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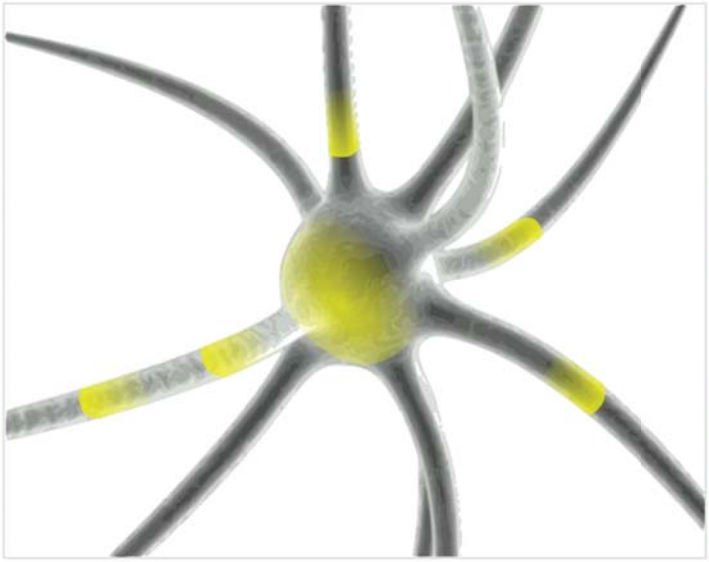
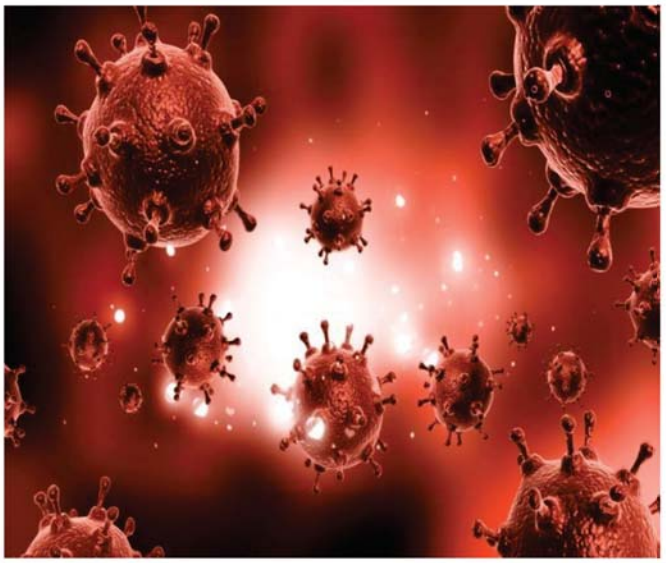
145 ГОДИНИ БЪЛГАРСКА АКАДЕМИЯ на НАУКИТЕ 1869-2014

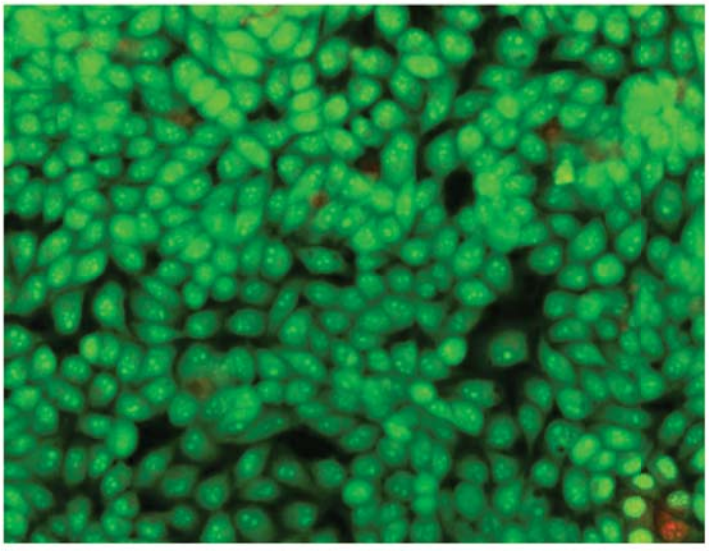
Institute of Experimental Morphology, Pathology and Anthropology with Museum

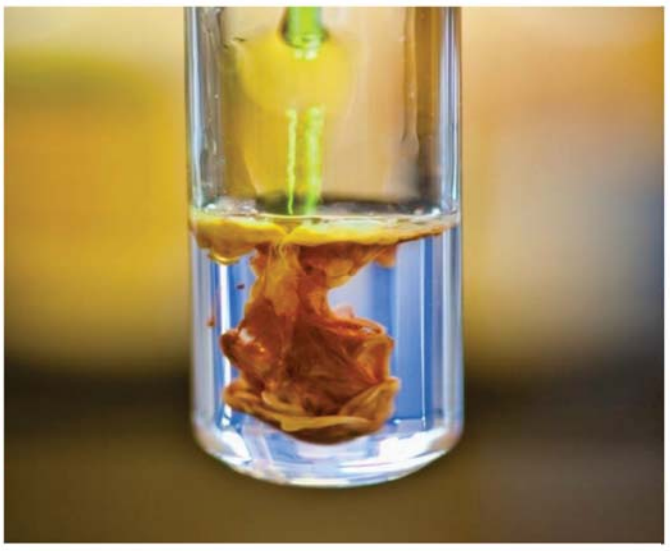
Европейски съюз

Proceedings of Vth Workshop on Experimental models and methods in biomedical research









April 7-9, 2014
Sofia, Bulgaria

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PROCEEDINGS

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

April 7 - 9, 2014

Institute of Experimental Morphology, Pathology and Anthropology with Museum

at the Bulgarian Academy of Sciences

Edited by: Dimitar Kadiysky and Radostina Alexandrova

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

THE FIFTH 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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STEFKA VALCHEVA-KUZMANOVA (FACULTY OF MEDICINE, MEDICAL UNIVERSITY - VARNA)

The responsibility for the content of published papers/abstracts belongs entirely to their authors

THE PROGRAM OF THE WORKSHOP

Monday, 7 April 2014

8.30 – 9.00 REGISTRATION
9.00 – 9.15 OPENING CEREMONY

Session A: Immunology

Chairpersons:
Prof. Reneta Toshkova, MD, PhD
Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences

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

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

9.15 – 9.30
AO1. GENERATION OF GENE-ENGINEERED CHIMERIC DNA MOLECULES FOR SPECIFIC THERAPY OF AUTOIMMUNE DISEASES
Vera Gesheva, Zsuzsanna Szekeres, Nikolina Mihaylova, Iliyana Dimitrova, Anna Erdei, Jozsef Prechl and Andrey Tchorbanov

9.30 – 9.45
AO2. SUPPRESSION OF DSDNA-SPECIFIC B LYMPHOCYTES REDUCES DISEASE SYMPTOMS IN SCID MODEL OF MOUSE LUPUS
Ivaylo Balabanov, Vera Gesheva, Nikola Kerekov, Kalina Nikolova, Nikolina Mihaylova, Todor Todorov, Maria Nikolova and Andrey Tchorbanov

9.45 – 10.00
AO3. FUNCTIONAL ELIMINATION OF AUTOREACTIVE T CELLS BY ANTIBODY THERAPY IN HUMANIZED SCID MODEL OF SYSTEMIC LUPUS ERYTHEMATOSUS
Petroslav Chipinski, Nikolina Mihaylova, Silvyia Bradyanova, Stela Chausheva, Ekaterina Todorova, Dobroslav Kyurkchiev, Fulvio D'Acquisto, Andrey Tchorbanov

10.00 – 10.15

AO4. FUNCTIONAL ELIMINATION OF AUTOREACTIVE T CELLS BY ANTIBODY THERAPY IN MRL/LPR MURINE MODEL OF SYSTEMIC LUPUS ERYTHEMATOSUS

Silviya Bradyanova, Nikolina Mihaylova, Stela Chausheva, Ekaterina Todorova, Dobroslav Kyurkchiev, Fulvio D'Acquisto, Andrey Tchorbanov

10.15 – 10.30

AO5. ЕПИГЕНЕТИКА ПРИ АВТОИМУННИТЕ ЗАБОЛЯВАНИЯ

Мария Младенова

10.30 – 10.50 Coffee Break

10.50 – 11.05

AO6. BUILT-IN ADJUVANTICITY OF GENETICALLY AND PROTEIN ENGINEERED CHIMERIC MOLECULES FOR TARGETING OF INFLUENZA A PEPTIDE EPITOPES

Desislava P. Hlebarska, Nikola S. Kerekov, Iva I. Ivanova, Nikolina M. Mihaylova, Maria Nikolova, Jozsef Prechl and Andrey I. Tchorbanov

11.05 – 11.20

AO7. INDUCTION OF IMMUNE RESPONSE TO A DNA VACCINE AGAINST HIV-1 IN A HUMANIZED MICE MODEL

Iliyan Manoylov, Maha Ghassan Moussa, Yahia Chebloune

11.20 – 11.35

AO8. МОНОКЛОНАЛНИТЕ АНТИТЕЛА – ПРОИЗВОДСТВО И ПРЕДИЗВИКАТЕЛСТВА ПРЕД СЪВРЕМЕННАТА МЕДИЦИНА

Кристияна Добрикова, Петя Генова-Калу, Даниела Пенчева

11.35 – 11.50

AO9. ЗА БЯГСТВОТО НА ТУМОРИТЕ ОТ ИМУННИЯ ОТГОВОР

Радостина Александрова

11.50 – 12.10

Poster Session

AP1. МОНОКЛОНАЛНИ АНТИТЕЛА В ДИАГНОСТИКАТА И ТЕРАПИЯТА НА РАКОВИТЕ ЗАБОЛЯВАНИЯ – УСПЕХИ И ПРЕДИЗВИКАТЕЛСТВА

Радостина Александрова, Никола Симеонов, Ивайло Данков, Симона Спасова, Георги Семовски, Шенол Чакър, Метин Мазгалджи

AP2. НАКРАТКО ЗА ТУМОРНИТЕ АНТИГЕНИ И ТУМОРНИТЕ МАРКЕРИ

Радостина Александрова, Ивайло Данков, Симона Спасова, Георги Семовски, Шенол Чакър, Никола Симеонов, Метин Мазгалджи

АР3. ПРИЛОЖЕНИЕ НА 3D ПРИНТЕРИТЕ В БИОЛОГИЯТА И МЕДИЦИНАТА
Георги Семовски, Пламена Димова, Емил Чиков

12.10 – 12.30
Session: FIRST STEPS

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

Session B.

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

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

Secretary: Metin Mazgaldzhi
Faculty of Medicine, Medical University-Sofia

13.30 – 13.45
BO1. TESTICULAR SERTOLI CELL FUNCTIONS. DIFFERENCES IN THE MODE OF PHAGOCYTOSIS OF BACTERIA BETWEEN MACROPHAGES AND SERTOLI CELLS
Elina Avramska, Soren Hayrabedyan

13.45 – 14.00
BO2. ИЗСЛЕДВАНЕ РОЛЯТА НА АНТИОКСИДАНТИ И БИОЛОГИЧНО АКТИВНИ СУБСТАНЦИИ (БАВ) ВЪРХУ СПЕРМАТОЗОИДИ ОТ БИЦИ СЛЕД КРИОКОНСЕРВАЦИЯ
Ирина Кирилова, Деница Даскалова, Десислава Градинарска, Кирил Лазов, Мария Иванова-Кичева

14.00 – 14.15

BO3. ОЦЕНКА НА ДЕЙСТВИЕТО НА SPIRULINA PLATENSIS ВЪРХУ РЕПРОДУКЦИЯТА НА СЕЛСКОСТОПАНСКИ ЖИВОТНИ

Десислава Абаджиева

14.15 – 14.30

BO4. EXPERIMENTAL MODEL FOR STREPTOZOTOCIN-INDUCED DIABETES MELLITUS NEONATALLY OR IN ADULTHOOD - COMPARATIVE STUDY ON MALE REPRODUCTION IN CONDITION OF HYPERGLYCAEMIA

Lakova E, Popovska S, Gencheva I, Donchev M, Krasteva G, Pavlova E, Dimova D, Atanassova N

14.30 – 14.50 Coffee Break

14.50 – 15.05

BO5. METHOD FOR DETERMINATION OF ESTROUS CYCLE IN RODENTS AND ITS APPLICATION FOR INVESTIGATION OF UTERINE ACTIVITY

S. Vylcheva, T. Georgiev, P. Hadzhibozheva, A. Atanasov, S. Yotov, A. Tolekova

15.05 – 15.20

BO6. COMPLEMENTARY MEDICINE IN GENERAL PRACTICE AND THE FAMILY DOCTORS, LITERATURE REVIEW

Staykova-Pirovska Y.

15.20 – 15.35

BO7. TOLL-LIKE РЕЦЕПТОРИ И ДИАБЕТ

Ваня Младенова

15.35 – 15.50

BO8. COMPARATIVE STUDY ON TWO DIFFERENT NUTRITIONAL MODELS FOR CREATING INSULIN RESISTANCE

Georgi Bogdanov, Nadka Boyadjieva

15.50 – 16.05

BO9. ЛЕЧЕНИЕ НА ТРУДНОЗАРАСТВАЩИ РАНИ

Метин Амид Мазгалджи, Владислав Стоянов, Драгомир Дарданов

16.05 – 16.20

BO10. OSTEOBLAST BEHAVIOR ON NANODIAMOND-MODIFIED THIN POLYMER FILMS

A. Ganeva, M. Keremidarska, E. Radeva, K. Elersic and N. Krasteva

16.20 – 16.35

BO11. ADHESION AND MORPHOLOGY OF MESENCHYMAL STEM CELLS CULTURED ON MODIFIED SILOXANE-BASED BIOMATERIALS

M. Keremidarska, A. Ganeva , E. Radeva, K. Elersic and N. Krasteva

16.35 – 16.50

BO12. POSSIBILITIES FOR APPLICATION OF ORAL MUCOSA EPITHELIUM IN LIMBAL STEM CELL DEFICIENCY

I. Valkova, I. Sainova, V. Pavlova, A. Georgieva, B. Alexieva, E. Nikolova

16.50 – 17.05

BO13. EFFECT OF GROWTH REGULATORS ON SOME PHYSIOLOGICAL CHARACTERISTICS OF *IN VITRO* PROPAGATED *ACHILLEA THRACICA* VELEN

Mariya Rogova, Nia Petrova

Tuesday, 8 April 2014

9.00 – 9.15

BO14. EXPERIMENTAL TUMOR MODELS DISPLAY DIFFERENT SUSCEPTIBILITY TO C-PHYCOCYANIN

Liliana Gigova, Sonia Apostolova, Liliya Yossifova, Ani Georgieva, Reneta Toshkova, Natalia Ivanova, Kaledona Minkova

9.15 – 9.30

BO15. BREAST CANCER

Nikola Simeonov

9.30 – 9.45

BO16. PROXIMITY EXTENSION ASSAYS (PEA)

Pavel Mitrenga, Gergana Taleva

BP1. ROLE OF GALECTINS AND CELL-SURFACE CARBOHYDRATES IN TUMOR CELL DISSEMINATION AND METASTASES

J. Stoyloff, S. Ivanov

BP2. FEW WORDS ABOUT MATRIX METALLOPROTEINASES AND THEIR TISSUE INHIBITORS

Radostina Alexandrova, Simona Spasova

Session C: Pharmacology and Toxicology

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

Stefka Valcheva-Kuzmanova, MD, PhD
Faculty of Medicine, Medical University - Varna

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

9.45 – 10.15
CO1. FATAL SUICIDAL POISONING DUE TO BORAX INGESTION – A CASE REPORT
Julia Radenkova - Saeva

10.15 – 10.30
CO2. EFFECTS OF CHLOROGENIC ACID ON EXPLORATORY BEHAVIOR AND LOCOMOTOR ACTIVITY IN RATS
S. Valcheva-Kuzmanova, A. Georgieva, S. Belcheva, R. Tashev

10.30 – 10.45
CO3. ANXIOLYTIC-LIKE EFFECT OF CHLOROGENIC ACID ADMINISTERED SUBCHRONICALLY TO RATS
S. Valcheva-Kuzmanova, A. Georgieva, S. Belcheva, R. Tashev

10.45 – 11.05 Coffee Break

11.05 – 11.20
CO4. CYTOTOXICYTY STUDIES AND FLUORESCENT IMMUNOLOCALISATION OF FUMONISIN B_1 ON DEC 99 CELL LINE
Katerina Todorova, Rositsa Milcheva, Simona Lazarova, Petar Dimitrov, Russy Russev, Rumen Dimitrov, Ani Georgieva and Ivan Ivanov

11.20 – 11.35
CO5. IN VITRO CYTOTOXICITY OF SILVER-MODIfiED NATURAL CLINOPTILOLITE
Z. Ivanova, B. Shivachev, L. Dimova, E. Shikova

11.35 – 11.50

CO6. XANTHATES (DITHIOCARBONATES): HEAVY METALS CHELATION PROPERTIES AND BIOLOGICAL EFFECTS

Tzveta Stoyanova, Viliana Todorova, Stanislav Yanev

11.50 – 12.05

CO7. *IN VITRO* ANALYSES OF ALVEOLAR SURFACTANT IN CLINICAL SAMPLES IN NORM AND PATHOLOGY

Maya Bangyozova, Albena Jordanova, Asya Tsanova, Y. Yamakova, Zdravko Lalchev

12.05 – 12.20

CO8. ANTIOXIDANT ACTIVITY IN BODY FLUIDS OF ATHLETES

Yasin Eroglu, Lubomir Petrov, Albena Alexandrova, Onder Daglioglu

12.20 – 12.35

CO9. RARE DISEASES AND ORPHAN DRUGS

Sava Todorov, Vera Kolyovska

12.35-12.50

CO10. MULTIDRUG RESISTANCE CELL LINES AS EXPERIMENTAL MODELS IN BIOMEDICAL RESEARCHES

Tanya Zhivkova, Lora Dyakova, Pavel Mitrenga and Radostina Alexandrova, Dana-Cristina Culita, Daniela Marinescu, Luminita Patron

12:50- 13:05

CO11. GLIOBLASTOMA MULTIFORME – В ТЪРСЕНЕ НА НОВИ ПОДХОДИ ЗА ЛЕЧЕНИЕ

Lora Dyakova, Tanya Zhivkova, Radostina Alexandrova, Dana-Cristina Culita, Gabriela Marinescu, Luminita Patron

CP1. P-GLYCOPROTEIN AND MULTIDRUG RESISTANCE

Radostina Alexandrova, Ivaylo Dankov, Metin Mazgaldzhi

13.05 – 13.30

Session: FIRST STEPS

Chairperson:

Assoc. Prof. Radostina Alexandrova, PhD

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

Session D: Microbiology and Virology

Chairpersons:
Prof. Elena Nikolova, MSc, PhD, DSc
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

14.00 – 14.30
DO1. MODELS OF HUMAN PAPILLOMAVIRUS-ASSOCIATED DISEASES
Evelina Shikova - Lekova

14.30 – 14.45
DO2. EFFECT OF COMBINED ADMINISTRATION OF PROTEASE INHIBITOR AND POLYPHENOLIC COMPLEX ON FUNCTIONS OF ALVEOLAR MACROPHAGES IN MICE, INFECTED WITH MINFLUENZA A VIRUS
S. Apostolova, L. Yossifova, E. Gardeva, R. Toshkova, J. Serkedjieva

14.45 – 15.00
DO3. DETECTION OF THE ERYTHROVIRUS B19 (EVB19) DNA IN STANDARD AND ALTERNATIVE DIAGNOSTIC SPECIMENS (SERUM SAMPLES AND DRIED BLOOD SPOTS, DBS)
St. Ivanova, A. Toshev, Z. Mihneva

15.00 – 15.20 Coffee Break

15.20 – 15.50
DO4. AVIAN RETROVIRUSES AND CANCER
Ani Georgieva, Anton Kril, Ivan Ivanov, Peter Hristov, Georgi Radoslavov

15.50 – 16.05
DO5. EFFECTS OF GROWTH TEMPERATURE ON THE MORPHOLOGY AND MOTILITY OF *ESCHERICHIA COLI* O157:H-
D. Borisova, V. Jordanova, Ts. Paunova-Krasteva

16.05 – 16.20

DO6. NEW STRATEGIES AND VACCINES FOR BORDETELLA PERTUSSIS PROTECTION

Mihail Mihailov, Tim Vladimirov, Nikolina Koleva, Elena Dragusheva

16.20 – 16.35

DO7. SIALIC ACID METABOLISM IN BACTERIA AND ITS RELATION TO PATHOGENICITY

Stefan Engibarov, Ignat Abrashev

16.35 – 16.50

DO8. BACTERIAL SIALIDASES – FEATURES, BIOLOGICAL ROLES AND PRACTICAL APPLICATIONS

Rumyana Eneva, Ignat Abrashev

16.50 – 17.30 Poster Session

DP1. ЧОВЕШКИ ПОЛИОМНИ ВИРУСИ ПРИ БЪБРЕЧНА ТРАНСПЛАНТАЦИЯ

Георги Тошев

DP2. БАКТЕРИОФАГИ – ПРИЛОЖЕНИЕ В СЪВРЕМЕННАТА МЕДИЦИНА

Галина Димитрова

DP3. ПОЛЕЗНИТЕ БАКТЕРИИ

Деяна Жекова, Тина Георгиева

Wednesday, 9 April 2014

Session E: Parasitology

Chairpersons:
Prof. Margarita Gabrashanska, DVM, PhD
Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences

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

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

9.00 – 9.30
EO1. THE ROLE OF THE PARASITE VARROA DESTRUCTOR AS VECTOR OF VIRUSES ON HONEY BEE APIS MELLIFERA
Delka Salkova

9.30 – 9.45
EO2. TRACE ELEMENTS IN BROILER CHICKENS INFECTED WITH *ASCARIDIA GALLI* AND TREATED WITH ZINC COMPOUNDS
M. Anissimova, M. Gabrashanska, St. Tepavitcharova, V. Ermakov

9.45 – 10.00
EO3. FASCIOLOSIS AND CARCINOGENESIS
N. T. Tsocheva-Gaytandzhieva, D. Salkova

10.00 – 10.15
EO4. OPISTHORCHOSIS, CLONORCHOSIS AND TUMOR GROWTH
N. T. Tsocheva-Gaytandzhieva, D. Salkova

10.15 – 10.30
EO5. *IN VITRO* METHOD FOR SOME SPECIES EIMERIA IN RABBITS
I. Vladov, M. Gabrashanska, M. Anisimova, D. Salkova

10.30 - 10.45
EO6. PARASITES ESCAPE THE HOST IMMUNE RESPONSE
Radostina Alexandrova

10.45 – 11.05 Coffee Break

11.05 – 11.30 Poster Session

EP1. КУЧЕШКА ТЕНИЯ
Шенол Чакър, Ардит Каси

EP2. TAENIA SAGINATA et TAENIA SOLIUM
Лилия Цветкашка, Катерина Петкова

EP3. РИБНА ТЕНИЯ И ОПИСТОРХИДИ
Светослав Славчев, Николай Спасов

EP4. BALANTIDIUM COLI
Евгения Ристовска, Стефния Йовинска

EP5. SOMETHING MORE ABOUT BALANTIDIUM COLI
Даниела Цветкова, Теодора Йорданова, Лина Абужамус

EP6. TRICHOMONAS VAGINALIS
Симона Спасова, Кристина Генкова

EP7. TRICHOMONAS VAGINALIS
Станислава Благоева, Даниела Иванова

EP8. TOXOPLASMA GONDII
Лина Абужамус, Теодора Йорданова, Даниела Цветкова, Мария Кръстева

EP9. TRICHINELLA SPIRALIS
Виктор Стойков, Йоанна Маламуси, Камелия Лазарова

11.30 - 12.30
Session: FIRST STEPS

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

ЦИРОЗА И НЕУСЕТНИЯТ ПРЕХОД КЪМ ТОЗИ ПЪТ БЕЗ ИЗХОД
Ирена Михайлова

РУСАЛКИТЕ: МИТ, РЕАЛНОСТ, КОНСПИРАЦИЯ
Бойка Андонова-Лилова

Session F: Neurobiology

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

Assoc. Prof. Lyubka Tancheva, MSc, PhD
Institute of Neurobiology, Bulgarian Academy of Sciences

Secretary: Desislav Dinev
Faculty of Biology, Sofia University "St. Kliment Ohridski"

13.30 – 13.45
FO1. INVESTIGATION OF ACTIVITY OF SOME GALANTAMINE PEPTIDES
Radoslav Klisurov, Dobrina Tsvetkova, Danka Obreshkova

13.45 – 14.00
FO2. EXPERIMENTAL RESEARCH ON PHARMACOLOGICAL AND TOXICOLOGICAL EFFECTS OF NEWLY SYNTHESIZED NEUROPEPTIDES WITH SHORT CHAINS
Stoeva S**.** L.Tancheva, A. Georgieva,T. Pajpanova, R.Kalfin

14.00 – 14.15
FO3. REVIEW OF PHARMACOLOGICAL ACTIVITY OF ELLAGIC ACID WITH FOCUS ON CENTRAL NEVOUS SYSTEM AND ANTIVIRAL EFECTS
Simona A. Aleksandrova, Lyubka P. Tancheva

14.15 – 14.35 Coffee Break

14.35 – 14.50
FO4. THE KETOGENIC DIET IN EPILEPSY-NEUROPHARMACOLOGY AND ANIMAL MODELS
E.Haritov, E. Angeleska, N. Boyadjieva

FP1. ANIMAL MODELS OF EPILEPSY AND IMPLICATIONS FOR DISEASE-MODIFYING EFFECT OF LEVETIRACETAM IN KAINAT MODEL OF EPILEPSY IN ADULT RATS
E. Haritov, E. Angeleska, N. Boyadjieva

FP2. COMPARATIVE STUDY ON NEONATAL EFFECTS OF LEVETIRACETAM, VALPROIC ACID AND DIAZEPAM ON BEHAVIORAL CHANGES AND BRAIN CYTOKINES IN NEONATAL KAINAT MODEL OF EPILEPSY
E. Haritov, E. Angeleska, N. Boyadjieva

15.15 – 15.30
FO5. INNOVATIVE METHODS IN NEUROBIOLOGY RESEARCH: CONNECTOMICS, BRAINBOW, OPTOGENETICS
S. Dimitrova, K. Dankov, M. Dimitrova, V. Goltsev

15.30 – 15.45
FO6. ALZHEIMER'S DISEASE – TREATMENT RESEARCH TRENDS
Stela Dragomanova, Marieta Georgieva, Simona Aleksandrova

15.45 – 16.00
FO7. ДА ИЗГОРИШ В СОБСТВЕНИЯ СИ ОГЪН - BURN OUT SYNDROME
Симона Спасова, Кристина Генкова и Мартин Григоров

16.00 – 16.15 Poster Session

FP3. БОЛЕСТ НА АЛЦХАЙМЕР
Ивайло Данаилов Данков

FP4. SPINA BIFIDA
Надежда Йорданова, Людмила Стоева

16.15 – 16.30 Closing Remarks

Session A. Immunology

Chairpersons:

Prof. Reneta Toshkova, MD, PhD
Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences

Assoc. Prof. Andrei Tchorbanov, MSc, PhD
Institute of MicrobiologyExperimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences

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

AO1. GENERATION OF GENE-ENGINEERED CHIMERIC DNA MOLECULES FOR SPECIFIC THERAPY OF AUTOIMMUNE DISEASES

Vera Gesheva[1], Zsuzsanna Szekeres[2], Nikolina Mihaylova[1], Iliyana Dimitrova[1], Anna Erdei[2†], Jozsef Prechl[2] and Andrey Tchorbanov[1]*

[1]*Institute of Microbiology, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria*
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Abstract

Systemic lupus erythemathosus (SLE) is an autoimmune disease characterized by B cell hyperactivity. Delivering of a self-epitopes to the auto-reactive B cells involved in the pathological immune response has a negative effect on their activation. The specific elimination of dsDNA - recognizing B cells is a reasonable approach for effective therapy of SLE.

We have previously constructed a protein chimeric molecule by conjugation of DNA-mimotope peptides to a monoclonal anti-mouse CD32 (FcrRIIb) antibody. Using this protein-engineered molecule for therapy of lupus-prone MRL/lpr mice we suppressed selectively autoreactive B-lymphocytes by cross-linking B cell surface immunoglobulins with the inhibitory IgG FcrRIIb receptors (citat EJI). In the present study we have created a chimeric gene-engineered DNA molecule, encoding a single-chain variable fragment (scFv) from a monoclonal antibody against FcγRIIb, coupled to dsDNA-like peptide as a B-epitope. Such a DNA construct inserted in the expression vector pNut was used as a naked DNA vaccine in a mouse model of lupus. The DNA construct is able to be expressed in eukaryotic cells and to cross-link cell surface receptors on DNA-specific B cells, delivering an inhibitory intracellular signal.

Groups of lupus-prone MRL/lpr mice were injected intramuscularly with plasmid DNA encoding the chimeric molecule. The administration of the recombinant DNA molecule prevented the increase of

IgG anti-DNA antibodies while in the control group they kept high levels. This result correlated with a low degree of proteinuria and preserved kidney histology in the chimera treated animals.
Keywords: Autoimmune therapy; Gene-engineered antibodies; Lupus;

AO2. SUPPRESSION OF DSDNA-SPECIFIC B LYMPHOCYTES REDUCES DISEASE SYMPTOMS IN SCID MODEL OF MOUSE LUPUS

Ivaylo Balabanov[1], Vera Gesheva[1], Nikola Kerekov[1], Kalina Nikolova[1], Nikolina Mihaylova[1], Todor Todorov[2], Maria Nikolova[3] and Andrey Tchorbanov*[1]

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Abstract

Self-specific B cells play a main role in the pathogenesis of lupus. This autoimmune disease is characterized by the generation of autoantibodies against self-antigens and the elimination of B and T cells involved in the pathological immune response is a logical approach for effective therapy. We have previously constructed a chimeric molecule by coupling a DNA-mimotope peptides to an anti-CD32 antibody. Using this protein molecule for treatment of lupus-prone MRL/lpr mice we suppressed selectively the autoreactive B-lymphocytes by cross-linking B cell receptors with the inhibitory FcγRIIb receptors. This approach was limited by the development of anti-chimeric antibodies in MRL mice. In order to avoid this problem, we established a murine SCID lupus model, allowing a long-term chimera therapy. Elimination of the double-stranded DNA-specific B cells by chimera therapy in MRL-transferred immunodeficient mice resulted in inhibition of T cell proliferation and prevented the appearance of IgG anti-DNA antibodies and of proteinuria.

AO3. FUNCTIONAL ELIMINATION OF AUTOREACTIVE T CELLS BY ANTIBODY THERAPY IN HUMANIZED SCID MODEL OF SYSTEMIC LUPUS ERYTHEMATOSUS

Petroslav Chipinski[1], Nikolina Mihaylova[1], Silvyia Bradyanova[1], Stela Chausheva[1], Ekaterina Todorova[2], Dobroslav Kyurkchiev[2], Fulvio D'Acquisto[3], Andrey Tchorbanov[1]

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Abstract

Systemic lupus erythematosus (SLE) is systemic autoimmune disease, characterized by the generation of autoantibodies specific for native (ds)DNA and nucleic acid–protein complexes, the formation of immune depositions, and inflammation in different organs and tissues. Current therapies are based mainly on immunosuppressive drugs such as corticosteroids and cyclophosphamide and are non-specific as well as coupled to side effects. A cure for the disease is not available so far and currently it is only possible to delay the development of lupus manifestations.

Annexin A1 (ANXA1) (37 kDa), known also as lipocortin 1, presumably acts as a second messenger of glucocorticoid pharmacological effects. Analysis of ANXA1 expression in T cells from patients suffering from rheumatoid arthritis showed higher levels of this protein compared to healthy control volunteers, providing clinical relevance to the role that ANXA1 might play in autoimmune diseases.

We hypothesized that it may be possible to down-regulate the activity of autoreactive T cells from SCID mice humanized with PBMC from lupus patients by treating them with a neutralizing monoclonal antibody against the ANXA1.

Therefore, *in vitro* and *in vivo* analyses of anti-ANXA1 antibody's effect were carried out. Data proven that both T and B cells express the protein. Furthermore, we observed reduction in the expression of the activation markers CD25 and CD69 on T lymphocytes incubated with the anti-ANXA1 antibody. Proteinuria levels of animals treated with the antibody showed lowered values during the middle of the course of treatment compared to controls treated with PBS only.

We hypothesized that annexin A1 plays a role in T cell activation and that blocking its activity by specific antibody could be used to reduce the number of autoreactive T cells, hence the manifestations of SLE in humanized SCID mice and eventually other autoimmune diseases that are related to T cells hyperactivity.

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2. Perretti, M., F. D'Acquisto. Annexin A1 and glucocorticoids as effectors of the resolution of inflammation. Nat. Rev. Immunol.. 2009, **9(1)**, 62-70. doi: 10.1038/nri2470.

AO4. FUNCTIONAL ELIMINATION OF AUTOREACTIVE T CELLS BY ANTIBODY THERAPY IN MRL/LPR MURINE MODEL OF SYSTEMIC LUPUS ERYTHEMATOSUS

Silviya Bradyanova[1], Nikolina Mihaylova[1], Stela Chausheva[1], Ekaterina Todorova[2], Dobroslav Kyurkchiev[2], Fulvio D'Acquisto[3], Andrey Tchorbanov[1]

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Abstract

Systemic lupus erythematosus (SLE) is the prototype systemic autoimmune disease, characterized by the generation of autoantibodies specific for native (ds)DNA and nucleic acid – protein complexes, the formation of immune depositions and inflammation in different organs and tissues. Annexin A1 (AnxA1) (37 kDa), was originally identified as a phospholipase A2 (PLA2)-inhibitory protein and second messenger of glucocorticoid pharmacological effects.

Analysis of AnxA1 expression in T cells from patients suffering from rheumatoid arthritis showed higher levels of this protein compared to healthy control volunteers, providing clinical relevance to the role that AnxA1 might play in autoimmune diseases.

We hypothesize that it may be possible to down-regulate the activity of autoreactive T and B cells from lupus-prone mice by treating them with a neutralizing monoclonal antibody against the AnxA1.

The immunomodulatory activity of the therapeutic antibodies was tested *in vivo* and *in vitro* at MRL/lpr model of lupus. The results show that the AnxA1 is expressed by both the B and T cells of the autoimmune mice. We have found a dose-dependent decrease in the expression of the activation markers CD25 and CD69 on splenocytes from MRL/lpr mice incubated in the presence of anti-AnxA1 antibody. The generated anti- Annexin A1 antibody has better therapeutic effect in the initial manifestations of the development of a lupus-like syndrome and retains significantly the levels of anti-dsDNA antibodies, secretion of IL10 and prevents the appearance of skin lesions in the lupus mice.

AO5. ЕПИГЕНЕТИКА ПРИ АВТОИМУННИТЕ ЗАБОЛЯВАНИЯ

Мария Младенова

Медицински факултет, СУ „Св. Климент Охридски"

AO6. BUILT-IN ADJUVANTICITY OF GENETICALLY AND PROTEIN ENGINEERED CHIMERIC MOLECULES FOR TARGETING OF INFLUENZA A PEPTIDE EPITOPES

Desislava P. Hlebarska[1], Nikola S. Kerekov[1], Iva I. Ivanova[1], Nikolina M. Mihaylova[1], Maria Nikolova[2], Jozsef Prechl[3] and Andrey I. Tchorbanov[1]

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Abstract

Highly purified, subunit, or synthetic viral antigens are known to be weakly immunogenic and potentate only the antibody, rather than cell-mediated immune responses. An alternative approach for inducing protective immunity with small viral peptides would be the direct targeting of viral epitopes to the immunocompetent cells by DNA vaccines encoding antibody fragments specific to activating cell surface co-receptor molecules.

Here, we are exploring as a new genetic vaccine, a DNA chimeric molecule encoding a T- and B-cell epitope-containing influenza A virus hemagglutinin peptide joined to sequences encoding an scFv antibody fragment specific for the costimulatory B cell complement receptors 1 and 2. This recombinant DNA molecule was inserted into eukaryotic expression vector and used as a naked DNA vaccine in WT and CR1/2 KO mice.

The intramuscular administration of the DNA construct resulted in the *in vivo* expression of an immunogenic chimeric protein, which cross-links cell surface receptors on influenza-specific B cells. The DNA vaccination was followed by prime-boosting with the protein-engineered replica of the DNA construct, thus delivering an activation intracellular signal. Immunization with an expression vector containing the described construct and boosting with the protein chimera induced a strong anti-influenza cytotoxic response, modulation of cytokine profile and a weak antibody response in Balb/c mice. The same immunization scheme did not result in generation of influenza-specific response in mice lacking the target receptor, underlining the molecular adjuvant effect of receptor targeting.

AO7. INDUCTION OF IMMUNE RESPONSE TO A DNA VACCINE AGAINST HIV-1 IN A HUMANIZED MICE MODEL

Iliyan Manoylov[1], Maha Ghassan Moussa[2], Yahia Chebloune[2]

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Immunodeficiency is that state of the organism in which its immune system can not protect it from disease causing agents and malignant cells. Acquired Immunodeficiency Syndrome (AIDS) is the most common immunodeficiency and is caused by infection with the Human Immunodeficiency Virus, type 1 (HIV-1). The infection leads to decline in the immune response – mainly caused by T cells depletion. The aim of our study was to test the effect on the immune response of the DNA vector CAL-SHIV-KU2-INT-.

In our study we designed the chimeric DNA plasmid CAL-SHIV-KU2-INT-, that is not capable to integrate itself into the cell genome. Next we evaluated the effect of the inoculation of the plasmid on cell cultures and in humanized mice. In order to do this we followed several parameters for a period of time – the levels of the CD4+ and CD8+ cells, the levels of IFNγ, the neutralizing activity of the mice serum antibodies.

The DNA vector CAL-SHIV-KU2-INT- is able to generate a measurable immune response in humanized and immunized mice. For the period of the study the immunization with the vector showed no negative effect on the studied mice.

The goals of our study were reached. We managed to generate a DNA chimeric plasmid, named CAL-SHIV-KU2-INT-. The inoculation with the plasmid had the expected effect on cell cultures. The immunization of humanized mice showed the positive effect of the plasmid on the activation of the immune system. In the future it would be interesting to see if the vector can induce the production of memory T and B cells, that are able to proliferate when recognizing the pathogen. It would, also, be positive to test its ability to protect the organism in a Macaque model.

AO8. МОНОКЛОНАЛНИТЕ АНТИТЕЛА – ПРОИЗВОДСТВО И ПРЕДИЗВИКАТЕЛСТВА ПРЕД СЪВРЕМЕННАТА МЕДИЦИНА

Кристияна Добрикова[1], Петя Генова-Калу[2], Даниела Пенчева[3]

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В последните години се наблюдава засилен интерес към моноклоналните антитела. В клиничната практика тези антитела намират приложение като терапевтични агенти. Няколко десетки моноклонални антитела се изпозват както срещу онкологични

заболявания, така и срещу автоимунни заболявания като артрит, астма, ревматизъм, множествена склероза, мускулен едем и др. Все повече моноклонални антитела се разработват или са в различни фази на клинично изпитание.

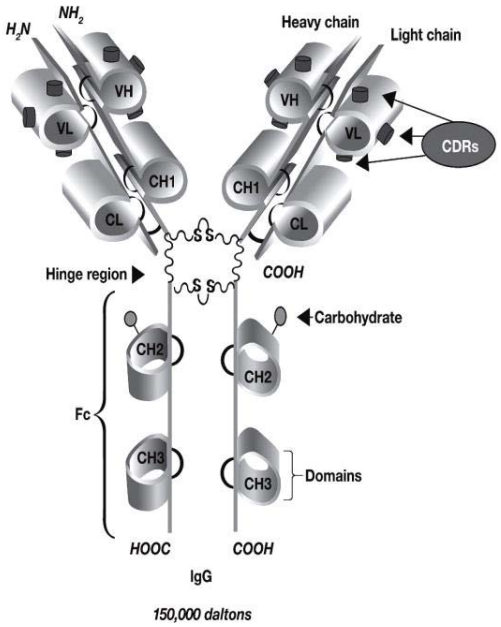
През 2005 г. тези терапевтични средства са донесли печалба на стойност 55 млрд, като през 2010 г сумата увеличава до 94 млрд долара. Приблизително 30 % от биотерапевтичните средства, използващи се в момента в клиничните проучвания , се заемат от антителата. Използването им в клиничната медицина се дължи на способността им да модулират естествения ход на болестта чрез въздействие върху критични патогенни молекули и способността им да стимулират имуно-медиирани ефекторни функции. Моноклоналните антитела стават възможни като терапевтични средства с развитието на хибридомната технология. Съществуват различни аналитични методи за характеризиране и пречистване на антителата. Сред тях широко застъпени са хроматографските методи - молекулно-ситова, афинитетна хроматография и електрофоретичните методи - главно SDS полиакриламидна електрофореза.

Моноклоналните антитела представляват нововъзникващ клас терапевтични средства, разработващи се в момента от много фармацевтични компании. През 2010 г. на световния пазар тези терапевтични лекарства носят печалба за 48 млрд. долара. Ежегодно от 2007 г. приблизително 40 нови моноклонални антитела са започнали клинични тестове [1].

В клиничната практика се използват няколко десетки моноклонални антитела в областта на онкологията и някои имуноалергични заболявания. Други антитела са в процес на разработване. Използването им в клиничната медицина се дължи на способността им да модулират естествения ход на заболяването чрез въздействие върху специфични патогенни антигени [2].

Моноклоналните антитела преставляват гликопротеини, които принадлежат към групата на имуноглобулините. Известни са пет класа имуноглобулини - IgG, IgA, IgM, IgE и IgD. От тях само IgG се синтезират за терапевтични цели с помощта на генно инженерни методи [1]. Молекулната маса на IgG е 150 000 D. Структурата е представена от две идентични леки вериги с молекулно тегло 23 000 D и две тежки вериги с молекулно маса 53 000 D. Всяка от леките вериги е свързана с тежката верига посредством нековалентни връзки и с един дисулфиден мост. Двете двойки вериги са свързани една с друга чрез дисулфидни мостове между тежките вериги. Молекулата наподобява буквата Y. Човешкият IgG се разделя на 4 подкласа в зависимост от различия в тежката верига - IgG1, IgG2, IgG3, IgG4 [3]. Всяка верига е изградена от структурни домени, които определят константни, вариабилни и хипервариабилни региони [1].

Във функционално отношение антителата съдържат два региона - Fc фрагмент, който се състои от тежките вериги и лесно кристализира, а другият - Fab фрагмент има антиген-свързваща активност и е съставен от леките вериги и N-края на тежките вериги [3].



Въз основа на механизма на действие съществуват три класа моноклонални антитела. Първият клас са директно действащи върху прицелния антиген. Тези моноклонални антитела блокират или стимулират специфични клетъчни мембранни молекули, като по този начин потискат туморния растеж или активират ефекторни клетки. Вторият клас моноклонални антитела са цитотоксични и имат способността да образуват имуноконюгати с различни цитотоксични молекули включително химиотерапевтици, радиоизотопи, някои клетъчни токсини, в това число дифтериен токсин, или биологични агенти като интерферони. Третият клас моноклонални антитела модулират имунният отговор на организма [2].

Моноклоналните антитела намират приложение като средство за лечение в онколкогията и при автоимуни заболявания като множествена склероза, ревматоиден артрит, ювенилен ревматоиден артрит, болест на Крон, астма, мускулен едем и деградация [2].

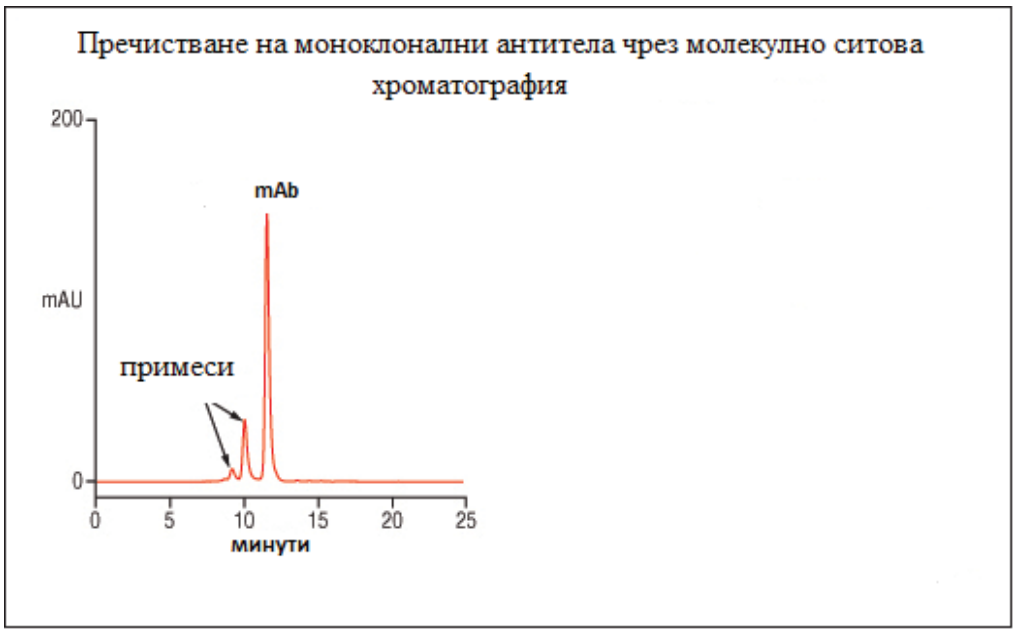
Моноклоналните антитела стават възможни като терапевтични средства с развитието на хибридомната технология от Кьолер и Милщайн през 1975 г. Този метод позволява създаването на специфични моноклонални антитела т.е антитела, които се синтезират само от един клон В-лимфоцити. Двамата учени доказват, че хибридизацията на соматични клетки може да бъде използвана за получаването на хибридобни клетки, които произвеждат моноклонални антитела. Тези клетки се получават, когато се слеят миеломни клетки и лимфоцити от слезка на имунизирано животно. В резултат на това се получава хибридомна клетка, която от В-лимфоцита е наследила способността да произвежда антитела, а от миеломната клетка - да се дели многократно. Миеломата е злокачественно заболяване, при което лимфоцитния клон започва безконтролно да се дели като синтезира един и същ имуноглобулин. Следователно по своята същност, миеломните имуноглобулини представляват моноклонални антитела.

Хибридомната техника включва два етапа. Първият етап представлява двукратно инжектиране на мишки с антиген през интервал от около месец. След няколко дни

имунизираните животни се убиват, като от слезката се изолират лимфоцити. Те се смесват с миеломни клетки, които са били култивирани *in vitro*. Двата изходни клетъчни типа заедно с формиралите се хибридни клетки се култивират на селективна среда, която позволява развитието само на хибридобните клетки. Те се изследват за секреция на желаното антитяло чрез имунологични тестове. Подбраните клетки се субкултивират *in vitro* като се използват специални методи за клониране, което да гарантира, че всяка от културите съдържа хибридомни клетки с моноклонални антитела. В този стадий хибридомните клетки могат да бъдат криоконсервирани. Във втората фаза на хибридомната техника, размножаването на клонирани хибридомни клетки може да се осъществи чрез подържане на растежа на клетките *in vitro* или чрез размножаването им *in vivo* във форма на асцитен тумор [4].

Задължително условие при производството на всяка една нова партида от моноклонални антитела е изследването им за съдържание на примеси и активност. Аналитично моноклоналните антитела се изследват за хетерогенност чрез SDS полиакриламидна електрофореза. За тяхното пречистване най-често се прилага афинитента хроматография. В редки случаи е наложително да се използват и други хроматографски методи, напр. молекулно-ситова хроматография, йоннообменна и др.

Молекулно-ситовата хроматография е метод, при който биомолекулите се разделят на базата на техния размер. Разделянето се основава на дифузия в порьозни гелове. Те съдържат пори, през които биомолекулите дифундират въз основа на различия в молекулните размери. Степента на задържане зависи от размера на разтворените молекули, отнесен към размера на порите. Малките молекули проникват в порите и се задържат в тях, докато по-големите молекули, поради невъзможността си да преминат през порите, проникват в стационарната фаза и се елуират първи [1].

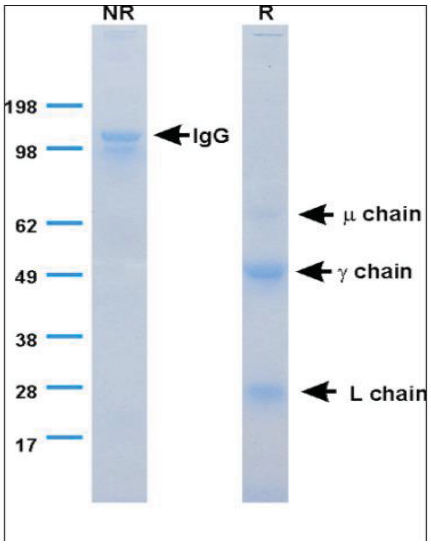


Афинитетната хроматография е метод, при който разделянето на биомолекулите се извършва на базата на обратимо взаимодействие на прицелната молекула с лиганда. При тази хроматография лигандната молекула, която има специфична разпознаваща способност, е свързана с хроматографската матрица. Този метод дава възможност да се извърши пречистване във основа на биологична функция. Молекулата, която трябва да се пречисти, се свързва с лиганда при преминаване на разтвора с изследваните молекули през хроматографската колона. След това прицелните молекули се елуират чрез използването на подходящи елуенти, включващи специфични разтворители, както и други лиганди. Елуирането може да се извърши с промяна на параметрите температура, pH, които водят до разкъсване на връзката между лиганда и прицелната молекула.

Описани са редица протеини, които специфично се свързват с IgG молекулата. От тях най-голямо значение има протеин А, който е локализиран на повърхността на клетъчната стена на грам-положителната бактерия *Staphylococcus aureus*. Този белтък е изграден от една полипептидна верига с молекулна маса 42 000 D и спада към биоспецифичните лиганди. Има способността при различните видове да се свързва с различен афинитен към Fc региона на IgG антителата. Специфичното взаимодействие между лиганда и белтъка се поддържа от водородни и хидрофобни връзки.

Към псевдоспецифичните лиганди се отнасят хистидин, триптофан, фенилаланин и други, които се използват за пречистване на разнообразни биомолекули. Представляват малки молекули, които са химически и физически по-стабилни. При пречистване на IgG моноклонални антитела, широко използван е хистидинът. Той се свързва с антитялото посредством водородни връзки, слаби хидрофобни и електростатични взаимодействия. Хидрофобните свойства на хистидина се дължат на имидазоловия пръстен. Различните физикохимични свойства са в резултат както на асиметричния въглероден атом. Тези свойства, в допълнение към ролята му в киселинно алкалната система, позволяват на хистидина да взаимодейства чрез различни механизми със съседни аминокиселини [5].

Стандартен метод, който се използва за определяне на еднородността или хетерогеността на моноклоналните антитела е полиакриламидната електрофореза. При този електрофоретичен медод под действие на електрично поле се осъществява движение на насочени заредени молекули. В структурно отношение много от протеините имат по няколко положителни и отрицателни групи. За да се осигури еднакъв старт на пробите е необходимо предварително да се натоварят с отрицателни заряди. Това се извършва от натриев додецил сулфат (SDS), който представлява анионен детергент. Следователно, разделянето на белтъчните проби се извършва на базата на молекулна маса, а не на електричен заряд. Заедно с пробите се нанасят и маркери, които представляват стандартни протеини с извества молекулна маса. При подаване на напрежение, молекулите ще мигрират през гела в зависимист от размера си, като големите молекули ще се придвижват по-трудно през порите на гела в сравниение с по-малките. След изключване на напрежението, разделените протеини в гела се визуализират като се използва специална боя. Приблизителната молекулна маса на изледваните протеини се изчислява като се сравнява дължината на миграция на стандартния белтък с пробата [6].



Моноклоналните антитела намират приложение в съвременната медицина като терапевтични средства както срещу онкологични заболявания, така и при заболявания като астма, множествена склероза, болест на Крон и др.

Астмата е хронично възпалително заболяване на дихателните пътища, характеризиращо с чести проблеми с дишането. Причините за заболяването е комбинацията между генетични фактори и факторите на околната среда. По данни на СЗО през 2011 г. 235-300 млн. души по целия свят са засегнати от астма. Стандартното лечение включва комбиниране на агонисти на бета-адреновите рецептори и стероиди. В последните години като лекарствено средство срещу астма все по-често се използват моноклонални антитела. Механизмът им на действие се състои в блокиране на цитокинов рецептор и последващите от това клетъчните взаимодействия. В момента се използват лекарствени средства базирани само на моноклоналното антитяло anti-IgE [7].

Моноклоналните антитела се използват и в борбата с различни видове тумори. Една част от тях се намират във фаза на клинични изпитания, докато други вече се одобрени за прилагане в клиничната практика.

Рецепторът на епидермалния растежен фактор (EGFR) има важна роля в растежа и разпространението на клетката. Нормално той се експресира във всички тъкани, но при настъпване на мутации в гена за рецептора, може да се стигне до неговото постоянно активиране. В следствие на това започва неконтролирано клетъчно делене, което води до развитие на различни типове тумори. Екстрацелуларният домен да растежния рецептор се свързва с TGFα и епидермалният растежен фактор, при което се активира интерцелуларният домен. Това от своя страна, довежда до активиране на различни сигнални пътища, които регулират клетъчния растеж. Моноклоналните антитела се свързват с EGFR, инхибирайки пролиферацията на клетките, синтезиращи TGFα – блокират един от най-важните пътища за подаване на сигнали, които участват в растежа на ракови клетки. Най-често използваните моноклонални антитела срещу различните видове тумори са *Erbitux® (cetuximab)*, *Rituximab*, *Panitumumab (ABX-EGF)*.

Най-успешното моноклонално антитяло е *Rituximab*. Представлява лекаствен продукт, който се използва за лечение на неходжкинов лимфом. Действа срещу антигена

CD20, който е локализиран на повърхносттта на B-лимфоцитите. *Cetuximab* представлява химерно антитяло IgG1, срещу лиганд-свързващото място на EGFR и
конкуретно потиска свързването на EGF, блокира свързването на ендогенни EGFR лиганди, като по този начин инхибира функцията на рецептора. Впоследствие cetuximab индуцира поглъщане на EGFR, което може да доведе до намаляване на рецепторната активност на EGFR. Cetuximab насочва и цитотоксичните имунни ефекторни клетки към EGFR-експресиращите туморни клетки Получава се чрез клониране на леките и тежките вериги на миши монолконално антитело M225 и съчетавайки ги с константните региони на човешка κ лека верига и γ1 тежка верига. Прилага се за лечение на пациенти с метастатичен колоректален карцином. Както при *in vitro*, така и при *in vivo* изследванията, cetuximab инхибира пролиферацията и предизвиква апоптоза на човешки туморни клетки, експресиращи EGFR. In vitro cetuximab потиска образуването на ангиогенни фактори от туморните клетки и блокира ендотелиалната клетъчна миграция. *Panitumumab* е рекомбинантно, изцяло човешко моноклонално IgG2 антитяло, произведено в клетъчна линия от бозайник чрез рекомбинантна ДНК технология. *Panitumumab* се свързва с висок афинитет и специфичност към човешките EGFR. Взаимодейства с лиганд-свързващия домейн на EGFR и инхибира рецепторното автофосфорилиране, индуцирано от всички познати EGFR лиганди. Прилага се при възрастни пациенти с метастатичен колоректален рак [8].

Моноклоналните антитела се прилагат като терапевтично средства и при автоимуните заболявания. Едно такова залобяване е множествената склероза, при което автореактивните Т-клетки преминават през кръвно-мозъчната бариера като атакува миелиновата обвивка на аксоните на главния и гръбначен мозък. Това причинява демиелинизация и образуването на лезии в мозъка. Множествената склероза вероятно е резултат от комбинацията на различни фактори: генетична предиспозиция, инфекциозни процеси и влияние на средата. *Natalizumab* е единственото одобрено моноклонално антитяло за лечение на множествена склероза. В ранен етап на заболяването B-лимфоцитите мигрират през кръвно-мозъчната бариера. Интегринът a4ß1, който е локализиран върху лимфоцитите се свързва с VCAM-1 на мозъка и гръбначните кръвоносни съдове. Това позволява преминаването им в централната нервна система. Механизмът на действие на Natalizumab се изразява в предотвратяване на това взаимодействие, в следствие на което лимфоцитите не могат да преминат в централната нервна система.

В клинично изпитание се намира Alemtuzumab. Причинява лизиране на лимфоцитите като се свързва към CD52, един силно експресиран, немодулиращ антиген, който се намира върху повърхността на практически всички B и T лимфоцитни клетки, както и върху моноцити и макрофаги. Лицензиран за лечение на хронична лимфоцитна левкемия. [9].

В последните години се наблюдава засилен интерес към моноклоналните антитела. В клиничната практика тези антитела намират приложение като терапевтични агенти Моноклоналните антитела представляват значителен напредък в клиничната медицина.

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АО9. ЗА БЯГСТВОТО НА ТУМОРИТЕ ОТ ИМУННИЯ ОТГОВОР

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АР1. МОНОКЛОНАЛНИ АНТИТЕЛА В ДИАГНОСТИКАТА И ТЕРАПИЯТА НА РАКОВИТЕ ЗАБОЛЯВАНИЯ – УСПЕХИ И ПРЕДИЗВИКАТЕЛСТВА

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АР2. НАКРАТКО ЗА ТУМОРНИТЕ АНТИГЕНИ И ТУМОРНИТЕ МАРКЕРИ

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АР3. ПРИЛОЖЕНИЕ НА 3D ПРИНТЕРИТЕ В БИОЛОГИЯТА И МЕДИЦИНАТА

Георги Семовски, Пламена Димова, Емил Чиков

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Session B.

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

Secretary: Metin Mazgaldzhi
Faculty of Medicine, Medical University-Sofia

BO1. TESTICULAR SERTOLI CELL FUNCTIONS. DIFFERENCES IN THE MODE OF PHAGOCYTOSIS OF BACTERIA BETWEEN MACROPHAGES AND SERTOLI CELLS

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Abstract

Mammalian spermatogenesis takes place in the luminal part of the seminiferous tubules. Sertoli cells are one of the two somatic cell types of seminiferous tubules, and together with germ cells, constitute the seminiferous epithelium. The seminiferous epithelium is surrounded by one or more layers of myoid cells: these somatic cells mark the outer limits of the seminiferous tubules [1]. Sertoli cells play a major role in spermatogenesis regulation and in altering the rates of spermatozoa produced. Sertoli cell functions include provision of structural support and nutrition to developing germ cells, phagocytosis of degenerating germ cells and residual bodies, release of spermatids at spermiation and pituitary hormone guided production of proteins that regulate the mitotic activity of spermatogonia.

Sertoli cells physically protect the seminiferous epithelium from the luminal space invasion and penetration of substances that may affect tissue and immune homeostasis by controlling by cell and fluid permeability controlling inter-Sertoli tight junctions. This mechanism is referred to as *blood-testis barrier*.

Sertoli cells possess the characteristics of immune cells; they express pattern recognition receptors, secrete antimicrobial proteins, and engulf dead or dying cells. The mechanism by which Sertoli cells engulf and kill bacteria has been compared to that of macrophages. Engulfed bacteria are left alive in Sertoli cells, while they were rapidly killed in macrophages, suggesting that Sertoli cells have means to eliminate bacteria that have invaded the seminiferous epithelium without evoking inflammation, unlike macrophages [2].

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ВО2. ИЗСЛЕДВАНЕ РОЛЯТА НА АНТИОКСИДАНТИ И БИОЛОГИЧНО АКТИВНИ СУБСТАНЦИИ (БАВ) ВЪРХУ СПЕРМАТОЗОИДИ ОТ БИВОЛСКИ БИЦИ СЛЕД КРИОКОНСЕРВАЦИЯ

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Резюме

Процесът на замразяване при ултраниски температури води до редица структурни и функционални нарушения в половите клетки. Предизвикват се увреждания на различни нива на плазмената мебраната (ПМ), цитозола и вътреклетъчните стуктури, което води до биологични промени в гаметите, свързани с понижаване на мотилитета, спромени в скоростните параметри и нарушаване интегритета на ДНК. Един негативен фактор на криоконсервацията е образуването на свободни радикали. Механизмите на крионарушенията на сперматозоидите след размразяване, които засягат мотилитета, тяхната жизнеспособност, целостта на плазмената и акрозомална мембрани и в последствие повлияват на фертилитета са многофакторни. В последните години голяма внимание се обръща на оксидативният стрес, които може да се окаже и основен фактор (Thomson et al., 2009). В нашите изследвания са направени проучвания върху ролята на антиоксиданти, върху структурното и функционално състояние на размразени сперматозоиди от биволски бици.

За целта е проучена ролята на ензимни и неензимни биологично активни вещества с антиоксидантно действие, при използване на два биотехнологични режима на замразяване – гранули и пайети. Анализът на резултатите показа, че след криоконсервация на семенна течност от биволи (гранули) достоверно по добри резултати се постигат при добавянето на кофеин към средата за размразяване (5 mg/ml) ($p<0.05$). При криоконсервирани сперматозоиди от биволи в пайети, добавянето на антиоксидантен микс (L-Glutathione, N-Acetyl Cysteine, vit. E, vit. C, Ca, Se и Zn) (mg/ml), към среда за размразяване, протектира в достоверно по висока степен скоростните параметри (VCL, VSL и VAP) и процента прогресивно подвижни сперматозоиди, в сравнение с контролите ($p<0.001$). Присъствието на естествена антиоксидантна защитна система като глутатион пероксидаза, супероксид дисмутаза, каталаза и др. при сперматозоидите (Partyka et al., 2012; F.J. Peña et al., 2003), не винаги може да компенсира негативната роля на свободните радикали. С настоящите изследвания се доказа, че допълнителното добавяне на БАВ с антиоксидантен ефект протектира плазмените мембраните на сперматозоидите чрез вероятно редуциране на липидната пероксидацияна ненаситените мастни киселини в ПМ, което предпазва сперматозоидите от бивол от морфологични и функционални нарушения. Резултатите са с практическа насоченост към използване на БАВ при криоконсервация на сперма от биволи.

Ключови думи: антиоксиданти, сперматозоиди, криоконсервация.

ВО3. ОЦЕНКА НА ДЕЙСТВИЕТО НА SPIRULINA PLATENSIS ВЪРХУ РЕПРОДУКЦИЯТА НА СЕЛСКОСТОПАНСКИ ЖИВОТНИ

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Резюме

Spirulina platensis е микроводорасло и потенциален фураж за много селскостопаски животински видове. Влиянието му върху развитието на животните произтича от хранителния й състав, богат на протеини, минерали, микро- и макроелементи. Основната цел на тази статия е да се направи преглед на констатациите до момента за използването на спирулина като допълнение на фуражите и нейното въздействие върху производителността на животните и здравето им. Резултатите от изследванията с водораслото отразяват положителния му ефект върху продуктивните и репродуктивните показатели на животните, в това число и на здравния статус. Въпреки това, настоящото равнище на познанията за отговора на животните към хранителната добавка *Spirulina platensis* е сравнително оскъдна и до голяма степен неизвестен.

Ключови думи: Spirulina platensis, селскостопански животни

EVALUATION ON INFLUENCE OF SPIRULINA PLATENSIS ON LIVESTOCK REPRODUCTION

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Abstract

Spirulina platensis is microalgae and potential food for many agriculture animals. His influence on the development of animals comes from its nutritional composition rich proteins, minerals, micro-and macronutrients. The purpose of this article is to review the findings to date on the use of spirulina as a supplement feed and its effect on animal health, performance and reproduction. Research results with algae showed its positive effect on productive and reproductive performance of animals, including health status. However, the present knowledge of the animal's response to nutrient *Spirulina platensis* is relatively scarce.

Key words: *Spirulina platensis, farm animals*

Въведение

В резултат на разширяване на пазара, особенно в развиващите се страни търсенето на продукти от животински произход се увеличава. Едновременно с това, се покачват и изискванията относно качеството на продукцията, като потребителският избор е насочен към безопасни за здравето храни [**7, 11**]. Това означава, че традиционната хормонална стимулация и добавянето на синтетични оцветители, антибиотици и др. в дажбата на животните, трябва да останат на заден план, тъй като излишъкът им се натрупва в месото и млякото. Поради тази причина идентифицирането на нови хранителни компоненти, е от решаващо значение за развитието на животновъдството. Новият ресурс трябва да има висока хранителна стойност, лесна асимилация, ефективно преобразуване, да съдържа витамини, микро- и макроелементи, растителни пигменти. Тези биологично активни вещества засягат редица физиологични процеси, като подпомагане детоксикацията на организма, повишаване на неспецифичната резистентност към неблагоприятни външни фактори, подобряване функцията на отделните органи и системи, в това число и репродуктивната. Натуралният продукт, отговарящ на горните критерии и способен да подсили функциите на селскостопанските животни е водораслото *Spirulina platensis* (СП). СП е влакнеста, спиралообразна цианобактерия, класифицирана като синьо-зелено водорасло. Хранителната стойност на СП се изразява в богат спектър на хранителни вещества, като незаменими аминокиселини, витамините B_{12}, вит. Е, вит. С, вит. Е, важните минерали К, Са, Cu, Mg, Mn, Zn, P, Se, Na и Fe. Освен около 90% белтъци, СП е богата още на въглехидрати, стероли. Състава й включва целия спектър от растителни пигменти, сред които ксантофили, β-каротини, зеаксантин, фитоцианиди. Също така е богат източник на мастни киселини, особено γ-линоленова киселина (GLA), която има ползи за здравето [**12**].

Днес производството на СП достига световни мащаби, като приблизително половината от добива й се използва в животновъдството. Добавката в комбинираните фуражи не влошава физикохимичния състав и качеството на месото, но увеличава относителния дял на месо в трупа, като намалява мазнините [**1**]. Водораслото подобрява функцията на кръвотворните органи, активира обмяната на веществата, което води до повишаване на броя на еритроцитите, съдържанието на хемоглобин, протеин, калций и фосфор, увеличава естествената устойчивост [**3**]. Освен горепосочените, СП има и антитерогенен ефект и се проявява в понижаване на общия холестерол, серумните триглицериди и липиди. Следователно, водораслото може да бъде полезно за предотвратяване на атеросклероза, намаляване на рисковите фактори за сърдечно-съдовови заболявания, за защита на клетките от липидна пероксидация и окислително увреждане на ДНК [**5, 6, 10**].

Основната цел на тази обзорна статия е да се направи преглед над констатираните до момента резултати след използване на добавката СП, за да се оцени нейното въздействие върху репродукцията на селскостопанските животни.

Ефекти върху репродукцията: Добавянето на СП в дажбата на бременни и кърмещи свине увеличава производителността на поколението, получено от тези майки. Нутриента води до повишаване теглото на новородените и до по-добро здравословно състояние. В периода на кърмене, добавка от 125 мг/ 1кг увеличава производството на мляко [**2**]. **Shimkiene et al**. (2010) откриват, че бременни овце, получаващи СП раждат по-тежки агнета (до 4 %), в сравнение с бременни овце от контролна група [**16**].

При нерези, влиянието на СП е установено върху количествените и качествените характеристики на сперма, като увеличава обема на еякулата, концентрацията на

сперматозоидите и преживяемостта им до 72 час [**14**]. Тези резултати съвпадат с получените от **Granaci** (2007) при изседване на сперма от бик [**9**].

Chainapong and Traichaiyaporn (2013) установяват, че СП като източник на каротини и провитамин А, има положителен ефект върху репродуктивните функции при женски сомове. Не е установена значителна разлика в качеството на хайвера между опитните и контролните риби, но въпреки това е наблюдавана тенденция към по-добри репродуктивни способности и здравен статус на животните, приемали добавката [**4**]. Добавена към пелети за сом, SP ускорява узряването на хайвера, повишава успеха на люпене и оцеляването на ларвите [**15**]. Добавката повишава плодовитостта на възрастни женски риби, които предоставят част от собствените метаболитни съединения към хайвера, а това води до подобрение в качеството и преживяемостта му [**8**].

Ефекта на биологично активните съставки в СП са били изпитвани *in vivo* при женски мишки преди овулация върху броя и качеството на ембриони. Доброто здравословно състояние на третираните животни рефлектира върху качеството и количеството на овулирали ооцити, в следствие от активираните метаболитни процеси. По-високата активност на цитохром С оксидаза в яйчниците на тези мишки затвърждават това предположение [**13**].

Заключение

СП е обещаващ нов ресурс в подкрепа на бъдещите нужди на животновъдството. Опити, използващи добавката СП към фуражните дажби на много селскостопански животински видове, вече са показали подобрения в репродуктивността, със запазване на добър здравен статус и качествена продукция. Въпреки това, литературните данни са ограничени и не представят последователна тенденция за полезността на хранителната добавка върху репродуктивността. Следователно, по-нататъшни изследвания с водораслото са необходими за да се изясни потенциалът му. Изследвания върху биологичните пътеки на действия на *Spirulina platensis* ще разширят познанията и бъдещото й приложения като безвредна добавка в животновъдството.

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BO4. EXPERIMENTAL MODEL FOR STREPTOZOTOCIN-INDUCED DIABETES MELLITUS NEONATALLY OR IN ADULTHOOD - COMPARATIVE STUDY ON MALE REPRODUCTION IN CONDITION OF HYPERGLYCAEMIA

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Abstract

Diabetes mellitus (DM) is one of the most common chronic diseases in nearly all countries. It has been associated with sexual dysfunction, both in males and in females. Diabetes is an established risk factor for sexual dysfunction in men, as a threefold increased risk of erectile dysfunction was documented in diabetic men, as compared with nondiabetic men [1, 2]. It has been found that DM effects on the metabolitic status of the testis, the expression of numerous spermatogenic genes and is associated with increased number s of sperm with nuclear DNA damage [3].Diabetes mellitus (DM) is in men of reproductive age. Reproductive dysfunction is a consequence of DM but the underlying mechanisms are poorly understood.

AIM: comparative evaluation of spermatogenesis in conditions of experimentally induced DM neonatally or in adulthood in relation to diabetes status.

Materials & Methods: DM – Single i.p. injection of streptozotocin (65 mg/kg) in adulthood (10 week-old rats) or neonatally on day 1 or 10 p.p. DM status confirmed by blood glucose > 15 mmol/l 2-3d after injection.

Testes sampled at 50d (neonatal treatments); 1 and 2 months after injection (adult treatment) are fixed in Bouin's fluid.

Germ cell development/Spermatogenesis was assessed by IHC for tACE – specific marker for spermatid elongation phase. Rabbit Poly Ab 1:500 (Santa Cruz); ABC-HRP technique.

Measurement of Plasma T levels by RIA.

Results

On d50 p.p. spermatogenesis completed; full GC complement present

d50DM/1d p.p – completion of Sd development and spermatogenesis; enlargement of ST lumen, shrinkage of ST in stage VIII.

d50DM/10d p.p – spermatogenesis is not completed, different degree of delay in Sd development (lack of Sd in stages IV-VIII or in early stages or total lost of Sd in all stages).

DM in adulthood – spermatogeneis grossly normal.

Conclusions

Neonatal testis is more affected by hyperglycaemia that adult testis.

Induction of DM on day 10 (1st proliferative wave started) affects GC development in a stronger extent compared to DM induced on day 1 (quiescent GC)

Neonatal testis/spermatogenesis is more vulnerable to DM at the time of proliferative phase of spermatogonia (d4.5-d12) than the time of their mitotic arrest/quiescent period before d4.5 p.p

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BO5. METHOD FOR DETERMINATION OF ESTROUS CYCLE IN RODENTS AND ITS APPLICATION FOR INVESTIGATION OF UTERINE ACTIVITY

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Abstract

The knowledge of the contractile patterns of the myometrium is a prerequisite for the understanding of physiological processes and the development of new therapeutic approaches to the reproductive disorders. Moreover, the uterine contractile activity plays an important role in the reproductive functions and might be a reason for common and important disorders such as infertility, implantation failure, dysmenorrhea, endometriosis, spontaneous miscarriage or preterm birth. Some studies revealed different behaviour of smooth muscle specimens from uterus, depending on the cycle phase.

In humans, the reproductive cycle, called the menstrual cycle, lasts approximately 28 days, while in rodents this cycle, called the estrous cycle, lasts approximately 4-5 days. This characteristic makes the rodents an ideal animal model for investigation of reproductive changes that occur during the estrous cycle. In mice and rats, the identification of the stage of estrous cycle is based on the proportion of cells types observed in the vaginal secretion: epithelial cells, cornified cells and leukocytes. By this method, the full estrous cycle can be divided into four stages: proestrus, estrus, metestrus, diestrus. As these stages are determinated from the hormonal balance of the organism – estrogen/progesteron ratio, this method could be useful for an indirect investigation of parameters, connected with the hormonal status.

In series of experiments with isolated rat uterine horns, the relation between the spontaneous and agonist-mediated uterine activity from one side, and the stage of the estrous cycle from the other, was found. This demonstrates the applicability of the method in a study of smooth muscle uterine contractions, as well as in the creation of various experimental models of reproductive disorders.

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BO6. COMPLEMENTARY MEDICINE IN GENERAL PRACTICE AND THE FAMILY DOCTORS, LITERATURE REVIEW

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Abstract

In the recent years, the alternative/complementary medicine is becoming more and more popular among the patients. Studies have shown that AM / CM is the fastest growing area in healthcare today. The family doctor is the first contact of the patient with the health system and should therefore be aware of the developments and the trends in the demand for health services from their patients concerning Complementary medicine.

Aim

The purpose of this report is to examine the awareness and availability of AM / CM by GPs in different countries and begin the research on this issue in Bulgaria.

Materials and Methods

Bibliographic study of scientific reports and materials from different countries where such studies have been carried out in recent years.

Results and Conclusions

During the study at first were found 7 million results for CM and Family medicine, scientific articles were much less, at a thorough search were prescreened 150 articles witch analyze CM in FM, 50 of them were articles in full text, in half of them the authors describe interest, offers, use of CM in General practice from perspective of family doctors, and other half perspective of patients. The subject on this article are GPs. After the materials were collected and analyzed, the conclusuion become clear that in many countries in Europe, America , Australia, etc. , GPs offer and discus various methods and treatments of AM / CM as a part of their constant care.

Keywords: GP, general practice, alternative medicine, complementary medicine, family medicine

Introduction

Family medicine or primary care is the gate keeper to the health system. The general practitioner (GP) is a specialist in general medicine in individual or group practice near my patients. He is their first contact with the health system and each first meeting, it must be well organized. GPs should be able to meet and predispose the patient, so that they can feel safe and relaxed with their doctor to be able to trust him and express their pain and suffering.

GP must have very good communication skills to deal with any type of temperament and personality characteristics GPs in their practice are facing with many different diseases caused by physical or psychological nature, heartaches, family problems and conflicts in which patients engage him, looking for advice, judgment for problem. Therefore GPs must have flexibility of mind and impartiality, but also empathy and most of all, he must continue seeking, learning and increasing their knowledge about their patients, morbidity, diseases, diagnosing process and treatment by all possible methods, which they acquired through participation in various specialized courses, seminars and workshops in order to optimize care for their patients.

Studies in recent decades have shown that, various alternative methods are increased their influence, the AM is the fastest growing area in healthcare today [2,8,9,10,11].

For Bulgaria alternative methods are licensed like unconventional methods of beneficial effects on individual health-Ordinance № 7 of the Ministry of Health in the Darjaven vestnik No. 22 from 2005 15mart. According to Art. 166, parag. 1 of 33 in unconventional methods are the following: use of non-pharmaceutical products of organic origin, use of non-pharmaceutical products of mineral origin, use of traditional physical methods, homeopathy, acupuncture and acupressure, iris, pulse and auricular research methods; dietetics and healing starving. When these non-conventional (alternative) methods complement traditional methods of diagnosis and treatment we are talking about complementary medicine (CAM). Using effectively wide range of methods from CAM, GP will be able to respond to the demand and needs of their patients, giving adequate and objective opinion without prejudices and subjectivity. GPs who are aware in CAM, can give advice to their patients about alternative medicine, and treat them by using additional set of proven effective treatments and diagnostics from CAM. Physicians who uses in their practice CAM becomes a holistic medical doctor with broad outlook and extensive knowledge in the management, healthy lifestyle, applying it and promoting it to their patients.

Aim

This study aims to analyze the level and the attitudes, knowledge and skills demand and supply of CAM, by GPs, using a bibliographic study of scientific articles and materials from different countries where such studies have been made already. For Bulgaria, they are no evidence for such studies, the performance of such analysis would be beneficial to understand what is the interest and applying of CAM in general practice on the territory of our country.

Methods and materials

The method used in this article is a bibliographic study of scientific publications and articles from different countries, in which have been made research about awareness, demand, supply and use of CAM by GPs.

Results

The literature review made to scientific material can be displayed in several major topics.

First we must consider what is the opinion of GPs towards CAM. A Canadian study showed that 56% of interviewed GPs believe that CAM has methods and techniques that can greatly help in conventional medicine [4, 7], another 54% work with alternative therapists, and the other group of GPs even apply some alternative technics in their practices [4]. Nearly 20% of GPs apply acupuncture, hypnosis or relaxation, and other 80% of GPs refer patients to therapists practicing AM showed study in Australia [9] U.S. results are similar [8, 12]. In Germany family doctors satisfy needs and expectations of their patients, 95% of GPs use a different form of CAM more often herbal medicine, homeopathy [2, 9]. The awareness and use of CAM by GPs in UK is widespread, even since 1995year, although their were applied only few techniques from CAM most often as acupuncture, chiropractic, hypnosis, herbal medicine, homeopathy, they are applied by a large group of GPs [5,7]. Awareness about CAM and its use by GPs in different countries varies from 16% in Canada and UK, 30% in New Zealand and 47% in Netherlands to 85% in Germany and 80% in Australia [9].

According to studies, the most commonly used alternative methods of treatment in general practice by GPs are: acupuncture, manual therapy, hypnosis, reflexology massage, meditation, and less homeopathy and herbal medicine (phytotherapy) for countries like England, America, Australia [4,5,8,9]. Germany GPs commonly used herbal medicine while homeopathy in Netherlands [9]. Another study involving a large number of European countries shows that the most commonly used alternative techniques in their General Practices are acupuncture, homeopathy, manual techniques, phytotherapy [11].

Does patients look for AM when they go to their GP? Studies show that more and more patients are turning to AM [2, 8, 9, 10, 11, 12, 15], 70% of patients in Germany says that they want their GP to apply and use more AM [7], while in U.S., despite the great interest in CAM from GPs, it appears that 70% of patients who have used AM doesn't tell their doctor about I it [9] which can be risky and led to side effects [15].

Is it conventional medicine effective enough? In article, Fisher P. and others investigated an interesting phenomenon an effectiveness gap (EG), according to them, this is an area of clinical practice in which available treatments are not completely effective. AM / CAM are not generally available through normal channels of health, therefore, if they are effective, we have the potential to further increase the efficiency achieved in health and community as a whole, so it's nice to be included and applied in conjunction with conventional medicine. The study says that EG is found in 68 of 78 cases studied. As diseases most often fall into this gap were musculoskeletal problems, depression, eczema, chronic pain and colon irritabile. From the collected data and the results, they conclude that a good response, and higher efficiency in treatment of above diseases, is by including various methods of AM [3].

Although there is interest in CAM, both by patients and GPs, there is a gap of efficiency caused by the mismatch of ability and willingness of GPs and patients needs about alternative methods when they go to GP [2].

There have been comparable research about what GPs, hospital specialists and students know of CAM, it turns out that GPs and specialists have similar levels of knowledge about the most common techniques of CAM, while students were with less knowledge, but they had much greater interestin it [6, 15]. GPs more often and earlier offered their patients unconventional therapies, compared with specialists [6].

Some studies have shown the interest of GPs to additional training in techniques of AM. GPs, who were not familiar enough with AM, found out that discussions with patients is difficult when it turn to AM, communication between patient and doctor, were not enough satisfactory [5, 6, 10]. In New Zealand 54% of GPs interested education and training in CAM, while in Israel was

88%, and 16% were already undergone such training. ¾ of Australian GPs interested in training at chiropractic, herbalism, naturopathy, and others [9]. A study in U.S. shows that AM have been teaching into medical schools and family practice since 1986 year [13] and in Europe, AM is widespread in many universities since 1997 [14].

Discussion

Literature review that have been made shows that in many countries in Europe, America, Australia, Canada and others, alternative methods are used in general practice. The majority of GPs are familiar with CAM and apply it at their practices. Patients are increasingly seeking unconventional methods of treatment, and in some case the GP failed to meet their expectations, in other cases patients did not report using AM to their doctor. As a final results using CAM should be expected improving diagnosis skills and knowledge at treatment in general practice, improving doctor-patient communication, regulating patient care, gradually shifting the focus from treatment to health promotion at the leading place.Therefore it is appropriate to undertake such an examination of Bulgaria, or at least a separate area of the country.

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BO7. TOLL-LIKE РЕЦЕПТОРИ И ДИАБЕТ

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Диабетът е заболяване, което е известно от древни времена, описван е най-вече като „болестта на жаждата". В днешно време е известно, че диабетът спада към към метаболитна група от заболявания. Тук са характерни няколко разновидности на диабета, но най-често са срещани тип 1 и тип 2.
Според Американското диабетно дружество диабетът има определени критерии за диагностициране и патологии, свързани със заболяването.
Главният симптом при захарния диабет е хипергликимията, която може да доведе до трайни увреждания на важни органи като сърцето и кръвоносната система, очите, бъбреците, нервите и други. Характерни за заболяването симптоми са – полифагия, полидипсия и полиурия. Двата най-разпространени типа на заболяването сред населението биват тип 1 или инсулин-зависим диабет и тип 2 или инсулин-независим диабет.
Диабет тип 1 се характеризира с разрушаване на бета-клетките, което от своя страна води до абсолютна инсулинова недостатъчност. Този тип се наблюдава предимно в юношестка възраст, но има и случаи на проява в зряла възраст. Този тип диабет е в резултат на клетъчно свързано автоимуно разрушаване на бета-клетките в панкреаса. Тази деструкция може да достигне до 90%. Тези нарушения изискват животоспасяващо лечение с инсулин и специална диета, която болният трябва да спазва [1]. Това автоимунно разрушаване на бета-клетките се предполага, че е в резултат на вирусни инфекции, активиране на heat shock протеини, невромедиатори и други, но каквито и да са причините при всички случаи има генетична основа.
Диабет тип 2 се описва още като инсулин-независим. Той се характеризира с инсулинова резистентност на периферните тъкани. При него поради инсулинова резистентност не се усвоява пълноценно глюкозата от кръвта, в резултат на което нивата на глюкоза и инсулин в кръвта остават високи продължително време. Тези високи нива на глюкоза са токсични за бета клетките от една страна, а от друга страна високите нива на инсулин в кръвта са сигнал за бета клетките да намалеят или спрат производството на инсулин. Това води до постепенно отмиране на бета клетките, което може да наложи лечение с инсулин при

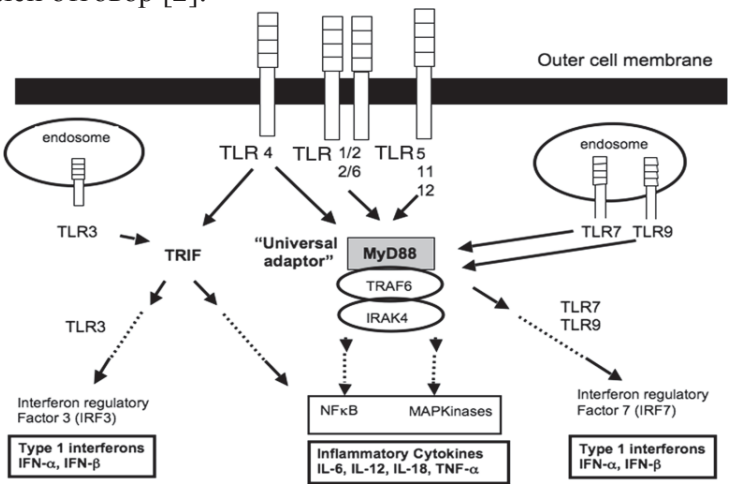
някои пациенти, но не винаги се налага лечение с инсулин, за да може да оцелее индивида. При тази форма на диабет също се наблюдава разрушение на бета-клетките, но не в такъв мащаб като при тип 1. Повечето пациенти с диабет тип 2 имат наднормено тегло и това е една от причините за инсулинова резистентност, но има и пациенти с нормално тегло които също страдат от заболяването. При тях има друг фактор на високо съдържание на телесни мазнини.

Тази форма на диабет е доста често срещана (до 95%). Често остава не диагностицирана поради не добре изразени симптоми и много от пациентите не разбират, че са болни. С напредване на възрастта симптомите стават по-ясно изразени или както ги наричат класически симптоми при диабет - силна жажда, намаляване на теглото, често уриниране и съпътстващите ги ретинопатии, бъбречна недостатъчност и други. Генетичното предразположение тук е много по-голямо от колко при първата автоимунна форма на диабет тип 1.

Диабетът има множество подформи, които се основават на генетично ниво. До момента установените аномалии съществуват в шест генетични локуси на различни хромозоми, като най-често откриваната е в хромозома 12 [1].

Toll-like рецепторите (TLR) са част от семейство рецептори, които участват в естествения имунен отговор. При животинския модел са открити около 13, а при човека - 11 рецептора. За първи път рецепторите са открити при Drosophila и там е установено, че те участват в неспецифичния имунен отговор. Човешкият TLR4 е трансмембранен протеин с екстацелуларен домен, притежаващ богати на левцин повтори и интраселуларен домен, хомоложен на рецептора за IL-1 [13].

Според някои автори [14] Toll-like рецепторите наред с други рецептори участват в първични имунни отговори на вродения имунитет при наличие на инфекции. Смята се, че сигналът подаван от Toll-like рецепторите активира NF-κB в ядрото. Повечето Toll-like рецептори използват във входа на действие адапторни протеини. Най-често те са MyD88 (миелоидна диференциация на първичния отговор 88). Този адаптор се води за един от главните, които свързват вътре клетъчната област на TLR, за да може да има бърз противовъзпалителен отговор [2].



Фигура 1. Сигнални пътища на Toll-подобни рецептори (TLRs), илюстрирани заедно с адаптерните протеини и сигнални пътища. (Информация, адаптирана от Akira et al. [4].

След множество проучвания в областта на диабета и Toll-like рецепторите е установено, че диабет тип 2 може да доведе до изменения в естествения имунен отговор [5, 6, 7]. Активирането на протеин киназата или инхибирането на В-киназата са генетично контролирани пътища и в следствие на това ядреният фактор NF-κB освен, че е отговорен за възпалителните пътища, той отговаря и за инсулина и глюкозния метаболизъм [8, 9]. Един от рецепторите, който играе много важна роля в естественият имунитет и имунния отговор е TLR4, тъй като участва във вродения имунен отговор и задейства механизмите за последващи противовъзпалителни процеси. TLR4 си взаимодейства с липополизахариди и други ендогенни лиганди, чиито нива при диабет са завишени [10, 11, 12]. Въз основа на проучените до сега изследвания е установено, че диабетът е метаболитно заболяване с много съпътстващи заболявания, които са свързани с имунната система и с вродения имунен отговор. Установено е че Toll-like рецепторите играят важна роля в естествения имунен отговор и следващите противовъзпалителни процеси. От това следва, че диабетът и Toll-like рецепторите са свързани с естествения отговор на имуната система за справянето с дадена инфекция. Toll-like рецептори са свързани с много заболявания. Те са едни от първите участници, които откриват и обезвреждат чуждите агенти, тъй като са важна част от вродения имунен отговор. С тяхното откриване са се дали отговори за детекцията и обезвреждането на микробални инфекции и противовъзпалителните процеси. Диабетът е заболяване, което има много голямо значение за населението, защото с диагностицирането му индивида трябва да промени начина си на живот и да живее с другите заболявания, които съпътстват диабета.

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BO8. COMPARATIVE STUDY ON TWO DIFFERENT NUTRITIONAL MODELS FOR CREATING INSULIN RESISTANCE

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Abstract

Insulin resistance (IR) is a widely spread physiological condition characterized by impaired answer of the body's cells to the action of insulin. The beta cells of the pancreas produce insulin but the cells of the body are resistant to its activity leading to increased level of glucose in the plasma. Consequently, the pancreas secretes even more insulin leading to hyperinsulinemia. Finally, IR might lead to the development of type II diabetes.

Different nutritional factors might lead to IR. We investigated the potential of refined palm oil (RPO) or fructose (F) to cause such impaired answer in adult female Wistar rats. In one of the models for creating IR we used RPO which was applied *per os* via gastric tube for six weeks. In the other model we applied *ad libitum* fructose solutions in different concentrations, at the beginning 15% for four months, and then 60% for three weeks. For assessing IR we performed the oral glucose tolerance test (OGTT).

The results demonstrated a clear diabetic activity of the RPO diet. On the other hand, the two different fructose diets did not alter the OGTT and showed no diabetic activity.

As a conclusion, a diet rich in refined palm oil might be used to create a model of insulin resistance and its pharmacological treatment can be further investigated.

BO9. ЛЕЧЕНИЕ НА ТРУДНОЗАРАСТВАЩИ РАНИ

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BO10. OSTEOBLAST BEHAVIOR ON NANODIAMOND-MODIFIED THIN POLYMER FILMS

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Abstract

As with all materials implanted into the body, polymers for bone regeneration must be biocompatible [1]. They should support cellular adhesion and growth, maintain cellular differentiation and functions. It is also crucial that these materials have mechanical properties similar to the native bone [2]. Several approaches, including addition of nanoparticles, have been investigated to improve mechanical properties of polymer materials. Recently, nanodiamond particles have received great interest because it is well known that nanodiamond is the hardness material in the nature as well as nanodiamonds possess lower cytotoxity compared to other carbon-based nanomaterials [3]. Therefore, in our work we have used nanodiamond particles to modify a polymer in order to study if it is possible while designing the bulk material characteristic with diamond particles to provide mechanical stability necessary for continued tissue used and to force specific cell adhesion and to guide adherent cell along a path of tissue regeneration [4].

We have used two methods of plasma polymerization (PP) to modify an organosilane with detonation nanodiamond (DND) particles. In the first one the monomer (hexamethyldisiloxane) has been mixed with DND particles immediately before PP, while in the second one - the monomer and the DND are deposited subsequently on a cover glass. Then, we have studied the effect of DND particles on surface characteristics of obtained thin composite films and cell adhesion, proliferation and differentiation of osteoblast-like cells cultured on such

prepared materials. The results showed that the composite HMDS-DND material, prepared by subsequent deposition of monomer and DND particles is more hydrophilic, with rougher surface and the osteoblast cells attached, grown and differentiated better in comparison with non-modified PPHMDS and the composite material, prepared by mix of monomer and DND particles.
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BO11. ADHESION AND MORPHOLOGY OF MESENCHYMAL STEM CELLS CULTURED ON MODIFIED SILOXANE-BASED BIOMATERIALS

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Abstract

Cell adhesion is a complicated, time-dependent process involving adsorption of proteins from the medium, attachment and spreading of cells and changes in cell morphology. It is widely known that physical and chemical characteristics of the materials clearly influence a cell's ability to attach and spread [1]. By modulating surface properties of a biomaterial we can regulate cell adhesion and thus can design biomaterials with im proved cell-contact properties.
In this work we evaluated the adhesion and morphology of rat mesenchymal stem cells on plasma polymerized hexamethyldisiloxane films (PPHMDS) modified additionally in amonia plasma and with nanodiamond particles. We chose plasma polymerization to create amine-rich and nanodiamond-rich thin film coatings because this technique allows deposition of well-adhering

polymer films on practically any type of substrate without the need for surface pre-modification which offers significant benefits in terms of feasibility, time and costs [2]. Different details about cell morphology was studied by different microscopic techniques. Cell adhesion was assessed by determining the number of attached cells and cell spreading area. Additionally, the effect of fibronectin (FN) was evaluated.

Phase-contrast pictures showed that on plain films rMSC had stelate-like, round or multipolar morphology. The best cell attachment was on PPHMDS films and lowest number attached cells-on DND films. However, the biggest average surface area was on DND while on PPHMDS it was much smaller. The pre-coating with FN improved cell attachment only on NH3-modified films and the cell spreading on PPHMDS. SEM pictures showed that rat mesenchymal stem cells had relatively large, multipolar shape with big, prominent nuclei and cytoplasmic extensions. Actin immunofluorescence demonstrated well developed stress fibers on FN-coated materials while on the plain ones the actin was found at the cell periphery in the site of focal adhesion contacts.

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BO12. POSSIBILITIES FOR APPLICATION OF ORAL MUCOSA EPITHELIUM IN LIMBAL STEM CELL DEFICIENCY

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Abstract

Possibilities for application of oral mucosa epithelium for development of novel therapeutic strategies in ocular limbal stem cell deficiency were examined. For this goal, techniques for substrate adhesion of the isolated cells and tissue explants from human oral mucosa were tested. All cells were characterized on the basis of their morphological characteristics: shape, presence or appearance of mitotic figures, as well as confluence and adherence ofn the substrate used. Formation of adherent and non-adherent cell sheets, consisting of cells with variable morphology and in different maturation degree, was observed. Future experiments in this direction are necessary, which should be connected mainly with cultivation of oral mucosa tissue explants and epithelial cells, on a bio-membrane in its role of appropriate biological substrate, about eventual

possibilities for future applications in construction of implants from “cell-membrane” type for the needs of reparative ophthalmology.

Key words: oral mucosa epithelium stem, *in vitro*-cultivation, limbal stem cell deficiency.

BO13. EFFECT OF GROWTH REGULATORS ON SOME PHYSIOLOGICAL CHARACTERISTICS OF *IN VITRO* PROPAGATED *ACHILLEA THRACICA* VELEN.

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Abstract

Plant growth regulators (PGR) are used for regulation of the physiological and biochemical processes in plants by controlling the primary and secondary metabolism [1, 2, 3]. The development of organs or structures and production of different compounds of interest in plants can be influenced by application of PGR.

The explants of *Achillea thracica* were cultivated *in vitro* at irradiance 60 $\mu mol.m^{-2}.s^{-1}$ at the top of the plants, 16 h day photoperiod, 25°C temperature and 60-70 % relative humidity. The effect of different concentration of 6-benzyl-adenine (BA) and indole-3-butyric acid (IBA) on some physiological parameters of *in vitro* propagated plants was studied.

After four weeks of cultivation the following parameters were estimated: stem length, number of stems produced from one explant, callus formation and rooting, fresh weight (FW). The amount of plastid pigments [4], total content of phenols (TP) [5] and flavonoids (TF) [6], and total antioxidant activity (TAA) [7] of the plants were measured.

Following results were obtained. By increasing the concentrations of BA the number and length of root decreased, the number of stems and callus formation were increased. Vitrification (water hyperacumulation) was not observed. The variants with maximum number of stems compared to the control were 0.6 and 1 $mg.l^{-1}$

IBA did not stimulate the formation of a large number of stems, but stimulated the root and callus formation. The addition of BA decreased the amount of plastid pigments especially at concentration 0.2 $mg.l^{-1}$. Indole-3-butyric acid - 1$mg.l^{-1}$, demonstrated a slight increasing of the amounts of plastid pigments compared to the control. IBA variants demonstrated negative effect on TP, TF and TAA. BA, however, influences the TP in positive direction in variants with 0.3, 0.5, 0.6 and 1 $mg.l^{-1}$ compared to the control. This cytokinin reduces the TAA at concentrations of 0.8-1 $mg.l^{-1}$.

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BO14. EXPERIMENTAL TUMOR MODELS DISPLAY DIFFERENT SUSCEPTIBILITY TO C-PHYCOCYANIN

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Abstract

Cells of myeloid *Graffi* tumor in hamsters and ascites tumor of *Guerin* in rats were grown in the presence of pure C-phycocyanin (C-PC) and their susceptibility to the treatment was compared. The changes in tumor cell proliferation, DNA integrity and activities of the main antioxidant enzymes were evaluated as markers for *in vitro* antitumor activity of C-PC. In the myeloid *Graffi* tumor in hamsters' model, C-PC significantly inhibited the growth of tumor cells and induced a DNA fragmentation, coupled with an increase in the cellular manganese superoxide dismutase and the glutathione reductase activities. In contrast to the promising activity against the cells from solid tumor, C-PC had no effect on the cells of ascites tumor of *Guerin*. This study showed a difference in the sensitivity of the cells from both types of tumors to the treatment and highlighted the meaning of the selection of experimental models when looking for new anticancer agents.

Introduction

C-phycocyanin is an accessory light-harvesting pigment in cyanoprokaryotes and two algal phyla (Rhodophyta and Cryptophyta). This water soluble and non-toxic biliprotein has many valuable pharmacological properties, such as anti-inflammatory, fibrinolytic [8], antidiabetic [16], anti-oxidant and free radical scavenging abilities [4], as well as antibacterial [18], antifungal, antiviral

[14] and anticancer [1, 10, 17] activities. Remarkably, the examined differently-sourced phycocyanins vary in their potential and mechanisms of action, which could be related to the differences in their structure, molecular weight and the biological characteristics of the used experimental models. Recently, it was shown that the purified C-PC, isolated from *Arthronema africanum*, possesses a promising *in vivo* antitumor activity in *Graffi* myeloid tumor in hamsters' model [9]. The aim of the present study was to evaluate and compare the *in vitro* effects of *A. africanum* C-PC on the cells derived from a solid and ascites tumors.

Materials and Methods

Isolation and purification of C-phycocyanin

Pure C-phycocyanin from *A. africanum* strain Lukavský 1980/01 was obtained by a modified rivanol-sulfate method of Minkova et al. [12].

Experimental models and isolation of tumor cells

Experimental myeloid *Graffi* tumor in hamsters was established and maintained according to Toshkova et al. [19]. Primary *Graffi* tumor cells were isolated from the tumor tissue under aseptic conditions and cultured in RPMI-1640 medium (Gibco BRL, Grand Island, NY, USA), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco BRL), 2 mM L-glutamine, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (Lonza, Basel, Switzerland). Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37°C.

Ascites tumor of *Guerin* was developed in the peritoneal cavity of Wistar white rats after intraperitoneal injection of 1×10^5 tumor cells. 7-8 days later, the resulting ascites fluid from the peritoneal cavity contained approximately 2×10^5 mL^{-1} viable tumor cells**.**

Ethical aspects

The animal tests were conducted in accordance with the principles for laboratory animal use and care as found in European Community guidelines.

Cell Proliferation Assay

The effect of C-PC on the viability of primary *Graffi* and *Guerin* tumor cells was assessed colorimetrically by the MTT assay as referred by Mosmann [13]. The antineoplastic drug Doxorubicin hydrochloride (DOX, Sigma-Aldrich) was used as a positive control. Cells, cultured only in the medium were used as negative controls.

Agarose gel analysis of DNA fragmentation

Graffi and *Guerin* tumor cells were treated with 50 and 100 μg mL^{-1} C-PC for 24 h. Treated and untreated tumor cells were lysed in 10 mM Tris (pH 8), 20 mM EDTA, 200 mM NaCl, 0.2% Triton X-100, and 100 μg mL^{-1} Proteinase K (Sigma-Aldrich, St. Louis, MO, USA). DNA was precipitated with isopropanol (1:1, v/v) and treated with 250 μg mL^{-1} RNase A (Sigma-Aldrich, St. Louis, MO, USA). DNA fragments (10 μg of total DNA) were separated by electrophoresis in 1.2% agarose gel at 80 V for 90 min in TBE buffer and visualized using ethidium bromide (1 μg mL^{-1}) staining and a UV transilluminator.

Preparation of tumor cell extracts and in-gel enzyme activity staining

Whole-cell extracts were prepared following Bravard et al. [6]. Protein concentration in the extracts was determined by the method of Bradford [5]. Equal amounts (20 μg) of protein from C-PC-treated and C-PC-untreated tumor cells were subjected to PAGE essentially as described by Okajima et al. [15], except that the SDS was omitted. Electrophoretic separation was performed on 10% polyacrylamide gels for 3–4 h with a constant current of 35 mA per gel. The in-gel activity staining of catalase (CAT) and glutathione reductase (GR) was performed on separate gels, following the methods of Chandlee and Scandalios [7] and Anderson et al. [2],

respectively. Localization of activity bands of superoxide dismutase (SOD) on gels and identification of enzyme metalloforms were performed according to Azevedo et al. [3]. Gel patterns were recorded immediately after the staining using the UVItec gel documentation system (Cambridge, UK). Image analysis of the gels was performed on a PC using Gel-Pro32 Analyzer software (Media Cybernetics Inc., USA). The activity (intensity) of each isoenzyme (band) was recorded as integrated optical density (IOD) in arbitrary units.

Results and Discussion

The treatment of *Graffi* tumor cells with 100 μg mL^{-1} C-PC caused a decrease in the number of viable cells to 51.4 ± 4.0% ($P<0.001$), while the same amount of C-PC did not exhibit any toxicity to Guerin tumor cells (cell viability was 97.1 ± 2.9%). The antineoplastic drug DOX (10 μg mL^{-1}) decreased only viability of the *Graffi* tumor cells (to 64.15 ± 1.33% from that of the untreated tumor cells, considered 100%).

Agarose electrophoresis of genomic DNA isolated from the *Graffi* cells treated with 50 and 100 μg mL^{-1} C-PC for 24 h showed a concentration-dependent fragmentation pattern (DNA ladder of 180-200 bp oligomers), typical for apoptotic cells. Internucleosomal DNA cleavage was not observed in the treated *Guerin* tumor cells, nor in the untreated *Graffi* and *Guerin* (control) cells (Fig. 1A).

Three bands of SOD activity (Fig. 1B) and two bands of GR activity (Fig. 1D) were visualized in the *Graffi* tumor cells. The upper, slow moving SOD band, was identified as manganese metaloform (MnSOD) according to its insensitivity to H_2O_2 inactivation, whereas the faster moving bands were sensitive to H_2O_2 (Fig. 1C) and KCN treatments (not shown), suggesting that they represented copper/zinc-containing (Cu/ZnSODs) activity.

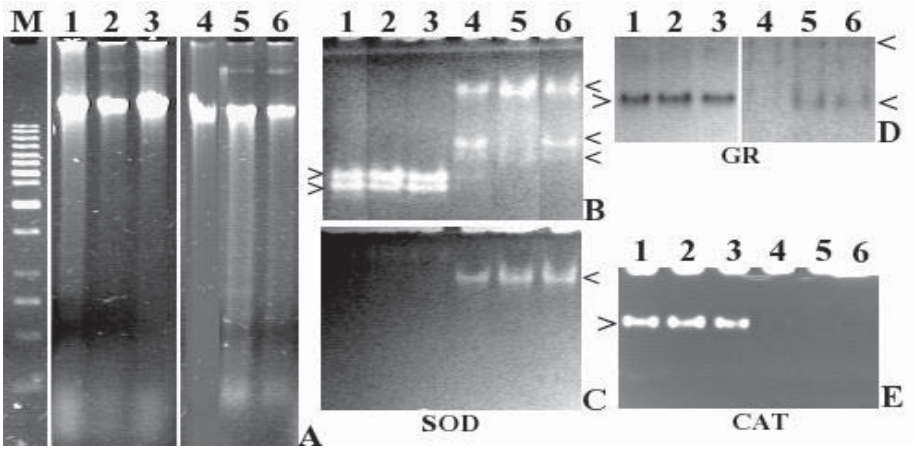

Figure 1. Effect of C-PC on DNA integrity and antioxidant enzyme activities of *Guerin* and *Graffi* tumor cells. **A**, DNA laddering; **B**, in-gel SOD activity; **C**, identification of SOD metaloforms by H_2O_2 treatment; **D**, in-gel GR activity; **E**, CAT activity. Lanes 1 to 3, *Guerin* cells, cultured only in the medium (control), treated for 24 h with 100 μg mL^{-1} C-PC and 50 μg mL^{-1} C-PC, respectively. Lanes 4 to 6, *Graffi* cells, cultured only in the medium (control), treated for 24 h with 100 and 50 μg mL^{-1} C-PC, respectively. In A, lane M, 1kb DNA Ladder, range 250 to 10 000 bp (Fisher Scientific, UK). Equal amounts of DNA (10 μg) and protein (20 μg) from

untreated and C-PC-treated tumor cells were subjected to agarose gel electrophoresis (A) and native polyacrylamide gel electrophoresis (B-E), respectively. Results are representative of three independent experiments.

The isoenzyme patterns were not affected by the C-PC treatments and did not show any change. However, the relative total activity of both SOD and GR clearly increased in the C-PC-treated cells in a concentration-dependent manner. SOD activity was enhanced after the application of 100 and 50 μg mL^{-1} C-PC by 114 and 60%, respectively (Fig. 1B, lanes 5 and 6) compared to the C-PC-untreated control (Fig. 1B, lane 4). The increases in the GR activity were approximately 2.6 and 2.1 times, respectively (Fig. 1D, lanes 5 and 6, vs. lane 4). Catalase activity was not detected in the control and C-PC-treated *Graffi* tumor cells (Fig. 1E, lanes 4-6). In the *Guerin* tumor cells, SOD activity was presented by two Cu/ZnSODs (Fig. 1B and C). The intensity of the slower moving band slightly increased (by only 12 and 20%, respectively) after treatment of the cells with 100 and 50 μg mL^{-1} C-PC (Fig. 1B, lanes 2 and 3). One GR activity band (Fig. 1D) and one band of CAT activity (Fig. 1E) were clearly seen on the gels in these ascites tumor cells. At the higher C-PC concentration, the activity of both enzymes was similar to the control levels. CAT and GR enzymes were by about 10% less active at the treatment with 50 μg mL^{-1} C-PC.

The antitumor potential of C-PC is well documented. The mainly studied C-PC, which is isolated from *Spirulina platensis* was shown to inhibit cell growth of various tumor cell lines. Its mechanisms of cell growth suppression, however, varied depending on the tested cell lane. For example, in HeLa cells, *S. platensis* C-PC treatment caused a decrease in DNA synthesis [20]; in rat histiocytic AK-5 tumor cells it induced apoptotic death through the down-regulation of Bcl-2 and generation of ROS [17]; in other models, this biliprotein inhibited the growth of tumor cells by no apoptotic pathways like a membrane destruction, leading to increased leakage of cell constituent, as in the case of *Ehrlich* ascites carcinoma cells [1], or stimulation of expression level of the proto-oncogene c-myc - in human chronic myelogenous leukemia K562 cells [11].

The present study revealed the ability of a highly purified C-PC from *A. africanum* to induce apoptosis in the myeloid *Graffi* tumor cells, as evidenced by the concentration-dependent ladder-like fragmentation pattern of the genomic DNA. In addition, this experimental model provided a new idea on the mechanism of the C-PC-induced apoptosis in which up-regulation of SOD, especially of MnSOD activity and the imbalance of antioxidant enzymes that favoured H_2O_2 accumulation could play a leading role. In contrast to its pronounced activity against the solid *Graffi* tumor cells, the C-PC of *A. africanum* did not show any effect on the cells of the ascites tumor of *Guerin*. Untreated and treated ascites cells maintained high and stable levels of their antioxidant enzymes SOD, CAT and GR, thus maintaining a cellular redox balance. Perhaps that was the cause for the unaffected by the treatments DNA integrity and cell vitality. The influence of the plasma membrane structure, in relation to low permeability and delivery of C-PC into the ascites tumor cells, can also be supposed.

In conclusion, our present and previous results show that the C-phycocyanin from *Arthronema africanum* has *in vitro* and *in vivo* antitumor activities and is a promising natural antitumor agent in experimental conditions. The present comparative investigation also focuses on the specifics of each experimental model and the importance of the selection of models when looking for new anticancer agents.

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BO15. BREAST CANCER

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BO16. PROXIMITY EXTENSION ASSAYS (PEA)

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BP1. ROLE OF GALECTINS AND CELL-SURFACE CARBOHYDRATES IN TUMOR CELL DISSEMINATION AND METASTASES

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Introduction

Neoplastic cells are characterized by specific molecular phenotype which is based on differential expression of a lot of molecular species. It is conceived now that oncogenes play an important role in formation of the tumor-specific phenotype by regulating gene expression. Glycosylation pattern of the tumor cells, formed by an array of glycosyltransferases, is not

exception from this rule. Carbohydrate-binding proteins are also differentially expressed, either as cell-surface receptors or in secreted/shed form.
Development of tumor cells metastases is a multi-step process. Many molecules, soluble and membrane-bound, play a role in dissemination of cancer cells and colonization of distant tissues. Metastatic cascade include: 1) development of tumor cells with metastatic potentials (clonal selection), 2) detachment of tumor cells having metastatic phenotype from the site of primary tumor and crossing the basement membrane, 3) dissemination of the metastatic tumor cells, generally through the vascular system (blood and lymph vessels), 4) invasion of the target organ and forming pre-angiogenetic metastatic foci and 5) growth of the metastatic mass requiring the concurrent development of blood vessels (neo-angiogenesis), which provide oxygen and various nutriments. In the process of metastases tumor cells elaborated mechanisms for preventing the immune system surveillance throughout all the steps of tumor cell dissemination.
Stromal microenvironment, in which neoplastic cells host, profoundly influences tumor cell's development, including their ability to form metastases. This influence is mediated, in large part, by bidirectional adhesive interactions leading to extravasation of the tumor cells. It is now perceived that metastasis is an inefficient process governed by several rate-limiting steps [1]. Failure to go out through all these steps can lead to tumor dormancy at any of the metastatic stages. Successful metastases lead to the development of vascularised tumor mass in the target organ. Other authors state that early steps in hematogenous metastasis may be quite efficient, but that regulation of cancer cell growth in secondary sites determines metastatic outcome. These authors have identified three key stages of tumor growth regulation: survival of a subset of single cells, proliferation of the subset of these cells to form pre-angiogenic micrometastases, and persistence of growth of the subset of these cells to form vascularized metastases [2]. Thus the formation of metastases implies the existence of tumor cells capable of successfully performing all the steps in the metastatic process: local invasion, lymphatic or hematogenous dissemination, arrest in the microvascular bed of the target organ, extravasation and growth of the secondary colony.

Role of glycosylation and carbohydrate-binding proteins in tumor cell metastases

Expression of terminal galactose (beta 1-3) N-acetyl galactosamine structures on cell surface glycoproteins could be associated with a high metastatic potential of human melanoma cells [3] In accordance with above data Chatterjee et al. have found higher galactosyltransferase activity in metastasizing variant of rat mammary carcinoma compared to the nonmetastasizing one [4] In another study it was shown that B16 metastatic murine melanoma cells exhibit a 5-fold higher cell surface galactosyltransferase activity than the nonmetastatic variant of B16 tumor cells [5] Human colon cancer with a high metastatic potential also express on their surface more poly-N-acetyllactosaminyl side chains with branched galactose residues, compared to the ones with low metastatic potential [6] However cell surface glycoconjugates of highly metastatic human colon cancer cells were found to be less fucosylated than glycoconjugates on the poorly metastatic cells [6] On the contrary, other authors found exclusive expression of fucosylated glycoconjugates on the metastatic variant of the rat tumor cells BSp73. The highly metastatic variant had also significantly more galactosyl, mannosyl and N-acetylgalactosylamine residues [7] Metastatic phenotype could also be correlated with incomplete glycosylation of cell surface glycoconjugates. Thus the number of galactose acceptor sites on the plasma membranes increase in parallel to the metastasizing capacity of rat mammary carcinomas [4] Highly metastatic variants of human colon cancer cells express on their cell surface more of the lysosomal membrane glycoproteins Lamp-1 and lamp-2, compared to the cancer cells with lower metastatic capacity [6] Avoiding the

mechanism of the programmed cell death (apoptosis) is essential for cancer cell survival and plays a role in tumor progression. Galectin-3 could act as anti-apoptotic molecule. Overexpression of galectin-3 in human breast carcinoma cell lines confer enhanced survival of the tumor cells upon exposure to different apoptotic stimuli, such as cytokines and radiation [8] Galectin-8 act as pro-apoptotic agent, and induces programmed cell death of human carcinoma cells [9] Expression of galectin-1 by means with cDNA transfection induces apoptotic cell death of colon cancer Colo201 cells [10].

Interaction between the tumor cells and the components of the extracellular matrix

All basal laminae contain a common set of glycoproteins, such as collagen IV and laminin. Cells generally do not bind directly to type IV collagen. The basal lamina is called also type IV matrix. Laminin component of the extracellular matrix (ECM) anchors the basal lamina to the cell surface. Moreover laminin has type IV collagen-binding site. Different cells surrounded by the basal lamina may utilize different cell surface receptors to bind ECM components. One class of laminin receptors is a member of the integrin receptor superfamily. Each integrin receptor binds to one or more matrix glycoproteins. The basal lamina is structured differently in different tissues. Different receptors for the basal lamina components may be used to cross this barrier. Fibronectins are matrix glycoproteins mediating cell attachment to extracellular matrices other than type IV. In many cases these matrices are loosely packed with several fibrous components and provide trail along which other cells can migrate. Fibronectins are present on the cell surface of non-transformed culture cells, but not on transformed cells. Metastatic tumor cells are transformed cells with a high affinity for basement membranes. Moreover these cells possess ability to produce basement membrane degrading enzymes which help them to traverse this critical barrier [11] Components of the extracellular matrix, which are glycoproteins, are used as attachment sites for tumor cell spreading and migration. Adhesive phenomena are mediated through a class of carbohydrate-binding proteins, called galectins, employing the terminal N-acetyllactosamine chains of the ECM glycoproteins. Transfection of colon cancer Colo201 cells with galectin-1 cDNA increases adhesion of these cells to fibronectin-coated dishes. The adhesion is dependent on the carbohydrate-recognition domain of galectin-1, since lactose act as inhibitor of the adhesion. Galectin-1 also mediates adhesion of Colo201 cells to laminin- and collagen-coated dishes. In these cases galectin-1 was thought to be expressed on plasma membranes of the colon cancer cells [10] Breast carcinoma cell line BT-549, transfected with galectin-3 cDNA also show increased adhesion to laminin- and collagen IV-coated dishes. In the case of fibronectin, used as adhesion substratum, galectin-3 transfected tumor cells adhered less than in the case of laminin- and collagen-coated dishes. Galectin-3 was found to be expressed on the cell surface in a punctuate manner [12] Overexpressing galectin-3 in human breast carcinoma cell lines lead to significantly enhanced adhesion to laminin, fibronectin exerted both directly and via increased expression of specific integrins, e.g. α-4 and β-7 [8]. Vitronectin is a blood plasma glycoprotein that mediate cell adhesion. Transfection of human breast carcinoma cell with cDNA for galectin-3 lead to increased adhesion to vitronectin-coated wells [14] Cancer cells can also specifically interact with the unique extracellular matrix protein, elastin. This interaction is mediated by galectin-3. Expression of galectin-3 is closely associated to the invasive/metastatic potential of various cancer types. Soluble elastin and/or peptide fragments of this protein are potent inhibitors of the metastatic spreading in experimental tumor models [15] Galectin-4 is composed of two carbohydrate recognition domains within the same peptide chain. Consequently galectin-4 does not need to form multimeric complexes in order to mediate tumor cells adhesion. It was found that extracellular galectin-4 mediate cell adhesion

[16] Treatment of B16-F1 and B16-F10 tumor cells with glycosylation inhibitor tunicamycin, lowered adhesion to endothelial extracellular matrix (basal lamina), and polyvinyl-immobilized fibronectin. Thus blocking of N-linked glycosylation of cell surface glycoconjugates could have anti-adhesive effect. Most probably N-acetyllactosamine chains of glycoproteins are involved in adhesion of tumor cells by mediation of secreted galectins [17].

The RDG (L-argininylglycyl-L-aspartic acid) sequence is present in many extracellular matrix proteins, such as fibronectin, laminin and collagen [18]. They are involved in adhesion between extracellular proteins and integrins. Novel RGD-containing peptides can be transformed into derivatives with improved pharmacological properties in cancer therapy [19] and [20].

Integrins are large and important family of adhesion molecules that promote stable interactions between cells and their environment. Some studies indicate that in human carcinoma (1299) cells galectin-8 inhibit cell adhesion to integrin-coated plates in a lactose dependent manner. α3 and β1 integrin derived from 1299 cells is a major galectin-8 binding-protein. Galectin-8 also interacts with λ6 and β1 integrins, but not with α4 or β3 integrins. Binding of galectin-8 could thus modulate integrin interactions with the extracellular matrix regulating cell adhesion and survival [9] Galectin-8 is a secreted protein. Secreted galectin-8 modulates cell adhesion by binding to integrins. Galectin-8-integrin complexes regulate negatively cell adhesion [21] The same authors found that galectin-3 associates with α1 and β1 integrin in a lactose dependent manner [14]. Transfection of breast carcinoma cell line, BT-549 with galectin-3 cDNA could modulate integrin expression. Thus higher surface expression of α6-β1 integrin was found on transfected clone, compared to non-transfected BT-549 cells [12]. Overexpression of galectin-3 in human breast carcinoma cells modulates synthesis of specific integrins, e.g. α-4 and β-7. Galectin-3 expression cells have significantly enhanced adhesion to laminin, fibronectin and vitronectin, exerted both directly and via increased expression of integrins [8]. Exogenously added galectin-1 also mediates adhesion of the tumor cells to dishes coated with fibronectin, laminin and collagen, probably by employing the cell-surface glycoconjugates [10]. Galectin-1 has one carbohydrate-binding domain in a single polypeptide chain. In order to mediate adhesion of tumor cells to ECM glycoproteins it must, at least, form dimmer structures under specific conditions. It is known that galectins require reducing agents in order to exert their biological functions. Such reducing environment usually exists inside the cell, in the cytosol. However outside the cell microenvironment is oxidative, which could help forming of disulfide bridges between individual galectin molecules, thus resulting in multimeric proteins. On the contrary, exogenously added galectin-3 reduces adhesion, in carbohydrate-dependent manner of various tumor cells to laminin-1, collagen IV and fibronectin glycoproteins [14].

Shedding and secretion of tumor-specific molecules

Shedding and/or secretion of tumor-specific molecules could modulate tumor spreading and metastases. Thus microvascular endothelium of metastasis-prone tissues undergoes activation in response to desialylated cancer-associated carbohydrate structures such as Thomsen-Friedenreich (TF) antigen (Gal-β1-3GalNAc) expressed on circulating glycoproteins and neoplastic cells [22]. It was found that colon cancer sera contain 10- to 30-fold higher quantities of haptoglobin beta subunit, identified as the major galectin-3 ligand. However it is not known whether this protein originate from the tumor cells and what is its role in tumor metastases [23] Another ligand for galectin-3, a 90 kDa protein, was found to be increased in the blood plasma from patients with adenomatous and adenocarcinomatous lesions [24] In the serum, sialyl Lewis (x) and soluble E-selectin were seen elevated in patients with advanced and recurrent breast cancer, especially in those with distant metastases [25] Gangliosides, shed by tumor cells could

be extremely potent enhancers of tumor formation *in vivo*. When 1 pmol of purified total gangliosides shed into the blood stream by highly tumorigenic cells where injected intradermally together with poorly tumorigenic cells, it was observed markedly increased tumorigenicity of the poorly tumorigenic cells in syngeneic normal mice [26] Tumor cells also release intact portions of their plasma membranes. It was found that membrane fragments were shed at a higher rate from the highly metastatic B16-F10 cells, than from poorly metastatic B16-F1 cells. In addition to quantitative differences in the protein composition of the shed membrane fragments 2 additional proteins were found in membrane fragment material from highly metastatic B16-F10. Shed membrane fragments consists of selected domains of the cell's plasma membrane glycoproteins [27] Highly metastatic cells secrete also more mucin into the culture medium, than poorly metastatic tumor cells. Thus LS LiM6, a highly metastatic colon carcinoma cell line secreted four- to five-fold more mucin into the culture medium, compared to poorly metastatic parental line LS174T [23]. Human melanoma cells secrete ligands for galectin-3, which are glycoproteins of 98 and 70 kDa molecular weights. It is not known what is the role of these glycoproteins, but they could mediate adhesion of the tumor cells to endothelial cells [28] T antigen-bearing glycoproteins are also capable of mobilizing galectin-3 to the surface of endothelial cells, thus priming them for harboring metastatic cancer cells [29] Thus secreted and/or shed molecules from the metastasizing tumors may be aimed at mediating adhesive phenomena or activating endothelial cells. In either of the two cases metastasizing cells help their spreading and metastasis to the target organs.

Keywords: metastases, galectins, glycosylation, cancer

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BP2. FEW WORDS ABOUT MATRIX METALLOPROTEINASES AND THEIR TISSUE INHIBITORS

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Session C: Pharmacology and Toxicology

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CO1. FATAL SUICIDAL POISONING DUE TO BORAX INGESTION – A CASE REPORT

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Abstract

Borax (sodium tetraborate hexahydrate or sodium borate) is a naturally-occurring mineral composed of sodium, boron, oxygen and water.

In this paper, a fatal case of toxicity due to borax ingestion for suicidal attempt is reported.

A 49 years, male patient was admitted to the Toxicology Clinic, Emergency University Hospital "N.I.Pirogov" with history of vomiting, greenish diarrhoea, abdominal pain, headache, dizziness, following suicidal poisoning of 200 g borax, dose exceeding - 10 times the lethal dose. According to the history of the case, it was revealed that he had been under psychiatric follow-ups and treatments due to paranoid schizophrenia and he had a previous suicide attempt. The patient was admitted to hospital on the third day after ingestion with hypotension, metabolic acidosis, oliguric renal failure, a generalized erythematous rash. Despite intravenous fluids, vasopressors, antibiotic therapy and resuscitation events, the patient's condition failed to improve. With a clinical signs of septic shock and multiple organ failure he died on 13 day of hospitalization.

The clinical features and treatment in such cases were discussed.

Key words: borax, suicidal poisoning, renal failure, sepsis

Introduction

Borax (sodium tetraborate hexahydrate or sodium borate) is a naturally-occurring mineral composed of sodium, boron, oxygen and water. Borax is a colourless, salt-like substance that can also be a white powder. Borax and boric acid have a number of legitimate applications and are widely used in manufacturing as detergents, water softeners and weak antiseptics. It is also used to make fertilisers, pesticides, and is sometimes found in pharmaceuticals [7, 15, 20].

Ingestion and other exposures to the chemical can cause various symptoms. The type and severity of symptoms varies depending on the amount of chemical involved and the nature of the exposure. Sufficient exposure to borax dust can cause respiratory and skin irritation. Ingestion may cause gastrointestinal distress including nausea, persistent vomiting, abdominal pain, and diarrhea. Effects on the vascular system and brain include headaches and lethargy, but are less frequent. In severe poisonings, a beefy red skin rash affecting palms, soles, buttocks and scrotum has been described.With severe poisoning, erythematous and exfoliative rash, unconsciousness, respiratory depression, and renal failure [3, 17, 21].

Documented cases of borate poisoning are now rare. The majority of documented borate - related deaths have occurred in infants. In this paper, a fatal case of toxicity due to borax ingestion for suicidal attempt was reported. The clinical features and treatment in such cases were discussed.

Case Report

A 49 years, male patient was admitted to the Toxicology Clinic, Emergency University Hospital "N.I.Pirogov" with history of vomiting, greenish diarrhoea, abdominal pain, headache, dizziness, following suicidal poisoning of 200 g borax, dose exceeding - 10 times the lethal dose. According to the history of the case, it was revealed that he had been under psychiatric follow-ups and treatments due to paranoid schizophrenia and he had a previous suicide attempt.

The patient was admitted to hospital on the third day after ingestion with hypotension, metabolic acidosis, oliguric renal failure, a generalized erythematous rash.

On examination, patient had mild dehydration, was disoriented, not obeying to oral commands. Baseline vital signs were heart rate -100 bpm, blood pressure- 80/40; 110/60 mm Hg, respiratory rate - 26/min, saturation of oxygen - 86 %. Examination of central nervous system revealed, disorientation, normal reactive pupils with exaggerated deep tendon reflexes, and rest of the findings being normal. Examination of cardiorespiratory system and per abdomen revealed no abnormality. Gastric lavage was performed, and activated charcoal was administered.

Intravenous fluids for correction of dehydration were administered. Forced alkaline diuresis was done to increase the elimination of borax. Corticosteroids, antibiotics, catecholamines was administered.

Foley's catheter was inserted for monitoring of urine output.

Table1: Laboratory results

Test / Day	**Hb** g/L	**WBC** x 10^9/l	**PLT** x 10^9l	**ASAT** U/l	**ALAT** U/l	**Creat.** μmol/l	**Urea** mmol/l
I	109	10,7	216	54	131	437	19,1
III	110	7,9	194	55	83	108	11,4
XII	87	3,6	78	60	49	87	8,4

Chest x - ray on admission - evidence of edema of the lung.

Arterial blood gas analysis revealed mild metabolic acidosis with respiratory alkalosis.

In the course of treatment, because of clinical signs of acute respiratory failure, the patient was transferred from the Clinic of Toxicology to the ICU - Intensive Care Unit. Despite intravenous fluids, vasopressors, antibiotic therapy and resuscitation events, the patient's condition failed to improve. With a clinical signs of septic shock and multiple organ failure he died on 13 day of hospitalization.

Discussion

Borate-containing compounds were formerly used as topical antiseptics and were components of many medicinal preparations including skin powders and ointments used for the treatment of burns and diaper rash. Boric acid is a commonly used as pesticide, disinfectant and wood preservative. Boron poisoning could be acute or chronic. Acute borax poisoning in adults has rarely been reported [1, 4, 11]. Acute boron poisoning could be suicidal or accidental. Most cases of boric acid poisoning have been reported to occur accidentally [10]. Nonetheless, boric acid ingestion for suicidal purposes has been quite rare [1]. Restuccio et al. reported a case of suicidal attempt in which a 45 year old man ingested approximately two cups of boric acid crystals dissolved in water. The patient developed nausea, vomiting and greenish diarrhea; and dehydration occurred shortly thereafter. He subsequently developed dysrhythmia and he failed to treatments administered and thus he died after 17 hours of admission [13].

Boric acid toxicity causes gastrointestinal irritation with vomiting and diarrhea that is bluish green in color [5]. Death in acute toxicity may be caused by shock resulting from dehydration and renal failure, though renal toxicity may not occur following ingestion of minimal amounts of boric acid [1].

Neurologic complications including agitation, seizure and encephalopathy may also occur. Dermal complications of boric acid toxicity includes erythrodermic rash similar to boiled lobster which could be generalized or localized to the palms of the hands, soles of the feet, buttock, scrotum or face followed by exfoliation after 2-5 days resembling toxic epidermal necrolysis or scalded skin syndrome of newborns [14, 19].

There could be a change in the condition of respiratory chain of microsomes following poisoning by boron and its compounds. Borate poisoning results in polyneuropathy with axonal degeneration with a decrease in the fibre density of the myelinated nerve fibres [2, 8].

Exposure to boron and its related compounds has been implicated as the potential cause of chronic kidney disease with impairment of renal function and structure. Treatment of boron poisoning is supportive and no specific antidote is available [6, 9]. Early decontamination with copious water ingestion could result in less severe form of disease [18]. Forced alkaline diuresis with a target achievable alkaline pH of urine, could increase the urinary elimination of boric acid (1, 16). Hypotension, metabolic acidosis and acute renal failure with death resulting from

circulatory collapse and shock are reported following boron poisoning [1]. Patient should be monitored for neurological signs and symptoms, oliguria, acidosis.

Conclusion

A fatal case of borax ingestion for suicidal attempt in patients with severe mental illness was reported. He was untreated for 3 days and presented with dehydration and renal function impairment. Death in acute toxicity may be caused by shock, resulting from dehydration and renal failure.

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CO2. EFFECTS OF CHLOROGENIC ACID ON EXPLORATORY BEHAVIOR AND LOCOMOTOR ACTIVITY IN RATS

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Abstract

Chlorogenic acid is one of the most abundant polyphenol compounds present in a variety of foods that are consumed daily, such as cherries, apples, kiwis, plums and coffee. It is one of the main bioactive substances in *Aronia melanocarpa* fruits.

The aim of the present study was to investigate the effects of chlorogenic acid on exploratory behavior and locomotor activity in male Wistar rats.

Chlorogenic acid was administered on a daily basis orally to different groups of animals (n=10) for 7, 14, 21 and 30 days at a dose of 20 mg/kg. Comparisons were made with saline-treated controls. At the end of each experimental period, the exploratory behavior and locomotor activity of rats were registered in an Opto Varimex apparatus (Columbus Instruments, USA). The number of horizontal and vertical movements recorded every minute for the first 5 min served as a measure of exploratory activity and habituation to the new environment. The total number of movements during the first 5 min and during the whole 10-min period of observation was used as a measure of locomotor activity.

It was found that after 7 and 14 days of treatment, chlorogenic acid did not significantly affect exploratory behavior and locomotor activity of rats compared to the saline-treated controls. After 21 and 30 days, it significantly decreased the number of horizontal and vertical movements

of the animals which might be the result of a sedative effect. At all testing periods, chlorogenic did not disturb habituation. As habituation is considered as an elementary form of learning, the present study suggested that chlorogenic acid did not disturb the memory and learning processes in rats.

Keywords: chlorogenic acid, exploratory behavior, locomotor activity, rats
Acknowledgements: This study was supported by Grant MU-Varna 2012/2014.

CO3. ANXIOLYTIC-LIKE EFFECT OF CHLOROGENIC ACID ADMINISTERED SUBCHRONICALLY TO RATS

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Abstract

Chlorogenic acid is a polyphenol found in various plant products such as coffee, beans, potatoes, apples, kiwis, plums and aronia fruits. Chlorogenic acid (5-O-caffeoylquinic acid) is formed by the esterification of caffeic and quinic acids.

The aim of the present study was to investigate the effect of chlorogenic acid on anxiety in rats.

The experiment was performed on 80 male Wistar rats divided in 8 groups (n=10). Chlorogenic acid was applied daily orally to different groups for periods of 7, 14, 21 and 30 days at a dose of 20 mg/kg. Comparisons were made with controls respectively treated with saline. At the end of each experimental period, the state of anxiety was evaluated using the elevated plus-maze test.

The results showed that for all testing periods chlorogenic acid significantly increased the number of entries into the open arms, the time spent there and the ratio of open/total arm entries as compared to the controls. At the same time, chlorogenic acid did not affect the locomotor activity assessed by the total number of arm entries. Thus, the increased exploration of the open arms not accompanied by increased locomotor activity indicated an anxiolytic-like effect which was not false positive.

In conclusion, the findings from the present study suggest an anxiolytic-like effect of chlorogenic acid in rats.

Keywords: chlorogenic acid, anxiety, plus-maze, rats
Acknowledgements: This study was supported by Grant MU-Varna 2012/2014.

CO4. CYTOTOXICITY STUDIES AND FLUORESCENT IMMUNOLOCALIZATION OF FUMONISIN B_1 IN DEC 99 CELL LINE

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Abstract

A new permanent cell line derived from duck embryos – DEC 99 (Ivanov and Kril, Patent № 63125/15.05.2001) was used for evaluation of the cytotoxic effect of the mycotoxin fumonisin B_1. For control test we used standardized BALB/c 3T3 mouse embryonic fibroblast cells (3T3 clone 31 (EC Commission Directive 2000/33 adapting to technical progress for the 27th time Council Directive 67/548/EEC). We demonstrated on ultrastructural level that fumonisin B_1 causes pathological alterations of the nucleus, nucleolus and cell organelles [1] . These alterations probably resulted from direct and indirect toxic effect [2, 3, 4]. There are very few data, about the fate and the target structures of the toxin after passing through the cell membrane [5].

The present study aimed to evaluate the cytotoxic concentrations of FB_1 in new *in vitro* model DEC 99 and to localize the intracellular distribution of the toxin by means of immunofluorescence.

Cytotoxicity tests were performed on DEC 99 and 3T3 cell lines and appropriate cultivation conditions were used. Concentrations of 400, 288, 207, 149, 107, 77, 56, 40 μg FB_1 per ml medium were testified for cytotoxicity in cell viability tests by neutral red uptake (NR) and propidium iodide – acridine orange asseys. Primary anti fumonisin B_1 monoclonal antibody (R-Biopharm AG, Darmstadt, Germany) and secondary FITC-labeled antimouse antibody (Santa Cruz Biotechnology, USA) were used in immunochistochemical assay for immunolocalization of the toxin within the cells. Non-treated and treated with 300 μg FB_1/ml for 48 h samples from DEC 99 monolayer cultures were stained according to a standard May-Grünwald -Giemsa protocol for morphological estimation. The observations were carried out by means of light and fluorescent microscope Leica DM 500B, (Wetzlar, Germany, microfilter cube I3, BP 450-490, dichromatic mirror 510, suppression filter LP515, LED wave – 470) and confocal microscope Leica TCS SPE, Wetzlar, Germany, 488 nm laser scanning, immerse X 40 (1,15 aperture), x 63 (1,30 aperture).

The values of the percentage of viable 3T3 cells at concentrations above 107 μg FB_1/ml were decreased but not statistically significantly. The percentage of viable DEC 99 cells was significantly affected by concentrations above 207 μg FB_1/ml, compared to controls (*$p<0.05$ for 207 μg FB1, **$p<0.01$ for 288 μg FB1 and ***$p<0.001$ for 400 μg FB1). Cells were rounded and detached, lysis of cells and free spaces between them were observed in cultures treated with concentrations above 207 μg FB_1/ml for 48h of incubation. May-Grünwald –Giemsa staining of treated with 300 μg FB_1/ml cells for 48h confirmed these results. Morphological examination revealed shrinked, cells with rounded shape and pycnotic nuclei, which were not firmly attached

– pointlace like cultures. Fluorescent microscopy revealed localization of FB1 within the cells after 48h of exposure. The observed fluorescence appeared to be situated in the cytoplasm as well as in the perinuclear spaces. The observed fluorescence was evidential near the nucleus but also diffuse or contrastly clusters of lightning was seen in the cytoplasm of cells treated with 300 and 400 μg FB_1/ml.

The results obtained by the cytotoxicity test and neutral red uptake showed that DEC 99 cells were more sensitive than 3T3 cells. The observed fluorescence within the cell after 48h of exposure was evidential for passing through cell membrane and accumulation of the toxin, presumably targeting cytoplasmic membrane structures and nucleolemma.

Keywords: cytotoxicity, fumonisin B_1, immunolocalization , permanent cell line DEC 99.

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CO5. IN VITRO CYTOTOXICITY OF SILVER-MODIfiED NATURAL CLINOPTILOLITE

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Abstract

Zeolites are natural aluminum silicates of volcanic origin with specific microporous structure. There are numerous investigations of their potential biological activities and application in medicine and cosmetics. In this study we tested *in vitro* the cytotoxic activity of two zeolites, the natural nonmodified clinoptilolite (CTP) and the natural modified clinoptilolite exchanged with silver ions (CPT-Ag) in tumor cell lines HeLa and HepG2 and in non-tumor cell line 3T3. The results show that CTP does not affect cell lines used in the study, but CPT-Ag has high cytotoxicity for all three cell lines, indicating the active component are the silver ions themselves and that the CPT-AG cytotoxicity is not selective for cancer cells.

Introduction

Zeolites are natural aluminum silicates of volcanic origin with specific microporous structure, pierced by channels and voids formed by 6 -, 8 -, 10 - and 12-membered rings and SiO44-AlO45-tetrahedrons. In the channels and holes are located charge compensating cations of the skeleton (Na +, K +, Ca2 +, Mg2 + and Ba2 +) and water molecules coordinating them. This structural configuration involves specific beneficial properties associated with ion exchange, selective sorption, catalytic activity, and dehydration (reversible) rehydration. It was found that zeolites have selective affinity to size and shape of ions and organic molecules. This could be used for reversible binding of small molecules such as oxygen, nitrous oxide and others. With these properties, zeolites can have significant application in the field of medicine and pharmacy [15]. Therefore zeolites are studied in terms of their potential biological activities, including antitumor effects [16]. Thus, it was shown that clinoptilolite exhibits antibacterial properties [10, 14]. There are a lot of studies on properties and application of zeolites in medicine and cosmetics [4, 5, 7, 8, 11-13, 17-20].

Micro- and nano-mesoporous silicate particles are considered as potential drug delivery systems because of their ordered pore structures, large surface areas and the ease with which they can be chemically modified [9]. On the other hand, silver in low concentrations has found application in medicine and cosmetics because of its antibacterial properties. Ag+ shows a strong affinity for sulphydryl groups and other anionic ligands of proteins, cell membranes, and tissue debris [3]. Further, anti-proliferative effect of silver nanoparticles on human glioblastoma cells (U251) in vitro was shown [1]. Dose-dependent cellular toxicity was observed after treatment of cell lines from lung adenocarcinoma A549 with silver nanoparticles and silver ions. [6]. Here we present our data on cytotoxicity of silver-modified natural clinoptilolite on cancer and non-cancer cell lines.

Materials and methods

Cell cultures

We used the cell lines from hepatocellular carcinoma (HepG2), cervical carcinoma (HeLa) and the nontumor cell line of murine fibroblasts (3T3). The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Gibco-Invitrogen, UK) supplemented with 10% fetal calf serum (Gibco-Invitrogen, UK), 100 IU/ml penicillin and 100 μg/ml streptomycin. All cell lines were maintained at 37^0 C in a 5% CO2 incubator.

Zeolites

The cells were treated with natural clinoptilolite (CPT) and natural, modified clinoptilolite exchanged with silver ions (CPT-AG). The source of natural clinoptilolite was the deposit of Beli plast, Kardzhali, Bulgaria. The zeolites were provided by the Institute of Mineralogy and Crystallography at Bulgarian Academy of Sciences.

Cytotoxicity assay

Neutral red (NR) cytotoxicity assay based on the protocol described by Borenfreund [2] was used to assess the cell viability. Briefly, cells were seeded in 96-well microtiter plates (Scientific Orange) at 10^5 cells/well and allowed to attach overnight. The cells were then treated for 24 h with different concentrations of each zeolite: 0.667mg/ml, 0.445mg/ml, 0.296 mg/ml, 0.198mg/ml, 0.132 mg/ml, 0.088 mg/ml, 0.058 mg/ml and 0.039 mg/ml. For the cytotoxicity assay 100μl of NR solution (50 μg/ml) was added to each well. After 3 h incubation at 37 ^{o}C and 5% CO_2, cells were destained with100 μl of acetic acid (1%)–ethanol (50%) (v/v). Untreated cells were used as controls, and wells with culture medium without cells were used as blank controls.

The optical density was measured at 570 nm with a microplate reader (TECAN, SunriseTM, Austria). The viability was represented as the percentage of viable cells after preincubation with zeolites, as compared to the untreated control group and was calculated for each concentration. The percentage of inhibition of the cells was estimate as follows: Cell viability (%)=OD570 (experimental)/OD570(control)x 100. All experiments were performed in triplicate.

Statistical analysis

The results from treated and control cells were compared using one-way analysis of variance (ANOVA) followed by Dunnett test. A *P*-value <0.05 was considered statistically significant. Concentration of each zeolite which showed 50% cytotoxicity (IC50) was calculated. The data were analyzed using *GraphPad Prizm, GraphPadSoftware Inc., USA, 2000.*

Results and discussion

We have studied *in vitro* the cytotoxic activity of two zeolites: the natural nonmodified clinoptilolite and the natural modified clinoptilolite exchanged with silver ions. The effect of zeolites on cell viability was tested in tumor cell lines HeLa and HepG2 and non-tumor cell line 3T3 by using NR assay.

Our results showed that CPT-AG had high cytotoxic activity for all three cell lines – tumor cell lines HeLa and HepG-2 and non-tumor 3T3 cells (Fig.1). The IC50 values of CPT-Ag in 3T3, HeLa and HepG-2 cells were 0,107 mg/ml, 0.170 mg/ml and 0,411 mg/ml, respectively. Cytotoxic effect in HeLa cells was higher than in HepG-2 tumor cell line, but was slightly weaker than in non-tumor 3T3 cells. There was no selectivity for cancer cell lines as CPT-AG exhibited higher cytotoxic activity for non-tumor 3T3 cells compare to tumor cell lines HeLa and HepG-2. CPT-AG shows significant cytotoxic effect for 3T3 cells even in concentration of 0.132 mg/ml.

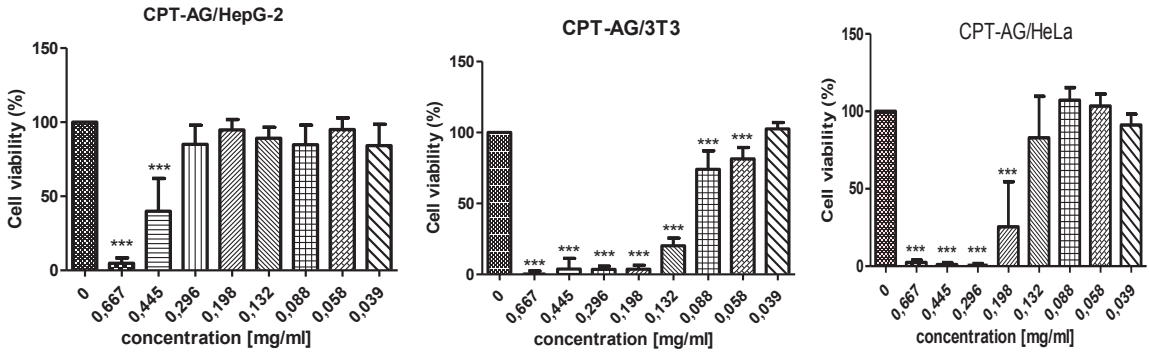

Fig.1. Cell viability assessed by the NR assay after treatment of HepG-2, HeLa and 3T3 cell lines with CPT-AG.

The natural non-modified CPT did not affect the cell viability at all concentrations in all cell lines used in the study (Fig.2). This indicates that the non-modified CPT which doesn't exchange any Ag^+ in the medium has no effect on the cells and the cytotoxic effect of CPT-AG is due to silver ions.

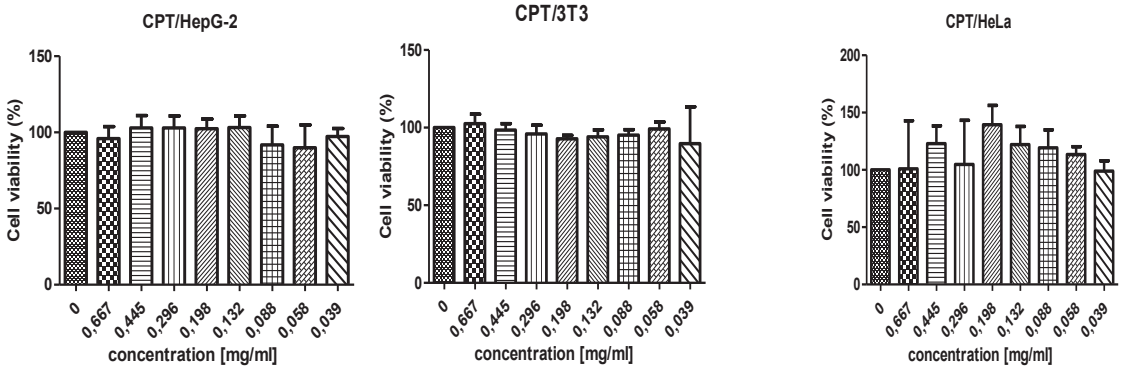

Fig.2. Cell viability assessed by the NR assay after treatment of HepG-2, HeLa and 3T3 cell lines with CPT.

In this study we tested *in vitro* the cytotoxic activity of natural zeolite clinoptilolite and its modification CPT-AG, where cations of naturel CPT were replaced by silver ions. The natural originating from Bulgaria nonmodified clinoptilolite doesn't affect HepG-2 and HeLa tumor cells and non-tumor 3T3 cells, but clinoptilolite exchanged with silver ions has high cytotoxicity for all three cell lines, indicating the active component are the silver ions themselves, not the carrier. The silver ions included in the pore system of natural clinoptilolite, transform it to material with high cytotoxic activity. CPT-AG cytotoxicity shows no cancer cell selectivity, it affects both non-cancer and cancer cells.

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CO6. XANTHATES (DITHIOCARBONATES): HEAVY METALS CHELATION PROPERTIES AND BIOLOGICAL EFFECTS

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Abstract

Different salts of aryl and alkyl derivatives of dithiocarbonic acid (known under the trivial names xanthogenates or xanthates) are well known from many years as powerful heavy metals chelators. Based on this chemical property xanthates are used with success in:

- Industrial flotation for production of cooper, zinc and lead.
- Environmental chemistry for removal of traces of heavy metals from wastewaters (mercury) or extraction of cadmium from soil.
- Analytical chemistry for spectrophotometric determination of metals (Cu, Mo, Zn).

In this short review we present data from our laboratory and by other authors for the biological effects of xanthates, which could be explained by chelation of biologically important metal ions in the body. With that their property could be explained:

- The in vivo antidotal effect after poisoning with heavy metals (chelation of mercury, cooper).
- The antiangiogenetic effect for inhibition of tumor growth (due to chelation of zinc in different metaloproteinases).
- CNS effects – inhibition of amphetamine-induced locomotor activity and decrease of brain noradrenaline levels (due to in vivo or in vitro inhibition of dopamine-β-hydroxylase (a cooper dependent enzyme).
- Antioxidant action – chelation of iron; chelation of cooper/zinc in the brain markedly ameliorate β-amyloid accumulation in Alzheimer's disease.
- Antiproliferative (antitumor) action – inhibition of phospholipase C (zinc dependent enzyme).
- Xanthates/heavy metals complexes with well-expressed antitumor action – gold (inhibition of thioredoxin reductase), platinum (thioplatin), palladium, bismuth, nickel; metronidazole/xanthate ^{99m}Tc complex as target delivery to hypoxic tumors.

CO7. *IN VITRO* ANALYSES OF ALVEOLAR SURFACTANT IN CLINICAL SAMPLES IN NORM AND PATHOLOGY

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Abstract

Alveolar surfactant (AS) is a complex lipoprotein mixture that covers the lung alveoli at the air-liquid interface. Its main function *in vivo* is to reduce the surface tension (γ, mN/m) in exhalation thus preventing the alveolar collapse. The insufficiency of AS in the lung as well as deviation from its optimal biochemical composition results in respiratory activity impairment.

The clinical therapy currently includes the application of various native or synthetic exogenous surfactant preparations which substitute human AS. Analysis of the composition and the properties of the alveolar surfactant are crucial for the assessment of lung maturity and optimal function and may prove the need of surfactant therapy application.

The aim of the present study was to estimate the physiology condition of lung surfactant by analysis of different clinical samples in norm and pathology. The analyzed clinical samples included gastric aspirates (GA) from prematurely born and full term infants, tracheal aspirates (TA) from unventilated and ventilated lung during surgery in patients with nonsmall cells lung cancer (NSCLC) and broncho-alveolar lavage samples from a patient with pulmonary alveolar proteinosis, taken after each stage of the applied whole lung lavage procedure (WWL).

In this regard we investigated the biochemical and biophysical properties of GA from 74 babies: 15 with NRDS, 6 prematurely born and 53 normally born and healthy; TA from 15 patients with NSCLC and 10 whole lung lavage samples, collected from a male patient during 15 WLL cycles. For determination of protein content in the samples Lowry protein assay (Peterson's modification) was used. The PL's concentration was determined via extraction by the method of Blight and Dyer. Thin-layer chromatography was used for determining the phospholipid profile of the individual phospholipid components. In addition, by using the method of Axisymmetric Drop Shape Analysis, the equilibrium and dynamic surface characteristics: maximal and minimal surface tension (γ, mN/m) during 10 cycles of compression-decompression, were determined.

Our results show that AS composition and behavior were changed by the different analyzed pathologies leading to alteration of phospholipid and protein concentrations as well as significant differences in the individual phospholipid profiles and surface tension characteristics, especially the minimal surface tension values ($\gamma_{min,}$ m/Nm).

The present study could find application into the clinical practice for fast surfactant maturity diagnostics in prematurely born children regarding lifesaving therapy with exogenous surfactants administration. It shows that lung cancer, hypoxia and inhalation anesthesia affect the biochemical and biophysical properties of AS which leads to changes in its composition and behavior. It will also be of great interest for the effective implementation of the procedure of whole lung lavage in the clinical practice.

CO8. ANTIOXIDANT ACTIVITY IN BODY FLUIDS OF ATHLETES

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Abstract

It is well known that physical exercise, especially with high intensity, can lead to oxidative stress. The body response to loading is commonly measured in blood. However in the last years an increased body of evidences suggests that saliva also could be used as an alternative source to investigate oxidative stress. The salivary glands contain mechanisms of protein and ion transportation from blood to saliva. The principle advantages of saliva over blood sampling are: easy and noninvasive collection, easy storage; possibility for repeated sampling; large quantities that can be obtained; collection on field. In saliva could be measured such oxidative stress biomarkers as thiobarbituric acid reactive substances, advanced oxidation protein products, as well as the activity of the antioxidant enzymes superoxide dismutase, glutathioine peroxidase and catalase, and the level of non-enzymatic antioxidants glutathione and uric acid. It will be of interest whether the salivary oxidative stress changes can reflect in the same manner the plasma markers of oxidative stress induced by acute exercise.

CO9. RARE DISEASES AND ORPHAN DRUGS

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Abstract

Most rare diseases are genetic and life-threatening - 75% of rare diseases are manifested in childhood - 30% of affected children do not survive to age of 5. Assessment for rare diseases and their impact on a particular country and worldwide use two main indicators, prevalence and incidence of disease. Most (80 %) of rare diseases are genetic rest can result from infections, allergies, degenerative and proliferative processes. Characterized by heterogeneous clinical picture with multiple symptoms that vary widely from one nosology to another. These diseases are chronic, progressive, degenerative, and often life-threatening. In most cases, no specific treatment. Work and research for specific therapy of rare diseases are significantly different from those of other drugs. The manufacture and development of orphan drugs is related to equivalent

and in many cases with higher costs since many of these drugs are biotechnological products, difficult to synthesize and produce. On the other hand, the small numbers of patients for whom they are intended not provide returns.
In order to stimulate research and development in the field of orphan drugs, public authorities implement initiatives for health and biotechnology industries. A group of diseases in which relatively intense work on the development of new drugs are lysosomal storage diseases. These are conditions in which a gene mutation resulting in enzyme deficiencies in the lysosomes of various cell in the result set substrates not degrade and build up in the different organs pathology and systems, which leads to their malfunction.

Rare diseases
Most rare diseases are genetic and life-threatening -75% of rare diseases are manifested in childhood - 30% of affected children do not survive to 5 years of age. There are different classifications of disease as "rare"; they differ significantly in America, Europe and Asia.
In the U.S., a disease is considered rare if it affects less than 200 000 people from the general population or 1: 1,500 affected.
In Japan - for rare disease accepted if occurs in less than 50 thousand people in the total population, or 1: 2,500 affected.
In Europe, a rare disease is considered if affects less than 5: 10,000.
To evaluate of rare diseases and their impact on a particular country and worldwide two main indicators are used - prevalence and incidence of disease.
Prevalence (number of patients at a time) it is used to assess the impact of the disease. According to Global Genes Project, rare diseases affecting about 350 million people worldwide. According to (EURORDIS) there are 6-7 thousand different rare diseases, affecting between 6 and 8 % of the population of Europe.
The incidence (number of born with a disease per year) of rare diseases can be varied within wide limits. For certain areas and communities they can be significantly more frequent, this is especially true for closed communities where the gene pool is not updated. An example is the frequency of Gaucher disease, which is 1: 40,000 in the general population but occurs in about 1: 10 people in the Ashkenazi community.
Such examples are there for entire countries, for example in the Netherlands observed extremely high incidence of Pompe disease.
Most (80 %) of rare diseases are genetic rest can result from infections, allergies, degenerative and proliferative processes. These diseases are chronic, progressive, degenerative and often live threatening. Lead to disability and significant deterioration in quality of life with loss of autonomy. Inflict physical and psychological suffering for the patient and his family. In most cases, there is no specific treatment. They affect people in varying degrees of severity, but always the life expectancy of the individual patient is greatly reduced.
Characterized by very heterogeneous clinical onset with a variety of symptoms, that varies widely from one nosology to another. In the presence of pathognomonic symptom, it is not present in 100% of patients, which further impedes diagnosis. Often a typical dysmorphic features are present. Patients visit different specialists before having a diagnosis. In general a significant period of time between onset of symptoms and diagnosis is observed, occasionally average about 8 years. Another challenge for differentiated diagnosis is different disease course not only between different patients as start and severity, but also within the same family. In addition, the clinical picture is often similar to other common nonhereditary disease. Rare

diseases are characterized with extremely varied onset - many severe courses starting in childhood and attenuated forms that are randomly discovered.
Inheritance of genetic diseases may be different.

Autosomal dominant inheritance - the mode of inheritance of a genetic disorder in which the individual receives only one altered copy (mutation) of a gene by altering gene dominates over the normal disease that is transmitted from generation to generation without skips one or two generations, and if one parent is affected by the disease then the likelihood the child to inherit and develop the disease is 50%, regardless of the sex of the child.
Autosomal recessive inheritance - a way of inheritance of a genetic disorder in which the individual receives two altered copies (mutations) of a gene, one from each parent, an individual with an altered gene is intact (healthy) carrier that is has a 25% chance the child to inherit genetic disease if both parents have the mutation that affects only one of the two genes. The parents are not sick as only one of their parents possessed mutation. Therefore, diseases that have this type of inheritance are the greatest challenge, as occur sporadically and are difficult to predict.
Inheritance related to sex chromosomes - in this type of inheritance mutation is in a gene localized on a sex chromosome X or Y, it can be both dominant and recessive.
In all types of inheritance particularly dominant and sex-linked, it is important to draw a family tree and make appropriate genetic consultation and if necessary to apply prenatal diagnosis of pregnancy.

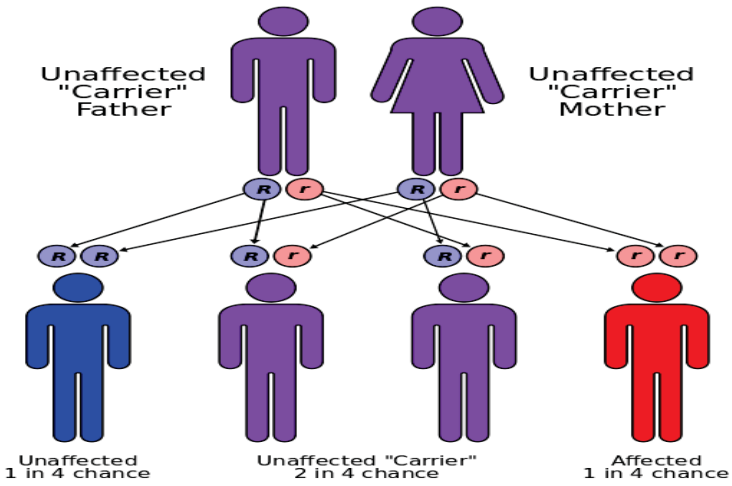

Fig.1: Autosomal recessive inheritance

Orphan drugs
Research and development for specific therapy of rare diseases is significantly different from those of other drugs. Firstly as shown by its name, they are designed for a very small number of patients to compare to drugs for the mass, making development not attractive for most manufacturers. The manufacture and development of orphan drugs is related to equivalent and in many cases with higher costs since many of these drugs are biotechnological products, difficult to synthesize and produce. On the other hand, the small numbers of patients for whom they are intended not provide returns.
In order to stimulate research and development in the field of orphan drugs, public authorities implement initiatives for health and biotechnology industries. It began in 1983 in the United States with the adoption of the orphan drugs, followed by Japan and Australia in 1993 and 1997,

in Europe in 1999 through the implementation of a common policy for orphan drugs in its Member States.
These measures provide exclusivity of production and sales of the company for a product for a certain period of time during this period; another may not produce the same drug. Exclusivity period is 10 years for Europe and 7 for U.S. Moreover, there are different requirements for conducting clinical trials with these products. This is taken into account the small number of patients and the inability to collect large groups a graded statistical significance. Another motivator is quick registration procedure due to reduced survival of most rare diseases.
Even with these concessions the development of orphan drugs remains limited. It involves a lot of research and development costs for the biotechnological production personally, it is evident from the lack of interest from other manufacturers to produce orphan drugs after the expiry period of exclusivity.
At present, in the presence of between 6 and 7000 different rare diseases has registered about 70 drug, as most of them are designed for malignancies that are rare or malignancies in children, which is a rare coupling clamp condition.
A group of diseases in which relatively intense work on the development of new drugs are lysosomal storage disease (LSD). These are conditions in which a gene mutation resulting in enzyme deficiencies in the lysosomes of various cell in the result substrates not degrade and build up in the different organs pathology and systems, which results in their not functioning. Most lysosomal diseases are with multi-organ involvement, and life-threatening. Characterized by diversities with disease severity according remaining enzyme activity. Total frequency of the LSD is 1: 6,000 to 1: 7,000 live births, all LSD are multisystem and progressive until there are more than 50 LSD.
Examples for lysosomal storage disorder with available specific therapy for the past 10-15 years.

Mucopolysaccharidosis (MPS)
MPS are eight different forms associated with a deficiency of a different enzyme leading to accumulation of glycosaminoglycans (mucopolysaccharides). Is currently available therapy for three of them - type 1 syndrome Hurler with three sub forms, type 2 Hunter syndrome, type 6 – Marataux - Lamy Syndrome.
MPS have overall incidence 1: 140,000, they are pan - ethnic disease incidence varies in different populations. Most are found in patients with a phenotype most severe manifestation due to manifest symptoms.
Mucopolysaccharidoses are autosomal recessive inheritance with the exception of Hunter syndrome, which is an X linked genes and affects exclusively boys.

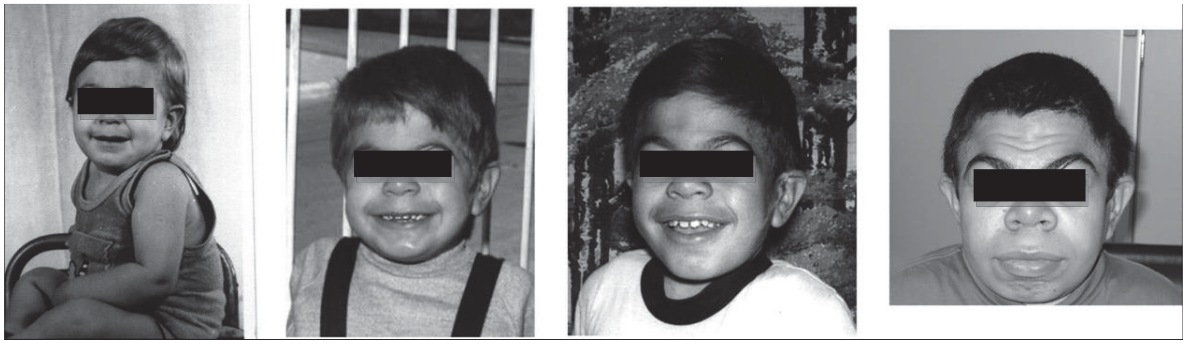

Fig. 2: **Development of the disease in MPS**

Pompe disease

An enzyme defect: reduced activity of the lysosomal enzyme α-glucosidase (GAA), leading to the accumulation of glycogen in tissues. The disease is with autosomal recessive inheritance and is characterized by progressive degeneration of the skeletal musculature and respiratory and infants - and the heart.

Incidence: 1: 138,000 infants, 1: 57,000 adults. There is a high incidence of infantile form in African - Americans and Chinese and later form in the Netherlands. Progression : - infantile onset - death to 1 year late start - slower progression, progression and severity of the onset of symptoms depend on the percentage of residual enzyme activity.

Fabry disease

Fabry disease is a progressive, multi- system, X- linked lysosomal storage disease caused by a defect in the gene encoding the enzyme α- galactosidase A. Partial or complete lack of activity of this lysosomal enzyme renders a certain degradation of glycosphingolipids and cause accumulation of the substrate in the lysosomes of various cell types. At the highest level is affecting the cells of the vascular endothelium, which may lead to tissue ischemia or infarction. Progression of the pathology present with symptoms involving the peripheral nervous system within a setting of age. As we age observed clinical picture including symptoms associated with kidney, heart and brain. Although the disease is an X - linked genes, heterozygous at many women experience high morbidity and premature mortality.

The clinical features associated with severe burning pain and paraesthesia in the extremities, reduced or absent sweating and tolerance to heat and cold as well as exercise. Other symptoms include abdominal pain, tinnitus and vertigo. Honest and very characteristic symptom is the appearance of clinical onset of angioceratoma. With progression of the disease and vascular injury, kidney failure, or early stroke and infraction are observed.

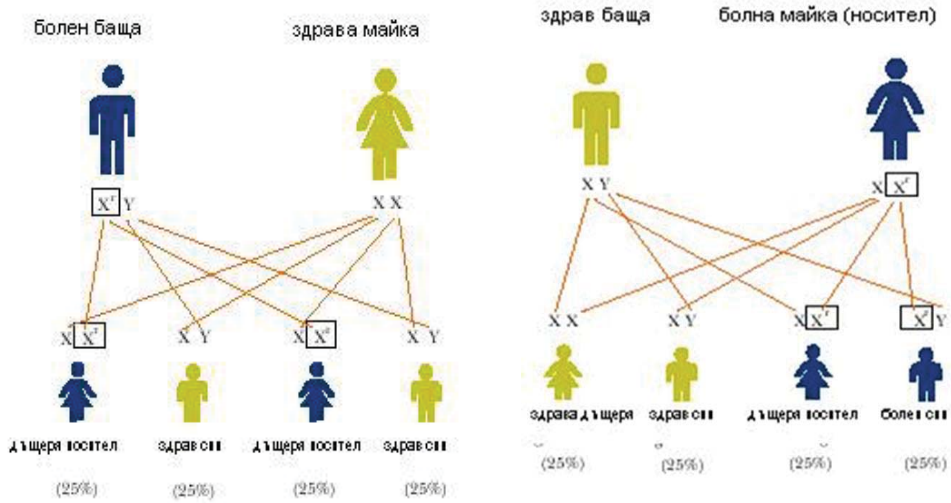

Fig. 3: Fabry disease is a X- linked lysosomal storage disease

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CO10. MULTIDRUG RESISTANCE CELL LINE AS EXPERIMENTAL MODELS IN BIOMEDICAL RESEARCHES

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CO11. GLIOBLASTOMA MULTIFORME - В ТЪРСЕНЕ НА НОВИ ПОДХОДИ ЗА ЛЕЧЕНИЕ

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CP1. P-GLYCOPROTEIN AND MULTIDRUG RESISTANCE

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DO1. MODELS OF HUMAN PAPILLOMAVIRUS-ASSOCIATED DISEASES

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Abstract

Human papillomaviruses (HPVs) are infectious agents with clinical importance. They are the causative agent for cervical cancer, carcinomas of vagina, anus, vulva, penis and oropharyngeal carcinomas. A range of different models have been developed to study HPV, particularly virus growth, virus-host cell interactions, virus-associated carcinogenesis and to test efficiency and safety of prophylactic and therapeutic vaccines. Here, we review the existing most important models: organotypic epithelial cultures, animal papillomaviruses as models to study HPV, murine transplantable tumors expressing HPV genes, and mice transgenic for HPV genes. Key differences between these model systems and natural HPV infection have limited their use and require development of better models in the future.

Introduction

The human papillomaviruses (HPVs) are ubiquitous human pathogens associated with the development of epithelial malignancies, especially cervical cancer. HPV also plays a causative role in most of oropharyngeal carcinomas, in the carcinomas of vagina, anus, vulva and penis. Keratinocytes in the basal layer of squamous epithelia are the initial targets for HPV infection and the virus life cycle depends on the differentiation status of infected cells. HPVs encode transforming proteins E6 and E7. In HPV-associated cancers the expression of E6 and E7 HPV oncogenes is up-regulated and correlates with the transforming potential of HPV. HPV oncoproteins E6 and E7 are responsible for immortalization and malignant transformation of infected cells through binding to the tumor suppressors p53 and retinoblastoma protein (pRb), respectively [23, 38]. When E6 interacts with p53, the function of p53 is disrupted resulting in

uncontrolled cell growth. After binding with E7, pRB is phosphorylated and becomes incapable of suppressing cell growth. Most of therapeutic vaccines have targeted E6 and E7 of HPV16 or HPV18.

HPV is strictly species and tissue-restricted and there is no simple monolayer cell culture system for analysis and propagation of the virus. In spite of these difficulties, great progress has been made in the elucidation of the molecular controls of virus gene expression, replication and pathogenesis. In addition, two prophylactic HPV vaccines have been in use since 2006 and a number of therapeutic vaccines have been tested in human trials. Much of these achievements in basic HPV research and vaccine development are based on studies in different model systems.

Here, we review the existing most important models, used to study HPV growth and pathogenesis and for developing and testing therapeutic strategies for HPV infection, including the novel human vaccines.

CELL CULTURE MODELS

Organotypic epithelial (raft) cultures

The study of HPVs has been hampered as most of currently available human cell lines fail to support HPV replication due to the differentiation-dependent aspects of virus life cycle – it is dependent on differentiation of squamous epithelial keratinocytes that the virus infects. The organotypic raft culture system has provided opportunities to develop an in vitro system that is capable of reproducing the entire HPV life cycle. Raft cultures allow primary human keratinocytes (PHK) to achieve stratification and differentiation in vitro [2]. These cultures can be prepared from normal keratinocytes, explanted epithelial tissue, or established cell lines. The keratinocytes grow to confluence on top of a collagen-fibroblasts matrix. Then the structure is lifted to a medium-air interface such that the upper surface of the epithelial cells is exposed to the atmosphere, while the dermal equivalent is kept moist and supplied with nutrients and growth factors secreted by the fibroblasts. As a result the PHK differentiate and within 10 days forms a full-thickness stratified epithelium [15]. These cultures can be sustained for about two weeks. Similar cultures are achieved by placing pieces of epithelial tissue biopsy directly on the collagen–fibroblast matrix and the type of epithelium that emerges is entirely comparable with the morphology of the source of the biopsy [39].

Organotypic cultures provided the first opportunities to propagate wild-type and mutated HPV genomes. HPV16, HPV18, and HPV31 virions were generated in raft cultures from transfected DNAs (23). Transfection with recombinant wild-type or mutant HPV genomes of PHKs isolated from different body sites (foreskin, larynx, cervix), followed by growing of transfected cells in organotypic cultures, is an approach to investigate the role of individual viral genes in different aspects of the viral life cycle. Due to the difficulties of PHKs transfection, retrovirus-mediated gene transfer has been used in many studies.

This system can also utilize the HPV immortalized cell lines or cell lines obtained from cervical pathological biopsies [6]. Normal PHKs stratify and differentiate in a manner similar to the normal squamous epithelial tissues, while transformed cell lines exhibit dysplastic morphologies similar to the lesions seen in vivo. Cell lines established from CIN lesions that harbored episomal HPV genomes (W12 and CIN-612) allowed viral amplification and packaging when grown in organotypic cultures [36]. HPV DNA replication has been extensively studied in the cell line W12 containing HPV-16 [17].

This culture system provides an essential tool for investigations of viral reproduction, virus-host cell interactions, for the genetic analysis, and for the evaluation of antiviral agents. Thus, the molecular functions and consequences of the expression of E6 and E7 oncogenes from high-risk mucosal HPVs were analyzed [4, 25]. The organotypic cultures were also used to produce virus

particles by applying different strategies and to test new immunotherapeutic approaches for squamous cell carcinoma, including factors contributing to the presence and function of immunocompetent cells within a neoplastic epithelium that develops on a mucosal surface [21].

ANIMAL MODELS

Animal papillomaviruses as models to study papillomavirus infections in human

Despite differences in genome organization and tissue tropism, animal papillomaviruses (PVs) have been widely used as models to study papillomavirus infection in humans and for development of HPV vaccines. Much of our understanding of PVs, their life cycle and other aspects of pathogenesis has derived from studies of animal PVs that cause lesions similar to those found in humans - bovine PV (BPV) infection of cattle, cotton-tail rabbit PV (CRPV) infection of domestic rabbits and canine oral PV (COPV) infection of dogs. Induction of papillomas and their neoplastic progression has been experimentally demonstrated and reproduced and virus-cofactor interactions have been elucidated in these systems. For example, CRPV has provided insights into the roles of viral gene products during productive and latent infection and during the progression to cancer [11, 20, 31, 40]. This model has been also used for the evaluation of drugs against papillomaviruses and of surgical treatments. In vitro studies with two animal PV early proteins, the transcriptional regulator E2 and the oncoprotein E5, have contributed to the elucidation of viral gene control and cell transformation. BPV E2 was the first viral product to be identified as a transcriptional regulator. Many of the function of E5 proteins have been first established for BPV E5 and later validated for HPV E5.

Animal PVs has been extensively used for the development of both therapeutic and prophylactic vaccines, as cattle, rabbit and dog provide the opportunity to study vaccination in the natural immunocompetent host. The studies showed that there was serum response to viral capsid proteins in infected animals and resistence to subsequent viral challenge in seropositive individuals. Neutralising antibody, directed to determinants on the L1major capsid protein, is generated in these individuals. It was suggested that a vaccine capable of inducing such responses must contain L1 protein in the correctly folded form, via L1 VLPs. Experimental studies in three animal models (dog, cow and rabbit) proved this idea and showed immunogenicity and efficacy of L1 VLP prophylactic vaccines. Studies to examine the ability of VLP vaccination to prevent papillomavirus infection have relied on CRPV in domestic rabbits (14), COPV in dogs [37], and BPV4 in cattle [27]. In each of these models, low dose intramuscular injection of VLPs generated excellent protection from experimental challenge with high-dose virus. Protection from virus challenge could be passively transferred using serum from vaccinated animals, indicating that antibodies were sufficient to confer protection [14, 37]. In addition, as type-specific and crossneutralizing titers were not greater after vaccination of animals with L1/L2 VLPs than with L1-only VLPs [35] all clinical trials of VLP vaccines have used L1 only VLPs. However, as there is no animal model for venereal transmission of PVs, the route of infection leading to cervical cancer, the preclinical studies cannot assess the levels of antibodies needed to prevent sexual transmission of oncogenic HPVs in women.

CRPV infection of domestic rabbits and COPV infection of dogs are the most widely used animal models for examining immunotherapies for warts. The COPV model is attractive because it involves mucosal lesions, but it has been difficult to study immunotherapies in this model because of the rapid rate of spontaneous regression [33]. It was shown that E2 may be the most attractive viral protein target for inducing immune-mediated canine warts regression [32]. Most immunotherapeutic approaches have not consistently induced regression of established CRPV warts [26]. Several studies have found E2 to be the most effective single target [13].

Mouse models

Murine transplantable tumors expressing HPV genes

In 1985, Kreider et al. used human cervix fragments infected with an extract of human condylomas and grafted them under the renal capsule of athymic mice to isolate the first HPV and reproduce some of the histologic features of HPV infection [28]. This isolate, HPV-$11_{Hershey}$, was subsequently passaged in other human tissues, primarily human foreskins, using the same animal model [22, 29].

Brandsma et al. were able to introduce with a gene gun HPV-16 DNA in human foreskin fragments that were subsequently grafted onto the flank of SCID mice [12]. Lesions with histologic features of HPV infection that contained HPV16 antigen and DNA developed, but the production of virions and the passage of the infection to uninfected tissue were not demonstrated.

A human xenograft SCID mouse model was developed that can produce infectious virus particles of HPV-6, HPV-11 and HPV-16 [8]. HPV-16 was repeatedly propagated in human foreskin fragments grafted in the SCID mouse and intraepithelial neoplasia in the implants were obtained. This model reproduces the natural HPV-induced lesions and thus permits the investigation of an authentic pathogenesis of HPV-induced premalignant lesions. Finally, it allows for the evaluation of antiviral strategies. The ease of isolating genital HPV strains in the human xenograft SCID mouse model contrasts with failed attempts using the athymic mouse [16, 28], indicating the advantages of the SCID mouse over the athymic mouse [7]. HPV-infected human xenografts (usually foreskin fragments) implanted in immunodeficient mice (e.g., athymic and SCID mice) were also used for *in vivo* studies of drugs against HPV.

Most animal studies of HPV therapeutic vaccines involve transplantable murine tumors - therapeutic vaccines are usually tested in mice with HPV-expressing tumor cells subcutaneously implanted into their flank. Subcutaneous injected C57BL/6 mice with murine TC-1 tumor cells, which continuously express HPV16 E6 and E7 oncoproteins, is the most commonly used model [30]. Immunization of C57BL/6 mice induced efficient immune responses and generated HPV16 E7-specific cytotoxic T lymphocytes. However, the therapeutic vaccines targeting E6/E7 tested to date have shown limited clinical efficacy in patients with HPV-associated genital neoplasia, indicating that vaccine-induced regression in mouse models with ectopic HPV-tumors is not predictive of clinical outcome. To assess HPV therapeutic vaccines in a more relevant setting, an orthotopic mouse model was established where tumors in the genital mucosa (GM) develop after an intravaginal instillation of HPV16 E6/E7-expressing tumor cells transduced with a luciferase-encoding lentiviral vector for in vivo imaging of tumor growth. Tumors remained localized in the genital tract, and histological analysis showed that most tumors grew within the squamous epithelium of the vaginal wall. In an orthotopic murine model for cervical cancer was shown that vaccination with HPV16-E7 polypeptide was able to induce E7-specific CD8 T-cells in the GM of mice and regression of small genital tumors [18, 19].

Mice transgenic for HPV genes

Numerous HPV transgenic mouse models have been generated to analyze *in vivo* biological properties of HPV genes, in particular E6 and E7 of HPV 16. Transgenic mouse containing the E6 and E7 genes from HPV16 under the control of the human keratin 14 (K14) allow expression of the virus genes in stratified epithelia, such as skin or mucosal tissues. K14-HPV16 mouse models develop carcinomas in the head and neck region, skin and cervix. While spontaneous tumors arise in the skin, cervical carcinomas appear in cooperation with estrogens. Characterization of K14-16E6, K14-16E7 and K14-16E6E7 mice has demonstrated that E6 is the main virus oncogene in skin squamous cell carcinomas and E7 in cervical carcinoma. It was also shown that tumors derived from K14-16E7 transgenic mice were primarily benign, while those in

K14-16E6 transgenic mice were mostly malignant, indicating that E6 alone not only is sufficient to induce tumor development but it also confers increased malignant potential *in vivo*.
The K14-HPV16 mouse did not develop spontaneous tumors in the cervix. Arbeit et al demonstrated that chronic estrogen administration induced cervico-vaginal squamous carcinomas in K14-16E6E7 animals and showed the important role of E7 in this process [1]. These mice developed a progressive disease that led to the formation of squamous carcinoma of the cervix over a 6-month period and closely reflected the histopathological characteristics of the progressive disease that leads to cervical cancer in humans. When treated chronically for 6 months with 17β-estradiol, the K14E7, but not the K14E6 or nontransgenic mice, developed cervical cancer. The E6 oncoprotein contributed to increased tumor size in estrogen-treated K14E6/K14E7 doubly transgenic mice. The validity of these HPV 16 transgenic mice as models for human cervical cancer has been demonstrated at several levels: The histopathological progression of disease in mice resembles that in human cervical cancer [34]; The role of estrogen as a co-factor in the development and progression of cervical cancers in mice [10] parallels the epidemiological evidence for a role of estrogen in human cancers; There is a close parallel in the expression pattern of biomarkers for human and murine cervical cancer [9]. The finding that the anti-estrogenic drug, indole-3-carbinol, inhibits cervical cancer in the HPV transgenic mouse [3] has led to its successful use in the clinical treatment of CIN2/3 [5].
In addition, the role of cellular targets of HPV oncogenes in carcinogenesis was determined using transgenic mice models. Thus, although pRb is the major E7 target, using hormone treated K14Cre;RbloxP/loxP animals it was shown that there are pRb-independent functions for E7-mediated carcinogenesis. Using knock-out animals, it was shown that cervical disease was significantly increased in p21 -/- mice compared with p21+/+ mice, showing that p21Cip1 can function as a tumor suppressor. It was shown that E6AP is absolutely required for E6 to cause cervical cancer. In addition, p53-independent activities of E6 also contributed to carcinogenesis, but these activities were manifested only in the presence of the HPV16 E7 oncogene.
Transgenic mice expressing HPV oncogenes in their epithelium can develop cervical cancer but are of limited use for testing therapeutic vaccines, because expression is wide spread in the tissue. These mice develop tolerance to the PV-encoded transgenic proteins, and are unable to generate PV antigen-specific cytotoxic T cells after immunisation. At the same time, although viral genes that are expressed as transgenes are considered as self-antigens, many studies have demonstrated that HPV transgenic mice can develop immune responses to E7 when stimulated appropriately.

Conclusions

Two types of model systems are currently in use to study papillomavirus infection in humans**.** The first uses organotypic raft cultures and the second type are in vivo models, including transgenic mice and models that make use of animal papillomaviruses. Analysis of different model systems has helped to understand the carcinogenic role of HPV. The models provided an essential tool for investigations of virus growth, virus-host cell interactions, for the genetic analysis of viral proteins and regulatory sequences, and for the evaluation of antiviral agents. Thus, the role of E6 and E7 as major oncogenes has been established and the contribution of each of them, as well other cofactors, has been analyzed. These models have also been instrumental in the development of HPV vaccines.
There is great potential for the development of improved diagnostic and prognostic tests, prophylactic and therapeutic vaccines, and traditional antiviral medicines. Efficient translation of basic research to clinical diagnosis and therapies depends upon the availability of appropriate

models. Thus, the advantage of raft cultures is that they are based on primary human keratinocytes but the disadvantage - the limited time to test therapies. On the other hand, mouse models allow testing long-term effects of new anti-HPV treatments in a living organism, but the differences between mouse and human species must to be taken into account. Better models, able to bypass these drawbacks of currently available systems are needed and must be developed in the future.

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DO2. EFFECT OF COMBINED ADMINISTRATION OF PROTEASE INHIBITOR AND POLYPHENOLIC COMPLEX ON FUNCTIONS OF ALVEOLAR MACROPHAGES IN MICE, INFECTED WITH INFLUENZA A VIRUS

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Abstract

Influenza is an acute, viral respiratory disease which remains a serious global threat to humans, causing significant morbidity and mortality each year. Current vaccination strategies and antiviral agents provide limited protection against the rapidly mutating influenza viruses. It has long been known that the natural products and their derivatives are invaluable sources of therapeutic agents. Many plant extracts and compounds of natural origin demonstrated considerable immunomodulatory and antiviral potential against influenza viruses. Modulation of innate immunity has an impact on the host's ability to respond to the influenza virus and determines a favorable outcome of the disease. In this study, we aimed to examine the combined therapeutic effect of protease inhibitor PISC-2002, obtained from *Streptomyces chromofuscus* and polyphenol extract from *Geranium sanguineum L.* on functions of alveolar macrophages from healthy and infected with influenza virus strain A/Aichi/2/68 (H3N2) mice.

Key words: bacterial protease inhibitor, plant polyphenolic complex, alveolar macrophages, influenza A virus infection.

Introduction

Influenza is an acute, contagious disease of viral etiology, caused by influenza virus from the *Orthomyxoviridae* family. Influenza A virus is the most pathogenic of the three types of influenza viruses (A, B and C). It leads to the occurrence of recurrent, seasonal epidemics and occasional pandemics, infects people of all ages and is a frequent cause of pneumonia and death, especially among the elderly and children. Influenza viruses are highly variable, enveloped RNA viruses with a single-stranded, negative and segmented genome [4,13]. The viruses infect mainly epithelial cells in the respiratory tracts and alveolar macrophages by attaching of the viral glycoprotein (HA) to a cellular receptor (sialic acid) on the host cell surface [5]. Alveolar macrophages are key effector cells of the innate immune system that protect the lungs from viral pathogens, regulate the inflammation and restrict the secondary bacterial infections. During influenza virus infection macrophages are activated and destroy the virus-infected cells by phagocytosis, simultaneously producing high levels of proinflammatory molecules, (cytokines, reactive oxygen and nitrogen species) [7,8]. Thereby, alveolar macrophages regulate the immune and inflammatory responses, enabling the host to overcome the viral infection. Fujimoto et al. (2000) found that the elimination of the infected cells by phagocytosis leads to inhibition of viral

growth *in vitro* [1]. Other investigators have reported that the influenza infection substantially depresses phagocytic and chemotactic activity of macrophages [2]. These findings give us grounds to consider, that the stimulation of phagocytic function of macrophages is crucial to overcome the viral infection. It is known that the excessive release of proinflammatory mediators can contribute to tissue damage. Therefore, the modulation of functions of alveolar macrophages enables the immune system to effectively control the viral infection, without causing lung tissue injury.

Due to the limited efficacy of the current vaccines and antiviral drugs, there is an increasing need for development of effective and safe agents against influenza viruses. A promising approach for the treatment of influenza virus is the use of viral inhibitors of natural origin, which possess immunostimulating activity, combining effectiveness and low toxicity to the body.

The aim of the present study was to investigate the combined effect of bacterial protease inhibitor PISC-2002 (PI), produced from *Streptomyces chromofuscus* and plant polyphenolic complex (PC) from *Geranium sanguineum L.* on functions of alveolar macrophages, isolated from healthy and infected with influenza virus strain A/Aichi/2/6 (H3N2), mice.

Materials and methods

In our experiments, we used male and female, inbred ICR mice (16-18 g) with induced lethal experimental influenza infection (EII), which were divided into 6 experimental groups and treated with protease inhibitor (PI) and polyphenolic complex (PC), as follows: group 1- mock-infected and PBS-treated –healthy control (HC) mice; group 2 - influenza virus-infected mice – virus control (VC); group 3 - PC and PI treated mice (PC+PI); group 4 – influenza virus-infected and PI treated mice (PI+VC); group 5 – influenza virus-infected and PC treated mice (PC+VC); group 6 – influenza virus-infected and treated with a combination of PI and PC, mice (PI+PC+VC).

PC was extracted from the roots of the medicinal plant *Geranium sanguineum* L. (Geraniaceae) and was administered by intranasal instillation, 3 hours before infection at a dose of 5μg/ml. The protease inhibitor PISC-2002 PI, produced by *Streptomyces chromofuscus*, (isolated from a soil sample) was administered at a dose equivalent to the optimal virus-inhibitory (1:8). Viral infection was induced under light ether anaesthesia by intranasal inoculation of A/Aichi/2/68 (H3N2) influenza strain, adapted to murine lungs with infectious titer of 10^7 TCID50/ml. To induce lethal hemorrhagic pneumonia, the mice were challenged with a dose of 10 LD50/0.05 ml PBS/mouse.

The isolation of alveolar macrophages (aMa) had been conducted on 2^{nd}, 6^{th} and 8^{th} day post-infection, according to the method of Holt et al. (1979) by 5 washing of the tracheal-bronchoalveolar cavity of mice with 1.0 ml cold (4^{o}C) RPMI-1640 medium. The spreading and phagocytosis of aMa in the presence of Zymosan were implemented by the method of Rabinovich and De Stefano (1973), with a modification of Passeti (1993) and the Spreading index (SI) and the Phagocytic index (PI) were calculated.

The myeloperoxidase activity of aMa was determined by a technique, described by Padmaja and Ramanadahm (1998). The nitric oxide production (NO) was measured in the cell culture supernatant of aMa by a method of Ding et al. (1988).

The optical density was measured in an ELISA spectrophotometer (TECAN, SunriseTM, Grödig/Salzburg, Austria) at a wavelength of 492 nm and 540 nm, respectively. The concentration of nitric oxide was estimated by using a standard curve $NaNO_2$ and expressed as nmol/2 x 10^5 cells.

The data are given as the mean ± standard deviation (SD). Significance testing was performed using one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test (GraphPAD 5.0 InStat; GraphPad Software, CA, USA). Values of *p<0.05, **p<0.01 and *** p<0.001 were considered statistically significant.

Results and discussion

The combined effect of protease inhibitor PISC-2002 and polyphenol extract from *Geranium sanguineum* L. on functions of alveolar macrophages, isolated from infected with influenza virus strain A/Aichi/2/68 (H3N2) mice were studied in dynamics (2nd , 6th and 8th day after the viral infection). Spreading, phagocytosis, NO production and myeloperoxidase activity were evaluated.

The results for spreading (Spreading index) and phagocytosis (Phagocytic index) of aMa, from influenza virus-infected mice, treated with combination of PI protease inhibitor and polyphenolic complex PC are shown in Fig.1 and Fig.2.

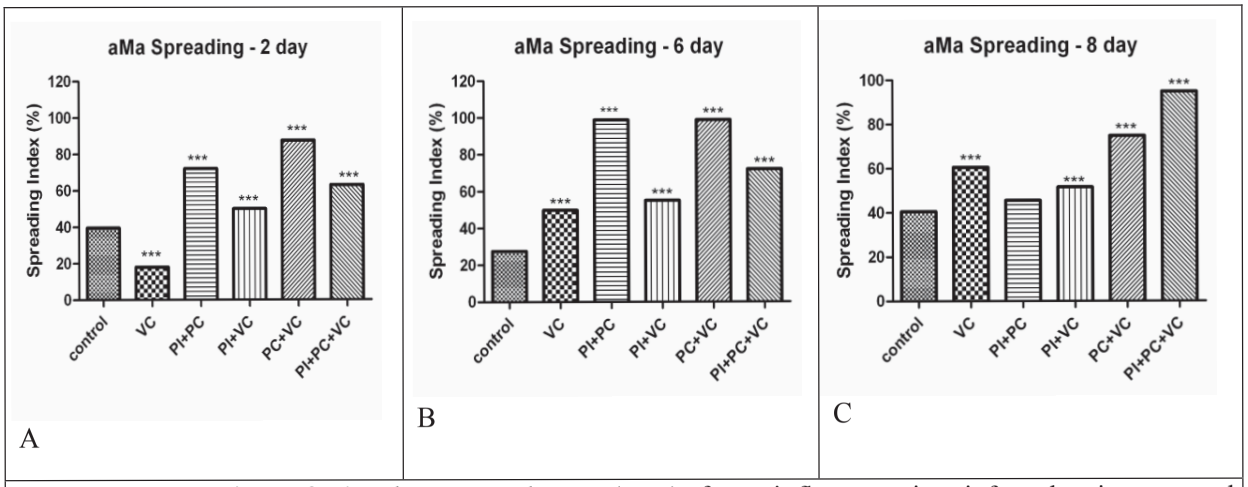


Figure 1. Spreading of alveolar macrophages (aMa) from influenza virus-infected mice, treated differently on 2nd, 6th and 8th day post-infection. Grp.1 – healthy control; Grp.2 - VC; Grp.3 - PI+PC; Grp.4 - PI+VC; Grp.5 - PC+VC; Grp.6 - PI+PC+VC

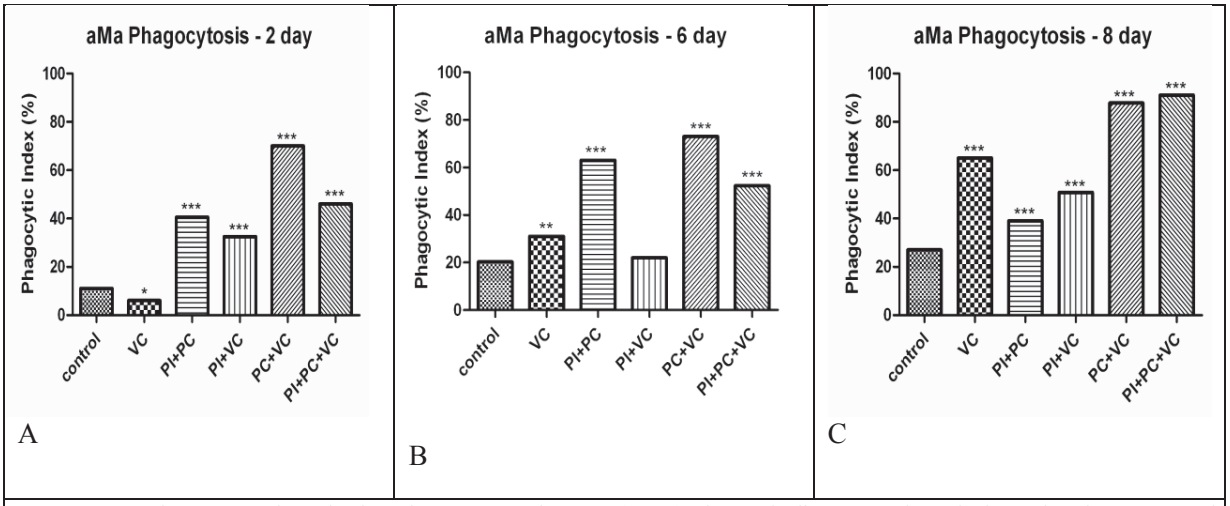


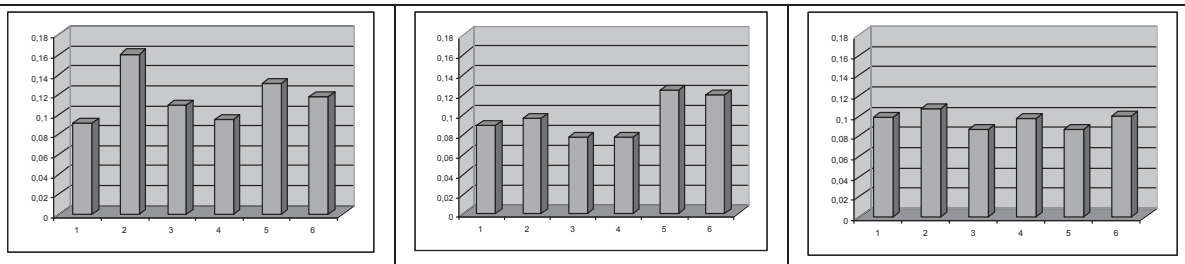
Figure 2. Phagocytosis of alveolar macrophages (aMa) from influenza virus-infected mice, treated differently on 2nd, 6th and 8th day post-infection. Grp.1 – healthy control; Grp.2 -VC; Grp.3 - PI+PC; Grp.4 - PI+VC; Grp.5 - PC+VC; Grp.6 - PI+PC+VC

As shown in Fig.1 and Fig.2 the influenza infection suppresses the spreading (Fig. 1A, Grp.2 - VC) and the phagocytic activity (Fig. 2A, Grp.2 - VC) of aMa on day 2 after the viral infection compared to the healthy controls. The administered therapeutic combination of PC and PI leads to enhancement of spreading and phagocytosis of alveolar macrophages at a different degree. The highest SI (94.75 ± 1.71) and PI (91.0 ± 2.58) values were recorded in the experimental group, treated with a combination of PC and PI on 8th day, after the infection (Fig. 1C and 2C, Grp.6 - PI+PC+VC). The phagocytic function of macrophages plays a major role in the elimination of foreign substances and apoptotic cells. Furthermore, the phagocytic macrophages are becoming competent antigen-presenting cells, which interact with T-cells and activate them.

Previous experiments have shown that infection with influenza virus A/Aichi 2/68 (H3N2) in mice, induces suppression of alveolar and peritoneal macrophages's phagocytic function [3]. The results from our experiments also confirm these data.

Recent studies have indicated that both natural compounds (PC and PI) demonstrated significant antiviral effect *in vitro*, as well as *in vivo* [11]. In addition to these findings, we established that the combined administration of PC and PI *in vitro* shows pronounced immunomodulating and immunorestorative effect on functions of influenza virus-infected macrophages.

In the current study, we also examined the effect of the administered therapy in influenza virus-infected mice on the levels of proinflammatory mediators - nitric oxide and myeloperoxidase of alveolar macrophages. NO-production of alveolar macrophages from the treated animals was measured in dynamics. The results obtained are shown in Fig.3.

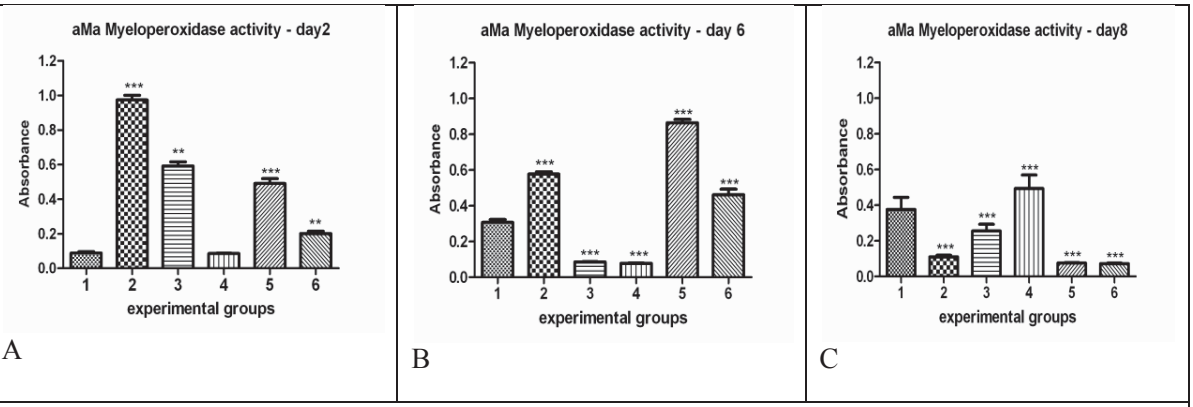


A-2nd Day | B-6th Day | C-8th Day

Figure 3. NO-production of alveolar macrophages (aMa), isolated from influenza virus-infected mice, treated differently on 2nd, 6th and 8th day post-infection. Grp.1 – healthy control; Grp.2 - VC; Grp.3 - PI+PC; Grp.4 - PI+VC; Grp.5 - PC+VC; Grp.6 - PI+PC+VC

The NO-production of influenza virus-infected alveolar macrophages (Grp. 2) is increased significantly on the second day of the research (Fig.3A). The administration of a combination of PI+PC has an immunorestorative effect in influenza virus-infected mice on the 2nd and 6th day post-infection (Fig. 3A и 3B). It is well known that activated macrophages after influenza infection released cytokines, reactive oxygen metabolites and proteolytic enzymes. This leads to an increased membrane permeability and microvascular leakage with the formation of reactive oxygen and nitrogen species [14], which can damage surrounding tissues, causing local and systemic inflammation.

The data for myeloperoxidase activity of alveolar macrophages, followed in dynamics on the 2nd, 6th and 8th day after EII in experimental animals are shown in Fig.4.



A | B | C

Figure 4. Myeloperoxidase activity of alveolar macrophages (aMa), isolated from influenza virus-infected mice, treated differently on 2nd, 6th and 8th day post-infection. Grp.1 – healthy control; Grp.2 - VC; Grp.3 - PI+PC; Grp.4 - PI+VC; Grp.5 - PC+VC; Grp.6 - PI+PC+VC

It has been found that myeloperoxidase is also involved in oxidative stress and inflammation, causing extensive tissue injury. This enzyme is stored in the azurophilic granules of polymorphonuclear neutrophils and macrophages and at the occurrence of inflammatory process

it is released into extracellular fluid [6]. The present experiments prove that myeloperoxidase activity of alveolar macrophages in influenza-infected mice is increased on the 2nd and 6th day after the viral infection (Grp.2) compared to healthy control, as shown in Fig.4.

According to many researchers, the polyphenol compounds, derived from higher plants possess antiviral and immunomodulating activity. In earlier studies, it has been proved that the polyphenol extract from medicinal plant *Geranium sanguineum* L. inhibits the viral replication of influenza viruses A and B *in vivo* and *on ovo* [10]. Toshkova et al. (2004), reported that PC from *Geranium Sanguineum* L., administered *in vitro* stimulates the phagocytic activity of murine polymorphonuclear leukocytes (PMNs) and peritoneal macrophages, and affects the spontaneous NO-production of macrophages [12].

The natural protease inhibitors are widely distributed in plants, animals, bacteria and some viruses. It has been found that suppression of the proteolytic virus activation by protease inhibitors can prevent the spread of influenza virus and the development of generalized viral infection [15]. Previous investigations have demonstrated that the bacteria from the genus *Streptomyces* produce protease inhibitors, which could be successfully used as antiviral agents [9].

The successful outcome of the progressive inflammatory reactions in the organism depends on the effective regulation of the functions of macrophages. Based on the results obtained, we confirmed that PC from *Geranium sanguineum* L. exhibits immunomodulating activity on aMa and we found that the immunostimulatory effect is stronger after administration of protease inhibitor and polyphenolic complex. The current studies also indicate that the administered therapeutic combination of PI and PC decreases or normalizes the NO-production and myeloperoxidase activity of alveolar macrophages.

Based on our findings, we can conclude, that combined therapy with PI and PC in influenza-infected mice has immunostimulating and immunorestorative effects on the functions of alveolar macrophages. Therefore, the combination of both natural products is a promising therapeutic approach for the treatment of influenza infection in experimental conditions.

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DO3. DETECTION OF THE ERYTHROVIRUS B19 (EVB19) DNA IN STANDARD AND ALTERNATIVE DIAGNOSTIC SPECIMENS (SERUM SAMPLES AND DRIED BLOOD SPOTS, DBS)

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Abstract

The **objective** of this study is to compare the specificity of two types of diagnostic source materials as etiological proof of erythrovirus B19: standard (serum based) and alternative (dried blood spots) by use of optimized protocols.

Materials and Methods. 13 serum samples and 13 dried blood spots (DBS) collected from ill individuals during acute and/or recent infection. The following methods were used: *serological* - indirect enzyme immunoassay (EIA) test for the presence of specific parvovirus B19 IgM antibodies and *molecular* methods - extraction and detection of B19-DNA. One-step PCR

technique, sequencing assay and genotyping of NS1-PCR product and electrophoresis in 2% agarose gel to visualize the PCR products were used, too.
Results and discussion. Positive IgM results were found in all tested sera samples (n=13). Positive PCR signals were detected in 11/13 (84.62%) serum samples and in 9/13 (69.23%) filter paper blood samples, respectively. Phylogenetic analyses were based on a NS1 region of 103 nt. All tested samples were genotype 1, subtype 1a.
Conclusion. In this study it is shown that blood samples spotted on filter paper are suitable for laboratory diagnosis, confirming the etiological agent and for surveillance of different diseases.

Introduction

The B19 virus was discovered by chance in 1975 by the Australian virologist Yvonne Cossart. The virus gained its name because it was discovered in well B19 of a large series of microtiter plates labelled in this way [1].

The virus generally referred to as parvovirus B19. Since 1995 erythrovirus B19, had been classified as the first known human virus in the Parvoviridae family, Parvovirinae subfamily, genus Erythrovirus. EVB19 is a ssDNA virus, 18–26 nm in diameter. B19 virus is most known for causing disease in the pediatric population. It is the classic cause of the childhood rash called fifth disease or erythema infectiosum, or "slapped cheek syndrome" [6]. However, it can also affect adults with toxo infection syndrome and arthropathy symptoms. The tropism of the virus and its different transmission (airborne, parenteral and transplacental) determines the clinical interest.

Viral replication takes place in human bone marrow cells (erythroid progenitor cells) and with a main receptor - blood group P antigen on the surface of a wide range cells. Based on the phylogenetic analysis of the DNA sequences of NS1-VP1unique junction region, human erythrovirus B19 is subdivided into three genotypes: B19V-related viruses corresponding to *genotype 1* (prototype strain Au), A6-related viruses corresponding to *genotype 2* (prototype strains Lali and A6) and V9-related viruses corresponding to *genotype 3* (prototype strains V9 and D 91.1), with no impact on the clinical course [4].

Laboratory confirmation of diseases with rash-febrile syndrome is an important differential diagnostic approach and it applies to these infections, especially in terms of mass vaccination when these diseases occur more frequently in a modified and atypical manner. Accurate laboratory confirmation depends on the proper collection, processing, shipment and storage of clinical samples, as well as the use of accurate tests by a proficient laboratory.

At this stage, it is assumed that the serum-based diagnostics is the "gold standard", while non-invasive specimens may be preferable when venous blood is difficult to collect, such as community-based or when sampling from young children and sustaining a cold-chain for samples is not feasible logistically.

The **purpose** of this study is to compare the specificity of two types of diagnostic source materials as an etiological proof of erythrovirus B19: standard (serum based) and alternative (dried blood spots) by use of optimized protocols.

Keywords: parvovirus/erythrovirus B19, serum samples, dried blood spots, one step PCR, sequencing, genotyping

Materials and Methods

Materials

1. Specimens: 13 serum samples and 13 dried blood spots (DBS) collected from ill individuals during acute and/or recent infection.

1.1. Serum samples

The blood samples were collected by venepuncture, used vacuette system and transported to the laboratory, usually under a cold chain. Storage: seven days at 2^0C - 8^0C; for longer period samples must be freezing (-20^{o}C).

1.2. Dried blood spot samples using Whatman FTA elute cards (FTA® is an acronym for fast technology for analysis of nucleic acids, a cellulose based matrix (filter paper))

Dried blood spots are collected using a sterile, autodisable lancet (1.5 - 2 mm) to obtain blood from the finger or heel of the suspected case. Blood drops are collected onto blood-collection filter-paper cards (4 circles, size 13 mm), prior labeled, and allowed to air dry 30 minutes thoroughly before being shipped (using postal service system) to the laboratory at ambient temperature in a ziplock plastic bag with desiccant packs. In the laboratory, the DNA is extracted from the blood spot using routine extraction procedure.

2. Methods

2.1. Enzyme immunoassay (indirect ELISA test) for the presence of specific parvovirus B19 IgM antibodies (*recom*Well Parvovirus B19 IgM, according to the manufacturers' instructions) was used.

2.2. Molecular methods:

2.2.1. Extraction of viral DNA from starting specimen (serum and DBS) with commercial test *NucleoSpin Blood*

Modified for DBS: Extraction procedure (pre-lyses) - punched of 6 mm disk (for each samples), put into 1,5 ml tube, add 180 ul 1xPBS, mix by pulse-vortexing for 15 seconds, incubation at 94^0C for 10 min, rpm [x10], cooling and following the standard extraction procedure.

2.2.2. One-step PCR technique *(KAPA Taq PCR Kits)* with consensus primers (concentration 20 p/mol)

*Forward Primer (**e1905f**): 5' TGCAGATGCCCTCCACCCA 3'*
*Reverse Primer (**e1987r**): 5' GCTGCTTTCACTGAGTTCTTC 3'*

Cycling parameters of NS1 parvovirus region - one step PCR:

1 cycle of 94°C for 6 min; 5 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min; 45 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s; and a final elongation step at 72°C for 7 min. Final: hold at 4°C

2.2.3. Electrophoresis in 2% agarose gel to visualize the PCR products (103 bp).

2.2.4. Purification of PCR products with *QIAquick PCR purification Kit*

2.2.5. DNA sequencing assay, with commercial kit *BigDye Terminator v3.1 cycle sequencing kit (Life Technologies)* and primers: *e1905f* and *e1987r* (concentration 5 uM).

Cycling parameters of DNA sequencing: 96°C for 2 min, 96°C for 1 min, 96°C for 1 sec, 50°C for 5 sec, 60°C for 4 min; Go to step 4 repeat 29 x. Final: hold at 10°C

2.2.6. Post sequencing PCR purification

2.3. Bioinformatic processing of results - *SeqScape v2.7, BioEdit v7.1 (Sequence Alignment Editor), MEGA v5.05*

Results and discussion

- Serological results

All sera samples (n=13) were tested by indirect EIA test for the presence of parvovirus B19 specific IgM antibodies, confirmed acute infection. 13/13 had positive IgM results.

- Qne step PCR

Viral RNA was extracted directly from the 13 serum samples and 13 DBS with modified protocol for DBS.
Positive PCR signals were detected in 11/13 (84.62%) serum samples and in 9/13 (69.23%) filter paper blood samples, respectively (Table 1 and Figure 1).

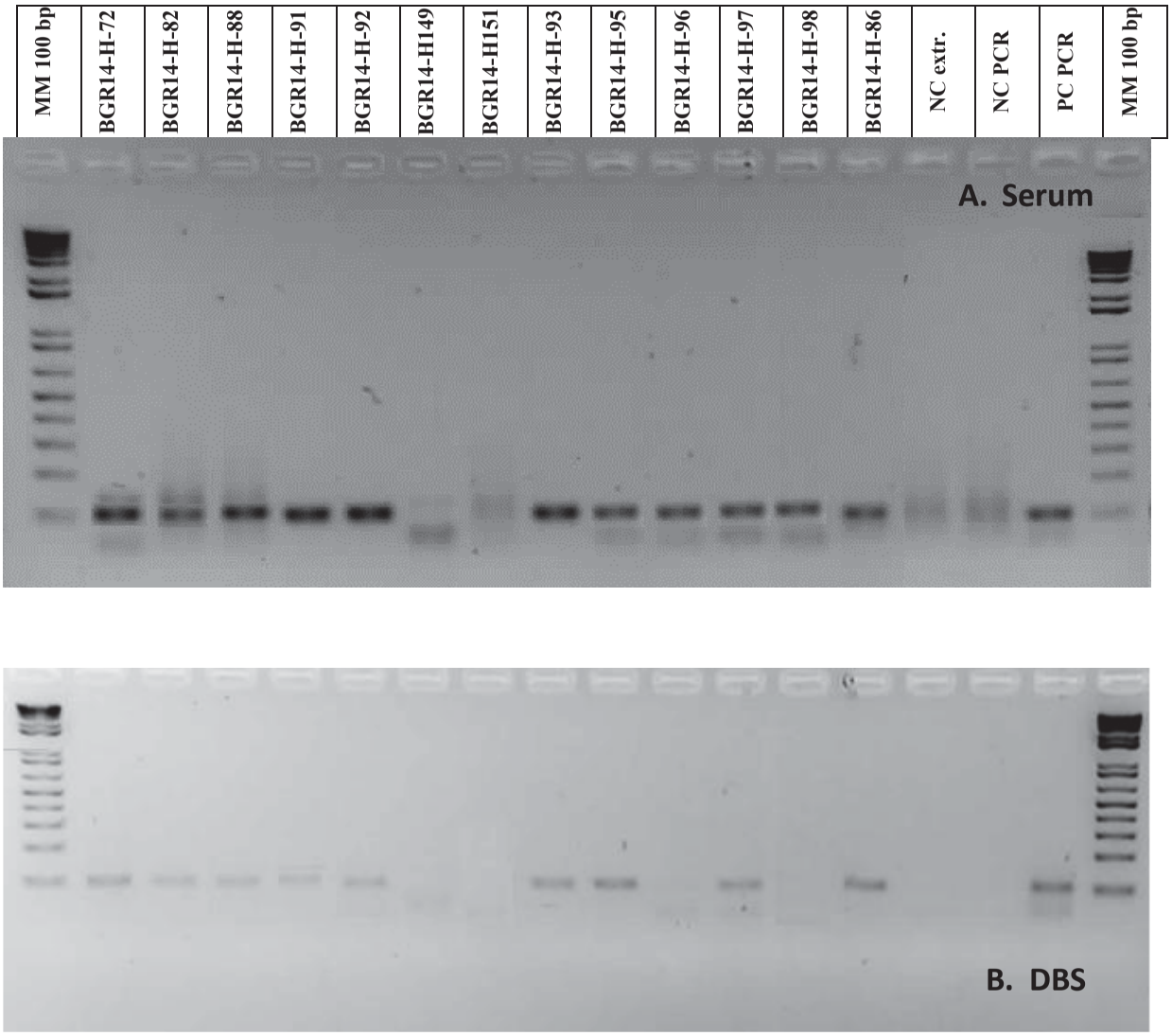
Table 1 Results of testing by one step PCR for erythrovirus B19-DNA

Tested specimens	Positive B19-NS1 region	Negative B19-NS1 region
Sera samples n=13	11	2
DBS n=13	9	4

Nucleic acid damage from nucleases, oxidation, ultraviolet light (UV) damage, microbes, and fungus is reduced when samples are stored on the FTA card which explains the high rate of detection of the B19-DNA in the DBS (2, 5).

In this study it is shown that blood samples spotted on filter paper are suitable for laboratory diagnosis of parvovirus B19 using PCR analysis.

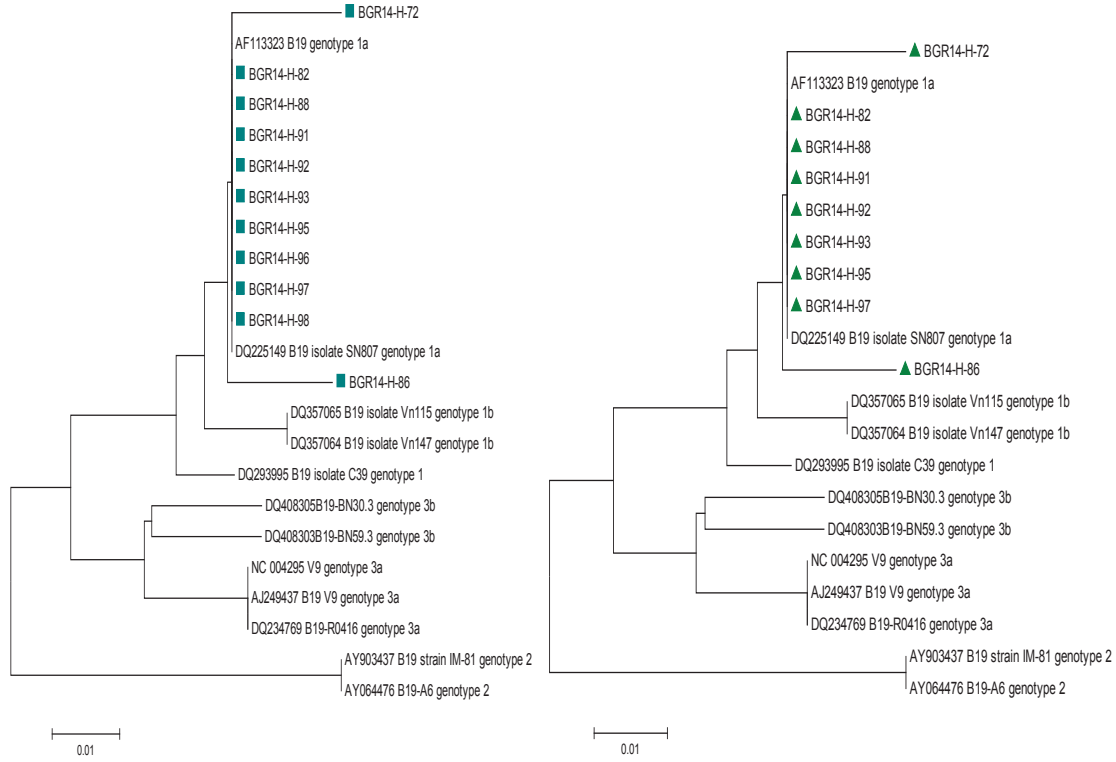
Figure 1 Electrophoresis assay (2% agarose gel) for visualization of parvovirus PCR amplicons (103 bp). Started material:
A. Serum: lanes 1, 18 - molecular marker (100bp); lanes 2-6 and 9-14 - positive samples; lanes 7, 8 – negative samples; lane 15 - negative extraction control; lane 16 – negative PCR control; 17 - positive PCR control
B. DBS: lanes 1, 18 - molecular marker (100bp); lanes 2-6, 9, 10, 12, 14 - positive samples; lanes 8, 9 - negative samples; lane 15 - negative extraction control; lane 16 - negative PCR control; 17 - positive PCR control



- DNA sequensing

Positive for NS1 region (serum and DBS based materials) samples were undergoing to sequence analysis and genotyping. All of tested samples are genotype 1, subtype 1a (Fig. 2). The study showed dominance of genotype 1. Other authors [3] report similar findings in a survey of B19 viral spread in Europe.

Figure 2 Phylogenetic trees of B19-NS1 positive samples



A. Serum based materials B. DBS based materials

Phylogenetic trees were based on: Fig. 2A - 23 sequences (11 obtained in this study and 12 downloaded from GenBank) and Fig. 2B - 21 sequences (9 obtained in this study and 12 downloaded from GenBank). The reference sequences are shown without symbols and are labeled with their GenBank accession number, name, and genotype designation. Phylogenetic analyses are based on a region of 103 nt and the neighbor-joining algorithm using the Kimura two-parameter model. Sequences are shown 3 different variants of genotype 1.

Conclusion

Both dried blood spots have been shown to have a potential role in laboratory diagnosis (confirming the etiological agent) and surveillance of different diseases.

Compared with serum sampling this sampling procedure:

. is easy to implement, although training is required;

 have good patient acceptance since the use of dried blood spots avoids venepuncture;

 have stability outside the cold chain for longer periods, easy storage and transportation.

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DO4. AVIAN RETROVIRUSES AND CANCER

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Abstract

The group of avian leucosis and sarcoma viruses (ALSVs) belongs to the *Alpharetrovirus* genus of the *Retroviridae* family. The retroviruses are enveloped RNA viruses that possess a specific enzyme called reverse transcriptase. The avian retroviruses induce various forms of leucosis, sarcomas, carcinomas and other neoplastic and nonneoplastic diseases in birds and thus have a great economic importance in the poultry industry worldwide. The *Alpharetrovirus* genus includes also the lymphoproliferative disease viruses (LPDVs) which are antigenically and genetically distinct from ALSVs and affect primarily domestic and wild turkeys. The reticuloendotheliosis viruses (REVs) are another group of oncogenic viruses isolated from birds, but they appeared to be more closely related to mammalian and reptilian retroviruses and are assigned to the *Gammaretrovirus* genus.

The present review is focused mainly on the ALSVs because they are the most common and well studied group of avian retroviruses. The genome and virion structure, strategies of replication and mechanisms of oncogenic action of the avian retroviruses are briefly described. The role of the avian retroviruses as a model system in the elucidation of the mechanisms of carcinogenesis is emphasized. The contributions of Bulgarian scientists in the field of avian retrovirology and the main directions of present studies on the viral strains Mc29 and Pts56 are outlined.

Key words: avian leucosis viruses, virus-induced carcinogenesis, myelocytomatosis, osteopetrosis, cancer models

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DO5. EFFECTS OF GROWTH TEMPERATURE ON THE MORPHOLOGY AND MOTILITY OF *ESCHERICHIA COLI* O157:H-

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Abstract

During its transmission, the intestinal pathogen *E. coli* O157 can come in contact with a variety of niches characterised by different temperatures, and of liquid or solid nature [1]. The aim of this study was to examine the effects of the growth temperature and the culture medium (liquid vs. solid, where tests permitted) on the morphology and motility of *E. coli* O157:H-, strain A2CK SS. The bacteria were cultivated at 20ºC or 37ºC, in nutrient broth (NB) or on nutrient agar (NA). To examine morphology, scanning electron microscopy (SEM) was applied [2]. It was shown that growth conditions influenced bacterial dimensions. In addition, when grown at 20ºC the bacteria released outer membrane vesicles. As predicted by the lack of flagellum, this H- strain was non-motile when inoculated in 0.3% motility-NA. Swarming motility was tested on swarmer agar - 0.6% NA supplemented with 0.02% glucose. The strain appeared to be a poor swarmer and moved no further than 0.5 cm apart from the point of inoculation. Nevertheless, it swarmed better at 20ºC when it formed a larger halo and two density waves. The presence of Type 4 pili was demonstrated by a twitching motility test [3]. This was performed by a tooth-pick inoculation into solid NA and cultivation, after which the agar was removed and the plate - stained with crystal violet. It was shown that this type of motility was significantly more expressed upon growth at 37ºC. The presence of Type 1 pili was examined by a test for mannose-dependent co-aggregation with *Saccharomyces cerevisiae* [4] The formation of aggregates including from 1 to 5 yeast cells was typical for the bacteria grown on NA at 20ºC, or in NB at both tested temperatures. When the bacteria were cultivated on NA at 37ºC, much larger aggregates were registered, involving from 5 to 20 yeast cells, indicating higher amounts of Type 1 pili.

The results show that culture conditions influence the phenotypic characteristics that are related with the surface structure of *E. coli* O157:H-.

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DO6. NEW STRATEGIES AND VACCINES FOR BORDETELLA PERTUSSIS PROTECTION

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Abstract

Pertussis is highly contagious respiratory disease. The first pertussis vaccine was developed in the 1930s and was in widespread use by the mid-1940s, when pertussis vaccine was combined with diphtheria and tetanus toxoids to make the combination DTP vaccine. It contained whole inactivated cell, which develops good immune response, mostly Th1 type. The first whole cell vaccine was high reactogenic, because of this it was replaced by new – acellular vaccine that contains reduced quantities of pertusis antigens. The latter vaccine stimulates mostly immune response of the Th2 type. All studies, connected to whooping cough, suggest that children, who were given the whole- cell vaccine, are more protected than those who have had the acellular vaccine. One of the most probable reasons for the high morbidity is adaptation and genetic changes of B. pertussis.

Nowadays, different strategies are being used for coping with the rising morbidity. A synthetic scheme was devised for preparing a conjugate vaccine composed of the B. bronchiseptica core oligosaccharide with one terminal trisaccharide. Conjugate- induced antibodies, by a fraction of an estimated human dose injected into young outbred mice as a saline solution, were bactericidal against B. pertussis, and their titers correlated with their ELISA values. Other strategy is a new pertussis vaccine for adults. For risk groups there is Cocoon strategy realization- a strategy that implies that all people, who take care of newborns, to be immunized, including women during pregnancy.

Key words: B. pertussis, vaccine, immunity

DO7. SIALIC ACID METABOLISM IN BACTERIA AND ITS RELATION TO PATHOGENICITY

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Abstract

Sialic acids are nine-carbon amino sugars which are terminal units of carbohydrate compounds of certain complex molecules (glycoproteins, gangliosides, glycolipids, mucins) (Fig. 1) [3]. They are located on the surfaces of many vertebrate cells. In eucaryotes, sialic acids participate in various cell-cell and cell-molecule interactions, including stabilizing glycoconjugates and cell membranes due to charge-charge repulsion, mediating cell-cell

regulation, acting as chemical messengers, regulating transmembrane receptor function, affecting membrane transport, etc [5].

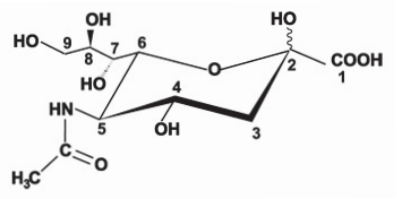

Fig. 1. Sialic acid (Neu5Ac)

Some bacteria (mostly pathogenic ones) use these substances in at least two different ways: they sialylate their surfaces thus protecting themselves from the host immune system, or they use them as carbon, nitrogen and energy source. There are two main pathways by which procaryotes obtain sialic acids: *de novo* biosynthesis or acquisition from host environment (Fig 2).

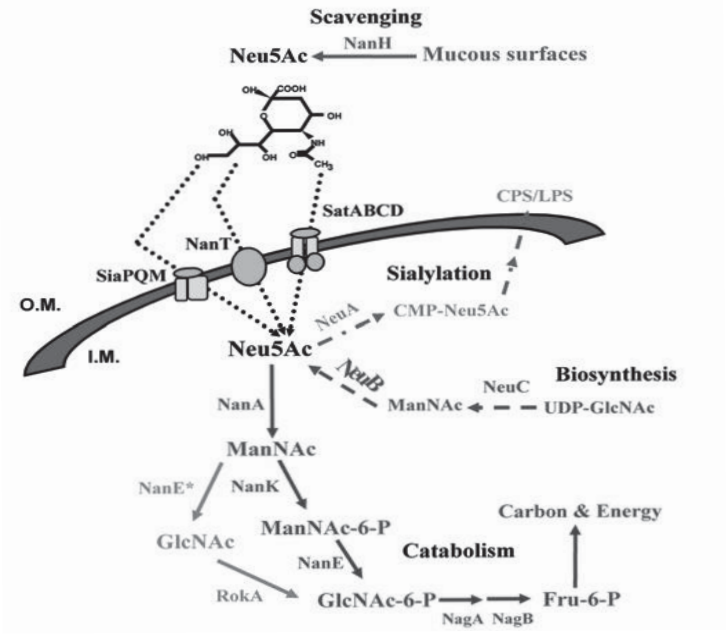

Fig.2. Sialic acid pathways in bacteria

The first route is a feature of some bacteria such as *Escherichia coli* K1, *Neisseria meningitidis*, *Campylobacter jejuni*. The precursor of sialic acid synthesis is uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc). The combined action of proteins NeuC and NeuB convert this metabolite to sialic acid via N-acetyl mannosamine (ManNAc) (Fig 2). Other bacteria acquire sialic acids directly from host environment. Some of them (*Vibrio cholerae*, *Clostridium perfringens*, *Erysipelothrix rhusiopathiae*) have specific enzyme (sialidase) which releases sialic acids from host sialoglycoconjugates. Others, which lack genes for sialidase (*Haemophilus*

*influenza*e), use sialic acids obtained by the action of sialidase-positive bacteria living in the same niche, or, as it has been hypothesized, by host sialidase, activated in the course of inflammation [4].

Regardless of the way in which they were obtained, the sialic acids enter the cell via variety of transport systems, including: classical secondary transporters, ATP-independent (TRAP) transporters, ATP-binding cassette (ABC) transporters etc (Fig 2). The effectiveness of these systems is of special importance for bacteria, such as *H. influenzae* and *Haemophilus ducreyi* which are unable to synthesize their own sialic acids and depend on external sources.

Sialylation

Once obtained (by either *de novo* biosynthesis or acquisition from the host), the sialic acid may be involved in anabolic process, which include sialylation and biosynthesis of cell surface structures. Pathogenic bacteria can "coat" the flagellum, the capsule polysacharide (CPS) or the lipopolysacharide (LPS) with sialic acids thus masking themselves from host immune system and changing host cell specificity [2]. The first step in sialylation is the synthesis of cytidine monophosphate-sialic acid (CMP-NeuAc, an activated form of sialic acid). The reaction is catalyzed by CMP-sialic acid synthetases. This compound is then added to appropriate acceptor by specific sialyltransferases. An important part of our knowledge about sialylation of cell-surface structures is based on the analyses of the polysialic (PSA) capsules synthesis process of *E. coli* and *N. meningitidis*. In *E. coli*, the activation of sialic acid prior to its incorporation into the capsule components is carried out by the enzyme NeuA. Another enzyme, NeuS, acts as polysialyltransferase adding sialic acid to oligosialic acid receptor thus forming the PSA capsule which is then exported from the cell (Fig. 2). LPS sialylation is a feature of some pathogenic bacteria including *H. ducreyi*, *H. influenzae*, *Haemophilus somnus* and *Pasteurella multocida* and this process is catalyzed by specific sialyltransferases, the main of which is Lic3A (Fig 2). This enzyme adds α-2,3-Neu5Ac and is thought to be required for bacterial survival. The Lic3A homologue Lic3B is an enzyme that adds mono- or disialic acid to the LPS acceptor. *C. jejuni* also possesses mono- or bifunctional LPS silyltransferases transferring either α-2,3-Neu5Ac or disialic acids. An interesting strategy for sialylation is used by *Neisseria gonorrhoeae* and *N. meningitidis*. These bacteria possess outer-membrane-associated sialyltransferase and use external CMP-sialic acid, which is present in small amounts as a normal constituent in human secretions (Fig 2).

Since sialic acids are common part of mammalian cell surfaces, it is hypothesized that sialylation of bacterial surface structures (LPS or capsules) represents "molecular mimicry" and helps the pathogens to overcome the action of host immune system. For example, the PSA capsule of *N. meningitidis* serogroup B and *E. coli* K1 is poorly immunogenic because of its similarity with PSA chains of the mammalian neuronal cell adhesion molecule, NCAM. In other sialo-positive serotypes the polysaccharides have no host analogs due to linkage differences and so form the basis of effective vaccines against bacterial meningitis [6]. The exact mechanisms by which the PSA capsule provides protection to the immune response are still not clarified. In meningococci, the capsule is required for resistance against the destroying effect of human serum, possibly by hindering the insertion of the complement membrane-attack complex (MAC) in the bacterial membrane. The sialylated capsule of *S. agalactiae* has inhibitory effect on phagocytosis and acts by impairing C3 deposition on the cell surface, thus preventing activation of the complement alternative pathway.

Similar to the effect of bacterial capsules, LPS sialylation also enhances the pathogenic potential of bacteria. In gonococci, sialylated LPS increases the binding to the bacterial cell surface of factor H (fH), an anti-activator of the complement alternative pathway. It is supposed

that this strategy resembles the effect that sialylation has on some eukaryotic cell membranes, which self-protect from C3 attack by displaying fH and results in higher serum resistance, although there is no strong evidence whether the sialylated LPS is an actual binding site for fH. In non-typable *H. influenzae* (NTHi), LPS sialylation inhibits deposition of C3 without requirement of fH binding. Restoration of virulence to an avirulent, sialic acid-free mutant of NTHi inoculated into complement-depleted chinchillas demonstrates the role for the complement in eliminating unsialylated *H. influenzae in vivo*. It is suggested that one possible binding site of C3 on unsialylated cell surfaces might be LPS, thus assuming the possibility that sialylation acts by masking those sites.

Catabolism of sialic acids

Some commensal or pathogenic bacteria have the ability to catabolize the sialic acids transported into cytoplasm. Among them are *C. perfringens*, *E. coli*, *P. multocida*, *H. influenzae*, *Bacteroides fragil*is [1]. The genes responsible for sialo-catabolism are organized in an operon (*nan*-operon). Once transported into the cell by NanT (permease), the sialic acid is degraded by NanA (aldolase) to N-acetylmannosamine (ManNAc) and pyruvate and the latter is ivolved in oxidative TCA cycle for energy production [6]. NanK (kinase) phosphorylates ManNAc yielding ManNAc-6-phosphate which is converted to N-acetylglucosamine-6-phosphate (GlcNAc-6-P) by NanE (epimerase). This product is further deacetylated and deaminated by the enzymes NagA and NagB to fructose 6-phosphate which enter the central metabolism [4]. The genes encoding NagA and NanG may be part of the *nan*-cluster (*H. influenzae*), or in most cases, can be scattered in the genome [1].

It is a question, how the bacteria regulate the ratio between anabolic and catabolic processes in which sialic acids are involved. There is evidence that in *H. influenzae* sialic acid catabolism competes with the LPS sialylation pathway for the sialic acids that have been transported in the cell. In *E. coli* K1 catabolism has the potential to compete with the polysialic acid (PSA) synthesis pathway [4].

There are data which relate sialic acid catabolism to pathogenicity. For example, the utilization of sialic compounds as carbon and energy source plays a significant role in colonization of the gut by *V. cholerae* isolates. It was established for *V. cholerae* and *Vibrio vulnificus* that *nanA* mutant strains form less CFUs when compared to wild-type and has decreased potential to colonize the human gut. Such strains demonstrate lower levels of cytotoxicity, reduced virulence, hampered ability of adherence to cell lines and less intestinal colonization than the wild-type parent strains. It is suggested that utilization of carbon and nitrogen source in gastro-intestinal tract is important for *in vivo* survival. A possible reason for such effects might be the interplay between synthesis of sialic acids, sialylation of the bacterial surface, and uptake and catabolism of sialic substances from the external environment [2].

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DO8. BACTERIAL SIALIDASES – FEATURES, BIOLOGICAL ROLES AND PRACTICAL APPLICATIONS

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Abstract

1. Biochemical function of sialidases.

Sialidases (exo-a-sialidases, neuraminidases E.C. 3.2.1.18.) are enzymes that selectively cleave terminal sialic acid residues from various glycoproteins, glycolipids, oligosaccharides, colominic acid and synthetic substrates. They hydrolyze α-glycosidic linkage between the second carbon atom of sialic acid and C_3, C_4, C_6, C_8 or C_9 of the carbohydrate component to which sialic acid is bound. The result of this action is the release of free N-acetylneuraminic (sialic) acid and carbohydrate containing molecule (Figure 1.).

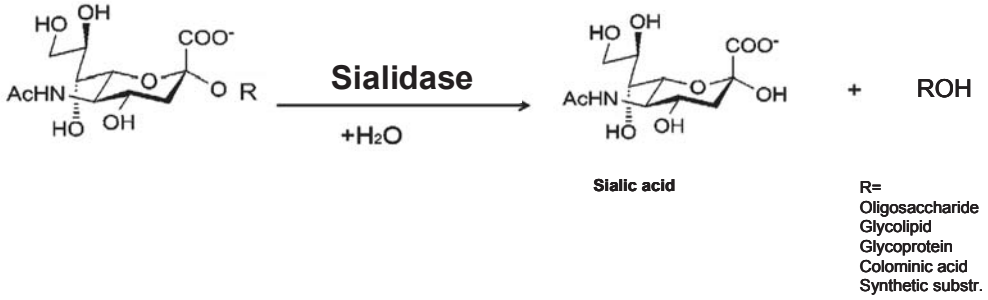

Fig. 1. Sialidase action

2. Distribution of sialidases in living organisms

Sialidase action was described for the first time in influenza viruses, when their hemaglutination effect was studied. An enzyme activity responsible for the release of influenza virus from the surface of chicken erythrocytes agglutinated by the virus particles was proposed.

Soon after this finding, it was demonstrated that culture filtrates from some bacteria (*Vibrio cholerae, Clostridium welchii*) have the same effect.

Up to now, many other microorganisms have been reported to produce sialidases. The majority of them, over 70 species, are prokaryotes from the orders *Pseudomonadales* and *Eubacteriales*. Some of the best studied producers are *V. cholerae, Clostridium perfringens, Pasteurella multocida, Streptococcus pneumoniae, Corynebacterium diphtheriae*, *Arthrobacter sialophilus*, *Bacteroides fragillis*, *Micromonospora viridifaciens*, *Salmonella typhimurium*, etc. Although rarely, the enzyme occurs also in actinomycetes and filamentous fungi [1].

Sialidases are common in animals of the *Deuterostomata* lineage (from *Echinodermata* through *Mammalia*) and in various microorganisms that are predominantly commensals or pathogens for the animals of this lineage. These enzymes and their substrates, sialoconjugates, appear to be absent from plants and most other metazoans. Sialidases are irregularly represented among bacteria and closely related species or even strains of one species differ in their ability to produce the enzyme. This unusual phylogenic distribution and some recent genetic studies implicate that sialidase genes are transferred between bacteria via horizontal gene transfer by phages.

It is suggested that sialidases have emerged in animals, which is confirmed by the presence of such homologous genes in regnum *Animalia*, hence the assumption that some microbes have acquired these genes during the coexistence with their animal hosts [3, 13].

3. Features of bacterial sialidases

Sialidases of different origin vary in their biochemical and biological properties. Most bacterial sialidases are inductive enzymes. The anaerobic representatives of the normal mammalian gut microflora are exception to this rule.

Some bacteria produce more than one sialidase isoforms with different biochemical properties and cellular location. For example, *S. pneumoniae* has three sialidases: NanA, NanB, and NanC, *A. ureafaciens* has four**,** *C. perfringens* and *P. multocida* have two sialidases, etc. One very interesting case is *M. viridifaciens*, which can express two sialidases with different molecular weights from one gene depending on the content of the nutrient media.

Most bacterial sialidases are extracellular enzymes but there are some incorporated in the cell membrane (*Haemophilus parasuis, Klebsiela pneumoniae, P. aeruginosa* or located in the cytosol (*C. perfringens, P. aeruginosa,* etc.). Typically, besides the extracellular form, there is intracellular one, and they may be products of a single gene or different genes [7].

There are five known α-glycosidic bonds in sialoconjugates: α(2,3), α(2,4), α(2,6), α(2,8) and α(2,9) [11]. Most bacterial sialidases hydrolyze α(2,3,6,8) bonds. Nevertheless, each bacterial sialidase demonstrates preference to a certain linkage or type of substrate. For example, *C. diphtheriae* has five-fold higher activity towards sialyllactose than that towards fetuin. Usually, the α(2,3) linkage is hydrolyzed to a higher extend. *Arthrobacter ureafaciens* sialidase hydrolyzes α(2,3,6,8,9) linkages, and its relative cleavage rates for different linkages are: α(2,6) > α(2,3) > α(2,8) = α(2,9).

Molecular weights of bacterial sialidases are within the range of 40-150 kDa**.** There are some exceptions like the sialidases of *E. rhusiopathiae* - 354 kDa, *P. multocida* – 250 kDa, *Clostridium chauvoei* –300kDa. Typically, larger sialidases are dimers. *B. fragilis* sialidase is composed by three subunits, 55 kDa each [1, 7].

4. Biological roles of bacterial sialidases

Most neuraminidase producing pathogenic bacteria utilize sialic acid compounds as carbon and nitrogen sources. Thus, any role of the enzyme in pathogenicity may be a secondary consequence of this trophic function. Although not obligatory, there is evidence for its

involvement in many infectious diseases like septicemia, meningitis, gas gangrene, pneumonia, cholera, and in the bacteria induced complications of other diseases like cystic fibrosis [2, 5].

Sialidase participation in the pathogenesis is in various ways. Cleaving terminal sialic acid residues of mucins it degrades the epithelial mucus layer – the first defense barrier of the host, enabling the microbe to reach the underlying tissue. In this way it becomes accessible for other enzymes that decompose host cells. Multiplication and invasion of bacteria in the tissues can be very rapid (for example, in *C. perfringens,* the causative agent of gas gangrene - 10 cm per hour). Removing sialic acids from glycoproteins located on cell surfaces, the enzyme exposes receptors for bacterial adhesins and toxins [5, 13].

In the recent years there are emerging data of bacterial sialidases influence on the host immune response. It has been demonstrated that the desialylation of leukocyte surface by *S. pneumoniae* sialidase enhances the immune activation of neutrophiles and causes inflammatory reaction. Sepsis, a common cause of mortality of hospitalized patients, is provoked by the massive over-reaction of the immune system towards bacterial pathogens what leads to tissue damage. The inflammatory reaction is enhanced by bacterial sialidases, which disrupt the inhibitory circuit in dendritic cells designed to keep immune responses in check when 'self' cells are damaged. Inhibition of sialidase activity leads to reduction in the inflammatory response and resulting morbidity in mice [10].

Another effect of microbial sialidases is anemia. Normally covered by a thick layer of sialic acids, erythrocytes loose part of this cover as a result of sialidase action. The underlying galactose receptors are a signal for the macrophages to detect such erythrocytes and destroy them. On the other hand, deprived from their negative surface charges of sialic acids, erythrocytes agglutinate what leads to thrombosis [13].

It has been established that neuraminidase contributes to the formation of biofilms. When sialidase deficient mutants of *S. pneumoniae* and *Porphyromonas gingivalis*, are used for experimental infection of mice tissues, biofilm formation is reduced [4].

A general nutritional role for bacterial sialidases can be proposed for human oral and enteric bacteria, and has been demonstrated directly in enteric microflora. Sialidase producing bacteria have been identified where no pathogenic role is expected. Many of these function in symbiotic relationships at mucosal surfaces, such as the human large intestine and the oral cavity. Others exist in situations where no role has been defined, such as in the soil bacteria *Arthrobacter* sp. Independently of sialidase relation to pathogenesis, it plays a key role in the catabolism of sialoconjugates in microbes. In this sense sialidases play dual role and, being nutritional enzymes, they may become instruments of the parasitic or pathogenic potential of bacteria [5].

As sialidases attack the 'front line'of host defense, they are a suitable target in the demand of inhibitors as antimicrobial agents. After the discovery of effective inhibitors of influenza virus sialidases, many scientists investigate their effect in prophylaxis and treatment of bacterial infections. Creation of selective inhibitors of microbial sialidases has not yet been as successful as of virus ones, because the inhibiting concentration IC(50) of most substances found until now is in the order of hundreds of micromoles. Among the great number of synthetic compounds tested, a reliable selective inhibitor of *V. cholerae* sialidase was identified recently. Potential inhibitors are sought among natural products, since they have various stereoisomers and often show highly specific biological activity [6].

5. Practical applications of bacterial sialidases

The diverse locations and functions of sialic acids in numerous structures and processes in animals and microorganisms determine the uses of sialidases in many practical aspects.

Commercial products of recombinant bacterial sialidases are available from the typical producers *Arthrobacter ureafaciens, Clostridium perfringens, Streptococcus pneumoniae, Vibrio cholerae*.

5.1. Bacterial sialidases in medicine and pharmacology

- Vaccines and neuraminidase antisera for therapy and diagnostics of infectious diseases. Bacterial sialidases have well defined antigenic properties. This feature allows the preparation of antisera of high titers after immunization of experimental animals. Monoclonal antibodies against *C. perfringens* sialidase are used for rapid diagnostics of gas gangrene. Streptococcal neuraminidase NanA, produced by all clinical isolates of pneumococci has appropriate antigenic properties and is proposed for the development of vaccines against pneumococcal infections [11].

- Glycosilation of therapeutic proteins. Basically, glycosidases cleave glycosidic linkages. Some of them, however, possess an additional activity and can catalyze a transglycosilation reaction, resulting in *formation* of glycosidic linkages. In the recent years the number of commercial products, containing therapeutic proteins has increased significantly and most of them are glycoproteins. The extent of sialylation is one of the major factors that determine the quality of the therapeutic proteins, since the terminal sialic residues prolong their half-live *in vivo*, protecting them from nonspecific or immune clearance. Various approaches are being developed to increase the sialic content of recombinant glycoproteins, one of which is enzymatic sialylation using sialyltransferases or sialidases that possess transsialidase activity [7]. Important therapeutic proteins whose efficacy in vivo is enhanced by glycosylation are medicines such as Ovidrel ® (for the treatment of infertility in women), Pulmozyme ® (for the treatment of cystic fibrosis), Xigris ® (for the treatment of severe sepsis), Cerezyme ® and Ceredase ® (for the treatment of Gausher's disease) and many other drugs [12].

- Applications of sialidases as therapeutic agents. Soon after obtaining the first relatively highly purified sialidases the first data on their effects on tumor cells were received. It was demonstrated that the enzymatic removal of sialic acid from the surface of the cell membrane enhances the immunogenicity of the tumor, and makes the experimental animals resistant to supralethal inoculations with untreated tumor cells. Considering these and other observations, Kline and Pendleton, (2010) patented a method of treating a wide variety of tumors by administering a neuraminidase solution in various forms [8].

- It was found that treatment of spinal cord injury with sialidase substantially improves the recovery of the nerve tissue and its functions [9].

5.2. Structural analyses of sialogonjugates

The linkage specificity of neuraminidases is a remarkable phenomenon that helps for determination of the structure of unknown glycoconjugates. Usually sialidases are used in combination with other exo- and endoglycosidases because of the complexity of the sugar component. Sialidases from *S. pneumoniae* and *S. typhimurium* are used mainly to analyze α,(2,3)-bound sialic acids. The broad spectrum of activity of *V. cholerae* and *A. ureafaciens* neuraminidases makes them suitable for experiments where total removal of all sialic groups is necessary in order to analyze the underlying sugar monomers. Structural characterization of oligosaccharides from proteins shows significant development since the introduction of mass spectrometry in the investigations. Preparation of samples for this sensitive method requires enzyme treatment for separation of sugar and protein components. Comparing the profiles of the sugar component and the desialylation products gives information on the types of linkages and the number of sialic residues in the sugar component [7].

5.3. Some applications of neuraminidases as diagnostic and analytical reagents in biology and medicine

- Neuraminidase from V. cholerae increases 16-fold the sensitivity of diagnostic erythro-ganglioside kits for cholera toxin by desialylation of di-and trisialogangliosides and increasing the amount of GM_1.

- An electrophoretic method has been developed for separation of the bone and the liver alkaline phosphatases in the blood plasma with the use of *V. cholerae* neuraminidase.

- Fast and efficient methods have been developed for the identification of *Actinomyces* species, capnophilic anaerobic bacilli and influenza virus through various neuraminidase tests based on the fluorogenic substrate MUN [1].

5.4. Enzymatic synthesis of homogenous sialoconjugates

Sialooligosaccharides can be isolated from natural sources but in this case they are usually a mixture of isomers with different linkage types of stereoisomers. The chemical synthesis of pure homogenous substances in sufficient quantity is difficult because of the numerous extraction steps. The presence of transsialidase activity in some sialidases makes them a desirable tool for enzymatic synthesis of pure sialoglycosides, by means of which the difficulty of chemical synthesis is avoided. Commercial products of sialidases from *A. ureafaciens, C. perfringens, V. cholerae* are applied in the synthesis of sialoconjugates using lactose and lactosamine as acceptors and sialic acid, its polymer (colominic acid) or its synthetic derivative pNP-α-Neu5Ac as donors [7].

Bacterial sialidases are enzymes which are involved in a number of vital processes in microorganisms and in their interaction with the environment, or the host. Clarification of their properties sheds light on the biology and ecology of many pathogenic and non-pathogenic bacteria and on the pathogenetic process of important diseases in humans and higher animals. Sialidases are widely applicable for various purposes in biochemistry, biology and medicine. There is an increasing interest in obtaining them in a pure form, especially from highly productive and non-pathogenic producers.

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DP1. ЧОВЕШКИ ПОЛИОМНИ ВИРУСИ ПРИ БЪБРЕЧНА ТРАНСПЛАНТАЦИЯ

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Резюме

Polyomavirus hominis 1, който е известен като ВК /BKV/ може да персистира в урогениталния тракт на човека без да предизвиква характерни клинични прояви. При определени неблагоприятни условия BKV може да премине в активна фаза на размножаване, която да доведе до органна увреда. Бъбречната трансплантация е хирургичнта интервенция ,съчетаваща различни рискови фактори за реактивация на BKV.Водещ рисков фактор е имуносупресивното лечение .В резултат от деактивацията на BKV може да се стигне до развитие на BKV асоциирана нефропатия /BKAN/. За доказване на активността на BKV след трансплрантацията се използват методи за изследване на урина-наличие на BKVв урината/виремия/ и персистиране на decoy клетки в седимента,PCR на плазмата.Резултатите показват,че 84 % от пациентите с бъбречна трансплантация отделят BKV в урината.Най-висок е този процент през първата година след трансплантацията,която е най-рискова за развитие на BKAN. Въпреки ,че процентът на ВК вирусна деактивация в началния период след трансплантация да е голям, в по-голямата част от пациентите не се установяват клинични изяви на заболяване.Тази група индивиди се определя като асимптомно отделящи BKV.Само 4% от пациентите развиват

нефропатия.Развитието на заболяването може да доведе до сериозни последствия-отхвърляне на бъбрека. От голямо значение е и откриването на BKVв плазмата на бъбречнотрансплантирани пациенти. Присъствието на ВК вирусна ДНК в плазмата говори за развитие на заболяване,тъй като BKV навлиза в кръвта след разрушаване на перитубуларния апарат на бъбрека.PCR на плазмата е чувствителен тест/100%/ за поставяне на диагноза BKAN.Изследванията показват статистически значима връзка между появата на ВК вирурия и провежданата имуносупресивна терапия.При пациенти на тройна имуносупресивна терапия вирурията е в по-голяма степен изразена, в сравнение с групата индивиди на двойна имуносупресивна терапия. Проучени са и други фактори-възраст и пол на пациента,вид на донора/жив или трупен /,наличие на съпътстваща CMVинфекция.Установено е , че нито един от тези фактори не оказва статистически значимо влияние за реактивацията на вируса .Резултатите показват , че единствено статистически достоверна връзка има между активирането на вируса и прилаганата медикаментозна схема- тройната имуносупресивна терапия усилва излъчването на вируса. Проведени са изследвания за BKV на донорно-акцепторни двойки преди трансплантация, поради факта ,че донорът може да бъде носител на латентна ВК вирусна инфекция. От друга страна при реципиента, подготвен за трансплантация също може да е налична реактивация на BKV , поради влошеното функционално състояние на собствения му бъбрек.Наличието на реактивация при част от реципиентите трябва да се има предвид и тези пациенти трябва да се проследяват и лекуват с повишено внимание.

Реципиентите трябва да бъдат обект на непрекъснат скрининг за уточняване на степента на вирусна репликация. Механизмът за повлияване на вирусната репликация е корекция на имуносупресивната терапия-намаляване,спиране или промяна на съответните терапевтични препарати.От изключителна важност е първоначалният скриниг за вирусна репликация в урината. PCR на урината в първите месеци след трансплантацията има важно диагностично значение.Положителният резултат показва наличие на вирусна репликация и трябва да се проследява и изяснява всяка промяна във функцията на трансплантирания орган.

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DP2. БАКТЕРИОФАГИ – ПРИЛОЖЕНИЕ В СЪВРЕМЕННАТА МЕДИЦИНА

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DP3. ПОЛЕЗНИТЕ БАКТЕРИИ

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Медицински факултет, СУ „Св. Климент Охридски”

Session E: Parasitology

Chairpersons:
Prof. Margarita Gabrashanska, DVM, PhD
Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences
Assoc. Prof. Svetlozara Petkova, MSc, PhD
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Secretary: Pavel Mitrenga, MSs
Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences

EO1. THE ROLE OF THE PARASITE VARROA DESTRUCTOR AS VECTOR OF VIRUSES ON HONEY BEE APIS MELLIFERA

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EO2. TRACE ELEMENTS IN BROILER CHICKENS INFECTED WITH *ASCARIDIA GALLI* AND TREATED WITH ZINC COMPOUNDS

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Abstract

The effect of dietary zinc from newly synthesized sources was investigated on the liver trace element content of *Ascaridia galli* infected chicks. Chick diet was supplemented with 0.12 g Zn^{2+} 7kg food either in the form of $2Gly.ZnCl_2.H_2O$; $Gly.ZnSO_4.5H_2O$ or $(Zn_xCo_{1-x})_4$.$(HO)_6.SO_4.2H_2O$ for 20 days. Experimental animals were divided into 8 groups: 1 - healthy and untreated; 2 - untreated and *A. galli* infected; 3 - uninfected and treated with $2Gly.ZnCl_2.H_2O$; 4 - infected and treated with $2Gly.ZnCl_2.H_2O$; 5 - uninfected and treated with $Gly.ZnSO_4.5H_2O$; group 6 - infected with *A. galli* and treated with $Gly.ZnSO_4.5H_2O$: 7 - uninfected and treated with

$(Zn_xCo_{1-x})_4.(OH)6.SO_4.2H_2O$; and 8 - infected and $(Zn_xCo_{1-x})_4.(OH)_6.SO_4.2H_2O$ treated. The contents of trace elements Co, Zn, Mn and Cu were established in liver tissue using an atomic absorption spectrophotometer. The levels of Co, Zn, Mn and Cu were reduced under *A. galli* infection in the liver tissue. The liver Zn level was significantly increased and musculature Zn level was unchanged in healthy checks treated with the Zn compounds. Excess dietary zinc in pharmacological doses restored the liver zinc losses in the infected chicks and did not influence the content of Co, Mn and Cu. The mineral balance of treated chicks were improved without any toxic signs.

Introduction

Mineral substances play an important role in the metabolic disturbances of helminthoses *Ascaridia galli* infection induces redistribution of trace elements between the internal organs and the blood [3]. Salts of these trace elements are used to correct mineral deficiencies during parasite infections. Supplemental Zn is used in diets for poultry in the form of neutral inorganic salts, however the prolonged use of these salts often leads to undesirable side-effects such as depressed growth, high mortality and others [6]. It is assumed that Zn derived from compounds with amino acids or proteins stimulates production of animals. Wedekind et al. (1992) [10] pointed to better bioavailability of Zn methionine (ZnMET) compared to that of its inorganic compounds in chickens. Recently, data exist that the treating mineral disbalance with basic salts of certain transitional elements or their mixtures that the application of basic salt of Zn is better tolerated by the chick organism than the normal salts. Benefits associated with the application of basic salts persisted even after more prolonged treatment of human and animal hosts [4, 6, 9]. Treatment with basic salts leads to improved effects on the growth, survival and the balance of trace elements in the host organisms in comparison with the normal salts.

The present study aims to examine the trace element contents in the liver of *Ascaridia galli* infected chicks after the treatment with 3 new zinc compounds: $2GlyZnCl_2.H_2O$; $GlyZnSO_4.5H_2O$ and $(Zn.Co_{1.x})4.SO_4.(OH)_6.2H_2O$.

Materials and methods

Three different sources of Zn were used {$2GlyZnCl_2.H_2O$; $GlyZnSO_4.5H_2O$ and $(Zn.Co_{1.x})4.SO_4.(OH)_6.2H_2O$}.

One day old male chick were divided into 8 groups: 1- controls (healthy animals); 2 – *A. galli* infected chicks; 3 - treated with $2GlyZnCl_2.H_2O$; 4 - *A.galli* infected and $2GlyZnCl_2.H_2O$ treated; 5 - treated with $GlyZnSO_4.5H_2O$; 6 -*A. galli* infected and treated with $GlyZnSO_4.5H_2O$; 7 - treated with $((Zn.Co_{1.x})4.SO_4.(OH)_6.2H_2O$; 8 - *A.galli* infected and $(Zn.Co_{1.x})4.SO_4.(OH)_6.2H_2O$ treated. All chicks were fed on a corn soybean meat diet formulated to meet the nutrient requirements of the growing chicks [7]. The chicks from groups 3,4,5, 6,7 and 8 received 0.1 g Zn^{2+} / kg food for 20 days starting 5 days post infection. Chicks from groups 2, 4, 6 and 8 were infected experimentally per os with 450 embrionated eggs at 14 days posthatching. Chicks were killed after 60 days. The determination of Zn^{2+}, Co^{2+} Mn^{2+} and Cu^{2+} in liver was made using an atomic absorption spectrophotometer Varian Techtran model AA 220 [1].

Results and discussion

The results of the experiment for the liver trace element content are summarized in Table 1. The induced *A. galli* infection in chicks leads to reduction of the liver trace element contents (Zn. Mn, Cu, Co). The quantity of liver Zn in infected chicks (group 2) was reduced by 43% compared to the controls. The $2GlyZnCl_2.H_2O$ supplementation increased Zn deposition in the livers of healthy chicks with 19% (P1/3 < 0.001) and $GlyZnSO_4.5H_2O$ - with 12%. The

application of basic Zn- Co salt increased the Zn level in healthy chicks with 10%. The reduced Zn content in the liver was partially restored by using of the new Zn compounds. The Zn-levels in the groups 4, 6 and 8 were higher than in group 2 (36%. 41% and 30% respectively). The liver Mn content in the infected chicks was reduced by *A. galli* infection with 34%. The supplementation of Zn did not influence Mn content in the control and infected chicks. The Co level in livers of infected chicks was reduced by 41%. The additional Zn from Zn-Gly compounds did not influence the Co liver level in healthy animals. The liver Co level was slightly increased in group 8 than that in group 2. The Cu-concentration in livers in infected chicks was reduced by 56% compared with the controls. The Zn^{2+} addition did not change the Cu-level in healthy and infected chicks.

Our results showed that the application of Zn compounds like Gly-Zn and Zn-Co basic salts per os elevated the liver Zn amount not only in healthy but in infected chicks too. Zn deposition in the liver in the infected chicks (with Zn deficiency) was higher than in the controls, received additional Zn. Gly-Zn and Co-Zn complexes may be utilized better than when there was Zn-depletion in the infected organs. These results are in agreement with the data of other authors (2, 8) about the trace elements application in diseases with elemental depletion. We found that Zn from the new Zn-compounds effectively influenced the liver Zn. No significant differences were found between the using of $2GlyZnCl_2.H_2O$ and $GlyZnSO_4.5H_2O$ as regarded tissue Zn concentration. The Zn-Co basic salt evaluates the Zn-liver concentration in healthy and infected chicks in a small extent than other compounds. It is due to its solubility between the neutral salts and the corresponding oxides or hydroxides.

Table 1. Trace element content in livers of *Ascaridia galli* infected chicks treated with Zn compounds (mg/g dry weight).

Trace Elements / Groups	Zn	Cu	Co	Mn
Group 1	163.33±16.68	27.51±1.40	1.72±0.09	5.89±1.12
Group 2	92.80±10.13 P<0.001	12.22±1.33 P<0.001	1.01±0.02 P<0.05	3.91±0.49 P<0.001
Group 3	195.07±21.19 P<0.05	26.09±3.17 P>0.05	1.66.±0.04 P>0.05	5.91±0.94 P>0.05
Group 4	126.34±15.52 P<0.05	10.98±2.11 P<0.001	1.20±0.01 P<0.05	4.02±0.08 P<0.001
Group 5	181.99±16.02 P<0.05	26.97±3.00 P>0.05	1.75±0.08 P>0.05	6.03±0.49 P>0.05
Group 6	131.40±12.48 P<0.05	10.94±1.25 P<0.001	1.12±0.05 P<0.01	3.25±0.09 P<0.001
Group 7	180.04±15.10 P<0.01	28.31±4.02 P>0.05	1.82±0.06 P>0.05	6.27±0.89 P>0.05

Group 8	119.98±21.04 P<0.01	11.05±2.27 P<0.001	1.05±0.09 P<0.01	3.42±0.78 P<0.001
Group 2 / Group 4	P<0.01	P>0.05	P>0.05	P>0.05
Group 2 / Group 6	P<0.0l	P>0.05	P>0.05	P>0.05
Group 2 / Group 8	P<0.05	P>0.05	P>0.05	P>0.05

The results of our study suggest that the addition of any of the newly applied Zn sources in pharmacological levels in chicks for a long time increases liver Zn content in healthy and *A. galli* infected chicks without any toxic signs. Zn^{2+} administrated either in the form of Gly-Zn or in the form of basic salt reduced to a large extent Zn-losses in infected with *A. galli* chicks.

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EO3. FASCIOLOSIS AND CARCINOGENESIS

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Some helminthoses are recognized as a significant factor in cancer development. However, the oncogenic potential of *Fasciola hepatica* is not certain. Data are reviewed about the combinations of fasciolosis and tumors in animals and humans. The interactions between fasciolosis and carcinogenesis are not well investigated and the received data are contradictory.

In comparison with other helminthoses, in natural conditions fasciolosis is very rarely complicated with neoplastic degeneration in humans and animals. Chapman (1999) [1] defines the chronic inflammation and the chronic alteration of the bile duct epithelium (which are observed under chronic infection with liver flukes) as a risk factor for the development of carcinogenesis of the bile ducts, but he considers that such a process is observed more rarely under *Fasciola hepatica* infection, in comparison with *Clonorchis sinensis* and *Opisthorchis viverrini* infections. The risk of the development of malignant diseases at the background of helminthoses is lower in children than in adults.

Often incorrect differential diagnosis is made of ectopic fasciolosis with malignant tumor disease.

Gavinet et al. (1997) [5] publish data about a case of tumour form of hepatic distomatosis in a 49-year woman treated by steroids for connective tissue disease. After the performing of right hepatectomy the presence of intraparenchymatous eggs (*F. hepatica*) have been found and reflected the ectopic migration of a mature fluke into the hepatic parenchyma.

A case of multiple myeloma that was under treatment with prednisolone and melphalan is reported. The patient is infected by *F. hepatica*, which involves many organs and the lesions are mistaken with metastatic ones [29].

Four human fascioliasis which are difficult to differentiate from hepatic malignancy were established in three patients, and this was misdiagnosed as common hepatic duct tumor in one patient [12]. Intrahepatic fascioliasis shows multiple ill-defined hypoattenuating lesions and filling defects of the lesion lumens on radiologic study.

Adnexal fascioliasis masquerading as ovarian cancer is diagnosed in a 49-year-old female patient admitted to our hospital with the complaint of occasional abdominal pain. Pelvic examination revealed a right adnexal mass [28].

A strange manifestation of ectopic fascioliasis mimicking a colon tumor is established [14].

Cervical tumor caused by the sexually mature stage of *Fasciola hepatica* is observed. Erratic localization of *F. hepatica* reveals a pathology involving chronic inflammation caused by a sexually mature parasite, although according to theory only immature parasites are located in ectopic lesions [16].

A rare cause of *F. hepatica* mimicking cholangiocarcinoma is reported [27]. It should be considered that the chronic phase of this zoonotic infection can be easily misdiagnosed as any other cause of obstructive jaundice.

Extra-hepatic fascioliasis with peritoneal malignancy tumor feature is observed [17]. Due to multiple clinical manifestation of extra-hepatic fascioliasis, its differential diagnosis from intraperitoneal tumors or other similar diseases should be considered.

Some authors suggest that *F. hepatica* belongs to the factors that represent neoplastic risk [6, 8]. Sriurairatana et al. (1996) [20] isolate human cholangiocarcinoma cell line (HuCCA-l) obtained from tumour of intrahepatic bile ducts with characteristics of adenocarcinoma, isolated from patient with fasciolosis. Maleewong et al. (1999) [15] isolate specific antibodies against *Fasciola gigantica*, which have been used for the diagnosis of the parasitic disease in humans. Sera from patients with other parasitic infections, healthy volunteers and with cholangiocarcinoma are also analyzed. The data indicate possible correlation of antibodies to *F. gigantica* with cholangiocarcinoma.

More of the available data about observations of malignant tumours in naturally infected with *F. hepatica* animals (mainly cattle) are very old. Galvez and Maglajlic (1956) [4] report data from different authors for cases of liver cancer in naturally *F. hepatica* infected cattle with fasciolosis but correlation between these diseases has not been proved for certain. Primary polymorphocellular anaplastic cholangiocarcinoma, within the little liver bile ducts, has been observed combined with biliar cirrchosis, fibrose cholangitis and pericholangitis at the background of chronic fasciolosis.

Vitovec (1974) [26] find out hepatocellular carcinoma in cattle under fasciolosis and its relationship to biliary cirrhosis of fasciolar origin.

Cornick (1988) [2] reports a case of a 5-year-old male llama (*Llama glama*) with gastric squamous cell carcinoma and generalized metastasis at the background of natural *F. hepatica* infection.

An increase of the mitotic activity of hepatocytes is established after the treatment with *F. hepatica* extract or implant [3, 9, 10].

A stimulation of the experimental diethylnitrosamine induced tumour growth is proved histologically, authoradiographically and statistically at the background of acute stage of fasciolosis [11, 21, 24]. Growth stimulating factor is isolated from the metabolite products of *F. hepatica* and investigated on cell cultures [13].

The CYP2A5 isozyme is known to be a participant in the metabolism of some carcinogens which are common polluters of the environment in the developing countries where the parasitic infections are prevalent. It is found an increased activity of this enzyme in the liver of *F. hepatica* infected mice [18].

Some new molecular and genetic investigations may reveal other mechanisms of the interaction fascioliaisis – carcinogenesis. Gentile et al. (1998) [8] investigate the possibilities of *F. hepatica* to provoke mutagenic events in the host tissues. When using Big Blue ® transgenic mouse assay, the authors find out that lacI mutations are twofold increased in the cells from *F. hepatica* infected mice compared with the control animals. The data present that the biological infections may enhance the genes' alteration in the surrounding host tissues. The presence of an aggressive inducing inflammation liver fluke *F. hepatica* could induce mutagenic events in mammalian tissues in mice [19]. The spectrum of the mutations in the liver of *F. hepatica* infected animals shows a significant increase in complex changes and multiple mutations (18.2%) when compared to those from uninfected control animals (2.8%).

In literature, theories are discussed about the involvement of the immune system in cancers and the possible relationship between the mammalian inflammatory response and parasite-associated cancers [7].

A cell growth inhibition of diethylnitrosamine induced experimental liver carcinogenesis is established morphologically and statistically during the chronic stage of fasciolosis in rats [21, 25]. The growth inhibiting effect of the mature *F. hepatica* is also confirmed on hepatoma 22 bearing mice [22]. Immunological and biochemical pathogenic mechanisms have been supposed for the interaction between parasitosis and carcinogenesis. Biologically active substances (BAS) (thermostabile and thermolabile), inhibitors of cell proliferation, of parasite or host origin, are isolated from *F. hepatica* tissues and the infected host liver and spleen, characterized and investigated *in vivo* on tumour bearing mice and *in vitro* on normal and tumour cell cultures (hepatocyte, lymphocyte, hepatoma MC29 and myeloma cells) [22, 23].

The cell growth inhibiting effects of BAS isolated from *F. hepatica* tissues and *F. hepatica* infected host livers are stronger than the effect of the substance isolated from the healthy rat liver. The strongest growth inhibiting effect is manifested by BAS isolated from *F. hepatica* tissues. The thermolabile BAS have the properties of immunosuppressors and specific inhibitors of cell proliferation with tissue specific and species non-specific activities.

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EO4. OPISTHORCHOSIS, CLONORCHOSIS AND TUMOR GROWTH

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Some of the chronic helminthoses are recognized as a significant factor in cancer development. Data are reviewed about the combinations of trematodoses (opisthorchosis and clonorchosis) and tumors in animals and humans. A relation between the infections and development of the tumors is established.

Opisthorchosis

The most important complication of liver fluke infection is an enhanced susceptibility to cholangiocarcinoma. After evaluating epidemiological studies, case series and case control studies, the International Agency for Research of Cancer (IARC) concluded that *Opisthorchis viverrini* is a definite human carcinogen whereas evidence for the carcinogenic effect of *O. felineus* and *Clonorchis sinensis* is more limited [8, 18]. In most regions of the world, cholangiocarcinoma is a very rare tumour. In areas where *O. viverrini* is endemic, however, the numbers of cases of cholangiocarcinoma generally outnumber those of hepatocellular carcinoma.

A number of cross-sectional or case-control studies on the association between *O. viverrini* infection and cancer of the liver have been reported from Thailand [8]. In the first large case study, an unusually high incidence of cholangiocarcinoma is observed in both the autopsy and biopsy materials taken from the patients with *O. viverrini* infection. The ratio between hepatocellular carcinoma and cholangiocarcinoma in autopsies without opisthorchiasis is 8:1, whereas the ratio is reversed among those with liver fluke infection. Similarly the ratio of these two malignancies in biopsies is 5:1 in non-infected patients and 1:2 in the presence of the fluke [8]. Similar results have been confirmed by other authors who show that the incidence of cholangiocarcinoma is almost twice that of hepatocellular carcinoma in endemic areas of *O. viverrini*, and the incidence in males is 2.4 times that in females. In another case control study, the *O. viverrini* infection increases the risk of cholangiocarcinoma fivefold [7, 8, 9, 15].

The incidence of liver cancer is observed to be correlated with the prevalence of infection with *Opisthorchis felineus* across four areas in the T'umen' region of north-west Siberia. Cases of both cholangiocarcinoma and hepatocellular carcinoma have been reported in people infected with *O. felineus* [8]. Another two patients from Siberia are reported suffering from an infection with the parasite *O. felineus* with complications in case 1 an eosinophilic leukemia and in case 2 a malignoma of the gallbladder [1].

The following possible mechanisms of carcinogenesis due to liver fluke infections are postulated:

Hyperplasia of bile duct epithelium and carcinogen exposure: Chronic irritation and chronic inflammation caused by the fluke results in hyperplasia and adenomatous changes of bile duct epithelium. These hyperplastic cells are vulnerable to carcinogen because the agent can easily induce DNA damage during active cell proliferation [8, 21].

Increased formation of endogenous carcinogen: Endogenous nitrosation caused by liver fluke infestation has been studied in both humans and animals. It is likely that N-nitroso compounds are formed in the area of chronic inflammation around the bile ducts as the result of

local generation of nitric oxide by inflammatory cells. Therefore, bile duct epithelial cells are exposed continuously to high concentrations of nitroso compounds leading to neoplastic transformation [4, 8, 21].

Activation of drug metabolizing enzymes: In male hamsters infected by *O. viverrini*, activities of hepatic cytochrome P-450 isoenzyme have been shown to be higher than those of controls especially in hepatocytes in the area of inflammation. N-nitrosodimethylamine, one of the products of endogenous nitrosation formed in the tissue, is significantly metabolized by cytochrome P-450. The product of this metabolism is a DNA methylating agent that can result in DNA damage, particularly in proliferating bile duct epithelial cells. There is a significant reduction in the levels of these enzymes after eradication of flukes by praziquantel treatment [8, 21].

Multi-factorial etiology of cholangiocarcinoma, mechanical damage, parasite secretions, and immunopathology may enhance cholangiocarcinogenesis [7, 19]. Animal studies show that in absence of carcinogens, cholangiocarcinoma is unlikely to develop in *Opisthorchis* infection. Generation of high yields of Syrian hamster cholangiocellular carcinomas and hepatocellular nodules by combined nitrite and aminopyrine administration and *O. viverrini* infection are established [20]. Promotion of N-nitrosodimethylamine-initiated bile duct carcinogenesis in the hamster by the human liver fluke, *O. viverrini* is established [3]. So these liver flukes are, for the most part, promoters and not initiators of cholangiocarcinoma [8].

Clonorchosis

There is ample evidence that *Clonorchis sinensis* is associated with cholangiocarcinoma [2, 6, 8, 9, 12]. Cases of cancer of the liver in association with infection with *C. sinensis* have been reported from China, Hong Kong, the Republic of Korea and Japan and in immigrants to North America from China and Laos. In Pusan, an area with extremely high prevalence of *C. sinensis*, the fluke increases the risk of cholangiocarcinoma sixfold. Another case control study in the same area showed that *C. sinensis* in the stool is significantly associated with cholangiocarcinoma with estimated relative risk of 2.7 [8].

Thirty-eight subjects from Hong Kong with chronic infestation by *C. sinensis* are studied. Ten of the patients die of hepatocellular carcinoma, seven of cholangiocarcinoma, and one each of carcinoma of the common bile duct and lymphoma [14].

A case is reported about a clinically unsuspected *C. sinensis* infection associated with cholangiocarcinoma in an elderly Chinese immigrant [16].

In a 32-year-old Laotian immigrant ultrasound examination reveals a posthepatic obstruction. Characteristic parasitic ova (*C. sinensis*) are present in bile fluid submitted for cytologic evaluation. Subsequent biopsy of the patient's bile duct lesion reveals a coexistent cholangiocarcinoma [13].

A case is reported of cholangiocarcinoma in a Laotian immigrant originally diagnosed with clonorchiasis [17].

A case of surgically resected intraductal variant of peripheral cholangiocarcinoma of the liver in a 46-year-old Korean man is described. Microscopically, the tumor is a well-differentiated papillary adenocarcinoma of large duct origin. Histologically *C. sinensis* infection is presented with adenomatous hyperplasia and dysplasia [10].

Cholangiocarcinomas have been successfully induced in experimental animals exposed to *C. sinensis* and treated with chemical carcinogens.

The origin and fate of small "oval" cells expressing different immunohistologic phenotypes and ultrastructural appearance are examined in livers of Syrian hamsters during cholangiocarcinogenesis induced by dimethylnitrosamine and promoted by *C. sinensis* infection.

It is proposed that the ductular-like oval cells are precursors of dysplastic ductular cells that give rise to cholangiocarcinomas after dimethylnitrosamine treatment and *C. sinensis* infection [11].

The effects of *C. sinensis* infection on induction of cholangiocarcinoma with N-2-fluorenylacetamide are investigated using female Syrian golden hamsters. The results suggest that *C. sinensis* infection has two important effects on the induction of cholangiocarcinoma with N-2-fluorenylacetamide: it increases the incidence of the tumor and it reduces the latent period for neoplastic formation [5].

Liver flukes *C. sinensis* are promoters and not initiators of cholangiocarcinoma [8].

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EO5. *IN VITRO* METHOD FOR SOME SPECIES EIMERIA IN RABBITS

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Abstract

Eimeriosis is one of the most common parasitic diseases in domestic rabbit (Oryctolagus cuniculus) causing high mortality. *In vitro* culturing in cells culture of Eimeria allows more accurate studies in the absence of a complex immune response of the host to decode a complex host - parasite interactions. The present study describes a methodology for the preparation of merozoites by oocysts of species from the genus Eimeria in rabbits. The HeLa cell culture proved to be good for the development of the stage.

Introduction

Eimeriosis is a disease in wild and domestic animals, and is caused by protozoa – genus Eimeria. It is one of the most common parasitic diseases in domestic rabbit (Oryctolagus cuniculus). It is widespread in all parts of the world, as in the European countries, the disease reaches 21-60 %. The invasion of the Eimeria reduces growth by 12 - 30%, causing diarrhea and a high death rate reaching 85%, which leads to large economic losses. The disease get sick rabbits of all ages, but it is the most serious and deadly in young aged 1 to 3 months. Eimerias are species - specific and may not cause disease in other animals.

Eimerias belongs to class Sporozoa and represent intracellular parasites which are in the epithelial cells of the intestine and bile ducts of the liver.

The first parasitic protozoa Eimeria stiedai (stiedae) were discovered by world-famous scientist Thonius Philips van Leeuwenhoek in 1674 in his unpublished letter to the Royal Society. Parasitic and pathogenic effect of Eimerias was proven in 1865 from Stieda and in the same year and from Lindeman [4, 11]. In wild and domestic rabbits are described more than 25 species Eimeria, but only 11 species have been isolated in pure culture and are well characterized [2, 5].

- *Eimeria exigua (Yakimoff, 1934)*
- *Eimeria perforans (Leocart, 1897)*
- *Eimeria piriformis (Kotlan et Pospesch, 1934)*
- *Eimeria flavescens (Marotel et Guilhon, 1941)*
- *Eimeria irresidua (Rissel et Jankiewiez, 1931)*
- *Eimeria stidae (Lindemaun, 1865)*
- *Eimeria intestinalis (Cheissin, 1948)*
- *Eimeria media (Kessel, 1929)*
- *Eimeria vejdovskyi (Pakandl, 1988)*
- *Eimeria coecicola (Cheissin, 1947)*
- *Eimeria magna (Pecard, 1925)*

In Bulgaria there are limited data on the species composition of the agents of eimeriosis in rabbits. Studies of Meshkov (1981, 1982) on eimeriosis in domestic rabbit in region of Burgas defines eight species eimerias [6,7].

Kostova (1989) conducted the most extensive research on the clinical picture, pathological, histopathological, haematological and biochemical changes in rabbits with experimental mixed infections of eimerias and spontaneous eimeriosis in rabbits. She has established the existence of six species eimerias [3].

In vitro cultivation of parasites is a method that reveals new aspects of parasitological research. The interaction between the parasite and the individual immune factors can be more easily studied in the cell cultures in the absence of the compound to the host immune defense.

For the first time Rutherford (1943) and Cheissin (1960) describe the intracellular development of Eimeria magna in rabbits, but *in vivo* observations [1, 8].

Speer and Hammond (1971) are the originators of *in vitro* investigation on the development of *Eimeria magna* to first and second generation schizonts [9]. They found that the sporozoites of E. magna after inoculation of the cell cultures were developed only to a mature second generation merozoites. One year later the same authors describe the *in vitro* development of the merozoites

to gametocytes and oocysts of *E. magna* isolated from rabbit intestine in cell cultures. Finally Speer and Hammond (1972) suggest that some of the intermediate steps required substances or conditions for further development of the shizonts, which may be found only in the natural host [10].

In Bulgaria a similar *in vitro* methodology for species of the genus Eimeria was not applied at all.

AIM

The aim of the study is the application of *in vitro* method for recreating the stage shizogony of merozoites to oocysts in some species of Eimeria in rabbits in heterologous culture systems.

Materials and methods

For the present study Eimeria oocysts were isolated and purified from the rabbit with procedure described by Coudert, Licois, Drouet-Viard (1995) [2]. The isolated oocysts were stored in a solution of 3% potassium dichromate. The oocysts were washed three times by centrifugation at 3000 rpm/min. for 5min. The supernatant was removed, and the precipitated oocysts were washed with 2 ml distilled water. Obtained samples were placed in a test tube of 2 ml. and 1 ml 13% sodium hypochlorite was added for 2 min to achieve additional sterility. The samples were washed three times by centrifugation at 3000 rpm/min. for 5 minutes. After that the supernatant was removed and the precipitated oocysts were washed to 2 ml with distilled water.

The counting was performed in the chamber of Thoma. In the sample for infection were used 1000 oocysts per 1 ml. With a magnetic stir bar they were stirred mechanically at 1000 rpm. / Min. For 5 min from the oocyst were released sporocysts, simulating gastrointestinal grinding. Samples were further treated with Streptomycin (100μg/ml) and Penicilin (200 UI) against fungi and bacterias.

In the experiment continuous cell lines HeLa (human cervical cancer cells) and MDBK (Madin-Darby bovine kidney cells) were used.

They were cultured in a liquid culture medium DMEM "low glucose" (1,000 mg / l) supplemented with 10 % FBS (fetal bovine serum) at 5 % CO_2, 70% humidity and a temperature of 37.5 degrees C.

In the experiment was used two groups of control of each cell lines (HeLa and MDBK) and two contaminated with Eimeria sp. Infection with Eimeria sp. was performed on confluent cell monolayer form both cell lines.

Photos of the cell cultures *in vitro* and the product were made by an inverted microscope OLYMPUS CR40 with lens 20x and 40x of the light box for 24h post infection.

Results

At the 24-th hour of the experiment schizonts and merozoites were revealed in HeLa cell culture (Fig.2). In addition, a reduction of the confluent cell monolayer was observed in comparison with that in the control (Fig.1).

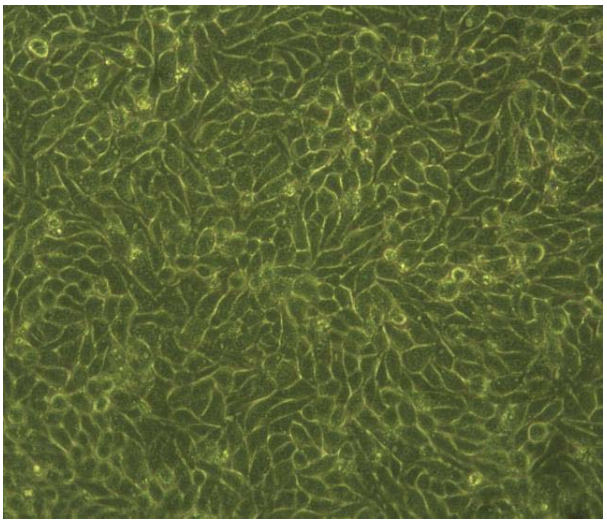

Fig.1 Control HeLa 20x - 24h

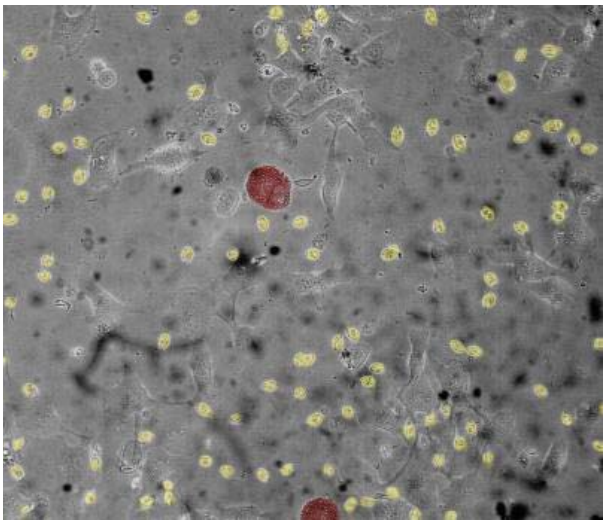

Fig. 2 HeLa infected with *Eimeria sp.* 20x - 24h

In MDBK cell culture schizonts and merozoites were observed again at 24h (Fig.4). In HeLa a noticeable reduction of the confluent cell monolayer was marked in comparison with control group (Fig.3).

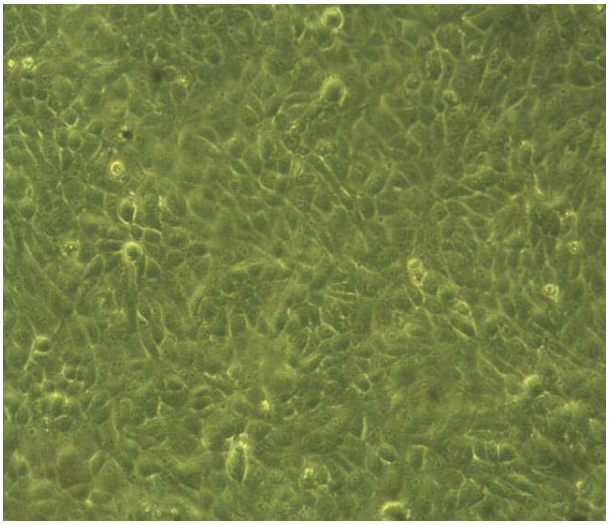

Fig. 3 Control MDBK 20x - 24h

Fig.4 MDBK infected with *Eimeria sp.* 20x - 24h

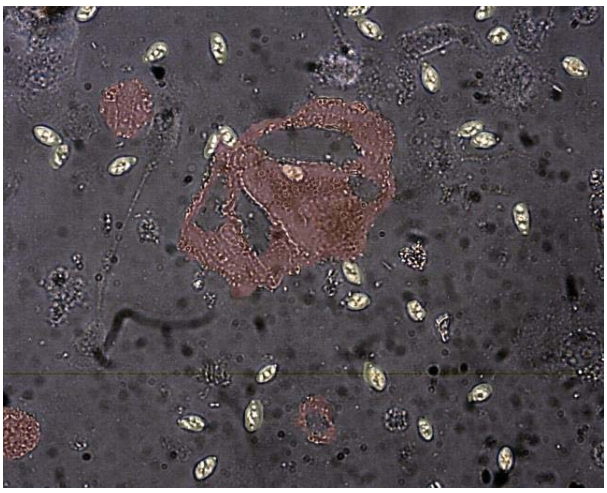

Fig. 5 HeLa infected with *Eimeria sp.* 20x - 48h

From 24 to 48 hours, there was a large number of merozoites, and after 48h the number was gradually reduced. This fact might be explained by the reduction of the cell monolayer due to the infection with eimerias (Fig.5).

Discussion

In both used cell cultures was recreated stage shizogony of merozoites to oocysts. In this experiment the HeLa cell culture proved to be good for the development of the stage. The groups were followed up to 96 hours. Macro- or micro-gamonts typical for the next intracellular stage of development of species of the genus Eimeria - Sporogony were not detected. This observation confirms received by Speer and Hammond (1971) results [9].

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EO6. PARASITES ESCAPE THE HOST IMMUNE RESPONSE

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EP1. КУЧЕШКА ТЕНИЯ

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EP2. TAENIA SAGINATA et TAENIA SOLIUM

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EP3. РИБНА ТЕНИЯ И ОПИСТОРХИДИ

Светослав Славчев, Николай Спасов

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EP4. BALANTIDIUM COLI

Евгения Ристовска, Стефния Йовинска

Медицински факултет, СУ „Св. Климент Охридски”

EP5. BALANTIDIUM COLI

Даниела Цветкова, Теодора Йорданова, Лина Абужамус

Медицински факултет, СУ „Св. Климент Охридски”

EP6. TRICHOMONAS VAGINALIS

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EP7. TRICHOMONAS VAGINALIS

Станислава Благоева, Даниела Иванова

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EP8. TOXOPLASMA GONDII

Лина Абужамус, Теодора Йорданова, Даниела Цветкова, Мария Кръстева

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EP9. TRICHINELLA SPIRALIS

Виктор Стойков, Йоанна Маламуси, Камелия Лазарова

Медицински факултет, СУ „Св. Климент Охридски”

Session: First Steps

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

ЦИРОЗА И НЕУСЕТНИЯ ПРЕХОД КЪМ ТОЗИ ПЪТ БЕЗ ИЗХОД

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РУСАЛКИТЕ: МИТ, РЕАЛНОСТ, КОНСПИРАЦИЯ

Бойка Андонова-Лилова

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Абстракт

Легендата за първата русалка датира от 1000 г. пр. Хр. и нейният произход е Близкият изток. Легендата разказва за богинята Атаргатис – майка на прочутата царица на Асирия Семирамида. Тя била влюбена в смъртен овчар. Един ден тя го погубила по невнимание. Тогава богинята от отчаяние решила да се хвърли в езеро и да прекара остатъка от живота си като риба. Но нейната божествена същност не позволила на водите да я трансформират изцяло и тя запазила тялото си на жена, но вместо крака получила рибешка опашка.). Легенди и митове за русалките се срещат във всички култури: древногръдска, египетска, европейска, арабска, славянска. За тези тайнствени същества четем в приказките на Шехеразада, гръцката митология, руската литература – Беляев „Човекът амфибия”, приказката за „Малката Русалка” на Андерсен и др. Мит или реалност са русалките?

През 1997 г. учени от Националната Агенция за Океаните и Атмосферата (НАОА), щата Вашингтон записват звук на непознат за науката морски вид, като го наричат „блуп”. [1]. Част от екипа изучавали звукът е д-р Брайън Маккормак. През

2000г. той започва проучване на влиянието на сонара върху морските бозайници. Той свързва изпитанията на сонарни технологий със самоубийства на китове. На 4 април 2004г. е най - масовото самоубийство на китове в шата Вашингтон. Д-р Брайън Маккормак и екипа му разследват самоубийствата. Учените предполагат, че сонарния звук плаши китовете и те излизат на плажа, като умират от задушаване. Но хистологичните изследвания на тъканите показват множество лезии, който най-верочтно се се получили от високочистотна инфразвукова вълна. При изследване на зуковия запис от звукоакустична станция близо до плажа на масовото самоубийство, отново е записан звука блуп. Д-р Маккормак иска спиране на сонарните изпитания, защото най - вероятно застрашават новият вид, но отговорът е спиране на Програмта за изледване на влиянието на сонарната технология върху морски бозайници под влиянието на военните сили на САЩ..

Д-р Родни Уебстър – спициалист по кмуникации на морски бозайници изследва звукът и остановява, че има много високи честоти, премахва звуците а китове и изолира само звукът блуп. Той прави откритие, че това не е звук на едно животно, а на поне 5-6 индивида между които тече комуникация..

Следващото голямо самоубиство на китове на 10 юли 2005 г. в Ю. Африка дава физическите доказатества за съществуването на новият вид.

Сред китовете на брега е изхвърлена човекоядна акула, в стомаха на която има останки от непознат вид. Около устата на акулата са открити пробождания, а в една от раните шип от мантия, който по късно се оказва част от сечиво (копие).

При изследване на тялото установяват, че гръдния кош е изграден от сгъваеми връзки като при повечето морски бозайници. Опашката наподобява опашка на ламантин, но с една основна разлика. При ренгеновото изследване установяват наличие на кости каквито в опашка на ламантинът липсват. Установяват наличие на таз и дълги бедрени кости. При изследване фалангите има много кости различни от тези на кит и делфин. По късно е установено, че това са кости от ръка. Важен е фактът, че на таза се открива илиачен ръб, които е характерен за животните ходещи на два крака – човекът. Тазът, дългите кости, ръка доказват, че това са костини от хуманоид, наш роднина, но не прародител, а съвременик.

При реконструкция на черепа се установяват големи орбити, характерни за дълбоководни животни. Също много по добре развити слухови органи, звуков отвор на върха на черепа за ехолокация.

Резултатите от ДНК доказват, почти пълно съвпадение с човешката. [2]

Всички тези доказателства са иззети от военните. Защо???

Най-обедителното доказателство за съществуването на този хуманоид идва от д-р Торстен Шмит – морски геолог, който картографира морското дъно на Норвежко море за сондажи на нефт и газ. Заснема с камера русалка на 6 март 2013г. Доказателства са приети от Датското правителство, че съществува нов вид бозайник и сондажите са спрени. [3].

1. http://ru.wikipedia.org/wiki/Bloop
2. http://www.sciencedirect.com/science/article/pii/0306987795902708
3. https://www.youtube.com/watch?v=BD1oACFz7zM

Session F: Neurobiology

Chairpersons:
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Institute of Neurobiology, Bulgarian Academy of Sciences

Assoc. Prof. Lyubka Tancheva, MSc, PhD
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FO1. INVESTIGATION OF ACTIVITY OF SOME GALANTAMINE PEPTIDES

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Abstract

Galanthamine is a drug often applied in neurological practice. Galanthamine possesses acetylcholinesterase and antioxidant activity, but didn'n inhibit γ – sectetase. Galantamine is considered to be highly toxic (1 - < 50 mg/kg) in accordance with Hodge Sterner scale based on its LD_{50} = 24 mg/mg in per orally treatment of mice.

For the improvement of its pharmacological profile, decreasing of toxicity and increasing of its bioavailability a new class derivatives: Galantamine peptides is developed.

Some of newly synthesized from prof. Vesenkov peptides: 6-O-N-[N-(3.4-dichlorophenyl)-D,L-Alanyl]-L-Leucyl-Glycil-Galantamine (GAL – LEU) and 6-O-N-[N-(3.4-dichlorophenyl)-D,L-Alanyl]-L-Valil-Glycil-Galantamine (GAL – VAL) possess both acethylcholinesterase and γ – secretase inhibitory activity and antioxidant properties. Toxicity of peptides is 3 times lower than Galantamine.

The aim of current study is the investigation of cytotoxic activity of peptides against different cell lines in vitro. In the experiment are used cell lines: HeLa, PC3 и 3T3. Peptide esters are applied in exponentially increasing concentration in accordance with standard MTT test of Moosmann.

Compounds exert good cytotoxic activity. The values of IC_{50} for GAL-LEU are: 23.63 μM (HeLa); 19 μM (3T3); 28.10 μM (PC3); and for GAL-VAL are: 31.95 μM (HeLa); 23.17 μM (3T3); > 30 μM (PC3). Results prove that both esters exerts cytotoxic activity against the examined cell lines and that in comparison with GAL – VAL, GAL – LEU possesses higher antiproliferative effect due to its lower IC_{50} values.

The effect of peptides is higher in comparison with the activity of Zidovudine against cancer cell lines. The mechanism of peptide cytotoxic activity is still unclear and more future investigations are needed.

The results for good cytotoxic potential of the examined compounds and the data for their low toxicity give the reason for continuation of investigations in connection with the aim for clarification of their exact mechanism of action.

FO2. EXPERIMENTAL RESEARCH ON PHARMACOLOGICAL AND TOXICOLOGICAL EFFECTS OF NEWLY SYNTHESIZED NEUROPEPTIDES WITH SHORT CHAINS

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Abstract.

The aim of the study is to investigate the basic pharmacological and toxicological effects of two new neuropeptides (P1 and P2), analogues of Tyr- MIF– 1 and nociceptine, synthesized by T. Pajpanova, (2013). Neuropeptides were applied on Albino male ICR mice in several doses (5, 10, 20 and 50 mg/kg b. wt. i.p.). Their activity on the CNS was studied as well as their effect on the hexobarbital sleeping time (HB– 100 mg/kg b. wt. i.p.). We studied the influence of the two compounds on the nociception in mice (test with acetic acid) when effective doses (4, 8 and 16 mg/kg b. wt. i.p.) were applied. The new neuropeptides demonstrated several suppressive effects on the CNS when a dose of 50 mg/kg b. wt. was applied, which disappeared within 48 hours. There was no mortality after the acute treatment – both on the 48th hour (acute toxicity) and on the 5th day (prolonged toxicity). Pathological changes in the internal organs of treated animals were not found. P1 and P2 have similar effects on HB narcosis (P1 shortens it by 40%, and P2 by over 50%), but the mechanism is unknown. It is possible that the substances accelerate the elimination of HB or have modulating CNS effect which could be related to their neuropeptidic nature. On the other hand the compounds had different effects on the nociception– P1 increases it (by 262%) and P2 has significant analgesic effect (by over 25%). The analgesic effect of P2 is dose-dependent. We suggest that it is related to its nociceptine structure and due to possible interaction with CNS receptors.

Keywords: toxicity, neuropeptides, hexobarbital, nociception

Introduction

Searching for biologically active peptidomimetics as a new direction in modern pharmacology requires complex, interdisciplinary researches. The modern drug design creates medicaments on the basis of well-known active peptides with improved pharmacokinetic properties [3], [4], [6].
Object of this study is two new short-chain neuropeptides, synthesized by T. Pajpanova, (2013), analogues of Tyr- MIF (P1) and Nociceptine (P2).

Our previous data found activity of similar compounds on central nervous system (CNS) [1], [5]. Having in mind their molecular design - very similar to some neurotransmitters in the central nervous system we decided to study their effects on central nervous system (CNS).
The aim of the study is to establish some basic pharmacological and toxicological effects of the new neuropeptides (P1 and P2) on laboratory mice.

1. Materials and Methods

1.1. Chemicals

The new compounds were synthesized by Pajpanova et al. (2013) in the Institute of Molecular Biology at Bulgarian Academy of Sciences. Acetic acid and Hexobarbital sodium salt were provided by SIGMA-ALDRICH.

1.2. Amimals

Albino male ICR mice (body weight 18–20 g) were supplied by Experimental Breeding Base-Slivnitza at the Institute of Neurobiology (Bulgarian Academy of Sciences). Animals were housed in plexiglass cages (6 per cage), under standard laboratory conditions (ambient temperature 20°C ± 2°C and humidity 72 % ± 4 %), water and standard pelleted food ad libitum. All performed procedures were approved by the Institutional Animal Care Committee and the principles stated in the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS123) were strictly followed throughout the experiment.

1.3. Methods

1.3.1. Toxicological studies

Some basic toxicological characteristics of neuropeptides applied on Albino male ICR mice (18-20 g) in several doses (5, 10, 20 and 50 mg/kg b. wt intraperitoneally- i.p.) were studied.
The observation on their effects and toxicity was till the 48th hour. The studies for prolonged toxicity continued till 5th day following changes in body growth, appetite and behavior. Dissection of the bodies was performed on the 48th hour and on the 5th day after compounds application.

1.3.2. Effects of the new compounds on CNS

The activity of the new compounds on the CNS was studied evaluating their influence on the hexobarbital sleeping time (HB – 100 mg/kg b. wt.- i.p.). Hexobartal was used as central nervous active agent, but also as known model substrate of hepatic cytochrome P-450 monooxygenases. An effective dose 5 mg/ kg i.p. of new compounds was applied 20 minutes before Hexobarbital administration. The changes in duration of HB sleeping time (in minutes) were estimated in the groups according to the reflex of reversal.

1.3.3. Studies for analgesic effect

The two compounds applied in an effective dose of 5 mg/kg were studied for analgesic activity using Acetic acid test [2]. The number of abdominal cramps for 20 minutes after acetic acid application was measured. Dose-effect analgesic activity of compound P2 was studied in doses 4, 8 and 16 mg/kg b. wt. i.p. according to the same method.

1.4. Statistical analysis

Results were performed using t-test of Student Fisher.

2. Result and discussion

Acute Toxicity

We established low acute toxicity of the both compounds. On the 48th hour after the application of the studied doses (over 50 mg/kg b. wt.) mortality was not observed. With an acute dose of 50 mg/kg b. wt. the compounds produced transit ataxia, respiration changes and sedation.We did not find any changes in the body growth, appetite and behavior of animals

after treatment with P1 and P2 (in doses 5, 10 and 20 mg/kg i.p.) compare to the controls. Compounds are pharmacologically active when a dose of 5 mg/kg i.p. is applied.

Studies for prolonged toxicity- on 5th day after administration

Prolonged toxicity was not established on the 5th day. Pathological changes in the internal organs of the treated animals were not observed either.

Influence of compounds on the HB-sleeping time

Surprisingly we established in our experiments that P1 and P2 decreased duration of HB narcosis (P1 shortens it by 40%, and P2 by over 50%), but the mechanism is still unknown (Fig. 1).

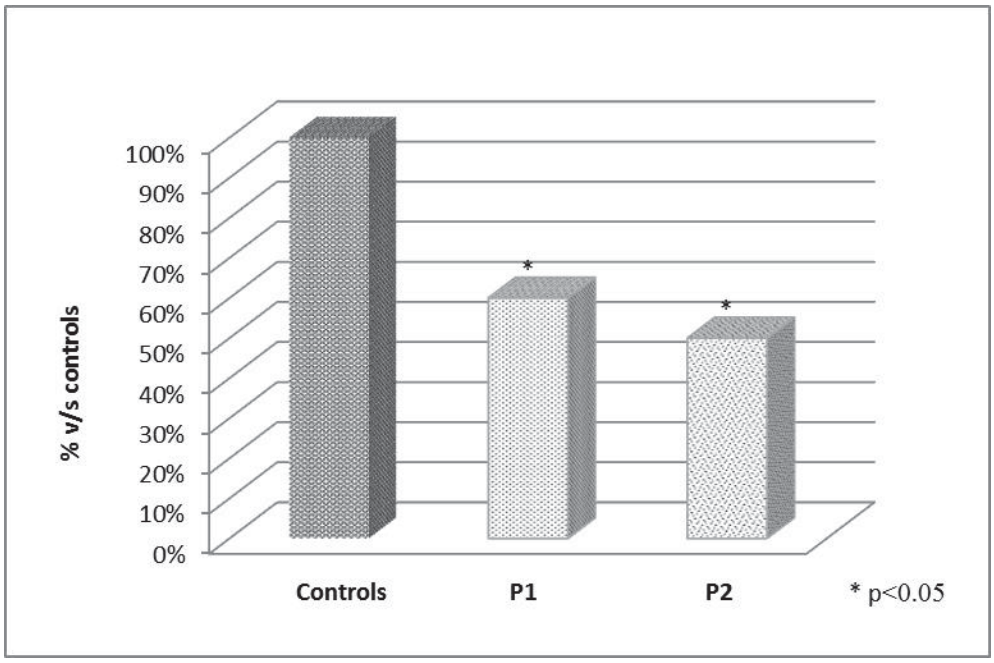

Figure 1. Effect of P1 and P2 on Hexobarbital sleeping time

It is possible that the new substances accelerate the elimination of HB via its hepatic metabolism. But we suggest that established drug interaction can be due predominately to functional antagonism between HB and new neuropeptides on the level of central nervous system and/or to receptor interactions. Only further experiments can clarify whether the mechanism of this interaction is on the metabolic level or on central nervous system level.

Analgesic effect

We established in our experiments that the new compounds had different effects on the nociception– P1 increases it (by 262%) and P2 decreased it (by over 25%). Established nociceptive effect of compound P1 is probably due to increased local irritation in animals after combine administration of the compound with acetic acid. Our experiments also demonstrated that compound P2 has a significant analgesic effect- and this effect is dose-related (Fig. 2 and 3).

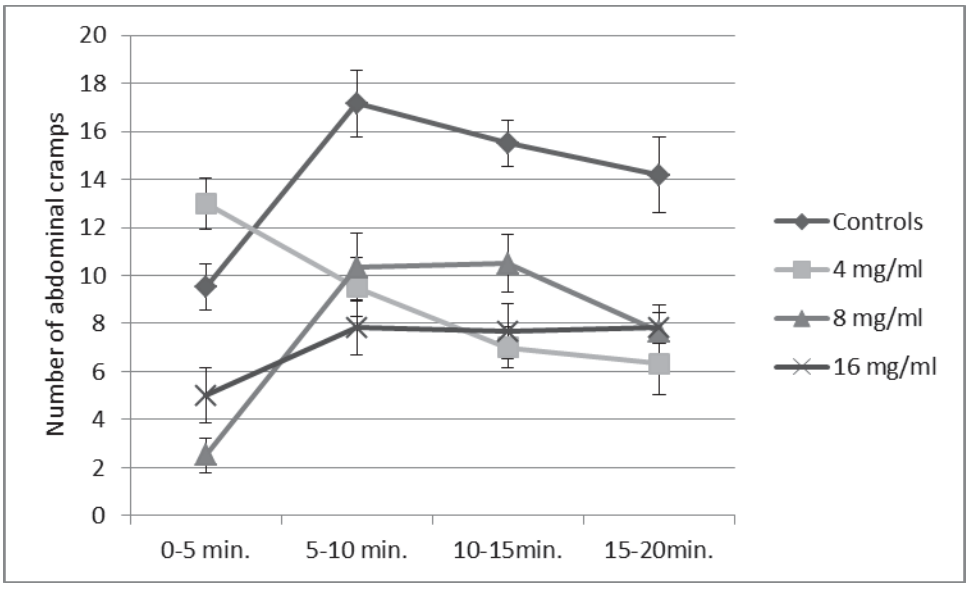

Figure 2. Dose dependent analgesic effect of P2 according Acetic acid test ($p<0.05$)

The analgesic effect (% vs controls) of the 3 doses of compound P2- (4, 8 and 16 mg/kg b. wt. i.p.) was significant and dose dependent in comparison to the control group (accepted for 100%)- Fig. 3.

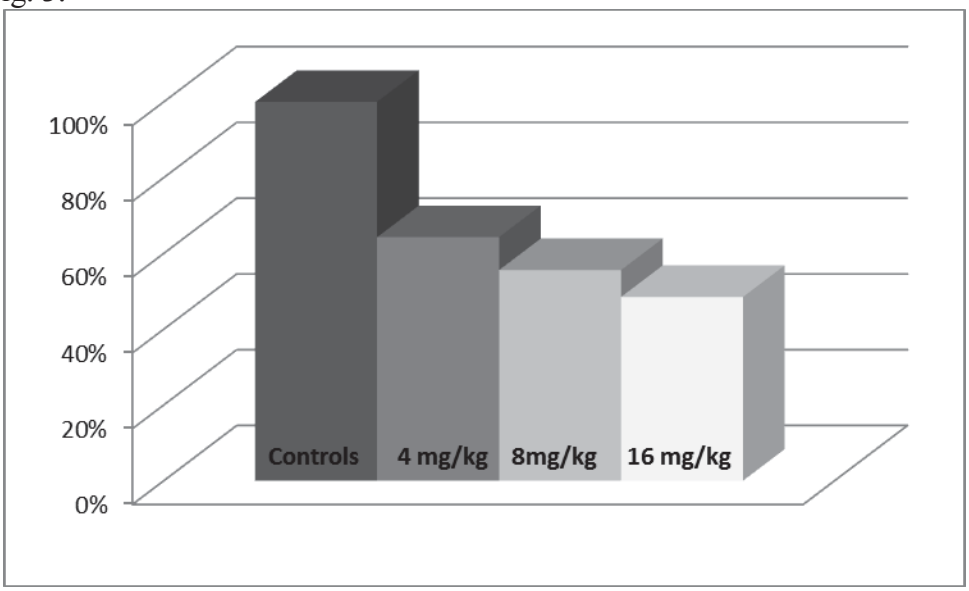

Figure 3. Dose-dependent analgesic effect of P2 (% v/s controls) $p<0.05$

The mechanism of the analgesic effect of P2 is not clear. We suggest that it may be related to the chemical structure of P2 close to this of mediator nociceptine. Pain modulation probably is a result of possible interactions with some CNS receptors and deserves further experimental studies (Mateeva et al. 2011, Pancheva et al, 2003).

3. Conclusion

Newly synthesized neuropeptides demonstrated low toxicity and significantly antagonized HB sleeping time in mice. Compound P2 also has a dose-dependent analgesic effect and as a derivative of nociceptine deserve further studies.

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FO3. REVIEW OF PHARMACOLOGICAL ACTIVITY OF ELLAGIC ACID WITH FOCUS ON CENTRAL NEVOUS SYSTEM AND ANTIVIRAL EFECTS

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Abstract

Pomegranate is one of the plants with proven beneficial health effects in various conditions; it contains very high concentrations of ellagic acid in all plant parts. Numbers of studies have been conducted demonstrating that the effects of pomegranate extracts are contributed primarily to ellagic acid.

Ellagic acid is a phenolic compound and is found in various plant species. Pharmacological properties of ellagic acid alone and ellagic acid-rich plant extracts have been scientific interest for decades. However, mechanisms of ellagic acid effects are not fully

studied. It is proven that the compound demonstrates strong antioxidant activity, thus having beneficial health effects against free-radical chronic diseases such as cancer and cardiovascular diseases. Effects of ellagic acid include anticancerogenic, antimutagenic, cardioprotective, hepatoprotective, anti-lipid peroxidative, anti-inflammatory, antiviral, as well as various central nervous system effects. Studies on influence of ellagic acid on central nervous system as well as its effect on viral infections are relatively new and very promising. This review covers the most studied ellagic acid effects with special focus on experimental studies on CNS-effects and antiviral activity.

Introduction

Ellagic acid (EA) is a natural phenolic lactone present in variety of plants and especially fruits. It is found at high concentrations in blackberries, cranberries, pecans, pomegranates, raspberries, strawberries, walnuts, wolfberries, grapes, walnuts and pecans. EA is found in significant concentration in *Punica granatum L.* – pomegranate. The fruit and extracts from different parts of the plant have been studied for their beneficial effects in various diseases. Part of these studies has recognized EA as primarily responsible for the beneficial effect of the extracts.

The most abundant polyphenols in pomegranate juice are the hydrolysable tannins called ellagitannins formed when EA binds with a carbohydrate. Pomegranate ellagitannins, also called punicalagins, are tannins with free-radical scavenging properties in laboratory experiments [21]. Due to the similar effects of EA alone and pomegranate extracts we will review some studies on CNS and antiviral effects of various pomegranate extracts.

Many studies have suggested that the biological functionality of EA is associated with its direct strong antioxidant activity which counters the negative effects of reactive oxygen species (ROS) that are generated during cellular metabolism and in response to chemical and environmental stresses. Different studies have also suggested that EA can aid in the regeneration of cellular antioxidants such as glutathione (GSH), activate Phase-II enzymes such as gluthathion-S-transpherase (GST), inhibit DNA topoisomerase, mediate cell cycle arrest and activate apoptotic pathways leading to reduction of cancer and other chronic diseases [1, 2, 20, 47].

Pharmacological properties of EA

Anticarcinogenic activity and underlying to this effect antioxidative property are one of the first properties of EA that have been studied and are subject of scientific interest for the past three decades. EA has been shown to be a potent anticarcinogenic agent [5], and one of the main mechanisms by which EA is proposed to have anticancer benefits is by modulating the metabolism of environmental toxins and therefore preventing the initiation of carcinogenesis induced by these chemicals as well as inhibiting the direct binding of carcinogens to the DNA. Moreover, EA significantly increases GST activity, thus enhancing the GST detoxification system and therefore demonstrating strong chemo protective effect [47]. Another mechanism of anticarcinogenic activity includes induction of G1 arrest and inhibition of overall cell growth and apoptosis in tumor cells [30]; inhibition of xenobiotic metabolizing enzymes and the induction of antioxidant responsive element-mediated induction of NADPH: quinone reductase and GST genes[1, 2] etc.

Antimutagenic activity. EA was found to inhibit the mutagenesis induced by aflatoxin B1 in *Salmonella* when incubated together with the aflatoxin B1. This supports the hypothesis that the mechanism of inhibition could involve the formation of a chemical complex EA on Aflatoxin B1 [24].

Anti-inflammatory and protective effects observed in model of Crohn's disease are proposed to be related to the reduction of neutrophilic infiltration in the colonic? mucosa accompanied by an increase in the production of mucus in goblet cells [37]. Protective effect against carrageenan-induced acute inflammation is observed as well [29].

Hepatoprotective effects were observed following orally administered EA against carbon tetrachloride [42] as well as against ethanol-induced toxicity in hepatic HepG2 cells [44]. Regulation of NO and TGF-β1 production in liver cells is the suggested mechanism of the later.

Cardioprotective and anti-lipid peroxidative properties are demonstrated EA's in isoproterenol-induced myocardial infarction in rats. This study provides evidence for the cardioprotective effect of EA based on its membrane stabilizing effect on lysosomes and membrane bond ATP-ases and also significant decrease of pro-inflammatory cytokines level [18, 19].

Anti-lipid peroxidative. EA has shown to be superior to vitamin E in study comparing the decreasing lipid peroxidation in embryonic and placental tissues in developmental toxicity and oxidative damage in embryonic/fetal and placental tissues [13].

Antiviral properties

Hepatitis B virus. Antiviral properties of EA alone have been described for their anti-Hepatitis B effects [18, 42]. The base of the studies describing EA as anti-hepatitis agent are the initial reports for the anti-Hepatitis B effects of extracts from different species of tropical plant *Phyllantus amarus (P. nuriuri)* - a plant widespread in India and China used for decades in treatment of hepatitis and jaundice. Studies on its effects [22, 50] show that the extracts down-regulate the transcription and replication of the virus via inhibiting Hepatitis B virus (HBV) polymerase and also inhibit *HBsAg–anti-HBsAb* in vitro [48].

Later study isolates EA from *P. urinaria* and identifies this substance as the carrier a pharmacological effect of the extract [41]. EA showed specific antiviral activity, by inhibiting *HBeAg* secretion, in HBV-infected cells. The results of the study suggest that HBV host tolerance could be overcome with this mechanism. Researchers later show additional data to that indicate that immune tolerance caused by *HBeAg* with high chronicity rates can be overcome by use of EA [17].

HIV. Studies on *Phyllantus nuriuri* extracts has identified that EA possesses the inhibitor activity for HIV-1-reverse transcriptase [32] and HIV-protease [31].

Influenza virus. A recent study on effects of *Aronia melanocarpa* in influenza virus attributes the main anti-infective role to EA and myricetin as components of *Aronia* extracts.

In this study, *Aronia* extract showed reactivity against different influenza viruses including oseltamvir-resistant H1N1. The antiviral properties of pomegranate polyphenols are attributed to inhibition of RNA replication of the influenza virus [10], however mechanisms of action are not completely well understood [47].

Effects on central nervous system (CNS)

Anxiolytic effect. Anxiolytic-like effects of dietary flavonoids are relatively well known [25]. The involvement of the GABA-ergic and serotonergic system in the antianxiety like activity of EA has been also. Compared to diazepam, the anxiolytic doses of EA did not prolong the duration of sodium thiopental-induced loss of righting reflex, indicating that this substance is non-hypnotic.

Study results demonstrated that acute and chronic administration of EA to mice has produced anxiolytic effect when tested in the elevated plus-maze. The tests with different receptor blockers suggested involvement of GABA-ergic system in the anxiolytic action of this flavonoid [15]. Another study shows reduction of nicotine induced withdrawal syndrome in mice [48, 49].

Antidepressant activity is reported for EA with acute or chronic administration to mice (25, 50 and 100 mg/kg p.o.) producing a significant reduction in the duration of immobility, with a profile comparable to that of fluoxetine. The study provides evidence that antidepressant activity of EA is dependent on the interactions between serotonergic and noradrenergic systems but does not involve the opioid system. Future experimental and

clinical trials may be needed to determine whether EA will produce similar therapeutic effect in depressed patients [7].

Antinociceptive effect. Dose related antinociceptive effect of EA in different animal models is reported in a recent study. Study results indicate that dose-related antinociceptive action of EA has both central and peripheral components which involve mediation by opioidergic system and L-arginine-NO-cGMP-ATP sensitive K^+ channels pathway. Antinociceptive effect was reached at dose of 302 mg/kg p.o. via intraperitoneal route of administration [27].

Alzheimer's Disease. A study revealed that EA promotes Aβ42 fibrilization and inhibits Aβ42-induced neurotoxicity [44].

Smaller soluble oligomers of β-amyloid oligomers play a critical role in the pathogenesis of Alzheimer's disease (AD). Selective inhibition of Aβ oligomer formation provides an optimum target for AD therapy. This study shows EA dose-dependently decreased levels of pathogenic Aβ oligomers and Aβ cytotoxicity, consistent with the hypothesis that plaques represent a protective mechanism for driving toxic Aβ oligomers to less toxic fibrils [9]. Moreover, these study results suggest a mechanism by which the intake of naturally rich in EA pomegranate juice decreases soluble Aβ42 levels and attenuates cognitive deterioration in [11].

Neuroprotection in Diabetes. EA has shown to attenuate oxidative stress on brain and sciatic nerve and to improve histopathology in streptozotocin-induced diabetic rats. The levels of oxidant/anti-oxidant parameters investigated were significantly decreased in EA treated diabetic rats in comparison to control group. According to histopathological results brain sections from diabetic rats (control group) showed nuclear hydropic degenerative changes in neurons and fibrillary degenerative changes in the tissue. Hemorrhagic focuses and damaged blood vessels were seen in the brains of all diabetic rats, however significantly decreased in diabetic rats treated with EA. Further investigations are needed to confirm EA potential to treated diabetes associated neurological complications [49].

Pomegranate juice

Pomegranate juice contains multiple phenolic compounds such as anthocyanins and ellaginannins (ETs). However, ETs comprise most of the polyphenols found in commercial pomegranate juice [54]. A study investigating the pharmacokinetics of ETs and EA in human plasma following oral administration of ellagitannins confirms EA absorption and presence in human plasma. However, no intact forms of ellagitannins were detected in any of the collected plasma samples [39].The pharmacokinetic profile of EA following oral administration in rat indicates poor but rapid absorption, rapid distribution and elimination [23, 51].

The goal of many pomegranate studies has been to identify the therapeutic components [16]. Different studies identify EA to be the marker compound of the various pomegranate extracts and exhibits powerful anticarcinogenic and antioxidant properties [12]. However, confirmation for superiority of pomegranate extract over EA has been obtained in a study investigating the topical anti-inflammatory and analgesic activities of standardized pomegranate rind extract in comparison with EA alone in vivo. The study results show that standardized pomegranate rind extract is superior over EA in terms of anti-inflammation and IL-β modulation [28].Studies also reveal data for synergism between EA and other polyphenolic compounds which serves as confirmation of the latter [38].

Pharmacological effects of pomegranate extracts

Pomegranate extracts (PE) and particular constituents have shown various pharmacological effects including antioxidant, anticarcinogenic, anti-inflammatory, maintenance of cholesterol levels through influencing of lipid peroxidation, [16] antiviral [10]

anti-malaria [43] etc. The current review will focus on effects of EA and other PE constituents on central nervous system.

Memory impairment. A randomized placebo-controlled, double blind trial of pomegranate juice in older adults with memory complaints suggests that 8 ounces (approximately 250 ml) of pomegranate juice taken daily for one month improves the verbal memory and influences neural activity during a visual source memory task. Methods included memory testing and functional brain activation (fMRI) as outcome measures. After 4 weeks, only PE group showed a significant improvement in verbal memory. Furthermore, compared to the placebo group, the PE group had increased fMRI activity during verbal and visual memory tasks. The results suggest a role for pomegranate juice in augmenting memory function as well as increases in task-related cerebral blood flow [3].

Alzheimer's disease. Pomegranate juice protective effects against amyloid load were investigated in a study involving transgenic mouse model - Amyloid precursor protein (APP) over expressing transgenic animals with early onset familial AD and Aβ deposition start at 7-8 months of age). The animals were given pomegranate juice since the age of 6 months and behavioral testing began at the age of 11.5 months without interrupting juice consumption.
Results of the study show improved behavioral performance of pomegranate juice fed APP mice in water maze compared to control APP group.

Pomegranate juice APP group also showed decreased Aβ and amyloid levels in the hippocampus. There has been non-significant but consistent trend for less neuritis cases associated with amyloid plaques in the dorsal cortex, hippocampus and corpus callosum.
Study results suggest that the mechanism by which pomegranate juice influences behavior and amyloid load is not by altering APP processing or Aβ production [11].

To examine the effects of *P. granatum* on $A_{\beta 1-42}$-induced learning and memory impairment in mice, in vivo behavioral tests were performed. The effect on dietary administration of *P. Granatum* on behavioral abilities was examined using and AD animal model based on intracerebro-ventricular Aβ injection.
The results of this study suggest that the ethanol extract of *P. granatum* mitigated H_2O_2-induced oxidative stress in PC12 cells. In addition, the extract inhibited neuronal cell death caused by Aβ-induced oxidative stress and Aβ-induced learning and memory deficiency [46].

Neuroprotection. Antioxidative and neuronal protective effects of *Punica granatum* extract were investigated against oxidative stress-induced cytotoxicity in PC 12 cells. The ethanol extracts of *P. granatum* protected PC12 cells from hydrogen peroxide (H_2O_2)-induced oxidative stress. Reduction assays revealed significant increase in cell viability when oxidatively stressed PC12 cells were treated with the PE [45]. Pretreatment with alcoholic and aqueous PEs, as well as with pomegranate juice of *in vitro* serum/glucose deprivation (SGD) model also showed significant and concentration-dependent increased cell viability following SGD insult for 6 and 12 h [6].

Interesting results from another study reveal that polyphenolic substances from pomegranate juice are detectable in the plasma of mice pups whose mothers drank pomegranate juice. The same study has provided evidence that dietary supplementation of pregnant mice with pomegranate juice protect against neurodegeneration in neonatal mice subjected to hypoxic-ischemic brain injury [26].

Convincing results are provided from a pilot clinical trial in patients undergone heart surgery. Memory dysfunction is a common complaint following heart surgery and may be related to a diffuse ischemic state induced by micro emboli dislodged during the procedure. Patients undergoing elective coronary artery bypass graft and/or valve surgery were given either 2 g of pomegranate extract or placebo per day from one week before surgery to 6 weeks after surgery. The patients were also administered a battery of neuropsychological tests to

assess memory function at 1 week before surgery (baseline), 2 weeks after surgery, and 6 weeks after surgery.

The placebo group had significant deficits in post-surgery memory retention, and the pomegranate treatment not only protected against this effect, but also actually improved memory retention performance for up to 6 weeks after surgery as compared to pre-surgery baseline performance [36].

Antiviral properties. The antiviral properties of pomegranate polyphenols are attributed to inhibition of RNA replication of the influenza virus. Punicalagin compounds with inhibitory concentrations of up to 40 mg/ml have been shown to be the most active in blocking viral RNA replication [10].

Viral inactivation by polyphenols is primarily attributed to virion structural damage. A study suggests that the virus variation in susceptibility to inactivation by pomegranate polyphenols is determined by envelope glycoproteins. Direct anti-influenza activity of pomegranate polyphenols is substantially modulated by small changes in these glycoproteins. The same study shows results for influenza viruses H1N1, H3N2 and H5N1 3-log reduction of viral titer at room temperature following pomegranate polyphenol application, but with lower activity against H5N1 influenza virus isolated from birds [46].

In addition, polyphenolic PE has proven to act synergistically to oseltamvir on influenza A virus [10]. Oseltamvir inhibits neuraminidase in the virus thus preventing it from emerging in cells.

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FO4. THE KETOGENIC DIET IN EPILEPSY- NEUROPHARMACOLOGY AND ANIMAL MODELS

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Abstract

Therapeutic options for management of medically refractory seizures in young children are limited. Despite the plethora of old and new anticonvulsant medications, up toone-third of children with epilepsy continue to have seizures, and many of these children

take multiple medications with concurrent intolerable side effects. Alternative treatments have included the vagus nerve stimulator and dietary approaches. The ketogenic diet (KD), a high fat, low carbohydrate, adequate protein formulation, has been used since early in the twentieth century for seizure control in refractory epilepsy. Clinical studies have verified the effectiveness of the KD. In general, at least half of all patients treated with the KD will exhibit a 50% or greater reduction in seizure frequency. Despite this effectiveness, the mechanisms by which the diet works are still unknown.

In our work we present mechanisms of action of ketogenic diet. *We summarize key insights published from experimental and clinical studies of KD, and focus particular attention on the role that ketone bodies, fatty acids, and limited glucose may play in seizure control*.

Metaboic changes likely related to the KD's action anticonvulsant properties include ketosis, reduced glucose and enhanced bioenergetic reserves.

Since the resurgence in clinical use of the KD in the 1990s, several laboratories have attempted to develop animal models to discern the mechanism of KD action and improve its formulation and effectiveness. In general, such models have met with modest success. Part of the challenge has been to replicate the effect of the KD in humans. However, the metabolism of rodents (the most commonly used model) differs in important ways from humans. During fasting, ketone bodies supply relatively more brain energy in humans than in rats. In the neonatal period, ketone bodies are used more by suckling rats than by humans. In addition, previous models have employed a wide variety of paradigms of seizure induction (kindling, pentylenetetrazole (PTZ), kainic acid, etc.), KD formulations, outcome measures, and assessments of metabolic parameters. Any mechanistic explanation for KD effectiveness in humans must account for certain clinical observations in animal models.

The fact that a fundamental modification in diet can have such profound, therapeutic effects on neurological disease underlines the importance of elucidating mechanisms of KD diet. Future studies will provide unique insights into how diet can affect the brain, both in health and disease, and likely provide the scientific basis for the development of potent new treatment strategies for the epilepsies.

Keywords: animal models, epilepsy, metabolism, ketogenic diet, seizures.

FP1. ANIMAL MODELS OF EPILEPSY AND IMPLICATIONS FOR DISEASE-MODIFYING EFFECT OF LEVETIRACETAM IN KAINAT MODEL OF EPILEPSY IN ADULT RATS

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Abstract

Epilepsy, a many-faced disease, can be modeled in different ways, depending on the aims of study. For example, the chronic disease conditions can be induced, or in contrast acute seizures may be elicited. The objective in inducing seizures in animals is to mimic human

epilepsy as close as possible. Animal models are used to study the pathophisiology of epilepsy and for the discovery of new anticonvulsants.

The major animal models are electrical stimulation, pharmacological models and genetic models. The pharmacological models are divided, depending on the way of convulsing application into systemic, intracerebral and topical application of convulsant. Systemic application includes administration of kainite, pilocarpine, pcrotoxin, bicuculin, penicillin.

Drugs acting selectively on absence seizures can be identified by animal screens, using either threshold pentylentetrazole clonic seizures in rats or mutant mice showing absence-like episodes (so-called lethargic, star-gazer, or tottering mutant). On the other hand, the maximal electroshock (MES) test, identifies drugs such as phenytoin, carbamazepine and lamotrigiune, wich are active against generalized tonic clonic seizures and complex partial seizures.

The KA model of epilepsy has been used for over three decades. The shared features between human epilepsy and animal KA model are that both seizures originate in and remain confined to the limbic system, and that similar neuronal loss and sclerosis occur in the vulnerable areas in the limbic system, for example in hippocampal pyramidal cell regions.

In the adult rats, KA-induced seizures are followed by latent phase of 4-8 weeks after wich the first spontaneous seizures occur, but epileptogenesis continues beyond this, and frequency of seizures increases in sigmoid fashion.

Recent findings in experimental models suggest the possible role of inflammation processes in the etiopathogenesis of seizures. Pro-inflammatory cytokines such interleukin 1beta, TNF-alfa have been shown to be overexpressed in experimental models of seizures in brain areas of seizure generation and propagation. Possible strategies that involve the pharmacological manipulation of inflammation can lead to the development of novel approaches for the treatment of epilepsy.

Present study aimed to compare the effect of levetiracetam (LEV), on the behavioral changes and level of hippocampal TNF-alfa and IL-1beata in kainic-induced model of epilepsy in adult rats.

Our data indicated that KA has a capacity to induce the levels of TNF-alfa in early stages development of the model of epilepsy. Significant anti-TNF-alfa effects were observed in LEV-pretreated rats. This could be involved in their a potencially antiseizure effects.

These data suggest that LEV may inhibite seizures by inhibiting pro-inflammatory cytokines in hippocampus and posses neuroprotective and antiepileptogenic properties.

The surch for new antiepileptic drugs has traditionally been directed to compounds that suppress seizures in a symptomatic fashion. There is enormous effort in the field to develop new strategies for antiepileptigenesis.

Possible strategies that involve the pharmacological manipulation of inflammation can lead to the development of novel approaches for the treatment of epilepsy with disease-modifying effect.

Keywords: animal models, epilepsy, kainic acid, levetiracetam, epileptoigenesis, TNF-alfa.

FP2. COMPARATIVE STUDY ON NEONATAL EFFECTS OF LEVETIRACETAM, VALPROIC ACID AND DIAZEPAM ON BEHAVIORAL CHANGES AND BRAIN CYTOKINES IN NEONATAL KAINAT MODEL OF EPILEPSY

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Abstract

Experimental evidence and clinical observations indicate that brain inflammation is an important factor in epilepsy. Inflammatory cytokines such asTNF-alfa have been shown to be over expressed in experimental models of epilepsy. Data exists that cytokines profoundly affect seizures in rodents.

Purpose: Present study aimed to compare the effect of levetiracetam (LEV), valproic acid (VPA) and diazepam (DZ) on the behavioral changes and level of hippocampus TNF-alfa and IL-1beata in kainic-induced model of epilepsy in pups.

Metods

Pups were devided into 5 groups of 5 animals each. In the first group, animals were given saline (i.p.) to serve as negative control. In the second group rats were injected i.p.with kainic acid (10mg/kg) to serve as positive control. In the other 3 groups-LEV, VPA, DZ were administered once daily between 10:00 and 11:00 a.m. for 3 succesive days (LEV-50mg/kg, i.p., VPA-50mg/kg, i.p., DZ-5mg/kg, i.p). On the thirth day, 2h after the last LEV, VPA and DZ injections, kainic acid was applied i.p.(10mg/kg).

Following the last administration all animals were observed during 3h. for assessment of convulsive behavior according to the rating scale of Racin.

To measure the levels of cytokines (TNF-alfa and IL-1beta) all rats were sacrificed 6 hours after the last injection they received. The levels of cytokines were determined by rat-specific sandwich- enzyme-linked immunosorbent aasay (ELISA).

Results

No behavioral changes were observed in the first group. In the second group, rats treated with KA showed well pronounced seizure syndrome. In the other 3 groups there were differences in the behavioral responses, but the seizures were less severes than KA group. The average symptoms rating in KA group reached a value of 5 in Racin's scale. DZ group reached value of 1-2. VPA group reached value of 2. LEV group show value of 3.

Levels of TNF-alfa and IL1beta were significantly raised in rats treated with KA, as compared with the 3 other studied groups. Levels of TNF-alfa and IL1-beta in LEV, VPA and DZ-groups were lower than group KA. The lowest level of cytokines was recorded in LEV group. Despite of lower cytokines levels, pups from these 3 groups also exhibited epileptiformic activity, but less severe than group KA.

Conclusion

Our data indicated that KA has a capacity to induce the levels of cytokines in early stages development of the model of epilepsy. LEV and VPA suppress cytokine's levels in pup's hippocampus. These data suggest that LEV may inhibit seizures by inhibiting pro-inflammatory cytokines in hippocampus and posses neuroprotective and antiepileptogenic properties. Taken together the results support the application LEV for neonatal seizures.

Working forward from animal models, and simultaneously backword from patients on the basis of their successful interventions by pharmacotherapy, data presented here could provide us with the best understanding of those forms of epilepsy with inflammatory mechanisms in pathogenesis.

Keywords: behavioral changes, epilepsy, kainic acid, seizures, TNF-alfa, levetiracetam, valproic acid, diazepam.

FO5. INNOVATIVE METHODS IN NEUROBIOLOGY RESEARCH: CONNECTOMICS, BRAINBOW, OPTOGENETICS

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The foundations of nowadays modern neuroscience are built by the discoveries of two great scientists – Camillo Golgi and Ramon y Cajal [4]. In 19th century Camillo Golgi discovered a staining technology which marks limited number of neural cells in black. The images he received – black cells on yellow background – were very clear and gave good contrast. Those were the first pictures of neural cells observed under light microscope. Even though Golgi was most certainly a brilliant scientist, till the end of his life he refused to believe that the structures he looked upon were actually separate cells in a typical tissue. He believed that an extraordinary machine, capable of so many different complex functions such as the brain must be made of a single network. Meanwhile Ramon y Cajal – a Spanish scientist, who used Golgi's staining reaction to observe neurons, raised another theory – the brain consists of different, separate cells that form networks by making connections between each other along their path through the nervous tissue. Nowadays, we know that Ramon y Cajal was right and indeed – neurons are distinguished cells consisting of a cell body (soma), axons and dendrites, which make synaptic connections and those connections lay beneath the many different functions that the brain exhibits.

Even though Golgi's method gave a lot of knowledge about the structure of the nervous system, it has a lot of disadvantages and the biggest is the fact that it cannot visualize synaptic connections as they are too small to be observed via light microscopy and that it makes it impossible to trace separate neurons along their network [4]. Those limitations build a great barrier to understanding the brain as a functional structure, because in order to comprehend the delicate mechanisms according to which it maintains all its functions, we need to have a real detailed view of brains' structure – a picture clear enough, allowing us to see what kind of connections are made, where and to whom. A parallel has to be made between the morphological structure of the neuronal network and the many functions, arising from it. In other words, we need a map of this wiring network. In this review we are going to introduce the latest techniques that made a breakthrough in modern neuroscience by finding the exact strategies to map parts of the brain functionally.

1. Connectomics

There are approximately 100 billion neurons in the adult human brain [4]. Each neuron makes thousands of connections to other neurons, resulting in an approximate 150 trillion connections in the human brain. Mapping all those connections is not an easy task. So far a full complete map of the whole brain does not exist. A team led by Thomas Morsic-Flogel at the University College of London mapped some of the connections in the mouse visual cortex by a method called Connectomics.

First, they choose a region of the brain which function is well known, than cut it into very thin slices – nanometer thin [4]. After detecting the structures, present on each slice using electron microscopy and after a software staining of those slices, they put them back together just the way they were and this reconstruction gives a very detailed 3D image of that particular brain region, where now we are able to see different synaptic connections, axon pathways and we can make conclusion about their functional meaning.

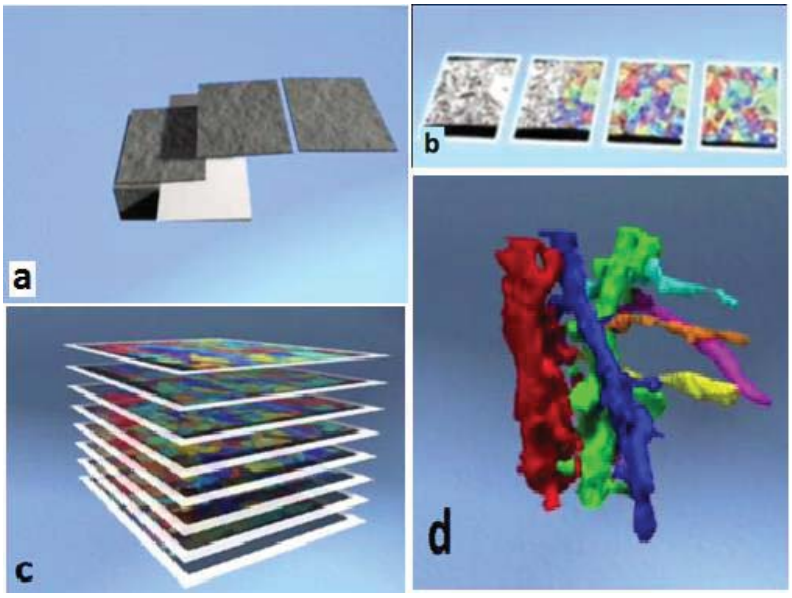


Fig. 1 The connectomics method: a) cutting thin slices of the brain region; b) staining the structures; c) rebuilding the slices back together and d) receiving an accurate 3D reconstruction of the brain part; picture taken from "Synapses, neurons and brains", www.coursera.com

2. Brainbow

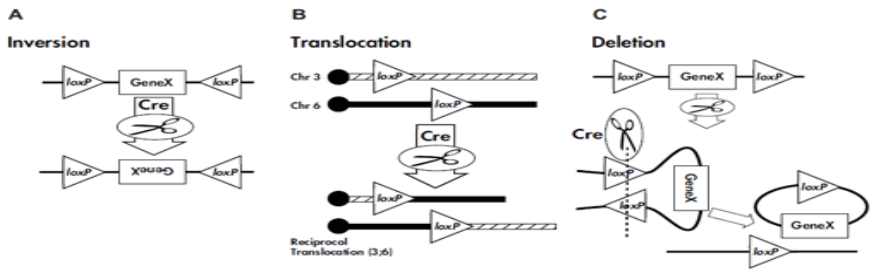
Most staining techniques give little information about the actual connections between neurons, because the wiring network of axons and dendrites is very messy and when cells are dyed in one color (like Golgi's staining, for example) they cannot be distinguished one from another [2,4]. Brainbow is a modern method which uses genetic engineering techniques to perform multicolored staining of nervous tissue. The number of colors stochastically expressed in a particular part of the brain can reach up to 166 different colors. This makes is easy to separate different neurons from each other and to trace their processes.

Brainbow uses stochastic expressions of different proportions of red, green and blue, produced by green fluorescent protein (GFP) in different neurons [2]. In cell and molecular biology GFP is exploited as a reporter protein. This means that genes, encoding GFP can be incorporated in the promoter sequence of a studied gene in order to be able to trace the genes' behavior – when and in what conditions it activated. GFP has several derivates, produced by modifications of its chemical structure – blue, cyan and yellow fluorescent proteins.

Brainbow follows 2 steps:

1. A specific gene construct that is able to recombine in many different sequences to produce one of 3 or 4 colors, based on the fluorescent protein (XFP) used.
2. Multiple copies of this transgenic construct are incorporated in the target organisms' genome and the result is a stochastic expression of different XFPs in different proportions, which leads to the ability of different neurons to exhibit variety of color cues.

Brainbow is based on a Cre/lox mechanism of recombination which can switch on and off the gene expression by excision of DNA, inversion and interchromosomal recombination [3]. The system consists of one single enzyme – Cre recombinase, which recombines short targeting sequences, named Lox-sequences. The Cre-enzyme and the original Lox (LoxP) are derived from a P1 bacteriophage. The proper positioning of the lox sequences allows scientists to activate, inhibit or replace genes. The expression of the Cre recombinase can occur in a defined cell type under the scientist's control, activated by external stimuli such as chemical signals or thermal shock. The Cre-lox recombination includes targeting of specific DNA sequences and their excision by the Cre-enzyme. The lox sequences, which contain specific sites for Cre, circle the studied sequence where the recombination occurs. The result depends on the lox sequences orientation. Two lox sites placed on one chromosomal arm lead to inversion, two loxes put together give deletions. If they are positioned on different chromosomes a translocation occurs.



1. **Fig. 2** The mechanism of Cre-lox recombination system, picture taken from http://cre.jax.org/introduction.html

Based on the Cre-lox recombination, scientists created a few different versions of Brainbow [3].

In **Brainbow 1** they produced a DNA construct where the arrangement of the lox sites allows two different recombination events to randomly express one of 3 colors: red (the original XFP), yellow and cyan. The proportion between the number of cells expressing each color are approximately equal to show that neither recombination event is preferential. **Brainbow 1.1** uses the same mechanism, but including 3 possible recombination events leading to the generation of 4 different colors – orange, red, yellow and cyan.

Brainbow 2 uses inversions. In neural cells they make a construct, consisting of RFP (red FP) and CFP (cyan FP) with parallel orientation. When the inversion event occurs some of the cells express the sense oriented sequence – RFP and others express the antisense oriented CFPs. In **Brainbow 2.1** two reversible DNA segments are positioned in tandem in order to generate more recombination events. Three inversions take place and in addition each excision can reduce the construct to 2 DNA segments, inverting multiply as long as the cell is supplied with Cre enzymes. Those different recombination events lead to the expression of 4 genes, producing green, yellow, red and cyan neurons.

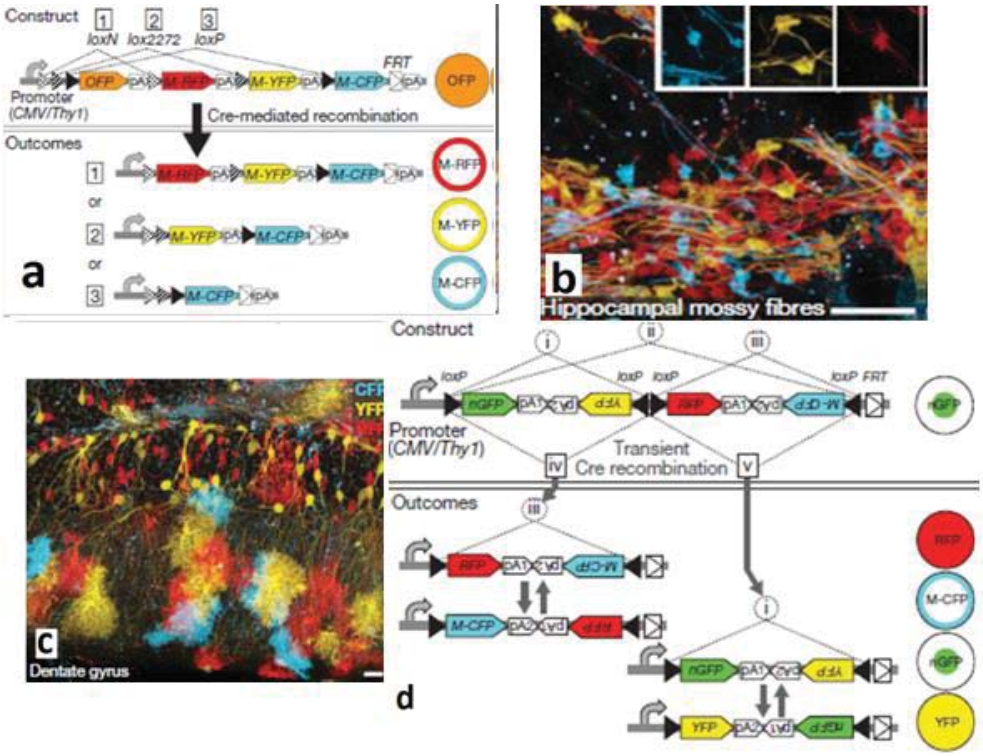


Fig. 3 a) Brainbow 1.1 mechanism and b) Fluorescent image of hippocampal mossy fibres in a rat brain, stained by Brainbow 11. C) Dentate gyrus neural cells stained by Brainbow 2.1 and d) Brainbow 2.1 mechanism, images taken from http://www.addgene.org/18723/, http://www.openoptogenetics.org and www.nature.com

Combinatorial expression of XFPs – In some mice lines individual neurons express only one of the XFPs, present in the transgene [2]. More commonly a coexpression of multiple cues is observed in the separate cells. How are the new colors produced? If, for example, 3 copies of a Brainbow 1 construct end up together they can recombine between each other and then at least 10 different color mixes are expected.

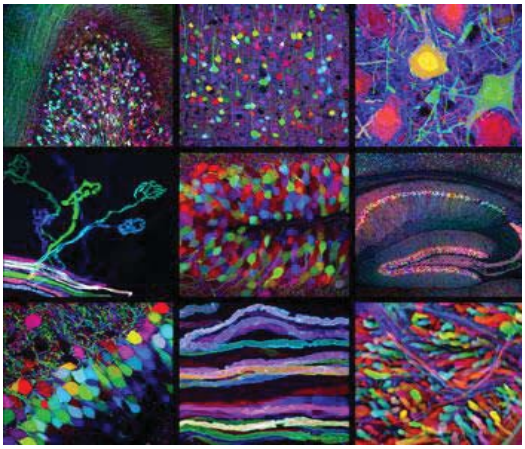


Fig. 4 Different brain regions, stained by combinatorial expression of XFPs, image taken from http://www.rsc.org/

3. Optogenetics

Optogenetics is a technique for controlling subpopulations of neurons in the intact brain using light [1,4,5]. This technique has the potential to enhance basic systems neuroscience research and to inform about the mechanisms and treatment of brain injury and disease.

In 1979 Francis Crick, taking note of the complexity of the mammalian brain and the fact that electrodes cannot readily distinguish different cell types, suggested that a major challenge facing neuroscience was the need to precisely control activity in one cell type while leaving the others unaltered [6]. Crick later speculated in lectures that light might be a relevant control tool, but without a concept for how this could be done.

Opsins are a group of light-sensitive 35–55 kDa membrane-bound G-protein coupled receptors found in photoreceptor cells of the retina [7]. They are involved in vision, mediating the conversion of a photon of light into an electrochemical signal, the first step in the visual transduction cascade. Opsins act as ion channels, sensitive to light with particular wavelength. For example, Channelrhodopsin is activated by blue light, which causes it to open and let positive Na^+ ions to enter the neuron thus causing depolarization – an activation of the neuron. There are other opsin proteins, altered at different wavelength, responsible for the transportation of different types of ions. For example halorhodopsin can inhibit the neurons' activity by allowing negative Cl^- ions to enter the cell, resulting in hyper polarization when illuminated with yellow light.

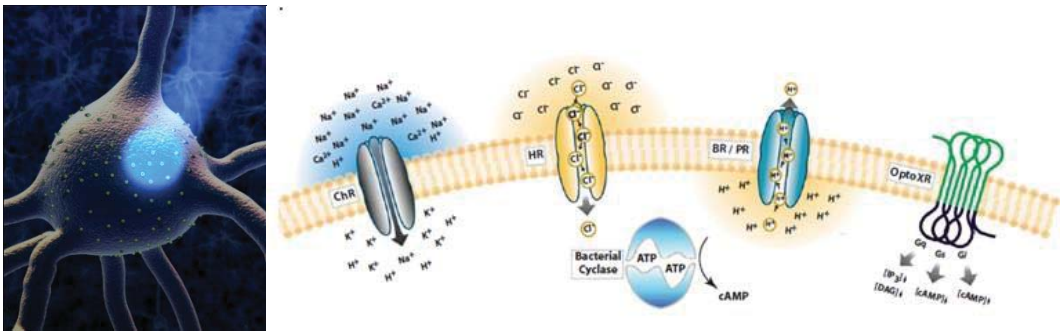


Fig.5 a) Opsins, incorporated in the neurons membrane. Image taken from http://web.mit.edu/ b) The opsin family of proteins, image taken from http://mysbfiles.stonybrook.edu/

Optogenetics uses viral vectors to include opsin genes in neurons in order to make them sensible to light [4,6,7]. Scientists first make a genetic construct, including a promoter sequence and an opsin gene, derived from bacteria. Using viral vectors they insert it into the studied animal's brain. The opsin protein expresses in the targeted neural cells. Light with specific wavelength is switched on trough a fiber-optic channel. Depending on the type of opsin, a different event takes place. The neuron is either activated, or deactivated.

The applications of such technique are endless. Controlling the brain with light gives huge possibilities for treatment of many different neurodegenerative and psychiatric brain diseases.

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FO6. ALZHEIMER'S DISEASE – TREATMENT RESEARCH TRENDS

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Abstract

Alzheimer's disease (AD) is a type of dementia causing problems with memory, thinking and behavior. Scientists have identified factors that increase the risk of AD - the most important are age, family history and heredity. Two abnormal structures have key role in damaging and nerve cells apoptosis: beta-amyloid plaques and tau tangles (twisted fibers).

Two types of medications are approved—cholinesterase inhibitors and memantine. The available treatments of AD are only symptomatic, cholinesterase inhibitors being the most widely used drugs. Several natural compounds with anticholinesterase activity can be used as leader compounds for the synthesis of new drugs.

Today, there is a worldwide effort (by WHO) to find better strategies of treating the AD, delay its onset, and prevent it from developing. Researchers are developing medications aimed at in amyloid processing. Recent studies have indicated the critical importance of tau in the pathomechanisms of neurodegeneration in AD and related tauopathies. There is a theory of novel anti-inflammatory treatments for AD. The results from several studies provide strong evidence in support of the hypothesis that AD represents a form of diabetes mellitus that selectively afflicts the brain.

Researchers hope various brain imaging techniques studies will soon provide methods to diagnose AD in its earliest stages — even before symptoms appear. Biomarkers may also offer better ways to monitor response to treatment.

Despite of that many theories and experimental efforts there still is an enormous need for more effective therapeutic strategies for the purposes of successful treatment of AD and other dementias.

Keywords: Alzheimer's disease, neurodegeneration, beta- and gamma-secretase, AChE, type 3 diabetes mellitus, β-asarone

Introduction

Azheimer's disease (AD) is a type of dementia causing problems with memory, thinking and behavior. It is most common form of dementia - a progressive disease which symptoms worsen over time. In its early stages, memory loss is mild, but with late-stages, patients lose the ability to carry on a conversation and respond to their environment.

- **Pathogenetic mechanisms**

Two abnormal structures have key role in damaging and nerve cells apoptosis:

- Plaquesare deposits of a protein fragment called beta-amyloid that build up in the spaces between nerve cells.

- Tangles are twisted fibers of another protein called tau that build up inside cells.

Most experts believe plaques and tangles somehow damageneurone communicationand nerve cell metabolism. The caused destruction and nerve cells apoptosis cause memory failure, personality changes, problems carrying out daily activities and other AD symptoms.

Elder individuals develop some plaques and tangles with aging. Those with Alzheimer's tend to develop far more and in a predictable pattern - starting in areas important for memory and spreading to other regions. (*Fig.1*) Using high-resolution functional MRI (fMRI) imaging in patients with AD and in mouse models of the disease, researchers have clarified three fundamental issues about Alzheimer's: where it starts, why it starts there, and how it spreads; this could improve early detection of the disease [25].

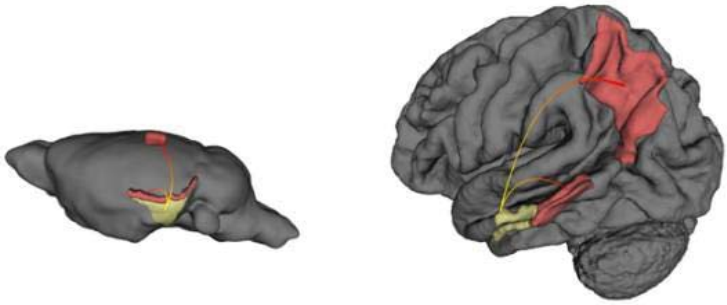

*Fig.1*Using fMRI in mouse (left) and human (right) brains, the researchers provide evidence that AD spreads from the entohrinal cortex (yellow) to other cortical regions (red) — the perirhinal cortex and posterior parietal cortex. Credit: Usman Khan/lab of Scott A. Small, MD, Columbia University Medical Center

- **Alzheimer's disease treatment guidelines (2011)**

So far two types of medications are approved—cholinesterase inhibitors and memantine. They affect the cognitive symptoms - memory loss, confusion, problems with thinking and reasoning. Three cholinesterase inhibitors are commonly prescribed: *Donepezil (Aricept)* in all stages of Alzheimer's; *Rivastigmine (Exelon)* and *Galantamine (Nivaline)* in mild to moderate Alzheimer's. Preventing the breakdown of acetylcholine supports communication among nerve cells by keeping acetylcholine levels high. *Memantine* improves memory, attention, reason, language and the ability to perform simple tasks. The drug regulates the glutamate activity (involved in learning and memory). It can be used as a monotherapy or combined with other Alzheimer’s disease treatments; delays worsening of

symptoms for some patients temporarily and its benefits are comparable to those of cholinesterase inhibitors.

- **New theories**

Scientists have identified factors that increase the risk of AD. The most important are age, family history and heredity**. Head trauma is** a possible link between serious head injury and future risk of AD, especially when it involves loss of consciousness. Another risk are conditions that damage the heart or blood vessels (high blood pressure, heart disease, stroke, diabetes and high cholesterol). Accumulating evidence suggested that dysregulation of cholesterol homeostasis might be a major ethiologic factor in initiating and promoting neurodegeneration in AD [31]. Cross-sectional reports suggest that statin users are less likely to have AD. Prospective studies have provided inconsistent evidence. It is unclear whether the association differs for lipohilic (which easily pass the blood–brain barrier) and hydrophilic statins. The use of statins, but not of non-statin cholesterol-lowering drugs, was associated with a lower risk of AD compared with never use of cholesterol-lowering drugs (the protective effect was independent of the lipophilicity of statins) [14]. Randomized, double-blind, placebo-controlled trial of *simvastatin* was conducted in individuals with mild to moderate AD and normal lipid levels. It had no benefit on the progression of symptoms despite significant lowering of cholesterol [24].

Swedish PhD student, Sara Bengtsson, theorises that the brain steroids that proliferate at times of stress(allopregnanolon) inhibit general brain activity and accelerate Alzheimer disease.This thesis shows that its chronically elevated levels accelerated the disease development and that high levels of beta-amyloids corresponded to dysfunction in brain synapses.

There is a theory of novel anti-inflammatory treatments for AD. Non-steroidal anti-inflammatory drugs (NSAIDs) affect inflammation by inhibiting cyclooxygenase (COX)-mediated signaling pathways including prostaglandins, which are the principal mediators of CNS. Over 30 epidemiological studies have been reported on NSAIDs in AD, most showing NSAID administration was associated with reduced incidence of AD. But NSAID toxicity has complicated efforts for long term prevention trials (including unexpected cardiovascular side effects).Main among these COX-dependent pathways are the prostaglandins and PGE2. Future studies are needed to determine which molecular components of the prostaglandin pathway can be targeted for as preventive or therapeutic interventions for AD while minimizing adverse side effects [28].

The results from several studies provide strong evidence in support of the hypothesis that AD represents a form of diabetes mellitus that selectively afflicts the brain. There is data about an experimental animal model with brain diabetes, cognitive impairment and molecular and pathological features that mimic AD (produced by intracerebral administration of a drug that is commonly used to produce T1DM (type 1 diabetes mellitus) or T2DM (type 2 diabetes mellitus) [10, 12] and a study showing that PPAR (peroxisome proliferator-activated receptor) agonists, which are used to treat T2DM, prevent many of the AD associated neurodegenerative effects of ic-STZ (intracerebral injection of streptozotocin) [29]. The data provide strong evidence that AD is intrinsically a neuroendocrine disease caused by selective impairments in insulin and IGF (insulin-like growth factor) signaling mechanisms, including deficiencies in local insulin and IGF production. Some of the most relevant data supporting this concept have emerged from clinical studies demonstrating cognitive improvement and/or stabilization of cognitive impairment in subjects with early AD following treatment with intranasal insulin or a PPAR(peroxisome proliferator-activated receptors) agonist [3, 16, 23].

- **New aproaches understanding dementia**

Beta-amyloid is the main component of plaques, one criterion of Alzheimer's brain abnormality. It is now clear how this protein fragment is clipped from its parent compound amyloid precursor protein (APP) by two enzymes — beta- and gamma-secretase. Inhibitors or modulators that target beta- and gamma-secretases as well as alpha-secretase activators are promising candidates for treatment of Alzheimer's disease [7].

Researchers are developing medications aimed at in amyloid processing. This includes blocking activity of aforementioned enzymes, preventing the beta-amyloid fragments adhesion into plaquesand using antibodies against beta-amyloid. *Solanezumab* (proposed INN) is a monoclonal antibody being investigated by Eli Lilly as a neuroprotector for patients with Alzheimer's disease. It binds to the amyloid-β peptides.2012 results of the EXPEDITION 1&2 phase III clinical trials showed only mildly encouraging but were said to be the "first evidence that targeting the amyloid cascade can slow the progression of disease."(SOURCE Eli Lilly and Company, INDIANAPOLIS, Oct. 8, 2012).

Tau protein is the main component of tangles related to Alzheimer's brain abnormality, which destroys cell transport systems. Clinical trials in symptomatic AD patients aimed at removal of Aβ load or lowering the Aβ production in the brain didn`t show any significant clinical efficacy (despite of reducing the Aβ load). So the tau protein is receiving more attention as a therapeutic target for these disorders. Recent studies have indicated the critical importance of tau in the pathomechanisms of neurodegeneration in AD and related tauopathies [32]. NAP (davunetide), a peptide derived from ADNP (activity-dependent neuroprotective protein), partly improved deficits relevant with ADNP deficiency. It provided potent neuroprotection in different neurodegenerative models, protecting the neuroglial cytoskeleton in vitro and inhibiting tau pathology (tauopathy) in vivo. Davunetide (NAP) is in a Phase II/III study of the tauopathy therapy [9].

Researchers identified the protein called metabotropic glutamate receptor 5 (mGluR5). It as another component in the protein chain that builds up in the brain and threafter cause reduced communication between brain cells and a cognitive deficit. A new drugs may be able to specifically target mGluR5 to break the chain of events leading to AD [26].

Growing evidence suggests that oxidative damage caused by the β-amyloid peptide in the pathogenesis of Alzheimer's disease may be hydrogen peroxide mediated. Many polyphenols, the most abundant dietary antioxidants, possess stronger neuroprotection against hydrogen peroxide than antioxidant vitamins. A test was made whether consumption of fruit and vegetable juices, containing a high concentration of polyphenols, decreases the risk of incident probable Alzheimer's disease. The conclusions are that they may play an important role in delaying the onset of Alzheimer's disease, especially among those who are at high risk for the disease. These results may lead to a new strategy in the prevention of Alzheimer's disease [6].

The generation of oxygen free radicals is involved in the pathogenesis of AD. A group of scientists investigated the relationship between AD and the intake of carotenes, vitamin C, and vitamin E in 980 elderly subjects who were free of dementia at baseline and were followed for a mean time of 4 years. Neither dietary, supplemental, nor total intake of carotenes and vitamins C and E was associated with a decreased risk of AD in this study [15].

- **Approaches for early diagnosis**

An alternative approach is brain scans. Scientists are using imaging technologies to directly measure the amount of amyloid plaques in the brain in living patients. This is known as amyloid-PET (positron emission tomography) and involves the injection into the bloodstream of a chemical that is radioactive for a very short time and seeks out and binds to amyloid deposits. However, the limitation is that PET scanners are few and far between and

the radioactive isotopes currently used are so short-lived that the chemists need to make them no more than half an hour or so before they are used [2].

Researchers hope various brain imaging techniques studies will soon provide methods to diagnose AD in its earliest stages — even before symptoms appear. Biomarkersmay also offer better ways to monitor response to treatment. A test has been produced to measure amyloid and tau in spinal fluid. Collection of spinal fluid has disadvantages due to patient discomfort and some minor side-effects [21].

Some scientists suggest that there are proteins which act as very early markers of AD pathology. They look for protein differences in the blood of people with Alzheimer's and elderly controls and concluded that the changes in the blood proteins called complement factor H (CFH) and alpha-2-macroglobulin (A2M) matched changes in the function of the brain in people not only with AD but also those with pre-Alzheimer states such as mild cognitive impairment [19, 27].

- **Perspectives**

Immunotherapy targeting the amyloid β (Aβ) peptide is a potential strategy to slow the progression of AD. Scientists tried to measure the safety and tolerability of CAD106, a novel active Aβ immunotherapy (vaccine), designed to induce N-terminal Aβ-specific antibodies without an Aβ-specific T-cell response [30].

Aβ immunotherapy is an experimental treatment for AD; yet, unexpected negative vascular side effects seen in early human clinical trials demonstrate that cognition of Aβ and AD pathogenesis is incomplete. Immunization with Aβ peptides neutralizes the amyloid trigger leading to neoangiogenesis and reverses hypervascularity in mice. This process resolves plaques suggesting that neoangiogenesis is a key mechanism in plaque formation. A meta-analysis demonstrated that hypervascular reversion in vaccinated Alzheimer's patients [4].

The metalloproteases meprin α and meprin β were discovered more than 30 years ago. Both enzymes were initially found to be highly expressed in kidney and intestine, but recently it has become clear that meprins are involved in angiogenesis, cancer, inflammation, fibrosis and neurodegenerative diseases. Different animal models and proteomics approaches for the identification of protease substrates revealed meprin activity in activation and release of pro-inflammatory cytokines. Meprin β might therefore be a promising therapeutic target in AD [11].

Scientists suggest that AMPA receptors (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor or quisqualate receptor for glutamate) may be potential pharmaceutical targets for the treatment of neurodegenerative diseases. Ampakines were developed as an attempt to improve the response of synapses. The drugs are designed to attach structures called AMPA receptors. Since cells communicate via the neurotransmitter glutamate, the drugs boost the signals. Research showed AMPAKINE®s can increase the production of the important neurotrophic factors BDNF (brain derived neurotrophic factor) and NGF (neuronal growth factor) in critical areas of the brain involved in memory [15]. Ampalex (Ampakine CX516) is an ampakine and nootropic agent that acts as an AMPA receptor positive allosteric modulator in patients with AD, schizophrenia and mild cognitive impairment.

3-(2,4-Dimethoxybenzylidene)-anabaseine is an analog of the paralytic alkaloid, anabaseine, from the ribbon worms Amphiporus sp. (Amphiporus lactifloreus, Johnston, 1828), that shows agonist activity on α7 nicotinic acetylcholine receptors. Anabaseine has shown its potentiality in Alzheimer symptomatological therapeutics by improving deficient sensory inhibition [18].

There are studies about effects of β-asarone, the major ingredient of Acorus Tatarinowii Schott, on cognitive function and neuronal apoptosis and its mechanism of action. Beta-

asarone attenuate Abeta (1-42)-induced neuronal apoptosis in hippocampus by reversal down-regulation of Bcl-2, Bcl-w (proteins that regulate apoptosis), caspase-3 activation, and JNK (c-Jun N-terminal kinases) phosphorylation. The results showed that it may be a potential candidate for development as a therapeutic agent to manage cognitive impairment associated with AD [17]. *Acorus calamus L.* shows neuroprotective effect against stroke and chemically induced neurodegeneration in rats. Specifically, it has protective effect against acrylamide induced neurotoxicity [23].

The available treatments of AD are only symptomatic, cholinesterase inhibitors being the most widely used drugs. Several natural compounds with anticholinesterase activity can be used as leader compounds for the synthesis of new drugs. Quinones and stilbenes are less well studied regarding cholinesterase inhibition, although some of them (sargaquinoic acid or (+)-α-viniferin) show promising activity. Among flavonoids, flavones and isoflavones are the most potent compounds. Xanthones and monoterpenes are generally weak cholinesterase inhibitors. The first known acetyl choline estherase (AChE) inhibitor was physostigmine, an alkaloid isolated for the first time in 1864 from Physostigmina venenosum Balf., which was used in therapy before the discovery of acetyl choline (ACh) as neurotransmitter (Rivastigmine is its synthetic lipophilic analogue). Berberine is an isoquinoline alkaloid isolated from the the dried rhizome of Coptis spp., Ranunculaceae, and has promising AChE inhibitory properties - inhibits β-secretase activity in a rabbit model of AD, reducing Aβ accumulation [13]. Clinical trial in which Salvia officinalis L. has been given to mild and moderate AD patients for 16 weeks, showed improved cognitive performance (1). Fruits, spices, nuts and herbs possess bioactive compounds necessary for the prevention and cure of various diseases without undesirable side effects. Resveratrol in grapes, curcumin in turmeric, gingerols in ginger, epicatechin -3-gallate in tea all possess anti-amyloidogenic activity in AD models. The neuroprotective effects of the withanoides, ginkolide, iridoid glycoside, and huperzineneeds to be studied. Most of these natural products act against Ab formation and/or tau hyper phosphorylation [8].

- **Nonpharmacological treatment**

The NeuroAD™ system, developed by Neuronix, is a new non-invasive treatment for patients with mild and moderate Alzheimer's disease. It is the first system to combine focused transcranial magnetic stimulation of the brain with cognitive training to simultaneously target specific brain regions affected by Alzheimer's disease. The NeuroAD™ stimulation, where magnetic impulses are transmitted to the brain (via a specially designed headset placed on the patient's head), is thought to induce 'long term potentiation' [22].

Despite the deserved enthusiasm and optimism for identifying means of halting the pathogenesis of AD, a clear need for more effective therapeutic strategies will continue for the purposes of successful pathogenic treatments.

Researchers have made progress in understanding healthy brain function and dementia correlated diseases. Today, there is a worldwide effort (initiated by WHO) under way to find better strategies of treating the AD, delay its onset, and prevent it from developing. An effective treatment that reverses, or delays, disease progression would obviously have a huge potential impact in this important group of patients. There may also be benefits in terms of reduced social care needs.

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FO7. ДА ИЗГОРИШ В СОБСТВЕНИЯ СИ ОГЪН - BURN OUT SYNDROME

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