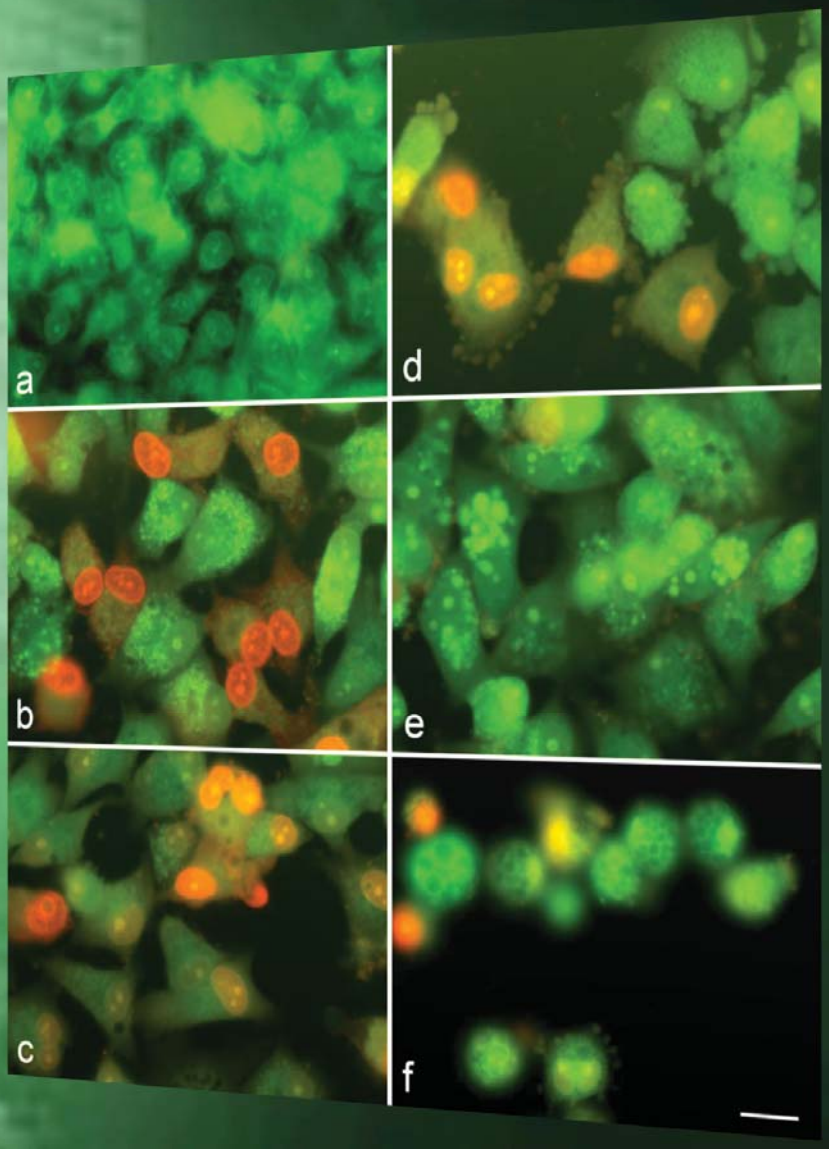
PROCEEDINGS

OF THE SIXTH WORKSHOP

ON EXPERIMENTAL MODELS AND METHODS IN BIOMEDICAL RESEARCH



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PROCEEDINGS

OF THE SIXTH WORKSHOP ON EXPERIMENTAL MODELS AND METHODS IN BIOMEDICAL RESEARCH

12-14 May 2015

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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THE SIXTH WORKSHOP

"EXPERIMENTAL MODELS AND METHODS IN BIOMEDICAL RESEARCH"

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

Tuesday, 12 May 2015

8.30 – 9.00 REGISTRATION
9.00 – 9.15 OPENING CEREMONY

Session A: Molecular Immunology and (Epi)Genetics

Chairpersons:
Assoc. Prof. George Miloshev, PhD
Institute of Molecular Biology, Bulgarian Academy of Sciences

Assoc. Prof. Andrey Tchorbanov, PhD
Institute of Microbiology, Bulgarian Academy of Sciences

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

9.15 – 9.45
AO1. SELECTIVE ELIMINATION OF ALLERGEN –SPECIFIC B LYMPHICYTES WITH CHIMERIC PROTEIN-ENGINEERED MOLECULE IN HUMANIZED SCID MODEL

N. Kerekov[1], A. Michova[2], G. Nikolov[2], M. Muhtarova[2], B. Petrunov[2], M. Nikolova[2] and A. Tchorbanov[1]

[1] Laboratory of Experimental Immunology, Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria;
[2] National Reference Laboratory of Immunology, National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria

9.45 – 10.00
AO2. SELECTIVE ALTERATION OF SELFREACTIVE B AND T CELLS BY CHIMERIC MOLECULES IN A HUMANIZED MOUSE MODEL OF TYPE 1 DIABETES

Iliyan Manoylov[1], Nelly Delcheva[1], Iliana Atanassova[2], Andrey Tchorbanov[1]

[1]Department of Immunology, The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria;
[2]Department of Endocrinology, SBALENG "Acad. I. Penchev", Sofia, Bulgaria

10.00 – 10.15

AO3. ANNEXIN A1 AS A TARGET FOR MANAGING PRISTANE-INDUCED SYSTEMIC LUPUS ERYTHEMATOSUS

S. Bradyanova[1], N. Mihaylova[1], P. Chipinski[1], S. Chausheva[1], M. Herbáth[2], F. D'Acquisto[3], J. Prechl[2] and A. Tchorbanov[1]

[1] Laboratory of Experimental Immunology, Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria;

[2]Immunology Research Group, Hungarian Academy of Sciences, Budapest, Hungary;

[3]Centre for Biochemical Pharmacology, William Harvey Research Institute, London, UK

10.15 – 10.30

AO4. TUMOUR NECROSIS FACTOR-RELATED APOPTOSIS INDUCING LIGAND (TRAIL) AS AN ARTHRITIC MARKER IN A MODEL OF OSTEOARTHRITIS

P. Ganova, V. Gyurkovska, N. Ivanovska

Institute of Microbiology, Bulgarian Academy of Sciences Sofia, Bulgaria

10.30 – 10.45

AO5. TARGETING OF VIRUS EPITOPES TO ANTIGEN PRESENTING CELLS VIA CHIMERIC MOLECULES IN HUMANIZED SCID MICE

D. Hlebarska[1], I. Ivanova[1], N. Mihaylova[1], I. Manoylov[1], D. Makatsori[2], A. Mamalaki[2], J. Prechl[3] and A. Tchorbanov[1]

[1] Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria;

[2] Hellenc Pasteur Institute, Athens, Greece;

[3] Immunology Research Group, Hungaria Academy of Sciences, Budapest, Hungary

10.45 – 11.05 Coffee Break

11.05 – 11.20

AO6. DEVELOPMENT AND APPLICATION OF A SPECIFIC FLUOROGENIC SUBSTRATE FOR THE CYTOCHEMICAL STUDY OF FIBROBLAST ACTIVATION PROTEIN ALFA

Mashenka Dimitrova[1], Ivan Iliev[1], Velichka Pavlova[1], Vanio Mitev[2], Stella Dimitrova[3], Doroteya Aleksandrova[2], Ivaylo Ivanov[2]

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

[2] Department of Medical Chemistry and Biochemistry, Medical University of Sofia, Bulgaria;

[3]Faculty of Biology, Sofia University "St. Kl. Ohridski", Sofia, Bulgaria

11.20 – 11.50

AO7. *SACCHAROMYCES CEREVISIAE* – A BRILLIANT MODEL FOR STUDYING AGEING

Matthew Serkedjiev, Dessislava Staneva, Milena Georgieva and George Miloshev

Institute of Molecular Biology "R. Tsanev", Bulgarian Academy of Sciences, Sofia, Bulgaria

11.50 – 12.05

AO8. **CHROMATIN - THE MAIN PLAYER IN THE AGEING PROCESS**

Dzhuliya Milcheva, Milena Georgieva, Dessislava Staneva and George Miloshev
Institute of Molecular Biology "Roumen Tsanev",Bulgarian Academy of Sciences, Sofia, Bulgaria

12.05-12.20

AO9. APPLICATION OF 3D QUANTITATIVE DNA METHYLATION IMAGING FOR STUDYING THE GLOBAL METHYLATION STATUS IN SERTOLI CELLS

Kristiyan Kanev, Elina Avramska, Krassimira Todorova, Soren Hayrabedyan
Institute of Biology and Immunology of Reproduction, Bulgarian Academy of Sciences, Sofia, Bulgaria

12.20 – 12.35

AO10. ЕПИГЕНЕТИЧНИ ПРОМЕНИ, СВЪРЗАНИ С БИОАКТИВНИТЕ ХРАНИТЕЛНИ ДОБАВКИ

Ваня Младенова
Институт по биология и имунология на размножжаването „Акад. Кирил Братанов", Българска академия на науките, София, България

12.35 – 12.50

Discussion

Session B: The Rainbow World of Medicine

Chairpersons:

Prof. Reneta Toshkova, MD, PhD

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

Assoc. Prof. Valya Grigorova, MD, PhD

Institute of Neurobiology, Bulgarian Academy of Sciences

Secretary: Desislav Dinev, MSc

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

13.30 – 13.45

BO1. BIOCHEMICAL MAKERS OF BONE METABOLISM IN EXPERIMENTAL ANIMALS

N.T. Tsocheva-Gaytandzhieva, V. Nanev, I. Vladov, M. Gabrashanska, P. Dimitrov, M. Alexandrov

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

13.45 – 14.15

BO2. **IN THE WORLD OF METALLOTHIONEINS**

Radostina Alexandrova[1], Tanya Zhivkova[1], Lora Dyakova[2], Abedulkadir Abudalleh[1], Desislav Dinev[1], Belma Turan[3], Danina Mirela Muntean[4]

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

[2]*Institute of Neurobiology, Bulgarian Academy of Sciences, Sofia, Bulgaria;*

[3]*Faculty of Medicine, Ankara University, Ankara, Turkey;*

[4]*Faculty of Medicine, Victor Babes University of Medicine and Pharmacy, Timisoara, Romania*

14.15 – 14.30

B03. БИОМАРКЕРИ ЗА РАННО ДИАГНОСТИЦИРАНЕ НА ОСТРО БЪБРЕЧНО УВРЕЖДАНЕ – NGAL

Жулиета Христова[1], Благославa Георгиева[2], Камен Цачев[3]

[1]*Катедра по медицинска генетика, Медицински факултет, Медицински университет – София, България;*

[2]*Фармацевтичен Факултет, Медицински университет - София, България;*

[3]*Катедра по клинична лаборатория и клинична имунология, Медицински факултет, Медицински университет - София, България*

14.30 – 15.00

BO4. **DIRECTIONAL ADAPTATION OF REACTIVE SACCADES IN PATIENTS WITH PRIMARY OPEN-ANGLE GLAUCOMA**

S. Borisova[1], V. Grigorova[1], P. Vasileva[2] , V. Miltenova[2]

[1]*Institute of Neurobiology, Bulgarian Academy of Sciences,Sofia, Bulgaria;*

[2] *Eye hospital ‘‘Prof. Pashev’’, Sofia, Bulgaria*

15.00 – 15.20 Coffee Break

15.20 – 15.50

BO5. **AWARENESS OF GPS ABOUT ALTERNATIVE MEDICINE – HOW CAN WE INITIATE IT**

Staykova-Pirovska Y., Despotova-Toleva L.

Department of Ophthalmology and Family Medicine, Faculty of Medicine, Trakia University, Stara Zagora, Bulgaria

15.50 – 16.05

BO6. WOUND HEALING

Desislav Dinev, Radostina Alexandrova

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

16.05 – 16.35

B07. MODERN TRENDS IN THE COMPLEX SURGICAL TREATMENT OF BURNS IN CHILDREN

M. Argirova, O. Hadzhiyski

Department of Burns and Plastic Surgery, MHATEM „N.I. Pirogov", Sofia, Bulgaria

16.35- 16.50. Poster Session

BP1. BETWEEN MESENCHYMAL STEM CELLS AND BONE IMPLANTS

Radostina Alexandrova[1], Tanya Zhivkova[1], Lora Dyakova[2], Abedulkadir Abudalleh[1], Boyka Andonova-Lilova[1], Desislav Dinev[1], Lylya Ivanova[1,3], Milena Fini[4], Orlin Alexandrov[5], Olafur Sigurjonsson[6]

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

[2]*Institute of Neurobiology, Bulgarian Academy of Sciences, Sofia, Bulgaria;*

[3]*Faculty of Biology, Sofia Iniversity "St. Kl. Ohridski", Sofia, Bulgaria;*

[4]*Instituto Ortopedico Rizzoli, Bologna, Italy;*

[5]*Health Service, Gorna Malina, Bulgaria;*

[6]*Department of Science and Engineering, Reykjavic University, Reykjavic, Iceland*

BP2. BRIEF OVERVIEW OF SOME GENETIC DISORDERS AFFECTING BONES

Orlin Alexandrov[1], Boyka Andonova-Lilova[2], Radostina Alexandrova[2]

[1]*Health Service, Gorna Malina, Bulgaria;*

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

BP3. GROWTH FACTORS IN BONE REPAIR

Orlin Alexandrov[1], Radostina Alexandrova[2]

[1]*Health Service, Gorna Malina, Bulgaria;*

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

BP4. WOUND HEALING IN MAXILLOFACIAL REGION

Orlin Alexandrov[1], Radostina Alexandrova[2]

[1]*Health Service, Gorna Malina, Bulgaria;*

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

16.50 – 17.05

Discussion

Wednesday, 13 May 2015

Session C: Cancer

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

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

Secretary: Delka Salkova, DVM, PhD
Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences

9.00 – 9.30
CO1. WHAT DO WE (NOT) KNOW ABOUIT METASTASIS?
Radostina Alexandrova
Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria

9.30 – 10.00
CO2. BRAIN METASTASIS IN *GRAFFI* HAMSTER EXPERIMENTAL TUMOR MODEL
V. Ormandzhieva, R. Toshkova
Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria

10.00-10.15
CO3. CELL CULTURES AS EXPERIMENTAL MODELS FOR LUNG CANCER
Tanya Zhivkova[1], Lora Dyakova[2], Abedulkadir Abudalleh[1], Radostina Alexandrova[1]
[1]*Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria;*
[2]*Institute of Neurobiology, Bulgarian Academy of Sciences, Sofia, Bulgaria*

10.15-10.30
CO4. CELL CULTURES AS EXPERIMENTAL MODELS FOR COLORECTAL CANCER
Lora Dyakova[1], Tanya Zhivkova[2], Abedulkadir Abudalleh[2], Radostina Alexandrova[2]
[1]*Institute of Neurobiology, Bulgarian Academy of Sciences, Sofia, Bulgaria;*
[2]*Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria*

10.30 – 10.45

CO5. CELL CULTURES AS EXPERIMENTAL MODELS FOR PROSTATE CANCER

Abedulkadir Abudalleh[1], Tanya Zhivkova[1], Lora Dyakova[2], Radostina Alexandrova[1]
[1]*Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria;*
[2]*Institute of Neurobiology, Bulgarian Academy of Sciences, Sofia, Bulgaria*

10.45 – 11.05 Cofee Break

11.05 – 11.20

CO6. ZEBRAFISH AS EXPERIMENTAL MODEL IN CANCER RESEARCH

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

11.20 – 11.35

CO7. *IN VITRO* ANTIPROLIFERATIVE ACTIVITY OF THE ALKYLPHOSPHOCHOLINE ERUFOSINE ON GRAFFI MYELOID TUMOR CELLS

A. Georgieva[1], R. Toshkova[1], S. Apostolova[2], V. Uzunova[2], M. R. Berger[3], R. Tzoneva[2]
[1]*Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria;*
[2]*Bulgarian Academy of Sciences, Institute of Biophysics and Biomedical Engineering, Sofia, Bulgaria;*
[3]*German Cancer Research Center, Department of Toxicology and Chemotherapy, Heidelberg, Germany*

11.35 – 11.50

CO8. FOUR NEWLY SYNTHESIZED METAL COMPLEXES WITH SCHIFF BASES – INFLUENCE ON VIABILITY AND PROLIFERATION OF VIRUS-TRANSFORMED CANCER CELLS

Elena Manlieva[1,2], Tanya Zhivkova[2], Lora Dyakova[3], Reneta Toshkova[2], Abedulkadir Abudalleh[2], Desislav Dinev[2], Gabriela Marinescu[4], Daniela-Cristina Culita[4], Luminita Patron[4], Radostina Alexandrova[2]
[1]*Faculty of Biology, Sofia University "St. Kl. Ohridski" , Sofia, Bulgaria;*
[2]*Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria;*
[3]*Institute of Neurobiology, Bulgarian Academy of Sciences;*
[4]*Institute of Physical Chemistry "Ilie Murgulescu", Bucharest, Romania*

11.50 – 12.05

CO9. POTENTIAL ANTI-CANCER EFFECT OF HEMOCYANINS ON BREAST CANCER CELLS

Veselina Uzunova[1], Sonia Apostolova[1], Krasimira Paunova[2], Yuliana Rainova[2], Maya Guncheva[2], Krasimira Idakieva[2], Rumiana Tzoneva[1]

[1]Institute of Biophysics and Biomedical Engineering, Bulgarian Academy of Sciences, Sofia, Bulgaria;
[2]Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria

12.05 – 12.20

CO10. STATINS AS ANTITUMOR AGENTS

S. Apostolova[1], R.Toshkova[2], R.Tzoneva[1]
[1]Institute of Biophysics and Biomedical Engineering, Bulgarian Academy of Sciences, Sofia, Bulgaria;
[2] Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria

12.20-12.35
Discussion

CP1. BRIEFLY ABOUT SOME CYTOTOXICITY ASSAYS

Radostina Alexandrova[1], Tanya Zhivkova[1], Abedulkadir Abudalleh[1], Lora Dyakova[2], Desislav Dinev[1], Boyka Andonova-Lilova[1]
[1]Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria;
[2]Institute of Neurobiology, Bulgarian Academy of Sciences, Sofia, Bulgaria

CP2. PROTEIN DETERMINATION BY THE METHOD OF BRADFORD: A BRIEF OVERVIEW

Dimitar Ivanov, Desislav Dinev, Radostina Alexandrova
Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria

CP3. MYC GENES AND BREAST CANCER

Radostina Alexandrova[1], Ivaylo Dankov[1,2], Iva Gavrilova-Valcheva[3], Maria Nacheva[4]
[1]Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria;
[2]Medical Faculty, Sofia University "St. Kl. Ohridski";
[3]National Specialized Hospital for Active Treatment in Oncology;
[4]UMHATEM "N. I. Pirogov"

CP4. SRC GENES AND BREAST CANCER

Radostina Alexandrova[1], Nikola Simeonov[1,2], Iva Gavrilova-Valcheva[3], Maria Nacheva[4]
[1]Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria;

[2]Medical Faculty, Sofia University "St. Kl. Ohridski";
[3]National Specialized Hospital for Active Treatment in Oncology;
[4]UMHATEM "N. I. Pirogov"

CP5. BRIEFLY ABOUT TUMOR SUPPRESSOR GENE P53 AND BREAST CANCER

Radostina Alexandrova[1], Orlin Alexandrov[2], Maria Nacheva[3]
[1]Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria;
[2]Health Service, Gorna Malina, Bulgaria;
[3]UMHATEM "N. I. Pirogov"

Session D: Microbiology, Virology and Parasitology

Chairpersons:
Assoc. Prof. Mariana Panayotova-Pencheva**, DVM, PhD**
Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences

Assoc. Prof. Evelina Shikova-Lekova, MD, 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

12.35 – 12.50

DO1. Intrauterine transmission of Trichinella and impact of the infection on the immunological status of the offspring.

Irina Odoevskaya[1], Sergey Movsesyan[2], Svetlozara Petkova[3], Valeria Dilcheva[3]
[1]Skriabin All-Russian Institute for fundamental and applied parasitology of animal and plants, Moscow, Russian Federation;
[2]Centre of Parasitology, A.V. Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences, Moscow, Russian Federation;
[3]Institute of Experimental Morphology Pathology and Anthropology with Museum Bulgarian Academy of Sciences, Sofia, Bulgaria

13.30 – 13.45

DO2. ПРОУЧВАНЕ НА СЕРОРАЗПРОСТРАНЕНИЕТО НА *TOXOPLASMA GONDII* СРЕД ДИВИ ЖИВОТНИ И КУЧЕТА В БЪЛГАРИЯ

Анетка Трифонова[1], Елеонора Кънева[2]

[1] Национална научноизследователска станция по ловно стопанство, биология и болести на дивеча, София, България;
[2]Национален център по заразни и паразитни болести, София, България

13.45 – 14.00

DO3. CELL GROWTH INHIBITING EFFECT OF THERMOLABLE BIOLOGICALLY ACTIVE SUBSTANCE ISOLATED FROM *FASCIOLA HEPATICA*-INFECTED RAT SPLEENS

Tsocheva-Gaytandzhieva N.T., R. Toshkova, D. Salkova, M. Gabrashanska
Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria

14.00 – 14.30

DO4. ANTIPARASITE REMEDIES APPLIED TO RUMINANTING ANIMALS FROM THE CERVIDAE FAMILY

M. Panayotova-Pencheva[1], D. Salkova[1], A. Trifonova[2]
[1]Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria;
[2]National Research Station of Game Management, Biology and Pathology, Sofia, Bulgaria

14.30 – 14.45

DO5. PRELIMINARY STUDIES ON THE SPREAD OF *VARROA DESTRUCTOR* AND *NOSEMA SPP.* IN CERTAIN AREAS OF BULGARIA

D. Salkova[1], K. Gurgulova[2], S. Takova[2], M Panayotova-Pencheva[1]
Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria;
[2]National Diagnostic & Research Veterinary Medical Institute „Prof. G. Pavlov“, Sofia, Bulgaria.

14.45 – 15.05 Coffee Break

15.05 – 15.20

DO6. SOME BIOCHEMICAL PROPERTIES OF NEURAMINIDASE FROM AEROMONAS STRAIN A40/02

S. Engibarov[1], R. Eneva[1], V. Kolyovska[2], I. Abrashev[1]
[1]Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria;
[2]Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria

15.20 – 15.50

DO7. PRELIMINARY SCREENING FOR HEPATITIS B VIRUS IN TREATED PATIENTS WITH RHEUMATOID ARTHRITIS AND ANKYLOSING SPONDYLITIS AND REVIEW ON THE LITERATURE

E. Golkocheva-Markova[1], M. Ivanova[2], S. Ivanova[3], I. Gekova[2], N. Stoilov[2], R. Stoilov[2], P. Teoharov[1]

[1]NRL Viral Hepatitis, Virology Department, National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria;

[2]Clinic of Rheumatology, University Hospital for Active Treatment "St. Ivan Rilski, Sofia, Bulgaria;

[3]NRL of Measles, Mumps and Rubella, Virology Department, National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria

15.50 – 16.20

DO8. NOROVIRUSES – THEIR LEADING ROLE IN NON-BACTERIAL GASTROENTERITIDES AND THE EXPERIENCE IN MONITORING THE INFECTION IN BULGARIA

Asya Stoyanova

Department of Virology, National Reference Laboratory of Enteroviruses, National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria

16.20 – 16.35

DO9. ONCOGENIC ACTIVITIES OF HPV PROTEINS

Zina Ivanova and Evelina Shikova

National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria

16.35 – 16.50

DO10. NOVEL HUMAN RESPIRATORY POLYOMAVIRUSES KIPYV AND WUPYV

Djanan Emin[1,2] and Evelina Shikova-Lekova[2]

[1]Faculty of Biology, Sofia University "St. Uliment Ohridski" , Sofia, Bulgaria;

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

16.50 – 17.05

DO11. SOME FEATURES IN EXPERIMENTAL INFLUENZA VIRAL INFECTION IN MICE AND ADEQUATE MARKERS USED FOR THEIR ASSAYING

A. Dimitrova[1], M. Mileva[1], D. Krastev[2], I. Drendarska[3], A.S. Galabov[1]

[1]The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences;

[2]Medical University of Sofia, Medical Colleague "Jordanka Filaretova";

[3]Military Medical Academy, Sofia

17.05 – 17.30 – Poster session
17.30 – 17.45 Discussion

DP1.ANTIBIOTIC RESISTANCE IN *STAPHYLOCOCCUS AUREUS*
Ivaylo Dankov
Medical Faculty, Sofia University St "Kl. Ohridski", Sofia, Bulgaria

DP2. ЧОВЕШКИ ПАПИЛОМА ВИРУСИ-ТИПОВЕ, ДИАГНОСТИКА, ПРОФИЛАКТИКА И ЛЕЧЕНИЕ
Георги Тошев
Биологически факултет „Св. Кл. Охридски", София, България

DP3. ЧОВЕШКИ ПОЛИОМНИ ВИРУСИ-ЛАБОРАТОРНА ДИАГНОСТИКА, ПРЕВЕНЦИЯ И ЛЕЧЕНИЕ
Георги Тошев
Биологически факултет „Св. Кл. Охридски", София, България

Thursday, 14 May 2015

Session E: Pharmacology and Toxicology

Chairpersons:
Assoc. Prof. Julia Radenkova-Saeva, MD, PhD
Toxicology Clinic, UMHATEM "N. I. Pirogov"

Assoc. Prof. Russy Russev, DVM, PhD
Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences

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

9.00 – 9.30
EO1. DISTRIBUTION OF ACUTE POISONING WITH PSYCHOACTIVE SUBSTANCES – ANALYSIS FOR THE PERIOD 2012 – 2014

J. Radenkova - Saeva
Clinic of Toxicology, Emergency University Hospital "N.I. Pirogov", Sofia, Bulgaria

9.30 – 10.00

EO2. EXPERIMENTAL MODELS FOR INVESTIGATION OF CADMIUM CHELATION

Svetlana Iovinska[1], Kamelia Lazarova[1], Yordanka Gluhcheva[2], Juliana Ivanova[1]
[1]*Faculty of Medicine, Sofia University, Sofia, Bulgaria;*
[2]*Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria*

10.00 – 10.15

EO3. GUINEA PIGS AS AN EXPERIMENTAL MODEL. TOXICITY OF PTAQUILOSIDE ISOLATED FROM BRACKEN FERN (*PTERIDIUM AQUILINUM* (L.) KUHN) IN GUINEA PIGS

Iliya Peev[1], Toni Todorov[1], Katerina Todorova[2], Simona Lazarova[2], Petar Dimitrov[2] and Russy Russev[2]
[1]*University of Forestry, Faculty of Veterinary Medicine, Bulgaria, Sofia. Bulgaria;*
[2]*Institute of Experimental Morphology, Pathology, Anthropology with Museum, Bulgarian Academy of Science, Sofia, Bulgaria*

10.15 – 10.30

EO4. ESTABLISHMENT OF THE REAL TIME DYNAMIC MODEL FOR ANALYSIS OF INTEGRITY AND PERMEABILITY OF THE BLOOD BRAIN BARRIER (BBB) IN VIVO- IN RATS

Lubomir Traikov[1]; Kristin Genkova [2*], Nevena Raikova [2], Kalliopi Negrou[1], Julia Petrova[2], Chiodji Ohkubo[3]
[1]*Department of Medical Physics and Biophysics, Faculty of Medicine, Medical University-Sofia, Sofia, Bulgaria;*
[2]*Department of Neurology; Faculty of Medicine; Medical University-Sofia, Sofia, Bulgaria;*
[3] *EMF Information Center 2-9-11 Shiba, Minato-ku, Tokyo, Japan*

10.30 – 10.50 Coffee Break

10.50 – 11.20

EO5. MODELING OF NUTRITION IN PATIENTS WITH RELAPSING REMITTING MULTIPLE SCLEROSIS WITH AIM TO REDUCE THE EXACERBATIONS

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

11.20 – 11.35

EO6. LIPID PEROXIDATION IN LIPOSOMES

A. Georgieva[1] , E. Tzvetanova[1], G. Nenkova[1], A. Alexandrova[1], M. Mileva[2]
[1]Institute of Neurology, Bulgarian Academy of Sciences, Sofia, Bulgaria;
[2]The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria

11.35 – 12.05

EO7. METALODENDRIMERS WITH BIOMEDICAL APPLICATIONS

B. Lazarov[1], S. Yordanova[1], E. Vasileva-Tonkova[2], I. Grabchev[3]
[1]Faculty of Chemistry and Pharmacy, Sofia University "St. Kliment Ohridski", Sofia, Bulgaria;
[2]Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria;
[3] Faculty of Medicine, Sofia University "St. Kliment Ohridski", Sofia, Bulgaria

12.05 –12.35 Poster Sessions

12.35– 12.50 General Discussion and Final Remarks

Session A: Molecular Immunology and (Epi)Genetics

Chairpersons:
Assoc. Prof. George Miloshev, PhD
Institute of Molecular Biology, Bulgarian Academy of Sciences

Assoc. Prof. Andrey Tchorbanov, PhD
Institute of Microbiology, Bulgarian Academy of Sciences

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

AO1. SELECTIVE ELIMINATION OF ALLERGEN –SPECIFIC B LYMPHICYTES WITH CHIMERIC PROTEIN-ENGINEERED MOLECULE IN HUMANIZED SCID MODEL

N. Kerekov[1], A. Michova[2], G. Nikolov[2], M. Muhtarova[2], B. Petrunov[2], M. Nikolova[2] and A. Tchorbanov[1]

[1] Laboratory of Experimental Immunology, Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria;
[2] National Reference Laboratory of Immunology, National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria
E-mail: tchorban@microbio.bas.bg

Introduction: Der p1 is allergenic molecule of Dermatophagoides pteronyssinus (Dpt) which causes house dust allergy. The pathological B cells produce allergen-specific IgE antibodies that mediate most of the allergic reactions. It may be possible to influence Der p1-specific B cells by administrating to them a chimeric molecule, containing an antibody against the inhibitory B-cell receptor CR1 coupled to a B/T cell epitopes from the Der p1 allergen. Co-crosslinking of the BCR and CR1 by this molecule is expected to deliver suppressive signal selectively silencing these B cells only.

Material and Methods: A synthetic peptide, Der p1 p52-71, and anti-CD35 monoclonal antibody were used for the construction of Der p1 chimera. We analysed the effects of the chimeric molecule *in vitro* and *in vivo* using PBMC from allergy patients transferred to the SCID mice. We measured Der p1-specific IgE antibody production by ELISA and determined the B-cell proliferation by ELISpot. We studied the effect of the chimeric molecules on apoptosis by flow cytometry.
Results: We observed significant inhibition of allergen-specific cell proliferation and reduction of specific IgE antibodies in the sera of humanized SCID mice. Expression of phosphatidylserine was changed in CD19+ and CD3+ cells from patients.

Conclusions: The constructed chimeric molecule binds Der p1 specific B-lymphocytes via their BCR and suppresses selectively their proliferation and the production of anti-Der p1 IgE antibodies by co-crosslinking of the inhibitory CR1 *in vitro* and *in vivo*. This way we could alter the allergic immune response towards a milder outcome.

AO2. SELECTIVE ALTERATION OF SELFREACTIVE B AND T CELLS BY CHIMERIC MOLECULES IN A HUMANIZED MOUSE MODEL OF TYPE 1 DIABETES

Iliyan Manoylov[1], Nelly Delcheva[1], Iliana Atanassova[2], Andrey Tchorbanov[1]

[1]Department of Immunology, The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Acad. G. Bontchev Str., Block 26, 1113 Sofia, Bulgaria;
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Autoimmune Diabetes Mellitus (ADM) is an organ-specific disease characterized by the autoreactivity of B and T cells. This leads to the generation of autoantibodies against a large array of self-antigens, related to the control of blood sugar, and as a result hyperglycemia and destruction of pancreatic beta cells are observed. GAD65, a membrane bound enzyme that transforms the Glutamic acid to Gamma Aminobutyric acid, is the main autoantigen in ADM. Autoreactive B cells secrete antibodies that recognize GAD65, but they are also important auto-antigen-presenting cells, needed for the activation of T cells. The elimination of self-reactive B and/or T cells is a reasonable approach for effective therapy of Diabetes Mellitus.
Complement receptor type 1 (CR1) on human B- and T-lymphocytes has a well known suppressive activity. The co-crosslinking of this receptor with the BCR on auto-antigen specific B cells can inhibit their activation and thus is an attractive new target for negative signal delivery.

We suppose that it may be possible to selectively eliminate GAD65 – specific B cells from ADM patients by using chimeric molecules, that contain an antibody against the B-cell's CR1, coupled to peptides identical to GAD65 B/T epitopes. These engineered molecules are expected to be bound selectively by the immunoglobulin receptors of the anti-GAD65 specific B-cells and to induce a strong suppressive signal.
We observed significant inhibition in the proliferation of specific GAD65 autoantibody-producing cells after treating them with the chimeric molecules. The co-culturing of these cells with the same chimeras increased the percentage of apoptotic B-lymphocytes.

AO3. ANNEXIN A1 AS A TARGET FOR MANAGING PRISTANE-INDUCED SYSTEMIC LUPUS ERYTHEMATOSUS

S. Bradyanova[1], N. Mihaylova[1], P. Chipinski[1], S. Chausheva[1], M. Herbáth[2], F. D'Acquisto[3], J. Prechl[2] and A. Tchorbanov[1]

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[2]Immunology Research Group, Hungarian Academy of Sciences, Budapest, Hungary;
[3]Centre for Biochemical Pharmacology, William Harvey Research Institute, Charterhouse Square, London
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Systemic Lupus Erythematosus (SLE) is a heterogeneous autoimmune syndrome. It is characterized by chronic inflamation, B and T-cell hyperactivity, production of autoantibodies against self-nucleat antigens and generation of immune complexes. Annexin A1 (Anx-A1) is Ca-dependent glucocorticoid-induced protein involved in FPR2 pathway, resolution of inflammation and inhibition of phosholipase A2 (PLA2) activity. Our hypothesis is that it is possible to suppress SLE symptoms by therapy with monoclonal antibody against Anx-A1.

The immunomodulatory activity of the therapeutic antibodies was tested *in vivo* and *in vitro* at Pristane-induced Balb/C model of lupus. Splenocytes were incubated with plate-bounded anti-CD3 and anti-CD28 antibodies and then incubated in ranging concentrations of anti-Anx-A1 antibody solution. We tested the effect of the antibody on expression of Annexin A1 and on CD25 and CD69 T cell activation markers. Proteinuria levels, survival and skin lesion formation were examine *in vivo.*

We have found that the AnxA1 is expressed by both the B and T cells of Balb/C mice with pristine-induced SLE and a dose-dependent decrease in the expression of the activation markers CD25 and CD69. Anti-AnxA1 antibody retains the levels of anti-dsDNA antibodies, prevents the appearance of skin lesions and decreases the levels of IgG and IgM against C1q in antibody treated animals compared to the PBS control group.

In conclusion we can say that the generated anti-AnxA1 antibody has a positive therapeutic effects at pristane-treated Balb/C mouse model.

Refferences:

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AO4. TUMOUR NECROSIS FACTOR-RELATED APOPTOSIS INDUCING LIGAND (TRAIL) AS AN ARTHRITIC MARKER IN A MODEL OF OSTEOARTHRITIS

P. Ganova, V. Gyurkovska, N. Ivanovska

Department of Immunology, Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria

Introduction

Osteoarthritis (OA) is one of the most common degenerative age-dependent joint diseases. Experimental models of arthritis are a necessary tool for studying the pathogenesis of these diseases as well as for the testing of new substances for their anti-arthritic activity [8]. Rodent models are very convenient for such studies because of the availability of genetically homogeneous inbred animal strains [1]. Intensive efforts have been made to define the relationships between inflammation and bone and cartilage destruction as well as between different markers and arthritis severity. TNF-related apoptosis-inducing ligand (TRAIL) is a type II transmembrane protein that belongs to the TNF super-family. Five receptors bind TRAIL defined as death (DR4 and DR5), decoy (DcR1 and DcR2), and osteoprotegerin (OPG). In mice only mTRAIL-R2 (mDR5) possesses apoptosis-inducing properties [5]. The potential role of TRAIL in joint inflammation has been proved by the experiments in collagen-induced arthritis induced in TRAIL deficient mice. They are sensitive to the development of joint inflammation, while the administration of soluble DR5 exacerbates arthritis [6]. However, none of the studies comprehensively investigated the role of TRAIL and all its receptors in the development of various types of arthritis. The intraarticular injection of collagenase in mice is

widely used animal model for analyzing molecular mechanisms of OA pathology that is comparable to humans [2]. The disease is characterized with articular degeneration after digesting collagen in cartilage, which caused articular instability, thereby reproducing the main events inherent for osteoarthritis onset and development. The aim of the present study was to elucidate the role of TRAIL in a model of osteoarthritis and to evaluate whether it might represent a reliable marker to characterize the development and prognosis of the disease.

Materials and methods

Collagenase-induced osteoarthritis (CIOA)

The experiments were conducted using 6-8 weeks old Balb/c and SCID mice. The arthritis was induced by intraarticular (i.a.) injection of 1U/10µl collagenase from *Clostridium histolycum* (Sigma-Aldrich, Germany) at days 0 and 2.

Determination of IL-6

Splenocytes and bone marrow cells from 6-week old mice were extracted and the quantity of IL-6 was measured by Mouse IL-6 ELISA MAX™ Standard kit (Biolegend, San Diego, UK) according to manufacturer's instructions.

Isolation of splenocytes and bone marrow cells

Splenocyte suspensions were prepared trough homogenizing the spleens and after lysis of erythrocytes cells were cultivated at a concentration of $2x10^6$/ml in α-minimal essential media (MEM) supplemented with 10% FCS, streptomycin 100 U/ml and penicillin G 100 µg/ml (Sigma-Aldrich). The splenocytes were stimulated with 10 µg/ml LPS or with a combination of 10 µg/ml phytohemaglutinin (PHA, Sigma-Aldrich) plus 10 ng/ml IL-2 (EuroClone). Bone marrow cells were isolated from femur and tibia and cells were resuspended at a concentration of $1x10^6$/ml in MEM supplemented with 10% FCS, streptomycin 100 U/ml and penicillin G 100 µg/ml. For osteoclast differentiation cells were stimulated with 30 ng/ml M-CSF plus 50 ng/ml RANKL (Biolegend, UK) in the presence or absence of 100 ng/ml TRAIL for another 3 days.

Flowcytometry

Synovial fluid cells were harvested and diluted to a concentration of $1x10^6$/ml and incubated with PE-conjugated anti-TRAIL mAb (CD253 clone: N2B2), and FITC-conjugated: anti-CD3e mAb (clone: 145-2C11), or anti-CD22 mAb (clone: OX-97), or anti-CD68 mAb (FA-11) (all from Biolegend). After 20 min cells were rinsed twice and resuspended in PBS.
Splenocytes ($1x10^6$ cells/ml) were incubated with Annexin V (FITC labelled; 5 µl, Biolegend) for 15 minutes and subjected immediately to flow cytometry.

Statistical analysis

Data represent mean ± SD. Statistical significance was assessed using two-way ANOVA, considering a p value ≤ 0.05 as significant.

Results

Effect of TRAIL on apoptosis of splenocytes from Balb/c and SCID mice

The number of apoptotic cells was higher after treating with TRAIL of splenocytes from SCID mice compared to Balb/c mice. TRAIL did not affect the apoptosis of splenocytes of Balb/c mice neither in the control group nor in the CIOA group (Fig. 1).

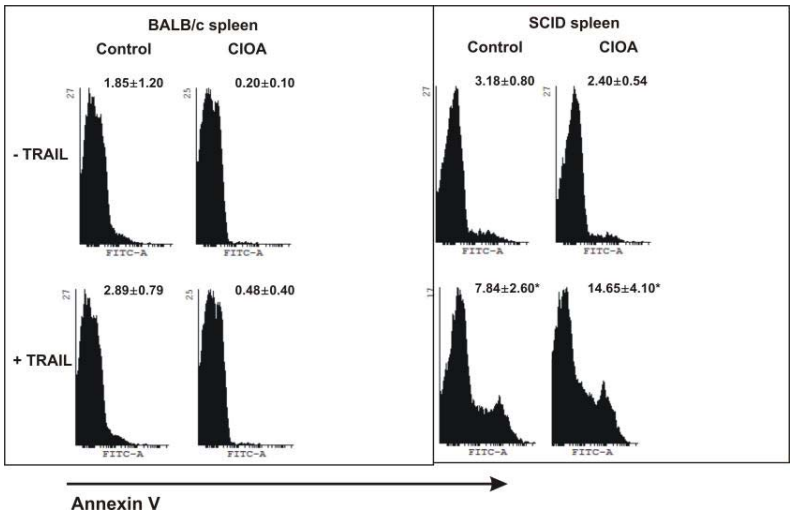


Fig. 1. *TRAIL-induced apoptosis of spleen cells from control and arthritic Balb/c and SCID mice. n=7, *p < 0.05.*

Determination of double positive cells in the synovial fluid at days 18 and 30 of CIOA

At days 18 and 30 the synovial fluids were isolated from control and CIOA mice and tested for expression of TRAIL by T cells (CD3ε), B cells (CD22) and macrophages (CD68) markers. At both time points CD68/TRAIL positive cells from CIOA mice showed significant increase compared to the control group (Fig. 2).

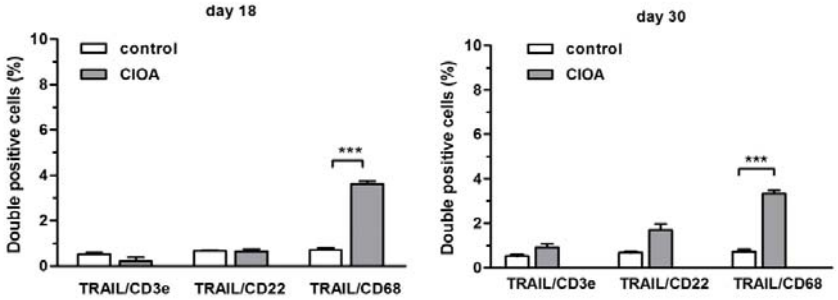
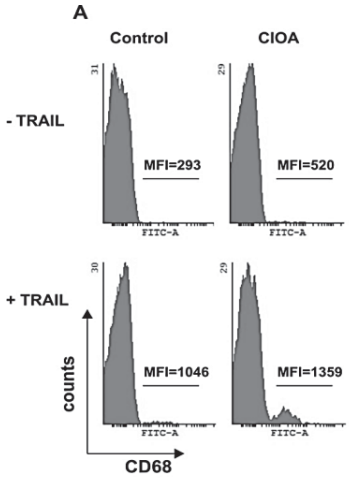


Fig. 2. *Double positive cells in the synovial fluid (n=7).*** p<0.001.*

Influence of TRAIL on differentiation of bone marrow cells

Bone marrow cells from CIOA mice were shifted to CD68 phenotype but not in non-arthritic mice. In the presence of TRAIL the cells expressing CD68 marker were increased in control group and yet more cells were observed in mice with CIOA (Fig. 1A, B).



B

Groups	- TRAL	+ TRAIL
Control	299.5±6.5	1046.0±109.0*
CIOA	520.0±50.0#	1359.2±184.4*

Fig. 3. *Differentiation of bone marrow cells from control and CIOA mice, after in vitro treating with TRAIL. n=7, $^{\#}p<0.05$ vs control, $^{*}p<0.05$ vs TRAIL-treated group*

Secretion of Il-6 by splenocytes and bone marow cells

Splenocytes (Fig. 4A) and bone marrow cells (Fig. 4B) were collected at day 7 of CIOA and cultivated in the presence of indicated stimuli. Unstimulated splenocytes from control and CIOA mice produced approximately equal levels of IL-6. Stimulated cells with LPS increased remarkably the production of IL-6 by both control cells and CIOA cells and the combination of PHA and IL-2 showed similar effect. Bone marrow cells cultivated in the presence of TRAIL showed increased production of IL-6 more expressed in CIOA group.

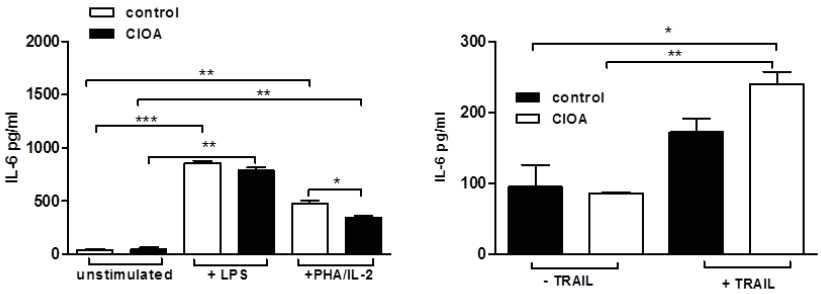


Fig.4. *Secretion of IL-6 by splenocytes **(A)** and BM cells **(B)**. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.*

Discussion

To date, there are only limited data regarding the role of TRAIL in systemic bone loss in arthritic patients but there are no comparative data on its effects on systemic markers or mediators of inflammation in OA. Therefore, the present study investigated TRAIL-mediated mechanisms in collagenase-induced model of arthritis. We tried to answer the question whether TRAIL might serve as a specific marker for OA. The progression of the disease is associated with an overwhelming number of non-fibroblastic inflammatory cells. Through regulation of apoptosis in these cells key disease processes might be modulated. Many data indicate that the effector mechanism in rheumatoid arthritis is T cell driven and leads to the development of aggressive synovial pannus formation. The synovial tissue is also an active site of B-cell accumulation where B cells secrete chemokines and cytokines and may function as antigen presenting cells [7]. Importantly, TRAIL triggered SCID splenocytes from non-arthritic mice to apoptosis, in contrast to healthy BALB/c mice. After double labeling we observed that TRAIL+/CD68+ cells represent relatively significant part at active and chronic phase of CIOA. This suggests that in a result of appoptic processes their pro-inflammatory potential might be limited. The remodeling of bone is a process that involves the removal of bone by osteoclasts and the formation of bone by osteoblasts. Osteoclast progenitors and other cell lineages are recruited from bone marrow to bone via circulating blood [3]. We found that TRAIL stimulated the differentiation of bone marrow progenitors towards CD68 phenotype when cells were isolated from healthy mice. Cells from arthritic mice also showed significant enhancement of M-CSF+RANKL induced CD68 differentiation when TRAIL was added to the culture medium. At the same time, TRAIL did not influence CD3 differentiation neither of control nor of arthritic BM cells (data not shown). One of the cytokines that are elevated in the peripheral blood and synovial fluid in patients with OA is IL-6 [4]. The increased expression of IL-6 under the action of TRAIL could promote osteoclast differentiation resulting in enhanced bone resorption. CIOA cells showed different sensitivity to nonspecific stimulation, being responsive

to LPS and in lower rate to PHA/IL-2 action. It should be noted that the stimulation correlated with an increase of IL-6 production. In conclusion, TRAIL although concerns the CIOA development is not firmly expressed as OA marker.

Acknowledgements
This work was supported by Grant № DFNI B-01/6 from the National Science Fund, Ministry of Education and Science, Bulgaria.

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AO5. TARGETING OF VIRUS EPITOPES TO ANTIGEN PRESENTING CELLS VIA CHIMERIC MOLECULES IN HUMANIZED SCID MICE

D. Hlebarska[1], I. Ivanova[1], N. Mihaylova[1], I. Manoylov[1], D. Makatsori[2], A. Mamalaki[2], J. Prechl[3] and A. Tchorbanov[1]

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[2] Hellenc Pasteur Institute, 127 Vassilissis Sofias Avenue 115 21, Ampelokipi, Athens, Greece;
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DNA vaccination is a novel strategy for protecting an organism against disease by injecting it with genetically engineered DNA to produce an immunological response. In the present study we have created a chimeric gene-engineered DNA molecule, encoding a single-chain variable fragment (scFv) from a monoclonal antibody to FcγRI coupled to the influenza hemagglutinin peptide HA317-41 and inserted into the eukaryotic expression vector system pTriEx. After expression *in vivo* the gene-engineered molecule is expected to bind selectively to FcγRI expressed by the antigen presenting cells and to deliver to them a strong activating signal via their surface FcγI (CD64) receptors.

Four different chimeric DNA molecules were constructed using a variety of molecular-biological methods as PCR, gene-engineering, cloning and plasmid amplifying. Plasmid DNA from all 4 clones was isolated and purified. Each chimeric molecule was introduced into the eukaryotic expression vector pTriEx3-Neo. Some of the gene constructs were transfected in CHO cell line which is capable of long-term foreign protein production. The expressed chimeric molecule was isolated, purified and concentrated by ultrafiltration.

Using flow cytometry it was confirmed the ability of the DNA chimera to interact with FcγRI as it competed successfully with a FITC-conjugated antibody with the same specificity for binding to cell line U937 that expresses FcγRI.

We hypothesize that it may be possible to generate protective serum levels of anti-influenza IgG and IgM antibodies and CTL activity against influenza virus-infected cells in humanized SCID mice. In the present study we shall investigate the response induced after immunization with the DNA chimeric molecule. The vaccination with naked DNA vaccine will be followed by immunization with the same expressed in vitro protein chimeric molecule.

AO6. DEVELOPMENT AND APPLICATION OF A SPECIFIC FLUOROGENIC SUBSTRATE FOR THE CYTOCHEMICAL STUDY OF FIBROBLAST ACTIVATION PROTEIN ALPHA

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Fibroblast activation protein alpha (FAPα, EC 3.4.21.B28) is a serine type transmembrane endopeptidase of the group of post-proline cleaving proteases. It is known to participate in the normal or pathological remodeling of extracellular matrix throughout its collagen cleaving capacity. The enzyme has a very low expression levels in normal tissues of humans and mammals. However, more than 90 % of the carcinomas and lots of sarcomas were shown to overexpress the enzyme both in the tumor reactive stroma and tumor cells. Moreover,

FAPα overexpression was recently detected also in chronic inflammatory conditions like rheumatoid arthritis, Crohn's disease and cirrhosis. Consequently, FAPα is considered as an important surface marker for activated fibroblasts as well as a valuable marker and a target for therapy of many tumor and inflammatory diseases. Studies on the expression of FAPα in normal and pathologically changed tissues and cells are usually performed using immunohisto-/immunocytochemistry. No cytochemical substrate for this enzyme is available thus far. In this study we present the development of a specific fluorogenic substrate for FAPα and its application for the fluorescent cytochemical localization of the enzyme in fetal and tumor mouse fibroblasts and in tumor cells from human mammary gland carcinoma. The cytochemical method for FAPα presented here can be useful for determination of the enzyme marker value in different diseases.

Introduction

Fibroblast activation protein alpha (FAPα, EC 3.4.21.B28) is a type II transmembrane glycoprotein, belonging to the small family of post-prolyl cleaving serine proteases [10]. The enzyme shows a very restricted normal tissue distribution - it is expressed only in embryonic fibroblasts, reactive fibroblasts in healing wounds and pancreatic islet A cells. However, over 90 % of epithelial tumors and lots of sarcomas were found to be FAPα-positive [11]. The enzyme is usually expressed in the surface of reactive stromal fibroblasts, but in some malignancies like breast ductal carcinoma, colorectal and gastric cancers and melanoma, it was found also in tumor parenchymal cells [4, 5]. FAPα has been recognized as an independent prognostic factor in breast and colon cancers. FAPα overexpression was recently detected also in other diseases like rheumatoid arthritis, liver and lung fibrosis, etc. Its marker value for these disorders is to be elucidated in future [for review see 6].

FAPα is known to possess both exo- and endopeptidase activities. As an exopeptidase, it acts on similar substrates as dipeptidyl peptidase IV (DPPIV, EC3.4.15.4). As an endopeptidase, it hydrolyzes collagen type I and exactly the collagenolytic activity governs the enzyme participation in tumorigenesis and tumor progression. Presently, there is considerable interest in identifying specific peptide substrates for FAPα, based on the following ideas: 1). such substrates would permit the *in situ* differentiation between FAPα and DPPIV activities using simple peptide probes; 2). since the inhibitors are usually structural analogues of the substrates, it would be possible to obtain specific FAPα inhibitors thus opening new possibilities for innovative anticancer therapies.

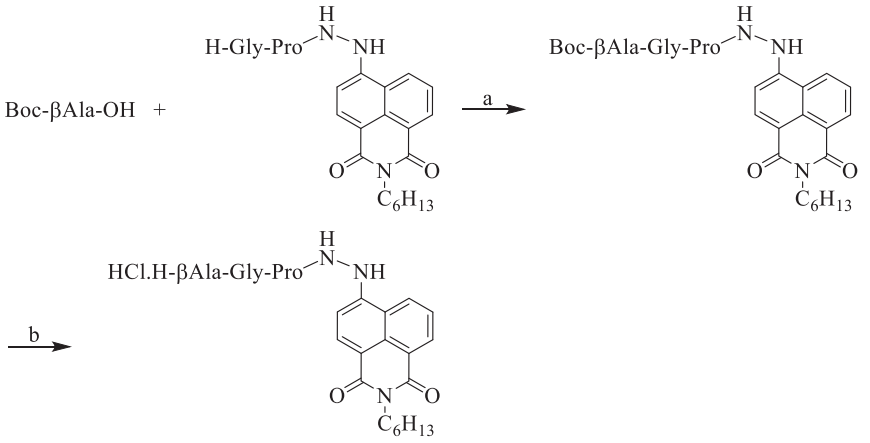
In the present paper we describe the molecular design and development of a specific peptide substrate for FAPα as well as its application for the imaging of the enzyme activity in preparation of cultured cells of different origin.

Materials and Methods

Molecular modelling. The structure of enzyme-substrate complex with docked substrate is presented on Fig. 1. This Michaelis complex was optimized by molecular mechanics calculations with the Drieding force field [9], based on the human FAPα structure [1] (PDB ID 1Z68).

Synthesis of the FAPα substrate 4-(β-Ala-Gly-Pro-hydrazido)-N-hexyl-1,8-naphthalimide (βAGP-HHNI). The chemical synthetic pathway of the FAPα substrate is

presented in Scheme 1. The N-tert-butyloxycarbonyl protected tripeptide hydrazide was obtained in high yields by applying the standard protocol with TBTU as coupling reagent. As a first step, the activation of the Boc-β-alanine was carried out by its conversion to benzotriazole active ester using TBTU. Subsequently H-Gly-Pro-HHNI [3] is added to the reaction mixture. The substrate for FAPα was obtained by cleaving off the protective group from the Boc-β-AlaGlyPro-HHNI by 4 M HCl/dioxane. The substrate and all the intermediates were purified by column chromatography and characterized by nuclear magnetic resonance. The precise reaction conditions, purification and results from the spectral analyses will soon be published elsewhere.



Scheme 1. Reagents and conditions*: (a) TBTU, HOBt, DIPEA (2 eq) in DMF, 1h, rt; (b) 4 M HCl/dioxane; H-Gly-Pro-HHNI was synthesized via the method reported previously [3].

*Abbreviations: TBTU - O-(Benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium tetrafluoro-borate; HOBt - 1-Hydroxybenzotriazole; DIPEA - N,N-Diisopropylethylamine; DMF – Dimethyl formamide; HHNI - 4-Hydrazino-N-hexyl-1,8-naphthalimide

Cell culturing and cell preparations. In the experiments the following cell lines were used: BALB/3T3 clone A31 (mouse embryonic fibroblasts), Meth-A (fibroblasts from methylcholanthrene-induced fibrosarcoma in mouse) and MDA-MB-231 (human highly metastatic carcinoma of the breast). The cells were cultured on cover slips (BALB/3T3 and MDA-MB-231) or in 75 cm^2 tissue culture flasks (Meth-A) in Dulbecco's Modified Eagle's Medium – high glucose 4.5‰ (DMEM), supplemented with 10% fetal calf serum and antibiotics in usual concentrations. Cell cultures were maintained at 37.5°C in a humidified atmosphere and 5% CO_2 until 95 % confluence in the case of adhesion cell lines (BALB/3T3 and MDA-MB-231). In the case of mouse fibrosarcoma cells, after the cultivation the cell suspension was centrifuged at 1500 rpm for 5 min and smears were made on cover slips. All the cell preparations were washed in PBS, air-dried and fixed in para-formaldehyde vapors for 4 min at room temperature. Then, they were covered with 0.5 % collodione (Sigma-Aldrich) for 30 sec immediately before use.

Incubation solutions and controls. The cells were incubated in solutions containing 0.5 mM FAPα substrate βAGP-HHNI and 0.5 mg/ml piperonal in 0.1 M phosphate buffer, pH 7.4, supplied with 100 mM NaCl at 37°C for 16 h. After the incubation, they were post-fixed in 4 % neutral formalin for 15 min at room temperature and embedded in glycerol/jelly. Control

samples were treated in the same manner but in the lack of substrate in the incubation solutions. All the preparations were observed under the fluorescent microscope Leica DM5000B (USA).

Results and discussion

FAPα expression in adult tissues is generally considered to be linked with pathological processes such as development of epithelial tumors, some sarcomas, inflammatory diseases, etc. The enzyme was found both in reactive stromal fibroblasts and tumor cells in many types of carcinomas. Its collagenolytic activity is believed to support tumor cell invasion and angiogenesis. However, FAPα overexpression in cancer is not exclusively connected with increased metastatic potential. Thus, in colon cancer the higher FAPα levels are associated with a poor prognosis, whereas in breast cancer those are related to a prolonged survival [6, 8]. In order to study the marker value of FAPα for different types of oncological and other diseases, specific peptide substrates for the enzyme are needed. The main difficulty in designing specific peptide probes for FAPα is its high structural similarity with DPPIV – another membrane-associated post-prolyl cleaving peptidase. According to the recent studies, the two proteases share 70 % catalytic domain homology [7]. The main difference in substrate specificities of FAPα and DPPIV is that FAPα has high endopeptidase and low exopeptidase activity, while exactly the opposite applies for DPPIV. The recently developed specific probes for FAPα show a high capacity of the enzyme to cleave off acetyl-Gly-Pro-based substrates [7]. However, such substrates are not suitable for enzyme cytochemical studies due to the very low substrate solubility in aqueous media.

Our novel cytochemical substrate for FAPα – βAGP-HHNI (Scheme 1) has the advantage of a better water solubility due to the free NH_2-group of β-Ala. On the other hand, β-Ala could not be recognized and cleaved off by other protease before the action of FAPα. Finally, this substrate is not appropriate for DPPIV as seen from the molecular modelling studies. The molecular model of the enzyme-substrate complex using the novel substrate, designed by us - βAGP-HHNI is presented in Fig.1. It shows that Oη atom of Tyr541 forms a hydrogen bond with carbonyl oxygen of proline of the substrate. The Oγ atom of Ser624 is at a distance of 3.42 Å from the carbonyl carbon of proline in P_1-position, favorably disposed for a nucleophilic attack. Nη1 atom of Arg123 forms hydrogen bonds with Oε1 of Glu203 and carbonyl oxygen of glycine of the substrate. Nα atom of glycine in position P_2 forms hydrogen bond with Oε1 of Glu203 and Nα atom of β-alanine in position P_3, forms hydrogen bond with Oη of Tyr124.

Our novel substrate was synthesized and tested for the localization of FAPα in cytochemical preparations of three types of cell lines (Fig. 2). Normal mouse embryonic fibroblasts showed a very low enzyme activity (Fig. 2A), whereas the tumor mouse fibroblasts had a visibly higher FAPα activity revealed by the substantially stronger fluorescent signal (Fig. 2B). This result corresponds to the previous findings that in fetal tissues the enzyme expression levels are lower than those in pathologically altered tissues and cells. A high FAPα activity was observed also in human tumor cells from the invasive ductal breast carcinoma (Fig. 2C) which is in accordance to the previous results that FAPα is expressed in breast carcinoma cells [2]. The cytochemical controls did not show any non-specific fluorescent precipitates of the samples incubated in the lack of substrate (Fig. 2D).

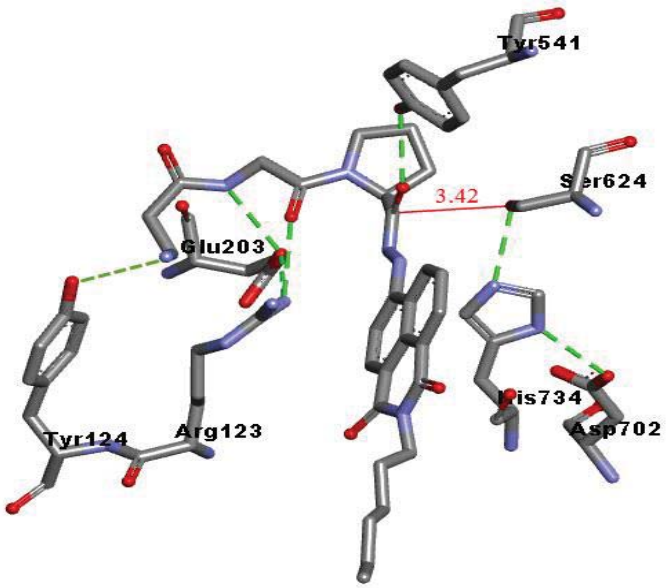


Fig.1. Possible mode of binding of βAGP-HHNI substrate in the active site of FAPα. The molecular model of the complex shows the catalytic triad Ser624–His734–Asp702 of the enzyme, Tyr541 constituting the oxyanion hole, Arg123, Tyr124 and Glu203.

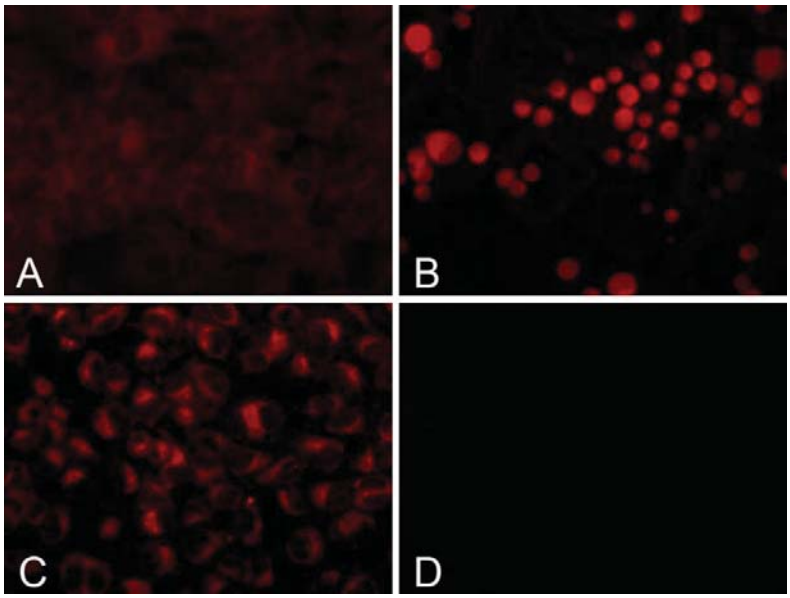


Fig.2. Fluorescent imaging of FAPα activity using the substrate βAGP-HHNI. Low enzyme activity in embryonic mouse fibroblasts of BALB/3T3 line (A). High activity in mouse fibrosarcoma fibroblasts of Meth-A cell line (B) and in human breast carcinoma cells – line MDA-MB-231 (C). Lack of non-specific fluorescent products in a control sample (D). Magn. 400X

In conclusion, we developed the first specific substrate for the *in situ* imaging of FAPα as revealed by the molecular model studies and enzyme localization results in different types of cultured cells. The cytochemical method for FAPα presented here can be useful for determination of the enzyme marker value in different tumor and/or other diseases.

Acknowledgements: This work is financially supported by the National Science Fund of the Bulgarian Ministry of Education and Science, Grant № T02/25 2014.

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AO7. *SACCHAROMYCES CEREVISIAE* – A BRILLIANT MODEL FOR STUDYING AGEING

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Abstract
The ever utilitarian yeast - *Saccharomyces cerevisiae* has set in stone its place in Molecular Biology as a model organism especially when bridging a given hypothesis to a more practical use in higher eukaryotes. *S. cerevisiae* is traditionally deployed as a model in many experiments because of its genetic tractability; because of its ability to exist as either haploid or diploid cells and due to its aptitude to form discrete colonies on a simple defined media. All this makes *S. cerevisiae* perfect for studies of organismal life cycle and metabolism, and importantly for the investigation of the many, quite dynamic and multifactor effects of ageing.
The purpose of this overview is to give examples of the study of biological ageing with the use of *S. cerevisiae* as a model organism by referencing experiments which clarify some of the hallmarks of ageing, and their elucidation through the use of this model organism. Specifically, this overview will focus on chromatin dynamics and its involvement in ageing. Collected data from *S. cerevisiae* mutants will be presented highlighting the significance of the interaction between the linker histone H1 and Arp4p, which is an essential subunit of chromatin remodeling complexes (INO80, SWR1 and NuA4). This interaction and its implication for chromatin structure maintenance will be discussed in the light of cellular lifespan and ageing.

Keywords: *S. cerevisiae*, ageing, linker histone, Arp4p, chromatin, model organisms, Molecular Biology

1. *Introduction*

There is a constant need for good biological model systems in in molecular biology studies. The real purpose of a model system is to overcome ethical and experimental constraints and at the same time to provide framework on which to develop and optimize different analytical methods. Concomitantly it has to bridge the process under evaluation to a practical purpose. Putting this into perspective we will review *Saccharomyces cerevisiae* a model which is convenient to study one of the most unsurmountable problems in modern life sciences known as ageing. There have been many articles related to ageing and most of them exploit the same paths in dealing with the multifactor nature of ageing. The extensive research has consequently followed many features of ageing prospecting the idea of nine key hallmarks that vanguard this

proses. The most important of these hallmarks are genomic instability, shortening of telomeres, epigenetic changes and metabolic deregulation. Exemplary is the genomic instability which can be manifested as the effect of exogenous chemicals, biological agents or errors in DNA replication and repair resulting in DNA damage. On the other hand, telomere shortening perquisites faster reaching of the proliferation limit. At the end, the epigenetic changes exhibit increased acetylation of histones H4 and H3 at H4K16, H4K20, H3K4 and H3K27 which also serve as markers for aging [1].

Here, we would like to explore the ageing pattern in a different way. The chosen platform for ageing based research is chromatin structural integrity with the main focus being on the role of linker histone H1 and Arp4p as the stoichiometric subunit of chromatin remodeling complexes.

2. *Chromatin architecture and remodeling*

We will briefly describe the basics of chromatin organization and its place in living beings. Chromatin is a DNA- protein complex through which DNA is compacted in the eukaryotic nucleus. The most basic building unit of chromatin is the nucleosome. Structurally the nucleosome is composed by four histones and a fragment of DNA. Histones are small proteins rich in the basic amino acid lysine and arginine. There are five types of histones in most eukaryotic cells H1, H2A, H2B, H3, H4. The four histones H2A, H2B, H3, H4 are associated in a compact structure known as the nucleosome. The histone - H1 differ from the other histone proteins in that it takes no part in the structural integrity of the nucleosome [2], but rather participate in the building and maintaining of the higher levels of chromatin organization (Finch, 1976; Happel, 2009; Thoma, 1979). This overview will shine more light on H1 linker histones effect on chromatin structural integrity and its role in cellular senescence and organismal ageing. The H1 linker histone is located on the outside of the nucleosome core and is adjacent to the place where DNA enters and exits the nucleosome. H1 possesses a globular core domain, short N-tail and a long C-terminal tail; the tails can interact with core and linker DNA. In the second level of chromatin compaction the nucleosomes form a “beads-on-a-string” like structure; this structure is known also as the 10nm fiber [3]. The next topologically preferable structure comes in the form of the 30nm fiber. The presence of the linker histone is important for the stabilization and formation of the 30nm chromatin fiber [4]. There are several models that describe higher-order structures of chromatin. One of them is the so-called “hierarchical helical folding model” which postulates that the 30nm chromatin fiber is folded into a bigger 100nm fiber and after that into 200nm in order to form chromatin loops and finally chromosome territories or mitotic chromosomes [5]. Proper chromatin dynamics ensures proper chromatin architecture which provides certain levels of gene regulation. Proper chromatin architecture is regulated by diverse remodeling complexes for example, INO80, NuA4 and SWR1. These complexes interact with the nucleosome and remodel the chromatin by sliding the nucleosomes along the DNA molecule or changing the histone proteins in the nucleosome. It has been hypothesized that the regulation of the nuclear processes is exerted through contacts with linker histones, which is possible through the Arp4p subunit. Arp4p is an actin-related protein and is a stoichiometric component of chromatin remodeling complexes such as INO80, NuA4 and SWR1.The importance of Arp4p was demonstrated when the mutated form was shown to cause impairment of the functions of these complexes [6].

3. *S. cerevisiae as an evergreen model system*

After this short summary of the chromatin basics we will give examples for the use of *S. cerevisiae* as a model system for the study of chromatin dynamics by referencing two of our articles from the recent four years. One of them provides results on the effects the linker histone

has on chromatin dynamics while the other solidifies those findings and investigates the interaction between the linker histone H1 and Arp4p. The model system of choice in both studies is one of the best models for ageing research in the name of the unicellular eukaryote *S. cerevisiae* [7, 8]. Yeast cells have been traditionally preferred as a model system for the study of organismal life cycle and metabolism. *Saccharomyces cerevisiae* was selected as a model system in these experiments for its genetic tractability. This allows the creation of mutants that either completely lacks a gene or even mutants that bear a mutation in important subunits of different chromatin proteins or complexes. These yeast cells could be easily selected by their ability to form discrete colonies on selective media [9]. In addition many of the stages and the profound molecular mechanisms related to ageing have been reveled in this yeast [10].

4. *Chromatin remodeling mutants exhibit explicit changes in chromatin architecture*

Notably, in contrast to higher eukaryotic cells which possess a dozen of H1 subtypes yeast cells possess only one copy gene (*HHO1*) for the linker histone. *HHO1* encodes for the protein known as Hho1p. And though that gene is not essential for the survival of the cells, its disruption alters higher-order chromatin structures and leads to premature ageing phenotypes [11, 12]). These results reveal a more intricate function exhibited by the association of H1 with chromatin especially when the ageing process is referred. The results from the experiments emphasize the role which *S. cerevisiae* linker histone Hho1p could play in the propagation of the ageing process. Both studies used yeast cells devoid of linker histones which resulted in slower growth of the cells in minimal media and in a pronounced delay of the mutants of their logarithmic growth accompanied with morphological abnormalities. As an emanation of these experiments it has been discovered that chromatin loops in the cells without the linker histone are longer in size than the chromatin loops in wild type chromatin and moreover, the mutants were with completely distorted higher-order chromatin organization which was more pronounced as the age of the cells increased [11]. These findings redirected the interest to chromatin remodeling complexes and their possible association with the linker histone. It was demonstrated that linker histone Hho1p in *Saccharomyces cerevisiae* interacts with Arp4p. The study used the yeast-two hybrid system in order to search for a possible protein interaction between Hho1p and Arp4p. This study analyzed the consequences of the Hho1p and Arp4p interaction by using a wild type, *hho1Δ*, *arp4* and *arp4 hho1Δ* mutant strains. Following the experimental assessment of the growing of cultures by measurement of their optical density at 600 nm it was shown that the four strains grew similarly until the single *arp4* mutant started to delay. At the end of the experiment it was established that the cells of *arp4* mutant were growing two times slower than the cell of other strains. It should be noted that the double mutant exhibited similar growth curve as the wild type and *hho1Δ* [13]. Previous experiments on the same mutants using Atomic Force Microscopy (AFM) revealed some interesting features of their chromatin. Isolated nuclei from the four strains were subjected to micrococcal nuclease digestion and further fractionation of the obtained chromatin fragments [12]. The isolated fragments were analyzed under AFM. The results clearly showed that when Hho1p is missing or Arp4p is mutated the chromatin structure is severely disorganized. When both mutations are joined in the double mutant the chromatin structure is totally disrupted.

5. *Conclusion*

Overall the reviewed studies revealed that the higher-order chromatin organization is abolished during ageing in the studied chromatin mutants which led to aberrant morphological phenotype. This pushed us one step further in the understanding of the epigenetic significance of the properly organized higher-order chromatin structures. Future studies will make possible to use

the gained knowledge in the solving of the practical issues regarding all aspects of the life and the ageing of living creatures.

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AO8. CHROMATIN - THE MAIN PLAYER IN THE AGEING PROCESS

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Abstract

Ageing is the major cause for diseases predominantly known as age-related diseases and also for the death in modern times. This process is characterized by accumulation of structural and metabolic aberrations which reduce cellular stress resistance and make organisms susceptible to environmental stimuli.

At least nine hallmarks of ageing are suggested to appear with the ageing of organisms, among which genome instability, shortening of telomeres, epigenetic changes, and metabolic alterations. Generally, genome stability is constantly attacked by exogenous chemicals as well as by intrinsic factors (errors in DNA replication and non-effective repair mechanisms) all this leading to DNA damage and thus to ageing. Chromatin as the platform for most epigenetic changes is the major constituent responsible for genome stability. Several lines of evidence have shown that the basic epigenetic role of chromatin in ageing is accomplished at all levels of its organization but the higher levels of compaction and their dynamics during ageing are yet poorly understood.

In the current experiments we have worked with mutant *Saccharomyces cerevisiae* cells whose chromatin structure has been disrupted. In these cells we have followed the role of the linker histone H1 and also of Arp4p, which is an important component of three chromatin remodeling complexes, during the process of chronological ageing of all studied yeast cells. We have further detected changes that occur in the cell cycle, cellular viability and the morphology of these cells after irradiation with ultraviolet light.

Keywords: chromatin remodeling complex, linker histone, Arp4, UVC, ageing

I. Introduction

Ageing remains one of the most studied and questioned biological processes for all times and its molecular base is being examined by numerous methods and different model organisms. A substantial amount of information on individual gene products, metabolic pathways, signal transduction cascades, environmental factors, and cellular mechanisms impacting ageing is available, but a deeper insight into how all these components interact under specific conditions and how they contribute to the process of ageing is yet not available. Amongst the many model organisms yeast cells have been the most exensively used model organism for identifying the genes and pathways involved in basic cellular processes such as the cell cycle, ageing, and stress response [1].

The genome of eukaryotic organisms is organised in chromatin. Chromatin is a nucleoprotein complex by which DNA is compacted and organised. It is involved not only in

packing, but also in the implementation of major regulatory effects on gene activity. It is normal to assume that during the process of cellular ageing the organization of chromatin and thus its regulatory role inevitably undergoes drastic changes. However, the transition of chromatin regulation from a young to an old cell is scarcely studied and yet far from being understood.

The purpose of this work was to study and characterize the process of ageing in *Saccharomyces cerevisiae* yeast strains, mutants in chromatin organization.

II. Materials and methods

1. Yeast strains:

In the current experiments we have used four strains of *Saccharomyces cerevisiae* with genotypes shown in Table 1.

WT (wild type)	MAT a *his 4-912 δ ADE-2 his 4-912 δ lys 2-128 δ can 1 trp1 ura3 ACT 3*
Δ*H1* (without the gene for the linker histone)	MAT a *his 4-912 δ ADE-2 his 4-912 δ lys 2-128 δ can 1 trp 1 ura 3 act 3 ypl 127C:: K.L. URA3*
arp4 (with a point mutation in the *ARP4* gene)	MATa *his4-912δ-ADE2 lys2-128δ can1 leu2 trp1 ura3 act3-ts26*
arp4 Δ*H1* (double mutant, with a point mutation in *ARP4* gene and without gene for the linker histone)	MATa *his4-912δ-ADE2 lys2-128δ can1 leu2 trp1 ura3 act3-ts26 ypl 127C::K.L.URA3*

Table 1. Genotypes of the used four yeast strains.

2. Cell growth

Cellular growth was assessed by measuring of yeast cultures density (OD) at 600 nm. Yeast cells have been cultivated in minimal media (2% dextrose; 1.7% yeast nitrogen base and the amino acids: lysine, adenine, histidine, tryptophan, and uracil for WT and mutant leucine for *arp4*, *arp4 /H1Δ*, at 30 ^{0}C at constant rotation. At three time points - 6th hour, 48th hour and at the 5th day the optical density of the cultures has been measured.

3. Assessment of the cell viability after irradiation with UV-C light

To follow the mutant's viability after UV-C stress, we cultivated the cells at 30 ^{0}C, with constant rotation. At certain time points aliquots were taken and 100 cells were seeded on agar containing rich YPD media (1% yeast extract, 2% dextrose, 2% peptone, 1,5 % agar). After seeding the cells were irradiated with different doses of UV-C light, received for 10 or 20 seconds, respectively. Irradiated cells were incubated for 48 hours at 30 ^{0}C, and colony forming units were counted.

III. Results and discussion

Ageing consists of naturally appearing alterations in the cells which are implemented by molecular programs written in the genome and in the epigenome at the same time. The organization of DNA in chromatin enables the epigenetic information transfer to nuclear

processes. Several lines of evidence have shown that the basic epigenetic role of chromatin in ageing is accomplished at its higher levels of compaction [2].

For the aim of our experiments we have worked with four yeast strains *Saccharomyces cerevisiae* – WT, *H1*Δ*, arp4, arp4/H1*Δ. WT is a wild type strain, progenitor of the other three mutants, *H1*Δ is a mutant strain with deletion of the gene *HHO1*, coding for the linker histone. [2-4]. The other mutant, *arp4*, has a point mutation in gene *ARP4*, which is essential for the integrity and function of at least three chromatin remodelling complexes (INO80, SWR1 and NuA4) [5]. The third mutant combines the mutations of the previous two and therefore was called the double mutant.

The kinetics of cultures' growth is presented in Figure1.

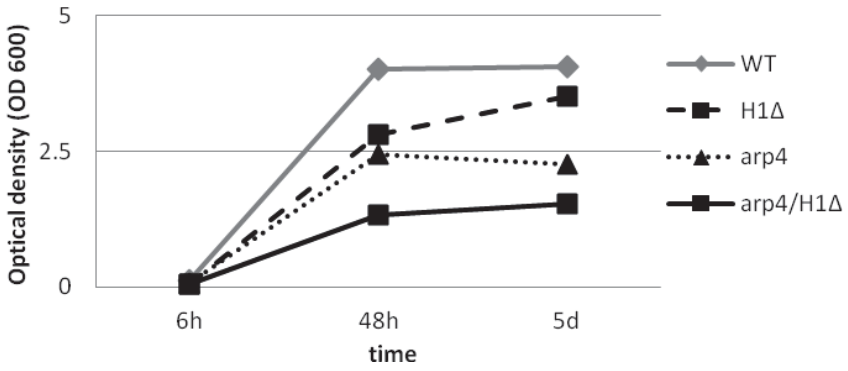
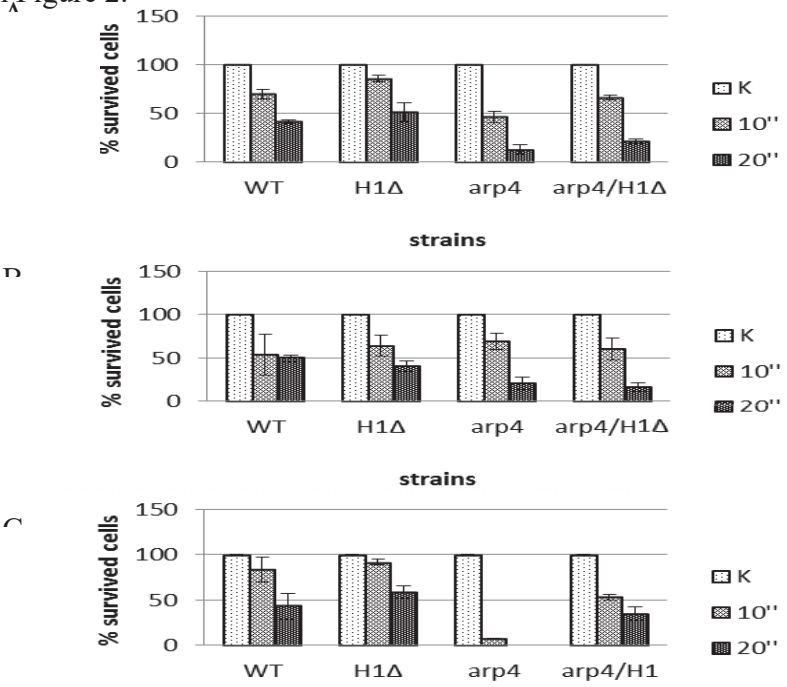


Fig1. A grap representing the yeast cell cultures' growth of the studied strains.

Results showed that all the three mutant strains grow slower than the WT. Notably, the double mutant *arp4/H1*Δ has the slowest growth in comparison with the other strains. Obviously, the impaired gene expression, caused by the severely disturbed chromatin organization in the double mutants, has slowed down the cell division in these cells.

In the next step of our investigation we have conducted a series of experiments to trace the survival of cells irradiated with UV-C light. The cells were cultivated in minimal media at 30 °C, at constant rotation and were UV-irradiated at three stages of their development – 6th hour, 48th hour and 5th day as was described in Materials and methods. The results are presented on Figure 2.



C

Figure 2. Cellular viability after UV-C light irradiation of the studied cells:
A- Rate of survival of the cells, irradiated with UV-C light at 6th hour of culture development;
B –cells irradiated at 48th hour;
C- cells irradiated at 5th day.

We have found that lower doses of UV-C radiation made no difference in the ability of cells to cope with this stress. However, for the three time points of the experiment, *arp4* mutant and the double mutant *arp4/H1*Δ showed lower survival rate in comparison with the WT and *H1*Δ, thus pointing to the fact that they are obviously more sensitive to irradiation with UV-C light.

The obtained results could be explained with the severely disrupted chromatin structure of these mutants, a phenomenon that has been shown in our previous publications [3]. Something more, this conclusion could be reinforced by the data obtained by other authors [6].

The characterization of these three mutants in chromatin structure leads to the suggestion that chromatin is important for the fine regulation of the cellular genome. This regulation can be broken if some of the proteins involved in these mechanisms are affected. As is in this case the result of such mutations could not necessarily fatal to the cells in short periods of time. In a longer perspective, however, such mutations can totally diminish cellular population by changing cellular morphology, by slowing the rate of cellular division, accelerating ageing, and making cells more sensitive to stress. Therefore, we argue that the linker histone H1 is one of the crucial players in the fine regulation of the genome stability and expression during ageing and under stress conditions.

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AO9. APPLICATION OF 3D QUANTITATIVE DNA METHYLATION IMAGING FOR STUDYING THE GLOBAL METHYLATION STATUS IN SERTOLI CELLS

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Sertoli cells are essential for the process of spermatogenesis, where they nourish and protect the developing sperm cells. Sertoli cells also act as phagocytes in order to eliminate apoptotic spermatogenic cells. In addition to their pivotal function in spermatogenesis, Sertoli cells play a major role in the testicular immune privilege. The bulk of testicular antigens lie behind the blood-testis barrier, which is created by highly specialized tight junction between adjacent Sertoli cells. In addition to their barrier function, Sertoli cells provide an immunoprotective environment for the developing sperm cells via the secretion of immunosuppressive cytokines (TGFβ1), inhibitor of granzime B mediated apoptosis (serpina3n) and inhibitors of the complement. Sertoli cells also express on their surface FasL and B7-H1. The former one can serve as a potential mechanism by which lymphocyte access to seminiferous epithelium can be prevented, while the latter one is a ligand inducing apoptosis in antigen-specific T-cells. Given their important role in both spermatogenesis and immune tolerance, any disturbances in Sertoli cell function could lead to impaired spermatogenesis and transient or permanent male infertility. DNA methylation is a crucial epigenetic modification, which is involved in regulating many cellular processes. It plays a key role in the regulation of gene expression, nuclear architecture and genome stability. Therefore, the higher genome organization of DNA provides an additional layer of information, which can shed light on the effect of different environmental factors and compounds on Sertoli cells function. We developed a protocol for 3D image based DNA methylation imaging, which was optimized to work in Sertoli cells, using classical immunocytochemical staining procedures followed by acquisition of Z-stacks via a confocal laserscanning microscopy. The image analysis was performed using the Tools for Analysis of Nuclear Genome Organization (TANGO) software. We found that the use of a primary monoclonal mouse anti-5-MeC antibody combined with a following FITC-conjugated polyclonal rabbit anti-mouse IgG and DAPI counterstaining produced high resolution 3D images, where the DAPI channel was used to construct the 3D form of the nuclei, while the FITC channel gave more than three thousand 3D globular like objects representing DNA methylation enrichment regions. Each 3D globular like object had its own coordinates, which could be used for comparison between Sertoli cells with normal and impaired function, as well as Sertoli cells treated with different compounds including activators of the innate immunity signaling and potential novel therapeutics. We concluded that this protocol for 3D image based DNA methylation imaging is favorable to research the global methylation status in Sertoli cells.

АО10. ЕПИГЕНЕТИЧНИ ПРОМЕНИ, СВЪРЗАНИ С БИОАКТИВНИТЕ ХРАНИТЕЛНИ ДОБАВКИ

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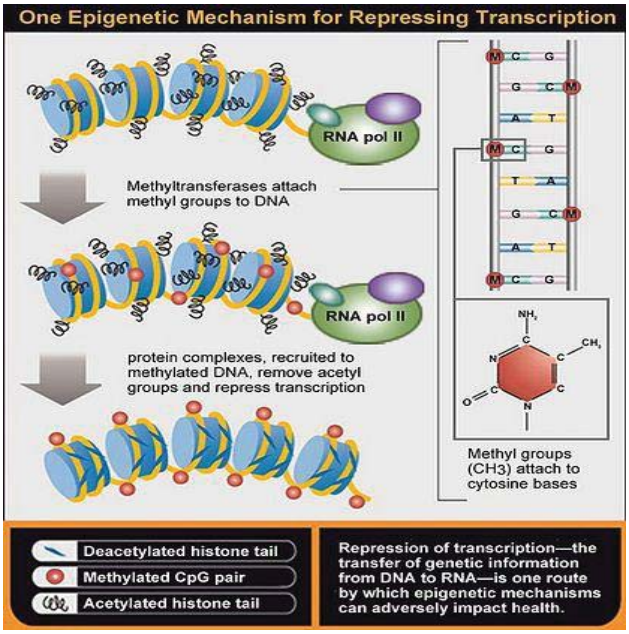
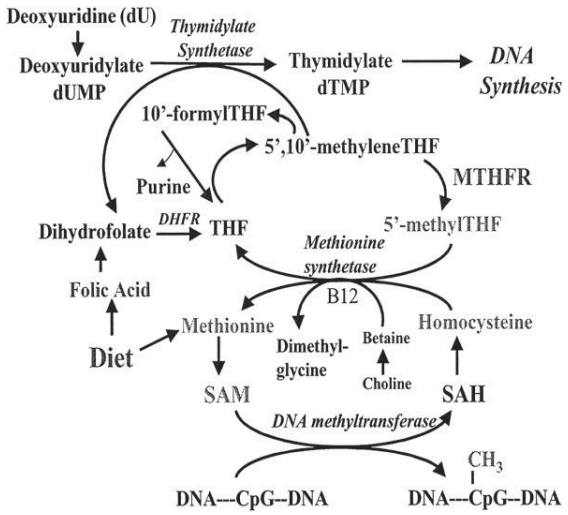
В днешното забързано ежедневие са навлезли най-различни хранителни навици и хранителни добавки, които подпомагат организма да се справи със стреса и натоварването на работната среда. Дали всички хранителни добавки са полезни? И дали те не водят до странични ефекти, за които не знаем? Могат ли те да доведат до епигенетични промени, които да се окажат вредни или полезни за организма? Това са много важни въпроси, които всеки човек трябва да си задава преди да започне да приема хранителни добавки и да спазва диети.

Като цяло епигенетиката, изследва взаимовръзката между генотипа и околната среда и промени в генната експресия без промяна на нуклеотидната последователност в ДНК [1]. Има няколко основни механизма в епигенетика, които са: промяна в метилиране на ДНК, модификации в хистоните и двата типа на кондензация на хроматина [1,2]. Най-често се наблюдава метилиране на цитозиновите бази, което се състои в добавяне на метилова група към въглеродния атом на 5-позиция от цитозиновия пръстен. Тази реакция може да се проведе и ускори от ДНК метилтрансферазите,които са няколко на брой [1,2,3,4].



Фиг. 1. Един епигенетичен механизъм за транскрипционно потискане, Bob Weinhold, Environmental Health Perspectives • VOLUME 114 | NUMBER 3 | March 2006
Организацията на хроматина и регулацията на транскрипцията се определя от хистоновите модификации. Хистоните са класифицирани в няколко групи H1, H2A,H2B,H3 и H4 [3,5].

Известно е, че биологично активните компоненти на храната повлияват активността на ензимите, които са отговорни за епигенетичните промени. Такиви растителни компоненти като гинестинът и катехинът действат върху ДНК метилтрансферазите, а резвератролът и куркума върху хистон диацетилаза и хистон ацетилтрасферази.
Основните епигенетични модификации са свързани с метилтрансферазите и всички от тях използват S-аденозилметионин (SAM), който е метаболит на 1-карбоновия метаболизъм и се явява като метилов донор за реакциите на метилирането. SAM е формиран от метилови групи, произлязъл от холин,метионин [12].



Фиг. 2. Опростена схема на метаболизъм на фолиева киселина, включващ ДНК синтеза и метилиране, Young-In Kim [13].

Епигенетичните промени могат да настъпят в следствие на влияние на различни фактори от околната среда [1,2,3]. Ние ще обърнем внимание на храненето и хранителните добавки, които са много значими външни фактори, предизвикващи епигенетичните промени.

Използването на с храни обогатени с метилови донори може да доведе до промени в епигенома и съотвено промени в метилирането и генната експресия [5]. Установено е ,че епигенетичните промени настъпват по време на гаметогенезата и това може да има последици за развитието на ембриона. Доказано е, че храненето на майката преди и по време на бременността допринася за тези промени [5].
Биоактивните хранителни добавки, могат да повлияват основните механизми в епигенетиката [6,7]. Много изследвания са свързани с промяната и добавяне на фолиева

киселина към диетата на майката [6,7], в резултат на което процентът на метилиране на гена IGF 2 DMR в поколението се е повишил. Има още данни, че животни в периода след отбиването от майката, оставени на диета с ниски количества на фолиева киселина, имат промени в метилирането, и в понататъшния здравен статус.. С това се доказва че промяна в храненето и добавяне на биоактивните компоненти към него, водят до установени промени в моделите на ДНК метилиране [7,8,9].

Добавянето на витамин B-12 към диетата също така повлиява върху хипермерилирането на ДНК , защото той участва като кофактор в синтезата на метионин [7,8]. По-горе споменатия холин е още един метиловия донор със съществено значение за ембрионалното развитие на индивида(7). Можем да направим следния извод, че при намалени нива на фолиева киселина, витамин B-12 и холин може да се промени метилирането на ДНК по време на ембрионалния преиод [7,8].

При установени модели на хранене с биоактивни вещества е доказано,че те имат голямо значение за превенция на заболяванията в детска възраст и като помощни средства за противоракова терапия [7]. Така полифенолите, съдържащите се в чая, гинестеинът от соя и изотиоцианати от растителни храни предпазват от рак чрез намаляване на хиперметилирането на ДНК в някои рискови гени свързани с канцерогенеза [7,10].

Епигенетика е обширно поле за изследване, защото много външни фактори водят до епигенетични промени. Храненето е един от тези фактори, който е от съществено значение за организма, защото чрез него дадения организъм си набавя всички необходими вещества, за да води пълноценен живот. Данните от литература убедително доказват, че и хранителния режим, и биодобавките повлияват основните механизми за епигенетичните промени, като променят активността на ензимите, отговорни за метилирането на ДНК и хистонивите модификации.

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Session B: The Rainbow World of Medicine

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BO1. BIOCHEMICAL MARKERS OF BONE METABOLISM IN EXPERIMENTAL ANIMALS

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Abstract

This study is a review of investigating on biochemical markers of bone metabolism in various experimental animals. The biochemical markers are investigated in animal serum, soft tissues around bone defects and bone implants. Alkaline phosphatise is investigated the most frequently as a biochemical marker of bone metabolism in experimental animals.

The bone tissues are hierarchical build composites with mineral-organic structure and complex architecture [4]. This organic-inorganic nature of the hard tissues ensures their unusual strength and partial elasticity.

The regeneration of harmed bones after disorder, trauma, operation, etc. is complicated and prolonged process. The bone relocation and fixation is not enough for the sufficiently regeneration in some fractures involving considerable loss of the bone substance. Materials exist which have similar to the bone chemical composition and supply mineral components for the osteogenesis.

Biochemical bone markers are widely used in the medicine for control of processes of fractures and bone disorders of the patients. In the veterinary medicine they are used more limited on dogs, horses, rats and cats for studying of the bone formation and resorbtion. The osteocalcin is an important index for bone formation. The alkaline phosphatase activity is an index for the osteoblaste activity. The bone alkaline phosphatase activity is considered for sensitive marker of the bone formation. These markers are routine indexes for the turnover of the bone matrix and bone supporting.

Biochemical markers of bone metabolism in animal serum

Sheep model

Study was made to examine the effect of Tramadol as an analgesic drug on serum bone alkaline phosphatase after dental implant in sheep model [1]. Data analysis by using Independent Samples Test showed that there were no significant differences between the mean levels of serum bone alkaline phosphatase in control group compared to treated group.

Dog model

Investigations were made to established reference values for a panel of serum markers of bone turnover in dogs of various ages [2]. Serum concentrations of the carboxyterminal propeptide of type-I procollagen, the aminoterminal propeptide of type-I procollagen and carboxyterminal cross-linked telopeptide of type-I collagen were measured by use of

commercial human radioimmunoassay kits. Serum osteocalcin concentrations and alkaline phosphatase isoenzyme activities were measured by use of techniques developed specially for dogs. Serum markers of bone turnover may be useful diagnostic and prognostic tools for management of dogs with musculoskeletal disorders.

A study was made to investigate the early events of bone regeneration of $Ca(OH)_2$ by studying biochemistry level of serum alkaline phosphatase as guide for bone regeneration in mandible of dogs [10]. The results showed that there was a high significant difference of the level of serum alkaline phosphatase between the different postoperative days.

Investigations were provide concerning biological response in dogs (definite by measuring some hematological and biochemical indices) after experimental reconstruction of missing teeth by titanium dental implants and combined technique for guided bone regeneration [3]. Alkaline phosphatase, acid phosphatase in serum, serum calcium and phosphorous levels, which are nonspecific indicators of osteointegration and osteolysis, were measures. The data proved that the used method of titanium dental implants and combined technique for guided bone regeneration did not have a negative influence on biological response of the organism of dogs.

Rabbit model

Investigations were made to evaluate the use of serum concentrations of biochemical markers of bone metabolism osteocalcin, bone-specific alkaline phosphatase and deoxypyridinoline to compare healing in infected versus noninfected fractures and in fractures with normal repair versus delayed repair in rabbits [16]. Measurement of multiple serum biochemical markers on bone metabolism could be useful for clinical evaluation of fracture healing and early diagnosis of osteomyelitis.

A study was made which compared the in vivo behaviour of three calcium phosphates (mono-phase Zn-β tricalcium phosphate, Mg-β tricalcium phosphate and a bi-phase mixture of hydrroxyapatite and β-tricalcium phosphate) in a paste form implanted in rabbits [7]. The serum bone markers calcium, phosphorus, magnesium, zinc, alkaline phosphatase and bone-alkaline phosphatase were measured using Human diagnostic kits. The mean values of the investigated biochemical markers varied non-significantly between different experimental groups.

An experimental study comparing the dynamics of several biochemical markers before and after osteosynthesis, utilizing implants coated with titanium and hafnium nitrides and non-coated implants made of medical steel on rabbits' bones [18]. The serum levels of alkaline phosphatase, calcium, phosphorus, total protein, glucose, liver transaminases ALT and AST were monitored for 60 days. The use of coated with titanium and hafnium nitrides implants, that possess high strength, thermal and chemical stability, was not accompanied by the development of additional reactive changes in the organism of experimental rabbits in comparison with the implants made of non-coated medical steel.

Rat model

A study was designed to investigate if local tissue and serum markers of bone remodelling reflect implant fixation following administration of lipopolysaccharide-doped polyethylene particles in a rat model [12]. Implant fixation strength was positively correlated with peri-implant bone volume and serum osteocalcin and inversely correlated with serum C-terminal telopeptide of type I collagen, while energy of yield was positively correlated with serum osteocalcin.

Composite bone scaffolds based on Zn modified β-tricalcium phosphate and mixture of xanthan gum, caragenan, saccharose and gelatine were investigated in a rat model [5]. Trace

element content of calcium, phosphorus and zinc and total alkaline phosphatise and bone alkaline phosphatise activity were evaluated in rat serum. Phosphatise activity was established by Human diagnostic kits using Screen master 588 LIHD 111. Osteocalcin content was determined by radioimmunologic kit. Calcium, phosphorus and zinc contents were determined by atomic absorption. The materials showed good biocompatibility and tissue tolerance to the new implants.

Efficacy of bone scaffolds – composite calcium phosphate/poly L-lactide acid (PLLA/HAP) was investigated in rats' model. For studying the process of bone formation were investigated the following serum indices: calcium, phosphorus, magnesium, zinc, total alkaline phosphatase and bone alkaline phosphatase. The data of the investigations showed that the values of investigated elements and the activity of the phosphatases were in normal physical values in all rats [15].

Biochemical studies were done on rats with implants on the base of Zn-modified-tricalcium phosphate and mixtures of xanthan gum, caragenan and gelatine. The serum bone markers calcium, phosphorus, zinc, total alkaline phosphatase and bone alkaline phosphatase were measured using Human diagnostic kits by the Screen master 588 LiHD 111. The levels of the investigated serum bone markers were in physiological values during the experiment [17].

Biochemical blood indices (calcium, phosphorus, total alkaline phosphatase, bone alkaline phosphatase and osteocalcin) in rats with implanted in tibia hydroxyapatite-gelatine-xanthan gum composite materials were studied. The result showed reduction of parameters under study on the 14th d p i and their increased on the 84 d p i. Based on the results obtained, both materials are believed to have the necessary properties for clinical use. Both materials can promote osteoconduction and are biocompatible [9].

A study was made to assess whether the presence of biphosphate pamidronate in the cement implanted onto the rat tibial bones had any effect on some biochemical markers in rat's serum characterising homeostasis [13]. Serum activities of alanine aminotransferase, aspartate aminotransferase and creatine kinase were determined six weeks after the surgery. The use of biphosphate pamidronate in the cement had some positive effect on the homeostasis of the rats after the surgery and a positive influence on the post operative regeneration process.

Biochemical markers of bone metabolism in animal tissues surrounding bone implants

Rat model

The study evaluated trace element content in the soft local tissue surrounding bone implants in rats. Bone effects were filled with authologous or Mg-beta tricalcium phosphate (Mg-β TCP) implants. The tissues were studied for iron, zinc, cuprum, cobalt, chrome, molybdenum and selenium using atomic absorption spectrometry. It was established higher zinc and molybdenum level in the rats with Mg-β TCP implants. Mg-β TCP did not cause any adverse effects in the surrounding tissue [6].

Study evaluated mineral content in the soft and hard local tissue surrounding bone implants was provided in rats [8]. Bone defects were created in femur and tibia of rats. The implants were zinc-modified beta tricalcium phosphate and a mixture of beta tricalcium phosphate and hydroxy apatite. Hard and soft tissues surrounding implants were studies for the content of several elements (calcium, phosphorus, magnesium, iron, zinc, copper, cobalt, chrome, molybdenum and selenium) using atomic absorption spectrometry. The results showed that the mineral composition of hard and soft tissue was quantitative similar but a bit qualitative different. The bone mineral composition was similar in rats from both investigated groups. The

similar chemical elemental composition in the muscle and bone tissue indicated good tissue tolerance. Applied new materials appear to be an appropriate material for further use because it has good biocompatibility in bone tissue.

Biochemical markers of bone metabolism in animal bone implants

Rat model

Bone that was virtually depleted of osteocalcin and reduced in concentration of its characteristic amino acid, γ-carboxyglutamic acid, was obtained from rats treated with warfarin [11]. Osteocalcin-dependent bone particles were resistant to resorbtion when implanted subcutaneously in normal rats. They were investigated for γ-carboxyglutamic acid, hydroxyproline, calcium and phosphorus. The date suggested that osteocalcin is an essential component for bone matrix to elicit progenitor-cell recruitment and differentiation necessary for bone resorption.

Ectopic bone formation by subcutaneously implanted demineralized bone matrix powder was assessed biochemically in rats of different ages [14]. The total calcium accumulation in implants was greatly depressed in older rats. The magnitude of alkaline phosphatase activity was decreased in older rats. The accumulation of the bone-specific vitamin K-dependent bone protein (bone gla protein) was decreased in the implants in older rats. Measurements of total calcium, alkaline phosphatase and bone gla protein at the site of demineralized bone matrix implants clearly demonstrates that bone formation decreases dramatically with increasing age.

Alkaline phosphatise is investigated most frequently as a biochemical marker of bone metabolism in experimental animals. Although total alkaline phosphatise activity is routinely measured in the most small animal biochemical analyses, little information exists in available literature about investigations of the biochemical bone markers in animals.

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BP1. BETWEEN MESENCHYMAL STEM CELLS AND BONE IMPLANTS

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BP2. BRIEF OVERVIEW OF SOME GENETIC DISORDERS AFFECTING BONES

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BP3. GROWTH FACTORS IN BONE REPAIR

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BO2. IN THE WORLD OF METALLOTHIONEINS

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BO3. NOVEL STRUCTURAL BIOMARKERS FOR ASSESSMENT OF RENAL FUNCTION AND EARLY DIAGNOSIS OF ACUTE KIDNEY INJURY - NGAL

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Summary: Acute kidney injury (AKI) is a common serious condition afflicting extremely heterogeneous patient population. AKI diagnosis is usually based on the accumulation of end products of nitrogen metabolism, such as serum creatinine. Unfortunately serum creatinine is an indicator which is not reliable enough for early diagnosis of AKI for its concentrations are

influenced by a variety of non-renal factors and clinically significant changes lag 1-3 days behind the initial structural damage.

Considering the limitations of serum creatinine, the demand for improved biomarkers of AKI is of intense interest to modern medical science. The discovery, translation, and validation of neutrophil gelatinase-associated lipocalin (NGAL) give new opportunities in searching a new approach for assessment of renal function during AKI. NGAL appears an excellent independent biomarker in plasma and urine providing both diagnostic and prognostic information.

Key words: acute kidney injury, early diagnosis, biomarkers, neutrophil gelatinase-associated lipocalin

БИОМАРКЕРИ ЗА РАННО ДИАГНОСТИЦИРАНЕ НА ОСТРО БЪБРЕЧНО УВРЕЖДАНЕ – NGAL

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Острото бъбречно увреждане (ОБУ) представлява внезапно отпадане на бъбречната функция. То е често срещано сериозно състояние, като заболеваемостта и смъртността, свързани с него, не са намалели през последните десетилетия. Засегнати са 5 – 7% от всички хоспитализирани пациенти. Сред критично болните около 6% са тези с изключително тежка увреда, налагаща бъбречно-заместителна терапия [25]. Обикновено ОБУ се диагностицира на база натрупването на крайни продукти от азотната обмяна (урея и креатинин) и/или намалена продукция на урина [5]. Серумният креатинин е късен и ненадежден показател за ОБУ поради редица причини [12]. Нивата му се влияят от редица извънренални променливи като пол, възраст, мускулна маса и метаболизъм, съпътстваща медикация, хранителен и хидратационен статус. Нерядко някои остри и хронични бъбречни състояния могат да се представят без повишение на серумния креатинин, благодарение на компенсаторните възможности на бъбрека- установено е, че серумният креатинин се повишава при отпадане на над 50% от реналната функция. Освен това концентрацията на серумния креатинин не отразява реалното понижение на скоростта на гломерулната филтрация в острата фаза, тъй като са необходими няколко часа до дни, за да се установи ново равновесие между предполагаемата стабилна

креатининова продукция и намалената му екскреция [17]. При тежки заболявания като сепсис измерените стойности на серумен креатинин намаляват и се оказват още по-ниски на фона на хемодилуцията в резултат на целенасоченото лечение с вливане на течности. Критично се явява и отдиференцирането на ОБУ от пренална азотемия или хронична бъбречна недостатъчност в момента на постъпване на пациента, с цел навременно предприемане на адекватни терапевтични мерки. Самостоятелното измерване на нивото на серумен креатинин не може да послужи за разграничаване на тези състояния [7]. Не на последно място повишението на серумния креатинин е късна индикация за функционални промени, касаещи степента на гломерулната филтрация, което забавя оценката на важни структурни промени в бъбреците в ранния стадий на ОБУ. От всичко изброено става ясно, че проследяването на промените в нивото на серумния креатинин е тест с недостатъчна диагностична ефективност за ранно диагностициране на влошена бъбречна функция [9].

Липсата на чувствителни и специфични биомаркери за ранна детекция на ОБУ силно затруднява диагностицирането и лечението на тези пациенти и има пагубен ефект върху терапевтичното поведение и клиничния изход от ОБУ [15]. Забавянето в диагностицирането на ОБУ, базирано на промяната в нивото на серумния креатинин, се явява сериозен проблем, тъй като при някои проучвания върху животни са установени редица интервенции, които могат да предотвратят и излекуват ОБУ, ако се приложат достатъчно рано в хода на заболяването, а именно- преди да започне повишението на серумния креатинин [13].

Като се имат предвид ограниченията на серумния креатинин, търсенето на по-надеждни биомаркери за ОБУ е от особен интерес за съвременната медицина. Идеалните биомаркери за ОБУ трябва да са достъпни, бързи и измерими, прецизни и точни, както в прогностично отношение, така и да определят тежестта на реналната дисфункция, специфични за бъбрека, да се повишават в ранния стадий на ОБУ, с висока чувствителност и специфичност [1]. През последното десетилетие по- доброто разбиране за ранния патофизиологичен отговор на бъбрека към стресови фактори откри редица гени и протеини, които бързо се индуцират в бъбрека [13]. Те участват в регулацията на нови пътища и механизми, които модулират бъбречната увреда. За щастие някои от тези бъбречни протеини са открити в урината и/или плазмата, очертавайки се като ранни неинвазивни биомаркери за ОБУ и неговия клиничен изход.

С откриването, тълкуването и валидирането на **неутрофилния гелатиназа-асоцииран липокалин (NGAL)** се разкриват нови възможности за търсене на нов подход за оценка на бъбречната функция при ОБУ. NGAL за оценка на ОБУ се разкриват и с определянето на се оказва отличен самостоятелен биомаркер в плазмата и урината, както в диагностично, така и в прогностично отношение. В контекста на увреден напълно развит орган, какъвто е бъбрекът, биологичната роля на NGAL е свързана със запазването на функцията, забавяне на апоптозата, както и усилен пролиферативен отговор [22,23]. проучванията на генната експресия при ОБУ показват бързо и мощно повишение на NGAL mRNA в дисталните сегменти на нефрона, по- специално в плътния възходящ край на бримката на Хенле и събирателните каналчета. Последващата синтеза на NGAL протеин в дисталния. нефрон и секрецията в урината формира основната част от уринния NGAL. Въпреки че плазменият NGAL свободно се филтрира през гломерула, той в голяма степен се реабсорбира в проксималния тубул. Това означава, че екскрецията на NGAL вероятно се дължи на бъбречна увреда със засягане на реабсорбцията в

проксималния тубул и/или индуциране на синтеза de novo на NGAL в дисталния тубул. По отношение на плазмения NGAL самият бъбрек не се явява основен източник. NGAL протеинът в кръвообращението произхожда и от други органи, каквито са черният дроб и белият дроб, а също и от активирани неутрофили, макрофаги и други клетки на имунната система. Освен това всяко намаление на степента на гломерулна филтрация намалява екскрецията на NGAL с последващо повишение в системното кръвообращение при пациенти с хронична бъбречна увреда. Предклинични проучвания върху животински модели определят NGAL като един от белтъците с най- висока индукция в бъбрека по време на разгърнат сепсис [14]. Последвали проучвания при хора определят NGAL като отличен биомаркер за ранно диагностициране, терапевтично мониториране и прогноза при често срещани случаи на ОБУ [19]. Разработването на стандартизирани клинични платформи за бързо и точно измерване на NGAL в урината и плазмата допълнително улеснява широкото използване и валидиране на NGAL като биомаркер [20].

NGAL се очертава като един от най- обещаващите биомаркери от ново поколение, както в клиничната нефрология, така и отвъд нея [7]. При проучване сред пациенти с установена ОБУ (с удвоени нива на серумен креатинин) с различна етиология се открива значително повишение на NGAL в серума и урината в сравнение с нормални контроли [23]. Нивата на уринния и серумния NGAL корелират с тези на серумния креатинин, а бъбречната биопсия показва интензивно натрупване на имунореактивен NGAL като чувствителен показател за установена ОБУ при хора. Най- добре е проучено поведението на NGAL при пациенти, при които ОБУ е очаквано усложнение. Сърдечните операции, включващи кардио-пулмонален байпас (КПБ) са сред най-често извършваните хирургични интервенции в болниците по света. В около 30% от случаите пациентите развиват постоперативно ОБУ с различна тежест, което е свързано със значително увеличение на неблагоприятните последствия и смъртността. Патогенезата на ОБУ вследствие сърдечна операция включва няколко механизма- наслагване на хемодинамични, възпалителни и нефротоксични фактори, което води до засягане на бъбрека [24].

Редица проучвания са фокусирани върху предиктивната стойност на NGAL, измерен в първите 2-6 часа след съдовата анастомоза при пациенти, подложени на КПБ. Стойностите на NGAL се сравняват с тежестта на ОБУ според критериите, включени в RIFLE класификацията (Risk, Injury, Failure, Loss, End-stage), като AUC-ROC показва по-добри стойности за разграничаване на категорията ОБУ: R (0.72), I (0.79) и F (0.80). Тъй като RIFLE критериите се основават основно на повишението на серумния креатинин спрямо изходните стойности, забавянето в промяната на този функционален показател средно с 1-3 дни след структурната увреда, нерядко може да бъде фатално в диагностично и терапевтично отношение. Освен това предиктивната роля на уринния NGAL след сърдечна операция спрямо изходната бъбречна функция дава най-показателни вариации при пациенти с нормална предоперативна бъбречна функция [20]. Още по- показателни са резултатите при деца, където отсъстват съпътстващи променливи като възраст, предшестващо бъбречно заболяване, удължено време за осъществяване на байпас, хронично заболяване или диабет.

При пациенти, постъпващи в спешно отделение с множествени травми, ОБУ е често срещано усложнение, обуславящо 40-60% от смъртността. Проучвания при критично болни новородени също показват висок процент на ОБУ и неблагоприятен клиничен изход [2]. Контраст-индуцираната нефропатия е дефинирана като повишение на серумния креатинин с 25% или повече в рамките на 48 часа след перкутанна сърдечна интервенция

[21]. Няколко проучвания определят и ролята на NGAL като прогностичен биомаркер за ОБУ вследствие приложение на контрастна материя [16, 4, 3, 18]. NGAL се очертава не само като отличен биомаркер за ранно диагностициране на ОБУ с различна етиология, но също така показва висока прогностична стойност по отношение тежестта на ОБУ. При различни пациентски групи е доказана корелацията между ранните постоперативни стойности и клиничния изход [10,6] . Проучвания сред хетерогенна група пациенти демонстрират най- високи стойности на NGAL в ранния постоперативен период при тези, при които в последствие се е наложила бъбречно-заместителна терапия.

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BO4. DIRECTIONAL ADAPTATION OF REACTIVE SACCADES IN PATIENTS WITH PRIMARY OPEN-ANGLE GLAUCOMA

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Abstract

When the eye movements of young and older subjects were adapted to double-step target displacement similar adaptive recalibration was demonstrated (Bock et al., 2014). Here we introduced the same paradigm in patients with bilateral primary-open glaucoma in order to investigate the directional adaptation of their reactive saccades and to compare it to that of control subjects (young and older) with healthy vision. Patients adapted to double-step targets requiring a change of response angle by -15 deg. The control groups showed comparable magnitude of recalibration. In contrast, the patients did not adapt to double-step targets. We suggest that the degraded adaptation of reactive saccade direction not due to aging but rather due to affected peripheral and central adaptive mechanisms, caused by glaucoma.

INTRODUCTION

Glaucoma is an optic neuropathy leading to progressive visual field loss [11]. As a result glaucomatous patients have difficulties executing some everyday vision based task like face recognition [3], walking and driving [5], which deteriorate the quality of their everyday life.

Loschky LC and McConkie (2002) suggested that people execute saccades of smaller amplitudes when their vision is degraded in order to avoid the effected areas. A study about face recognition in patients with primary open glaucoma of both eyes [4] reported that larger saccades, done by the patients in order to adapt their eye movements, were associated with better face recognition. Lamirel et al. (2014) reported that patients with primary open glaucoma exposed impaired accuracy and latency of their saccades during the exploration of complex visual scenes. Smith et al. (2012) revealed that patients with primary open glaucoma of both eyes executed less number of saccades compared to normally-sighted people when viewing images of everyday scenes in laboratory conditions. The authors explained the result with degradation of peripheral vision leading to inability to detect items and execute saccades towards them. In a driving task, patients with primary open glaucoma of both eyes showed significantly more saccades than control subjects when viewing a driving scene as a compensation for their restricted field of view [2]. All these studies established that saccadic eye movements are changed in patients with glaucoma. However, we did not meet in literature studies about saccade adaptation. It reveals due some physiological (growing or aging) or pathological (oculomotor or brain lesions) processes to compensate for occurring asymmetry between the two eyes.

The present study investigates whether patients with bilateral primary open glaucoma can adapt direction of their reactive saccade eye movements. For this purpose, we applied modified double–step paradigm [8] in laboratory conditions.

Materials and METHODs

Eight young (20-35 age range) and eight older subjects (50-66 age range) with normal or corrected to normal vision participated in the study. All of them had normal or corrected to normal vision and had no eye adaptation experience. Six patients (57-68 age range) with

primary open angle glaucoma (POAG). Diagnosis of POAG was done in SOBAL "Prof. Pashev" by ophthalmologic examination, perimetry, optical coherent tomography and gonioscopy. The study was approved by the Ethics Committee of the Institute of Neurobiology, Bulgarian Academy of Sciences.

The participants sat in a semidarkened room in front of 17 ``computer monitor situated 40 cm ahead. In order to avoid the spontaneous movements of the subjects' head was stabilized by a chinrest. In single-step trials, the target (a grey square with size of 0.7 cm) appeared in the center of the screen for 760-1500 ms and was displaced in one of eight possible loci along an imaginary circle of 11 cm radius about the center (0 degree is rightward). 760 ms later the target came back to the center of the circle. In double-step trials, the first displacement of the target was followed by a second displacement, 200 ms later. The size of the target` step was - 15 deg clockwise. 640 ms later the target came back to the center of the circle. All participants were instructed to look at the target with their eyes.

DC-electro-oculography (EOG) with a bandpass filter of 0.08-100 Hz was used to register the eye movements. The signal was digitized at 100 Hz with a resolution of 0.01_/bit, and was calibrated about once every 100 saccades.
The experiment consisted of 29 episodes, each one with 20 target presentations. Two baseline single-step episodes were followed by 25 double-step episodes (adaptation phase). Aftereffect phase duplicated the baseline phase and finished the experiment.
A software defined saccadic direction as angular difference between first target step and primary saccade. Saccade latencies were defined as the interval between the target appearance and onset of primary saccade. The mean direction and reaction time of saccades for each episode and subject was calculated.

RESULTS

Fig. 1 demonstrates eye directions in the groups of controls and patients. The baseline directions were similar in all groups that was confirmed by one-way ANOVA ($p>0.05$). During the adaptation phase both control groups gradually changed the response directions towards second target step and reached a stable level at the end of adaptation. On the contrary, patients` adaptive performance was poorer and consisted of more positive eye displacements than negative. This discrepancy was confirmed by an ANOVA with between factor *Group* and within factor *Episode* (25 adaptation episodes), which yielded significance for *Episode* ($F(24,408) =3.08$, $p<0.001$) and *Group* ($F(2,17) =9.91$, $p<0.01$). Post-hoc decomposition confirmed that the adaptation magnitudes of both young and older control groups differed significantly from the adaptation magnitude reached by the patients (-4. 5 and -5.5 vs. + 0.67).
Saccade directions in aftereffect phase, showed corresponding (negative) magnitudes to those in adaptation phase in both control groups, but positive magnitudes in patients` group. One-way ANOVA of aftereffect phase yielded a significant effect of Group ($F(2,41) =6.97$, $p<0.05$). Post–hoc decomposition revealed that eye angle directions in both young and older control groups differed significantly from the eye directions in patients` group (-2.8 and -3.1 vs. +2.22).

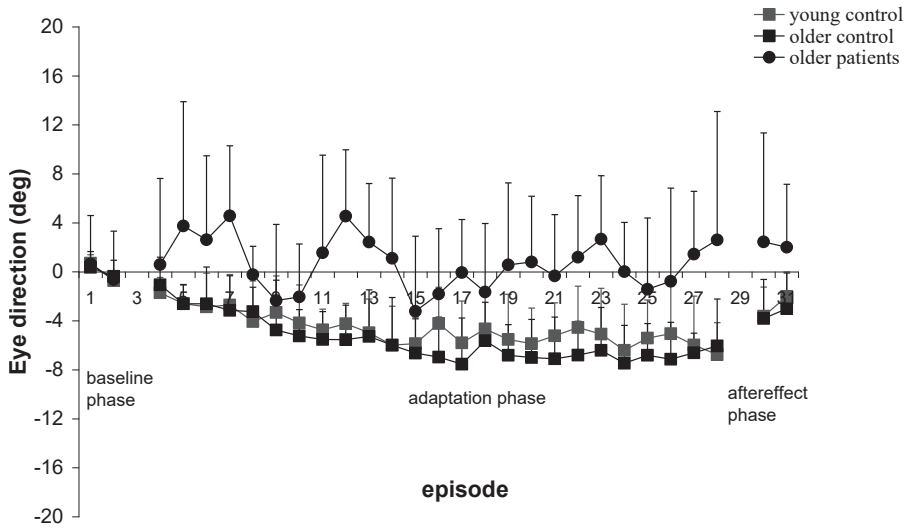

Fig. 1. Eye directions in all 3 groups during baseline phase (first two episodes), adaptation phase (25 episodes) and aftereffect phase (last two episodes). The groups adapted to double-step targets of -15 deg size. Each symbol is the across–subject mean of one episode, and each error bar the pertinent intersubject standard deviation.

Fig. 2 reveals that baseline reaction times of the eyes were shorter in the young control group compared to the reaction times in both control and patient older groups. The same tendency was demonstrated in the adaptation phase. An ANOVA of the adaptation phase yielded significance for Episode ($F(24,360) = 1.73$, $p<0.05$) and Group ($F(2,15) = 11.17$, $p<0.05$). Post-hoc decomposition confirmed the lower reaction time of the young control group (171.63 ms) vs. those of the older controls group (197.54 ms) and patients (219.34 ms).

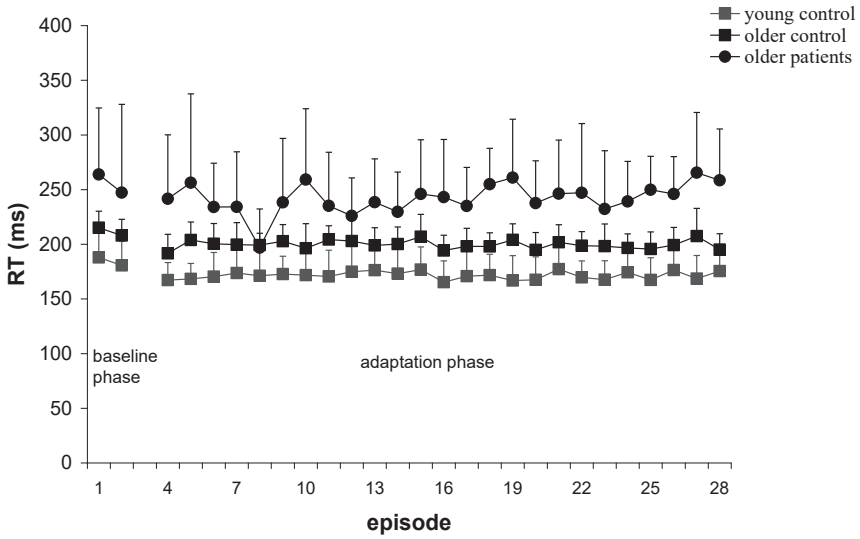

Fig. 2. Eye reaction times during the baseline and adaptation phase in all groups. The groups adapted to double-step targets of -15 deg size. Each symbol is the across–subject mean of one episode, and each error bar the pertinent intersubject standard deviation.

DISCUSSION

The present results revealed that adaptive changes in both control groups were similar. The magnitudes of saccade directions in the aftereffect phase, were also comparable for both age healthy groups. Since aftereffects reflect sensorimotor recalibration [10, 9] the results demonstrate that adaptive recalibration is not degraded in older subjects. All these findings confirm age-independence of saccadic adaptation [1]. Therefore, examined elder patients with bilateral primary-open glaucoma revealed degraded adaptation of reactive saccade direction not due to aging but rather due affected peripheral and central adaptive mechanisms by glaucoma. Most likely dysfunction in the retina in turn impairs subsequent stages, including visual cortical processing and visuomotor activity. As in our glaucomatous patients both eyes are impaired, no central compensation is possible, i.e. not only sensorimotor recalibration but also strategic components involved in saccadic adaptation is affected that leads to full adaptive inability. The present study suggest that rehabilitation programs of primary open angle glaucoma should be applied in earlier stages of glaucoma, mainly when only one of both eyes is impaired. These patients could probably adopt new oculomotor strategies to compensate for their visual field defect.

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BO5. AWARENESS OF GPS ABOUT ALTERNATIVE MEDICINE – HOW CAN WE INITIATE IT

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Abstract

Background
Studies have shown that AM/CM becomes more and more popular among patients. The number of doctors using AM/CM is raising, most of all among family doctors. General practice is the place of the first contact of the patient with the health care system. GPs should know patient's wishes, thoughts and doubts, understand the therapy options and help them to make informed decision. This understanding is a key point for the GPs to meet patient's expectations. That is why we focused our attention to the awareness of patients and GPs in the field, using two types of questionnaires devoted to these two target groups. Here we discuss the process of creating questionnaires and some preliminary result.

Aim of the study
The aim of this part of the study is to evaluate the awareness of Bulgarian family doctors regarding AM/CM focusing on some large cities.

Methods and materials
Based on literature search and analysis of our and foreign experience and published data in recent years, we designed 2 questionnaires especially created and adapted for Bulgarian GPs.

Results
Analyzing privies scientific data from other countries, taking into consideration our health care system and the features of Bulgarian primary care we consider our research method to be a

questionnaire type. The points for this decision are based on the method price (it is cheap), access to large groups of people at same time and convenient for respondents. We give the respondents enough time to think, to ask and discuss. The questionnaires cover four main areas: [1] socio-demographic with questions about age, residence, sex, type of general practice etc; [2] awareness and knowledge about different types of available/offered alternative therapies; [3] revealing the use/desire of AM/CM for patients and GPs, and [4] willingness to learn more about the opportunities and benefits of Alternative and Complementary medicine in General Practice setting. On the second stage of the pilot study we will cover some large cities, pointing out the advantages and disadvantages, the response rate and some variables.

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AWERNESS OF GPS ABOUT ALTERNATIVE MEDICINE – HOW CAN WE INITIATE IT?

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Abstract

Studies have shown that AM / CM is more and more popular among patients. The number of doctors using AM and CM is raising, most of all among family doctors 8 They are the first contact of the patient with the health system and GPs should know their wishes, thoughts and understanding about their therapy. Therefore it is important to understand what want patients and if GPs meet their expectations. Thus we made by creating two types of

questionnaire for patients and for GPs. At this report we discuss the creating process and final result of GPs questionnaire.

Aim

The purpose of this report is to perform the creating of a suitable method of research about awareness of family doctors to AM/CM for our country especially for some large towns.

Methods and materials

Based on literature search and analysis of our and foreign experience and published data in recent years, we designed the first for Bulgaria questionnaire, especially created and adapted for Bulgarian GPs.

Results

Analyzing privies scientific data from other countries and take into consideration our health system and primary care we choose our research method to be questionnaire. It is cheap, only printing paper, can include large group of people at same time,convenient for respondents - they have more time to think, easier for administer and etc. So we create questionnaire which questions cover four main areas: socio-demographic with questions about age, residence, sex, type of general practice etc; second is awareness and knowledge about different types of available/offered alternative therapies; the third -revealing the use/desire of AM/CM for patients and GPs, and the forth -willingness to learn more about the opportunities and benefits of Alternative and Complementary medicine in GP. The second stage is pilot study to be made for the territory of some large town, and to see advantages and disadvantages, response rate and reviews from that kind of research.

Key words: general practice, questionnaires, research method, patients, study, alternative medicine

Introduction

Scientific studies and researches in different countries shows that Alternative medicine and Complementary medicine (AM / CM)are more and more popular among patients [3,6,13].The number of doctors using AM and CM is raising every year, the number of countries in which AM and CM are making a way among people and society are growing too[2,3,4].The spread of AM and CM among doctors from different clinic directions is interesting and indicative- the majority are family doctors, the group of anesthesiologists are second but mostly for using acupuncture [6,8,10]. Family doctors are the first contact of the patient with the health system and they should know their wishes, thoughts and understanding about their therapy. Therefore it is important to understand what want patients and if GPs meet their expectations. Many researches shows that most of the patients have great thrust in their family doctor [6]. The doctor must be well trained not only in conventional medicine, studied in universities, but he must have opinion and knowledge about different alternative therapies that are most popular among patients and have proved that are effective in healing process. Although there are few systems and therapies from CM that are studied in some universities already in some countries [2,8,14]. Their are so many scientific data about this topic AM/CM in general practice for different countries, but about Bulgaria data are insufficient, there are only for hospitals and private pediatric practices [20]. We are very interested in this topic which seems to be new for Bulgaria and its exploring can be useful in many directions- for doctors, patients, health system and prosperity of the society. This research is second stage of PhD thesis about AM/CM in General practice. The first stage was literature review of that kind scientific data made in other

countries in resent years. Now we focus our attention for creating the most suitable research method about knowledge and use of AM/CM in GP taking in consider of local factors in health system, general practices and others particularities in Bulgaria. So we create two types of questionnaire for patients and for GPs. At this report we discuss the creating process and final result of GPs questionnaire.

Aim

The purpose of this report is to perform the creating of a suitable method of research about awareness of family doctors and patients to AM/CM for our country especially for some large towns.

Methods and materials

Based on literature search and analysis of our and foreign experience and published data in recent years, we designed the first for Bulgaria questionnaire, especially created and adapted for Bulgarian GPs.

Results

During the search we discuss different options in creating the suitable instrument for our study. We focus upon tree big types of research instruments – questionnaire, semi-structure interview and mixed methods. They all have advantages and disadvantages [1,11,12]. Our job was to analyzing the scientific articles and data from literature and foreign experience and to choose the most appropriate method for our study. After analyzing we stop upon questionnaire because it is– cheap method – only printing paper, can explore large territory for short time, it is research method easy to administrate, suitable for respondents- they have more time to think, it can be anonymous, much quicker and there is no subjective influence of the researcher, than other research methods like semi-structure interview for example[1]. But we cannot miss easily that it's not perfect instrument, there can be risk of missing data, like unfilled and unanswered questions, for some reason if the question is not clear or is confusing. We can never be sure that this is the right person if he is not doing that in front of us, also this research method is not so flexible[11,12], but for our purpose is acceptable, even more if we include it in mixed method[1]. When the instrument was chosen we start searching studies that have already made with this kind of research instrument for general practitioners and patients. In most cases they were presenting only few questions or some groups of questions, for example Moshe Frenkel and his collogues in their report present some questions from questionnaire to patient[7], most of their questions have four or five types of answer of choice. While another study from UK presents some example of questions to family doctors, but here we see more open-ended and with self-filling structure questions[9]. The majority of this studies are using questionnaires sent by mail and includes some big cities or regions[2,3,6,7,9,10].

The questionnaire that we made after analyzing the literature and other foreign experience is two types for our two target groups- GP and patients. It is anonymous questionnaire and questions are nearly 30, with only two or three open- ended. Questionnaires cover four main areas:

1. Socio-demographic with questions about age, residence, sex;
2. Awareness/knowledge of different types available/offered alternative therapies;
3. Revealing the use/desire of AM/CM for patients and GPs;
4. Willingness to learn more of AM/CM in GP.

Same structure of the both types questionnaires we have but questions are transformed according to target group.

For example – questions to GP – Do you recommend different alternative therapies to your patient? Yes No
And to patient- Does your GP have ever recommended to you any alternative therapies?
Yes No
Some questions have two types answer yes and no, but others are with more possibilities:
Your attitude to alternative medicine:
□ enthusiastically
□ receiving
□ Failure
□ indifferent

Indicate the reasons why you don't use AM in your practice
□ it's effective
□I'm not familiar enough with it
□ not safe

Do you think that AM is:
□ additional to conventional medicine
□ be used alone for prevention
□ alone for the treatment and prevention
□ to conventional medicine for prevention
□ additional to conventional medicine for treatment and prevention
□ unscientific and has no place in your work
□ should not apply

And some open- ended questions like -Please tell, what alternative method would you like to study in more detail?
The questionnaires must be well design and structured for the purpose of the study, to find out is our research method is appropriately, understandable and well -structure pilot study first must we made. This pilot study will help us to find out if is any misunderstanding or confusion in questions, or there are new suggestions and point of view to be in help for our further study.

Conclusions:

This study presents creating process of finding the most suitable research method and the final result of it -two types questionnaires**,** one for GP and second for patient, covering four main areas, questions are transformed according to the target group. Pilot study is next step where the questionnaires will be tested.

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BO6. WOUND HEALING

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Over the ages, many agents have been placed on wounds to improve healing. Until now nothing has been identified that can accelerate the healing process in a normal individual. Many hinder the healing process. The main issue in wound management is to create an environment where the healing process can proceed optimally. The first step of wound healing is inflammation. Immediately after injury, intense vasoconstriction leads to blanching, a process mediated by epinephrine, neurotropil elastase and prostaglandins released by injured cells. After 10 minutes it is reversed by vasodilatation. Leukocytes now migrate into the wound by diapedesis, they adhere to endothelial cells, to wounded tissues. Alteration in pH from breakdown products of tissue and bacteria, along with swelling causes pain. Neutrophils, macrophages and lymphocytes come into the wound. Macrophages eat bacteria, dead tissue, they secrete matrix metalloproteinases, that break down damaged matrix. Being a source of cytokines, macrophages stimulate fibroblast proliferation, collagen production. The second stage of healing is proliferation which is mediated by fibroblasts. Proliferation includes Mesenchymal cell chemotaxis, cell proliferation, angiogenesis and epithelialization. Epithelization is the process of epithelial renewal after injury. The sequence of events here are cellular detachment, migration, proliferation, differentiation. Epithelization is followed by wound contraction. The process begins approximately 4-5 days after wounding and it represents centripetal movement of the wound edge towards the center of the wound. The last process of wound healing is Remodeling. It increases the strength of the new tissue after wounding. During that process, the number of intra and intermolecular cross-links between collagen fibers increases dramatically. The process continues for up to 12 months. Excessive healing results in a raised, thickened scar, with both functional and cosmetic complications, for it can be a hypertrophic scar or a keloid. No modality of treatment is predictably effective for these forms of lesions.

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BP4. WOUND HEALING IN MAXILLOFACIAL REGION

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BO7. MODERN TRENDS IN THE COMPLEX SURGICAL TREATMENT OF BURNS IN CHILDREN

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Abstract

Burns are the second major cause for death from injury in childhood. They not only affect the body physically, but also cause long-lasting psycho-emotional, functional and aesthetic consequences. Thermal burns are the most frequently occurring type of burns in

children, followed by chemical and radiation ones. Almost a half of the burns in childhood occur in the age group 0-4 years. Children are more vulnerable to burn injury in comparison with adults because of their anatomical and physiological features. Because of the complex therapeutic behaviour, burns need to be treated in a specialized burn centre. Thus, competent and timely treatment is provided and disease complications are reduced. During the last 25 years the technological development has taken the knowledge in the field of pathophysiological and pathohistological mechanisms of burns and their evolution on a new level. This has changed the usual clinical course and treatment of the burn disease and introduced a qualitatively new treatment approach and behaviour. The modern local treatment of burns, the new operative techniques, the ample choice of temporary skin substitutes, the modern intensive care and resuscitation provide conditions for great reduction of the burn death rate, shortening of the hospitalization time and achieving of excellent cosmetic and functional outcomes. Until recently, patient survival after extensive burns was a principal measure for successful treatment outcome. Now, when an increasingly great part of the extensive burns are treated successfully, the good functional and aesthetic outcomes as well as the complete social adaptation are the new measures for success. The main treatment goal is to return the child to the family and to his/her natural environment, and to restore the normal child's everyday activities which correspond to his/her age.

Session C: Cancer

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

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

Secretary: Delka Salkova, DVM, PhD
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CO1. WHAT DO WE (NOT) KNOW ABOUIT METASTASIS?

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CO2. BRAIN METASTASIS IN *GRAFFI* HAMSTER EXPERIMENTAL TUMOR MODEL

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Abstract

Brain tumors can be classified into two major classes, namely, primary brain tumors that start in the brain and secondary brain tumors that are generated by the cancer cells that migrated from tumors developed in other parts of the body. In the present study were observed changes in the morphology of the brain vessels, choroid plexus epithelial cells and blood vessels (on the 10th and 30th day) and metastatic foci in the brain tissue in hamster *Graffi* experimental tumor model (TBH). In the present study were observed metastasis near the brain ventricles and remarkable changes in the brain vessels in all tumor bearing hamsters. The most significant increased luminal diameter of the blood vessels was observed in TBH on the 30-th day of examination. These changes in the brain microvasculature and plexus choroideus are evidence for alteration of the blood-brain barrier and blood-cerebrospinal fluid-barrier and probably were result of secondary brain tumors.

Key words: brain metastasis, experimental hamster *Graffi* tumor model, blood-brain barrier and blood-cerebrospinal fluid-barrier

INTRODUCTION

Secondary brain tumors are more common than primary ones and are the most common cause of tumors in the intracranial cavity. This means that a cancerous neoplasm has developed in another organ elsewhere in the body and that cancer cells have leaked from that primary tumor and then entered the lymphatic system and blood vessels. They then circulate through the bloodstream, and are deposited in the brain. How the metastatic process is regulated also largely remains a mystery.The development of new therapeutic approaches for these diseases is a difficult challenge, and there is no effective treatment for almost all the brain diseases. In most of the cases, the major cause of the failure in the development of drugs to treat brain diseases is the presence of blood-brain barrier [2]. The cerebrospinal fluid (CSF) circulatory system is involved in the neuroimmune regulation, cerebral detoxification, and delivery of various endogenous and exogenous substances [3]. The barriers of the brain play critical roles in controlling the movement of various methabolites, but also drugs, between the blood and the brain (Blood-Brain Barrier) and the blood and the CSF (Blood-CSF-Barrier). Fundamental to all brain barrier mechanisms is the presence of intercellular tight junctions between intimately opposed cells comprising these interfaces (endothelial cells of the brain vesels - BBB and choroid plexus epithelial cells – B-CSF-B) [7]. Plexus choroideus is highly vascularised

structure in the brain ventricles. It produces cerebrospinal fluid and involves in the synthesis and transport of numerous CSF components. Choroid plexus has an important role in the homeostasis of nutrients in the CSF [1]. The transplantable myeloid tumor used in this study originated as a Graffi murine leukemia virus-induced tumor in newborn hamsters, adapted and maintained to mature Golden Syrian hamsters [4, 8].
The ***aim*** of the present study is to investigate the morphological changes of the brain structures and blood vessels in the hamster *Graffi* experimental tumor model.

MATERIAL AND METHODS

Experimental hamster Graffi tumor model: Golden Syrian hamsters, 2 months old, were used in experiments. The experimental Graffi tumor was primary created by the Graffi-virus in new-born hamsters, and maintained monthly *in vivo* by subcutaneous transplantation of live tumor cells (2×10^6/ml PBS) in the interscapular area of hamsters, for keeping the tumor's survival [4, 8, 9]. The tumor is 100% cancerous, and the animals die usually up to the 30th day after transplantation. The animals were kept under standard conditions with free access to food and water.
Histopathologycal examination: Brain samples from control (healthy) and tumor bearing hamsters (TBH) were taken, fixed in Carnoy's solution and embedded in paraffin using routine histological practice. Tissue sections (5-7μm) were stained by hematoxylin-eosin and examined under light microscope Leica DM5000B.
All studies were performed in accordance to the Guide for Care and Use of Laboratory Animals, as proposed by the Committee on Care Laboratory Animal Resources, Commission on Life Sciences and National Research Council, and a work permit No. 11130006.

RESULTS AND DISCUSSION

For the first time brain was examined in experimental hamsters with Graffi tumor to establish metastases and morphological changes of brain structures in the ventricles during tumor progression. The histopathological studies of the brain were carried out on the 10th (Fig. 2.) and the 30th day after tumor implantation (Fig. 3-4). At the same time brain samples were taken from control hamsters (Fig. 1). Massive accumulation of tumor metastatic cells were detected in the brain tissue, lateral ventricle and under ependyma in TBH on the 30-th day of examination. Tumor cells have a morphology similar to that described morphology of the primary tumor, reported in our previous studies. It was found that the tumor metastasizes lymphatic and haematogenous route. In our previous studies metastases were observed in regional and non-regional lymph nodes, in the lung, liver and spleen of TBH. Tumor cells in metastatic lesions are morphologically identical to the primary solid tumor [8, 10].
Remarkable changes were found in the morphology of brain vessels in all tumor bearing hamsters. The most significant increased luminal diameter of the blood vessels was observed in TBH on the 30-th day of examination. Most of the endothelial cells of the blood vessels near by metastasis were destroyed.
Many brain vessels and choroid plexus cappilaries with destructive changes were seen in the present study. Some endothelial cells were destroyed. There were many macrophages in the apical part of the choroid plexus epithelial cells and in the CSF and many dark epithelial cells. Most blood vessels in the plexus choroideus are wide-calibre (10-15 μm) fenestrated capillaries,

wich included non-fenestrated endothelial segments with some vesicles [5]. Similar changes in the rat choroid plexus blood vessels and epithelial cells we observed in our preliminary investigations after low doses ionizing irradiation [6].

Experimental study of the TBH revealed that the tumor appearance and development led to the morphological damages in the brain tissue and vessels and choroid plexus and violation of the permeability of the blood-brain barrier and blood-CSF-barrier.

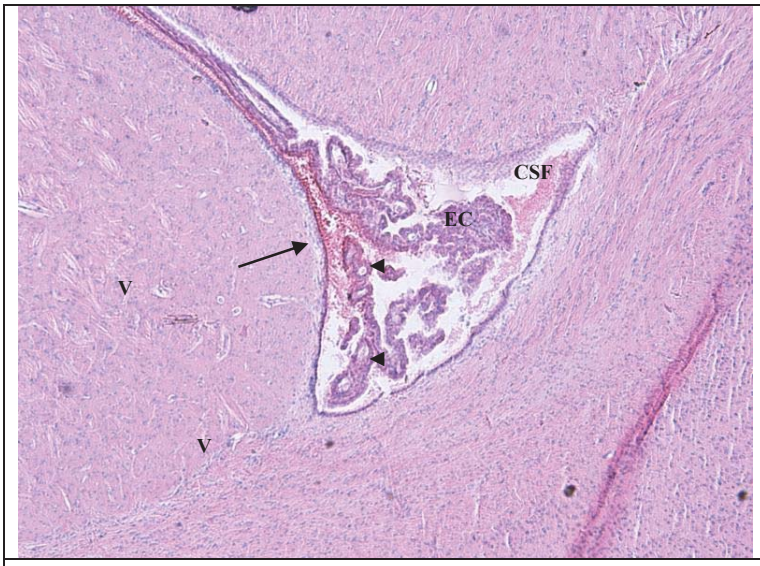

Fig. 1. Light microscopic micrograph of control hamster cerebrum near by the lateral ventricle (→). Brain blood vessels (V); Choroid plexus epithelial cells (EC) and blood vessels (◄), cerebrospinal fluid (CSF). H&E stain. X 5

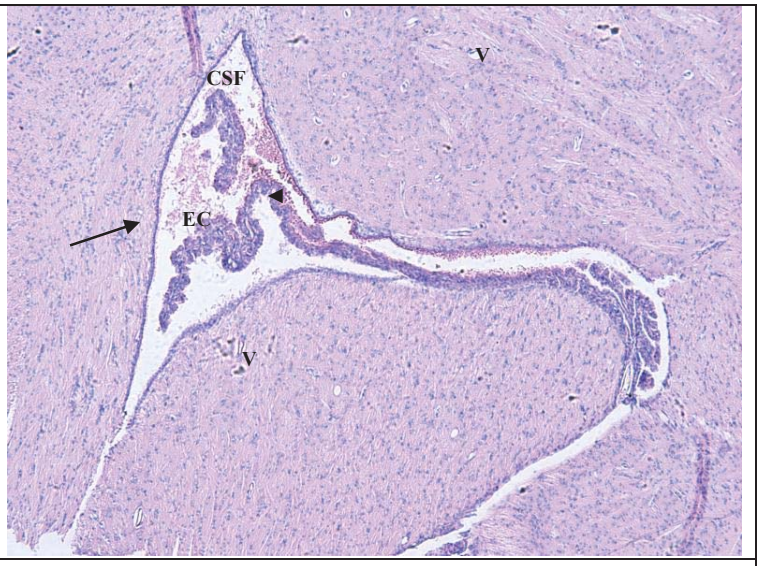

Fig. 2. Light microscopic micrograph of tumor bearing hamster (10 days after tumor inplantation) cerebrum near by the lateral ventricle (→). Brain blood vessels (V); Choroid plexus epithelial cells (EC) and blood vessels (◄), cerebrospinal fluid (CSF). H&E stain. X 5

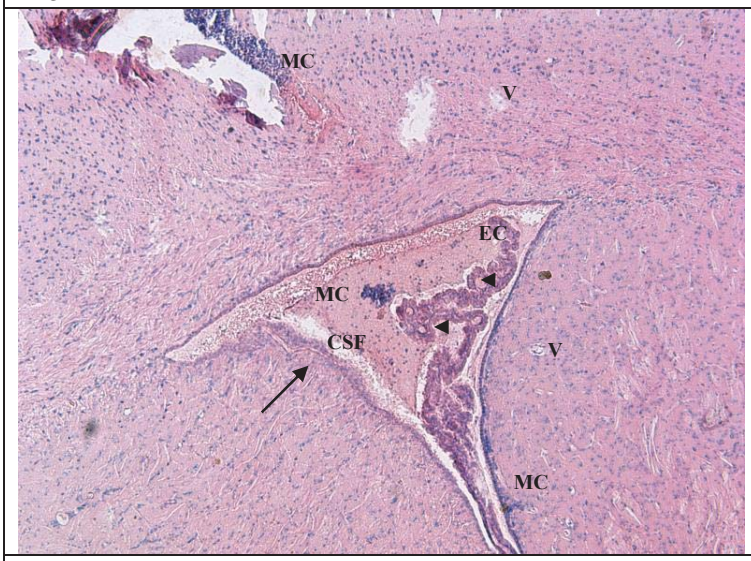

Fig.3. Light microscopic micrograph of tumor bearing hamster (30 days after tumor inplantation) cerebrum near by the lateral ventricle (→). Brain blood vessels (V);

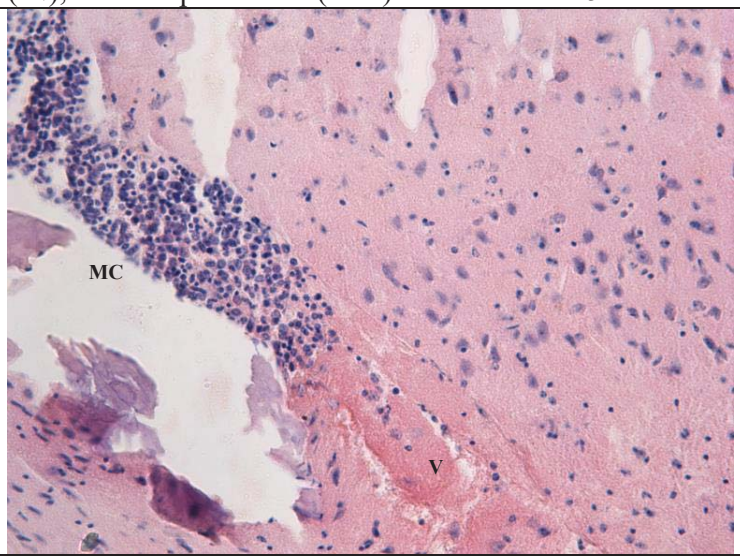

Fig. 4. Light microscopic micrograph of tumor bearing hamster (30 days after tumor inplantation) cerebrum near by the lateral ventricle. Massive accumulation of tumor

Choroid plexus epithelial cells (EC) and blood vessels (◄), cerebrospinal fluid (CSF); Massive accumulation of tumor metastatic cells (MC) in the brain tissue, lateral ventricle and under ependyma. H&E stain. X 5	metastatic cells (MC); blood vessels (V). H&E stain. X 20

Conclusion: The obtained results clearly demonstrate that the increased luminal diameter of the brain capillaries, the destructive changes and atrophy of epithelial and endothelial cells of the plexus choroideus in the brain ventricles are processes which probably contribute to the emergence of metastases.

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CO3. CELL CULTURES AS EXPERIMENTAL MODELS FOR LUNG CANCER

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CO4. CELL CULTURES AS EXPERIMENTAL MODELS FOR COLORECTAL CANCER

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CO5. CELL CULTURES AS EXPERIMENTAL MODELS FOR PROSTATE CANCER

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CO6. ZEBRAFISH AS EXPERIMENTAL MODEL IN CANCER RESEARCH

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CO7. IN VITRO ANTIPROLIFERATIVE ACTIVITY OF THE NOVEL ANTI-CANCER AGENT ERUFOSINE ON GRAFFI MYELOID TUMOR CELLS

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Abstract

Alkylphosphocholines (APCs) are synthetic phospholipid analogues with a pronounced antineoplastic activity mediated by an interference with the lipid metabolism of the tumor cells and modulation of cellular signaling pathways regulating the proliferation, differentiation and apoptosis.

In the present study the antiproliferative activity of the novel APCs erucylphospho-N, N, N-trimethylpropanolamine (erufosine) on *in vitro* cultured Graffi myeloid tumor cells was determined by a (3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) (MTT) dye reduction assay. The dose- and time-dependence of the antineoplastic effect was assessed after exposure to four different concentrations of erufosine. The tested anti-cancer agent was found to exert a clear dose-dependent antiproliferative effect on Graffi myeloid tumor cells. The IC_{50} value of the erufosine was calculated. The morphological changes in the Graffi tumor cells cultured in the presence of erufosine were analyzed by live/dead staining with acridine orange and ethidium bromide and were visualized by by ZOE™ Fluorescent Cell Imager. The microscopic observations support the claim that the erufosine causes death of Graffi tumor cells through induction of apoptosis. Based on these results we could suggest that the erufosine appears to be a promising agent in the treatment of haematological malignances.

Key words: alkylphosphocholines, erufosine, Graffi myeloid tumor cells, antiproliferative activity

Introduction

Cancer has become one of the most significant health challenges of our time. Systemic chemotherapy is the most common therapeutic approach, however, considerable limitations exist including the toxicity to healthy tissues and low achievable drug concentrations at the tumor site. These discrepancies of contemporary anticancer therapy define the need for the development and implementation of novel therapeutics and treatment strategies.

Alkylphosphocholines (APCs) are phosphocholine esters of long-chain aliphatic alcohols that represent a new and promising class of anti-cancer agents [6]. The mechanism of the antitumor action of APCs substantially differ from those of the conventional cytotoxic agents, which target mainly the DNA of the tumor cells [1]. The antiproliferative activity of the APCs is due to the disruption of lipid metabolism of the tumor cells, primarily by inhibition of the synthesis of phosphatidylcholine and modulation of lipid-mediated signal transduction involved in the control of cell proliferation, differentiation and death [14].

The class of APCs comprises of various compounds, from which one, hexadecylphosphocholine (miltefosine, HePC) has found an application in clinical practice for treatment of skin metastases in patients with breast cancer [1] and others are currently investigated in clinical and preclinical studies [6].

The unsaturated alkylphosphocholine erufosine (ErPC3) belongs to the third generation APCs and it is the first compound of this class that can be administered intravenously, because it does not cause haemolysis and myelotoxicity [6]. The ability of erufosine to cross the blood-brain barrier makes it a potential therapeutic agent for the treatment of brain tumors [6].

In vitro, the antiproliferative effect of erufosine was demonstrated in various cell lines of human haematological malignancies [4, 9, 10] as well as solid tumors [7, 8]. Preclinical studies have also shown a pronounced antiproliferative effect of erufosine on various *in vivo* model systems - glioblastoma and chemically-induced mammary carcinoma [6].

In the present study, primary cell cultures derived from Graffi myeloid tumor in hamsters (GMTH) were used to confirm the antineoplastic effect of the erufosine on haematological neoplasm. The GMTH is a transplantable, rapidly growing tumor with very aggressive behavior, showing no spontaneous regression. The tumor was originally induced by Graffi murine leukemia virus in newborn hamsters [5] and adapted to grow in a solid form after subcutaneous inoculation [12]. The GMTH is a reliable model for studing of the antitumor effects of natural and synthetic chemical substances, showing precise and reproducible results [13]. Cell cultures derived from the tumor represent a valuable *in vitro* model system for primary screening and selection of potentially active antineoplastic agents.

Materials and methods

Test compound

Erufosine was synthesized at the Max Planck Institute for Biophysical Chemistry, Göttingen, Germany, and was kindly provided by Prof. Eibl. Erufosine was dissolved in PBS (phosphate buffer saline) pH 7.4 and ethanol at a volume (1:1, v/v) and was stored at 4°C.

Cell cultures and cultivation

Primary Graffi cells were isolated from the solid myeloid tumor tissue from Syrian golden hamsters under aseptic conditions. The cells were grown as monolayers in 25cm^2 tissue culture flasks in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U mL^{-1} penicillin and 100 µg mL^{-1} streptomycin. The cultures were maintained at 37ºC in a humidified atmosphere with 5% CO_2. All culture reagents were purchased from Gibco/BRL (Grand Island, NY).

MTT-dye reduction assay

The *in vitro* antiproliferative activity of erufosine was studied on Graffi myeloid tumor cells using the standard MTT-dye reduction assay, described by Mosmann [11]. The trypsinized tumor cells adjusted to a density 1×10^5 cells/mL RPMI-1640 containing 10% FBS were plated

(100 µL/well) in 96-well flat-bottomed microplates (Orange Scientific) and allowed to adhere for 24 h. The cells were then treated with erufosine at concentrations of 10 µM, 20 µM, 40 µM and 80 µM (six wells per concentration) for 24 h, 48 h and 72 h. Untreated tumor cells were used as negative controls. The plates were observed by inverted light microscope (Olympus CK40, Japan) to identify changes in the growth characteristics and cytopathological alterations of the treated cells. At the end of treatment period, the culture medium was discarded, the cells were rinsed with PBS and 100 µL of MTT (3-(4,5-dimethylthyazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution at a concentration of 0.5 mg/mL were added to each well. The plates were incubated for 3 h at 37.5°C in a humidified atmosphere and 5% CO_2. Formazan crystals were dissolved by adding 100 µL/well of an absolute ethanol/DMSO (1:1 v/v) solution and the absorption was measured using a microplate reader (TECAN, Sunrise TM, Groedig/Salzburg, Austria) at 580 nm.

Morphological study

The identification of apoptosis or necrosis was carried out by a double staining with acridine orange (AO) (3,6-dimethylaminoacridine) and ethidium bromide (EB) (3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide) method [2, 3]. Briefly, Graffi tumor cells were seeded in 96-well plates and after 24 h were treated with ErPC3 at concentrations that does not exceed the IC_{50} values established by the MTT test (7.5 µM, 15 µM and 30 µM). Control cells were incubated for the same period in culture medium. After 72 h tretment, the cells were washed with phosphate buffer saline (PBS), pH 7.4 for 1 min to remove culture media and 5 µL of fluorescent dye solutions in PBS (AO 10 µg/mL and EB 10 µg/mL) were added to each well. The cells were examined by ZOE™ Fluorescent Cell Imager (Bio-Rad Laboratories, Inc.) within 10 minutes before the fluorescence signal starts to fade.

Statistical analysis

The relative cell viability, expressed as a percentage of the untreated negative controls was calculated for each concentration. The statistical significance was assessed by One-way ANOVA followed by Bonferroni's post hoc test (GraphPad Prism software package). $p < 0.05$ was accepted as the lowest level of statistical significance. Probit regression analysis (Statistica 5.5 software package) was applied to determine the concentrations inducing 50% inhibition of the cell growth (IC_{50} values).

Results and discussion

Light microscopic investigation of erufosine-exposed GMTH cells showed cytopathic alterations such as shrinkage, rounding up and detachment from the substrate (Fig. 1). After 24 hours of treatment, cytopathic alterations were evident only in the cells exposed to 40 µM and 80 µM erufosine. The observed morphological changes progressed in a concentration- and time-dependent manner and after 48 h and 72 h were visible at all tested dose levels.

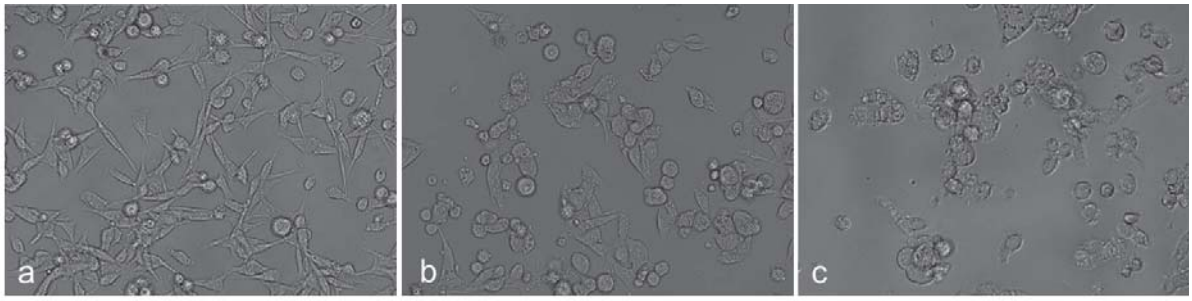

Figure 1. Cytopathic alterations in Graffi myeloid tumor cells exposed to erufosine for 72 h, visualized by light microscopy. **a**-control culture; **b**-culture treated with 20 μM erufosine; **c**-culture treated with 40 μM erufosine.

The results from the MTT dye reduction assay revealed a concentration-dependent antiproliferative effect of erufosine on *in vitro* cultures of Graffi tumor cells (Fig. 2).

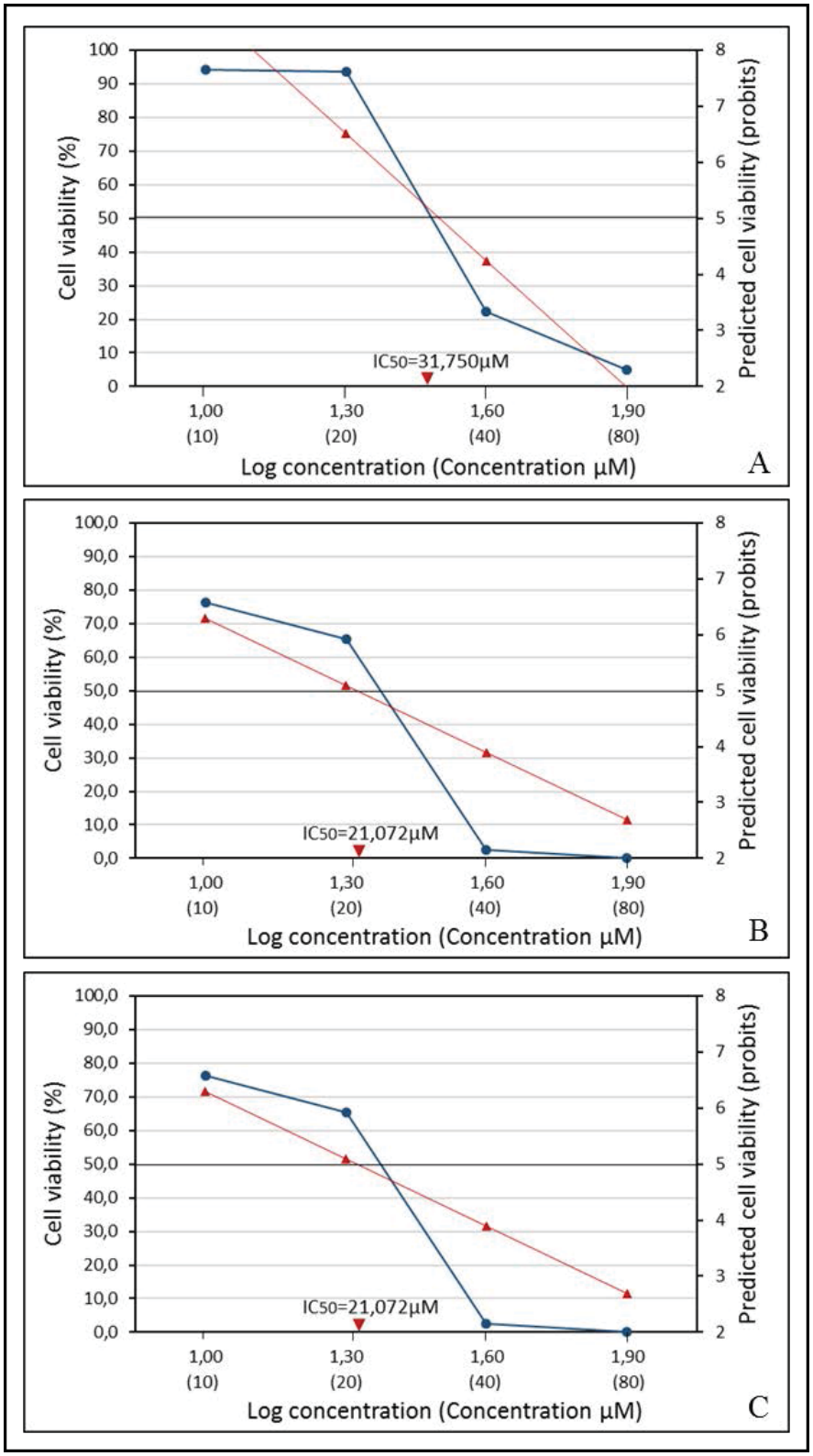

Figure 2. Antiproliferative effect of erufosine on Graffi myeloid tumor cells after 24 (A), 48 (B) and 72 (C) hours of exposure.

The exposure period of 24 hours was sufficient for reduction of the viability of the Graffi myeloid tumor cells. Statistically significant decrease of the cell viability ($p < 0.001$), as compared to the untreated controls, was established at the concentrations higher than 20 μM. After 48 h and 72 h the reduction of the cell viability was significant ($p < 0.001$) at all used concentrations of erufosine. The IC_{50} values of erufosine, determined after 24 h, 48 h and 72 h of exposure were 31,750 μM, 21,072 μM and 23,005 μM, respectively.

To determine whether the inhibitory activity of the ErPC3 is associated with induction of apoptosis, morphological alterations of Graffi cells were assessed by fluorescent microscopy after AO/EB double staining. Untreated Graffi cells were with homogenous pale green nuclei and bright green nucleolus. Erofusine-treated Graffi cells showed rounding, retraction of the pseudopods, blebbing of the cellular membrane, reduction of the cellular and nuclear volume (picnosis) and fragmentation of the nucleus (karyorrhexis), which are morphological features of apoptosis (Fig. 3).

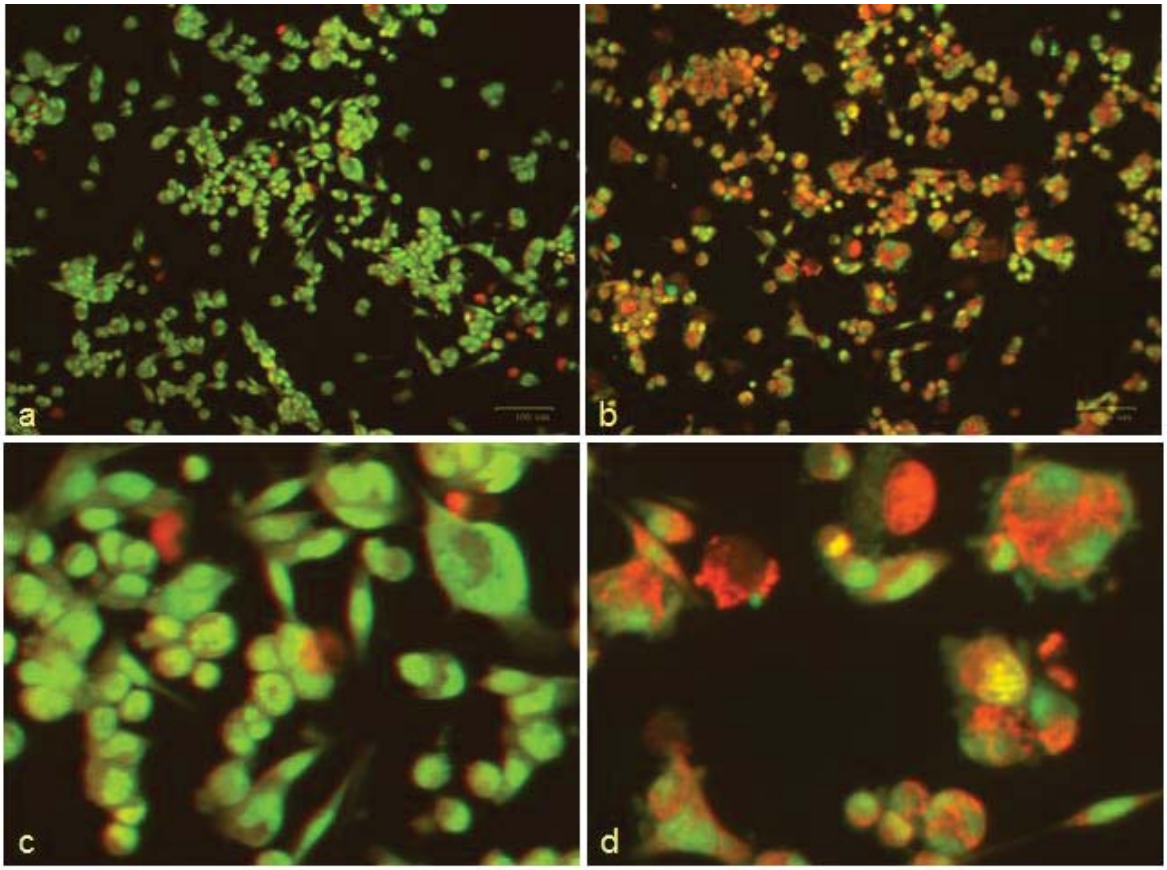

Figure 3. Fluorescent images of Graffi myeloid tumor cells exposed to erufosine and double stained with AO (green) and EB (red). **a**-control cells; **b**-cells treated with 30 μM erufosine; c, **d**- details from **a** and **b**.

Early apoptotic cells with bright green nucleus and condensation of chromatin observed as dense green areas, late apoptotic cells sowing chromatin condensation and orange colored nucleus as well as formation of apoptotic bodies were found in cell cultures exposed to erufosine at all concentrations used.

The results of the current study reveal that ErPC3 possesses promising antiproliferative properties and apoptogenic effects against Graffi tumor cells. The cell viability results showed an obvious decrease of viable cells in ErPC3-treated cultures. Graffi cells treated with ErPC3 had typical morphological changes that implicate apoptosis.

Results presented clearly demonstrate the *in vitro* antitumor activity of the tested ErPC3 on Graffi tumor cells and are a premise for further assessment of its *in vivo* antineoplastic potential.

Acknowledgements

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CO8. FOUR NEWLY SYNTHESIZED METAL COMPLEXES WITH SCHIFF BASES – INFLUENCE ON VIABILITY AND PROLIFERATION OF VIRUS-TRANSFORMED CANCER CELLS

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The aim of our study was to evaluate the influence of four complexes of Zn/Ag and Zn/Au with Shiff bases Salampy and Saldmen on viability and proliferation of cultured cells from the myeloid tumor of Graffi in hamster as well as of lymphocytes and bone marrow cells of the same tumor-bearing animals. The compounds were applied at a concentration range of 0.05 – 20 μg/ml for 48 h and 72 h. The number and viability of the treated cells were determined by trypan blue dye exclusion technique using automated cell counter.

Acknowledgement: This study was supported by Granr № DFNI Б 02 30 from 12.12.2014, Fund "Scientific Research", Ministry of Education and Science, Bulgaria.

CO9. POTENTIAL ANTI-CANCER EFFECT OF HEMOCYANINS ON BREAST CANCER CELLS

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Cancer is one of the major reasons for human death. This requires the use highly specific targeted anti-cancer agents. It is known that hemocyanins are very promising class of antitumor therapeutics based on their immunogenic properties.

The aim of the present research was to investigate the anti-proliferative activity of Hemocyanin obtained from *Rapana thomasiana* (RtH) and the contribution of cholinium salts of aminoacids (ChAAs) for conformational stability of the hemocyanin molecule on cancer and non-cancerous cells.

ChAAs were synthesized in two steps, namely cholinium chloride was exchange to hydroxide anion on the ion exchange resin, and then cholinium hydroxide was reacted with the corresponding amino acid. The target compounds were isolated, purified and identified using H^1-NMR (400 MHz). The hemocyanin used in this study was isolated from freshly obtained hemolymph of the gastropods *Rapana thomasiana* (RtH) by centrifugation at 180 000 x g for 3h at 4°C, and subsequent purification using gel chromatography. In this study prior to use RtHs were incubated with a certain amount of ChAAs for 1 h at RT.

Two cell lines were used: *MCF-7* (breast cancer cells) and *3T3* murine fibroblasts as control cells (all from ATCC). Cell viability was determined by MTT proliferation assay. Briefly, the cell lines were seeded in 96-well plates ($1x10^4$ cells/well). Different concentrations of RtH ranging from 100 to 700 μg/ml were added to the cell culture media. While ChAAs and RtH-ChAAs complexes were tested at concentration of 0.67-4.7 mM and 100-700 μg/ml, respectively. Cells without treatment was used as a control. After incubation for 24 hours at 37°C, 5% CO_2 MTT reagent was added to each well and incubated for another 3 h. The cell viability was determined by measurement of O.D. at 570 nm.

The results of analysis do not indicate cytotoxicity of the ChAAs in both cell lines. Inhibitory effect of cancer cell growth after treatment with RtH at a concentration of 250-700 μg/ml and incubation 24h was observed. In contrast, the same concentration range of RtH promotes high proliferation on fibroblasts. Visible reduction in cell survival was observed for cancer cells treated with the modified RtH. Viability of MCF-7 cells decreased more than 50%. In contrast, the viability of 3T3 fibroblasts was not affected by treatment with the RtH-ChAAs.

Conclusion: The present study demonstrated that the effect of RtH-ChAAs on cell proliferation is cell specific. Since ChAAs play role for conformational stability of RtH and for enhanced anti-proliferative activity of cancerous cells for non-cancerous cells no such effect was obtained.

Acknowledgements: This study was supported by grants DNTS/Germany 04/01 and DFNI-BO 02/5.

CO10. STATINS AS ANTITUMOR AGENTS

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Abstract

High plasma cholesterol is one of the major reasons for cardiovascular diseases. Statins are the common drug used to reduce elevated total cholesterol and low density lipoprotein (LDL) cholesterol levels. Statins decrease hepatic cholesterol production by competitively inhibiting HMG-CoA reductase in mevalonate pathway. Besides a reduction of the cholesterol it has recently been found that statins possess anticancer properties. A number of experimental and clinical studies have demonstrated that they induce anti-proliferative, proapoptotic, anti-invasive and radiosensitising effects in many different tumor types. The precise molecular mechanisms by which statins exert their antineoplastic effects are not fully understood. PI3K signalling pathway is very often upregulated in different cancers. Recent studies indicate that anticancer activity of statins is due to the inhibition of the PI3K/AKT signalling pathway.
Due to the marked anticancer effects and low toxicity statins may be successfully used as novel therapeutic agents for the treatment of cancer alone or in a combination with other anticancer agents. However, further studies are needed to evaluate their optimal dose and highest effectiveness and better understand the cellular antitumor action of statins.

Key words: HMG-CoA reductase inhibitors, antitumor effects, cancer treatment

CP1. BRIEFLY ABOUT SOME CYTOTOXICITY ASSAYS

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The aim of our study is to present some of the most popular assays used to evaluate the influence of compounds on viability and proliferation of cultured cells. The advantages and pitfuls of MTT test, neutral red uptake cytotoxicity assay and crystal violet staining will be discussed.

Acknowledgement: The study was supported by Grant № ДФНИ Б 02 39 from 12.12.2014.

CP2. PROTEIN DETERMINATION BY THE METHOD OF BRADFORD: A BRIEF OVERVIEW

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Bulgarian Academy of Sciences, Sofia, Bulgaria

CP3. MYC GENES AND BREAST CANCER

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CP4. SRC GENES AND BREAST CANCER

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CP5. BRIEFLY ABOUT TUMOR SUPPRESSOR GENE P53 AND BREAST CANCER

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Session D: Microbiology, Virology and Parasitology

Chairpersons:
Assoc. Prof. Mariana Panayotova-Pencheva, DVM, PhD
Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences

Assoc. Prof. Evelina Shikova-Lekova, MD, PhD
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Secretary: Tanya Zhivkova, MSc
Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences

DO1. INTRAUTERINE TRANSMISSION OF TRICHINELLA AND IMPACT OF THE INFECTION ON THE IMMUNOLOGICAL STATUS OF THE OFFSPRING

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The influence of the immune status of the offspring of pregnant rats experimentally infected with Trichinela spirallis was traced in order to explore the migration of Trichinella larvae from mother to the offspring. For tracking the vertical transmission of migratory T. spiralis larvae, blood samples and smears from the viscera and muscle tissue of newborn rats were examined. During the experiment the growth dynamics of antibodies specific to Trichinella antigens was monitored in adult rats. The level of specific antibodies in the serum samples of the newborns in the control group was significantly lower than that in the animals born and milked by already infected rats as well as in the case of the infected adult rats. This trend had been observed throughout the entire experiment.

It was established that antibodies produced by mother in answer to migratory larvae are aimed at effective protection of foetus against infestation and increase the immune status of the offspring providing effective protection against re-infestation with larvae of T. spiralis [1,2]

The analysis of the results of the experiment were based on the recent immunological knowledge of the leading role of the inborn immunity in the formation of the immune response to the acquired immunity. It was proven that in protection against infections and other biologically active agents the inborn immunity would be playing a leading role inducing the start of the acquired immunity and its further activity. The immunoglobulin receptors contain particular proteins which recognize only their complementary pathogenic structures

The interaction between the inborn immunity and the acquired one is carried out by the dendritic cells and various mediator molecules [3, 4].

Obtained data demonstrate that passive immunity transmitted from mother to offspring can influence the course of the development of epidemiological and epizootic processes in natural and synanthropic foci of trichinellosis and in their progeny, which is subsequently infected with larvae of T. spiralis.

Key words: Trichinellosis, intrauterine transmission of trichinellosis, immune status

Acknowledgements: This work has been supported by RAN, project № 14-16-00026.

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DO2. SEROPREVALENCE SURVEY OF TOXOPLASMA GONDII AMONG WILD ANIMALS AND DOGS IN BULGARIA

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Abstract

Serum samples from 65 brown hares (*Lepus europaeus, P.*) obtained from 31 hunting areas of 12 administrative regions in Bulgaria during 2012-2013 were tested for presence of specific antibodies to the protozoan parasite *Toxoplasma gondii* using passive haemagglutination test (BulBio – NCIPD, Sofia). To investigate the environmental and public health significance of disease, by the same method, were tested serum samples by 102 home and stray dogs from three areas of the country, as well as ten samples from wild boars, roe deer, foxes and jackals. Presence of specific anti - *Toxoplasma* antibodies were found in 6.15% of brown hares, 42.16% of dogs, in roe deer, jackals and one wild boar.

This study complements and updates the available epizootiologial data on the prevalence of *Toxoplasma gondii* in intermediate hosts among wild animals and dogs in our country.

Key words: wild animals, brown hare, dogs, *Toxoplasma gondii,* Bulgaria.

Introduction

Toxoplasmosis is a protozoan disease with wide geographic distribution. The causative agent is the unicellular parasite *Toxoplasma gondii* (Family Toxoplasmatidae) which infects mammals, birds and humans. Definitive hosts are carnivores of the family Felidae. The intermediate hosts become infected congenital, by contact, but mainly alimentary - by taking food and water contaminated with sporulated oocysts or consumption of tissues from infected animals. In different wild hosts toxoplasmosis infection is unnoticeable or provoke mild symptoms, without manifestation of pathognomonic signs. This makes it difficult to diagnose and requires a study be made of materials obtained after hunting animals. In recent years, there have been reported cases of fatal toxoplasmosis in wild animals is the northern part of Europe [14,15]. Sedlak et al. experimentally established high sensitivity of hares to primary toxoplasma infection [18]. Jokelainen found that acute generalized toxoplasmosis is the reason for the death of 14 of 173 studied hares (8.1%) from Finland [15]. The diagnosis was confirmed by immunohistochemical methods, and through the genetic method (PCR) defined genotype of *T.gondii* - genotype II [15]. An interesting fact is that this parasite is able to cause illness and even kills its host, but not infrequently causes subclinical or asymptomatic infections [11], with an established seropositivity. Latent infections can be detected through a study of the immune response of the host for this parasite. Hares make up the immune response, as the presence of antibodies settles between 7 and 12 days after infection [18].

In Spain, using Modified agglutination test, antibodies to *T.gondii* were found in 14.2% of the studied hares, such as spread of infection was significantly higher in animals in the forest areas of Catalonia (53.8%) while those living in arid areas level of seropositive ranged from 6.1 to 14.6% [4]. High levels of seroprevalence are reported in populations of hares from Germany [11]. Frölich et al. identify positively reacted on the Sabine-Feldman test 46% of the studied hares from the four areas of the province Schleswig-Holstein [11]. European hares from the Czech Republic, Slovakia and Austria are tested for serum antibody by indirect fluorescent assay. In studied hares with detected *T.gondii* antibodies are 14% of them, and seropositivity for individual countries is respectively 21%, 6% and 13% [7].

The literature data about the prevalence of *T.gondii* infection among wild animals in Bulgaria are scarce. Angelov et al. in 1956 by means of serological tests detected for the first time toxoplasmosis in hares [1]. In 1968 and 1971 Nancov, by complement fixation test (CFT), found that 15.7% and 14.8% of the studied animals react positively for the disease, in various areas the seropositive levels is range from 7.4% to 33.3% [2,3]. Although toxoplasma infection is widespread in the hare in Bulgarian [2,3], severe epizootics in this species of game, with a high mortality, have not been reported.

In recent years there have been increasing reports of the presence of *T.gondii* antibodies in wild animals. Positive titers are found in the 33.9% of the tested deer in Norway [21], 24% in the Czech Republic [6], 13.7% and 21% in Spain [13,17]. Toxoplasma infection in feral pigs is serological identified in a Spain, Portugal and Slovakia, as levels the seropositive animals are respectively 38.4%, 20.6% and 8.1% [5,12].

In dogs, infection with *T.gondii* is usually asymptomatic and the detection of specific antibodies shows that they have been infected. Reports indicate that the free living dogs, in close contact with wild birds and rodents, in rural areas, are at higher risk for infection. Specific *T.gondii* antibodies are found in 23% of the tested dogs in Sweden [20] and 26% in Austria [22]. Serological testing in these animals in Bulgaria is made and reported by Kostova in 1999 and after performing passive haemagglutination test (PHAT) specific antibodies are found in 27.78% of them, the highest percentage seropositivity is observed in stray dogs [16].

People usually become infected by ingestion of oocyst from the environment, the tissue cysts or tachyzoites in undercooked meat or viscera of many different animals, or unpasteurised milk. It is not known which of these routes is epidemiologically more important [8,19]. Study of Elmore et al. shows that direct contact with cats is not a major risk for infecting people [9]. In the past, the consumption of raw or undercooked meat, has been regarded as a major route of transmission to humans [19]. It has been found relationship between serological identified cases in animals and those in people living in the same area [2,10]. The dogs probably play a role in mechanical transmission of infection to humans [10]. In America, recent outbreaks of acute toxoplasmosis in humans have been associated with oocyst environmental pollution [8]. Therefore, knowledge about possible routes of horizontal transmission to humans and the sources of infection in nature is a prerequisite for the development of effective strategies for the prevention of infection.

The **purpose** of our study is testing of serum samples from wild animals (hares and some game species) and dogs for the presence of anti-*T.gondii* antibodies in reaction passive haemagglutination and investigation of *Toxoplasma* seroprevalence among these intermediate hosts, inhabiting the wild nature or living near the settlements in different regions of Bulgaria.

Materials and Methods

Materials: In the 2011-2013 were collected blood samples from 65 brown hares (*Lepus europaeus, P.*) shot by hunters and one caught and dead in lowland agricultural areas of 12 administrative regions in Bulgaria: Blagoevgrad, Burgas, Vidin, Vratsa, Lovech, Pazardzik, Pleven, Plovdiv, Silistra, Sliven, Stara Zagora and Yambol (Table 1). The age of the hares was determined by Stroh's method and they were classified as subadult and adult.

Gathered were blood samples from other game species (n = 10): wild boar-3, roe deer-3, red deer-1, jackals - 2 and one fox.

In the study were included blood samples from 102 adult dogs (stray dogs and home dogs living in a yards) obtained in carrying out veterinary activities in 2012-2013 in the regions of Veliko Tarnovo, Zlatograd (Smolyan) and Pancharevo (Sofia-city).

Blood Sera were separated following centrifugation immediately after their receipt in the laboratory and stored to conduct serological tests in temperature -20ºC

Methods: Serum samples were tested for the presence of specific *T.gondii* antibodies in the passive haemagglutination test (PHAT) with toxoplasmic erythrocytic diagnosticum (commercial kit BUL BIO - Ltd. Bulgaria), according to the manufacture instructions.

Results and Discussion

The study of the 65 serum samples from brown hares established the presence of specific *T.gondii* antibodies in four of them (6.15%). Serological titer of 1:20 is obtained in three hares shot in hunting area Kameno (Burgas), Kalitinovo (Stara Zagora) and Kiril and Methodi (Stara Zagora).

In one of animals (caught in the land of the area Belozem, Plovdiv region) found a high serological titre (1:80). On the literary data brown hares with proven acute form of infection with *T.gondii*, show high titre of antibodies [15]. The diagnosis in this case has not been confirmed by other methods.

Table 1. Results of serological examination of serum samples from brown hares obtained from different areas of Bulgaria

Regions	Hunting areas	Number of testing samples	Positive results (n)	Titre		
				1:20	1:40	1:80
Blagoevgrad	1	1	0	0	0	0
Burgas	3	8	1	1	0	0
Vidin	1	1	0	0	0	0
Vratsa	2	4	0	0	0	0
Lovech	2	2	0	0	0	0
Pazardzik	1	1	0	0	0	0
Pleven	1	1	0	0	0	0
Plovdiv	4	12	1	0	0	1
Silistra	2	3	0	0	0	0
Sliven	5	11	0	0	0	0
St. Zagora	7	18	2	2	0	0
Yambol	2	3	0	0	0	0
Total	31	65	4	3	0	1

The established seroprevalence of toxoplasmosis amongst hares are close to those living in some dry territory to Spain and Slovakia and significantly lower from those in Germany, Czech Republic, Austria or forest areas in Spain [4,7,11]. Serological studies show less prevalence of *T.gondii* among brown hares (6.15%) in comparison with the levels established by Nankov in 1968 and 1971 (15.7% and 14.8% respectively) [2,3]. The difference is probably due a long period of time between the two surveys (about 45 years), methods of testing, changes in the game management practices, and a serious reduction of the number of this species of game in Bulgaria.

Studies of many scientists show that there were no significant differences between the levels of seropositive animals and their sex and age [4,12]. Our results show differences in terms of sex of tested animals. It was found that reacted positively were predominantly female hares - 7.9% of the test, and 3.7% of males. No significant difference was observed in seropositivity for both age groups (adults, 6.4% and subadults 5.6%).

Testing of serum samples from wild animals for the presence of specific anti-*T.gondii* antibodies show a serological titre of 1:80 in one of three tested wild boars. With a positive result were and three roe deer (one with a serological titre of 1:20, and two with 1:80). In both jackals is established 1:20 titer, while in fox were not found specific antibodies. The results cannot be compared with data reported by different scholars [5,6,12,13,17], but show the presence of seroreagents among different species of wild animals show the presence of this infection in the forest areas of the country.

Serologic testing for toxoplasmosis at 102 samples of free-living dogs (home and stray) detects *T.gondii* antibodies in 43 of them (42.16%) - Table 2. Seropositive dogs showed titers from 1:20 to 1:320 and presented in percentages as follows: 20.93% have titer 1:20; 1:40 – 51.16%; 1:80 - 9.30%; 1:160 – 11.63% and 1:320 – 6.98%.

These levels at the home and stray dogs, are higher than those reported for Bulgaria 27.78% [16], Sweden – 23% [20] and in Austria - 26% [22].

Table 2. Results of the serological test (PHAT) of serum samples from home and stray dogs

Regions	Dogs	Number of testing samples	Positive results (n)	Titre					Seropositive %
				1:20	1:40	1:80	1:160	1:320	
Veliko Tarnovo	Home	3	1	1	0	0	0	0	33.33
	Stray	8	3	3	0	0	0	0	37.50
	Total	**11**	**4**	**4**	**0**	**0**	**0**	**0**	**36.36**
Smolyan (Zlatograd)	Home	8	4	0	1	1	0	2	50.00
	Stray	8	7	4	0	0	2	1	87.50
	Total	**16**	**11**	**4**	**1**	**1**	**2**	**3**	**68.75**
Sofia (Pancharevo)	Home	23	6	2	2	1	1	0	26.09
	Stray	52	22	12	6	3	1	0	42.31
	Total	**75**	**28**	**14**	**8**	**4**	**2**	**0**	**37.33**
	Home	34	11	3	3	2	1	2	32.35
	Stray	68	32	19	6	3	3	1	47.06
Total		**102**	**43**	**22**	**9**	**5**	**4**	**3**	**42.16**

In this study was observed higher percentage of seroprevalence among the stray dogs 47,06% (min 37.5%, max 87.5%) in comparison with those, living in the yards - 32.35% (min 26.09%, max 50%). Such results have been reported from Kostova in 1999 [16]. Probably as a result of their diet, contact with infected animals (domestic and wild) and access to the environment contaminated with *T.gondii* oocysts.

Seroprevalence of *T.gondii* is highest in dogs by the region of Zlatograd – 68.75%. With approximately the same levels are the animals in regions of Veliko Tarnovo and Pancharevo (Sofia), respectively - 36.36% and 37.33%. In the literature data there is a connection between the number of seropositive animals and certain factors such as the presence of definitive hosts, favorable climatic conditions, agricultural activity and the type of feeding [4,12]. This gives us reason to believe that high levels of seropositive dogs registered in the region of Zlatograd (87.5% from stray and 50% from home dogs) are the result of the complex interaction of these factors.

The results of the present investigation showed the presence of antibodies against *T.gondii* in the tested different wild animals species in Bulgaria (brown hare, roe deer, wild boar and jackal). The observed seropositivity among brown hare and roe deer is an evidence for environmental contamination with infective oocysts.

The results of our study indicate the presence of specific anti-*T.gondii* antibodies in wild animals inhabiting the lowland agricultural areas. *Toxoplasma* infection is serologically established in different species of game, inhabiting forest areas, where brown hare present, but due to its low density, is not an object of intense hunting activity. The results indicate that infection with *T. gondii* is present in nature, which could give public health impact, if infected wild animals are used for food.

The high percentage of seropositive dogs - 42.2% focuses on their importance as a likely source of human infection. Stray dogs in their role as a liaison between wildlife and humans can be an indicator for spreading of *T.gondii* in the environment.

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DO3. CELL GROWTH INHIBITING EFFECT OF THERMOLABLE BIOLOGICALLY ACTIVE SUBSTANCE ISOLATED FROM *FASCIOLA HEPATICA*-INFECTED RAT SPLEENS

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ABSTRACT

Thermolable biologically active substances (BASes) were isolated from healthy and *Fasciola hepatica* infected rat spleens. The effect of the newly isolated BASes was studied *in*

vitro on nonactivated lymphocyte cell cultures. An inhibiting immunomodulating effect of the BAS isolated from *F. hepatica* infected rat spleens was demonstrated.

A new property of mature *Fasciola hepatica* L. to inhibit experimental liver carcinogenesis was established in our previous investigations [6]. This formed the basis for a hypothesis on the possible roles of some biologically active substances of parasite and host origin in the pathogenesis of this interaction. Thermolable and thermostable biologically active substances were isolated from the tissues of *F. hepatica* and from normal and *F. hepatica* infected host liver tissue, which were proved as inhibitors of cell proliferation [8, 9, 10].

The aim of the present work is to isolate thermolable biologically active substances (BASes), inhibitors of cell proliferation, from the spleens of healthy and *F. hepatica* infected rats, and to investigate their effect *in vitro* on the leukocyte cell proliferation.

MATERIALS AND METHODS

Wistar male rats aged 30 days were orally infected with 20 metacercariae of *F. hepatica*. The animals were sacrificed 4 months later when the chronic stage of fasciolosis has been developed. The spleens of the infected animals were processed to obtain the BAS. The BAS isolated from spleens of healthy animals bred under the same conditions were used as controls.

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

The BASes were isolated by the modified method of Verly et al. [11] by ethanol precipitation from aqueous tissues homogenate. The fraction obtained between 70% (v/v) and 87% (v/v) ethanol saturation was investigated.

Protein estimation of the newly isolated BASes was carried out according to Bradford [1].

The dried extracts of the BASes dissolved in PBS were added to the lymphocyte cell cultures at a dose of 20 μg per well.

The spleen lymphocyte cell cultures were obtained by gradient centrifugation on Polysep (Pharmachim, Bulgaria – 1.077 g/cm^{-1}). The cell number was adjusted to 1 X 10^6 cells/ml and applied to U-bottomed microtiter plates (Nunc) in RPMI 1640 medium (Flow) containing 10% fetal calf serum.

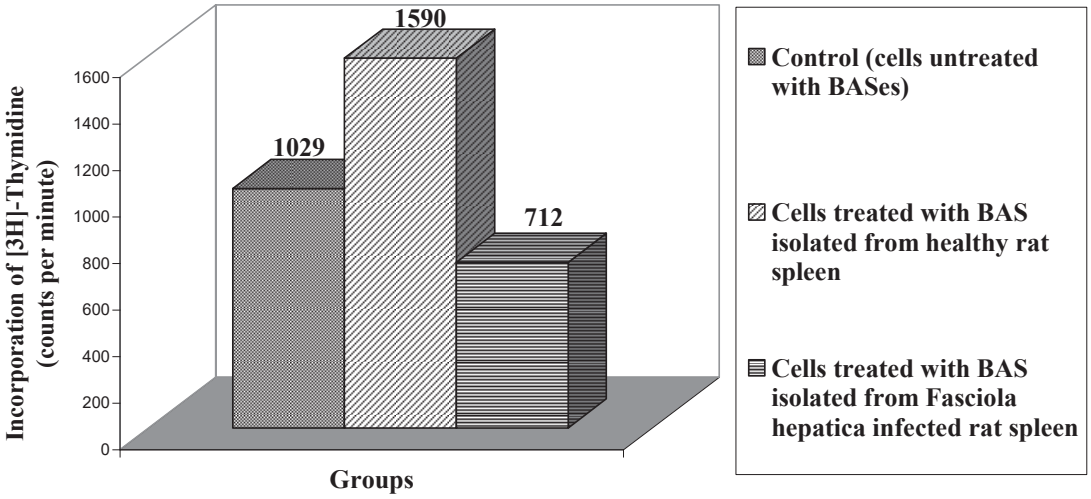
The BASes were added on the 0 h to nonstimulated lymphocyte cell cultures. The cells were cultured for 24 h at 37ºC in a humidified 5% CO_2 atmosphere. 1.5 μCi ^{3}H-thymidine (UVVVR- Prague) was added to each well and the incubation continued for an additional 18 hours. The cells were harvested on NC filters (45μ) (Millipore) and counted in a scintillation counter (Intertechniques). The results are statistically processed by variation analysis after the Student-Fisher's method by t-criterion.

RESULTS AND DISCUSSION

The effects of the BASes isolated from healthy and *F. hepatica* infected rat spleens are investigated on nonstimulated lymphocyte cell cultures. The data are presented on Figure 1.

Fig. 1

INCORPORATION OF [3H]-THYMIDINE IN NONSTIMULATED LYMPHOCYTE CELL CULTURES AFTER TREATMENT WITH BAS ISOLATED FROM *FASCIOLA HEPATICA* INFECTED RAT SPLEEN



The treatment of the lymphocyte cell cultures with the newly isolated from *F. hepatica* infected rat spleens BAS leads to 1.44 fold decreasing of [^{3}H]-Thymidine incorporation in comparison with the control untreated cell cultures (Fig. 1). The percent of the inhibition is 30.8%. Statistically significant decreasing of the cell proliferation under the effect of this BAS is established ($P<0.01$). The treatment of the cell cultures with the BAS isolated from healthy rat spleens does not inhibit the cell proliferation of the lymphocyte cell cultures.

The newly isolated BAS from the spleens of *F. hepatica* infected rats show immunosuppressive effect in contrast to the BAS isolated from healthy rat spleens.

Phase immune alterations in humoral and cell immunity are established in *F. hepatica* infected host concerning migration, development and localization of parasites in the body [2, 3, 4, 5]. In previous paper we reported data about a stimulation of cell immunity under the experimentally inhibited tumor growth *in vivo* at the background of chronic fasciolosis [7]. The received data and the literature information suggested us about the possible involving of some immune mechanisms and endogenous immunomodulators in the pathogenesis of the anti-tumor effect of chronic fasciolosis.

The present results support our hypothesis for possible stimulation of the production of inhibitors of the cell proliferation in some tissues of *F. hepatica* infected host.

There are no literature data available for isolation and characterization of similar BASes from the spleens of *F. hepatica* infected hosts.

Newly isolated BAS from *F. hepatica* infected rat spleens has different effect on the cell proliferation in comparison with the BAS isolated from healthy rat spleens. Future investigations will reveal more about their role in complex immunological mechanisms in the body of infected and non-infected hosts.

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DO4. ANTIPARASITE REMEDIES APPLIED TO RUMINANT ANIMALS FROM THE CERVIDAE FAMILY

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ABSTRACT

The present study represents a review of the literature on the experience accumulated in the antiparasite treatment of the ruminant animals of the Cervidae family distributed in Bulgaria as well as of species close to them. The summed-up literature data show that among the tested anthelminthics a good effect against the gastrointestinal parasites have displayed oxfendazole, albendazole, thiabendazole, mebendazole, fenbendazole, luxabendazole and tetramisolum. Febantel is also one of them having shown a relatively positive effect also against the dictyocaulus infection. No so good effect reaching up to unsatisfactory one against the lung helminths, especially protostrongylids, has been demonstrated by the preparations cambendazole, levamisole and rintal. Ivermectin has been most efficient in the struggle both against the gastrointestinal and lung nematodes. Its efficacy in the fight against the pulmonary helminthoses has been better when applied at higher doses such as 400µg/kg body weight and in its subcutaneous and pour on administration as compared to the per oral one. The attempts for controlling the extrapulmonary protostrongylidoses with levamisole and rintal have proven to be unsuccessful. Ivermectin has been in that respect the best of the rest of the anthelminthics but also at the high dosage (400µg/kg body weight) though even in these cases its effect has been incomplete. The treatment of trematode infections with albendazole has shown rating of efficiency between 20 and 80 per cent in per oral and almost 100 per cent in the intraruminal administration. Niclosamide has been effective in monesiosis. Ivermectin has been tested also as a remedy for fighting the external parasites in the deer. It has been highly effective in nasopharyngeal and subcutaneous myiasis, ticks, mange mites and lice.
Key words: parasite treatment; deer; Cervidae

INTRODUCTION

Parasitoses in animals, including in wild ones, display an array of negative effects. Most of them become a pre-disposing factor for secondary deficiencies and infections, others are detrimental to reproduction and some would lead to lethality of the hosts. Consequently, they are in direct dependency of the numbers of their populations. On the other hand wild animals present an epidemiological niche for parasites common with the ones in the domestic animals and are often an important source of hazardous parasitic zoonoses. That is way taking measures for fighting parasitic diseases amid the wildlife is not only an indication of a good management in game-breeding and hunting industry but is also a healthcare necessity. The application of anti-parasitic remedies in the wild animals including the deer has however, its specifics. For example, Mason [17] indicates that important point is that deer are not sheep or cattle, and the efficacy (and toxicity) of any pharmaceutical product, be it a drench or some other type of drug, has to be established independently in deer. In this connection the goal of the present study was set and it is to carry out a review of the literature of the experience with regard to the anti-parasitic treatment of ruminant animals from the Cervidae family and in this way to help experts in the field with information which would make it easier for them to take action in the control and prevention of the parasitic diseases among them.

RESULTS AND DISCUSSION

Treatment in cases of gastrointestinal and lung nematodoses

The study of Presidente et al. [22] is among the earliest concerning treatment of parasitoses in cervids. The authors have observed an outbreak of dictyocaulosis among a captive herd of black-tailed deer fawns. A transient decrease in output of *Dictyocaulus viviparus* larvae in feces has been occurred after treatment with levamisole hydrochloride given as a drench at the rate of 16 mg/kg of body weight. Lungworm larvae have not recovered in feces 6 days after cambendazole given as a drench at 40 and 50 mg/kg of body weight. Larvae have been again recovered in feces from these fawns between post-treatment days 15 and 23. Output of larvae in feces has increased when fawns have been confined on a contaminated grass pasture that has been intensively grazed. Deteriorating physical condition of the fawns has necessitated additional treatment with cambendazole and movement to a woodlot where reinfection by ingestion of larvae has been probably minimized. A non-infected deer has been placed on the contaminated pasture 75 days after the infected herd has been removed. After 55 days, lungworm larvae have been recovered in feces from this deer. Then 29 days later, 20 fawns have been placed on this pasture. Four of six of these fawns that have been subsequently necropsied have harbored light burdens of *D. viviparus*. Small numbers of lungworm larvae have been recovered in feces from five of eight remaining fawns.

Later Foreyt and Drawe [6] have established that albendazole at dose rates of 11 to 54 mg/kg (mean of 25) of body weight has been highly effective (greater than 99%) against *Haemonchus contortus, Ostertagia odocoilei, O. mossi, Trichostrongylus askivali* and *Nematodirus odocoilei* in 22 white tailed deer in southern Texas.

Tetramisolum given at 10 mg/kg live weight orally 2–3 times a year to reindeer kept in an enclosure has eliminated the parasitic invasions, in spite of the established infection with *Dictyocaulus* and *Trichostrongylus* species during a period of six years [11].

In the Mahtra forest, Estonia, 24 *Capreolus capreolus* with natural helminthiases have been treated with tetramisole 20% granules at 5, 10 or 15 mg/kg body weight once or at 10 mg/kg twice with an interval of 2 weeks. In every case, the granules have been mixed in feed. Considerable decreases in the parasite burdens have been achieved (except with the 5 mg/kg dose) for gastrointestinal nematodes, especially the dominant species *Ostertagia leptospicularis* and *Chabertia ovina* [33].

Oxfendazole at 1.7 mg/kg body-weight, thiabendazole at 44 mg/kg, both on 3 consecutive days, and mebendazole (10% granulated) at 10 mg/kg for 3 or 5 days have been tested on 5000 roe deer. All 3 anthelminthics have given good efficacies. The cure rates for the most important gastrointestinal helminths have been 78 to 100%, except *Trichuris* spp. with 54.6 to 72.3%. The most effective has been the 5-day mebendazole treatment [3].

Fenbendazole at 5 mg/kg body-weight mixed in feed has been given to 28 *Cervus elaphus* with mixed nematode infections. Six days after treatment the faeces have been free of eggs. Autopsy of 5 treated and 8 untreated deer have showed that treatment has cured 2 animals of *Spiculopteragia assmetrica* and *S. boehmi*, 3 of *Capillaria bovis* and 4 of *Nematodirus filicollis* and has given good reductions in worm burdens [29]. In other studies Schultz et al. [28] have provided fenbendazole to captive and free-ranging white-tailed deer to determine effects on gastrointestinal nematode burdens. They established that fenbendazole reduce gastrointestinal nematode burdens of captive and free-ranging white-tailed deer too.

The efficacy of mebendazole on nematode infections in farmed fallow-deer has been studied by Szokolay and Rehbinder [31]. The substance has been administered as medicated feed, 6 mg/kg body weight during 10 days. The effect of mebendazole on *Chabertia* has been good while the effect on *D. viviparus* has been incomplete probably due to arrested L5-larvae, which have been unaffected by the anthelmintic during arrested state.

Mackintosh et al. [15] have investigated the efficacy of febantel and ivermectin in six-month-old red deer calves. Five calves have received febantel by mouth at 7.5 mg/kg, five have received a subcutaneous injection of ivermectin at 200 μg/kg and five have been controls. All calves have been killed seven days later and total lung and gastrointestinal worms have been count. Febantel has been 85 and 99.8% efficient in removing immature and mature *D. viviparus*, respectively, and ivermectin has been 100% efficient in both cases. There has no gastro-intestinal nematodes in any of the treated calves, compared to an average of 619 in the control calves.

Bowie et al. [2] have performed the following experiment. Thirty eight newly weaned hinds have been randomly allocated to one of two equal groups. One group has received ivermectin, the other, oxfendazole at dose rates of 0.2 mg/kg and 4.5 mg/kg, respectively. All deer have been drenched four times and have been grazed on pasture. Both anthelminthics have reduced *D. viviparus* faecal larval counts to low levels 20 days after dosing, but the mean larval output and the proportion of deer shedding *D. viviparus* larvae at 27 and 33 days after treatment, have been significantly lower in the ivermectin treated group. This study suggests that ivermectin prevents reinfection with *D. viviparus* for approximately 14 days longer than oxfendazole.

Naturally infected fallow deer have been treated with pellets luxabendazole in feed (2 mg/kg/day for 5 days). Based on post mortem examinations, the drug has been >90% effective against intestinal nematodes [10].

Three groups of ten 4-month-old red deer calves naturally infected with lungworms (*D. viviparus*) have been treated with either oral ivermectin (200 μg/kg), topical ("pour-on") ivermectin (500 μg/kg) or oral oxfendazole (5 mg/kg). Faecal larval counts have been undetectable or very low for 14 days after treatment with oxfendazole, 28 days after treatment with oral ivermectin and for 49 days after treatment with topical ivermectin. This study suggests that the topical formulation of ivermectin has been very effective against lungworm and has had a more persistent action than the oral ivermectin formulation in young red deer [16].

Ivermectin solution (at 0.2 mg/kg) has been added to the feed of red deer kept in a reserve, with a second dose a month later. Treatment has been highly effective against *D. viviparus, Varestrongylus sagittatus* and various other strongylid nematodes [12].

The anti-parasitic effect of the orally administered paste formulation of ivermectin in reindeer has been evaluated by means of a trial designed to compare the efficacies of orally and s.c. administered ivermectin at the same dosage (0.2 mg kg^{-1} body weight) in naturally infected adult reindeer. Oral treatment has been less efficacious than s.c. injection against parasitic nematodes [21].

Andrews et al. [1] have evaluated the use of ivermectin in red deer hinds. Their results have demonstrated that ivermectin, when used at 400 micrograms/kg body weight, proved to be more efficient than 200 μg/kg body weight although positive worm egg counts together with the isolation of lungworm (*Dictyocaulus* species) larvae have been recorded from hinds having received the anthelmintic at the higher dose.

One hundred and fifteen young red deer, heavily infected with lungworms (*D. viviparus*) and lightly infected with gastro-intestinal nematodes, have been divided into three groups. One group has been treated with one adult sheep dose of a slow-release albendazole capsule, another has been dosed orally five times with liquid albendazole and the third has been left untreated. The capsule has eliminated faecal lungworm larvae during the 103-day trial period. There has been a highly significant difference in faecal larval counts between the capsule-treated and control group [25].

Mason [17] has pointed that levamisole, one of the three major drench families available, is not work satisfactorily against lungworm in deer. According to him this is that because it has metabolized too quickly and hence has not presented for sufficient time at therapeutic levels.

Connan [4] has established that subcutaneous ivermectin at 400 μg/kg has removed 100% of the adult and developing worms by *Ostertagia* spp., but although it also has removed 95% of hypobiotic larvae, some larvae have remained in every treated animal. The efficacy of fenbendazole at 15 mg/kg given over five days has been 75% effective against hypobiotic larvae and although the long interval after treatment has obscured the result, it has been probably much higher against later stages.

Rehbein and Visser [24] have conducted trial to evaluate the persistent anthelmintic effect of ivermectin as a topical treatment at 500 μg/kg against induced infection with *D. viviparus* in red deer. The results have showed a highly significant anthelmintic activity for at least 28 days against a newly acquired infection with *D. viviparus* (> 99% efficacy).

Six red and six wapiti hybrid deer, naturally infected with lungworm and gastro-intestinal parasites have been treated with pour-on moxidectin at 500 μg/kg body weight and slaughtered 14 or 16 days later [32]. Total worm counts have been performed on the lungs, abomasum and abomasal digest of each deer. The efficacy of moxidectin pour-on has been 100% against adult and immature lungworms (*D. viviparus*) in red deer, and 100% and 99.7% effective against adult and immature lungworm in wapiti hybrid deer. The efficacy of moxidectin pour-on has been 100, 100, 99.9 and 99.9% respectively against adult, fifth stage, late fourth stage and early fourth stage larvae of *Ostertagia*-type nematodes (assumed to be *Ostertagia, Spiculopteragia, Skrjabinagia* and *Apteragia* spp.) in both red and wapiti deer. According to the authors **t**he pour-on formulation of moxidectin, at 500 μg/kg body weight, is highly effective against mature and immature lungworms and abomasal nematodes in wapiti hybrid deer and equally effective in red deer.

Rintal 2.4% premix in single dose 7.5 mg of active substance on kg body weight has been given to the calves of red and fallow deer [9]. Effectiveness of deworming against nematodes from family Trichostrongylidae has been 85.2% in red and 97.8% in fallow deer. Against namatodes from genus *Trichocephalus* the effectiveness has been higher extending in red deer 98.1%, and in fallow deer 99.5%. Anthelmintic has not been effective against nematode *Varestrongylus sagittatus*.

Foreyt et al. [7] have treated Columbian black-tailed deer infected with *D. viviparus*, *Parelaphostrongylus* sp., *Trichuris* sp., *Moniezia* sp., *Eimeria* spp., and gastrointestinal strongylids with ivermectin subcutaneously at doses between 0.2 and 1.3 mg/kg of body weight monthly for four consecutive months. All nematode eggs and larval stages in feces have been eliminated or greatly reduced following treatment.

Treatment in cases of extrapulmonary protostrongylidoses

Four anthelmintic preparations (Fenthion, Fenbendazole, Mebendazole and Ivermectin) have been tested against brainworm *Elaphostrongylus rangiferi* by Nordkvist et al. [20]. The benzimidazole compounds have been highly effective, with mebendazole a bit ahead. Ivermectin has had a moderate effect and Fenthion has not been effective on this parasite. According to the study ivermectin has been the overall most effective anthelmintic in this test.

Nichols et al. [19] have reported for a case in which *Parelaphostrongylus tenuis* has been occurred in 6 *Rangifer tarandus tarandus* at the National Zoological Park's Conservation and Research Centre, USA. Clinical signs have begun with ataxia of the rear legs, often progressing to paraplegia. The front legs have been affected to a lesser degree. Treatment of 3 *R. tarandus* with dexamethasone at 1 mg/kg i.v. and levamisole at 5.5-11.0 mg/kg s.c. has given initial mild improvement, but eventually all animals either have died or have been euthanized.

Ivermectin has been injected subcutaneously at 200 and 400 μg/kg of body weight into seven white-tailed deer in an attempt to control the muscle nematode *P. andersoni* [27]. Counts of first-stage larvae in feces have dropped to zero at 17 to 18 days post-treatment. Larvae have been reappeared in feces 1.5 to 6 wk later in six deer. Four deer have been treated again approximately 9 wks. after the first treatment; larval counts have dropped to zero in 12 to 18 days. Larvae have been reappeared in low numbers 45 to 55 days after the second treatment. Because deer have been held indoors on cement and the prepatent period of these worms is approximately 2 mo., according to the authors the reappearance of larvae has not be due to reinfection by accidental ingestion of gastropod intermediate hosts. Results have suggested that ivermectin at dosages of 200 or 400 μg/kg of body weight suppressed larval production by adult female nematodes for several weeks or destroyed first-stage larvae in the lungs.

Ivermectin solution (at 0.2 mg/kg) has been added to the feed of red deer kept in a reserve, with a second dose a month later [12]. The author has established that the drug has not been effective against *Elaphostrongylus cervi.* Demiaszkiewicz et al. [9] have given Rintal 2.4% premix in single dose 7.5 mg of active substance on kg body weight to the calves of red and fallow deer. They have found that this anthelmintic also has not been effective against nematode *E. cervi*.

Treatment in cases of parasitoses due to flatworms

According to study of Foreyt and Drawe [6] albendazole at dose rates of 11 to 54 mg/kg of body weight is 38% effective against mature and immature deer liver flukes (*Fascioloides magna*). Later Qureshi et al. [23] also have tested this anthelminthic against *F. magna.* Adult white-tailed deer naturally infected with the trematode have been captured and randomly assigned to four groups. Each group has been fed pelleted feed coated with albendazole for each of seven consecutive days to deliver the drug at a dose rate of approximately 0.0, 5.0, 8.5, or 16.5 mg/kg bodyweight/day. At 7 wks. post-treatment, each animal has been euthanized and necropsied. Effects of albendazole treatment have included significant reduction in parasite egg count per gram of feces and increase in serum albumin concentration. Smaller parasites or remains of dead parasites have been seen at the end of migratory tracks in the treated groups. Efficacy of the drug has been 82 to 84%.

Treatment of fascioloidosis in Croatia has been conducted in red deer over a period of three years [30]. The free-ranging animals have been treated with a mixture of triclabendazole and standard deer salt brick components at a dose of 60 mg/kg body weight per deer, twice in seven days. Captive deer have been treated individually by intraruminal application of triclabendazole using a needle or esophageal tube. Coprological control has been made 30 days after administration of antiparasite preparation. Positive results (findings of *F. magna* eggs in

faeces) in the free ranging deer population have varied from 20% to 80% respectively. In captive deer the authors have recorded almost ideal efficiency of triclabendazole (after treatment 95.5% of all faecal samples have been free of eggs).

Forejtek and Chroust [5] have examined roe and red deer in the South-Moravian forests, Czechoslovakia, for parasites before and after treatment with Taenifugin (niclosamide). Taenifugin has been added to food and has been 100% effective against both *Moniezia expansa* and *M. benedeni.* Lungworms and intestinal worms have not been affected by the treatment.

Treatment in cases of external parasites

Miller et al. [18] have studied the influence of ivermectin administered orally to white tailed deer parasitized with lone star ticks, *Amblyomma americanum*. They have established that daily oral doses of 35 and 50 μg/kg/d has provided 100% control of adult and about 90% control of nymphs that have been placed on treated fawns. A single oral dose of 50 μg/kg has given >90% control of adult and nymphal ticks attached to treated fawns at the time of drug administration and 70% control of ticks placed on treated deer three days thereafter. When ticks have been placed on fawns treated with a single dose of ivermectin (50μg/kg) the engorgement period has been longer, ticks have been lighter in weight, and females have laid fewer eggs than ticks detaching from control fawns. A single oral dose of ivermectin at 20 μg/kg has prevented about 60% of the adult and nymphal ticks attached at the time of drug administration but has not affected other ticks placed on the animals after treatment. Finally, the authors have concluded that ivermectin is highly effective against ticks *A. americanum*.

Garris et al. [8] have described a case of *Psoroptes cuniculi*, the ear mite of domestic rabbits, which has been found on captive white-tailed deer. In addition to moderate infestations in their ears, two 4-yr-old bucks, two 3-yr-old does, and seven 4-yr-old does have showed patchy areas of alopecia along the sides and brisket. Both bucks also have had patchy areas of alopecia around the base of antlers. Ear mites have been eradicated from all deer except from one doe by providing ivermectin treated corn to the deer at a rate of 1,000 g (equivalent to 200 mcg/kg of ivermectin)/day/deer for several days. The ear mite infestation in the one doe has been eradicated by intramuscularly injection with ivermectin at 400 μg/kg. After treatment with the ivermectin and eradication of the mites, the alopecia has improved and eventually has been eliminated.

A survey of nasopharyngeal and subcutaneous myiasis affecting roe deer has been conducted in the Czech Republic [26]. The deer have been treated each winter across the board with ivermectin pulvis (150 mg/kg). Parasites found have been the larvae of *Hypoderma diana* and *Cephenemyia stimulator*. The results have showed that through the help of treatment with ivermectin, it is possible to keep parasite levels low.

The anti-parasitic effect of the orally administered paste formulation of ivermectin in reindeer has been evaluated by means of a trial designed to compare the efficacies of orally and s.c. administered ivermectin at the same dosage (0.2 mg kg^{-1} body weight) in naturally infected adult reindeer. Both formulations have been 100% effective against larvae of the warble fly, *Oedemagena tarandi* [21].

Lamka et al. [14] have tested per oral administration of ivermectin with respect to the control of larval stages of hypodermosis (*H. diana*) in roe deer. Ivermectin has been administered for two days at a daily dose of 0.30 mg/kg body weight during winter game feeding. The shot deer have been checked for the presence of larvae throughout the year. Prevalence and intensity of infection have been determined. Prevalence and intensity of infection have been very low in comparison with the situation before treatment and with the

control group. These results have documented the high efficacy of mass per oral ivermectin administration in the control of warble fly larvae. According to the authors the ivermectin is the first drug suitable for the treatment of roe deer hypodermosis.

In Austria ivermectin has been used to control oestrinosis and hypodermosis in red- and roe deer since many years [13]. In his work the author points that Ivomec-Praemix 0.6% has been examined and it has turned out that the treatment has been extremely successful. In practice Ivomec-Praemix 0.6% has to be given preference due to its stability. The most favorable dosage for red- and roe deer has been 2 x 0.4 mg ivermectin/kg body weight administered at an interval of one week, although equally good results could be obtained with 1 x 0.3-0.4 mg ivermectin/kg body weight. With the above mentioned dosages a 100% success of treatment could be achieved against *Hypoderma actaeon*.

Foreyt et al. [7] have used ivermectin in cases of black-tailed deer parasitized with lice. The deer have been treated with ivermectin subcutaneously at doses between 0.2 and 1.3 mg/kg of body weight monthly for four consecutive months. Lice have been eliminated following treatment.

Conclusion

The summed-up literature data show that among the tested anthelminthics a good effect against the gastrointestinal parasites have displayed oxfendazole, albendazole, thiabendazole, mebendazole, fenbendazole, luxabendazole and tetramisolum. Febantel is also one of them having shown a relatively positive effect also against the dictyocaulus infection. No so good effect reaching up to unsatisfactory one against the lung helminths, especially protostrongylids, has been demonstrated by the preparations cambendazole, levamisole and rintal. Ivermectin has been most efficient in the struggle both against the gastrointestinal and lung nematodes. Its efficacy in the fight against the pulmonary helminthoses has been better when applied at higher doses such as 400µg/kg body weight and in its subcutaneous and pour on administration as compared to the per oral one. The attempts for controlling the extrapulmonary protostrongylidoses with levamisole and rintal have proven to be unsuccessful. Ivermectin has been in that respect the best of the rest of the anthelminthics but also at the high dosage (400µg/kg body weight) though even in these cases its effect has been incomplete. The treatment of trematode infections with albendazole has shown rating of efficiency between 20 and 80 per cent in per oral and almost 100 per cent in the intraruminal administration. Niclosamide has been effective in monesiosis. Ivermectin has been tested also as a remedy for fighting the external parasites in the deer. It has been highly effective in nasopharyngeal and subcutaneous myiasis, ticks, mange mites and lice. The analysis of the studies presented shows that antiparasitic remedy with the widest spectrum and best efficiency in the Cervidae is Ivermectin. In any case, before undertaking treatment with it as well as with any other cure it is advisable to perform investigations aimed at the up-to-date parasitological status of the animals thus allowing for the most precise dosages and regimes of administration.

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DO5. PRELIMINARY STUDIES ON THE SPREAD OF VARROA DESTRUCTOR AND NOSEMA SPP. IN CERTAIN AREAS OF BULGARIA

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ABSTRACT

Biodiversity of ecosystems is largely provided by pollinator activity of honeybees. The parasitoses in bees caused by *Varroa destructor* and *Nosema spp*. play a very important role in colony losses, thus attracting the attention of many scientists. In the present work are made preliminary studies on the spread of varroosis and nosemosis in 66 samples from 11 regions of the country. Methods of OIE -MDTVTA are used for the identification of *Varroa destructor* and *Nosema spp*. and determining the degree of invasion in samples of bees from colonies died in the winter-spring period of 2015.

It is found that the *Varroa destructor* is established in 16,6% of samples, and the degree of invasion in the bees is in the range 0.5% to 100%. Spores of *Nosema spp*. is demonstrated in 41%, the degree of invasion per bee is in the range of 3.10^5 / bee to 26.10^6 / bee. Mixed infections of both parasites are observed in 24,2% of samples analyzed. 18.2% of samples were negative for both parasites.

Studies on the project continued in other areas of the country, by including methods for identification and distinguish species *N. apis* and *N. ceranae*.

Keywords: *Apis mellifera*, *Varroa destructor*, *Nosema spp*., spread, colony losses.

INTRODUCTION

The phenomenon of ”decline of honey bees" have not been decided, despite numerous studies. However, there is consensus that pests and pathogens are the most important cause of unexplained losses of bee colonies. Recent attention of researchers focuses on selected bee pathogens - mostly parasites that have shown interest in the losses of colonies in different regions of the world, and which are considered current threats to honey bees and beekeeping.

Today *Varroa destructor* can be found everywhere around the world where are kept colonies of honey bee Apis mellifera and hardly it is possible to find not infested colonies. The only exception is Australia, where *V. destructor* is not established so far and still considered exotic parasite in bees [1]. The ectoparasitic mite *V. destructor* as the bee pathogen feeding on the haemolymph of its host produces of haemocytes and protein content reduction. In Germany *V. destructor* still has been the main cause of winter losses, confirming that no other pathogen has a comparable impact on beekeeping. In addition, two honey bee pathogenic viruses known to be

associated with V. destructor, DWVand ABPV, were significantly related to the observed winter losses [6].

Microsporidia *Nosema apis* and *N. ceranae*, are pathogenic for adult honeybees. Recently, it became evident that *N. ceranae* is also widespread in the *A. mellifera* population throughout the world [7, 8, 13]. Reports on the impact of *N. ceranae* infections on honey bee health and colony survival are contradictory. In Spain, *N. ceranae* causes an unusual form of nosemosis eventually leading to colony collapse [9, 11]. The worldwide distribution of *N. ceranae* [4, 13, 14], which is not inevitably accompanied by the symptoms described by Higes et al. [9], also suggest that *N. ceranae* killing honey bee colonies might be a regional problem rather than a global phenomenon. The observed virulence of *N. ceranae* in Spain has been explained by the better adaptation to elevated temperatures of *N. ceranae* compared to *N. apis* [5].

Some studies had tested hypothesis that the stress caused by *V. destructor* parasitism helped *N. apis* breeding, but the connection between both species could not be confirmed be influenced of climate conditions [2].

The purpose of this study was to make preliminary studies on the project that was further to cover the whole country, to get a picture of current situation on the prevalence of the two parasitoses - varroosis and nosemosis.

MATERIAL AND METHODS

The investigation was conducted on 66 samples from 11 different regions of the country in Winter-Spring period of 2015.

Sample collection

The bee samples were collected from the hive bottom of died colonies in 11 regions of the country. Every sample contained about 200 bees.

Methods of investigation

The methods were used of *OIE Terrestrial Manual, 2008 - Chapter 2.2.7*. for *Varroa destructor* identification, and *OIE Terrestrial Manual,2013 - Chapter 2.2.4.* - for the identification of *Nosema spp*., respectively [10].

Investigation for *Varroa destructor*

The dead bees from the hive bottom were floated with industrial alcohol, stirred continuously for around 5-10 minutes. The mites that floated to the surface were identified and observed [3]. Than the contents were placed of the sieve on a bright plate, where the mites can be easily identified and counted. The percentage of infestation level was calculated by following formula:

% *V. destructor* = (Number of foretic mites / number of adult bees) x 100

Investigation for spores of *Nosema spp*.

The abdomens of 20 bees of each sample are obtained in 20 ml of distilled water. Of the suspension are made smears on a glass slide. Smears were air-dried, ethanol-fixed and were stained with Giemsa's stain (10% in 0.02 M phosphate buffer) for 45 minutes. *Nosema spp*. spores had a distinctive appearance, with thick unstained walls and an indistinct blue interior, without visible nuclei. To quantify the average infection level spores were counted and were calculated per bee by method of Oliver [12].

RESULTS

The results of the percentage of positive samples infected with parasites and negative samples were shown in Figure 1. It appears that the most positive samples were for *Nosema spp.* – 41%. The second place takes the samples with mixed invasion – 24.2% and the lowest percentage of the positive samples were infested *Varroa* – 16.6%. The negative samples were 18.2%. Frequent occurrence of mixed invasion with *V. destructor* and *Nosema spp*. is confirmed by the research of other scientists.

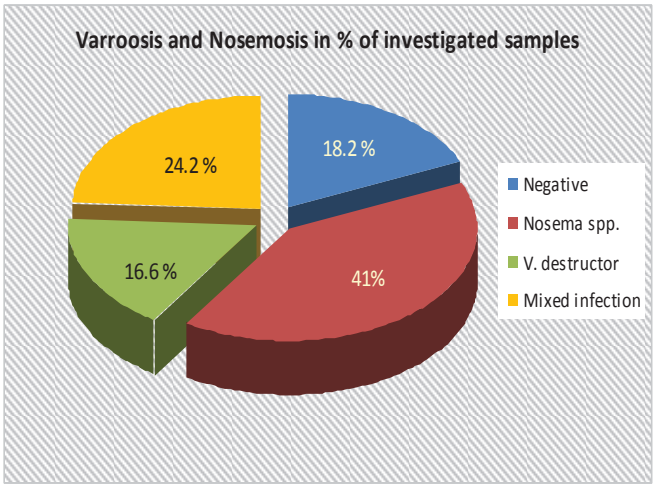

Fig.1. The results of investigated samples

Study results for different regions are shown in Table 1.

Table 1. Disease prevalence in different regions of Bulgaria: Varroosis and Nosemosis (number of positive samples)

Region	Number of samples	*Varroa destructor*	*Nosema spp.*	Mixed invasion	Negative
Blagoevgrad	5	1	4	-	-
Burgas	1	-	-	1	-
Varna	1	-	-	1	-
Veliko Tarnovo	5	-	4	-	1
Pazardzik	8	2	4	1	1
Pleven	25	2	12	6	5
Ruse	2	-	1	1	-
Sliven	1	-	-	-	1
Smolyan	1	-	-	1	-
Sofia-grad	9	6	1	1	1
Sifia-oblast	8	-	1	4	3
Samples Total number	66	11	27	16	12

The results from samples analyzed by region shown that the affected with Varroa areas were from Sofia-grad, Pazardzhik and Pleven. The most infested with Nosema spp. samples

come of Pleven. Samples from Blagoevgrad, Veliko Tarnovo and Pazardzhik were equally invaded. The most samples with mixed infections with both parasites were from Pleven and region Sofia-oblast. The high prevalence of nosemosis compared with varroosis can be explained by a wet spring and prolonged rainfall in 2015.

The degree of infestation with spores of *Nosema spp*. is given in Fig. 3. and the degree of infestation with mite *Varroa destructor* are given in Fig. 4.

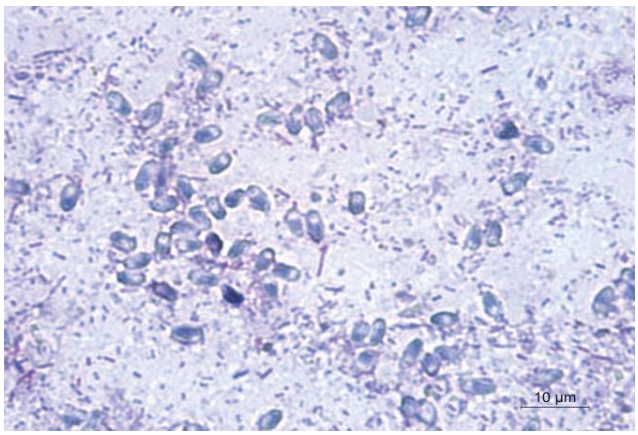

Fig. 2. Spores of *Nosema spp.*

In our studies we found that the highest percentage of positive samples with spores of Nosema spp. (fig.2) had a degree of invasion between 1 and 10 million - 39%. A degree under 1 million showed 17% of the samples and high invasion was established in 9% of the infected samples, respectively (fig.3).

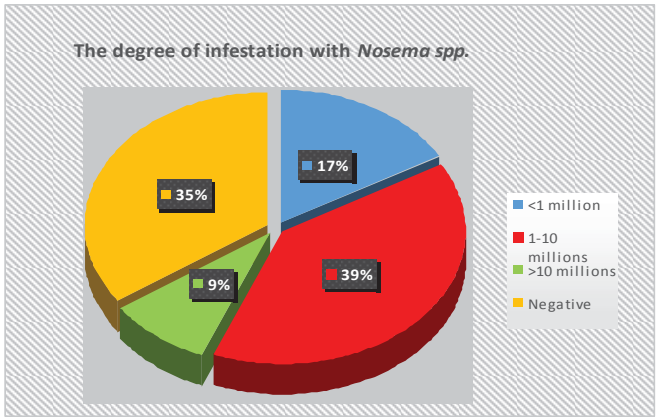

Fig. 3. The degree of infestation with *Nosema spp.* in investigated samples from different region of Bulgaria during Winter-Spring period of 2015

Similarly, as in nosemosis the largest percentage of the infected with *Varroa* mite (fig.4) were the samples with a mean invasion - between 5 and 20%. The highest degree of invasion (> 20%) was established in 9% of samples invaded and the lowest (<5%) - in a small proportion of samples - 3%, respectively.

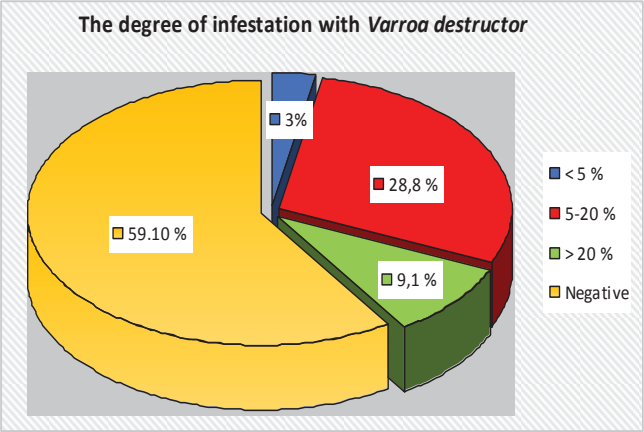

Fig. 4. The degree of infestation with *Varroa destructor* in investigated samples from different region of Bulgaria during Winter-Spring period of 2015

CONCLUSION

In our preliminary studies we found that most of the tested samples of dead in the Winter-Spring period of 20115 were infected with *Nosema spp.* The most infested with *Nosema spp.* samples come of Pleven. The affected with *Varroa* areas were Sofia-grad, Pazardzik and Pleven. The most samples with mixed infections with both parasites were from Pleven and the region Sofia-oblast.

The largest percentage of the infected with *Nosema spp.* and *Varroa* mite were the samples with a mean invasion.

Our studies on the project will continue in other regions of the country to establish the prevalence of *Nosema spp.* and *Varroa destructor.*

Acknowledgements: This work is related to a project between IEMPAM- BAS and NDRVMI - BFSA (Study the spread of the causes of parasitic diseases in bees Varroa destructor and Nosema spp. in different regions of Bulgaria)

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DO6. SOME BIOCHEMICAL PROPERTIES OF NEURAMINIDASE FROM AEROMONAS STRAIN A40/02

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Neuraminidase is an enzyme which cleaves terminal α-glycosidically bound sialic acids, which are mostly found in complex structures such as glycoproteins, glycolipids, oligosaccharides. The enzyme is considered to be of virulence significance in some pathogenic bacteria.
There are few works concerning neuraminidase production among *Aeromonas* representatives [1, 2], which are pathogens in fish, amphibians, and in humans. The aim of the work is to

investigate for the first time some biochemical features of neuraminidase from *Aeromonas* strain.
The inducibility and substrate specificity of the enzyme were studied. Various low- and high-molecular compounds (N-acetylglucosamine, N-acetylmannosamine, fetuin, transferrin) stimulate the production of the A40/02 neuraminidase, however sialic acid exerted the highest inductive effect. The enzyme demonstrate broad substrate specificity and was active against complex compounds containing α2-3, α2-6 and α2-8 glycosidically bound sialic acids.
The purification procedures were carried out using ammonium salt precipitation and chromatography with DEAE cellulose and Q-sepharose. The use of fluorogenic substrate in native PAGE determined the molecular weight of approximately 130 kDa.
Although the pathogenicity of A40/02 was not investigated, some characteristics of the strain (strong proteolytic production, the uptake of Congo red dye, resistance to some antimicrobial agents) suggest a pathogenic capability. A possible role of neuraminidase in such capability could be assumed.

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DP1. ANTIBIOTIC RESISTANCE IN *STAPHYLOCOCCUS AUREUS*

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Medical Faculty, Sofia University "St. Kl. Ohridski"

DO7. PRELIMINARY SCREENING FOR HEPATITIS B VIRUS IN TREATED PATIENTS WITH RHEUMATOID ARTHRITIS AND ANKYLOSING SPONDYLITIS AND REVIEW ON THE LITERATURE

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Abstract

The prevalence of hepatitis B virus (HBV) infection in the world is still high despite the existence of vaccine. Bulgaria is a country with the intermediate endemicity for HBV with predominance in adult's between 40 and 49 years. Rheumatoid arthritis and ankylosing spondilitis are musculoskeletal conditions that cause severe long term pain and disability which prevalence increased with ageing. Hepatitis B virus infection can be asymptomatic and self-limited or as consequence to chronificate. At the same time after resolved infection the small concentration of viral DNA can be stored in liver or serum. Reactivation of HBV infection with increased of HBV DNA concentration is not infrequent in patient with reactive arthritis or ankylosing spondilitis during the treatment. In the present work the short review of epidemiology, course of infection and possible predictors for reactivation of HBV were proposed. The results from preliminary HBV screening of target patients with rheumatoid arthritis and ankylosing spondilitis were shown.

Epidemiology of HBV infection in Bulgaria

The geographic distribution of HBV infection is characterised with areas of high endemicity (prevalence of HBV surface antigen (HBsAg) in the general population >7%) these are South East Asia, China, Africa, the Middle East and the Amazon Basin; intermediate (prevalence between 2% to 7%) - Eastern and Southern Europe, the Middle East, central Asia, South Asia, and parts of South America; and areas with low endemicity (up to 2%) - North America, Northern and Western Europe and Australia [13]. For Bulgaria acute HBV infection is following by prevalence (15.5%) hepatitis A infection (77.12%) [18]. Kojouharova et.al., studied the main serological marker for HBV in the general population, established the prevalence of 3.87% for HBV surface antigen (HBsAg), and 23.59% for antibodies against HBsAg (anti-HBsAg) and against HBV core antigen (anti-Hbc), which is a marker for a resolved infection. The highest prevalence of 5.72% for HBsAg was detected in adults aged between 40 and 49 years [9]. The main roughs of HBV transmissions are trough contaminated blood or blood product, sexually and during child labor. For Bulgaria the sexual transmission is the most prevalent route with 48% in a case of acute infection, followed by nosocomial transmission– 26% [18]. Hepatitis B virus shares common routes of transmission with human immunodeficiency virus and for this reason co-infection can occur. The prevalence of HBV co-infection among Bulgarian HIV positives varies between 10% and 13.9% for the years 2010 - 2013 [7].

Course of HBV infection

Hepatitis B infection can be asymptomatic, with development of acute infection or chronic infection, the latter resulting sometimes in cirrhosis and liver cancer [8]. The development of chronic infection depends on the immune status of the infected person and the age when infection is acquired. If the newborns are exposed to HBV during the births in 90% they become persistently infected, while up to 92% of the HBV infection acquired during adolescence is self-limited and cleared [16]. The HBV infection consists of four phases:

1. Immune tolerance phase is characterized by minor immune response to the virus. Patients present with normal or minimally elevated alanine aminotransferase (ALT) levels, normal liver

functions or minimal histological activity. Presence of hepatitis B e antigen (HBeAg) and HBV DNA is detected in the serum. Patients are highly contagious due to the high level of viremia [1, 20]. The immune response is characterized with T-cell hyporesponsiveness, due to the ineffective antigen processing and transport to the major histocompatibility complex class I molecules [17].

2. Immune clearance phase is characterized with elevated or fluctuating ALT levels and fluctuating but progressively decreasing HBV DNA levels, accompanied by histological inflammation activity. Seroconversion to anti-HBeAg with lost of HBeAg occurs in the majority of patients, about 90% of them [1, 20]. The immune response is characterized by secretion of various cytokines – interferon-α, interferon-β, interferon-γ, tumor necrosis factor-α or interleukin-6 [5].
3. Immune control phase is characterized with complete HBeAg seroconvertion, decreasing levels of HBV DNA, normal ALT and inactive liver histology [5]. Patients, who developed HBsAg seroconversion with production of antibodies - anti-HBs, are diagnosed as having resolved hepatitis. A small proportion of these patients are found to have very low levels of detectible HBV DNA in their serum that was observed occasional. The third phase may last lifelong, with HBsAg seropositivity and it is defined as inactive hepatitis or occult carrier stage. Less than 1% per year of inactive carriers undergo spontaneous HBsAg seroconversion [4].
4. Reactivation phase is common in chronic hepatitis B infection. In these phase some patients are HBeAg negative and anti-HBe – positive, but with increased serum HBV DNA levels, increased transaminase levels and liver necro-inflammatory changes [1, 5].

Reactivation of HBV in rheumatoid arthritis patients

Hepatitis B reactivation can be transient, with no symptoms, but in many cases the reactivation might be severe with a quite high morbidity – 5% to 40% [6]. It occurs during immunosuppressive therapy, cancer chemotherapy, sudden withdrawal of antiviral therapy, progressive immunodeficiency in HIV co-infection, following liver transplantation, due to past HBV exposure of the organ donor, or spontaneous [15]. The increased risk for HBV reactivation, indicated by increased/or detected HBV DNA, during corticosteroids treatment is due to the presence of glucocorticoid response element in HBV DNA [21]. It is established that in rheumatoid arthritis patients the prevalence of HVB reactivation is up to 3.3% [3]. In this case reactivation can occurs: 1) in patients with chronic HBV, who are HBsAg-positive and taking tumor necrosis factor agents (TNFA), 2) in patients with resolved HBV infection, who are HBsAg-negative, but with low levels of HBV replication in the liver and in peripheral blood mononuclear cells. During reactivation HBsAg-positivity reappears [22].

There are various recommendations for managing patients with chronic HBV, but there are not exact criteria for the patients with a past HBV infection. Some authors regard all anti-HBc-positive and HBsAg-negative individuals as inactive carriers and in the same time occult infection can be found in patients without any serological HBV markers [12]. The aim of the presented study is to investigate the serological and virological evidence of HBV reactivation in patients with ankylosing spondylitis (AS) and rheumatoid arthritis (RA) and the incidence of its reactivation under treatment with antirheumatic drugs. At the same time patients are tested serologically for the presence of IgM and IgG class antibodies against parvovirus B18 (B19V) as it is reported that the B19V infection triggers autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis [19].

Results of preliminary HBV screening of patients with AS and RA

Patients: The study started in January 2015 and included 9 patients, among whom 3 were diagnosed with AS - according to the modified New York criteria [23], and 6 - with RA according to ACR criteria (1987) [2]. Clinical assessment included collecting demographic data and information regarding therapy applied and disease activity parameters. Disease activity score (DAS) in AS patients was assessed using the ASDAS-CRP [10], and in those with RA by the DAS28-CRP (14). ASDAS-CRP <1.3 was defined as inactive disease [11], DAS28-CRP <2.6 was considered consistent with remission [24]. The magnitudes of the parameters over these cut off values signified disease activity.

Serological and virological evolution for HBV: Sera samples from all patients were tested by enzyme-linked immunosorbent assay (ELISA) for the presence of HBsAg (SURASE B-96 ELISA kit GmbH, Germany) and anti-HBc (Competitive ELISA for HBcAb in human serum and plasma, DIA.PRO, Italy) according manufacturer instructions. Sera samples were screened also by ELISA for the presence of IgG and IgM class antibodies (indirect ELISA kit *recom*Well Parvovirus B19 IgM/IgG) against parvovirus B19 (B19V).

Serum HBV viral load was determined by COBAS AmpliPrep/COBAS TaqMan HBV Test (Roche Diagnostics GmbH), with an analytical measurement range from < 2.00E + 01 IU/ml to > 1.70E + 08 IU/ml. The conversion factor between HBV copies/ml and HBV IU/ml is 5.82 copies/IU, using the WHO International Standard for HBV DNA for nucleic acid technology assays testing – NIBSC 97/746.

Results: The target cohort included patients from 24 to 66 years old, with a mean age of 45±14 years. Two of the patients were males and 7 – females, where the male to female ratio was 2:7. Three (33.3%) of the patients were diagnosed with AS and 6 (66.7%) – with RA. The time that has elapsed between the appearance of the first symptom and the diagnosis was between 2 and 37 years. Disease activity score for the patients with AS varied from 2.8 to 5.0, and for those with RA – from 2.2 to 14.3. Four (44.4%) of the patients have been treated with synthetic disease-modifying antirheumatic drug (DMARDs) - Methotrexate and 5 (55.6%) have been receiving only symptomatic therapy. Main patients' characteristics are shown on Table 1.

Table 1. Patients' characteristics

Patient	Age (45±14)	Sex	diagnosis	Disease duration (years)	Disease activity		Treatment
					RA (DAS28-CRP)	AS (ASDAS-CRP)	
1	32	M	AS	6	-	5.0	NSAIDs
2	45	F	RA	5,5	2.93	-	DMARDs
3	55	M	AS	37	-	2.8	NSAIDs
4	49	F	RA	4.3	2.62	-	DMARDs
5	66	F	RA	2.2	6.32	-	DMARDs
6	24	F	AS	17	-	3.2	NSAIDs
7	25	F	RA	11	5.09	-	NSAIDs
8	50	F	RA	6.4	4.62	-	DMARDs
9	59	F	RA	14.3	6.85	-	NSAIDs

Legend: M – male; F – female; AS - ankylosing spondylitis; RA - rheumatoid arthritis; DMARDs - synthetic disease-modifying antirheumatic drugs; NSAIDs – nonsteroidal anti-inflammatory drugs.

In two (22.2%) of nine patients presence of a serological markers for HBV were established. Patient number 1 is anti-HBc positive, that is a marker for a past HBV infection, but he is negative for HBV DNA (Table 2). The patient suffers from AS, possesses the highest DAS 5.0 and is treated with nonsterodal anti-inflammatory drugs (Table 1). Patients number 3 is positive for HBsAg, anti-HBc and HBV DNA with concentration of 182 copies/ml (Table 2). This patient is diagnosed with AS, possesses the lowest DAS (2.8) and is treated with nonsterodal anti-inflammatory drugs (Table 1). The rest 7 (77.8%) patients are negative by serological markers and HBV DNA. For the parvovirus B19V, all sera samples are IgM negative but 5 of 9 (55.6%) are IgG positive, that is a marker for a past infection with B19V.

Table 2. Virological status of the patients

Patient	**HBV**			**B19V**	
	HBsAg [OD]	anti-HBc IgG	HBV DNA [copies/ml]	IgG	IgM
1	negative	+	Negative	+	negative
2	negative	negative	Negative	+	neative
3	+	+	182	negative	negative
4	negative	negative	Negative	negative	negative
5	negative	negative	Negative	+	negative
6	negative	negative	Negative	+	negative
7	negative	negative	Negative	negative	negative
8	negative	negative	Negative	negative	negative
9	negative	negative	Negative	+	negative

Legend: HBV – hepatitis B virus; B19V – parvovirus B 18; HBsAg – hepatitis B virus surface antigen: anti-HBc – antibodies against hepatitis B virus core antigen

As this is the initial screening of the patients involved in the study, at time point 0, the results are still incompleted. The patients will be followed for a longer period during their anti-rheumatoid treatment and the increase or possible reappearance of HBV will be tested on the two additional time points – the 3th and the 6th months.

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DO8. NOROVIRUSES – THEIR LEADING ROLE IN NON-BACTERIAL GASTROENTERITIDES AND THE EXPERIENCE IN MONITORING THE INFECTION IN BULGARIA

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Abstract

Noroviruses are the most common etiological agent causing large outbreaks of non-bacterial gastroenteritis affecting all age groups worldwide. Usually noroviruses cause acute diarrhea, often accompanied by vomiting, abdominal cramps, fever, and fatigue. Most of the diseased recover within 2-3 days, but the illness could be more severe in vulnerable populations, i.e. the extreme age groups, immunocompromized, etc. About 1/3 of the infected individuals could be asymptomatic but capable of transmitting the infection. Transmission occurs primarily through contaminated food or water, through person-to-person contact and exposure to fomites. Noroviruses are highly contagious, extremely stable in the environment and resistant to common disinfectants. That is the reason outbreaks to frequently occur in semi-closed communities such as nursing homes, hospitals, kindergartens, schools, military settings, cruise ships, etc.

Noroviruses comprise one of the genera in *Caliciviridae*, which are naked viruses with icosahedral symmetry of the capsid and a linear non-segmented positive-sense, single-stranded

RNA genome. Noroviruses are genetically diverse and the genus is subdivided into 5 genogroups (GI, GII, GIII, GIV and GV). Genogroups are further subdivided into >30 distinct clusters or genotypes. Noroviruses display a wide degree of genetic variability – members within a genogroup differ by 45–61% in their capsid genes, members within a genotype differ by 14–44%, and strains within a genotype differ by 0–14%. The high degree of variability is undoubtedly one of the factors complicating the protective norovirus immunity which is also believed to be short-termed.

The Bulgarian experience in detecting and monitoring norovirus infections is in its initial phase and is still insufficient. Nevertheless, a RT-PCR based method for norovirus detection and identification is developed and implemented in the routine laboratory practice using a commercial kit. Approximately 200 fecal specimens from diarrheal patients suffering from non-bacterial gastroenteritis have been tested and 23.9% of them turned out to be positive for noroviruses with the prevalence of genogroup GII.

1. Epidemiology of Norovirus Infection

Gastroenteritis is a major health problem worldwide caused by a number of infecting agents, including bacteria, viruses, and parasites. Noroviruses are the key viral pathogens associated with almost a fifth of all acute cases of gastroenteritis and are now recognized as the leading cause of viral diarrheal outbreaks both in developed and developing countries [7;8]. Severity of symptoms – diarrhea, often accompanied by vomiting, abdominal cramps, fever, and fatigue – vary to a great extent. Diarrhea may lead to varying degrees of dehydration, which in children and in the elderly, as well as in those with other underlying illnesses, may be a life-threatening event. Most of the diseased recover within 2-3 days. About 1/3 of the infected individuals could be asymptomatic. Despite the lack of disturbing symptoms asymptomatic individuals are capale of successful transmission of norovirus infection. Transmission occurs primarily through contaminated food or water, through person-to-person contact and exposure to fomites. Noroviruses are highly contagious [19] extremely stable in the environment and resistant to common disinfectants. There is a prolonged shedding of virus from both symptomatic and asymptomatic individuals [1]. That is the reason outbreaks to frequently occur in semi-closed communities such as nursing homes, hospitals, kindergartens, schools, military settings, cruise ships, etc. What is more, there is a lack of lasting immunity with no cross-reactivity between different genotypes [11].

2. Molecular biology of Noroviruses

The prototype norovirus, Norwalk virus, was first identified as the cause of a gastroenteritis outbreak in an elementary school in Norwalk, Ohio, USA in 1972 [13].

Noroviruses comprise one of the genera in *Caliciviridae*, which are naked viruses with icosahedral symmetry of the capsid and a linear non-segmented positive-sense, single-stranded RNA genome, containing three open reading frames. Noroviruses are genetically diverse and currently the genus is subdivided into 5 genogroups (GI, GII, GIII, GIV and G5) [20] based on the amino acid sequence of the largest structural protein (VP1) [21]. Genogroups GI, GII and GIV infect humans, but noroviruses infect other species as well: pigs (GII), cattle and sheep (GIII), mice (GV). More recently, a novel norovirus identified in domestic dogs with diarrhoea

has been proposed to represent a new genogroup, GVI [9]. Genogroups are further subdivided into >30 distinct clusters or genotypes with at least eight genotypes belonging to GI and 21 genotypes belonging to GII [21].

Noroviruses display a wide degree of genetic variability – members within a genogroup differ by 45–61% in their capsid genes, members within a genotype differ by 14–44%, and strains within a genotype differ by 0–14% [21]. The high degree of variability is undoubtedly one of the factors complicating the protective norovirus immunity which is also believed to be short-termed.

Among all human noroviruses, genogroup GII, genotype 4 is reported as the most common etiologic agent in gastroenteritis outbreaks (about 70-80%), although both GII and GI are detected in routine outbreak investigations [16], [7].

The norovirus genome ranges in size from 7.3 to 7.5 kb across the genus [20]. The 5' end of the genomic RNA is covalently attached to a virus-encoded protein, VPg, and the 3' end is polyadenylated. There are UTRs at either ends, which are typically short [20]. The UTRs contain evolutionarily conserved structures that are important for viral replication, translation and pathogenesis [17]. The genome is organized into three conserved open reading frames (ORFs). ORF1 encodes the nonstructural polyprotein that is cleaved by a viral protease into probably 6 proteins, one of them being the viral RNA-dependent_RNA-polymerase [2]). ORF2 and ORF3 encode the major (VP1) and minor (VP2) capsid proteins, respectively [10]. Most of the cellular interactions and immune recognition are thought to be located in a sub-domain of the capsid VP1 protein that extends above the viral surface and has the most sequence divergence in the genome [4].

The inability of human noroviruses to be cultured *in vitro* in cell culture [18] greatly hampers the characterization of the virus life cycle. The major experimental system for studying norovirus replication nowadays is the Norwalk virus RNA replicon system, where cells stably maintain Norwalk virus RNA [5].

Similar to other positive-strand RNA viruses, norovirus replication occurs in close association with host-derived membrane complexes in the cytoplasm. Genome replication occurs via a negative-sense RNA intermediate and is performed by the viral RNA-dependent-RNA-polymerase (RdRp). The processes behind viral assembly, encapsidation and the exit of noroviruses are largely unknown. [20].

3. Methods of Detection of Noroviruses

The lack of an efficient cell culture system for propagating human noroviruses significantly hurdles both molecular biology and diagnostics of noroviruses. Another major reason for the difficulties met is the genetic variability, which complicates RT-PCR-based detection assays.

Electron microscopy (EM) was the first tool used for identifying noroviruses but this is an expensive and time-consuming procedure, which is rather insensitive and usually rarely available outside of research units. EM incapable of differentiating between different norovirus genogroups and genotypes. The latter disadvantage could be overcome by the use of specific antisera, i.e. by applying immuno-EM, which significantly increases the specificity and sensitivity of the technique.

Direct serotyping based on the neutralization assay, is not possible because no cell culture system has been established for growing human noroviruses yet. Nevertheless successful immunological methods for detection of noroviruses do exist due to the expression

of the capsid protein in a baculovirus system. There are commercial kits for norovirus immunoassay capable of simultaneous detection of noroviruses of both prevalent genogroups. These kits possess a relatively high sensitivity (83%) and specificity (98-100%), but the sensitivity is not sufficient in sporadic cases and negtive specimens according this technique should be checked otherwise.

Antigen-capture ELISA kits are developed in which anti-norovirus antibodies are immobilized on the bottom of polystyrene plates which would react specifically with the norovirus antigens if present in the fecal specimen of the patient. These commercial kits are capble of detecting homologous viruses, but they fail to distinguish antigenic variants. Their sensitivity is also relatively high (83%) and the specificity is 100%. Enzyme immunoassay (EIA) offers an attractive alternative to the expensive and technically demanding molecular detection assays. However, the development of a broadly reactive EIA for noroviruses has been challenging because of the number of antigenically distinct norovirus strains and the high viral load required for a positive signal by these assays. In evaluations, the sensitivity of these kits when compared with RT-PCR has ranged from 36% to 80%, and specificity has ranged from 47% to 100% [6].

Consequently, reverse transcription-polymerase chain reaction (RT-PCR) and genomic sequencing have become the major means for diagnostics and characterization of viruses, and nowadays are considered as the "gold standard". Accumulated sequence information has been especially useful for viral diagnosis and genotyping. Most investigators choose primers from conserved regions, such as the RdRp gene in ORF1, in order to detect the greatest number of the diverse norovirus strains [12]. In contrast to EIAs, the extreme sensitivity of RT-PCR permits the detection of very low titers of virus that might be present in samples from persons without disease, i.e., this makes asymptomatic infection to be also detected and monitored [15].

For strain identification detection primers should be directed to more variable regions, i.e. the region encoding the capsid protein. Conventional RT-PCR assays for genotyping have been already proposed using four different regions of the genome [14].

However, the gold standard for genotyping norovirus strains is full capsid sequencing. To determine or confirm norovirus genogroup/genotype, as well as proving the existance of norovirus recombinant forms, the genes encoding RdRp and the major structural protein should be sequenced and matched.

Today the majority of clinical virology laboratories perform real-time RT-PCR assays for norovirus detection. Positive samples can be typed subsequently by DNA sequencing of RT-PCR products, thus genotyping is performed.

4. Our experience in Detecting and Monitoring Norovirus Infections in Bulgaria

Norovirus detection in stool specimens of patients suspected for non-bacterial gastroenteritis has been performed since several years at The National Reference Laboratory for Enteroviruses of the National Center of Infectious and Parasitic Diseases in Bulgaria.

For studying norovirus infections in both sporadic cases and outbreaks, modern molecular biological techniques have been implemented. First, stool specimens undergo the viral RNA extraction step. This is carried out either by the classical procedure described by Boom [3] or by using a commercial kit. RNA extraction is followed by a two-step polymerase chain reaction (the first step being reverse transcription of viral RNA to DNA, followed by a second step of the conventional PCR). The norovirus detection PCR is directed to the gene encoding viral RNA-dependent RNA-polymerase, and group identification is carried out by

primer targeting to the main structural protein VP1. Among 193 fecal samples tested, 46 were positive for noroviruses as genogroup II dominated (91.3%; 42/46), norovirus genogroup I were demonstrated in 8.7% (4/46) and one case was a mixed infection of both norovirus genogoups (2.17%). This initial results confirm the leading role of norovirus infections in non-bacterial gastroentirtides.

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DO9. ONCOGENIC ACTIVITIES OF HPV PROTEINS

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Abstract

Over the past several decades, knowledge of the biology and epidemiology of human papillomavirus (HPV) infection has increased. The virus has been identified as a necessary causal agent for cervical squamous neoplasia and has been linked to the development of neoplasia in several other mucosal sites. The viral oncoproteins E5, E6 and E7 were identified as the major players in HPV induced carcinogenesis. Here we summarise the current information on HPV proteins and their functions, emphasizing on HPV oncoproteins and their role in neoplastic transformation.

Introduction

Over the past 30 years, human papillomavirus (HPV) has been shown to play a role in the development of various cancers. Most notably, HPV has been linked to malignant progression in neoplasms of the anogenital tract, including cervical, vulvar, vaginal, penil and anal cancers. However, HPV has also been suggested to play a significant role in the development of cancers in other anatomic locations, such as the head and neck, lung, breast and bladder [9]. There are about 200 different types of HPV. HPVs have been found in the Alpha-, Beta-, Gamma-, Mu- and Nu-genera. Alpha-HPVs are clasificated into two groups termed “high-risk” (16, 18, 31, 33,

35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 53, 73, 81, 82) and “low-risk”(6, 11, 42, 43, 44, 40, 61, 54, 55, 70, 57, 71, 72, 84, 26) based on the frequency of their association with cervical cancers. Papillomaviruses have circular double-stranded DNA genomes with sizes close to 8 kb (Fig.1). In spite of their small size, their molecular biology is very complex. In short, the HPV genome can be divided into three regions as determined by their functions: the non-coding long control region (LCR), the early (E) and the late (L) coding regions. The early region encodes for nonstructural proteins E1, E2, E4, E5, E6, and E7, involved in regulation of replication and transcription, cell growth and maturation and virus release, as well as malignant transformation. The late region encodes for the structural proteins L1 and L2 that compose the viral capsid [34].

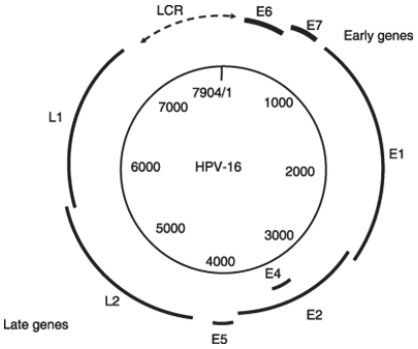

Fig.1.The genetic map of HPV16

HPV proteins

L1

The major capsid protein L1 is involved in formation of a 55-60 nanometer capsid, composed of 72 star-shaped capsomers. Each capsid contains 360 L1 molecules. It plays a key role in viral entry into the host cell and interacts both with cell receptors and L2. L1 is expressed exclusively in non-transformed cells and is highly immunogenic. Self-assembled virus-like particles composed of L1 are the basis of prophylactic HPV vaccines.

L2

The minor capsid protein L2 is less abundant than L1 with around 12 copies per virion. This protein performs several important functions, including facilitating the packaging of the viral genome into nascent virions as well as the entry of the virus into new host cells. L2 is of interest as a possible target for more broadly protective HPV vaccine.

E1

E1 protein is essential for replication of HPV genome. E1 protein binds to the viral origin of replication in the long control region of the viral genome. E1 uses ATP to exert a helicase activity that forces apart the DNA strands, thus preparing the viral genome for replication by cellular DNA replication factors [5, 27].

E2

E2 protein regulates viral transcription from the early promoter and is essential for viral replication. E2 facilitates the binding of E1 to the viral origin of replication. E2 also utilizes a cellular protein known as Bromodomain-4 (Brd4) to tether the viral genome to cellular chromosomes [29]. This tethering to the cell's nuclear matrix ensures faithful distribution of viral genomes to each daughter cell after cell division. E2 protein has pro-apoptotic activity [2, 3]. It is thought that E2 serves as a negative regulator of expression for the oncogenes E6 and E7 in latently HPV-infected basal layer keratinocytes. In late stages of carcinogenesis, the genital HPV genome is integrated into the host chromatin, with the preferential disruption of

the E2 gene [8, 10] tend to increase the expression of the E6 and E7 oncogenes, resulting in cellular transformation and possibly further genetic destabilization.

E4

Although E4 proteins are expressed at low levels during the early phase of viral infection, expression of E4 increases dramatically during the late phase of infection. The E4 protein of many papillomavirus types is thought to facilitate virion release into the environment by disrupting intermediate filaments of the keratinocyte cytoskeleton. E4 facilitates DNA replication. E4 has also been shown to participate in arresting cells in the G2 phase of the cell cycle. E4 proteins from diverse papillomaviruses interact with the serine-arginine-specific protein kinase SRPK1, a kinase important in the replication cycles of a diverse range of DNA and RNA viruses. E4 inhibits SRPK1 phosphorylation, not only of cellular SR proteins involved in regulating alternative splicing of RNA but also the viral transcription/replication regulator E2. There are findings that reveal a potential E4 function in regulation of viral late gene expression through the inhibition of a host cell kinase [32].

Viral oncoproteins: oncogenic activities of E5, E6, and E7

E5

The E5 protein induces aberrant cell proliferation by stimulating cell growth through stabilization of growth factor receptors (EGFR, PDGFR beta and CSF1R), by enhancing EGF signaling and by inhibiting apoptosis. E5 inhibits transport of major histocompatibility complexes to the cell surface and thereby helps the virus to evade immune response.

Not all HPV genera code for an E5 protein [1]. For instance αHPVs encode and express E5 but β HPVs do not. E5 is a weak transforming protein in vitro and its effects are seen best when in co-operation with the other viral oncoproteins: 16E5 together with E6 can induce the formation of koilocytes, a well known morphological marker of HPV infection [26]. The development of koilocytotic vacuoles may be linked to the E5-induced relocalization of calpactin I to the perinuclear region promoting perinuclear membrane fusion [25]. 16E5 oncoprotein binds and inhibits the activity of the 16 kDa subunit of V-ATPase, altering the endosomal acidification and degradation of EGF-R [14, 36] thus enhancing its recycling to the plasma membrane. 16E5 can also delay EGF-R degradation by interfering with membrane trafficking and the fusion of early and late endosomes [37]. The EGF-R signalling pathway can be activated by 16E5 through either EGF-dependent or EGF-independent processes. 16E5 activates mitogen-activated protein kinase (MAPK) p38 and ERK1/2 in human keratinocytes in an EGF-independent manner [12]. Two different pathways, a receptor tyrosin kinase-mediated pathway and a protein kinase C (PKC)-dependent pathway, are involved in the MAPK activation [11, 18] which increases the transcription of c-fos and c-jun [4, 6, 7], forcing the cells through the cell cycle and stimulating transcription of the viral oncogenes E6 and E7. Due to the integration of HR HPV genome during malignant progression, the E5 gene is not expressed in cervical tumours but both 16/18 E5 mRNA and protein have been detected in anogenital LSIL [21, 35] supporting the possibility that E5 plays a role in early steps of HPV infection to protect infected cells from apoptosis. HPV E5 proteins are not thought to play a role in the later steps of malignant progression because in high-risk HPV infections that progress to cancer the viral DNA typically integrates into the host genome often resulting in the loss of the E2 and E5 genes [39]. However, in contrast to other high-risk HPVs, HPV-16 DNA can exist in integrated, episomal or integrated and episomal form in malignant lesions of the cervix. Nevertheless, the fact that a substantial proportion of the tumours do not express E5 indicates that the protein is not essential for HPV-16 mediated tumour progression.

E6

HR-HPV E6 protein can block apoptosis, promote proliferation, activate telomerase, disrupt cell differentiation, adhesion and polarity, and reduce immune recognition.

E6 inactivates the main tumor suppressor protein p53 which role is to monitor the integrity of the cell genome by the induction of cell cycle arrest or apoptosis. The oncogenic potential of HPV E6 depends not only on its ability to target p53 for degradation but also on its interaction with a variety of other cellular factors [17, 23]. E6 in combination with a cellular E6-associated protein (E6AP) binds and marks p53 tumor suppressor protein, leading to degradation of p53 by proteasomes, genetic instability and progression to malignancy [28]. E6 has also been shown to target other cellular proteins, thereby altering several metabolic pathways. One such target is NFX1-91, which normally represses production of telomerase, a protein that allows cells to divide an unlimited number of times. When NFX1-91 is degraded by E6, telomerase levels increase, inactivating a major mechanism keeping cell growth in check [22]. Additionally, E6 can act as a transcriptional cofactor—specifically, a transcription activator - when interacting with the cellular transcription factor, E2F1/DP1 [19].

Activation of telomerase contributes to immortalization of malignant cells. E6 can activate telomerase in epithelial cells independent of p53.

All these mechanisms in concert result in genomic instability, accumulation of mutated cells and development of malignant phenotypes. Moreover, E6 has been shown to help the virus to avoid the immune defenses by interacting with two proteins that are part of the innate immune response to viral infection: Interferon regulatory factor-3 (IFR-3) and Toll-like receptor 9 (TLR9).

E7

E7 protein is the major transforming protein needed for maintenance of a transformed phenotype. E7 induces abnormal cell proliferation, interacts with histone acetyl transferases and with negative regulators of the cell cycle and tumor suppressors, primarily the retinoblastoma (Rb) family.

E7 inactivates the main tumor suppressor, retinoblastoma-protein pRb. pRb is important for the correct transition from G1 phase to S phase of the cell cycle. E7 competes with pRb, binding and inactivating retinoblastoma protein by proteasomal degradation. As a result, the transcription factor E2F is released, stimulating the expression of genes involved in cell cycle progression and DNA synthesis, thus pushing the cell cycle forward.

Under physiological conditions, free E2F leads to induction of p16 ink4, a tumor suppressor protein inhibiting cyclin-dependent kinase binding to Cyclin D which regulates the G1 cycle checkpoints and leads to cell cycle arrest in the G1 phase [13]. In cells infected with HPV, however, E6 blocks the inhibitory effect of p16 ink4 showing a synergistically interaction with E7, and resulting in altering cell differentiation by maintaining the keratinocytes in a suitable condition for the replication of the viral genome, reactivation of DNA synthesis, stimulation of progression of the cell cycle and inhibition of apoptosis [28, 30].

HPV proteins: mechanisms of neoplastic transformation

The E5, E6, and E7 oncoproteins have proliferative properties and mediate progression to malignancy. They cause DNA damage as well as chromosome instability during HPV-associated carcinogenesis. E6 and E7 action is more efficient when the two act in concert. In addition, E5 might potentiate the transforming activity of E7.

The mechanism by which HPV infection produces neoplastic transformation has been progressively elucidated and consists of at least three components [31]. The first is direct effects of the viral oncoproteins on the cell cycle, mediated via interactions between E6 and E7 oncoproteins of high-risk HPVs and the p53 and Rb proteins respectively. Direct influence of these oncoproteins on other cell-cycle regulators such as cyclin E has also been demonstrated [31]. The second effect is also mediated via viral oncoproteins and consists of abnormalities in centrosome duplication, leading to genomic instability, subsequently reflected in progressive allelic imbalance [15, 16]. The latter include alterations in 3p and specific amplifications at 3q25–27 [20, 33]. The third component is mediated via E6 and consists of telomerase upregulation and a disruption of normal replicative senescence [24].
It was recently revealed in lung cancer cases that HPV 16/18 E6 and E7 oncoproteins can mediate expression of multiple target genes and proteins, such as p53/pRb, VEGF, HIF-1α, cIAP-2, and hTERT, and contribute to cell proliferation, angiogenesis and cell immortalization through different signaling pathways [38].
Deregulation of E6 and E7 expression is the critical event in neoplastic dedifferentiation. The levels of E6 and E7 are regulated by E2. In many tumors, the viral DNA genome is integrated within the host cell genome, causing a disruption in the E2 gene and leading to increased levels of E6 and E7 and a more malignant phenotype. It is important to note that the presence of E6 and E7 in a cell is not enough to cause the development of cancer. Additional changes in cellular genes are needed and this is the reason for the long delay, often several decades, between HPV infection and the appearance of malignant tumors.

Conclusion

HPV can be regarded as an environmental factor in tumor development. There is a relationship between HPV infection and some neoplasms of the anogenital tract, including cervical, vulvar, vaginal, penil and anal cancers but also lung, breast and others. Viral oncproteins are the main factor for HPV induced carcinogenesis. Although the recently introduced prophylactic HPV vaccines are effective in preventing initial HPV infections, they have no effect on existing lesions and cancer. Elucidating the role of HPV ocoproteins in different types of tumors is important for development and assessing the potential impact of therapeutic HPV vaccines on HPV-related diseases.

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DP2. ЧОВЕШКИ ПАПИЛОМА ВИРУСИ - ТИПОВЕ, ДИАГНОСТИКА, ПРОФИЛАКТИКА И ЛЕЧЕНИЕ

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DP3. ЧОВЕШКИ ПОЛИОМНИ ВИРУСИ - ЛАБОРАТОРНА ДИАГНОСТИКА, ПРЕВЕНЦИЯ И ЛЕЧЕНИЕ

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DO10. NOVEL HUMAN RESPIRATORY POLYOMAVIRUSES KIPyV AND WUPyV – BASICS CHARACTERISTICS AND POTENTIAL ROLE IN HUMAN PATHOLOGY

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Abstract

Two novel human respiratory polyomaviruses, KIPyV and WUPyV, were discovered in respiratory specimens from children with acute respiratory infections. However, so far definitive evidences of their role in human pathology are lacking. In this review we describe aspects of virological background and emphasize on current knowledge on potential etiopathogenicity of these agents.

Introduction

Viral infections associated with respiratory syncytial virus, influenza virus A or B, parainfluenza virus, rhinovirus, or adenovirus are among the leading causes of respiratory diseases and are responsible for significant mortality and morbidity worldwide. However, about 30% of all cases with respiratory diseases cannot be attributed to known agents, suggesting that additional respiratory pathogens are likely to exist. Advances in molecular technologies enabled identification of novel human viruses and in 2007 two new human polyomaviruses (HPyVs), KIPyV and WUPyV, have been identified in patients with respiratory infections.

Discovery of KIPyV and WUPyV

The first HPyVs, JC polyomavirus (JCPyV) and BK polyomavirus (BKPyV), were discovered in 1971 in severely immunosuppressed patients. In 2007, two novel viruses, KIPyV and WUPyV, were identified in Sweden and the USA by screening of human respiratory samples with large-scale sequencing and bioinformatics [3, 19]. These new viruses were named after the

institution where the discovery was made: Karolinska Institute polyomavirus (KIPyV) and Washington University polyomavirus (WUPyV).
Today the human polyomavirus family consists of thirteen members (Table 1): BKPyV, JCPyV, KIPyV, WUPyV, MCPyV, HPyV6 and HPyV7, TSPyV, HPyV9, MWPyV, STLPyV, HPyV12 and NJPyV.

KIPyV/WUPyV genomics and phylogeny

All polyomaviruses are small viruses (~45 nm in diameter) containing a single circular double-stranded DNA genome. HPyVs have a similar genome size (4.5–5.4 kbp), organization, and share DNA sequence similarity. Polyomavirus virions are non-enveloped, and the capsid consists of 72 pentamers of the major structural protein VP1, each of which is associated with a single molecule of VP2 or VP3.
The genome size of KIPyV and WUPyV is within the range of polyomaviruses, 5 040 bp and 5 229 bp, respectively. In genome organization KIPyV and WUPyV are typical polyomaviruses (Figure 1) [3, 19]. The genome can be divided into three functional regions: the early region, the late region, and the non-coding control region (NCCR). The early region encodes the alternatively spliced large T-antigen and small T-antigen, regulatory proteins involved in viral DNA replication and gene expression. The late region encodes the structural viral proteins VP1, VP2 and VP3, which form the viral capsid. It was shown that, in its native form, VP1 of KIPyV and WUPyV is highly immunoreactive. The early and late regions are separated by NCCR, containing the early and late promoters and the origin of viral DNA replication. KIPyV and WUPyV like other novel HPyV genomes do not encode agnoprotein.

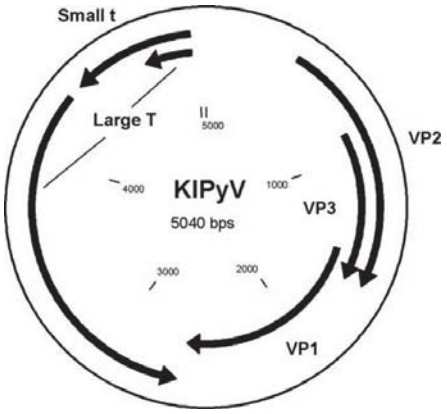

Fig. 1. Genome organization of KIPyV. Putative coding regions for VP1 to VP3, small T-antigen, and large T-antigen are marked by arrows [3].

Phylogenetic analysis indicates that KIPyV and WUPyV are closely related and define a novel branch within the Polyomaviridae family. They share significant homology with BKPyV, JCPyV and other polyomaviruses with regard to genomic organization. However, the genomes and proteins of KIPyV and WUPyV display little sequence homology with the previously known HPyV. While the nonstructural proteins have substantial amino acid sequence similarity to those of the other primate polyomaviruses, the structural proteins have extremely high sequence divergence as compared to other known polyomaviruses. So, in amino acid sequence the WUPyV structural proteins are only 15–28% identical to those of BKV and JCV (Table 2). At the same time, the late regions of KIPyV and WUPyV genomes show 64–71% amino acid sequence identity.

Table 1. Human polyomaviruses

Full name	Abbreviation	Year of discovery	Source of isolation	Reference
BK polyomavirus	BKPyV	1971	Urine	18
JC polyomavirus	JCPyV	1971	Urine, brain	36
Karolinska Institute polyomavirus	KIPyV	2007	Nasopharyngeal tissue	3
Washington University polyomavirus	WUPyV	2007	Nasopharyngeal tissue	19
Merkel cell polyomavirus	MCPyV	2008	Lesion	17
Human polyomavirus 6	HPyV6	2010	Skin	39
Human polyomavirus 7	HPyV7	2010	Skin	39
Trichodysplasia spinulosa-associated polyomavirus	TSPyV	2010	Lesion	27
Human polyomavirus 9	HPyV9	2011	Skin, blood, urine	40
Malawi polyomavirus	MWPyV	2012	Stool, wart	13, 42, 44
St Louis polyomavirus	STLPyV	2013	Stool	25
Human polyomavirus 12	HPyV12	2013	Metastatic liver tissue	23
New Jersey polyomavirus	NJPyV-2013	2014	Endothelial cells (myositis, cut. necrosis)	28

Table 2. Homology of Predicted WU Proteins [19]

Gene	Predicted Size (aa)	% Amino Acid Identity to:				
		KIPyV	JCPyV	BKPyV	SV40	MuPy
STAg	194	68	40	41	38	23
LTAg	648	70	48	49	49	32
VP1	369	65	27	28	28	25
VP2	415	71	16	17	17	12
VP3	272	64	15	15	16	11

Prevalence and etiopathogenicity of KIPyV and WUPyV

The widespread of KIPyV and WUPyV infection in human population has been revealed by extensive molecular and serological investigations. Seroepidemiological studies showed that seropositivity varies between 55% and 90% for KIPyV and 64% and 97.5% for WUPyV [29] and there is a strong increase in KIPyV and WUPyV seroprevalence with age [21]. It was suggested that primary exposure occurs during childhood, establishing a persistent infection, after which infection is sustained by close inter-human contacts [2, 22, 34, 37]. KIPyV and WUPyV were detected throughout the year although seasonal variations were found. There was

intensive search for these viruses in clinical specimens to determine their natural history and role in human pathology, but the results have been inconclusive [12].
The genuine host cells for KIPyV and WUPyV have not been established. Although viral DNA is predominantly detected in respiratory specimens, it has also been found in tonsils, lungs, blood, CNS and lymphoid tissue [5-7, 9, 10, 11, 29, 41] and the detection frequency increased in immunosuppressed individuals [4, 41].
KIPyV and WUPyV were identified first in respiratory specimens from children with acute respiratory infections, which suggested the respiratory tract as a possible site of infection, transient or persistent [3, 19]. In a number of subsequent studies respiratory tract diseases have been associated with the detection of KIPyV and WUPyV in respiratory samples. The viruses have been detected more frequently in nasopharyngeal secretions [1, 24, 26, 31, 32]. At the same time, the number of patients with respiratory symptoms who were positive for WUPyV and/or KIPyV was low. The detection rates of KIPyV and WUPyV range from 1% - 16%, and 0.5%-8%, respectively, in various studies of healthy individuals and patients with respiratory symptoms [8]. Furthermore, co-infection with one or more respiratory viruses is a common finding in individuals who are positive for KIPyV or WUPyV. Only in a small number of children KIPyV or WUPyV was the only virus detected, which suggests that these viruses might cause respiratory disease independently [2, 16]. So far, however, the etiopathogenicity of WUPyV and KIPyV in respiratory diseases remains speculative [35]. It is possible KIPyV and WUPyV could play a role in respiratory diseases only in immunocompromised patients [14].
WUPyV or KIPyV DNA has been found sporadically in fecal specimens from patients with acute nonbacterial gastroenteritis and in some hospitalized infants [38, 43]. However, no clear association between virus detection and gastrointestinal symptoms could be shown so far [5, 7, 12, 30, 33].
Little is known about the oncogenicity of KIPyV and WUPyV [5, 15]. To date, KIPyV and WUPyV have not been associated with a specific malignancy, but viral DNA sequences have been found in lung cancers and lymphoma [7, 15, 20]. WUPyV and KIPyV DNA was detected in paraffin-embedded tonsils from adult patients with a wide spectrum of benign and malignant conditions [5], indicating that these viruses can infect and establish a persistent infection in tissues that are rich in B cells.

Conclusions

After discovery in 1971 of first human polyomaviruses BKPyV and JCPyV, eleven more HPyVs, including KIPyV and WUPyV, have been identified in last decade due to the new techniques for viral detection. Although KIPyV and WUPyV share certain characteristics with the well-studied BKPyV and JCPyV, they show important differences such as little sequence homology of genomes and proteins, differences in viral life cycle, pathogenicity and so on. KIPyV and WUPyV are widespread among human population and have been detected more often in respiratory specimens worldwide. However, at this time there is no firm evidence for an etiological role of these viral agents in respiratory or any other disease and their clinical significance remains to be established. In addition, there are gaps in our understanding of WUPyV/KIPyV tissue tropism, routes of acquisition, persistency and reactivation etc. It is expected further studies to shed light on all these issues.

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DO11. SOME FEATURES IN EXPERIMENTAL INFLUENZA VIRAL INFECTION IN MICE AND ADEQUATE MARKERS USED FOR THEIR ASSAYING

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Free radicals, reactive oxygen species and oxidative stress

A free radical is every particle or a part of it (ion, molecule, group of atoms, etc.) that has an unpaired electron and is therefore unstable and highly reactive. In normal concentrations, they play an important role as mediators in the regulation of signaling processes. The paradox of aerobic life is the vital oxygen is at the same time deadly toxic. The reason is the electronic structure of the oxygen molecule - biradical which determines its one-electron reduction to oxygen radicals and other reactive oxygen species. Free radicals and other reactive oxygen species (ROS) - superoxide radical ($O_2^{\cdot -}$) nitrogen oxide (NO), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH) exist in living tissues in low but measurable concentrations which are determined by the balance between the speed of the formation of radicals and speed of their elimination (1).

Except for anaerobic microorganisms, all living organisms require molecular oxygen as an electron acceptor for efficient production of energy. However, oxygen is a strong oxidant,

and it is impossible to avoid secondary oxidations not involved in physiological metabolism. These random oxidations would have deleterious consequences if they were not neutralised by an efficient antioxidant system (14). 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 (12).

So called "pro-oxidants" promote oxidative damage in laboratory model systems whereas antioxidants inhibit (9).

Oxidative stress corresponds to an imbalance between the rate of oxidant production and that of their degradation. The complete four-electron reduction of molecular oxygen occurs within mitochondria and produces water, at the end of the respiratory chain (Figure 1). Sometimes molecular oxygen is partly reduced instead of the proteins of the respiratory chain, and superoxide and various reactive oxidant intermediates are produced, leading to secondary oxidations (14).

Defence of the organism

Antioxidants are molecules which can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged. Some endogenous radical protectors are: superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), glutathione peroxidase (GSH-Px), glutathione reductase (GR), etc. Since free radicals have very short live scientists measure the activities of the antioxidant enzymes and compounds. These are the biochemical markers of oxidative stress. They are used in order to determine the effect of influenza virus infection in experimental model in mice.

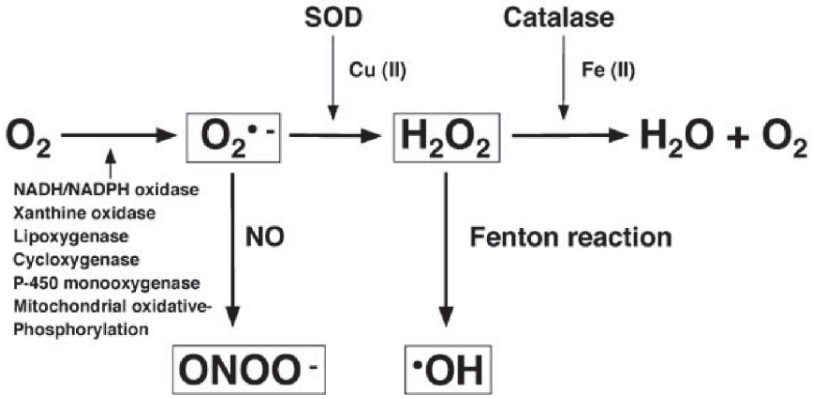

Figure 1. Sources of reactive oxygen species (ROS) generated endogenously *(Palamara, A., 2011)*

How does Influenza virus cause oxidative stress?

Viruses are parasitic pathogens that replicate in living cells, exploiting multiple intracellular pathways in their hosts for their own advantage and growth. Numerous studies have reported that viral infection is often associated with redox changes characteristic of oxidative stress (12).

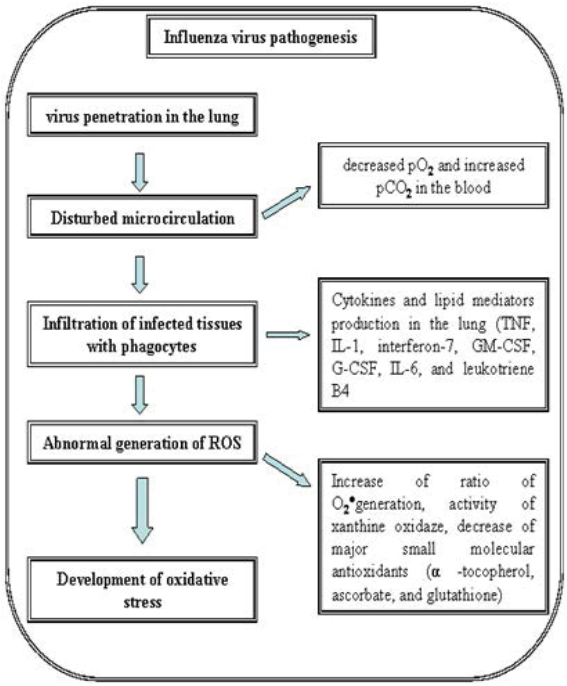

Figure 2. The pathway of influenza virus pathogenesis (*Mileva, M. et al., 2012*)

Influenza viruses possess RNA as their genome and belong to the family Orthomyxoviridae. They cause acute respiratory inflammation in humans and symptoms such as high fever, body aches, and fatigue (7). Intermittently and unpredictably new viruses arise that are capable of causing pandemics. Pandemics are a regular occurrence. Historical accounts suggest that there have been more than 20 since the 16th century, typically occurring every 10 to 40 years. The 1918 pandemic (H1N1) is widely regarded as the single biggest natural disaster documented to have affected our species, resulting in approximately 40 million deaths worldwide (WHO).

The genome of influenza virus type A (IAV) consists of eight RNA segments, encoding HA, neuraminidase (NA), nucleoprotein (NP), M1, M2, nonstructural protein (NS)1, NS2, polymerase acidic protein (PA), polymerase basic (PB) 1, PB1-F2, and PB2. Recently, research has focused on using reverse genetics to elucidate the role of each viral protein in the pathogenicity of influenza viruses. The range of severity of diseases caused by genetically similar IAV in humans is extremely wide, indicating that host conditions play an important role in determining the pathogenesis of IAV. I IFN is a key molecule in the innate immune responses to infection with influenza virus and the magnitude of the type I IFN response influences the pathogenicity of the virus. Thus, the pathogenesis of influenza virus infection in humans depends on a combination of virus and host factors (7)(Figure 3).

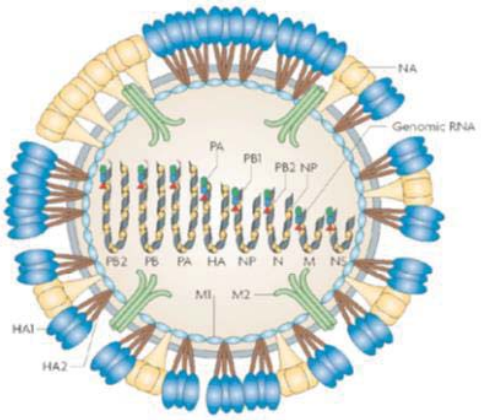

Figure 3. Schematic diagram of influenza A virus virion (*Palamara, A., 2011*)

IAV inhibits cellular respiration (mitochondrial O2 consumption),diminishes cellular glutathione (GSH) and triggers apoptosis (1). Reactive oxygen species and reactive nitrogen species play a pivotal role in the regulation of the immune response to infection (10). 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 dithiocarbamate, *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 (16).

Biological markers of oxidative stress during influenza infection

The ideal biomarker should have the following characteristics:
(i) Sample collection and analysis is simple and reliable;
(ii) The biomarker is specific for a particular type of exposure;
(iii) The biomarker only reflects a subclinical and reversible change;
(iv) Relevant intervention or other preventive effort can be considered;
(v) Use of the biomarker is regarded as ethically acceptable.

The purpose of this study was to find the pathologic features caused by influenza virus infection in the histological structure of different organs in experimental mice model.

Materials and methods

As a material for the present morphological study were used organs from experimental animals - mice. For the purpose of the investigation were used male mice, line ICR (14-16 g).

Experimental design:

The animals were divided into two groups:

Group I - control group (healthy non-infected animals);

Group II - mice infected with influenza A/Aichi/2/68 H3N2 (2.5 to LD50) by intranasal inoculation.

The animals were housed in cages with free access to water and food, at 22-24 °C with a 12 hour light/dark cycle. Experiments were conducted by the standard protocol approved by the Bulgarian Academy of Sciences. The animals were anesthetized and decapitated on the 5th day after virus inoculation. Tissues were fixed with 10% formalin solution and prepared for light microscopy by standard protocol of the Medical University in Sofia. In this study were used routine methods of stain Nissl. Light microscopic pictures of lungs, liver, small intestine, kidney were made.

Hematoxylin and eosin (H&E) stains have been used for at least a century and are still essential for recognizing various tissue types and the morphologic changes. Hematoxylin has a deep blue-purple color and stains nucleic acids by a complex, incompletely understood reaction. Eosin is pink and stains proteins nonspecifically. In a typical tissue, nuclei are stained blue, whereas the cytoplasm and extracellular matrix have varying degrees of pink staining (6). The first step is removing the wax in which the object is fixed. Xylene is used. After de-waxing the side is passed through alcohol and after that water to remove the xylene. Then hematoxylin is applied. After 2-3 min it is rinsed with water. Next eosin is applied for 1-5 min and rinsed with water. The object is then dehydrated in ascending alcohol solutions (50%,70%,80%,95%, 100%) (13).

Results and discussion

We used experimental mice model to define the histological features of infected lungs, kidneys, stomach, guts. The non infected animals showed normal structure of lungs, liver, small intestine, kidney (Fig. 4 A, Fig. 5 A, Fig. 6A, Fig. 7 A). In infected with influenza virus animals showed pathological changes in the investigated organs, expressed in edema, changes in the histologic structure, dilatation of blood vessels.

The morphological studies on mouse lungs 5 days after inoculation with influenza virus A/Aichi/2/68 (H3N2) have suggested that this model of experimental influenza virus infection causes progressive damage of the alveolar cells, with acute inflammatory reaction and development of massive bronchitis and probably pneumonia(Fig. 4 B).

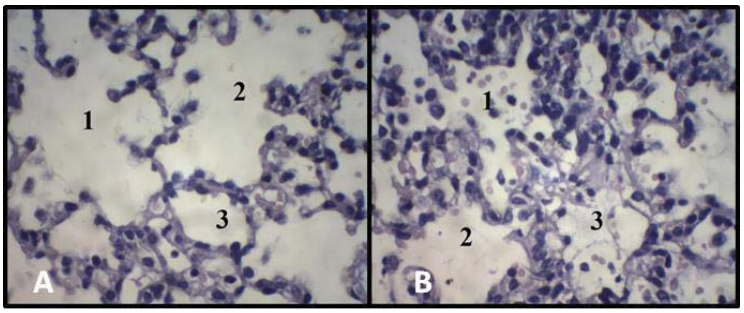

Figure 4. Light microscopic picture of lungs. Staining: HE
A. Non infected animal: 1 and 2 - alveolar sacks with normal histology; 3 alveolar canal.
B. Infected animal: 1 and 3 - sacks with impaired alveolar histology, intracellular edema and the presence of red cell infiltration, 2- alveolar sac with preserved structure

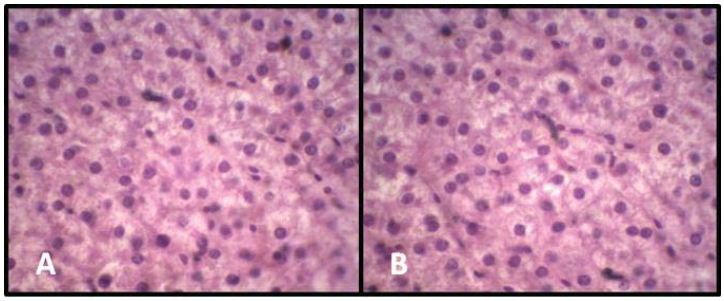

Figure 5. Light microscopic picture of liver. Staining: HE

A. Liver of non infected animal.
B. Liver of infected animal- the structure is more visible. There is oedema of the heptocytes.

In infected liver there is edema of the hepatocytes. The histological structure is more expressed (Fig. 5 B).
In the small intestine the histological picture reveals edema, dilatation of the blood vessels and stasis (Fig. 6 B).

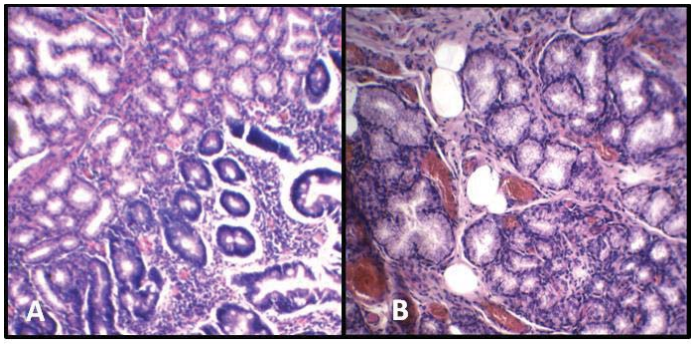

Figure 6. Light microscopic picture of small intestine. Staining: HE

A. Non infected animal. Normal histologic picture of the epithelium covering the mucous membrane of the small intestine.
B. Infected animal. Many dilated vessels variating in size could be seen. There is venous stasis and interstitial edema.

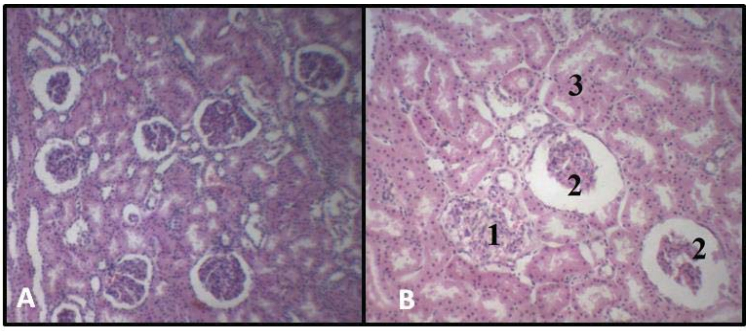

Figure 7. Light microscopic picture of kidney. Staining: HE

A. Non infected kidney. Malpighian bodies and canals could be seen.
B. Infected kidney;1- dilatation of vessels in the glomerule; 2- glomeruli with vessel colapse; 3- intracellular edema of the canal system.

One of the most probable reasons for the histologic changes in the invested organs is the development of oxidative stress in the course of the influenza virus infection. ROS and reactive nitrogen species (RNS) modulate the permissiveness of cells to viral replication, regulate host inflammatory and immune responses, and cause oxidative damage to both host tissue and virus progeny. ROS and RNS readily enter into reactions with macromolecules into the cells, particularly with key components of membranes and nucleic acids. A decrease in GSH levels and general oxidative stress have been demonstrated during influenza virus infection in both in vivo and in vitro experimental models (12).

Oxidative stress can be defined by measuring the activity of the antioxidant enzymes.

SOD- catalyzes the conversion of superoxide anion radical ($O_2^{\cdot-}$) to less reactive species.

$$2O_2^{\bullet} + 2H^+ + SOD \rightarrow H_2O_2 + O_2$$

To determine the activity of SOD the method of Beauchamp & Fridovich (1971) could be used. Superoxide radicals generated photochemically reduce nitroblue-tetrazolium salt (NBT) to insoluble formazan blue. Spectrophotometrically at 560 nm is measured the inhibition of the reduction of NBT in the presence of the enzyme preparation. The unit of enzyme activity is the amount of enzyme, performing 50% inhibition of the reduction of NBT (5).

Catalase catalases the reaction of the decomposition of hydrogen peroxide to water and oxygen. There are two types of catalases: Type A and Type B. The enzyme is localized mainly in peroxisomes, less in mitochondria and endoplasmic reticulum (2).

For measurement of Cat activity the method of Aebi is (1974) used. The enzyme catalyzes the following reaction:

$$2H_2O_2 + Cat \rightarrow 2H_2O + O_2$$

The decrease of absorbance at 240 nm corresponds to the decomposition of H_2O_2 and serves as a measure of the activity of the catalase (3).

Glutathione peroxidases are a family of selenium- dependent and-independent antioxidant enzymes and can be divided into two groups: cellular and extracellular. Glutathione peroxidase reduces H_2O_2 to H_2O by oxidizing GSH. Re-reduction of GSSG is then catalyzed by glutathione reductase through the glutathione cycle:

$$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$$

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$$

The oxidation of NADPH is measured by the reduction of the absorbance at 340 nm, which serves as a measure of the activity of glutathione peroxidase. This is the method of Gunzler et al. (8).

For measurement of total glutathione the method of Tietze (1969) is used. At the beginning of experience reference standard with oxidized glutathione should be made. The reaction is started with NADPH, which converts oxidized glutathione into reduced in the presence of glutathione reductase. GSH reacts with DTNB and colored compound is received with a peak of absorption at 412 nm. Values are expressed in ng/mg protein (15).

Conclusion

Influenza virus infection starts in the airways and turns into a disease of the whole organism. The oxidative stress that causes affects not only the lungs but the whole body. Histological

changes are visible in all investigated organs. The function of the antioxidant enzymes is upregulated because of the increased production of reactive oxygen species. Using antioxidants and/or biologic response modifiers in the treatment could help preventing the consequences of the infection.

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Session E: Pharmacology and Toxicology

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Toxicology Clinic, UMHATEM "N. I. Pirogov"

Assoc. Prof. Russy Russev, DVM, PhD
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EO1. DISTRIBUTION OF ACUTE POISONING WITH PSYCHOACTIVE SUBSTANCES – ANALYSIS FOR THE PERIOD 2012 – 2014

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ABSTRACT
OBJECTIVE: To investigate the incidence of acute monotoxic poisonings with psychoactive substances /PAS/ as well as combined intoxication at the Clinic of Toxicology, Emergency University Hospital "N.I. Pirogov " during the period 2012 - 2014, to characterize the type of PAS, to present the distribution of patients by sex and age; to make an analysis of epidemiological data - distribution, dynamics, trends.
MATERIAL AND METHODS: The records of the Toxicology Clinic, Emergency Hospital "N.I.Pirogov" were reviewed retrospectively for all psychoactive substances poisonings during a 3 years period - from January 1, 2012 to December 31, 2014.
THE RESULTS of the study show the following:
1. A decrease of heroin poisoning was shown, however an increase in marijuana, amphetamines and methadone poisoning was observed during this 3 years period. .
2. The highest incidence of monotoxic poisoning with psychoactive substances was found in 2013. The men predominate over the women. The largest number of patients was in the age group - 26-35 years, followed by those in the age group 16-18 years old.
The lowest number of patients was in the age group older than 35 years.
3. The highest incidence of mixed poisoning was found in 2012 and 2014. The men predominate over the women. The largest number of patients was in the age range - 26-35 years, followed by patients in the age group over 35 years. The lowest number of patients was in the age group under to 15 years old.

Key words: intoxication, psychoactive substances, heroin, marijuana, amphetamines, methadone

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EO2. EXPERIMENTAL MODELS FOR INVESTIGATION OF CADMIUM CHELATION

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For a better understanding of the mechanism of cadmium (Cd) toxicity and its chelation from the living organism different experimental models were designed. To investigate how the chelation of Cd can be achieved, Cherian M [1] applied the following experimental model. Experimental animals were divided into 10 groups of 6 rats each. They were injected intravenously with 1 mg Cd/kg as $CdCl_2$. Each group was injected intraperitoneally with 10 different chelating agents in equimolar amounts (400 μmol/kg). Bile and urine samples were collected after 3 hours. The results showed that all dithiol compounds containing adjacent thiol groups could mobilize Cd from the hepatic metallothionein (MT) and increase the biliary excretion of Cd. Because of their high toxicity these chelating agents have poor therapeutic value [1]. It has been reported that mono- and diesters of DMSA have higher efficacy as chelating agents. Monomethyl DMSA (MmDMSA) and Monocyclohexyl DMSA (MchDMSA) have been used by Jones et al in their *in vivo* study on male albino mice exposed to Cd for seven days. The administration of MmDMSA and MchDMSA reduced significantly whole body Cd levels and no redistribution of Cd in the brain was observed [4]. Recent investigations

indicated that the polyether ionophorous antibiotic monensin was even more effective than the traditional chelators. An animal model was applied to confirm the ability of the tetraethylammonium salt of monensic acid to act as an antidote for subacute Cd intoxication. ICR male mice were divided into three groups – normal control group, toxic control group (intoxicated with Cd(II) acetate) and group treated with tetraethylammonium salt of monensic acid as antidote. The antidote was applied after the Cd-intoxication. [3]. The results demonstrated that the Cd concentration in the lungs and spleen of the third group animals was reduced by 80% and by 55% in the testes compared to the Cd-treated controls. A similar animal model but with a higher dose of Cd(II) acetate was applied to investigate the effect of the tetraethylammonium salt of monensic acid on Cd-induced toxicity. The histopathological analyses revealed that the tetraethylammonium salt of monensic acid significantly recovers the normal structure of the organs [2]. The animal model is suitable for comparative assessment of the potential application of the polyether ionophores as antidotes for Cd-poisoning.

Acknowledgment. The financial support by Sofia University "St. Kliment Ohridski" Fund for Scientific Research is gratefully acknowledged (grant 64/2015).

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EO3. GUINEA PIGS AS AN EXPERIMENTAL MODEL. TOXICITY OF PTAQUILOSIDE ISOLATED FROM BRACKEN FERN (*PTERIDIUM AQUILINUM* (L.) KUHN) IN GUINEA PIGS

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Abstract

Guinea pigs (*Cavia porcellus*, genus *Cavia*) are rodents, belonging to the family Caviidae, tamed as early as 5000 BC by South American tribes. They originated in the Andes and were brought to Europe by the Spanish conquistadors. Biochemical and genetic studies revealed that guinea pigs are domesticated descendants of a closely related species of cavy such as Cavia aperea, Cavia fulgida and most likely C. tschudii. Biological experimentation on guinea pigs has been carried out since the 17th century, but nowadays have been displaced by rats and mice. However this experimental model is irreplaceable, due to some characteristic features as: life span, anatomy and size, reproductive specifics, nutritional requirements and habits, polymorphism, size of gene pool compared to man, friendly temper, state of health, ect.

Guinea pigs are considered to be a suitable model for fundamental and applied biomedical researches focused on: ulcerative colitis; cholelithiasis; giant cell pneumonia (Cytomegalic inclusion disease); experimental porphyria, resemble different types of hepatic porphyria in man; lymphoblastic leukemia; acute-tubular-basement-membrane-antibody tubulointerstitial nephritis; complement deficiency - C4, associated with systemic lupus erythematosis-like syndrome; endocrine disorders as diabetes mellitus and Di George Syndrome (Congenital Thymic Hypoplasia in children); pregnancy toxemia (Preeclampsia); osteoarthritis; nervous diseases as Demyelinating optic neuritis, seen in multiple sclerosis, Creutzfeldt-Jacob Disease; musculoskeletal (Achondroplasia); urogenital (Genital herpes simplex infection), allergic and metabolic diseases. Guinea pigs are extensively used for studies on infectious disease as tuberculosis, syphilis, brucellosis, diphtheria, anthrax and various parasitic invasions. These rodents are often used in many toxicological tests.

The present study analyzes some hematological, biochemical, histological and urinalysis changes in guinea pigs treated with norsesquiterpen glycoside ptaquiloside derived from Bracken fern (*Pteridium aquilinum* L. Kuhn). Hematology revealed statistically significant abnormalities of leukocytes, lymphocytes, erythrocytes, platelets, packed cell volume and mean levels of hemoglobin in erythrocytes. The tests on the biochemistry profile determined statistically significant changes in the levels of gamma glutamyl transferase, total protein and albumin. In urinalysis proteinuria was the most common finding. Ptaquiloside applied in doses of 5 mg/kg bw inflicted histopathological changes in kidneys and bladder of treated animals.

Key words: guinea pig, experimental model, ptaquiloside toxicity

EO4. ESTABLISHMENT OF THE REAL TIME DYNAMIC MODEL FOR ANALYSIS OF INTEGRITY AND PERMEABILITY OF THE BLOOD BRAIN BARRIER (BBB) IN VIVO- IN RATS

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The blood–brain barrier (BBB) is a diffusion barrier, which impedes influx of most compounds from blood to brain. Three cellular elements of the brain microvasculature compose the BBB—endothelial cells, astrocyte end-feet, and pericytes (PCs). Tight junctions (TJs), present between the cerebral endothelial cells, form a diffusion barrier, which selectively excludes most blood-borne substances from entering the brain. Astrocytic end-feet tightly ensheath the vessel wall and appear to be critical for the induction and maintenance of the TJ barrier in the mammalian brain.

TNF-□ and proinflammatory substances and specific disease-associated proteins often mediate BBB dysfunction. Despite seemingly diverse underlying causes of BBB dysfunction, common intracellular pathways emerge for the regulation of the BBB structural and functional integrity. Better understanding of tight junction regulation and factors affecting transport systems will allow the development of therapeutics to improve the BBB function in health and disease.

The laboratory animals that were used and monitored are preliminary operated and implanted open cranial window (CW) in order to observe directly microvasculatory bed of the brain. 30 Male Wister rats (n=30). Twenty of them were treated with glioma tumor cells. Ten were treated with inhibitor of VEGF (experiment). The other ten were treated with carrageenan to induce inflammation, and 10 were left as pure control.

Preparation of cranial window. Rats were anesthetized with 40 mg/kg body wt i.p. ketamine.

A catheter was placed into a tail vein for injection of the intravascular tracer, fluorescein iso- thiocyanate albumin (FITC-albumin; molecular weight=69,000 Da).

Permeability of the blood-brain barrier was evaluated using two methods. First, extravasation of FITC-albumin was indicated by measurement of tissue fluorescence through fluorescent microscopy and by means spectrophotofluorometer (Shimadzu FL-260) for evaluation of the ratio of FITC-albumin fluorescence between blood plasma and cerebrospinal fluid-CSF.

EO5. MODELING OF NUTRITION IN PATIENTS WITH RELAPSING REMITTING MULTIPLE SCLEROSIS WITH AIM TO REDUCE THE EXACERBATIONS

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Key words: multiple sclerosis, nutrition

Multiple sclerosis (MS) is the most common chronic immune-mediated inflammatory disease of the central nervous system in young adults which is characterized by inflammatory demyelination and neurodegeneration with unpredictable effects lasting for the rest of their lives. Neurological symptoms include sensory disturbances, optic neuritis, limb weakness, ataxia, bladder dysfunction, spasticity, cognitive deficits and fatigue.

The new concept of MS as a neurodegenerative disease has important clinical implications. There are specific treatment strategies that act within the central nervous system to prevent neurodegeneration and they need to be provided from the earliest stages of the disease [5].

Currently there is no definitive treatment of MS but the aim of the medication is to reduce the power of attacks and symptoms to be more rarely. Early diagnosis is crucial as well as early neuroprotective therapy and preservation of myelin. The most important for early diagnosis are ophthalmologists, gynecologists and general practitioners. Now there are many drugs that are able to maintain a good quality of life in a single condition – early diagnosis and rapid drug intervention. We also have many publications on this topic.

Now I am presenting a publication presented in April at the 67th American Academy of Neurology (AAN) Annual Meeting in Washington, DC [1]

Compared to healthy controls, women with multiple sclerosis have lower levels of folate, magnesium, vitamin E, and other nutrients that may have important anti-inflammatory or antioxidant properties, a new study shows [3]

"It's unclear if deficiencies in these nutrients cause MS or are a consequence of it," said lead researcher Sandra D. Cassard, research associate, Johns Hopkins School of Medicine, Baltimore, Maryland. More research is needed to clarify the relationship, she said.

The study included 27 patients with relapsing-remitting MS and 30 healthy controls who were participants in a vitamin D3 supplementation study. Participants were white women aged 18 to 60 years with 25-hydroxyvitamin D levels of 30 ng/mL or less and body mass index (BMI) less than 30 kg/m^2 at screening.

At baseline, participants completed the validated Block 2005 Food Frequency Questionnaire, which asks about diet and nutrition over the previous year. They then began taking oral vitamin D3, 5000 IU/day, for 90 days.

This analysis focused on intake of five nutrients:

Folate: This water-soluble vitamin B found naturally in foods such as beans and lentils helps in formation of red blood cells.

Magnesium: A mineral found in foods such as raw spinach, nuts, seeds, and mackerel fish, magnesium helps to keep muscles and nerves healthy.

Lutein-zeaxanthin: Found together in green leafy vegetables and other foods, they are potent antioxidants. Occurring in especially high concentrations in the eye, they are thought to be crucial for healthy vision.

Quercetin: A flavonoid phytoestrogen with antioxidant and anti-inflammatory properties, this nutrient, found in such foods as capers and onions, has potential immunomodulatory and neuroprotective effects.

α-Tocopherol: The dietary form of vitamin E is found in vegetable oils, wheat germ, and other foods. This antioxidant has the potential to attenuate demyelination.

The researchers found that compared to controls, participants with MS had a significantly lower mean intake of all of these nutrients (Table 1).

Table 1. Food intake of nutrients in patients with (with) MS vs controls

Nutrient	Patients With MS	Controls	*P* Value
Food folate (µg)	243.9	321.4	.01
α-Tocopherol (mg)	6.7	8.1	.03
Magnesium (mg)	254.3	321.2	.01
Lutein-zeaxanthin (µg)	3634.7	5384.6	.01
Quercetin (mg)	5.8	11.6	<.001

While the most significant finding was for the comparison of quercetin between the groups, stressed Dr Cassard.

However, lower levels of these nutrients in the diets of MS patients compared to healthy controls suggest areas warranting further research.

Not only did patients with MS have lower intakes of certain nutrients than their healthy counterparts, but they fell below recommended dietary allowances (RDAs) for many of these nutrients. However, all study participants had lower intakes of folate and α-tocopherol than the RDAs. Patients with MS also fell below RDA intake of magnesium.

Patients with MS had lower mean percentage of kilocalories from fat than controls (36.7% vs 40.3%). Dietary sodium intake was similar between the two study groups. Dr Cassard noted that the results are unadjusted for BMI. Patients had a higher BMI than controls (25.3 vs 23.6 kg/m^2). Dr Cassard speculated on how the lack of certain nutrients might affect the disease process in MS. "Free radicals are believed to play a role in demyelination and axonal damage in MS," she explained. "Some of these nutrients have antioxidant properties, which may interact with free radicals, inhibiting potentially damaging effects." It's possible, said Dr Cassard, that MS itself depletes the body of nutrients or in some way inhibits their absorption. However, she pointed out that the differences found in the study were based on self-reported diet history over the previous year, not on measurement of nutrients from body fluids [1]

Results are preliminary and further research is needed before neurologists start recommending dietary modifications to patients with MS. The larger vitamin D study should shed more light on the possible link between vitamin D deficiency and MS, said Dr Cassard. "Those results are being reported separately and the manuscript is currently under review." [2,4]

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It is presented at the 67th American Academy of Neurology (AAN) Annual Meeting, April 18-25, 2015.

Omega-6 fatty acids (11-23 g/day linoleic acid) had no benefit in 75 relapsing remitting (RR) MS patients (progression at two years or in 69 chronic progressive (CP) MS patients. Linoleic acid (2.9-3.4 g/day) had no benefit in CPMS (progression at two years.

Slight decreases in relapse rate and relapse severity was associated with omega-6 fatty acids in some small studies, however these findings are limited by the limited validity of the endpoints.

Omega-3 fatty acids had no benefit on progression at 12 months in 14 RRMS patients or at 24 months in 292 RRMS patients [3]

The most common dietary interventions are supplementation with polyunsaturated fatty acids, allergen (gluten and milk)-free diets, vitamins, and micronutrients and antioxidants such as selenium, *Gingko biloba* extracts, coenzyme Q10.

The main conclusion is that nutrients do not affect directly to improve the state of MS patients. The aim is to help in any way with drugs, with rehabilitation and physical therapy, through diet and lifestyle. Data from our scientific observations indicate that there is no direct link between eating habits and the occurrence of seizures in MS patients, but that does not mean that one does not affect the other (Fig. 1).

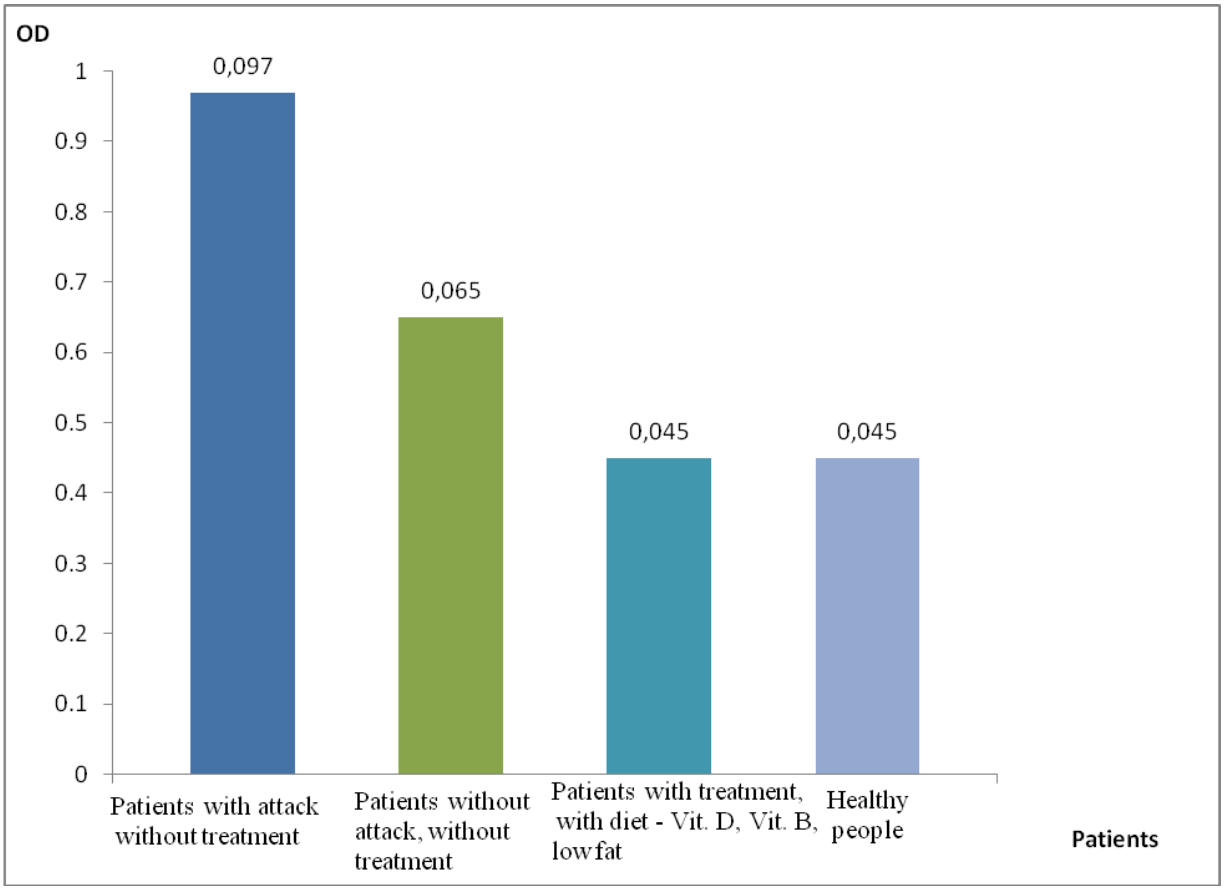

Figure 1. Serum IgG anti-GM1 antibodies in RRMS patients in different phases of treatment and diets in the case of early beginning of the treatment

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EO6. LIPID PEROXIDATION IN LIPOSOMES

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Abstract

Lipid peroxidation in biological membranes is considered to be one of the major mechanisms of cell damage in aerobic organisms exposed to oxidative stress. Free radicals which are formed by various mechanisms induced cascade of oxidative processes, thereby they change arrangement of lipids and proteins in the membranes. As results are changes of hysical properties and permeability of this membranes, and their function are affected. The efforts of many scientists are dedicated to understanding the factors that govern the processes of peroxidation, and relationship with peroxidation inducers. Biological membranes are complex structures and these processes are difficult to quantify. This requires the use of liposomal membranes that are simplified models of biological membranes. A liposome is a tiny bubble (vesicle), consists of the same material as a cell membrane. Structurally, liposomes are vesicles in which an aqueous volume is entirely enclosed by a membranous lipid bilayer. Membranes are usually consisting of phospholipids, which are molecules that have a hydrophilic head group and a hydrophobic tail group. The head is attracted to water, and the tail, which consists of a long hydrocarbon chain, is repelled by water.

The results obtained from peroxidation of liposomal lipids could be help to explain the mechanisms of the physico-chemical interaction of liposomal lipid with every one substance, candidate to be an effective chain-breaking antioxidant.

The kinetics of peroxidation induced by "external" generators of free radicals is determined by the balance between the effects of membrane properties and rate constant of propagation (Kp) and termination (Kt) of the free-radical peroxidation in the membrane were involved oxidizable lipids; peroxidation induced by transition metal ions depends on additional factors, including the binding of metal ions to the lipid - water - surface and formation of metal ions-hydroperoxide complex; antioxidants are capable to increase or inhibit lipid peroxidation. These processes depend on composition of membranes, as well of the properties of the peroxidation inducer. Although, peroxidation in model systems differs from peroxidation in biological membranes, results obtained in model membranes may be useful for understanding the issues that unable tested in biological membranes and gives an opportunity to predict radical scavenger effect of various new substances.

Introduction

Under normal physiological conditions in the aerobic organisms active oxygen species (ROS) are generated continuously. Some of them have a radical nature. As radicals these particles are highly reactive and are capable to oxidize virtually all biomolecules. They are eliminated by the antioxidant defense system that maintains the pro/antioxidant balance in cell.

In various pathological conditions, however, their generation could be intensified and the antioxidant capacity of biological system may be insufficient to eliminate their production. This could result in attacking and damaging cell structures. Condition associated with abnormal generation of a free radical and / or reduced antioxidant protection is referred to as oxidative stress (OS).

There are two important conditions for existence of the cell: its autonomy and the exchange of substances and energy with environment. The balance between these two conditions is ensured by properly functioning biological membranes. Because of the presence of polyunsaturated fatty acids (PUFA) in their structure, they are particularly prone to attacks of ROS. This could induce chain branching oxidation reactions, resulting in various intermediates and end products [6; 17]. The process is known as lipid peroxidation (LP) and is considered one of the main causes of damage to biological membranes [9; 13; 16].

Although the mechanisms of LP are widely studied, the complexity of natural membranes and complex mechanisms of their oxidation in biological systems, together with the lack of universal quantitative indices of OS is often linked to serious contradictions between the results of different studies. Some of these complications can be avoided by using simpler model systems, such as liposomes, in which structure predominate phospholipids like natural cell membranes.

Liposomes are described for the first time by the English haematologist Alec D. Bangham when monitoring negatively stained dried phospholipids [1]. A mixture of phospholipids and water spontaneously form a suspension of vesicles (liposomes), composed of a double lipid layer, which is the most advantageous structure in terms of thermodynamics.

In research liposomes are used as a biological membranes model for studying the effect of different water-soluble and lipid-soluble substances on processes related to cellular activities, including oxidative stress.

Influence of chemical and physical factors on liposomal lipid peroxidation

The ratio of lipids containing unsaturated and those containing saturated fatty acids (i.e., oxidizable and nonoxidizable lipids) in the composition of the liposomes affect their membrane properties. This affects the mechanisms of LP [4; 10].

The advantage of monolayer liposomes made by synthetic lipids is the possibility their composition to be determined by the researcher. This feature is important in studying the relationship between the kinetics of LP, used inductor and physicochemical properties of liposomes. While lipids, extracted from biological samples, are complex structures, which adds additional factors of influence and reproducibility of the results is low.

Classification of LP inducers

In designing an LP the most common inducers are copper ions, iron ions (often tested in the presence of ascorbic acid), hypochlorite (HOCl), which is generated by myeloperoxidase reactions, as well as "organic" radical generators – water-soluble (e.g. AAPH azo-bis-aminopropanhydrohlorid) and lipid-soluble (e.g. AMVN-azo-bis dimethylvaleronitrile) [2; 3; 14; 4; 11].

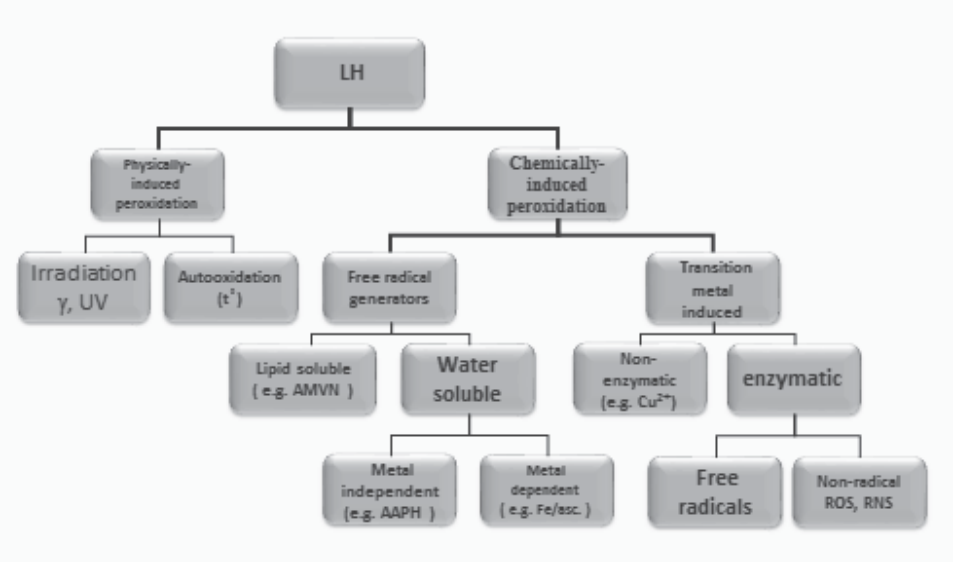

Fig. 1 Classification of the inducers of lipid peroxidation [15]

The mechanisms responsible for the oxidation induced by different types of inducers (as shown in Fig. 1) vary considerably: when water-soluble inducers are used to initiate peroxidation, the free radicals are formed in the solution and attack the liposomes from the external environment [8]; the lipid-soluble inducers (e.g. AMVN) undergo a thermal degradation in the liposome bilayer, resulting in generation of free radicals that could induce chain reactions. The peroxidation of liposomal PUFA by transition metal ions as inductor is quite different from the above mentioned mechanisms. The transition metals bind to liposome surface forming free radicals upon reduction from lipid-soluble reducing agents such as tocopherol [18] or hydroperoxides [12].

The main attributes of an LP study are: inducers of peroxidation; composition and physical properties of liposomes (ratio of the oxidizable and non-oxidizable lipids); inhibitors of peroxidation, if present. The evaluation of the effect of each of these factors requires the comparison of peroxidation in systems which differ only by a single factor studied.

When the kinetics of LP is tested in presence of inhibitors, their effect dependents on the physical properties of liposomes, the nature of the inducer, and the concentration of components in the test system [7; 5] These complexities limit the possibility of reaching generalizations.

Relationship between peroxidation kinetics, liposomes composition and peroxidation inducer

Peroxidation of liposomal lipids is a complex function of the liposomal composition. In particular, the composition influences the susceptibility to oxidation on the respective mechanisms (Fig. 1). The physical properties of liposomes are determined by their composition depending on the ratio of the oxidizable and non-oxidizable lipids. In turn, the physical properties of liposomes may affect their sensitivity to oxidation by various inducers.

The oxidation is carried out by different mechanisms, depending on the oxidant. Oxidizing lipids affect the peroxidation only through their effect on physical properties. But the change in the composition of the oxidizing lipids affects the kinetics of peroxidation by affecting both the physical properties of the liposomes, and the chemical properties of the

oxidizable lipids - the number of double bonds in PUFA, the number of bis-allylic hydrogen atoms, the position of the double bonds with respect to the liposomal surface and geometrical isomerism of the double bonds.

Dependence of the peroxidation on the physical properties of the liposomal lipid bilayers

The most complicated factor in the preparation of liposomal suspension is the lipid bilayer packing. This process is driven by thermodynamic forces and defines all experimentally detectable physical properties. In homogeneous structures, the dense packing increases the bilayers thickness, order parameter and micro-viscosity. With increasing density of the packing the viscosity of the lipid bilayer increase, but other measurable parameters such as surface area per phospholipids' polar group, fluidity (which is the reciprocal of the micro-viscosity) and the rate of lateral diffusion decrease.

The LP is a sequence of diverse (monomolecular or bimolecular) reactions. The monomolecular reactions are much less influenced by the physical properties of the bilayers than bimolecular reactions.

The rate of oxidation in a homogeneous membrane is experimentally detectable. The rate constants of bimolecular reactions reflect the rate of lateral diffusion, which is a function of the packing of the lipids in the bilayers. The later is strongly dependent on the lipid composition. In particular, the rate constant for bimolecular reaction is an increasing function of the rate of lateral diffusion of oxidizable lipids in bilayers. This means that the increasing rate of lateral diffusion (more "fluid" bilayers are less "hard") may favor the increase of both the speed of propagation of free-radical chain reactions and the rate of outage of free-radical processes by biradical fighting. These two processes have opposite effects on the overall rate of peroxidation. Therefore, the total effect of lateral diffusion on the peroxidation speed can hardly be uniquely predicted.

The PUFA residues in the membrane are susceptible to peroxidation, because their hydrocarbon chains contain polar-related H (bis-allylic) atoms. They are relatively easy to attack by exogenous free radicals. In the presence of molecular oxygen, the principal products of peroxidation are lipid peroxides. These molecules may be subjected to heat-induced and/or catalyzed by transition metal ions decomposition to produce "secondary" free radicals. Thus, oxidative chains are further conceived. As a result of hydroperoxides decomposition, complex mixtures of relatively stable products of peroxidation (unsaturated aldehydes, ketones and alcohols) are produced.

The mechanism of chain peroxidation of oxidizable lipids (LH), occurring upon the attack of exogenous radicals (X•) is developing as follow:

1. extraction of a reactive hydrogen atom from the bis-allylic groups of PUFA hydrocarbon chain

$$LH + X^{\bullet} \rightarrow L^{\bullet} + HX$$

2. Formation of peroxyl radicals ($LO_2^{\bullet}$) at extremely fast reaction of lipid radicals ($L^{\bullet}$) with dissolved molecular oxygen:

$$L^{\bullet} + O_2 \rightarrow LO_2^{\bullet}$$

3. Formation of lipid hydroperoxides (LOOH), through the rate-limiting reaction of peroxyl radicals and oxidizable lipids:

$$LO_2^{\bullet} + LH \rightarrow LOOH + L^{\bullet}$$

The stoichiometry of the last two reactions of the chain peroxidation is:

$$LH + O_2 \rightarrow LOOH$$

4. Termination of the chain peroxidation by the reaction of two peroxyl radical:

$$LO_2^{\bullet} + LO_2^{\bullet} \rightarrow NRP \text{ (non-radical products)}$$

LP can be induced by exogenous free radicals $X^{\bullet}$ (e.g. radical formation resulting from either thermal decomposition of organic substances generating free radicals or radiation). LP may also be induced by free radicals formed within the system (e.g. metal-catalyzed destruction of preformed hydroperoxides).

The rate constant of LP is given by Halliwell and Gutteridge (1999):

$$V=Kp[LH]\sqrt{\frac{Ri}{2Kt}}$$

Where:

$\sqrt{\frac{Ri}{2Kt}}$ refers to the equilibrium concentration of free radicals, which is the square root of the ratio between the speed of free radicals production (Ri) and speed constant of chain reaction termination (Kt).

Kp is the rate constant of the free-radical chain reaction propagation.

LH is the concentration of oxidizable lipids in the bilayers or in areas of the double lipid layer with oxidizable lipids when laterally separation of the phases is carried.

In a kinetically simple case, where Ri is constant (for example in an AAPH induced peroxidation), and the membrane is homogeneous, the dependence of the peroxidation of membrane properties is associated with the rate constants Kp and Kt. For a given composition, the both rate constants refer to bimolecular reactions and, therefore, peroxidation is expected to increase with an increase in the rate of lateral diffusion of the oxidizing lipids in the bilayers. This, in turn, leads to an increase in both the rate constant of propagation (that accelerates the overall peroxidation) and the rate constant of termination, which slows the rate of peroxidation. Therefore, even in this relatively simple case, it is difficult to predict the overall effect of the membrane packing (incl. fluidity, viscosity, other parameters and measurable factors) on the LP speed because both KP and KT change with the same magnitude, but have opposite effects on the rate of peroxidation.

Conclusion

Peroxidation in liposomes differs from peroxidation in biological membranes, but the results obtained in such model membranes may be useful for understanding problems that cannot be tested in natural membranes. Despite the relative simplicity of LP in model systems, the reactions taking place in them are complex, since they depend in a complex manner on the composition and physical properties of the liposomes, as well as of the properties of the inductor and the inhibitor (if present). The results obtained in this model can serve to predict the peroxidation in biological membranes. The in vitro study of LP inhibitors in liposomal model systems is only a preliminary step towards the clarification of their antioxidant properties.

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Lipid peroxidation in biological membranes is considered to be one of the major mechanisms of cell damage in aerobic organisms, exposed to oxidative stress. Free radicals, formed by various mechanisms, induce peroxidation that consequently alters arrangement of lipids and proteins in membranes. In turn these alterations change the physical properties and permeability of the membranes, which affect their function. The efforts of many scientists are dedicated to understand the factors that govern the processes of peroxidation, to clarify the membrane's composition and properties, as well as to reveal the features of peroxidation inducers. Biological membranes are complex structures and is very difficult to quantify the processes occur. This requires the use of simplified biological membrane models such as liposomes. Although the peroxidation in model systems differs from the peroxidation in biological membranes, the results obtained in model membranes may be useful for understanding the issues that cannot be tested in biological membranes. Despite the relative simplicity of lipid peroxidation in liposomes, the reactions occurring in them are complicated because they depend on the different interactions between the peroxidation inducer, the composition and physical properties of the liposomes. According to the literature data the liposomal lipid peroxidation mechanisms could be explain with their physicochemical properties. In particular they involve the following options: 1) the kinetic of peroxidation induced by "external" generators of free radicals is determined by the balance between the effects of membrane properties and the rate constant of propagation (Kp) and termination (Kt) of the free-radical peroxidation in the relevant membrane area where oxidizable lipids present; 2) the peroxidation, induced by transition metal ions, depends on additional factors, including binding of metal ions to the lipid-water surface and formation of metal ions-hydroperoxide complex; 3) the antioxidants are capable to increase or inhibit the lipid peroxidation, depending on the membrane composition and the properties of the peroxidation inducer.

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EO7. METALODENDRIMERS WITH BIOMEDICAL APPLICATIONS

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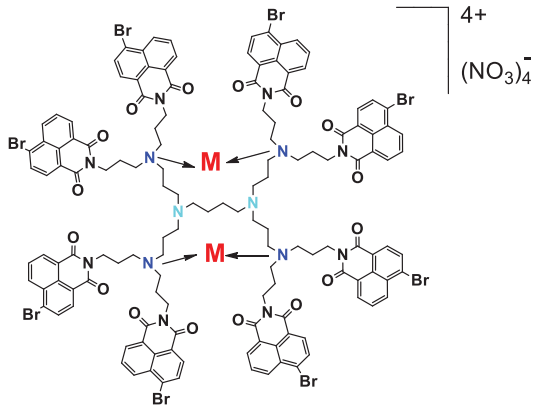
Over the past years dendrimers are of significant scientific interest due to their unique structure and special chemical and biological properties. They have a three-dimensional structure and reactive functional groups that allow their design with desired properties. One of the possibilities is to modify dendrimer periphery with fluorescent units which greatly expands their application in various scientific fields such as biology, medicine, environmental protection and in high technology as sensors, biosensors and photochemical molecule devices. In our laboratory, fluorescent dendrimers modified with 1,8-naphthalimide have been synthesized and investigated.



Possible complexation of metal ions with dendrimer D

In this study, the synthesis of a new poly(propylenamine) dendrimer of second generation containing eight 4-bromo-1,8-naphthalimide units in the dendrimer periphery and their Zn(II) and Cu(II) complexes was described. The photophysical characteristics of the dendrimer free of metal ions and the metal complexes in organic solvents of different polarity was investigated. An excimer fluorescence emission has been observed in DMSO and chloroform and it has been demonstrated than this emission has been retained after metal ion complexation. The chemical structure has been investigated by FT-IR and surface enhanced Raman spectroscopy (SERS) and basic vibrational characteristics of the dendrimer as ligand and metal complex are presented. *In vitro* antimicrobial screening of the newly synthesized metallodendrimers showed promising antibacterial activity against some pathogenic Gram-positive and Gram-negative bacteria and good antifungal activity against the yeast strains *S. cerevisiae* and pathogenic *C. lipolytica.* The results also indicate that the nature of the metal ion does not affect the microbiological activity of the complexes. Therefore, the new metal complexes could find application in designing new antimicrobial preparations to control the spread of infections caused mainly by *Acinetobacter*, *Micrococcus* and *Candida* species.

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