption. Third, the body can withdraw calcium from bones. The body activates these processes by three main hormones:

Parathyroid Hormone (PTH)

PTH is the primary regulator of blood calcium levels, responding rapidly to minor changes in calcium levels. When calcium levels drop below normal range (hypocalcemia), PTH is released to decrease urinary calcium excretion and mobilize calcium from bone.

Calcitriol

This hormonal, functionally active form of vitamin D is produced by the kidneys in response to stimulation by the parathyroid hormone. Calcitriol (1,25(OH)2 D) increases calcium absorption from the intestine and limits calcium excretion when blood calcium levels are low. It also affects bone directly, by mobilizing the deposition of calcium and phosphorus.

Calcitonin

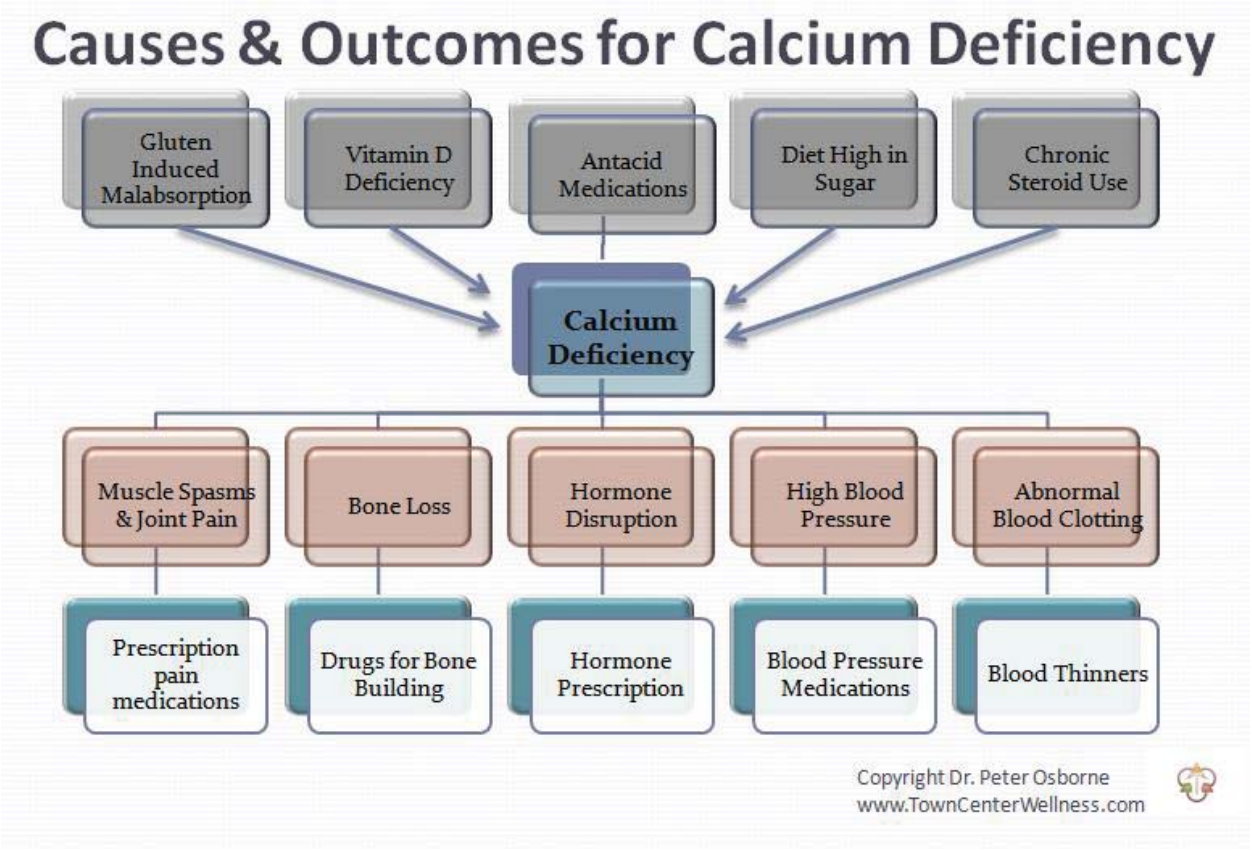
An increase in blood calcium stimulates calcitonin, a hormone produced by cells in the thyroid gland. This hormone lowers blood calcium by inhibiting bone resorption. Calcitonin's physiological function as a regulator of blood calcium levels is relatively minor compared to the actions of PTH and calcitriol.

Pregnancy

A baby begins to accumulate calcium in bone during the third trimester of pregnancy. Accumulation of calcium in bone continues until its peak in early adulthood. Then the amount of bone, as well as the calcium level in bone, begins its gradual decline at the rate of 1 percent per year.

Even though only 1 percent of the calcium in the body is found outside of bone, this form of calcium is critical for many functions in the body. Therefore, its level is maintained in a narrow range in the blood and tissues. Consider some of the key non-bone functions of

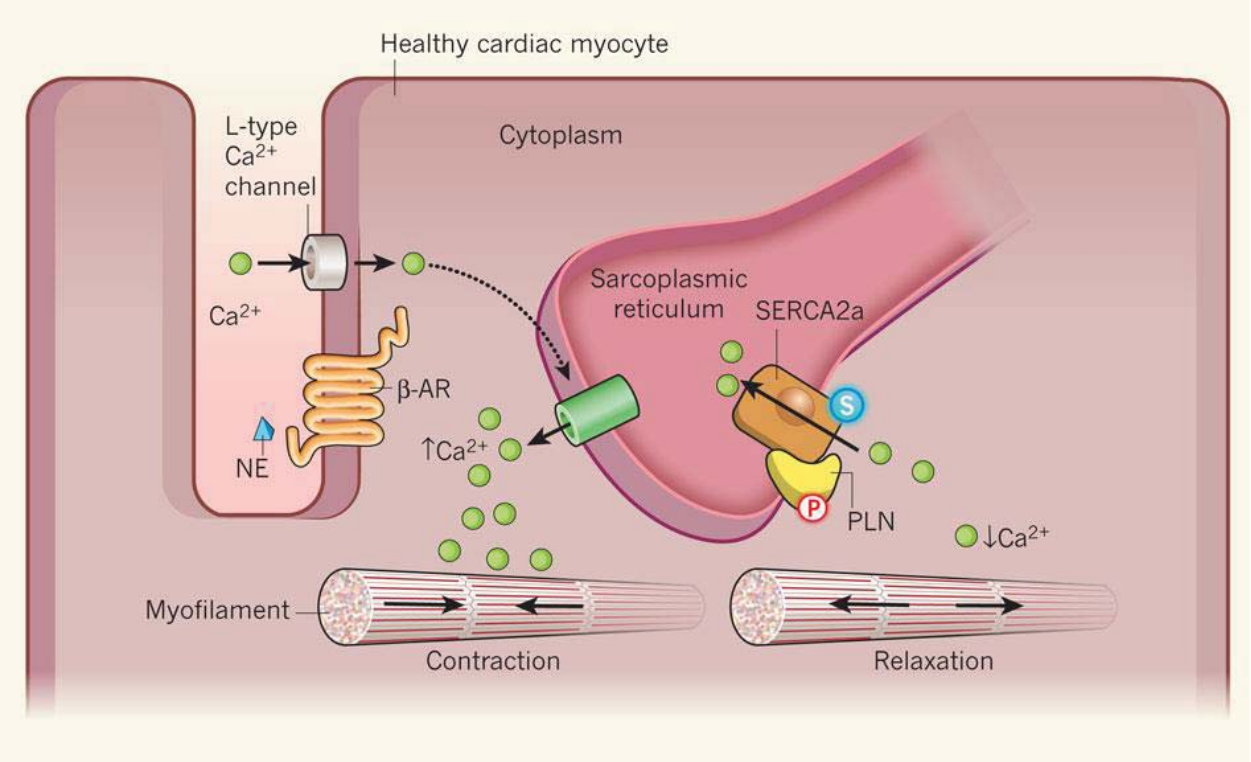
calcium:



- It's essential for blood clotting.
- It stabilizes blood pressure.
- It contributes to normal brain function.
- It's critical for communicating essential information among cells.

Normally, the amount of calcium inside a cell is very low relative to the amount that's in your blood. Cells let calcium inside in response to a large number of chemicals, such as hormones. This chemical stimulus of calcium rushing into a cell makes them perform all sorts of critical functions. For example, it helps insulin open cells to glucose, it is needed for the release of chemicals that transmit a signal from a nerve cell to a target cell, facilitates the actual process of contraction of the muscle cell, assists the movement of sperm into an egg to fertilize the egg.

Mechanism by heartcells - summerized



The signalling function of Ca2+ demands a very low ionic concentration of the cation within heartcells. This is achieved by two mechanisms, the reversible complexation of calcium by non membranous (protein) ligands, and its binding and transport by transmembrane proteins. The second mechanism is more efficient, since the complexation by soluble proteins is limited by their amount in heart sarcoplasm or within heart organelles, whereas membrane proteins can regulate calcium efficiently, even if present in low amounts, if they 'return' rapidly in the uncomplexed form after each binding and transport cycle. There are four basic transport modes: ATPases, Na+/Ca2+ exchangers, channels, electrophoretic uniporters. They have either low or high calcium affinity, thus serving different purposes in the various phases of the functional cycle of heart cells. On an integrated level, sarcoplasmic reticulum can be considered as the organelle presiding over the rapid and fine regulation of Ca2+ linked to the contraction/relaxation cycle. Sarcolemma regulates Ca2+ with both low and high affinity, but handles a quantitatively minor amount of Ca2+ (trigger Ca2+. Mitochondria are low-affinity organelles, whose primary role probably is the regulation of Ca2+ in their matrix, rather than in the sarcoplasm.

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FO2. CALCIUM PHOSPHATE CEMENTS DERIVED FROM ANHYDROUS DICALCIUM PHOSPHATE AND TETRACALCIUM PHOSPHATE

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Abstract
Formation of calcium phosphate cements starting from DCPA (dicalcium phosphate anhydrous) and TTCP (tetracalcium phosphate) powders was designed by thermodynamic modeling. The theoretical results were confirmed by experimental studies. The starting calcium phosphate powders were obtained from commercial DCPD by thermal dehydration (for DCPA) and high-temperature reaction with $CaCO_3$ (for TTCP) followed by mechanical milling. The reacting liquid phases used in the experiments were solutions of acids (acetic, α-amino acetic, lactic, tartaric, citric, ascorbic or salicylic acid) and K_2HPO_4 applied in different concentrations. Modifiers as xanthan gum and glycerin were used to improve the cement manipulation. The cement samples were characterized as regards phase composition (XRD), manipulation features (initial and final setting times), and influence on the micro environment (*in vitro* studies). The best results were found using not concentrated (30-40%) lactic, citric and acetic acids. *In vitro* testing of the best cement samples in SBF showed that they are prospective as biomaterials for bone defects filling.

Introduction
Calcium phosphate cements (CPC) are considered as a second generation of bioactive materials for bone regeneration. They are prepared from one or several mixed calcium phosphate powders [1-3] upon interaction with various liquid phases – water or aqueous solutions of different substances [4-7]. Upon mixing of the solid and liquid phases, reactions of dissolution/crystallization or hydrolysis take place on the grain boundaries as a result of which less soluble calcium orthophosphates are obtained. The main advantages of calcium phosphate cements are: ability for *in vivo* self-setting; good suitability for injection application using non-invasive surgery; good osteoconductive and partly osteoinductive properties; good processing; excellent biocompatibility and bioactivity; non-toxicity; low price. Their drawbacks are: low mechanical strength; solubility in body fluids; not well-defined micro- and macroporosity necessary for the formation of interconnected pores [8].

The aim of this study was to design, by thermodynamic modeling, DCPA and TTCP derived calcium phosphate cements and on this theoretical base to perform experimental studies on the preparation of calcium phosphate cements with good manipulation characteristics and micro environmental behavior.

Thermodynamic simulations
The ion-association model, computer program PHREEQCI v.2.14 [9], was used to simulate the solubility/crystallization processes in the systems TTCP-DCPA-H_2O, TTCP-DCPA-K_2HPO_4-H_2O and TTCP-DCPA-citric acid-H_2O. The solid/liquid ratios and the solutions concentrations were varied. The saturation indices (SI) were calculated for all solid phases

which can be formed under the experimental conditions and their values were used as an indicator for predicting phase transformations in stable and metastable systems.

Materials and experimental methods

Materials

Commercial DCPD and $CaCO_3$ (Sigma Aldrich, for analysis) were used for the preparation of the starting DCPA and TTCP, respectively. Aqueous solutions of K_2HPO_4, acetic, α-amino acetic, tartaric, lactic, citric, salicylic and ascorbic acids in different concentrations were used as starting liquid phases (Table 1). Merck reagents with an analytical grade quality were used. Xanthan gum and glycerin were used as additives in the systems.

Experimental methods

Preparation of starting powders

DCPA was prepared from DCPD by thermal dehydration at 200°C; TTCP was prepared by sintering an equimolar mixture of DCPA and $CaCO_3$ at 1500°C for 5 h. The solid phases thus prepared were ball milled for 5 h.

Preparation of cements

Equimolar mixtures of DCPA and TTCP powders with particle size less than 28 μm and activated surfaces (by milling) were subjected to prolonged mixing with the liquid phases at a solid/liquid ratio of 2.6 g/ml to form a plastic mass. The latter was molded in rubber-molds with diameter of 10 mm and height of 5 mm and dried in air for 24 h.

Characterization of cements

Phase characterization - X-ray diffraction was applied for phase registration of the dried cements. A Bruker D8 advance XRD apparatus was used. It was operating at 40 kV and 40 mA with CuKa radiation and SolX detector within the 2θ range of 10–90°2θ with a step of 0.04°2θ and counting time of 1 s/step.

Mechanical characterization - the initial and final setting times of the cement samples prepared in the rubber-molds were determined by the Vicat needle method [10]. Formation characteristics, as well as final mechanical properties of the cements were visually checked.

Micro environmental behavior - in vitro testing of the air-dried cement samples in simulated body fluid (SBF) was performed. Conventional simulated body fluid (SBFc, pH 7.2-7.4) was prepared according to Kokubo [11]. Cement samples air-dried for 24 h were taken out from the rubber-molds and were immersed in a solution at a solid/liquid ratio of 1:20. The experiments were conducted at room temperature under static conditions. The pH values of the liquid phases were measured permanently to check the cement influence on them.

Results and Discussion

The stable and metastable equilibria in the systems TTCP-DCPA-H_2O, TTCP-DCPA-K_2HPO_4-H_2O and TTCP-DCPA-citric acid-H_2O were thermodynamically modeled with a view to predict the behavior of the calcium phosphate cements under study. K_2HPO_4 was used to simulate the behavior of the cement systems in the presence of a salt with a common ion (PO_4^{3-}) in the solution. Citric acid was used as a model organic acid. It was chosen because of the availability of thermodynamic data for citrate complexes, including calcium citrate tetrahydrate as a solid phase.

The results showed that the thermodynamic equilibria did not depend on the solid/liquid ratio or the concentration of K_2HPO_4 or citric acid. In all systems DCPA and TTCP dissolved (SI<0) and transformed into HA, OCP, ACP, or CaCitr (calcium citrate tetrahydrate) (Fig.1). HA was the only crystallizing phase in all stable inorganic systems (Fig. 1a, 1b). OCP and/or ACP crystallized in all metastable systems. HA, CaCitr and DCPA in

various ratios crystallized in the stable DCPA – TTCP - citric acid - H_2O system (Fig.1c) depending on the Ca concentration.

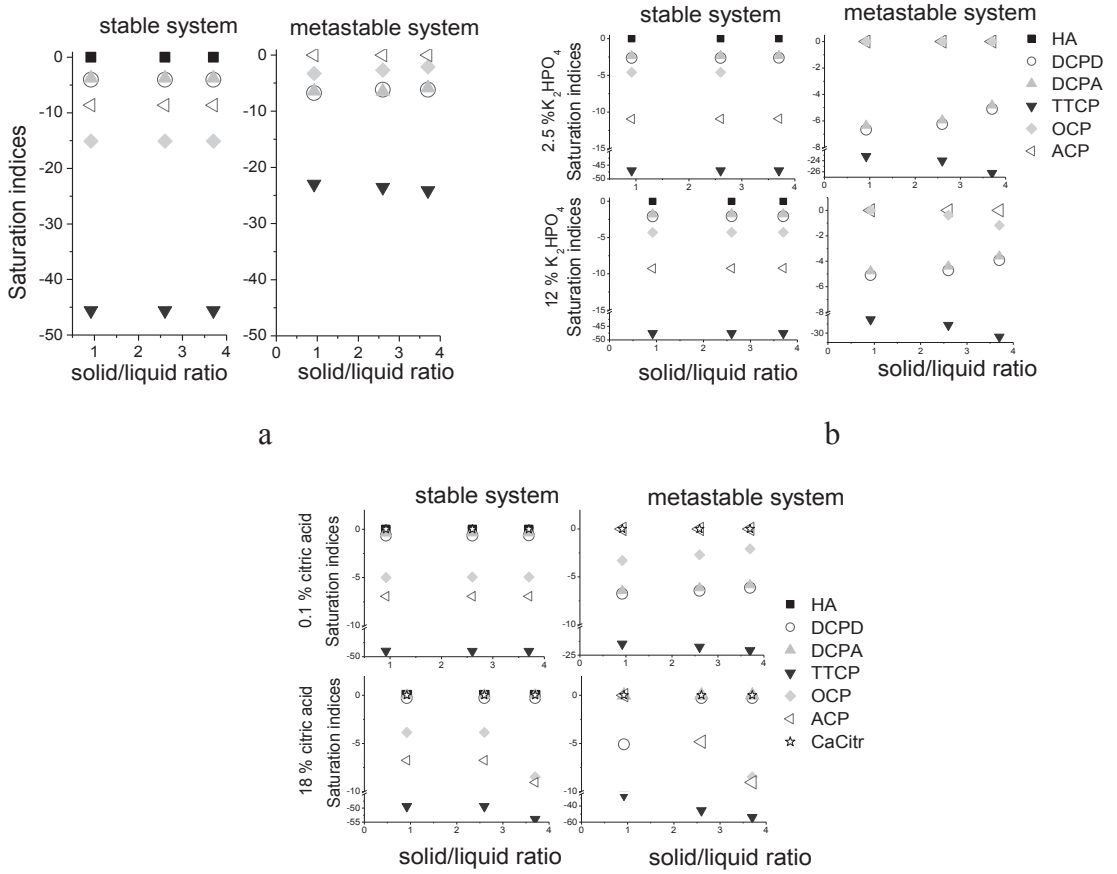


Fig. 1. Saturation indices in the systems: (a) TTCP-DCPA-H_2O, (b) TTCP-DCPA-K_2HPO_4-H_2O and (c) TTCP-DCPA-citric acid-H_2O. HA-hydroxyapatite, TTCP-tetracalcium phosphate, DCPA-dicalcium phosphate anhydrous, TCP-tricalcium phosphate, ACP-amorphous calcium phosphate, OCP-octacalcium phosphate, and CaCitr – calcium citrate tetrahydrate

Within a given concentration range of citric acid or K_2HPO_4 the saturation indices became more negative upon decreasing the solid/liquid phase ratio, i.e., the solubility of the solid phase increases due to the increased amount of liquid phase. On increasing the concentration of citric acid, the SI values of DCPA and calcium citrate tetrahydrate significantly increased and reached 0, i.e. they were in equilibrium with the solution. The values of SI for TTCP, DCPD and ACP slightly increased, i.e. their solubility did not change.

A series of cements (Table 1) based on DCPA and TTCP and different liquid phases (aqueous solutions of K_2HPO_4, acetic, α-amino acetic, tartaric, lactic, citric, salicylic and ascorbic acids in different concentrations) were experimentally prepared. In order to compare their setting characteristics, a solid/liquid ratio of 2.6 g/ml was used in all cases. In Table 1 the manipulation and mechanical characteristics of the cements are presented. The results reveal that cement

Table 1. Influence of the liquid phase on the manipulation and mechanical characteristics of TTCP и DCPA based cements

Liquid phase	pH	Manipulation characteristics			Mechanical characteristics of the dried cements
		Initial setting time, min	Final setting time, min	Formation	
A Inorganic liquid phase K_2HPO_4					
0.69% K_2HPO_4	9.01	10	30	poor	compact
59.9% K_2HPO_4	9.2	7	12	plastic	cracky
B Organic liquid phase					
Acetic acid $C_2H_4O_2$					
2% $C_2H_4O_2$	3.35	10	20	plastic	compact
42% $C_2H_4O_2$	1.41	6	10	poor	compact
α-Amino acetic acid $C_2H_5NO_2$					
2% $C_2H_5NO_2$	6.88	15	45	plastic	cracky
18% $C_2H_5NO_2$	6.39	15	60	plastic	compact
Lactic acid $C_3H_6O_3$					
2% $C_3H_6O_3$	1.98	20	39	plastic	cracky
10% $C_3H_6O_3$	1.66	20	36	plastic	cracky
18% $C_3H_6O_3$	1.49	12	30	plastic	compact
40% $C_3H_6O_3$	**1.14**	**3**	**6**	**plastic**	**hard, compact**
80.2% $C_3H_6O_3$	0.43	3	30	poor	compact
Tartaric acid $C_4H_6O_6$					
2% $C_4H_6O_6$	1.72	12	40	plastic	cracky
18% $C_4H_6O_6$	1.20	10	24	plastic	hard
18% * $C_4H_6O_6$	0.98	10	60	plastic	cracky
57.08% $C_4H_6O_6$	0.81	9	cracky	-	-
Citric acid $C_6H_8O_7$					
2.05% $C_6H_8O_7$	1.86	10	36	plastic	cracky
14.3% $C_6H_8O_7$	1.52	10	30	plastic	compact
14.3% * $C_6H_8O_7$	1.33	14	60	poor	compact
18.22% $C_6H_8O_7$	1.12	4	9	plastic	hardening
18.22% ** $C_6H_8O_7$		**4**	**9**	**plastic**	**hard, compact**
18.22% *** $C_6H_8O_7$	1.18	3	30	poor	compact
42% $C_6H_8O_7$	0.9	5	10	plastic	compact
Ascorbic acid $C_6H_8O_6$					
2% $C_6H_8O_6$	2.13	9	60	plastic	cracky
18% $C_6H_8O_6$	1.60	15	51	plastic	compact
18% * $C_6H_8O_6$	1.50	15	60	plastic	compact
24.81% $C_6H_8O_6$	1.58	12	45	plastic	compact

Additives: *2% xanthan gum and 5% glycerin; ** 2% xanthan gum; *** 5% glycerin

samples with K_2HPO_4 liquid phase have poor mechanical strength and plasticity. The solution of K_2HPO_4, even rather dilute (0.69 %), provides a high pH (>9) that favors the formation of basic calcium phosphates.

The reduced intensity of DCPA and TTCP peaks and the presence of an amorphous halo in the X-ray diffraction pattern of the sample obtained with 59.9% K_2HPO_4 as liquid phase indicate the formation of ACP but not of the thermodynamically stable HA (Fig. 2a).

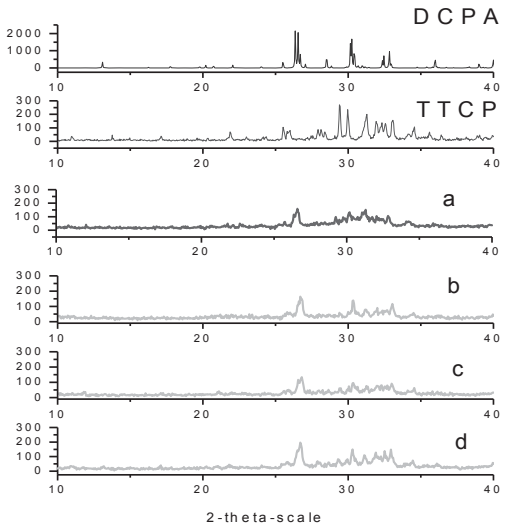
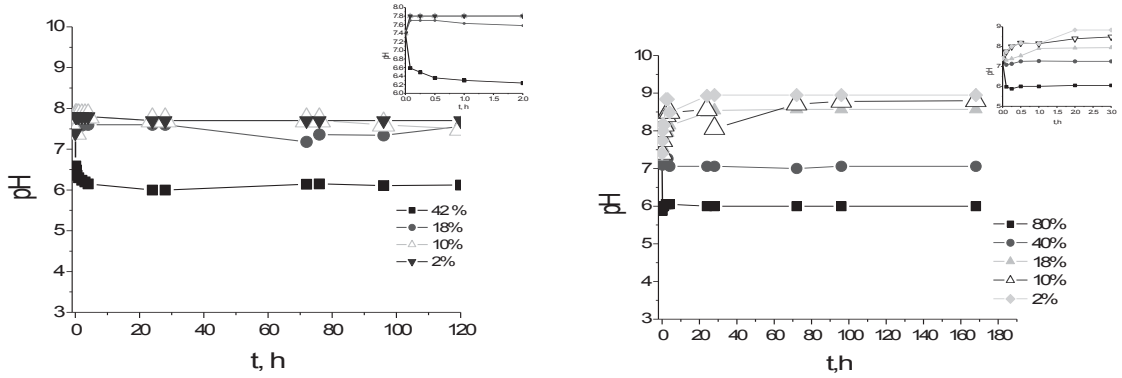


Fig. 2. X-ray powder pattern of DCPA, TTCP and cement samples obtained with 59.9% K_2HPO_4 (a) and lactic acid in different concentrations: (b) 80%; (c) 40%; (d) 18%.

Different biocompatible organic acids were used in the subsequent set of experiments. The dilution of the organic acids, with the exception of α-amino acetic acid, increased the initial and final setting time, but favored the plasticity of the cement. The use of the additives xanthan gum and glycerin lengthened the manipulation time due to their hydrophobicity, but did not considerably improve the plasticity and mechanical properties of the cements, especially in the case of glycerin application. The best results were achieved using 40% lactic acid and 18.22% citric acid, which reduced the initial setting times without affecting the formation characteristics and mechanical properties of the cements. The X-ray analysis of cement samples obtained with lactic acid in different concentrations did not reveal the presence of a phase other than the starting TTCP and DCPA. The only differences were the decrease in peaks intensity (Fig. 2b, 2c) and the appearance of a weak amorphous halo (Fig. 2d). This fact could be related to the surface dissolution of the starting materials and the precipitation of an amorphous phase.

The results from the *in vitro* testing of the best cement samples prepared with different concentrations of citric and lactic acids in SBF (pH 7.2-7.4) are presented in Fig. 3. In both cases, a change of pH in the first 2 hours occurred, after which the systems reached equilibrium. The changes of SBF's pH depend on the acid concentrations in the cements – concentrated acids provoked a decrease in pH below the physiological value (7.2-7.4) while dilute acids increased the pH. Best preservation of the physiological pH and thus best microenvironmental behavior was achieved for cement samples prepared with 40 % lactic acid. We explain the established regularity with the probability of excess acid deposition on the TTCP and DCPA grains surface, as well as with the formation of different calcium complexes. The solubility of the latter in SBF caused the changes in its pH. As the content of the newly formed phases was low, they could not be identified by X-ray analysis.



a b

Fig. 3. Influence of the cement samples prepared with (a) citric acid and (b) lactic acid on pH of SBF during *in vitro* testing (pH of the initial SBF is 7.2-7.4).

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FO3. APPLICATION OF NANOTECHNOLOGY IN TISSUE ENGINEERING AND REGENERATION

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Abstract
Tissue engineering applies the principles of engineering and life sciences to create biological replacements for damaged tissue and organs [20]. Although tissue engineering holds great potential, more research is required to provide adequate tissue replacements and determine their effect on human health. The recent advance of nanotechnology may hold the key to resolving these problems. Nanomaterials have the unique property to mimic surface properties. Moreover, nanomaterials have been observed to exhibit superior physicochemical properties compared to conventional materials. These distinct characteristics of nanomaterials help improve tissue growth. This paper summarizes some of the recent scientific progress of nanotechnology in bone, neural and vascular tissue engineering. It also discusses the potential risks and challenges that nanomaterial synthesis poses.

Introduction
The increasing prevalence of diseases and organ malfunctions has compelled scientists to develop new ways of restoring damaged tissue. The field of nanotechnology offers a new way of improving traditional tissue engineering materials.
Cell proliferation and differentiation are regulated by signaling molecules in the extracellular matrix (ECM). Moreover, the extracellular matrix plays a critical role in shaping tissue structure. Nanomaterials can mimic the extracellular matrix and its functions due to their similar size. Additionally, the surface area, surface roughness and surface area to volume ratios of nanomaterials can be increased in order to enhance their physicochemical attributes (i.e. mechanical, catalytic and magnetic properties) [2]. The biomimetic features of nanomaterials as well as their excellent physicochemical parameters have a crucial part in facilitating cell growth and tissue regeneration.
Currently, scientists focus on developing nanomaterial scaffolds for tissue regeneration. In this paper, the recent advances in the use of nanomaterials in bone, neural and vascular tissue regeneration will be reviewed. The risks and challenges that nanotechnology holds will be covered as well.

Electrospinning
The most widespread method for processing nanomaterials in tissue engineering applications is the electrospinning process. The main advantage of electrospun scaffolds is that their parameters (i.e. surface tension, conductivity, viscosity etc.) can be tuned [21]. In this sense, electrospinning allows the creation of nanofibrous scaffolds with desirable properties.
Electrospinning apparatus consists of a polymer-filled syringe, two electrodes, power supply and a rotating collector plate.
The polymer is pumped through the tip of the needle. By applying high voltage to the system, an electric field is created between the tip of the needle and the collector plate. When the surface tension of the droplets in the tip is overcome by the force of the electric field, a charged jet injection that moves towards the collector is formed, thus generating electrospun scaffolds [11].

A pivotal feature of electrospinning is the large surface area-to-volume ratio it provides, which improves diffusion and cellular uptake [16]. Furthermore, the technique produces patterned fibers which can mimic extracellular functions and influence cell orientation and function [10, 26].
The ability to control the properties of nanofibrous scaffolds, such as their hydrophilicity, biocompatibility, biodegradability and strength, holds great potential in biomedical applications. These parameters are determined by the chemical compositions of the polymer. By selecting proper components and adjusting their ratio, nanofibrous scaffolds can be modified to fulfill a variety of functions [11].

Bone Reconstruction

Traumatic bone damage happens frequently. The drawbacks of traditional implants have driven scientists to find new ways of reconstructing damaged bone. It is clear that a new generation of cytocompatible bone replacements must be researched to regenerate bone defects.
Bones are made from organic substance (collagen fibers, laminin, fibronectin) and inorganic components such as hydroxyapatite (HA) [19]. These substances are all nanometer in dimension. The dimensional similarity between the bone ECM and nanomaterials means that the latter is suitable for bone tissue reconstruction.
Nano-hydroxyapatite is a type of nanophase material with growing popularity in bone reconstruction due to its ability to promote mineralization [13]. The nanometer sizes and high surface fraction in nanophase materials have been shown to increase osteoblast functions [13]. For example, some in vitro studies demonstrated that nanophase HA significantly increased osteoblast adhesion, while inhibiting competitive fibroblast adhesion [23]. The increased vitronectin absorption on nanophase HA may explain the improved osteoblast adhesion [23].
The utilization of carbon nanotubes (CNTs) in bone tissue reconstruction has also demonstrated promising results. The success of CNTs is due to their superior cytocompatible, mechanical and electric properties [12]. In a recent study by Price et al. CNTs exerted a significant enhancement in osteoblast adhesion, while decreasing competitive cell adhesion [18]. The study resulted in improved osteointegration. Other research groups have demonstrated that CNTs can stimulate osteoblast proliferation and differentiation [9].

Nerve Regeneration

In addition to bone reconstruction, nanomaterials can help in nerve cell regeneration. Repairing nerves and achieving full recovery of nerve functions is a challenging task, considering the complexity of the nervous system.

The ideal materials for nerve regenerations must have both excellent biocompatibility and physicochemical properties. Otherwise, nanomaterials may provoke inflammation and may prove unable to guide tissue regeneration. Nanotechnology provides an excellent platform for designing nanotubes for neural tissue regeneration [13].

Biodegradable polyesters such as Polyglycolic acid (PGA) and Polycaprolactone (PCL) are commonly used in tissue engineering. In fact, several PGA and PCL nerve guidance conduits have been approved for clinical use. One such example is Neurotube® (Synovis
Micro Companies Alliance, Birmingham, AL) - a PGA mesh tube. Its effectiveness in peripheral nerve reconstruction has been demonstrated by a large clinical trial, reporting very encouraging results [27]. Neverthless, Neurotube® has a high rate of degradation which leads to the creation of acidic degradation deposits [13].

Carbon nanotubes are commonly used to guide axon growth and improve neural activity due to their similar nanoscale dimensions as neurites [13]. For example, Mattson et al. found that neurons grew on multi-walled carbon nanotubes (MWCNTs) [15]. The research showed considerably increased neuron length and branches. Lovat et al. demonstrated that MWCNTs potentially boosted electrical signal transfer of neuronal networks [26].

Furthermore, CNTs can be combined with stem cells in order to treat neural damage. Studies have shown that CNTs can contribute to selective stem cell differentiation [1]. For example, Lee et al. injected CNTs filled with stem cells into damaged neural tissue in rat brains [1]. Histological results showed that stem cells successfully differentiated into neurons without any glial formations around the scaffolds. It is clear, that CNTs played an important role in delivering stem cells to injury sites as well as promoting their differentiation into neurons.

Vascular Regeneration

The structure of vascular tissues contains numerous nanosized structures such as collagen and elastin which makes it excellent for reconstruction using nanomaterials. Moreover, evidence suggests that nanomaterials may inhibit processes such as thrombosis and inflammation [13].

Results have demonstrated that nanostructured titanium can greatly improve vascular cell adhesion and proliferation compared to conventional titanium [22]. Additionally, nanostructured titanium induces increased endothelialization on nanostructured stents. According to these results, it can be concluded that titanium nanomaterials could eliminate one of the main problems concerning vascular stents – overgrowth of smooth muscle cells compared to endothelial cells.

Further research into vascular tissue regeneration has shown that nanostructures created through chemical itching and cast-mold technique tend to enhance cell proliferation [5-7]. Based on the results, scientists have created 3-D polymer and nanofibrous scaffolds for vascular applications. For example, a study by Xu et al. managed to fabricate a nanofibrous scaffold which mimicked the structure of the medial layer in arteries [3]. The research yielded that the smooth muscle cells interacted favorably with the scaffold, stimulating tissue repair.

Challenges and Risks

Although the usage of nanomaterials in regenerative medicine and tissue engineering has achieved tremendous progress, it is important to note that the research of nanomaterials in diverse tissue engineering applications is still in its infancy. In order to promote cell growth, nanomaterials must meet several criteria such as biocompatibility, adequate porosity, appropriate nanotopography etc.

The influence of nanomaterials on human health is still not very well studied. Toxic responses generated from nanomaterial degradation have been reported [13]. The cellular uptake of nanoparticles by alveolar macrophages, endothelial cells or intestinal epithelium may present a problem in the field [13].

However, science is unable to give an adequate answer about nanomaterial toxicity. Further investigation is needed to determine the exact effects of nanomaterials on human health.

Conclusion

Advances in the field of nanotechnology are rapid. Nanomaterials show much promise in the tissue engineering applications due to their feature to interact with the extracellular matrix and mimic it, thus stimulating cell growth. Moreover, the ability to control nanomaterial properties can lead to the creation of scaffolds with desirable attributes. The potential of these biomaterials has been demonstrated both in vitro and in vivo. However, despite the promising results, further research is required in order to determine the mechanisms of nanomaterial interaction and their effect on human health. Despite the challenges that lie ahead, scientists are actively looking for new methods of creating ideal nanomaterials. Perceiving how nanomaterials interact with cells will change our understanding of modern medicine.

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FO4. Brief journey in the world of tissue engineering

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FO5. Bone tissue engineering

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Abstract

The repair of large segmental bone defects that can appear due to trauma, inflammation and tumor resection still remains a major clinical problem. Bone tissue engineering represents one of the most challenging new fields for scientists and clinicians. The use of autologous stem cells, and appropeiate scaffolds and biological signals offers the possibility to overcome the disadvantages of classical tissue reconstruction - donor site morbidity and limited amount in the case of autologous grafts, immunogenicity of allogenic grafts and loosening of alloplastic implants.

Introduction

Bone disease is a serious health condition that directly impacts on the quality of life of sufferers, the frequency and severity of the disease increases with age. Bone defects often result from tumor resection, congenital malformation (such as osteogenesis imperfects, osteopetrosis), trauma, fractures, surgery, or periodontitis in dentistry, as well as from diseases, such as osteoporosis or arthritis. As a result of the limited physiological regeneration of bone and unsolved problems facing modern medicine in this area many people suffered serious bone injuries never return to their normal physical activity and quality of life. All this has serious social, financial and psychological consequences [28].

The techniques used to repair damaged bones

Autografts (transferred from healthy parts of the bones of the same patient) remains the "gold standard" for stimulating bone repair and regeneration and are widely used because they show high performance. However, there are problems related to a severely limited amount of tissue being available and the procedure to harvest the material is associated with complications (post-operative continuous pain, hypersensitivity, pelvic instability, infection, and paresthesia) that affect 10% to 30% of the patients [9, 10, 16].

Allografts (transferred from other people) are the next best alternative at present and are also widely used. Unfortunately, they have disadvantages related to not only limited availability but also with foreign body reactions and infections (for example risks of virus transmission to the recipient), moreover they do not have osteogenetic properties (10, 16, 24, 25]. In general allograft bone has a higher incidence of nonunion or delayed union than autografts [7, 14]. In addition, an experienced group of orthopedic surgeons working at an

institution with access to a large volume of patients, and a reliable, modern bone bank to select the appropriate graft for each individual have to be available for successful bone allograft transplantation [23].

Increasing incidence of bone damage due to injury, disease, or tumor resection has given rise to a growing need for bone grafts and several biomaterials (metals, calcium phosphate ceramics, bioactive glasses, polymers, composites) have been developed with more or less clinical success. However, artificial materials implanted into bone defects are generally encapsulated by a fibrous tissue and became isolated from the surrounding bone. That is why, in orthopaedic surgery and traumatology there is a significant need and demand for the development of bone substitute materials that are bioactive and exhibits material properties (mechanical and surface) comparable with those of natural, healthy bone; that do not damage healthy tissue, do not pose any infectios (viral, bacterial) risk to patients, and can be supplied at any time, in any amount. These materials must possess some important properties, such as osteoinduction, osteoconduction, osteogenesis.

The physiology of bone grafting

- Osteoconduction (phenomenon of new bone formation on the surfaces of biomaterial) is the physical property of the graft to serve as a scaffold for viable bone healing.
- Osteoinduction is the ability of graft material to recruit mesenchymal stem cells from the surrounding tissue and to induce them to differentiate into mature bone cells. Growth factors such as bone morphogenic proteins (BMP's - multifunctional growth factors that belong to the transforming growth factor beta superfamily), interleukins and platelet-derived growth factor (PDGF) can influence the recruitment and differentiation of mesenchymal stem cells.
- Graft Osteogenesis is represented by the cellular elements within a donor graft, which survive transplantation and synthesize new bone at the recipient site.
- Osseointegration is the stable anchorage of an implant achieved by direct bone-to-implant contact [1, 2, 14, 20, 27].

Tissue engineering

Tissue engineering offers new opportunities for reparation of large bone losses. This technology is based on the combined use of cultured living cells and 3D scaffolds and is able to deliver vital cells to the damaged site of the patient. Bone tissue engineering is a complex and dynamic process that initiates with migration and recruitment of osteoprogenitor cells followed by their proliferation, differentiation, matrix formation along with remodeling of the bone. The approaches in tissue engineering can ensure delivery of biologically active molecules (growth factors, cytokines), drugs, as well as gene expression, which facilitates recovery of the defect [4].

Scaffolds

A key component in tissue engineering for bone regeneration is the scaffold (three-dimensional porous structures) that serves as a template for cell interactions and the formation of bone-extracellular matrix to provide structural support to the newly formed tissue. The general criteria for an ideal scaffold for such purposes are:

i. *Biocompatibility* – that means ability to support normal cellular activity including molecular signaling systems without any local and systematic toxic effects to the host tissue. An ideal bone scaffold must be osteoconductive and osteoinductive as well as able to form blood vessels in or around the implant within few weeks of implantation to actively support nutrient, oxygen and waste transport.

ii. *Mechanical properties* – the ideal scaffold should exhibit mechanical properties similar to those of the bone repair site and the material must keep its structural integrity during the first stages of the new bone formation.
iii. *Pore size* – the scaffold must possess a highly interconnected porous network, formed by a combination of macro- and micropores that enable proper tissue ingrowth, vascularization and nutrient delivery. Unfortunately, porosity reduces mechanical properties such as compressive strength, and increases the complexity for reproducible scaffold manufacturing.
iv. B*iodegradability* at a rate commensurate with natural remodeling, with degradation products that are non-toxic and that can be easily excreted by the body. The degradation behavior of the scaffolds should vary based on applications such as 9 months or more for scaffolds in spinal fusion and 3 to 6 months for scaffolds in cranio-maxillofacial applications.
v. Potential to be commercially producible and sterilizable to the required international standards for clinical use [4, 13, 30].

Scaffolds made with different materials (bioglass based, polymeric, composite, metallic, etc) that have been tested under *in vitro* and *in vivo* conditions [4].

Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are multipotent progenitor cells found in various tissues (bone marrow, muscle, trabecular bone, dermis, adipose tissue, peryosteum, pericyte, blood, synovial membrane), most commonly bone marrow and adipose tissue. MSCs are capable of differentiating into all the appropriate differentiation lineages (osteoblastic, myoblastic, adipocytic, chondrocytic, endothelial, and neurogenic). For the osteogenic lineage, mesenchymal stem cells sustain a cascade of differentiation steps as described by the following sequence: Mesenchymal stem cell → immature osteoprogenitor → mature osteoprogenitor → preosteoblast → mature osteoblast → osteocyte or lining cell or apoptosis. In bone marrow osteoprogenitor cells represent a very small percentage (e.g. < 0.005%) of nucleated cell types in healthy adult bone.

In recent years, much progress has been made in understanding the factors that regulate the gene expression program that underlies the induction, proliferation, differentiation, and maturation of osteoblasts. A large and growing number of transcription factors make important contributions to the precise control of osteoblast formation and function [12, 17, 31, 32].

The master switch that determines osteoblast fate is the transcription factor RUNX2 (Runt-related transcription factor 2, also known as Cbfa1 or AML3). RUNX2 is a member of the RUNX family of transcription factors, also containing the RUNX1 and RUNX3 proteins. During the early stage of osteoblast differentiation, Runx2 regulates the expression of major bone matrix protein genes [15]. Overexpression of RUNX2 in osteoblasts blocked G1 to S phase progression [31]. The critical dependence of RUNX2 on osteoblast differentiation is demonstrated with *Runx2* deficient mice. These knockout mice have neither osteoblasts nor endochondral and intramembranous bone [26]. Mutations in *RUNX2* were identified as the cause for the human autosomal dominant condition cleidocranial dysplasia (CCD) that is characterized by hypoplastic clavicles, persistently open calvarial sutures and dental abnormalities [21]. CCD patients are haploinsufficient for *RUNX2* and it is unclear why only certain mineralized tissues are affected [8]. A similar phenotype of *Runx2* deficient mice is seen with deletion of *Osterix* (Sp7, Osx), a downstream target of RUNX2 [22]. Osx is an osteoblast-specific transcription factor required for bone formation. Osx was first discovered as a bone morphogenetic protein-2 inducible gene in mesenchymal stem cells. Osx knock-out

mice lack bone completely, and cartilage is normal [32]. RUNX2 also cooperates with other critical osteoblast factors such as Msh homeobox 2 (MSX2), Twist 1 (Basic Helix-loop-Helix factors), BMPs, WNTs and Hedgehog proteins. Activating transcription factor 4 (ATF4) and zinc-finger protein 521 (ZFP521) acting as cofactors of Runx2 are also important [3, 8, 17, 19].

Multiple signaling pathways are able to individually activate RUNX2 expression. Well-studied activators of osteoblastogenesis are the bone morphogenetic proteins (BMPs), members of the transforming growth factor-β superfamily (TGF-β). These glycoproteins act as a disulfide-linked homo- or heterodimers, being potent regulators of bone and cartilage formation and repair, cell proliferation during embryonic development and bone homeostasis in the adult. BMPs also promote the angiogenesis, regulate the activity/ affect the production of some growth factors. Signaling transduction by TGF-β/BMPs is specifically through both canonical Smad-dependent pathways (TGF-β/BMP ligands, receptors and Smads) and non-canonical Smad-independent signaling pathway (e.g. p38 mitogen-activated protein kinase pathway, MAPK). Following TGF-β/BMP induction, both the Smad and p38 MAPK pathways converge at the Runx2 gene to control mesenchymal precursor cell differentiation. The coordinated activity of Runx2 and TGF-β/BMP-activated Smads is critical for formation of the skeleton. BMPs have been considered as the most potent growth factors that can promote the bone regeneration [5, 6, 8, 11, 17].

Open questions

Two of the hot questions that engage the mind of scientists and clinicians in the recent years are:

1) Is it possible to use allogenic instead of autologous MSCs for the needs of bone tissue engineering;
2) Whether the banking of human bone marrow-derived mesenchymal stem cells (MSCs) could be made possible, in other words - if cryopreserved autologous MSCs (harvested from the patient's own bone marrow), could be cultured, expanded with the patient's own serum and can be thawed and cultivated for grafting at a later date [18, 29].

These questions are not occasional because of various reasons. For example, it deserves to be noted that the number of MSC with osteogenic potential decreases early during aging in humans, and, in certain diseases (e.g. myelodysplastic syndrome), the patient's bone marrow may be damaged or the healthy cells reduced in number [18, 29].

Conclusion

Bone tissue repair is one of the major concerns of regenerative medicine. Significant basic and applied research and development is needed to realize the full clinical potential of bone tissue engineering. Improved methods for isolation and processing of mesenchymal stem cells and better understanding of the molecular mechanisms involved in their osteogenic differentiation as well as development of advanced materials for 3D scaffolds are among the main problems that have to be solved in the near future.

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FP1. Развитието на терапевтичните ваксини в борбата с различни заболявания

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FP2. Карцином на млечната жлеза – Her2 рецептор

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