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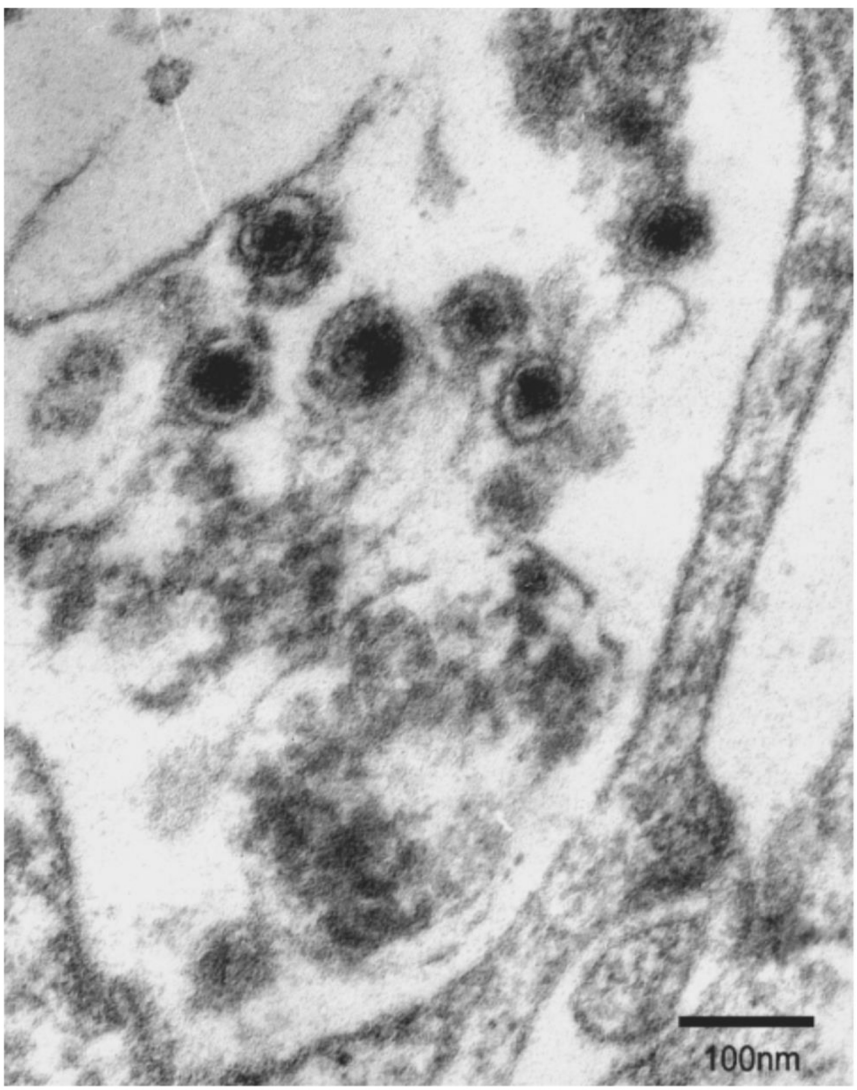
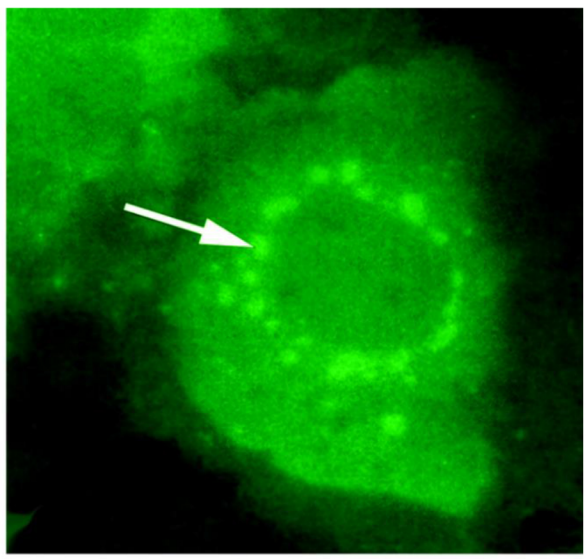
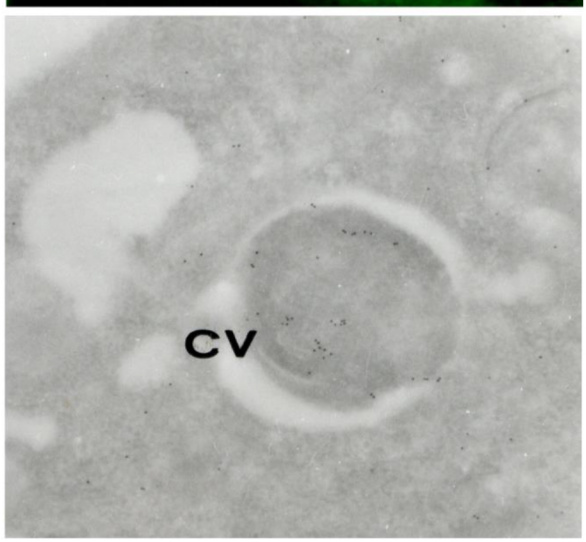
PROCEEDINGS

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







MAY 27–29, 2013

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PROCEEDINGS

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

May 27 – 29, 2013

Institute of Experimental Morphology, Pathology and Anthropology with Museum

at the Bulgarian Academy of Sciences

Edited by: Dimitar Kadiysky and Radostina Alexandrova

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

THE FORTH 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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P R O G R A M M E

Monday, 27 May 2013

9.00-9.45 - Registration

9.45 – Opening Ceremony

Session A.

Chairpersons:

Assoc. Prof. Evelina Shikova, MD, PhD
Assoc. Prof. Russy Russev, DVM, PhD

Secretary: Lora Dyakova

10.00-10.20

AO1. THE RETROVIRUSES THAT CHANGED THE WORLD OF SCIENCE

Radostina Alexandrova

10.20 – 10.40

AO2. AURORA PROJECT: A EUROPEAN NETWORK ON CERVICAL CANCER SURVEILLANCE AND CONTROL IN THE NEW MEMBER STATES

E. Shikova I. Ivanov, G. Ganchev, D. Mircheva

10.40 – 10.50

AO3. HPV DNA TESTING FOR IDENTIFICATION OF HPV INFECTION

E. Shikova and Z. Ivanova

10.50 – 11.10 Coffee Break

11.10-11.30

AO4. PAPILLOMAVIRUS-LIKE PARTICLES FOR HPV VACCINES DEVELOPMENT

E. Shikova, Z. Ivanova

11.30 – 11.50

AO5 **BLV INFECTED RATS AND RABBITS AS A MODEL OF HUMAN LYMPHOCYTIC LEUKAEMIA**

Petar Dimitrov, Katerina Todorova, Rositca Milcheva, Evgeny Gabev and Russy Russev

11.50 – 12.10

AO6. PLANT SUBSTANCES ARE PROMISING INHIBITORS OF HERPES VIRUS INFECTIONS

Neli Milenova Vilhelmova-Ilieva

12.10 – 12.20

AO7. BIOLOGICAL PROPERTIES OF ELLAGITANNINS IMPORTANT FOR HUMAN HEALTH

Neli Milenova Vilhelmova-Ilieva

12.20 – 12.30 POSTER SESSION A

AP1. ВИРУСИ - ЛЕКАРСТВО ЗА МОЗЪЧНИ ТУМОРИ

Тим Владимиров Владимиров

12.30 – 12.35

AP2. БАКТЕРИОФАГИТЕ – АНТИБИОТИЦИ НА БЪДЕЩЕТО

Яна Славейкова

12.30 – 13.30 LUNCH TIME

13.30 – 14.00

ПРЕДСТАВЯНЕ НА НОВИТЕ ПРОГРАМИ И СТИПЕНДИИ НА НЕМСКАТА ФОНДАЦИЯ "АЛЕКСАНДЪР ФОН ХУМБОЛДТ"

проф. Илза Пъжева

Session B.

Chairpersons:

Prof. Reneta Toshkova, MD, PhD
Assoc. Prof. Svetlozara Petkova, PhD

Secretary: Assist. Prof. Petar Dimitrov, DVM, PhD

14.10 – 14.30

BO1. INVESTIGATIONS ON SOME HEMATOLOGICAL PARAMETERS IN SHEEP EXPERIMENTALLY INFECTED WITH *HAEMONCHUS CONTORTUS*

V. Nanev, I. Vladov, D. Hrusanov, M. Gabrashanska

14.30-14.40

BO2. MODEL OF *EIMERIA TENELLA* INFECTION AFTER BASIC COPPER SALT SUPPLEMENTATION

M. Anisssimova, I.Vladov, V. Koinaski, M. Gabrashanska, V. Ermakov, S. Tyutikov

14.40 – 14.50

BO3. COMPARISON OF ALLOZYME ANALYSIS OF SIX TRICHINELLA ISOLATES BY PAAE

Valeria Dilcheva, Svetlozara Petkova, Ivan Iliev

14.50 – 15.10

BO4. SERUM SIALIC ACID LEVELS IN TRICHINELLA SPIRALIS INFECTED RATS

D. Ivanov, M. Gabrashanska, R. Gavazova, S. Petkova

15.10 – 15.30 Coffee Brfeak

15.30 – 15.50

BO5. THE NURSE CELL – *TRICHINELLA SPIRALIS* COMPLEX AS AN EXPERIMENTAL MODEL FOR INVESTIGATIONS ON THE GLYCOSYLATION IN SKELETAL MUSCLE TISSUE

R. Milcheva, B. Izrael-Vlková, D. Ivanov, S. Petkova, P. Celec, P. Janega, R. Gavazova, R. Russev, P. Babál

15.50 – 16.10

BO6. EFFECT OF BIOLOGICALLY ACTIVE SUBSTANCES ISOLATED UNDER FASCIOLOSIS ON LYMPHOCYTE CELL CULTURES

Tsocheva-Gaytandzhieva, N., E. Nikolova, D. Salkova

16.10 – 16.20

BO7. EFFECT OF THERMOLABILE BIOLOGICALLY ACTIVE SUBSTANCES UNDER FASCIOLOSIS ON TUMOR CELL CULTURE

N. Tsocheva-Gaytandzhieva, R. Toshkova, A. Filchev, D. Salkova

16.20 – 16.40

BO8. NOSEMOSIS – THE PESTS OF THE 21th CENTURY IN EUROPE

Delka Salkova Salkova

16.40 - 17.00

POSTER PRESENTATION - SESSION B

BP1. THE DARK SIDE OF TOXOPLASMA GONDII

Deyana Jekova

BP2. ENTAMOEBA HISTOLYTICA

Кристина Антонова Генкова

BP3. КУЧЕТО - ПРЯТЕЛ ИЛИ ВРАГ НА ЧОВЕКА. КУЧЕШКА ТЕНИЯ

Кристин Ботева

BP4. ECHINOCOCCUS GRANULOSUS

Мартин Григоров, Ради Фетиани, Тамара Саад

BP5. ВНИМАНИЕ: ТЕНИИ!

Венета Димитрова , Ива Политова, Стефка Георгиева

BP6. SCHISTOSOMA

Мартин Григоров, Тамаара Саад, Ради Фетияни

Tuesday, 28 May 2013

Session C.

Chairpersons:

Prof. Elena Nikolova, PhD, DSc
Assoc. Anton Kril, DVM, PhD

Secretary: Tanya Zhivkova

9.45 – 10.05

CO1. EFFECTS OF LEAD AND SALINOMYCIN ON SOME ORGAN WEIGHT/BODY WEIGHT INDICES IN FEMALE MICE, SUBJECTED TO SUBACUTE LEAD INTOXICATION

Juliana Ivanova, Yordanka Gluhcheva, Silvia Stoykova, Vasil Atanasov, Sonja Arpadjan

10.05 – 10.15

CO2. CHANGES IN MICE SPERM COUNT AFTER SUBACUTE INTOXICATION WITH LEAD AND SALINOMYCIN DETOXICATION - PRELIMINARY DATA

Ekaterina Pavlova, Donika Dimova, Juliana Ivanova, Yordanka Gluhcheva, Nina Atanassova

10.15 – 10.35

CO3. IN OVO STUDY ON THE GENOTOXIC AND CARCINOGENIC POTENTIAL OF ANTHRACENE-DRIVED SCHIFF BASES AND ANTHRACENE-CONTAINING AMINOPHOSPHONATES

A. Kril, A. Georgieva, I. Iliev, I. Tsacheva, E. Vodenicharova, E. Tashev, T. Tosheva, I. Kraicheva, K. Troev

10.35 – 10.55

CO4. CRITERIA FOR MECHANISM-BASED ENZYME INACTIVATION

Tzveta Stoyanova, Viliana Todorova, Stanislav Yanev

10.55 – 11.05

CO5. ACYLPEPTIDE HYDROLASE AS A BIOMARKER OF EXPOSURE TO ORGANOPHOSPHATES

Vassil Komsalov, Stanislav Yanev, Bozhidarka Pandova, Viliana Todorova

11.05 – 11.15

INCREASED TOXICITY OF NEWLY SYNTHESIZED PEPTIDOMIMETICS BY DIMETHYL SULPHOXIDE AS SOLVENT IN RODENTS

Klisurov R., E. Encheva, L. Tancheva, M. Novoselski, D. Tsekova, M. Genadieva, Sv. Stoeva

11.15-11.20

POSTER PRESENTATION - SESSION C

СР1. СИНТЕТИЧНА БИОЛОГИЯ: ПРИЛОЖЕНИЕ В МЕДИЦИНАТА

Мария Минчева и Вивиан Петрова

11.20 – 11.40 Coffee Break

Session D.

Chairpersons:

Prof. Margarita Gabrashanska, DVM, PhD
Assoc. Prof. Diana Rabadjieva, PhD

Secretary: Abedulkadir Mahdi Abudalleh

11.40 – 11.50

DO1. BIOCHEMICAL STUDIES IN RATS WITH EXPERIMENTAL DEFECTS FILLED WITH DIFFERENT HYDROXYAPATITE SUBSTITUTES

M. Gabrashanska. R. Ilieva, M. Alexandrov, K.Sezanova, V. Nanev, I. Vladov

11.50 – 12.10

DO2. MURINE BONE MAROOW CELLS AND CULTURES FROM BONE EXPLANTS IN THE EVALUATION OF BIOCOMPATIBILITY OF CALCIUM PHOSPHATE MATERIALS FOR BONE IMPLANTS

Radostina Alexandrova, Tanya Zhivkova, Boyka Andonova-Lilova, Lora Dyakova, Gergana Taleva, Diana Rabadjieva, Stefka Tepavitcharova

12.10-12.30

DO3. DEVELOPMENT OF NOVEL *IN VITRO*-CULTIVATION TECHNIQUES FOR DERIVATION OF CORNEAL EPITHELIAL EXPLANTS

Ilina Valkova, Iskra Sainova,Ani Georgieva, Anatoli Neronov, Marin Bratanov, Elena Nikolova

12.30 – 13.30 Lunch Time

13.30 – 13.50

DO4. ПРОУЧВАНЕ ВЪРХУ ВЛИЯНИЕТО НА КРИОКОНСЕРВАЦИЯТА, ПО ОТНОШЕНИЕ ПОДВИЖНОСТТА И ДНК ФРАГМЕНТАЦИОННИЯ ИНДЕКС НА СПЕРМАТОЗОИДИТЕ, ПРИ СВЕЖА И ОБРАБОТЕНА СЕМЕННА ПРОБА

Л. Илиева, Н. Айвазова, В. Рилчева, Д. Мартинов, Е. Конова

13.50- 14.10

DO5. ASSESSMENT OF FROZEN-THAWED HUMAN SPERM USING AN ANNEXIN V BINDING AND DNA INTEGRITY TEST BY FLOW CYTOMETRY

V. Rilcheva, N. Ayvazova, L. Ilieva, Tz. Lukanov, E. Konova

14.10 – 14.20

POSTER PRESENTATION - SESSION D

DP1. СТЕРИЛИТЕТЪТ ПРИ МЪЖЕТЕ – ПОГЛЕД ОТ ВСИЧКИ СТРАНИ

Радостина Димитрова, Милица Симов

DP2. IN VITRO MATURATION OF HUMAN OOCYTES

Galina Nenkova

DP3. МЕТОДИ ЗА ОПЛОЖДАНЕ IN VITRO

Даниела Гулева, Александра Борисова, Николай Димитров

Session E.

Chairpersons:

Assoc. Prof. Radostina Alexandrova, PhD
Assoc. Prof. Ivayla Pantcheva-Kadreva, PhD

Secretary: Assoc. Prof. Elena Gardeva, DVM, PhD

14.20 – 14.40

EO1. SYNCHROTRON RADIATION CIRCULAR DICHROISM AND ITS APPLI CATION FOR BIOMEDICAL PURPOSES

I. N. Pantcheva, M. Mitewa

14.40 – 15.00

EO2. COMPLEXATION OF OBIDOXIME WITH RESPECT TO ITS REACTIVATION ABILITY

A. Nedzhib, S. Stoykova, I. N. Pantcheva

15.00 – 15.20 Coffee Break

15.20 – 15.40

EO3. ANTITUMOR ACTIVITY OF *ARTHRONEMA AFRICANUM* C-PHYCOCYANIN IN *IN VIVO* EXPERIMENTAL MODEL

Elena Gardeva, Reneta Toshkova, Liliya Yossifova, Kaledona Minkova, Liliana Gigova

15.40-16.00

EO4 EFFECT OF ISOXICAM AND ITS COPPER COMPLEX ON VIABILITY OF CULTURED HUMAN BREAST CANCER CELLS

Lora Dyakova, Tanya Zhivkova, Dzhem Farandzha, Daniela-Cristina Culita, Gabriela Marinescu, Luminita Patron, Radostina Alexansdrova

16.00 – 16.20

EO5. BIOLOGICAL ACTIVITY OF RGD PEPTIDES

A. Balacheva, R. Detcheva, T. Pajpanova

16.20 – 16.40

EO6. BIOLOGICAL PROPERTIES OF GLYCOSAMINOGLYCANS

V. Pavlova, E. Nikolova

16.40 – 17.00

EO7. EXPRESSION OF CARBOHYDRATE-BINDING PROTEINS IN CULTURE MEDIUM FROM MCF-7 ADENOCARCINOMA CELLS TREATED WITH LANTHANIUM-CHOLATE COMPLEX

J. Stoyloff, R. Alexandrova, D Culiţa, L Patron, S. Ivanov

17.00 – 17.30

POSTER PRESENTATION – SESSION E

EP1. BRIEFLY ABOUT BRCA GENES AND HEREDITARY BREAST CANCER

R. Alexandrova, I. Gavrilov, D. Tsingilev, M. Nacheva

EP2. FEW WORDS ABOUT TRIPLE NEGATIVE BREAST CANCER

R. Alexandrova, M. Taushanova, K. Timcheva

EP3. BREST CANCER CELL LINES AS EXPERIMENTAL MODELS IN ONCOLOGY

R. Alexandrova, I. Gavrilov, D. Tsingilev, M. Nacheva

EP4. APPLICATION OF HUMAN TUMOR CELL LINES FOR EVALUATING ANTITUMOR PROPERTIES OF SALINOMYCIN AND ITS METAL COMPLEXES

Tanya Zhivkova, Lora Dyakova, Ivayla-Pantcheva-Kadreva, Mariana Miteva, Radostina Alexandrova

EP5. VIRUS-TRANSFORMED RAT SARCOMA CELLS IN SEARCHING FOR NEW ANTITUMOR AGENTS

Abedulkadir Abudalleh, Tanya Zhivkova, Boyka Andonova-Lilova, Lora Dyakova, Dimitar Ivanov, Marin Alexandrov, Pavel Mitrenga, Radostina Alexandrova

EP6. CHLORELLA IS A HEALTH CARE

Zhale Dogan, Maria Mladenova, Boyka Stilyanova

EP7. НИЕ НЕ ЖИВЕЕМ, ЗА ДА ЯДЕМ, А ЯДЕМ, ЗА ДА ЖИВЕЕМ

Силвия Петрова, Луция Кулкина

EP8. КОМПЛИМЕНТИ ЗА КОМПЛЕМЕНТА

Марина Димчева

EP9. БИОСИНТЕЗ НА ПЪРВИЧИНИ И ВТОРИЧНИ МЕТАБОЛИТИ ЗА ПРИЛОЖЕНИЕ В МЕДИЦИНА

Боян Пашов, Галина Димитрова, Тина Георгиева

Wednesday, 29 May 2013

Session F.

Chairpersons:

Prof. Diana Wesselinova, DVM, PhD, DSc
Assoc.Prof. Ivan Goshev, PhD

Secretary: Milena Georgieva, PhD

9.45 – 10.05

FO1. DETERMINATION OF ANTIOXIDATIVE PROPERTIES

Milan CIZ

10.05-10.25

FO2.LUMINOMETRIC EXAMINATION OF OXIDATIVE BURST OF PHAGOCYTE

Antonin LOJEK

10.25 – 10.45

FO3. **ANTIOXIDANT AND *IN VITRO* ANTICANCER ACTIVITY OF NEW ANALOGS OF OCTREOTIDE**

Svetlana Ts. Staykova, Ivan Goshev, Diana W. Wesselinova, Liubomir T. Vezenkov, Aleksandra D. Kalistratova, Emilia D. Naydenova

10.45 – 11.05

FO4. ANTIOXIDANT ACTIVITY OF SOME BENZIMIDAZOLE DERIVATIVES TO DEFINITE TUMOR CELL LINES

Goshev I., A. Mavrova , B. Mihaylova , D. Wesselinova

11.05 – 11.25 Coffee Break

Session G.

Chairpersons:

Assoc. Prof. Albena Alexandrova, PhD
Assoc. Prof. George Miloshev, PhD

Secretary: Delka Salkova, DVM, PhD

11.25 – 11.45

GO1. CHROMATIN COMET ASSAY – A POWERFFUL TOOL FOR MONITORING CHROMATIN ORGANIZATION DURING AGEING

K. Uzunova, M. Georgieva and G. Miloshev

11.45 – 12.05

GO2. CHANGES IN THE CHROMATIN STRUCTURE REVEALED BY CHROMATIN COMET ASSAY (CHCA) IN THE FRAGILE X CHROMOSOME SYNDROME MODEL *DROSOPHILA MELANOGASTER*

Milena Georgieva, Maria Petrova, Ginka Genova, George Miloshev

12.05 – 12.15

GO3. IAPs, SMAC AND THEIR ROLE IN APOPTOSIS

M. Georgieva, R. Detcheva, T. Pajpanova

12.15 – 12.25

GO4. ESTABLISHMENT OF STABLY TRANSFECTED MDCK CELL LINE EXPRESSING WILD TYPE OF HBEST1 PROTEIN

Kirilka Mladenova, Veselina Moskova-Doumanova, Petya Koleva,
Tanya Topouzova-Hristova, Jordan Doumanov

12.25-12.45

GO5. VISUALIZATION OF SYNAPTIC CIRCUITS

Albena Alexandrova

12.45 – 13.00

POSTER PRESENTATION – SESSION E

GP1. ПРЕЦИЗЕН МЕТОД ЗА ИЗВЛИЧАНЕ НА ДНК ОТ БИОЛОГИЧНИ СЛЕДИ ВЪРХУ АМУНИЦИИ ЗА ЦЕЛИТЕ НА ГЕНЕТИЧНОТО ПРОФИЛИРАНЕ

Лилия Николова и Катя Попова

GP2. ГЕННА ТЕРАПИЯ И ДИАБЕТ

Мария Панчева

Session H.

Chairpersons:

Prof. Reni Kalfin, PhD
Assoc. Prof. Anna Tolekova, MD, PhD

Secretary: Assist. Prof. Dimitar Ivanov, PhD

14.00 – 14.20

HO1. EFFECTS OF LOW SELENIUM INTAKE ON ENDOCRINE SYSTEM

Ivelina Himcheva and Boryana Ruseva

14.20 – 14.40

HO2. URINARY BLADDER MOTILITY IN EXPERIMENTALLY INDUCED DIABETES MELLITUS

T. Georgiev, P. Hadzhibozheva, R. Kalfin, A. Tolekova

14.40 – 15.00

HO3. СУБМАКСИМАЛНА ИЗДРЪЖЛИВОСТ И ВРЕМЕ ДО ИЗТОЩЕНИЕ НА ПЛЪХОВЕ, ПОДЛОЖЕНИ НА КОМБИНИРАНА ВИСОКО ЛИПИДНА И ВИСОКО ВЪГЛЕХИДРАТНА ДИЕТА БЕЗ ДОБАВЕН ХОЛЕСТЕРОЛ

П. Ангелова, Н. Бояджиев, К. Георгиева, П. Атанасова, П. Хрисчев

15.00-15.20 Coffee-Break

15.20 - 15.40

HO4 EXPERIMENTAL MODEL OF PARKINSON'S DISEASE – PROTECTIVE ROLE OF VASOACTIVE INTESTINAL PEPTIDE

Reni Kalfin, Maria Lazarova, Iren Belcheva

15.40 - 15.50

HO5. ANIMAL MODELS OF DEPRESSION

Almira Pavlova Georgieva

15.50 – 16.10

HO6. PHARMACOLOGICAL MODULATION OF COGNITION WITH NEW PEPTIDOMIMETICS IN SOCIALLY ISOLATED RATS

E. Encheva, M. Novoselski, R. Klissurov, L. Tancheva, D. Tsekova, N. Belova

16.10 – 16.30

HO7. BEHAVIORAL EFFECTS OF CHRONIC LOSARTAN TREATMENT AFTER KAINIC ACID STATUS EPILEPTICUS

Natasha Ivanova, Daniela Pechlivanova, Jana Tchekalarova

16.30 - 16.50

HO8. INTERSTRAIN DIFFERENCES IN THE EFFECT OF LONG-TERM TREATMENT WITH MELATONIN ON KAINIC ACID-INDUCED STATUS EPILEPTICUS, OXIDATIVE STRESS AND THE EXPRESSION OF HEAT SHOCK PROTEINS

Zlatina Petkova, Milena Atanasova, Daniela Pechlivanova, Jana Tchekalarova

16.50 – 17.00

POSTER PRESENTATION – SESSION H

HP1. ГЕННА ТЕРАПИЯ И ДИАБЕТ

Мария Панчева

17.00 – 17.15 Closing Remarks

Session A.

Chairpersons:

Assoc. Prof. Evelina Shikova, MD, PhD
National Center of Infectious and Parasitic Diseases;
Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences

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

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

AO1. THE RETROVIRUSES THAT CHANGED THE WORLD OF SCIENCE

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AO2. AURORA PROJECT: A EUROPEAN NETWORK ON CERVICAL CANCER SURVEILLANCE AND CONTROL IN THE NEW MEMBER STATES

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Abstract

Over 34 000 new cases and 16 000 deaths due to cervical cancer (CC) are reported annually in Europe-27. However, huge differences are observed among European countries in CC incidence and mortality. Geographical variations in CC burden are mainly due to the influence of cytology-based screening programmes. As far as today many European countries have developed population-based screening programs for CC, although they differ greatly in terms of organizational characteristics, implementation stage, coverage and quality assurance.

AURORA is a European programme carried out in 11 European countries (Bulgaria, Cyprus, Czech Republic, Greece, Hungary, Italy, Latvia, Poland, Romania, Slovakia, Slovenia) to support the exchange of information and expertise on good practices in promoting CC prevention in the New Member States, and in particular, addressing hard to reach populations (HTRP). The first step of AURORA Project was the analysis of the local context and the identification of local HTRP groups. The second step, the collection and analysis of good practices, regarded the evaluation of gold standard intervention and the collection of local promotional interventions in the field of CC prevention. The analysis showed high grade of variability in epidemiology and cervical cancer screening (CCS) programmes among AURORA partners. The burden of CC is particularly high in the New EU Member States with the highest rates in Romania, followed by Bulgaria. In all AURORA countries, the CC incidence rates were higher than in EU15 except Cyprus, Greece and Italy. However, in Cyprus, there were no reliable epidemiological data for CC and in Greece, a large underreporting of cancer morbidity data has been estimated. In countries were CCS is not yet implemented, the general population has been regarded as HTRP while in others countries some age groups, or women living in particular regions, or in particular settings were selected as HTRP. Analysis of the selected local projects as good practices allowed to drow some general conclusions on how to better promote the prevention of CC.

Collaboration and cooperation among European Community Member States providing an exchange of information on strengths and weaknesses of the previous experiences in implementing CC prevention will avoid duplications of efforts and will improve the cost effectiveness of the activities. The synergy among EU countries is expected to accelerate cervical cancer prevention improvements and therefore the epidemiological figures situation.

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AO3. HPV DNA TESTING FOR IDENTIFICATION OF HPV INFECTION

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Abstract

The link between persistent infection with high-risk HPV and occurrence of cervical cancer resulted in the development of a number of test systems to detect HPV infection. Most of currently available tests are based on the detection of HPV nucleic acids, mainly on HPV DNA detection. HPV DNA detection methods include target amplification assays and signal amplification techniques. The first type mainly comprises PCR-based methods. There are two ways to perform PCR for HPV DNA detection: broad-spectrum PCR for detecting all mucosal HPV types and type-specific PCR using type specific primers which amplify a single HPV genotype. Hybrid Capture 2 is the most widely used test based on signal amplification technique. HPV DNA testing is recommended as a primary screening tool for women over 30, for the triage of women with equivocal cytological results and for the follow-up after

treatment. HPV DNA testing is more sensitive than cytology, but there is concern that HPV testing is less specific than Pap testing. There are expectations that new approaches, such as mRNA-based detection of HPV E6/E7 oncoproteins and detection of specific host markers may improve identification of HPV infections associated with neoplasia.

Introduction

Over 100 types of HPV exist, of which more than 40 can be sexually transmitted, of them 15 are high-risk HPV (hr-HPV) types and can cause cervical cancer. The hr-HPV DNA can be detected in up to 99.7% of cervical squamous cell carcinomas [2, 22] and in 94% to 100% of cervical adenocarcinomas and adenosquamous carcinomas [21, 24]. Infection with hr-HPV is common, especially in women under age of 35 years, however, the vast majority of HPV infections will regress spontaneously. Cervical cancer is a complication of persistent infection with hr-HPV.

The recognition of the strong causal relationship between persistent infection with hr-HPV types and occurrence of cervical cancer resulted in the development of a number of test systems to detect HPV infection. HPV cannot be cultured reliably in a laboratory setting, it is a cell-associated virus and there are little or no infectious HPV particles in malignant tissue. Therefore, HPV detection methods are based on the detection of HPV nucleic acids and the most widely used HPV testing methods rely exclusively on the detection of HPV DNA.

HPV DNA detection methods

Target amplification assays

Target amplification assays mainly comprise PCR-based methods. There are two ways to perform PCR for HPV DNA detection: broad-spectrum PCR and type-specific PCR.

In type-specific PCR type-specific primers which amplify a single HPV genotype are used. In this case multiple type-specific PCR reactions must be performed separately [15] which is labor-intensive, costly and time consuming [15, 23].

Broad-spectrum PCR methods are capable of detecting all mucosal HPV types and use consensus primers that are directed to a highly conserved region of the HPV genome [1] Most laboratories use consensus primers targeting the L1 region, since it is the most conserved part of the genome. Multiplex systems targeting different regions for different types in one reaction have been also described.

The most commonly used consensus primers (shown in Table 1) are: GP5/6 [19] and its extended version GP5+/6+ [4, 8], the MY09/11 degenerate primers [13] and its modified version, PGMY09/11 [6, 7]. Both PCR systems target a conservative region within L1, generating 150bp (GP5/6- and GP5+/6+-PCR) and 450bp (MY09/11 and PGMY09/11) PCR fragments, respectively. GP5/6-PCR and GP5+/6+-PCR assays use a pair of single, unmodified, primers that are applied under low stringency PCR conditions. MY09/11 uses degenerate primers, and PGMY09/11 is based on the use of overlapping primers targeting the same regions as MY09/11. A short fragment PCR system, the SPF10 assay, was developed targeting a small (65 bp) region within L1, thereby potentially increasing the sensitivity of the assay [9].

Table 1. The main primer sets used in PCR amplification

Primer sets	**Characteristics**	**Amplified fragment length**
MY09/11	Amplify a highly conserved L1 region	~450 bp
PGMY09/11	Modified version of MY09/11	

GP5/6	Amplify a highly conserved L1 region	~150 bp
GP5+/6+	Extended version of GP5/6	~150 bp
SPF-PCR	Amplify a smaller region of L1	~65 bp

Analysis of a PCR product can be done in several ways: gel electrophoresis, enzyme immunoassays using microplates (EIA), reverse hybridization assays, real time PCR. Most widely used are the reverse hybridization assays [10, 11, 20] - labeled PCR products hybridize to oligonucleotides immobilized to various solid supports such as strips, filters, microarrays and microsphere. The advantage of these assays is that in a single analysis genotyping for a broad spectrum of HPV types can be performed. EIA is usually limited to the detection of HPV types as a pool (group detection). Real-time PCR assays are based on fluorescent-labeled probe(s) added to a standard PCR reaction that can hybridize with the amplicon during the annealing step.

The sensitivity and specificity of PCR based methods vary, depending mainly on the primer set, the size of the PCR product, the reaction conditions and efficacy of the DNA polymerase used in the reaction, the spectrum of HPV types amplified.

Signal amplification techniques

Signal amplification methods have a liquid-phase or an in situ hybridization format. Hybrid Capture 2 (Digene Corporation) is an example of the liquid-phase format and is the first FDA-approved HPV DNA detection test. Hybrid Capture 2 test is CE-marked, clinically validated commercial HPV test available worldwide. The assay relies on hybridization in solution of RNA probes to the single-stranded HPV DNA and subsequent chemiluminescent detection of the RNA/DNA hybrids [18]. RNA probes are complementary to the genomic sequence of 13 high risk (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) and 5 low risk (6, 11, 42, 43, 44) HPV types, which are used to prepare a high and low probe mix that are used in two separate reactions. The assay does not distinguish specific types. The clinical sensitivity of the test is very high (85 to 100%). The analytical sensitivity of the HC2 assay is at the picogram level of HPV DNA, whereas that of the PCR assays is at the subpicogram level.

Application of HPV DNA testing

Primary screening tool in all women over 30 alone or in combination with cytology

Up to 80% of sexually active women have been infected by one or more genital HPV types at some point throughout their life. HPV prevalence is highest in young women shortly after their sexual debut. As most of HPV infections are transient and are cleared spontaneously in about 90% of women, after the age of 30 years the rate of HPV positivity declines and reaches a relatively stable level. Therefore, in order to exclude transient positive cases, HPV screening should be initiated after the age of 30 years. HPV testing in women older than 30 years has an average sensitivity and specificity of 89% and 90% with negative predictive value greater than 97% [16] Women found positive for HPV can then undergo colposcopy with directed biopsy for confirmation of disease and appropriate management.

Triage of women with equivocal cytological results

Atypical squamous cells of undetermined significance (ASC-US) are squamous cells on a Pap smear that do not have normal appearance but are not clearly precancerous. Women with ASC-US require further testing because some of these women have precancerous lesions of the cervix. There are three options for evaluation of an ASC-US result - HPV testing, repeat Pap smear, colposcopy. It was found that HPV testing of women with ASC-US

detected 96.3% of women with previously undiagnosed CIN3 or cancer. This would significantly reduce referrals to colposcopy, particularly if the management strategy is to perform colposcopy on all women found to have ASC-US on cytologic evaluation.

HPV DNA testing for follow-up after treatment

Although currently available techniques for the treatment of cervical cancer precursors are considered very effective, in approximately 5-15% of cases the precursor lesions may persist or recur. It was found that treated women remained at increased risk of cervical cancer for at least 8 years compared to the general female population [17]. HPV testing has been used as an alternative to cytology and colposcopy for the detection of persistent or recurrent disease. If HPV DNA is undetectable 6 to 8 months post-treatment, the likelihood of posttreatment persistence or recurrence of disease is negligible. It was found that the sensitivity, specificity, and negative predictive value of HPV testing for the post-treatment detection of CIN 2/3 are 96.5%, 77.3% and 98.8%, respectively [12].

HPV DNA Testing vs. Cytology

Multiple studies indicate that HPV testing is more sensitive and has a higher negative predictive value compared to cervical cytology for identifying cervical cancer and its precursors [14]. A meta-analysis of studies from the United Kingdom (HART, Hammersmith), France (Reims), Germany (Hannover and Tuebingen, Jena), the Netherlands (Amsterdam) and Canada found that the sensitivity for detection of CIN2+ disease by HPV testing was 96% and by cytology was 53% , while the specificity was 92% and 97%, respectively [3]. The performance of HPV testing was found to be similar in different areas of Europe and North America while cytology was with limited reproducibility. In addition, testing for hr-HPV DNA has a very high negative predictive value (NPV), with most studies reporting values greater than 99 % and some reporting 100% [5].

Conclusion

HPV DNA testing is more sensitive than cytology, but there is concern that HPV testing, especially in young women, is less specific than Pap testing and with poor positive predictive value because cannot differentiate between women who test positive and who are truly at risk of high-grade lesions and cancer. Therefore, better indicators of HPV infection associated with increased risk of progression to neoplasia than detection of HPV DNA are needed. There are expectations that new approaches, such as involvement of mRNA-based detection of HPV E6/E7 oncoproteins and detection of specific host markers may improve identification of HPV infections associated with neoplasia.

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AO4. PAPILLOMAVIRUS-LIKE PARTICLES FOR HPV VACCINES DEVELOPMENT

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Abstract

In EU cervical cancer is the second most common cancer after breast cancer affecting women 15–44 years of age. The primary cause of cervical cancer is persistent infection with high-risk types of human papillomavirus (HPV), HPV 16 and HPV 18 being the most common high-risk types, responsible for about 70% of all cervical cancer cases. Low-risk HPV types, most commonly HPV 6 and HPV 11, have a causal role for development of approximately 90% of all cases of condylomata acuminata. This link between HPV infection and specific diseases has stimulated the development of HPV vaccines.

The discovery that the major capsid protein of papillomaviruses, L1, could self-assemble into virus like particles (VLPs) was the most important event for the development of current prophylactic papillomavirus vaccines. VLPs do not contain DNA, so they are noninfectious and nononcogenic, but are able to induce generation of high titers of virus-neutralizing antibodies. The vaccines, currently in use in Bulgaria and throughout the world, were independently developed and tested by two companies, GlaxoSmithKline (GSK) and Merck. The GSK vaccine, Cervarix, is bivalent and based on VLPs of HPV-16 and -18, produced in insect cells. Merck's vaccine, Gardasil/Silgard, is quadrivalent, composed of VLPs from HPV6, -11, -16, and -18, produced in yeast. Both vaccines demonstrate also a certain degree of cross-protection towards HPV types genetically and antigenically related to those included in the vaccines. Clinical trials demonstrated that currently available prophylactic HPV vaccines are safe, well tolerated, and highly efficacious in preventing persistent infections and cervical diseases associated with vaccine-HPV types. The limitations include the need for multiple parenteral doses, the lack of protection against some HPV types

that cause cervical cancer, and a relatively high cost. To overcome these limitations second-generation vaccines based on L2 minor capsid protein of HPV are under development.

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AO5. BLV INFECTED RATS AND RABBITS AS A MODEL OF HUMAN LYMPHOCYTIC LEUKAEMIA

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Abstract

Human T leukaemia is known to be quite identical with enzootic bovine leukosis due to common genome organization of their aethilogical agents - Human T leukaemia virus (HTLV) and Bovine leukaemia virus (BLV). Because of the very long period of the latency and the pathogenesis of the illness in cows, we tried to infect rabbits and rats with BLV. The inoculated material was derived from FLK cells permanently producing BLV. The viral presence in the inoculum used was proved by PCR, transmission electron microscopy and light and electron immunocytochemistry.. About 1/3 of the infected animals sustained BLV seropositivity during the experiment and developed symptoms of lympholeukaemia – immunosuppressive clinical condition, marked leukocytosys (predominantly lymphocytes and lymphoblasts) and lymphoid cell accumulations in most viscera. BLV DNA detected in diseased animals by PCR indicated the role of BLV as an aetiological factor of lympholeukaemia. The pathological changes in rats were more pronounced than those in rabbits proved by the statistical analyzes of results from haematological studies and survival. The results proved these two species of laboratory animals and especially the rats to be a suitable model to study the leukaemogenesis due to BLV/HTLV infections.

Keywords: bovine leukaemia virus, pathology, rats, rabbits

Introduction

The enzootic bovine leukosis (EBL) caused by the RNA bovine leukaemia virus (BLV) is spread worldwide due to the easy transmission of the infection both in the vertical and horizontal way. Economic losses associated with control and eradication of the disease are significant but the research interests are directed to the infectious agent because of the genetic similarity of BLV to a large group of retroviruses responsible for leukaemia in humans, such as HTLV-I, HTLV-II, and HIV [1, 16]. As for as the genome of BLV and HTLV I and II there is over 50% similarity of their nucleotide sequences [10].

It has been discovered that BLV affects other species as well. Sheep and goats are strongly sensitive. Pigs, rabbits, rats, cats, dogs, and some primates are also susceptible to BLV infection [7]. Sensitivity of human cells of neural origin to this infection has been proven, too [2].

The successful study of the EBL as a model of some human leukaemias is dependent to a great extent on the possibility of modelling the disease in experimental animals and creating easily manipulated animal model with a shorter incubation period. However, the attempts for reproducing the ailment in small laboratory animals were unsuccessful. In most cases, BLV antibodies were produced without manifestation of visible pathological processes [4]. Restricted pathological changes, resembling the lesions occurring in the human in the course of acquired immune deficiency syndrome (AIDS), have been reported by Altanerova et al. (1989) [3] and Todorova et al. (1995) [17] in rabbits infected with BLV.

The aim of the present investigation was to obtain additional clinical and haemathological data and to describe histopathological characteristics of the disease in rats and rabbits infected with BLV.

Material and Methods

Infection material. FLK cells from a culture permanently producing high amounts of BLV were tested for viral activity by immunofluorescence, immunogold labelling, transmission electron microscopy (TEM), and PCR analyses:

Immunofluorescence. The production of viral Tax protein was visualised by indirect immunofluorescence in FLK-BLV cells..

Electron microscopy. The monolayer culture from FLK-BLV cell line was embedded in Epon and observed under JEOL 1200 EX electron microscope.

For immunogold labelling, cells from the same line were embedded in antigen activity preserving resin Lowikryl K4M (Serva) [8] and proceeded for Tax protein visualization.

PCR analysis. PCR was used to detect BLV in peripheral blood mononuclear cells (PBMCs) of the diseased animals. Primers to the Pol and Tax regions of BLV were used for amplification.

Laboratory animals. Thirty-two Wistar rats of both sexes, at the age of 1-2 months, with a weight at about of 50 g, 18 New Zealand White rabbits and six California rabbits of both sexes, aged 1-2 months, weighing 300-400 g, were used in the experiments and as control animals. The experimental animals were infected twice at 10 days interval.

All animals were kept under standard conditions [11] and were sacrificed at the end of the second year.

Haematology. Blood samples from all animals were collected every 4 months starting 2 weeks after the last inoculation. Leukocytes were counted using the Bürker camera. Blood smears were also prepared and stained with May–Grünwald–Giemsa method. Haematologic reference ranges described by Harknes and Wagner (1995) [13] were used.

Serology. All blood serum samples were tested for gp51 and p24 antibodies, using the single radial immunodiffusion method originally described by Manchini et al. [14].

Pathomorphology. Autopsies were performed on all dead or sacrificed experimental and control animals. Samples from the liver, spleen, lymph nodes, kidneys, and lungs were collected from all autopsied animals for microscopic observation.

Statistical analysis. Prisms 2 (GraphPad Software, Inc., San Diego, CA, USA), Ky Plot (KyensLab, Inc., Tokyo, Japan) and Excell (Microsoft Corp., Inc., USA) were used for the statisitical analysis of the data. Briefly, the following analyses were performed:
Test of Kolmogorov-Smirnov vereified the Gaussian distribution of data.
On-line Grubb`s test [12]; Variation analysis; Student`s-Fisher t-test; Pearson`s correlation analysis; Linear-regression analysis; Density estimation (Density); Kaplan-Meier (survival) analysis.

Results

Inoculum. TEM revealed numerous C-type virus particles in the intercellular spaces of the FLK-BLV culture (Fig. 1a). BLV protein production was proved by immunofluorescence (Fig. 1b) and immunogold labelling (Fig. 1c).

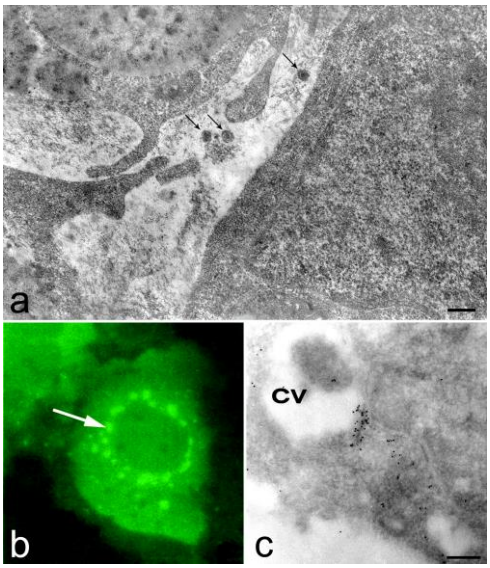

Fig. 1. BLV viral particles in intercellular space (a), perinuclear pearl-like accumulation of BLV Tax protein in cytoplasmic vacuoles (CV) (b), and Tax immunogold labelling (c). Bar 200 nm.

Serology. The results from radial immunodiffusion test showed that 34% of the rats and 22% of the rabbits inoculated with BLV-containing material reacted positively 2 weeks p.i.. The gp51 and p24 antibodies persisted untill the end of the experiment in almost 90% of the cases.

PCR analysis. PCR was applied to detect DNA extracted from PBMCs, obtained from rats and rabbits with symptoms of lympholeukaemia, developed after inoculation with cells of BLV producing FLK cell line. The diseased animals were positive for BLV DNA. PCR products of 709-bp and 1008-bp were amplified using BLV pol and tax primers, respectively (Fig. 2). No BLV DNA was detected in PBMCs of non-infected animals.

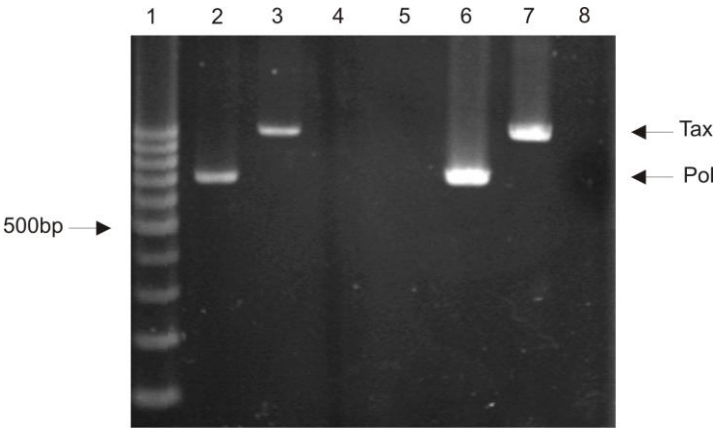

Fig. 2. BLV DNA detection by PCR in PBMCs of diseased rats: amplification of 709 bp fragment of pol gene (line 2) and 1008 bp fragment of tax (line 3). PBMC DNA of non-infected rats amplified by BLV pol (line 4) and tax (line 5) primers was used as negative control. BLV producing FLK cell line was used as positive control – amplification with BLV pol and tax primers (lines 6 and 7). Line 1 - molecular weight marker (100 bp)

Clinical findings. During the experiment and especially after the first year, 60% of the rats and no more than 25% of the rabbits developed disorders such as weight loss, alopecia, pruritus, erythema, conjunctivitis, rhinitis, and pneumonia.

In the second year, by the end of the experiment, the most of the treated rats were already dead. All control animals were in a good health and no mortality was observed.

Haematology. The groups of the infected animals from both experimental species showed increase white blood cell (WBC) number, more pronounced in rats, with a peak in months 14-18 *p.i.* The elevation of WBC number was due to the appearance mainly of lymphocytes and lymphoblasts in the peripheral blood (Fig. 3). No alterations of WBC number were observed in control animals untill the end of the experiment.

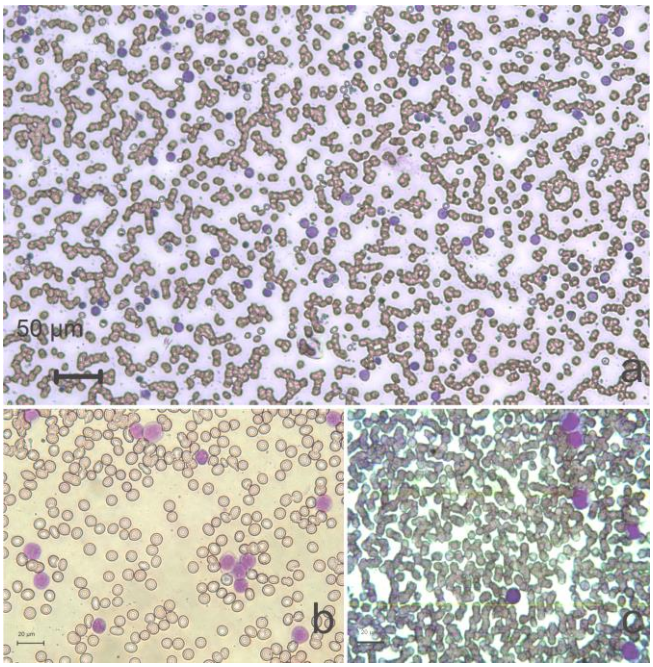

Fig. 3. Lymphocytosis in rat inoculated with BLV (**a**). Young and mature lymphocytes in rat (**b**) and rabbit (**c**), 20-th month p.i.

Gross pathology. Enlarged lymph nodes were sometimes observed in experimental rats and rabbits (Fig. 4). They were of fat consistence and homogeneous cut surface appearance. Alterations in the other viscera were not found.

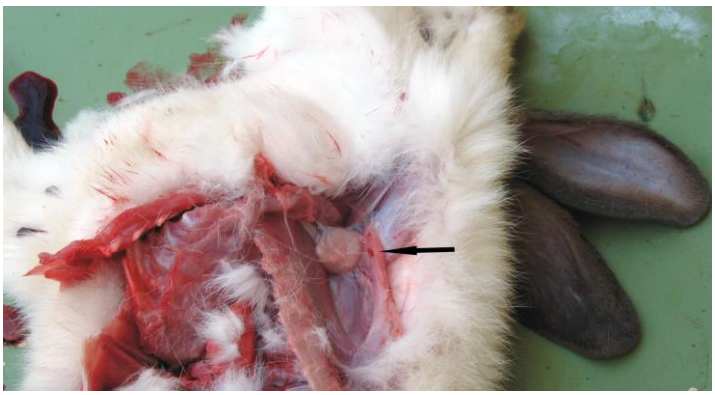

Fig. 4. Enlarged prescapular lymph node in a BLV-inoculated rabbit.

Microscopically perivascular lymphoid infiltrations were observed in the liver (Fig. 5) and kidney of the seropositive animals. The histopathological lesions in the lungs were manifested by haemorrhages, laceration of alveoli due to lymphocyte infiltrations, and presence of focal serous or purulent pneumonia (Fig. 6).

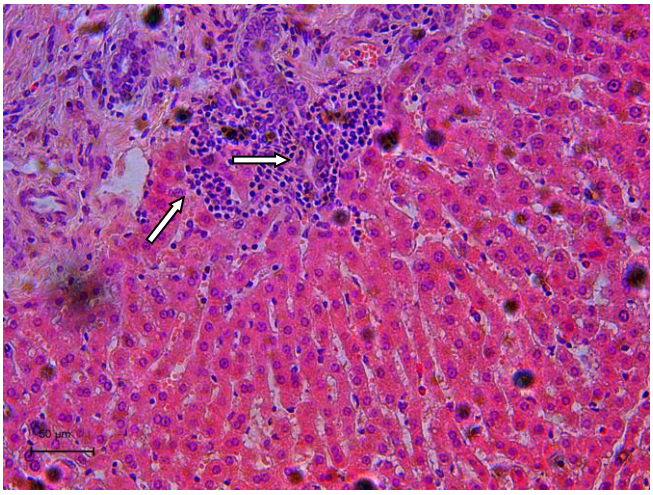

Fig. 5. Liver from BLV-infected rabbit; perivascular lymphoid infiltrations. H&E. Perivascular lymphoid infiltrations and holangiocyte hyperplasia ⟹ (B, C), 23-24 months p.i.

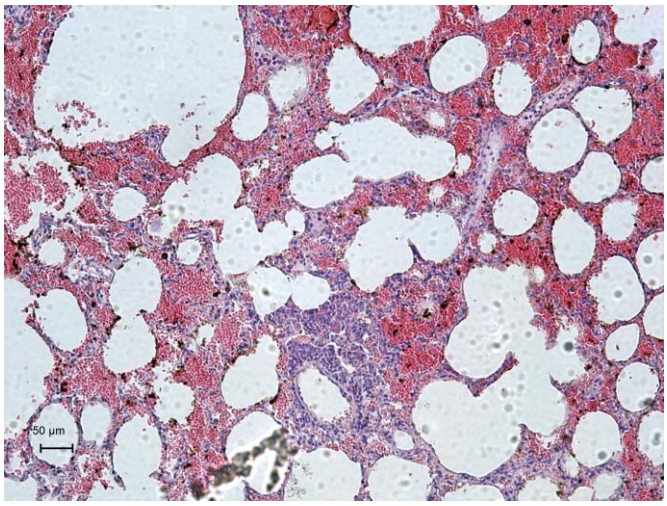

Fig. 6. Lung tissue from a BLV-infected rat. Large haemorrhagic areas and lymphocyte infiltration. H&E.

In the spleen the white pulp was spatially reduced, but relatively rich in cells, and in some cases displayed well-shaped germinal centers. The sinuses were packed with erythrocytes. The lymph nodes showed histiocytosis with many blast cells in mitosis.

Statistical analysis of the results from the hematological investigations

The total number of leukocytes and the percentage of lymphocytes of the experimental rabbits and rats were submitted to statistical analysis. The Kolmogorov-Smirnov showed a Gaussian distribution; therefore parametric analysis was applied in next calculations.
According the range analysis (on-line test of Grubb) the analyzed data did not contain outline values ($P = 0.01$). The linear regression analysis did not show linear dependence of the data.

Statitical analysis of the total number of leucocytes and % lymphocytes in rabbits.

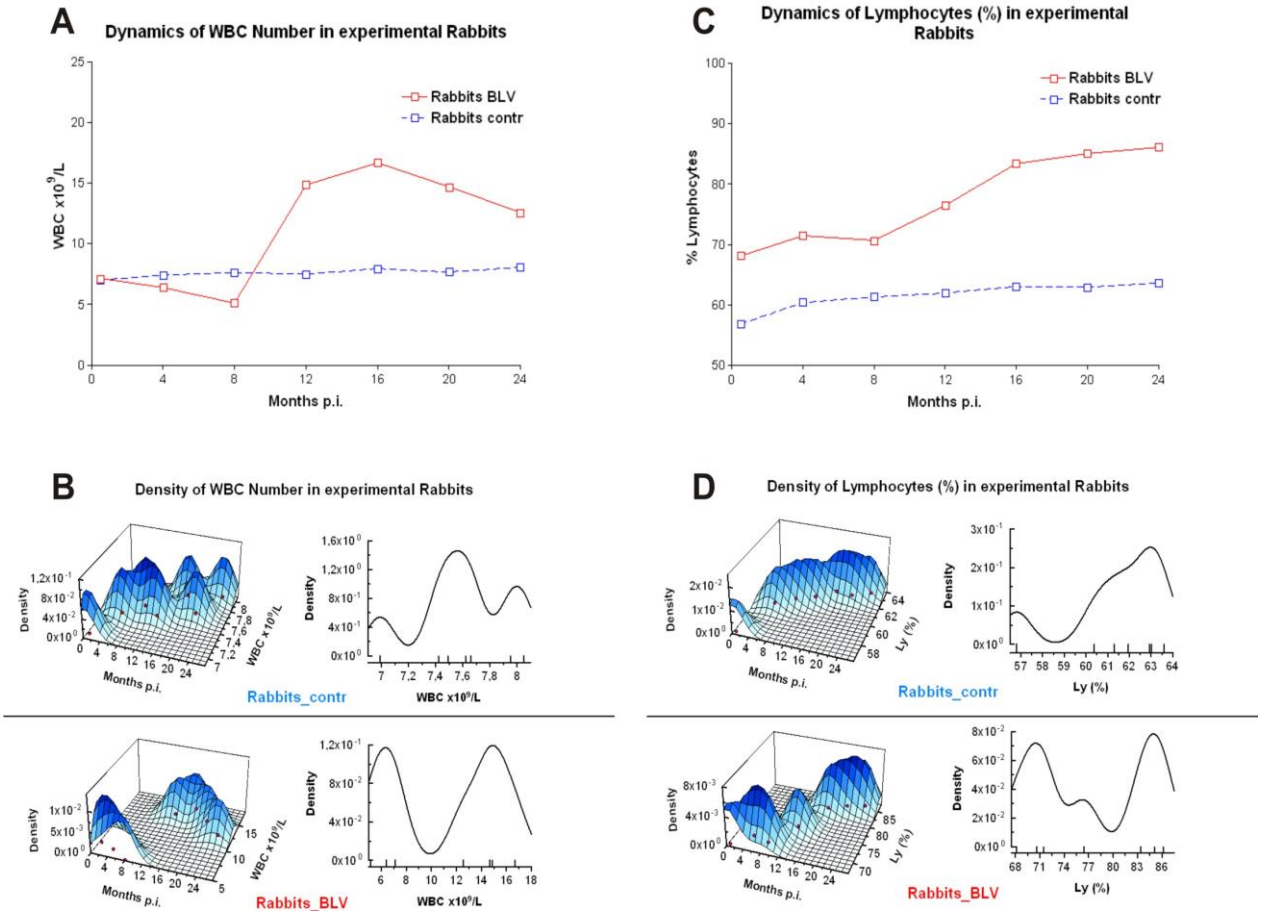

Fig. 6. Dynamics and density of the total number of leucocytes and % of lymphocytes in the expremental rabbits.

The interpretation of the dynamics and the density of the WBC number and the comparison of data with selected referent values (7.5-13.5 x 10^9 / L) showed that the BLV infected rabbits develop leukocytosis between 11 and 22 month *p.i.* (Fig. 6A-red line). According to the Fisher (F) criterion ($P<0.0001$) there is a statistical significant difference between the groups of the infected and control animals, as shown by the statistical analysis of the dynamics in the WBC number.

Regarding the changes in % lymphocytes a constant increase during the first 8 months p.i. was observed in the inoculated rabbits. This increase overpassed the referent limits (55-80%) after the 14 month (Fig. 6C-red line). Thereafter, the increase in the dynamics curve of the % lymphocytes coincides with the probability density function peak with values 83-86% (Fig. 6D-Rabbits_BLV) and could be determined as leukocytosis. There is a statistically significant difference between the two groups as determined by Student-Fisher test (Student $P=0.0002$; Fisher $P<0.006$).

Statistical analysis of the total number of leucocytes and % lymphocytes in rats

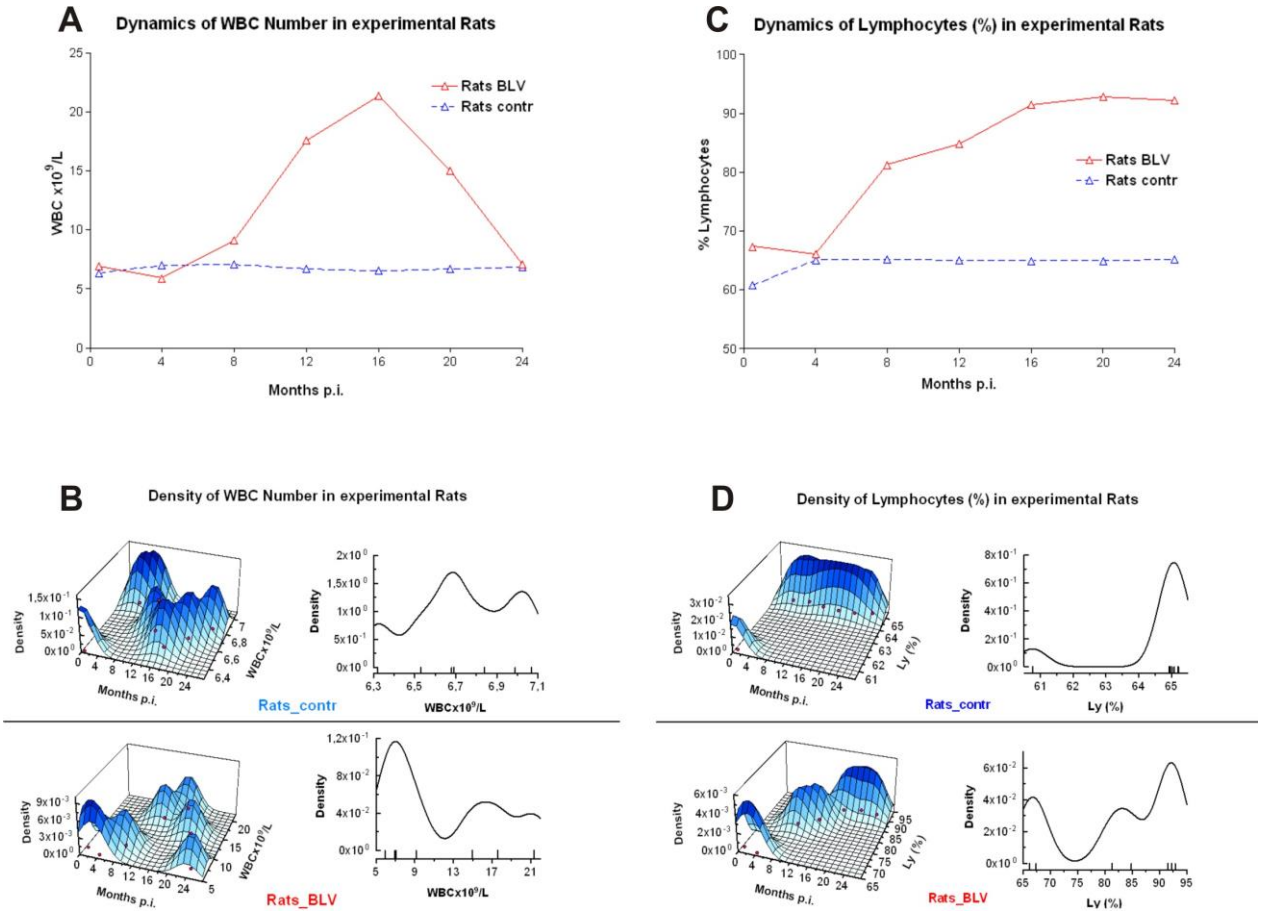

Fig. 7. Dynamics and density of the total number of leucocytes and % lymphocytes in the experimental rats.

Taking into account the dynamics (Fig. 7A-red line) and density (Fig. 7B-Rats_BLV) of the WBC number it could be concluded that during the period 12-th – 19-th month *p.i.* the infected rats developed leukocytosis (referent values 6-17x10^9 / L). The results of the statistical analysis showed statistically significant difference between the groups of infected and control rats according to Student-Fisher test (P = 0.046 Student and P <0.0001 Fisher). According to the test of Pearson there was no statistically significant correlation to the number of WBC between the two groups of rats (r = 0,34; P = 0.45).

In infected rats the % of lymphocytes after a slight decrease in the fourth month began to rise sharply, causing (after 12-th month) lymphocytosis (reference range 65-85%), persisting until the end of experiments (Fig. 7C-red line). Student-Fisher test showed that the two groups have a statistically significant difference. According to the criterion of Student P = 0.0015, and according to that of Fischer P <0.0001. Correlation between the values of the compared groups was not found (Pearson r = 0,56, P = 0.19).

Discussion

The obtained data showed that treatment of rats and rabbits with BLV-producing cells of the FLK-BLV permanent line leads to infection in approximately 1/3 of the experimental animals. The electron-microscopically observed virus particles, the BLV-protein (Tax) production, and the BLV DNA detection by PCR revealed the infectivity of the FLK -

inoculum used. The result is a successful infection of the inoculated laboratory animals proved by the presence of specific viral antibodies in the serum. These data are in conformity with reports from other authors, who demonstrated the infection with BLV by the detection of antibodies not only in rabbits and rats but also in other experimental animals [6, 7].

In our study, the BLV infection leads to a primary disease with clinical, haematological, and histopathological findings, which were not detected in the control animals. Alopecia, accompanied by pruritus and erythema, rhinitis, and pneumonia, more pronounced during the second half of the experiment, could be explained by the well known immunosuppressive properties of BLV [15]. The clinical course of the disease, described in this study could be interpreted as AIDS-like symptoms and confirms partially the findings of Altanerova et al. [3].

The observed changes in the total number of leukocytes and % of lymphocytes in the infected with BLV groups from both species are statistically significantly different from their respective control groups. In the animals from the infected groups the blood cell count showed that rabbit leukocytosis, which develop during the second half of the trials were due to the a progressive lymphocytosis. This, expressed to a greater extent, was also applied to the rats. The permanent increase of % of lymphocytes in the rats over the normal level (over 90%) is more strongly expressed, as compared to the reference values either, or to those in the control animals, as confirmed by the statistical analysis. Therefore, after infection with BLV this kind of laboratory animals reacts with strong deviations in the white blood cells count, developing a high-grade lymphocytosis compared to rabbits. The hematologic changes observed in the infected animals from both laboratory groups too closely resemble a leukocytosis due to persistent lymphocytosis, which is one of the most common forms of the EBL in cattle. Moreover, an increase of lymphoid cells proportion was found, which had characteristics of malignancy - young various types, more basophilic cytoplasm, different shape and size of nuclei, segmentation and granularity of the nuclei and prominent nucleoli.

With approaching 24-th month p.i., the WBC count decreased sharply in the infected rats, while the % of the lymphocytes was retained in stable levels. It could be assumed that after the end of the experiments, the infected rats tend to develop leukopenia accompanied by strong relative lymphocytosis. Such a condition is sometimes associated with the terminal phase or appears as a complication of certain neoplastic diseases, particularly hematologic. Dearden (2008) [9] reported neutropenia as one of the complications in patients with chronic lymphocytic leukemia (CLL).

To summarize the discussed so far clinical and hematological abnormalities in the wo laboratory species infected with BLV, except for an immunosuppressive effect in the second half of the time course of the experiment, a carcinogenic effects of the virus was also observed, manifested by significant lymphocytosis and presence of immature and malignant cells in the peripheral blood.

Moreover, enlarged lymph nodes, histological lesions in the viscera, such as lymphocyte infiltrations and activation of the reticuloendothelial system, appearred to be similar to the alterations induced by the field strain of BLV in cows. BLV DNA response, detected in diseased rats and rabbits by PCR, indicates the role of BLV as an aetiological factor of lympholeukaemia, developed in these animals after BLV infection. These alterations were more pronounced in rats, which, to our opinion, are more suitable as a laboratory model of EBL than rabbits. Altanerova et al. [6] reported for BLV production in a spleen cell line issued from infected with BLV rats. In these spleen cells BLV DNA were detected. Positive BLV infection was proved by PCR (BLV pol primers) and in rabbits inoculated with FLK BLV cells and with BLV SGV provirus pU5gag-pol-env [5].

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AO6. PLANT SUBSTANCES ARE PROMISING INHIBITORS OF HERPES VIRUS INFECTIONS

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Abstract

Recently, the literature reports that some traditional medicine plants have anti-herpetic activity. Many molecules, such as essential oils, terpenes, alkaloids, phenols, flavonoids, sugar-containing components, etc. have been proven in extract of plants. They are characterized by a variety of biological activities, as well as antiviral activity against a variety of viruses, including herpes.

Introduction

Members of the family *Herpesviridae* are isolated from all classes of vertebrates as well as invertebrates (molluscs). The family includes over 150 representatives, eight of which attack humans (herpes simplex virus 1 and 2 – HSV-1 and HSV-2; varicela zoster virus – VZV; human cytomegalovirus – HCMV; Epstein-Barr virus – EBV; human herpesvirus 6, 7 and 8 – HHV-6, HHV-7 and HHV-8) [11]. These infectious agents identified as HHV (human herpes viruses), cause diseases of varying severity and varied clinical expression - from asymptomatic and self-limiting infections such as mucocutaneous lesions of the oral cavity and the urogenital tract, keratitis, keratoconjunctivitis, severe generalized infections, encephalitis, meningitis, and malignant neoplasms.

The main problem facing, the development of effective anti-viral chemotherapeutic agents comes from the close relationship between the viral replicative cycle and the host cell. Therefore, effective targets for antiviral agents are that virus-specific structures and functions that determine viral replication, but which do not exist or are not essential for the cell, respectively, for the host organism.

The virus - specific targets for chemotherapeutic agents are:

1. Extracellular virions: reducing the possibility of virions to infect susceptible cells is based on the ability of certain compounds to nonspecifically inactivate virions outside the cell by denaturing viral proteins and / or by causing structural changes in the lipids of the envelope.

2. Attachment (adsorption): reducing the possibility viral attachment protein to recognize specific cell receptors, which may be proteins, carbohydrates or lipids.

3. Fusion: at this stage fusion of the viral envelope with the cell membrane is prevented thus stopping the virus to penetrate inside of the cell.

4. Intracellular replicative cycle: in the infected cell herpes viruses induce a number of virus-specific enzymes that duplicate the action of similar cellular enzymes, but differ from them in many physico-chemical characteristics: - molecular weight, requirement for certain cations, sensitivity to inhibitors, substrate specificity.

5. Assembly of new viral particles and leaving the host cell.

In herpesvirus infections, chemotherapy was developed based on the application of nucleoside analogues, that reduces the duration of symptoms and lead to faster healing of the lesions. The disadvantage of nucleoside analogues is the relatively rapid selection of resistant mutants [1]. Patients with a compromised immune systems, and in the first place - HIV positive ones are with the highest risk [29].

Due to the frequent failure of treatment with nucleoside analogues, there is a need for developing new inhibitors of herpes viruses [14]

Plant substances inhibitors of herpes virus infections

Hundreds of years people used a variety of plants for the treatment of various diseases. In recent decades, huge number of extracts of these plants are tested for activity against many viruses including herpes. Significant part of these extracts showed remarkable anti-herpesvirus activity. Grape, apple, strawberry juices and extract of *Azadirachta indica* possess anti-HSV activities. Aqueous extract of *Carissa edulis* root from Kenya show *in vitro* and *in vivo* antiviral activity against acyclovir (ACV) sensitive and ACV resistant strain of HSV-1 and HSV-2. Extract of *Ceratostigma willmattianum* demonstrate suppression of viral adsorption, replication and transcription of HSV-1 and HSV-2 [5]. Essential oils of ginger, thyme, hyssop and sandalwood effectively inhibit drug-resistant clinical HSV-1 strain [23]. Extract from *Cardamine angulata*, *Conocephalum conicum*, *Lysichiton americanum*, *Polypodium glycyrrhiza* and *Verbascum thapsus* exhibit ntiviral activity against HSV [13], extract of the Taiwan folk remedy *Boussingaultia gracilis* and *Serissa japonica* and extract of Senna petersiana used for sexually transmitted diseases [5]. Methanolic crude extract from *Mallotus Peltatus* possesses weak anti-HSV activity [3]. *Eucalyptus globulus* is a traditional herb in Iran used from many years ago. Methanolic extract of this plant inhibits HSV-1 replication in cell culture in various dilutions [8]. Acetone, ethanol and methanol extracts of *Phyllanthus urinaria* inhibit HSV-2 infection *in vitro* [28]. An extract of *Ribes nigrum* L., known as blackcurrant in Europe and Kurokarin® in Japan inhibits HSV-1 attachment on the cell membrane and inhibits virus replication of HSV-1, HSV-2 and VZV by suppression of protein synthesis in infected cells in the early stage of infection [25]. Ethanolic extract of *Rheum officinale* and methanol extract of *Paeonia suffruticosa* inhibit attachment and penetration of HSV-1 and other studies show that aqueous extract of *P. suffruticosa* and ethanolic extract of *Melia toosendan* affect attachment and replication of HSV -1 and HSV-2. Garlic extract and extract of *Terminalia chebula* showed anti-HCMV activity [5], as well as extracts from *Euphorbia australis* and *Scaevola spinescens* [13].

For extensive study of different plant species for their antiviral activity, folk medicine has turned attention to marine species, extracts of which also reveal notable activity against herpes viruses. Water extracts from *Haslea ostrearia* and *Polysiphonia denudate* from the Bulgarian Black Sea coast indicates anti-HSV activity and affected adsorption of virus and the intracellular stages of viral replication [22]. Crude extracts from Brazilian seaweeds showed activity against HSV-1 and HSV-2 [24].

The extracts of all plant species contain a large number of secondary metabolites that possess anti-herpesvirus activity. These are mainly essential oils, terpenoids, alkaloids, lignans, coumarins, polysaccharide, saponins, proteins and peptides, phenolics, polyphenolics, flavonoids and tannins.

Essential oils (volatile oils, ethereal oils) are containing volatile aroma from plants. In recent years they are used in aromatherapy due to their healing properties. Eucalyptus sandalwood oils [13], oils of *Melissa officinalis*, *Artemisia douglasiana* and *Eupatorium patens* [5] manifested anti-HSV activity. Some studies show that the activity is due to a direct virucidal effect, others – due to the inhibition of cell-to-cell transmission [21].

Terpenes are a class of hydrocarbons, produced by many plants and some insects. Terpenes are referred to as terpenoids when possess additional elements like oxygen or are chemically altered by some rearrangement in the carbon skeleton. They are the main component of any plant resin or essential oils. Moronic acid from *Rhus javanica*, sclerocarpic acid from *Glyptopetalum sclerocarpum* and lupenone from *Euphorbia segetalis* are terpenes with anti-HSV activity. Meliacine from *Melia azedarach* L.inhibit DNA synthesis, nucleocapsids assembly and late stages of HSV replication [5]. Ursolic acid isolated from *Mallotus peltatus* displays high activity against HSV replication, probably in an early stage [3].

Alkaloids are compounds that contain mostly basic nitrogen atoms but may also contain oxygen, sulfur and more rarely other elements such as chlorine, bromine and phosphorus. The majority of alkaloids are known for their hallucinogenic, stimulant or tranquilize properties. Six aporphine alkaloids demonstrated activity against HSV-1, three of them oliverine HCL, pachystaudine, and oxostephanine interfere with the viral replicative cycle [18]. Acridone alkaloid citrusinine-I inhibits HSV-1, HSV-2, resistant to ACV strain of HSV-2 and HCMV by suppressing DNA synthesis [27].

Polysaccharides are carbohydrates with repeated monomer units joined by glycosidic bonds that may be amorphous or insoluble in water. Monosaccharides in the composition of the polysaccharide may be of one type (homopolysaccharide or homoglycan) or different (heteropolysaccharide or heteroglycan). Sulfated polysaccharide galactofucan and polysaccharide from *Prunella vulgaris* L. demonstrated anti HSV activity [5].

Saponins are amphipathic glycosides composite of one or more hydrophilic glycoside combined with a lipophilic triterpene or steroid. They have a bitter flavor and the ability to produce soap-like foaming when shaken in aqueous solutions. The saponins spirostane, tomatidane, solasodane, nuatigenin, ergostane and furostane dimers manifested anti HSV-1 activity [5].

Peptides are short chains of amino acid monomers (approximately 50 amino acids or less) linked by peptide bonds, and by covalent bonds formed between the carboxyl group of a given amino acid and the amino group of another amino acid. 2kD peptide was extracted from *Sorghum bicolor* that inhibits the initiation and the spread of HSV-1 infection [9]. The peptide meliacine of *Melisa azedarach* affects uncoating and budding of HSV-1 [4].

Phenols (phenolics) consist of hydroxyl group bonded to an aromatic hydrocarbon group and depending on the number of phenol rings phenols are divided into phenols and polyphenols [2]. Phenols are produced by plants and microorganisms as a result of various

external stressors and may have variations in their structure depending on the type of organism from which they are produced [12].

Flavonoids (or bioflavonoids) are polyphenolic compounds of low molecular weight and they are the most important plant pigments for flower coloration. Flavonoids amentoflavone and robustaflavone were isolated from medicinal plants *Rhus succedanea* and *Garcinia multiflora* manifested significant anti-HSV-1 and HSV-2 activity and andrhusflavone and succedaneflavanone that demonstrated anti-VZV effect. Morin extracted from *Maclura cochinchinensis* show inhibitory activities against HSV-2 [13]) and ent-epiafzelechin-(4α→8)- epiafzelechin extracted from *Cassia Javanica* prevent penetrating of virus in the cells and affect replication at the late stage [7].

Coumarins are double-ring polyphenols that belong to the benzopyrones family characterized by its fragrance. The anti-herpes activity of some coumarins and / or their derivatives is a proven fact. Resveratrol and oxyresveratrol inhibit herpesvirus replication [10]. An additional study demonstrates that resveratrol affects the expression of essential immediate-early, early and late genes and the synthesis of viral DNA.

Lignans are phenylpropanoids and are one of the major classes of phytoestrogens. Lignan yatein isolated from *Chamaecyparis obtuse* inhibits the expression of alpha gene ICP0 and ICP4, arrests DNA synthesis and structural protein synthesis of HSV-1 [5].

Tannins are polyphenols that are devided into two groups: hydrolyzable and condensed. In the structure of the hydrolyzable ones the sugar residue is esterified by gallic acid or other polyphenolic acids. Condensed tannins are mainly composed of flavonoid monomers, mostly (+) catechin or (-) epikatehin connected with each other through the carbon-carbon bonds [20]. Various tannins were tested for antiviral activity in recent years. Three hydrolysable tannins, i.e. castalagin, vescalagin and grandinin extracted from powdered pedunculate oak (*i.e., Quercus robur*) manifested significant anti-HSV-1 and HSV-2 activity, and also showed synergistic antiviral effect *in vitro* when concomitantly administered with ACV [26]. Chebulagic acid and punicalagin – two hydrolysable tannins isolated from *Terminalia chebula* Retz. inactivated HSV-1 entry and cell-to-cell spread as their targets are HSV-1 glycoproteins [17]. Seven ellagitannins isolated from *Phyllantus myrtifolius* and *P. urinary* and eugeniflorin D (1) and D (2) isolated from *Eugenia uniflora* L. are active against DNA polymerase of EBV [16]. Ellagitannins geraniin possess virucidal effect against herpesviruses [19]. Hydrolyzable tannin casuarinin from *Terminalia arjuna* Linn prevent attachment of HSV-2 in the cell, and also violates the late stages of infection [6]. Eugeniin (from *Geum japonicum* and *Syzygium aromaticum*) showed significant inhibitory effect on activity of DNA polymerase of HSV-1 [15].

Conclusion

Plant species from almost all plant families are at the heart of alternative medicine in different countries. Roots, stems, bark, leaves, flowers, fruits and grains possess medicinal properties from which are isolated substances with different structure as essential oils, terpenes, alkaloids, saponins, peptides, phenols, flavonoids, tannins, polysaccharides and other. They possess various biological activities, including anti-herpetic activity results in a damage at different stages of the virus replicative cycle. Further research remains to be conducted more research to discover new active substances, as well to establish the exact mechanism of action and the optimal concentrations in which active ingredients can be applied. Nature by its diversity provides a vast world in which future antiviral agents are hiding.

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AO7. BIOLOGICAL PROPERTIES OF ELLAGITANNINS IMPORTANT FOR HUMAN HEALTH

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Abstract

Hundreds representatives of group of the ellagitaninns have been isolated and characterized in the last 40 years. Interest in them is caused by the healing properties of plants of which they have been isolated. Many teams are working to clarify the biological properties of the substance and to determine the exact mechanism of their activity.

Introduction

Tannins are group of antioxidant polyphenols which are divided into hydrolysable that can be hydrolyzed to glucose and gallic acid, and condensed tannins (proanthocyanidins) composed of flavonoids. Ellagitannins refer in the group of hydrolysable tannins made up of two galloyl units connected together and form the basic structural unit of ellagitannins – hexahydroxydifenic acid (HHDP). They are important secondary metabolites of plants because of their property to induce resistance against microbes due to their ability to bind with proteins and polysaccharides, and thereby block their growth.

Many ellagitannins are isolated and structurally characterized from plant species used in traditional medicine. Grape, strawberry, cranberry, blueberry, pomegranate, chestnut, walnut, brasilian nut, pecan nut are with the highest content of ellagitannins [3].

Biological properties of ellagitannins

This group of plant products attracted the attention of more and more research teams due to their multifunctional properties that make them promising therapeutic agents.

Antioxidant activity

Oxidative stress play significant role in the development of cancer, cardiovascular, neurodegenerative and inflammatory diseases. Ellagitannins have the ability to prevent the formation of free radicals, as well as reduced damage in the cells caused by them [20,15]. This effect is stronger when ellagitannins are containing a greater number of phenolic hydroxyl groups [5]. Products derived from the metabolism of ellagitannins also possess antioxidant properties [6].

Antidiabetic activity

High level of glucose in the blood can cause damages to eyes, kidneys and heart. *C*-glycosidic ellagitannins lagerstroemin, flosin B and reginin A from *Lagerstroemia speciosa* used for treatment of diabetes in the Philippines are activators of glucose transport [21].

Antihypertensive activity

Castalagin, chebulinic acid and corilagin isolated from the leaves of Lumnitzera racemosa are hydrolysable tannins with pronounced antihypertensive activity [9].

Antitumor activity

One of the most remarkable abilities of ellagitannins is their activity against various tumors as cervical cancer, prostate cancer, malignant cells in skin, breast, stomach, lung, esophagus, liver and others [3].

Many experiments have been conducted, the results of which lead to several possible mechanisms of action. Ellagitannins possess the ability to bind to proteins located on the surface of the cell membrane and thus preventing the proliferation of metastatic cells. They can induce apoptosis in tumor cells by inhibiting factors responsible for the formation of metastases. Another mechanism suggests that during the DNA replication ellagitannins bound carcinogens in complex and so it can not cause mutation [15]. In literature has many data for ellagitannins with antitumor activity – geraniin, corilagin [13], oenothein A and B, woodfordin C, D and F [10].

There is evidence that ellagitannins reduce the negative effects of chemotherapy and mitigates the effects of radiation exposure in anticancer therapy [17].

Immunomodulatory activities

Ellagitannins show immunomodulatory activities using different mechanisms as a promoter of the formation of catechin-polysaccharide complex that is a potential immunostimulant [11]; enhance the functionality of macrophages [16]; stimulates the secretion of cytokines IL-1 , IL-β2 and αTNF- by human peripheral mononuclear cells [19].

Enzyme inhibitory activity

Representatives of this group are also effective inhibitors of certain enzymes. Woodfruticosin (woodfordin C) is with atni-topoisomerase II activity; eugeniflorin D1 and D2 isolated from *Eugenia uniflora* L. and oenothein B effectively inhibit Epstein-Barr virus (EBV) DNA polymerase. Enzymes 5α-reductase and aromatase have an important role in the development of benign prostatic hyperplasia, which potential inhibitors appear oenothein A and B isolated from *Epilobium* species. It has been suggested that an important factor in the expression of genes, DNA replication and differentiation of cells is enzyme poly(ADP-ribose) glicohydrolase that can be inhibited by oligomeric ellagitannins oenothein B and nobotanin B, E and K. Enzyme α-glucosidase (maltase) possibly important in the development of type-2 diabetes are inhibited from chebulagic acid (isolated from *Terminalia chebula*), tellimagrandin I and eugeniin (casuarictin) [21].

Cell proliferation and differentiation activity

Geraniin and furosin isolated from *Phyllanthus muellerianus* stimulated proliferation and differentiation of human keratinocytes and dermal fibroblasts as well as biosynthesis of collagen [1].

Antimicrobial and antiparasitic activity

Many ellagitannins exhibit activity against various bacteria, fungi and parasites. The growth of bactaria *Escherichia coli*, *Candida albicans*, *Crypotococcus neoformans* and fungus *Aspergillus fumigatus* are affected by the action of pomegranate, punicalagin, punicalin, gallagic and ellagic acid. Derivatives of ellagic acid demonstrated activity against bacteria *Klebsiella pneumoniae*, *Bacillus cereus*, *Salmonela typhi* and *Salmonela pyogenes* [3]. Ellagitannins manifested antiparasitic activity against intracellular amastigotes of *Leishmania Donovan* [7].

Antiviral activity

In recent years more and more often been reported antiviral activity of various ellagitannins. Ellagitannins are potential inhibitors of various enveloped viruses due to its capabilityits to bind with different proteins and altering their structure to inactivate them.

Many studies have been conducted with ellagitannins against the replication of human immunodeficiency virus (HIV) and the results of the various teams indicate that targets for action of elagitaninite in replicative cycle of HIV are several. There is evidence for ellagitannins that suppressed HIV replication by inhibiting reverse transcriptase [2, 12]. Other authors reported for ellagitannins (geranin and corilagin) that reducing HIV replication by inhibiting HIV-1 protease and HIV-1 integrase enzyme [12] and ellagitannins isolated from *Tuberaria lignosa* inhibit entry of HIV in MT-2 cells [4].

Replication of human, porcine and duck influenza A virus in vitro is prevented by hydrolysable tannin strictinin [14].

Studies were conducted with herpes viruses and again the results show that there are different targets for action.

As mentioned above replication of EBV are suppressed by eugeniflorin D1, D2 and oenothein B via inhibition of DNA polymerase.

Three nonahydroxyterphenoil-bearing C-glucosidic ellagitannins castalagin, vescalagin and grandinin manifested significant anti- herpes simplex virus-1 (HSV-1) activity and the highest activity of the three substances showed castalagin. They show well-defined but relatively low activity against HSV-2 replication [18].

Activity of eugeniin against HSV replication was studied *in vivo* and *in vitro* and was found that most influenced is the stage of DNA synthesis and in particular that exhibits specificity for inhibition of viral DNA polymerase in comparison to cellular DNA polymerases [8].

Conclusion

For many years people are aware of the healing properties of plants and used them to treat various diseases. In recent years the development of technology made it possible to identify the active ingredients of which are due to these properties of plants. One of the largest groups of substances isolated from plants are polyphenols. Ellagitannins are type of polyphenols and have already proven different biological activities important for human health, as antioxidant, antitumoral, antimicrobial, antiviral and others. As an integral part of many food products ellagitannins are included in the daily diet of many people suffering from various diseases. Ellagitannins can naturally enhance the body's ability to cope with various infections and diseases, improving immune response and also have a positive effect on heart disease and high blood pressure. which makes them promising therapeutic agents. All of the activities described above convert ellagitannins in promising therapeutic agents.

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АР1. ВИРУСИ - ЛЕКАРСТВО ЗА МОЗЪЧНИ ТУМОРИ

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АР2. БАКТЕРИОФАГИТЕ – АНТИБИОТИЦИ НА БЪДЕЩЕТО

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Session B.

Chairpersons:

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

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

Secretary: Assist. Prof. Petar Dimitrov, DVM, PhD
Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences

BO1. INVESTIGATIONS ON SOME HEMATOLOGICAL PARAMETERS IN SHEEP EXPERIMENTALLY INFECTED WITH *HAEMONCHUS CONTORTUS*

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Abstract

The large stomach worm, *Haemonchus contortus*, commonly known as "the barber's pole worm", is a blood-sucking nematode found in the abomasa of sheep and goats. *Haemonchosis* is a serious health problem which causes lower production due to high morbidity, mortality, and cost of treatment and control measures. A range of hematological measures were significantly different between infected groups and uninfected controls at one or more time points. The concentrations of packed cells volume, erythrocyte sedimentation rate, blood eosinophil number were increased, while the concentration RBC, haemoglobin, and some erythrocyte indices were significantly decreased ($P<0.05$) in haemonchus infected animals. It was concluded that decreased hemoglobin concentration, total serum proteins, total RBC and some erythrocyte indices were important indicators of haemonchosis in sheeps.

Introduction

Small ruminants play an important socio-economic role within traditional farming system in many developed and developing countries. Gastrointestinal nematodes impose severe economic constraints on the sheep and goat production worldwide. Losses occur due to subclinical parasitism through mortalities, reduced production, decreased weight [7, 8] and decreased fertility [4] and milk yield [6].

Haemonchus contortus is a major pathogen primarily affecting the abomasa of sheep and other small ruminants. Adult worms feed on blood and can cause severe anemia and even death. The purpose of the present study was to examine pathological changes associated with experimental infection of *Haemonchus contortus* in sheep.

Material and Methods

In the study were used 16 male 6-month-old lambs of the Black head Pleven breed with a middle weight 26-27 kg. The lambs were divided into two groups (eight lambs in a group): Group 1 – controls and Group 2 – lambs infected with *Haemonchus contortus* larvae. The second group was infected twice one days with 1800 *H. contortus* larvae (L3) per a lamb. The Baermann technique was used to extract the L_3 larval stages of *Haemonchus contortus* intestinal nematodes and counted under a dissecting microscope to determine the larval counts. Before experimental infection animals were kept in collective pens located in the vivarium of the Institute of Experimental Morphology, Pathology and Anthropology with Museum, BAS, for a month for adaptation to the diet and to the experimental environment. During this period the lams were treated with antihelmintics – combination of closantel and albendazole. Individual blood samples were taken by jugular venipuncture using evacuated tubes with EDTA on days −1, 10 and 48th of the study. Packed cell volume (PCV), RBC, haemoglobin, mean corpusculare volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and eosinophils in peripheral blood were determined by using automated hematology analyzer HEMA-SCREEN 18588 LiHD113. Animals were killed on day 48 post-infection by intravenous barbiturate followed by exsanguination. Animals were handled following European Union recommendations for animal welfare under the supervision of the local IEMPAM-BAN ethics committee.

Results and Discussion

Our results show a statistically significant difference in follows parameters between infected group compared to the control group: Hgb, MCH and MCHC. The comparing of RBC and PCV between control group and invaded with *H.contortus* also found decreased, but not statistically significant.

The results are shown in figures 1,2,3 and 4.

Fig.1. Hemoglobin concentration in blood of lambs noninfected and infected with *H.contortus*

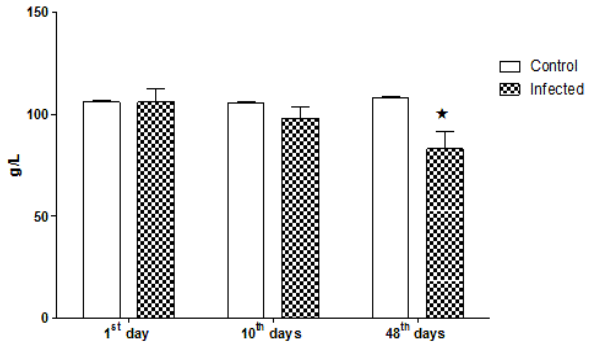


Fig. 2. Mean corpuscular hemoglobin in blood of lambs noninfected and infected with *H.contortus*

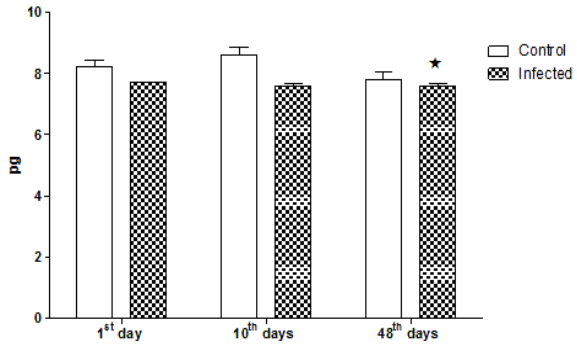


Fig.3.Mean corpuscular hemoglobin concentration in blood of lambs noninfected and infected with *H.contortus*

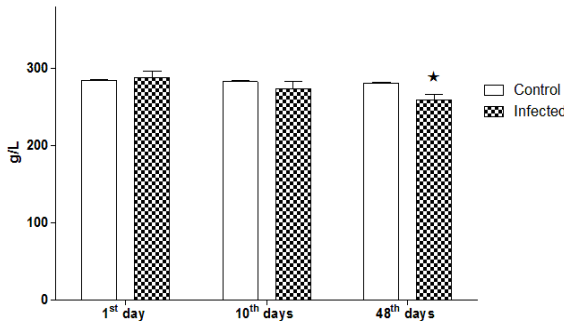
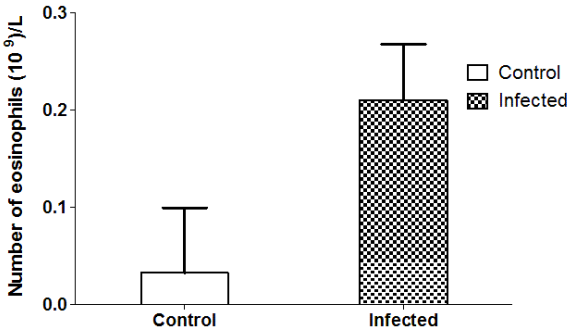


Fig.4. Whole blood eosinophil count in lambs noninfected and infected with *H.contortus*



The reduced RBC counts, Hgb and PCV values in infected group may be attributed to the bleeding of abomasa due to injuries caused by the parasites similar to that described by [1,3]. However, marked blood loss in the infected animals is to be attributed to the blood sucking activities of *Haemonchus contortus.* Eosinophillia observed in present investigation is in agreement with the findings of [2], who concluded that eosinophilia is associated with antigenic stimulation or parasitic burden. Sheep eosinophils are potentially lethal to *Haemonchus contortus* infective larvae in vitro [9,10]. In vivo studies have described the close association between eosinophils and death of *H. contortus* larvae in the abomasal mucosa of sheep [5].

Conclusions

Alteration in hematological parameters observed in the present study carry importance as they may indicate the extent of abomasal damage and help in better understanding of the pathogenesis of anemia.

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BO2. MODEL OF *EIMERIA TENELLA* INFECTION AFTER BASIC COPPER SALT SUPPLEMENTATION

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Abstract

The effect of tribasic copper chloride (TBCC) on broiler chickens experimentally infected with *Eimeria tenella* (Protozoa) was investigated. Body performance, parasite burden, liver and plasma vitamin E and copper level were measured. The results showed improved body weight gain, reduced feed conversion ratio, elevated liver vitamin E and Cu content. The data from this experiment indicate that TBCC is a safe product and well available to broiler challenged with *E. tenella* without any negative indices.

Keywords: TBCC, *Eimeria tenella*, vitamin, Cu, broiler performance

Introduction

Avian eimeriosis is one of the most common diseases in countries with industrial poultry breeding. One of the most frequently and pathogenic protozoas in chicks is *Eimeria tenella*. Its pathogenic influence on avian organism has been studied in various aspects. Metabolic disorders take part place in the pathogenesis of eimeriosis [8]. Reduction of some small molecular weight antioxidants (vitamin A, C and E) was established in chick eimeriosis by Coles et al [1] ; Georgieva et al [4].

The essentiality of copper for poultry is well known [2]. Luo et al [9] reported that the copper in tribasic copper chloride $Cu_2(OH)_3Cl$ (TBCC). TBCC occurs naturally as the mineral

atacamide and was the first discovered in the Atacama Desert. This form is a secondary mineral that is formed by the oxidation of other Cu- containing deposits. TBCC is more available to broilers than of copper sulfate when supplemented to diets. The studies have indicated that the Cu in TBCC is as available and as save as that in feed grade copper, however more studies are needed to evaluate the interactions between chickens infected with *E. tenella* and TBCC [10].

The following study was conducted to investigate the interactive effects of TBCC and *E. tenella* infection on growth, feed intake, parasite status, liver copper and vitamin E concentration of chickens.

Material and methods

The study was performed on 60 clinically healthy broiler chickens, aged 20 days, weighted 335-370 g. Up to 11 days, the birds were housed in cages on slat floors under conditions excluding further Eimeria infection. At 11 days experimental groups of 20 birds each were established. The 1st experimental group comprised healthy, untreated and un-infected birds. The birds from gr, 2 and 3 were infected once with 8.10^4 sporulated *E. tenella* oocysts on day 13. The fodder chickens from gr. 3 was supplemented with 400 mg Cu^{2+}/kg food for 7 days – 2 days before and 5 days post the inoculation. The experiment lasted 7 days p.i. On day 1 four chicks were killed to establish the level of vitamin E and Cu. On day 7 birds were killed and the content of copper and vitamin E was determined. The level of Cu was established by atomic absorption spectrophotometry. The vitamin E content was established by HPLC. Body weight gain, feed intake and liver Cu and vitamin E were checked on day 1 and 7 p.i. Lesion scores and oocyst index were checked on day 7.

Results and discussion

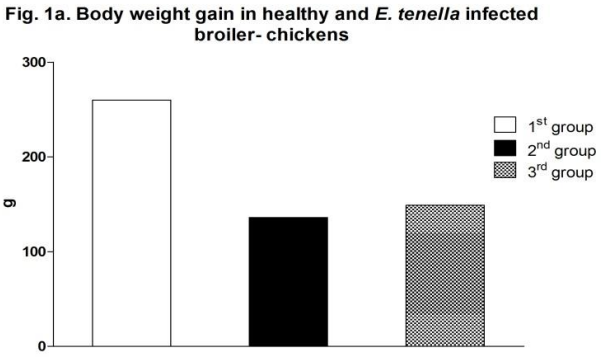


Fig. 1a, 1b presents the live body weight, the weight gain and the feed conversion ratio (FCR) in chickens infected and uninfected with *E. tenella*, infected with *E. tenella* and supplemented with copper. The results evidenced that at the beginning of the experiment, the body weight in all groups was equal but at end, it was significantly lower in the infected birds (gr.2) compared to the healthy chickens. This decrease was obviously due to the relatively low body weight gain in all infected birds compared to healthy controls. After Cu supplementation weight gain was found to significantly higher, compared to infected un-supplemented birds. At the same time, FCR was higher in the infected chickens, compared to the controls. After supplemented with TBCC, it was found that FCR to be lower, compared to infected un-supplemented birds.

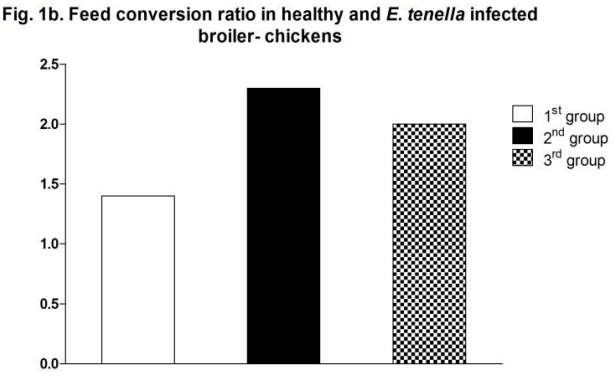


Fig.2. presents the lesion scores and the oocyst index. Our results showed that 7 days, post challenge, *E. tenella* produced substantial injures in the caeca manifested by visible erosions of the mucosa, extremely tinned mucosa and blood on the intestinal lumen as well as enlargement in the size of the intestine. The oocyst index in infected but non-treated birds as expected, indicated that oocysts had been released in significant amounts by chickens infected with *E. tenella*, demonstrating the development of infection. It was find that the oocyst index and lesion scores in infected and Cu supplemented 20-day-old chickens significant decreased, compared to the birds in gr. 2.

Fig. 2. Lesion scores and the oocyst index in healthy and *E. tenella* infected broiler- chickens

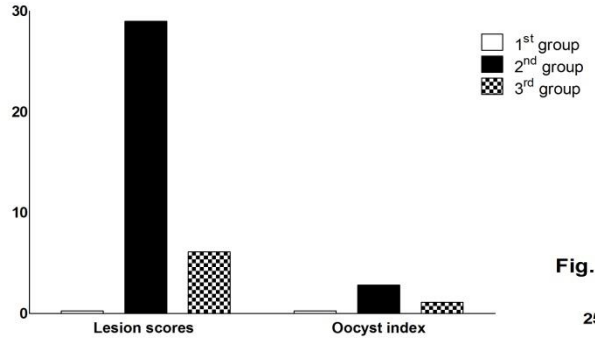


Fig 3 presents Cu level. It was reduced in the infected chickens compared to the healthy ones. Cu concentration was increased after Cu supplementation.

Fig. 3. Liver copper levels in healthy and *E. tenella* infected broiler- chickens

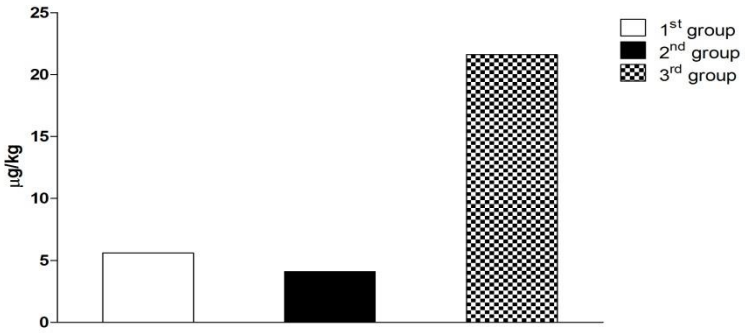
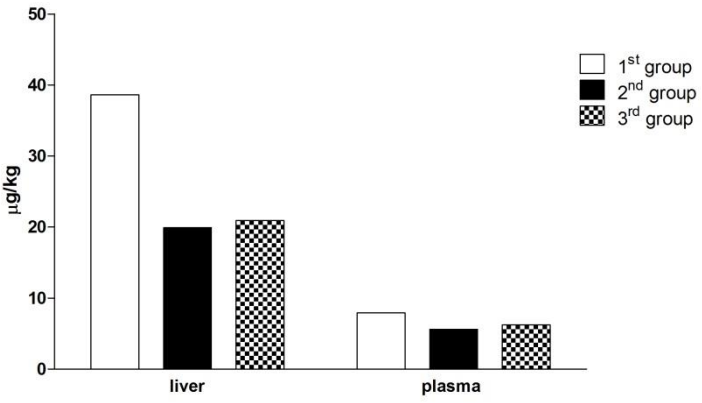


Fig.4. Liver and plasma vitamin E concentration in healthy and *E. tenella* infected broiler- chickens



The liver and plasma vitamin E levels are presented in fig.4. TBCC increased significantly liver vitamin E content in the infected with *E. tenella* birds.

Discussion

Cu supplementation applied in physiological norm affected FCR and body weight gain positively. Body weight gain was similar in the infected, supplemented and controls. The lack of a detrimental effect on body weight by TBCC supplementation in chickens was established by Luo et al [9]. The authors showed positive effect of TBCC on broiler performance, relative copper bioavailability and oxidation stability of vitamin E. Vitamin E was increased after TBBCC supplementation in broilers. Liver Cu concentration was elevated in chickens

consuming diets that contained from 150-450 mg/kg supplemented copper [10]. Increased Cu level could influence positively the activity of Cu, Zn superoxide dismutase, acting as an antioxidant enzyme [3]. So that both antioxidants (Cu,Zn-SOD and vitamin E were increased in the liver in chickens challenged with *E. tenella* [5] and treated with copper. In that case TBCC was increased antioxidant defense system in the chickens infected with *E. tenella.*

Our data with regard to the lesion scores and the oocysts index value showed that by day 7 p.i. E. *tenella* provoked significant injuries to the intestinal mucosa. Alterations in the cecum and oocyst production were indicative of a severe infection involving pathogenic oxidative stress.

The established changes in caeca and the oocyst production in the birds in gr, 3 were indicative of beneficial effect of TBCC on the lesions scores and the oocyst index on eimeriosis infection. Combined effect of Cu and the infection on the lesion scores and oocyst indexes was significantly presented. They were reduced probably due to Cu supplementation.
TBCC possesses several chemical characteristics that make it desirable as a Cu source in production, such as small particle size, excellent flow, and low water solubility [10]. It was established that TBCC was less active than copper sulfate in promoting the oxidative stress [7, 8]. TBCC could be used in poultry as additive compound during eimeriosis.

The World Association for the Advancement in Veterinary Parasitology (WAAVP) requires the determination of the parameters live body weight, weight gain and FCR for evaluation of the severity of infection [6].In compliance with those necessities we observed a stunted growth in chickens infected with *E. tenella* and the increased FCR indicated severe infection. The lower body weight was due on one part to the worsened FCR and probably to impaired antioxidant defense, on the other.

Our results are suggestive of the beneficial effects of TBCC on bird performance and the prevention of lesions and oocysts caused by *E. tenella*.
The data from this experiment indicate that TBCC is a safe product and well available to broiler challenged with *E. tenella* without any negative indices.

Acknowledgement: This study was supported by a bilateral project between Bulgarian and Russian Academy of Science and by the European Social Fund and Republic of Bulgaria, Operational Programme “Human Resources Development” 2007-2013 framework, Grant № BG051PO001-3.3.06-0048 from 04.10.2012.

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BO3. COMPARISON OF ALLOZYME ANALYSIS OF SIX TRICHINELLA ISOLATES BY PAAE

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Absract

Six isolates of the Trichinella genus (ISS03, ISS13, ISS10, ISS02, ISS029, ISS035) were tested to establish their identity and genetic variability using 10,5% PAAE and isoenzyme comparative analysis. The aim is to obtain new evidence for species identification. For typifying five enzyme systems were used (REP, EST, MPI, ACP and AK). Each of the isolates tested in most of the studied enzyme systems showed different electrophoretic mobility. These data are proof and support for different types of data belonging to the Trichinella genus. The isoenzyme method can be successfully applied and used to identify and prove the species belonging to different isolates of the Trichinella genus.

Keywords: Trichinella species, PAAE, allozyme analysis

Introduction

The taxonomic complexity of nematodes of the Trichinella genus is well documented on the basis of numerous biochemical, biological, epidemiological, clinical and immunological studies and established eight species and three genotypes.
Gel electrophoresis is a well-known biochemical technique for measuring genetic variation within and between populations of a species [1].

The electrophoretic study of the gene-enzyme systems has been providing data on a great number of problems in zoology, genetics and parasitology. Electrophoretic studies of gene-enzyme systems make it possible to: a) estimate the amount of genetic divergence between populations; b) analyze the patterns of genetic variation in natural populations; c) detect sibling species; d) investigate gene flow; e) investigate natural hybridization among

populations and species; and f) analyze genetic drift and natural selection phenomena [9].

The enzymatic profiles generated in gel electrophoresis often illustrate the presence of isozymes (or isoenzymes) which are defined as "multiple molecular forms of a given enzyme occurring either in a single individual or in different members of the same species" [5].

The use of biochemical methods for the analysis of genetic relationship among morphologically similar taxa is well accepted in systematics [8].

Allozyme analysis as a method for studying the genetic composition of Trichinella was first reported by Flockart et al. 1982 [4]. He has studied four enzyme systems and has established enzyme polymorphism in the Trichinella genus, without being able to distinguish between T. nativa and T. nelsoni.

The objective of this study was to identify genetic similarities and differences in some populations of six Trichinella isolates using isozyme analysis.

Material and methods

Trichinellae: For infecting the experimental animals and the obtaining Trichinella larvae the six isolates (courtesy of Dr. E. Pozio) from the world reference laboratory for trichinellosis - Rome, Italy were used. The isolates were maintained by passaging in mice and rats.

Experimental animals and yield of Trichinellae from muscle mass: 60 white Balb/C mice (six groups of 10) were used. Each mouse was infected with 200 Trichinella larvae from the corresponding isolate. On day 50 from the beginning of the experiment the animals were sacrificed in accordance with the ethical norms for humane attitude towards the animals. Following the definitive digestion of the muscle mass by pepsin and hydrochloric acid the Trichinella larvae were utilized for the production of the antigen.

Antigens (total extracts from muscle Trichinellae): The suspensions of muscle trichinellae in PBS (1:2) from the six Trichinella isolates were thrice homogenized in the glass homogenizer of Poter (the procedure is unique for each isolate). The supernatant following its protein content being defined after Lowry et al. 1951 [6], was divided into small quantities which were kept at -20°C, for periods of up to 1 mo retained their enzyme activity.

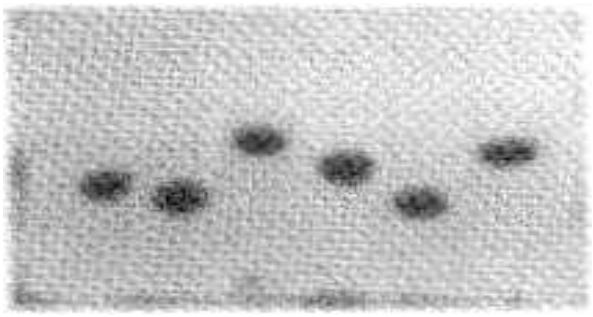
Thin-layer polyacrylamide electrophoresis [3]. The thin-layer PAAE was performed on an apparatus for vertical electrophoresis.

Results and Discussion

Six Trichinella isolates: ISS03 (T1), ISS02 (T2), ISS13 (T3), ISS10 (T4), ISS029 (T5), ISS035 (T6) were tested for their identity and variability by isozyme methods. The concentration of protein in the samples of soluble proteins was between 5 and 10 mg / ml. The following five isoenzymes were compared: peptidase, mannose - phosphate - isomerase, esterase, acid phosphatase and adenylate kinase. The results of electrophoretic studies exhibit both ostensibly identical similarities and differences in the location of the fractions. Zymograms from 5 enzymes exhibiting polymorphism are presented in Fig. 1.

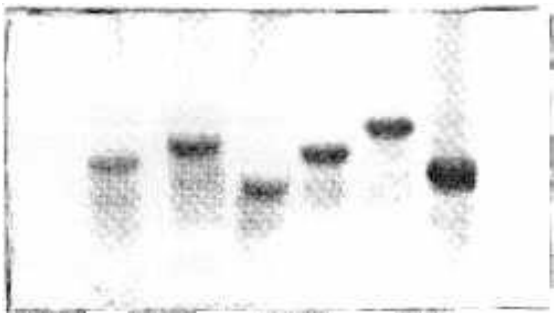
The peptidase shows a similarity in the location of the fractions for isolates № 1 and № 2 and a difference for each of 3, 4, 5 and 6. For esterase enzyme, respectively similarity between 1 and № 2 and a difference between № 3, 4, 5 and 6. For enzyme mannozophosphateisomerase the similarity is between № 1 and № 2 and the difference is between № 3, 4, 5 and 6. For enzyme alkalinephosphatase the similarity is between № 3 and 6, and the difference is between 1,2,4 and 5. For enzyme adenylatekinase differences were recorded among the six isolates.

PEP



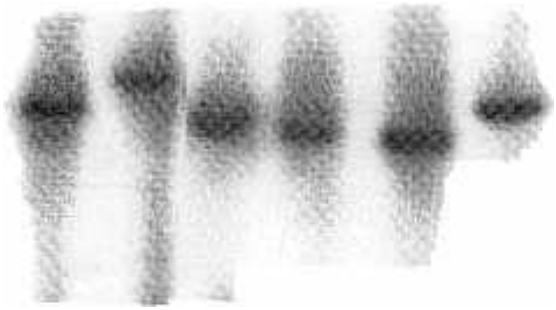
1 2 3 4 5 6

EST



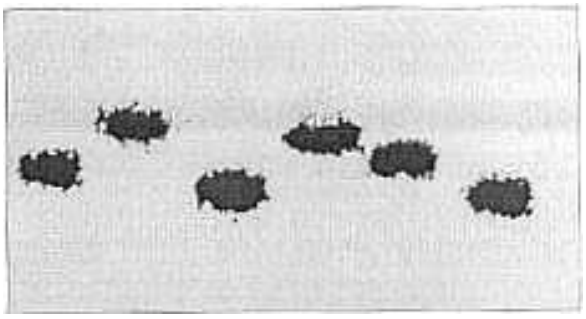
1 2 3 4 5 6

MPI



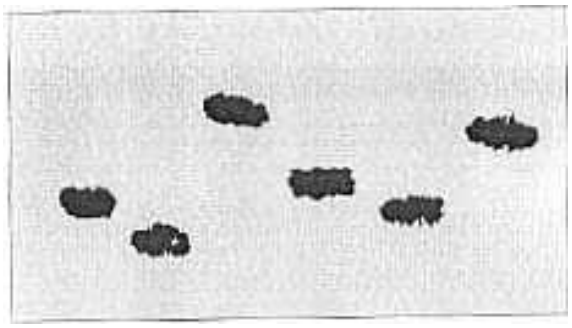
1 2 3 4 5 6

ACP



1 2 3 4 5 6

AK



1 2 3 4 5 6

Fig.1. Electrophoregrams of five enzymes (peptidase, esterase, mannozophosphateisomerase, Alkalinephosphatase,adenylatekinase) presenting the polymorphism of 6 Trichinella isolates following PAAE.

The results of the isoenzymatic analysis support the concept that the Trichinella genus is genetically heterogeneous displaying at least 8 genotypes. According to Bullini L.1985 [2] the presence of sibling species in helminths and parasites is well-known and we need various criteria and a series of tests until conclusions on taxonomic rank can be drown.

The genetic differences of six Trichinella isolates were defined by using standard genetic identity and distance standards Nei M. 1972 [7].

The obtained results from the electrophoretically - isoenzyme studies provide further evidence and show the existence of six differentiated species in the Trichinella genus.

The discrimination among the different genotypes in the Trichinella genus is made by the method of cluster analysis "Simple matching". This type of analysis is designed for different types of data and in the case of the present study it was chosen because of the limited sample size and further transformation into discrete variable levels of the enzyme mobility.

Based on the differences established in the protein components of each of the six isolates under study we can assume that they possess characteristic protein components for each isolate, which shows genetic differences between the studied helminths. These data support the concept of a polytypic genus of Trichinella presented in the work of Pozio et al. 1992, [10].

On the basis of the results of the isoenzyme studies and previous studies we can conclude that the investigated six Trichinella isolates belong to the following species: T. spiralis, T. britovi, T. pseudospiralis, T. nativa, T. nelsoni, T. murrelli.

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BO4. SERUM SIALIC ACID LEVELS IN TRICHINELLA SPIRALIS INFECTED RATS

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Abstract

Sialic acids comprise N- and O-acyl derivatives of the neuraminic acid and they are a component of the glycoproteins and glycolipids. They are biologically important and essential of the glycoconjugates and they are reported to be altered in patients and animals, suffering from various inflammatory and infectious diseases. Studies have shown that the serum sialic acid evaluation can be a valuable indicator for diagnostic and prognosis of many inflammatory diseases. In the present study we investigated the content of free, lipid and protein bound sialic acid in normal serum and in serum of rats with Trichinella spiralis infection in different phases of the disease. The experiment covered 8 weeks post infection. The data analysis disclose that the serum sialic acid concentrations in the Trichinella infected rats were significantly higher (at week 8) than those found in the healthy controls. A strong positive correlation was observed between the serum levels of different forms of sialic acid and the development of the disease.

Introduction

Sialic Acid is the generic term given to a large family of nine-carbon 3-deoxi-2-keto sugars containing a carboxyl group. They are usually attached to the terminal positions of oligosaccharides glycoproteins and glycolipids [19]. As a result of their location and their negative carboxylate functionality, the sialic acids play important roles in a variety of biologic processes, such as cell-cell communications, cell-matrix interactions, adhesions and protein targeting [18].

Trichinellosis is a disease, caused by parasitic nematodes, which belong to genus Trichinella. These parasites infected a broad range of mammals, birds and reptiles. They alternate during their life cycle between enteric stages and skeletal muscle stages within their hosts. The muscle phase is associated with disruption of myofibrils enlargement and centralization of host muscle nuclei, inflammation around infected cells and elevated expression of two host markers: acid phosphatase activity and syndecan I [1]. The infection induces changes in the activities of some antioxidant markers as superoxide dismutase and glutathione peroxidase [16].

Sialic acids are found in many body fluids, such as serum, urine, breast milk, saliva, semen, cerebrospinal fluid and pleural effusion [9, 14]. Their concentrations usually change during diseases such as neoplastic tumors, myocardial infarction, inflammatory disorders and diabetes mellitus [16, 5, 4]. Serum sialic acid values were analyzed in many inflammatory and infectious disease [7,10]. The results show that at the beginning of inflammatory reactions the serum sialic acid concentrations increase rapidly. There are no published reports about the

concentration of serum sialic acid in Trichinella spiralis infected rats. Therefore, the aim of the present study was to investigate the free, lipid and protein bound sialic acid levels and whether serum sialic acid concentrations correlated with the different stage of the disease.

Material and methods.

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

Male Wistar rats, weighting 100 g each where used. They where allocated into two groups: group 1, control, healthy and group 2, experimentally infected with T. spiralis.

The rats were housed individually in cages constructed of polypropylene and glass. All animals were kept under conditions of controlled lighting with alternative dark (1800–0600 hours) and light (0600–1800 hours) cycles.

Each rat from groups 2, was orally infected with 1,000 T. spiralis muscle larvae. The larvae (code ISS03) were obtained from the International Trichinella Reference Centre, Rome, Italy. Acording the method of Wakelin and Lloyd (1976), the T. spiralis infective larvae were isolated by digestion of skeletal muscles from eviscerated, skinned, and minced carcasses of mice in 0.5% HCl and 0.5% pepsin at 37°C. These mice had been infected about 4 months ago.

Blood samples were taken after anesthesia with ketamine, collected without anticoagulant and serum was obtained by centrifugation at 3000g four 15 min at 4^0C.
The protein content was determined by the method of Bradford [2].

Lipid bound sialic acid was determined by a method according to Katopodis and Stock [11]. This method involved extraction of serum glycolipids with chloroform-methanol, precipitation of the glycolipids using phosphotungstic acids, and estimation of sialic acid using the resorcinol technique. The blue color that developed was measured at 580nm.

Free sialic acid concentration was determined colorometrically using the periodate-thiobarbituric acid assay described by Warren, (1959). [21]. The measurements of the content of bound sialic acids in sialoglycoproteins were carried out according to the acidic ninhydrin method described by Yao et al. [12].

Results and discussion

In the last years different reports have demonstrated about the correlation of serum sialic acid with acute phase proteins in various inflammatory disease or injury. Most of these studies reported that the sialic acid concentration increases the following inflammatory process. Some of them indicate that the infectious with various parasites are also associated with elevated serum sialic acid concentrations [10, 6] .

This study was conducted to examine the effect of Trichinella infection on free, lipid and protein bound serum sialic acid concentrations. Our results show a positive correlation between Trichinella infections and the sialic acid levels. Two week after the infections, we observed an increase of the sialic acid concentrations. At week 7 the sialic acid levels in the infected rats (group 2) were significantly higher compared to those in the control group (fig. 1,2,3).

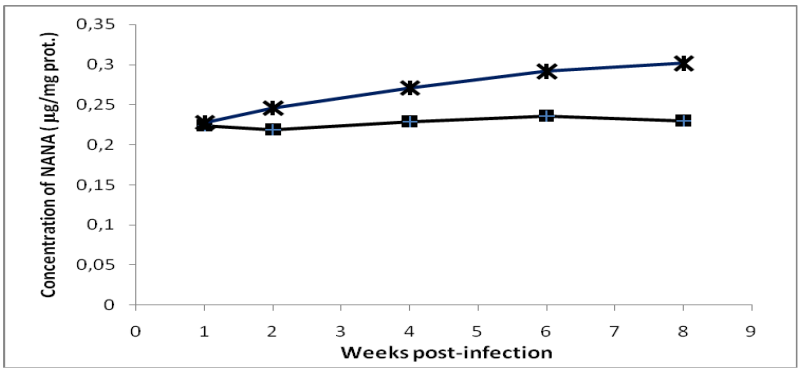

Fig1 Time dependence of free sialic acids content in serum of Wistar rats. *Square* group 1 (healthy), Stars group 2 (infected)

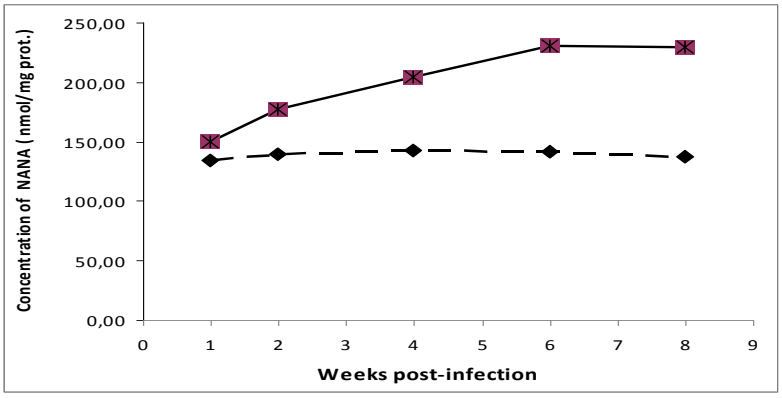

Fig2 Time dependence of protein bound sialic acids content in serum of Wistar rats. *Rhomb* group 1 (healthy), Stars group 2 (infected)

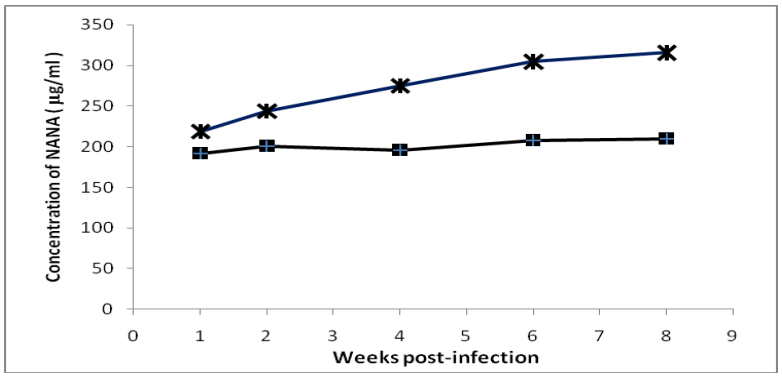

Fig3 Time dependence of lipid bound sialic acids content in serum of Wistar rats. *Square* group 1 (healthy), Stars group 2 (infected)

It is obvious, that this is a parallel augmentation in the sialic acid quantity in the serum, which correlated with the development of the disease. Elevated sialic acid concentration has also been observed in several inflammatory and infectious diseases. Chatterjee et al. [3] reported a significantly increased O-acetilated sialic acid from the serum of visceral leishmaniasis patients. In another study, conducted by Karagenc at al. [9] they showed, that the increase in serum sialic acid in cattle with natural tropical theilerosis was highly compared with the control group.

Infection with T. spiralis leads to inflammation around infected cells [20]. The inflammation is the major and complex reaction of the body against infection upon tissue

injury. The local inflammatory response is later accompanied by a prominent systematic response, known as acute phase response. This response increased the leucocitosis and altered the production of large number of proteins, produced in liver, known as acute phase proteins [8]. Many of the serum acute phase proteins are typical secretable glycoproteins and some have sialic acid residues localized within their carbohydrate side-chains . The most of them like α1 acid glycoprotein and heptoglobin increase about 5-10-folds, other, like c-reactive protein and serum amiloid A increase by about 100- fold [13]. In this context the increase in serum protein sialic acid concentration in T. spiralis infected rats (0,001) may be attributable to elevated serum acute phase proteins during inflammatiom. Free and lipid bound sialic acid concentrations of infected rats were also significantly increased in serum compared to the control animals (P<0,001). The mechanism including sialic acid is not clearly understood, however the mechanism is very complex and can be related to the intensified cell metabolism including sheding of sialic acid into the circulation as a result of cell membrane damage, tissue proliferationq tissue destruction and inflammation. This study attempted to elucidate the potential of sialic acid as a serum chemistry parameter in T spiralis infected rats. The results suggest that serum sialic acid might be a useful parameter as an indicator of inflammation in trichinellosis.

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BO5. THE NURSE CELL – *TRICHINELLA SPIRALIS* COMPLEX AS AN EXPERIMENTAL MODEL FOR INVESTIGATIONS ON THE GLYCOSYLATION IN SKELETAL MUSCLE TISSUE

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Introduction

Sialic acids occupy the terminal position on the oligosaccharide chains of glycoconjugates situated on the outer cell membranes. They serve a diverse variety of functions referring to almost all types of recognition phenomena and adhesion mechanisms [1]. The knowledge about glycoproteome in skeletal muscle is limited and most of the information comes from detailed studies on aberrant glycosylation in some inherited muscle diseases [2]. This work describes for the first time the intracellular changes in sialylation of skeletal muscle fiber during the process of its transformation into a nurse cell after occupation by the parasitic nematode *Trichinella spiralis*.

Material and Methods

Sample preparations

BALB/C mice, 3 males in each group, were inoculated with 500 infective *Trichinella spiralis* larvae (code ISS03). The mice were sacrificed at defined time points (0, 10, 14, 16, 25 and 40 days post infection (d.p.i.)). Skeletal muscle samples were frozen or fixed in modified methacarn (MMC) fixative or 10% NBF for 48 hours and routinely processed in paraffin. Parallel sections were stained with haematoxylin and eosin for routine histological evaluation.

Glycoprotein analyses

MMC fixed muscle tissue sections from all intervals were stained with lectins specific for sialic acid – TML (Calbiochem), MAL and SNA (Vector), N-acetylgalactosamine – HPA (Sigma) and VVL-B4 (Vector), and D-galactose – PNA (Sigma) (Table 1). Sugar specificity of lectins was confirmed by neuraminidase pretreatment of sections or pre-incubation of the lectins with the corresponding sugars (all products of Sigma). The level of glycoprotein-bound sialic acid was determined in homogenized muscle tissue samples by color acidic ninhydrin reaction and the absorbance was measured at 470 nm. Sialic acid was expressed in terms of nmol N-acetylneuraminic acid/mg protein.

Lectin	Abbreviation	Carbohydrate specificity	Applied dillution
Arachis hypogea agglutinin	PNA	Galβ(1,3)GalNAc	1:50
Helix pomatia agglutinin	HPA	GalNAc-α	1:50
Maackia amurensis lectin-II	MAL-II	SiAα(2,3)Galβ	1:1000

Table 1. The lectins used in this study, their abbreviations, carbohydrate specificity, and applied dillutions. SiA –sialic acid, Gal – galactose, GalNAc – N-acetyl-D-galactosamine (referenced by 17)

		(1,4)GlcNAc	
Sambucus nigra agglutinin	SNA	SiAα(2,6)Galβ(1,4)GlcNAc Siaα(2,6)GalNAc-α-O-Ser/Thr	1:2000
Tritrichomonas mobilensis lectin	TML	SiA	1:100
Vicia villosa lectin – isoform B_4	VVL-B_4	GalNAc-α-O-Ser/Thr	1:300

Sialyltransferase analyses

Total sialyltransferase activity was measured in homogenized muscle tissue samples by incorporation of CMP-N-[^{14}C]-acetylneuraminic acid (Amersham) on acceptor asialofetuin (Sigma). Radioactivity was counted in liquid-scintillation spectrometer. Sialyltransferase activity was expressed in terms of counts-per-minute (cpm)/mg protein. Total RNA was extracted using RNazol®RT (Molecular Research Center) and fragments of the reference gene peptidyl prolyl isomerase A (PPIA, accession number NM_008907.1), beta galactoside α-2,6-silalyltransferase 1 (ST6Gal1, NM_145933.3) and [α-N-acetyl-neuraminyl-2,3-β-galactosyl-1,3]-N-acetylgalactosaminide α-2,6-sialyltransferase type 1 (ST6GalNAc1, NM_011371.2), -2 (ST6GalNAc2, NM_009180.3) and –3 (ST6GalNAc3, NM_011372.2) of *Mus musculus* were amplified by two step real-time RT-PCR (Fermentas, Qiagen, Eppendorf). Normalization of target gene expression versus the expression of the reference gene was calculated using the $2^{\Delta Ct}$ method according to the formula:

$$2^{(Ct(\text{reference gene}) - Ct(\text{target gene}))} = 2^{(\Delta Ct)}$$

where Ct is a cycle threshold value, and the resulting $2^{\Delta Ct}$ (in relative expression units) were plotted against the timeline of the experiment.
NBF fixed muscle tissue sections from all intervals were treated with three rabbit polyclonal antibodies against α-2,6-sialyltransferase IV (ST6GalNAc4) and α-2,3-sialyltransferases II and IV (ST3Gal2, 4) (Aviva Systems). The substrate specificities of all sialyltansferases analysed in this study are listed in Table 2.

Enzyme (short name)	Substrat specificity	Type of created linkage
ST3Gal2	**Gal**-β-1,3-GalNAc-	α-2,3-
ST3Gal4	**Gal**-β-1,3-GalNAc- **Gal**-β-1,4-GlcNAc-	α-2,3-
ST6Gal1	**Gal**-β-1,4-GlcNAc-	α-2,6-
ST6GalNAc1 ST6GalNAc2	**GalNAc**-α-1-Ser/Thr Gal-β-1,3-**GalNAc**-α-1-Ser/Thr SiA-α-2,3-Gal-β-1,3-**GalNAc**-α-1-Ser/Thr	α-2,6-
ST6GalNAc3 ST6GalNAc4	SiA-α-2,3-Gal-β-1,3-**GalNAc**-	α-2,6-
ST8SIA2	**(SiA-α-2,8)**$_n$-α-2,3-Gal-β-1,4-GlcNAc-	α-2,8-

Table 2. The substrate specificities of the sialyltransferases investigated in this study and the types of the formed linkages. SiA –sialic acid, Gal – galactose, GalNAc – N-acetyl-D-galactosamine, GlcNAc – N-acetyl-D-glucosamine, Ser – serine, Thr – threonine. The monosaccharides in bold indicate a residue onto which a SiA is transferred (referenced by 17).

Statistical analysis of semiquantitative evaluation of the gene expressions

Statistical evaluation of the data was performed using GraphPad Prism 5.03 software (San Diego). Non-parametric one-way analysis of variance (Kruskal-Wallis test) with Dunn`s Multiple Comparison Test (significance level 0.05) was computed to detect statistically

significant differences in the yield of the real-time PCR products between the control and infected samples.

Results

Detection of sialylated glycoproteins

The sarcoplasm of the healthy skeletal muscle fibers did not react with any of the lectins used in the study. The areas of the occupied sarcoplasm were reactive towards TML, MAL and SNA during the whole process of transformation and within the mature Nurse cell. The occupied sarcoplasm was reactive with HPA, PNA and VVL-B4 only after pretreatment of the sections with neuraminidase (Table 3). With small exceptions (VVL-B4), the intensity of the staining of the sarcoplasm with all of the lectins used in the study increased progressively in the time course of Nurse cell formation. The level of bound sialic acids increased from 18.2 nmol/mg protein in control samples up to 52 nmol/mg protein in infected samples (data not shown).

	Days post infestation		
Lectin	10	14	40
HPA	-	++	-
HPA-Neu	+	++	++
MAL	++	+++	+++
MAL-Neu	-	+	-
PNA	-	-	-
PNA-Neu	++	+++	+++
SNA	++	+++	+++
SNA-Neu	-	+	++
TML	+	+++	++
TML-Neu	-	-	-
VVL-B4	-	-	-
VVL-B4-Neu	-	++	+

Table 3. Evaluation of the intensity of intra-cellular staining within the skeltal muscle fibers occupied by *Trichinella spiralis* after application of lectins with different carbohydrate specificity, with and without pretreatment with neuraminidase (Neu). The results were interpreted as negative (-) and weak (+), moderate (++) and strong (+++) positive.

Siayltransferase activity

The rates of incorporation of isotope-labelled sialic acid were significantly enhanced at day 10 p.i (data not shown). The specificity of the primers used in the study was evident by the single peaks of the melting curves indicating a single product of apmlification. The mRNAs for ST6Gal1, ST6GalNAc2 and ST6GalNAc3 were expressed in healthy mouse skeletal muscle tissue. Concerning the mRNA for ST6GalNAc1, the real-time PCR resulted in very high Ct values (Fig. 1). The relative quantification of mRNA species showed statistically significant increase of expression of ST6GalNAc1 on days 16 and 25 p.i., and significant decrease in expressions of ST6GalNAc2 and 3 respectively on days 10 and 16 p.i. The expression of ST6Gal1 remained unchanged during the time course of experiments. ST6GalNAc4 antibody did not react against normal or invaded mouse skeletal muscle fibres (data not shown). A transient increase of the sarcoplasmic expression of ST3Gal2 and 4 was observed at day 14 p.i. followed by complete absence of staining at days 16 and 25 p.i. The expressions of both enzymes were significantly diminished within the Nurse cell (40 d.p.i.) (data not shown).

Predicted oligosaccharide structures which came forward into the sarcoplasm of mouse skeletal muscle after invasion by T. spiralis

The intense staining with SNA, and with VVL-B4 and HPA after neuraminidase treatment, accompanied by increased amplification of ST6GalNAc1 mRNA suggested appearance of sialyl-Tn-Ag (Siaα2,6GalNAc-O-Ser/Thr). The increased intensity of SNA staining might be also due to 6`-sialyl lactosamine (Siaα2,6Galβ1,4GlcNAc) even if the level

of amplification of ST6Gal1 mRNA remained unchanged throughout the time course of the study. Accumulation of sialyl-T-Ag (Siaα2,3Galβ1,3GalNAc-O-Ser/Thr) was taken into consideration because of the intense staining with PNA after neuraminidase treatment and the transient increase of expression of ST3Gal2 and 4. The manifestation of MAL accompanied by increase of ST3Gal4 expression suggested accumulation of 3`sialyl lactosamine (Siaα2,3Galβ1,4GlcNAc) (Table 4).

Our findings	Predicted carbohydrate structure
↑SNA, ↑VVL-B4-Neu, ↑HPA-Neu, ↑ST6GalNAc1	Sia-α-2,6-GalNAc-α-1-Ser/Thr Sialyl-Tn-Ag
↑SNA, unchanged level of ST6Gal1	Sia-α-2,6-Gal-β-1,4-GlcNAc 6`-sialyl lactosamine
↑PNA-Neu, ↑ST3Gal2, ↑ST3Gal4	Sia-α-2,3-Gal-β-1,3-GalNAc-α-1-Ser/Thr Sialyl-T-Ag
↑MAL, ↑ST3Gal4	Sia-α-2,3-Gal-β-1,4-GlcNAc 3`-sialyl lactosamine

Table 4. Summary of the predicted carbohydrate structures, which came forward into the sarcoplasm after invasion by *Trichinella*, based on the investigation of expression of different types of sialyltrasferases by immunochistochemistry and PCR, and the lectin-based glycan analysis.

Discussion

After invasion by *Trichinella*, the occupied cytoplasm of the muscle cell gradually dies by apoptosis. Satellite cells are activated, proliferate and differentiate into cytoplasm of the newly-formed nurse cell that persists for years [3]. The invaded portion of sarcoplasm loses its contractile capabilities but not its adherence [4], which suggests an increased expression of proteins responsible for its integrity. The results from our study proposed accumulation of sialyl-Tn-Ag, sialyl-T-Ag, 6`- and 3`-sialyl lactosamine oligosaccharide structures into the occupied sarcoplasm.

Recent pioneer studies indicated that the processes of gene expression and cell differentiation are associated with increased biosynthesis of sialylated glycoconjugates and are actually a result of it; however the mechanism of their regulatory function is still not known [5, 6, 7]. An overexpression of uridine diphospho-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase, which is the key enzyme of the sialic acids biosynthesis, was recently reported in muscle injury and regeneration [8, 9]. One of the mutations of the human GNE gene is responsible for hereditary Inclusion Body Myopathy (hIBM), which was characterised by hyposialylation of skeletal muscle glycoproteins from patients with hIBM [10]. Skeletal muscle tissue expresses relatively low levels of the enzyme in comparison to other tissues [11], and its selective involvement as a result of this particular mutation of GNE still remains very enigmatic [10]. Hence, it is tempting to speculate that the skeletal muscle fibres are very sensitive to losses of sialic acid, which might be essential in sustaining their proper structure and function.

The question about the mutations of the glycosyltransferases and how they alter the proper structure and function of the skeletal muscle tissue has been a subject of study by many researchers [12-16]. The paradox is that even if we know the particular mutation and the resulting disease, we still have very little information about the substrates of the mutated glycosyltransferases, because of the limited knowledge about the glycoproteome of the skeletal muscle tissue. Thus, in the presence of appropriate methodology, we propose the Nurse cell – *Trichinella spiralis* complex as a suitable model for investigation of sialylated glycoproteins in skeletal muscle tissue because of their rich accumulation within the Nurse cell.

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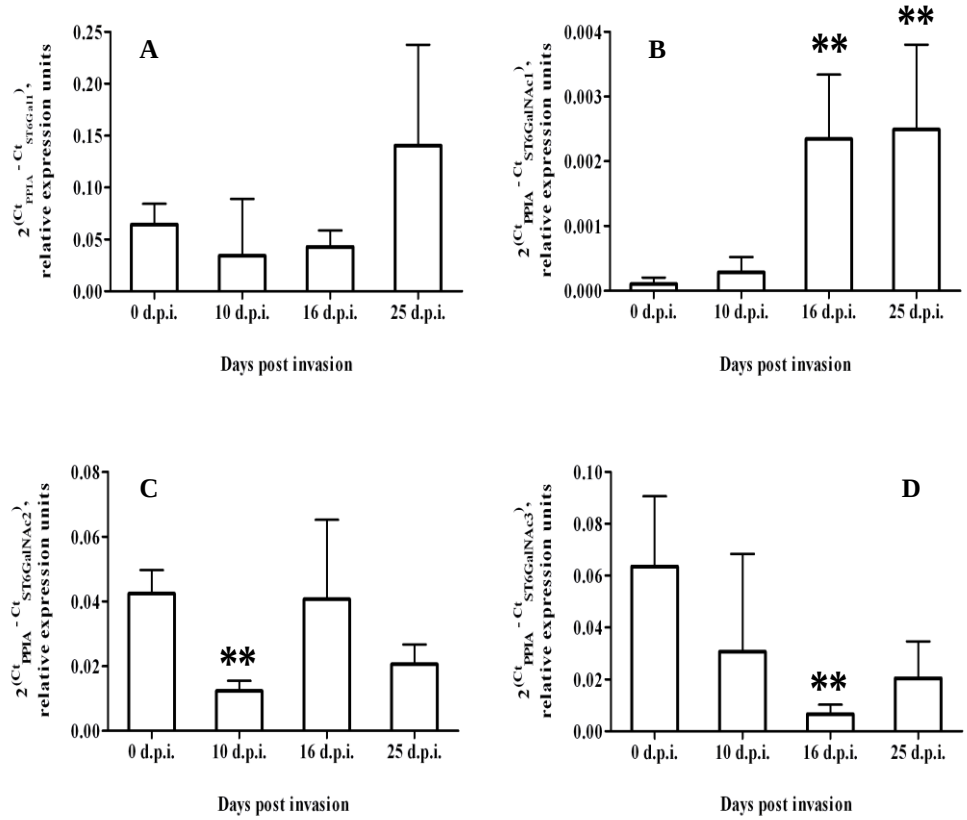

Fig. 1. Relative quantification of the expression of ST6Gal1 (A), ST6GalNAc1 (B), ST6GalNAc2 (C) and ST6GalNAc3 (D) mRNA through normalization against the reference gene PPIA in mouse skeletal muscle tissue in the time course of development of the Nurse cell –*Trichinella spiralis* complex.
The stars indicate statistically significant difference of mRNA expressions of the invaded tissues in progress versus the non-affected skeletal muscle used as a control, ** P< 0.01.

BO6. EFFECT OF BIOLOGICALLY ACTIVE SUBSTANCES ISOLATED UNDER FASCIOLOSIS ON LYMPHOCYTE CELL CULTURES

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Abstract

Thermolabile biologically active substances (BASes) were isolated from the tissues of the helminth *Fasciola hepatica* and from healthy and *F. hepatica* infected rat liver. The effect of the newly isolated BASes was studied on nonactivated and mitogen activated lymphocyte cell cultures. An immunomodulating effect of the newly isolated BASes was demonstrated.

Biologically active substances (BASes) inhibitors of cell proliferation from parasite and host origin were isolated and their growth inhibiting effect had been established *in vitro* on primary hepatocyte cell cultures (5, 6). The newly isolated biologically active substances were two types: 1. from the tissues of the helminth *Fasciola hepatica* and 2. from *F. hepatica* infected rat liver.

In the present work we aimed to study the effect of the newly isolated BASes on lymphocyte cell cultures, obtained from the spleens of healthy rats.

Materials and Methods

Wistar rats were orally infected with 20 metacercariae of *F. hepatica*. Mature parasites were obtained from the rat bile ducts 4 months later [4]. The parasites and the livers of the infected animals were processed to obtain the BASes. Livers of healthy Wistar rats bred under the same conditions were used for controls. Healthy rat spleens were removed aseptically and used to make lymphocyte cell cultures.

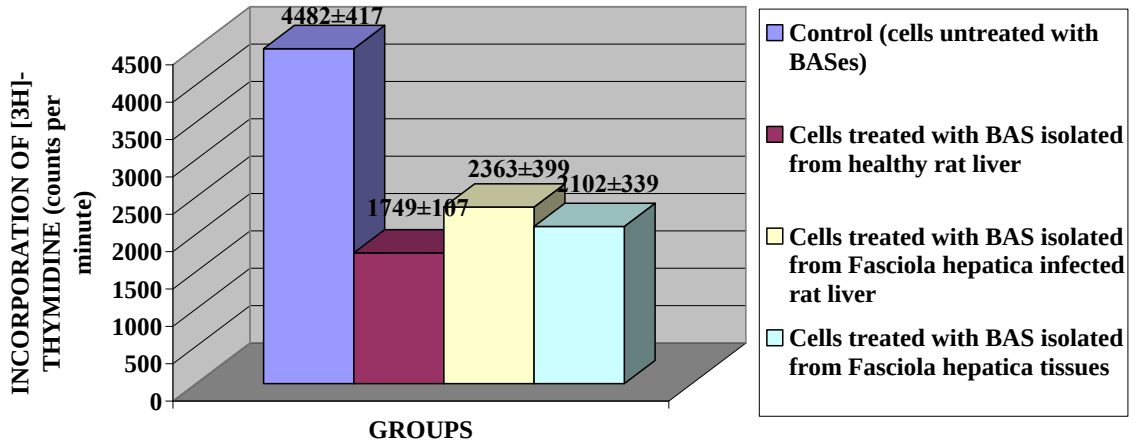
The BASes were isolated from livers of healthy and *F. hepatica* infected rats and tissues of *F. hepatica* by ethanol precipitation of aqueous tissue homogenates using the fraction with final concentration of ethanol between 70% and 87% (v/v) by the modified method of Verly et al. for obtaining liver chalones [7]. Protein estimation of the newly isolated BASes was carried out according to Bradford [2]. The dried extracts dissolved in PBS were added to the lymphocyte cell cultures at a dose of 20 μg per well.

The spleen lymphocyte cell cultures were obtained by gradient centrifugation on Polysep (Pharmachim, Bulgaria – 1.077 g/cm^{-1}). The cell number was adjusted to 1 X 10^6 cells/ml and applied to U-bottomed microtiter plates (Nunc) in RPMI 1640 medium (Flow) containing 10% fetal calf serum. The above described cultures were stimulated with phytohemagglutinin (PHA) (Difco) – 5 μg/ml, Pokeweed mitogen (PWM) (Difco) – 22 μg/ml and lipopolysaccharide (LPS) (Difco) – 50 μg/ml. The BASes were added on the 0 h to mitogen activated or nonactivated cultures. The cells were cultivated for 3 days at 37ºC in a humidified 5% CO_2 atmosphere. 1 μCi ^{3}H- thymidine (UVVVR – FPrague) was added to each well and incubation continued for an additional 18 h. The cells were harvested on GF/C (Whatman) filters and counted in a scintillation counter (Beckman). All cultures were triplicated and the results were given as mean cpm (counts per minute) from the triplicates ± SD.

Results and Discussion

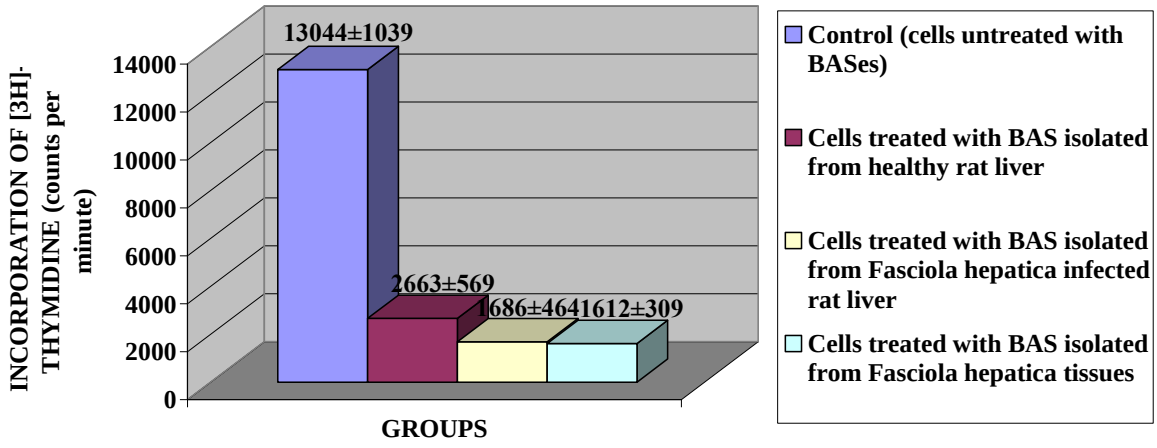
The treatment of spleen lymphocyte cell cultures with the newly isolated BASes caused a twofold reduction of ^{3}H-thymidine incorporation in the cells compared to the untreated control (Fig. 1). Slight differences were noted between the effects of the three types BASes ($P<0.02$ or $P<0.05$).

FIG. 1. INCORPORATION OF [3H]-THYMIDINE IN NONSTIMULATED LYMPHOCYTE CELL CULTURE FROM HEALTHY RAT SPLEENS



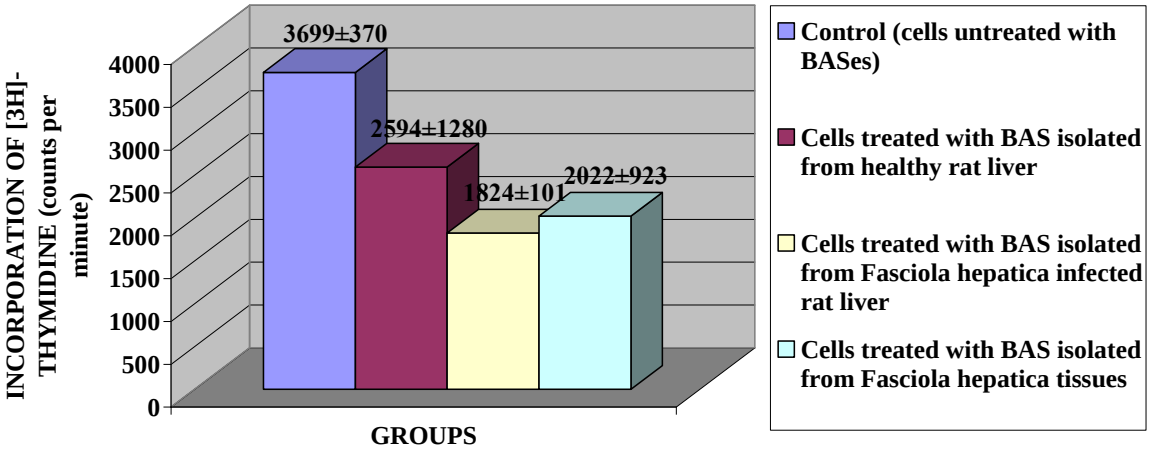
However, the BASes treatment on the background of PHA stimulation caused stronger suppression of the blast transformation of the spleen lymphocyte cell cultures ($P<0.1$) (Fig. 2). The values were are about 2 times lower compare to untreated nonstimulated control and from 3.4 to 8 times lower in comparison with PHA stimulated control. The strongest effects of inhibition were seen in BAS isolated from *F. hepatica* infected rat liver and BAS isolated from *F. hepatica* tissues.

FIG. 2. INCORPORATION OF [3H]-THYMIDINE IN PHA MITOGEN STIMULATED LYMPHOCYTE CELL CULTURE FROM HEALTHY RAT SPLEENS



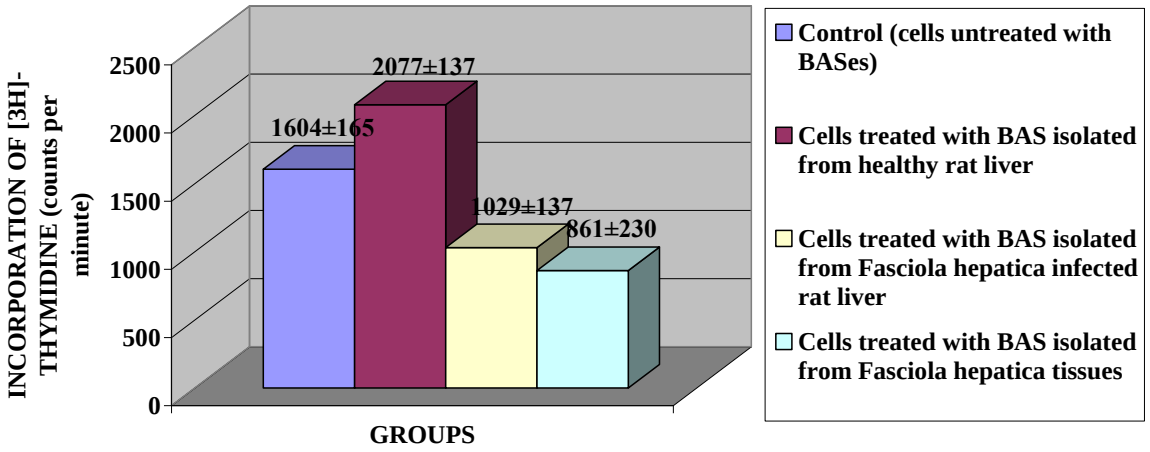
Mitogen stimulation with PWM and LSP combined with BASes treatment caused a slight effect of inhibition of DNA-synthesis in the spleen lymphocyte cell cultures. The values were from 1 to 2 times lower, compared both to the mitogen-stimulated controls and to the untreated controls (Fig. 3, 4).

FIG. 3. INCORPORATION OF [3H]-THYMIDINE IN PWM MITOGEN STIMULATED LYMPHOCYTE CELL CULTURE FROM HEALTHY RAT SPLEENS



Inhibitors of DNA synthesis of their own cells were isolated biochemically from various types of tissues [1, 3]. No data are available in the literature for the isolation of chalones from parasite infected host organs or chalone-like extracts from *F. hepatica*. There were data concerning preparation of chalone-like substances from the parasites *Taenia crassiceps* and *Ascaris suum* [3]

FIG. 4. INCORPORATION OF [3H]-THYMIDINE IN LPS MITOGEN STIMULATED LYMPHOCYTE CELL CULTURE FROM HEALTHY RAT SPLEENS



The liver chalones isolated from normal liver were known as effectors on the DNA synthesis and as immunomodulators (1). Our present data pointed to an inhibitory effect on the DNA-synthesis not only by the control BAS isolated from normal liver but also by BASes isolated from *F. hepatica* infected liver and from the tissues of *F. hepatica*. The strongest was

the inhibiting effect of the BASes isolated from *F. hepatica* tissues and from the infected liver on the blast transformation of the spleen lymphocyte cell culture. Kudrna & Prokopic [3] suggested that the inhibition of DNA synthesis by means of the extracts of helminth tissues prepared in the same way as chalones of mammalian tissue might indicate that the regulating mechanism of DNA synthesis in helminthes are similar to that in mammals.

The present study demonstrated an immunomodulating effect of the newly isolated BASes and suggested the possibility for their practical use in medicine for therapy of some tumoral and autoimmune diseases and in the surgery in the transplantation of some tissues and organs.

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BO7. EFFECT OF THERMOLABILE BIOLOGICALLY ACTIVE SUBSTANCES UNDER FASCIOLOSIS ON TUMOR CELL CULTURE

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Abstract

Thermolabile biologically active substances (BASes) were isolated from the tissues of the helminth *Fasciola hepatica* and from healthy and *F. hepatica* infected rat liver. The effect

of the newly isolated BASes was studied on hepatoma MC29 tumor cell cultures. The strongest inhibiting effect on cell proliferation was by BAS isolated from *F. hepatica* tissues. The growth inhibiting effect of BAS isolated from *F. hepatica* infected rat liver was stronger than the effect of BAS isolated from normal liver tissue.

Introduction

A new property of mature *Fasciola hepatica* was established in previous investigations – its inhibiting effect on experimental liver carcinogenesis [6, 7]. This formed the basis for a hypothesis on the possible roles of some biologically active substances (BASes) of parasite or host origin in the pathogenesis of this interaction.

The aim of the present work is to study the effect of newly isolated thermolabile BASes from the tissues of the helminth *F. hepatica* and from healthy and *F. hepatica* infected rat liver on DNA synthesis of tumor cell cultures.

Materials and Methods

Wistar rats were orally infected with 20 metacercariae of *F. hepatica*. Mature parasites were obtained 4 months later from the rat bile ducts. The animals were sacrificed with the correspondence with the ethical standards of human approach to the animals. The parasites and the livers of the infected animals were processed to obtain the BASes. The livers of healthy Wistar rats, bred under the same conditions were used as controls.

The thermolabile BASes were isolated by the modified method of Verly et al. [8] for chalone isolation by ethanol precipitation from aqueous tissue homogenates using the fraction with final concentration of ethanol between 70% and 87% (v/v). Protein estimation of the newly isolated BASes was carried out according to Bradford [3]. The BASes isolated from normal liver tissue, from *F. hepatica* infected liver and from the tissues of mature *F. hepatica* were investigated on hepatoma MC29 cultures. The dried extracts dissolved in PBS 1:1 (w/v) were added to the tumor cell cultures at a dose of 20 μg per well at the beginning of the experiment (20 μl of the solution per well).

The cell cultures were obtained from hepatoma MC29 in chickens [5]. The tumor tissue pieces were treated with 0.2% (w/v) trypsin solution. The cells obtained were washed with PBS and their viability was not less than 90-95% as estimated by the trypan-blue exclusion test. The cell number was adjusted to 1 X 10^5 cells per well applied to U-bottomed microtiter plates (Titertek) in E199 medium (Difco) containing 20% (v/v) fetal calf serum. The cells were cultured for 24 h at 37°C in a humidified 5% CO_2 atmosphere. 1.5 μCi ^{3}H-thymidine (UVVVR- Prague) was added to each well and the incubation continued for an additional 18 hours. The cells were harvested on NC filters (45μ) (Millipore) and counted in a scintillation counter (Intertechniques). All cultures were triplicated and the results were given as mean cpm from the triplicates ± SD.

Results and Discussion

The results are summarized in Fig. 1 and Table 1.

The BASes have a strong inhibiting effect on DNA synthesis in the hepatoma MC29 cell cultures. The treatment with the BAS, isolated from a normal liver tissue causes a well marked decrease in the ^{3}H-thymidine uptake in the culture, which is 2.8 time lower compared to the untreated control ($P<0.01$). The percentage inhibition of DNA synthesis is about 64.3. The ^{3}H-thymidine uptake of the tumor cells treated with the BAS isolated from *F. hepatica* infected rat liver is 4.7 fold lower compared to the untreated control ($P<0.001$) and about 2 times lower compared to the effect of the BAS isolated from normal liver tissue. The percent inhibition of the DNA-synthesis is 78.8. The strongest inhibiting effect is with the BAS isolated from the tissues of mature *F. hepatica*. The ^{3}H-thymidine incorporation into the

hepatoma MC29 cells in this case is 2.8 times lower compared to the effect of the BAS isolated from *F. hepatica*-infected rat liver, 4.7 times lower compared to the effect of BAS isolated from healthy rat liver and 13 times lower in comparison to the untreated control ($P<0.001$). The effect of inhibition of DNA-synthesis is about 92.4 percent.

The trypan blue staining of the cells after cultivation shows slight increased percentage of the dead cells in all experimental groups (from 18% to 24%) than in the control group (about 14%).

Fig. 1. INCORPORATION OF [3H]-THYMIDINE IN HEPATOMA MC29 CELL CULTURE AFTER TREATMENT WITH THE NEWLY ISOLATED BASes

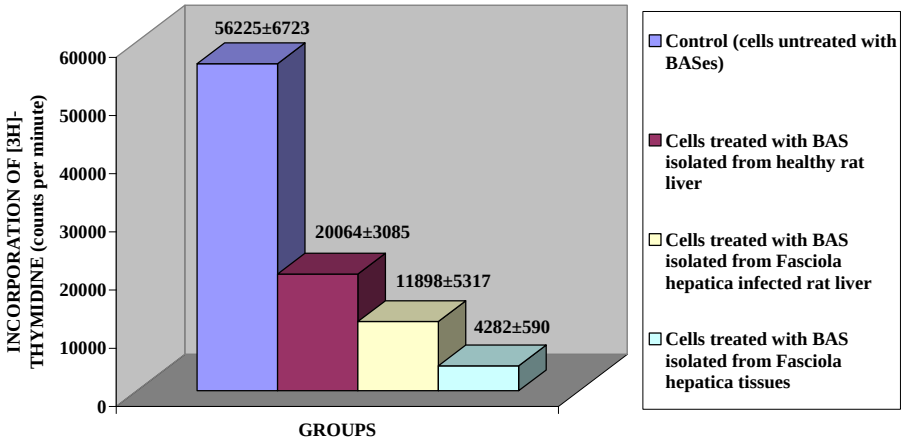

Table 1. Percent of inhibition of hepatoma MC29 cell cultures after treatment with the newly isolated BASes

Groups	Percent of inhibition
Control cells (untreated with BAS)	
Treated with BAS isolated from healthy rat liver	64.3
Treated with BAS isolated from *F. hepatica* infected rat liver	78.8
Treated with BAS isolated from mature *F. hepatica* tissues	92.4

The results support our presumption about the possible role of some BASes inhibitors of cell proliferation from the parasite and host origin in the mechanisms of the interaction between *F. hepatica* and experimental liver carcinogenesis.

Some characteristics of the newly isolated BASes, which are important for their characterization [1, 2] are established in the present investigation.

Tissue specific activity is established for both of the BASes isolated from normal and *F. hepatica*-infected liver tissue. They both inhibit the DNA-synthesis in hepatoma MC29 cells. The BAS isolated from the tissues of mature *F. hepatica* shows no tissue specific activity. The strong inhibiting effect of the BAS on the hepatoma MC29 cells might be explained by the processes of evolutionary adaptation of the parasite to the host tissue in which it develops and inhabits.

Our data confirm another characteristic of the newly isolated BASes – their species non-specificity. The newly isolated BASes influence cell cultures from chicken tissues, although they are isolated from rat liver tissues and from the tissues of lower animal – parasitic worm. Kudrna and Prokopic [4] suggest that the inhibition of DNA synthesis by extracts of helminth tissues prepared as the same way as chalones from mammalian tissue might indicate that the regulating mechanisms of DNA synthesis in helminthes are similar to those in mammals.

The present study demonstrates a well expressed inhibiting effect on cell proliferation in hepatoma MC29 tumor cell cultures by the newly isolated BASes from *F. hepatica* tissues and *F. hepatica* infected rat liver and offers a future possibility for their practical use for therapy in soma branches of medicine.

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BO8. NOSEMOSIS – THE PESTS OF THE 21th CENTURY IN EUROPE

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Abstract

In this review is presented an emerging disease caused by *Nosema ceranae*. Two microsporidian parasites are described from honey bees, *Nosema apis* and *Nosema ceranae*. *N. apis* was isolated in the European honey bee (*Apis mellifera*). *Nosema ceranae* is a microsporidian parasite described from the Asian honey bee, *Apis cerana.*
Microsporidiosis caused by infection with *Nosema apis* or *Nosema ceranae* has become one of the most widespread diseases of honey bees and can cause important economic losses for beekeepers. Honey can be contaminated by spores of both species and it has been reported as a suitable matrix to study the field prevalence of other honey bee sporulated pathogens.
Since 2006, beekeepers have reported increased losses of *Apis mellifera* colonies, and one factor that has been potentially implicated in these losses is the microsporidian *Nosema ceranae*. Within a few years after it was detected in Spain in 2005, the rest of European countries that had technical capacity to differentiate *Nosema apis* from *N. ceranae* reported its presence.

Keywords: *N. ceranae, N. apis, Apis meliffera, nosemosis*

Introduction

Nosema ceranae is a microsporidian parasite described from the Asian honey bee, *Apis cerana*. *N. apis* was isolated in the European honey bee (*Apis mellifera*) [35] and *N. ceranae* was isolated from the Asian honey bee (*Apis cerana*) in China [11]. The parasite is cross-infective with the European honey bee, *Apis mellifera*. It is know that both parasites are cross-infective across host species, but that N. apis develops less well in *A. cerana* compared to *N. ceranae* in *A. mellifera* [10,13]. It is not known when or where *N. ceranae* first infected European bees, but *N. ceranae* has probably been infecting European bees for at least two decades. In 1994, a microsporidian similar to *N. apis* was described in Asian honey bees from China. This parasite, called *Nosema ceranae,* was subsequently detected in European honeybees (*Apis mellifera*) in Taiwan. Recently, natural infections of N. ceranae in *A. mellifera* were detected in Taiwan and in Spain [16, 23].

Previously, nosemosis in European honey bees was attributed exclusively to *N. apis* [6], with the recent exception of disease in regions of Asia and Europe that were ascribed to the closely-related *Nosema ceranae* [16, 23]. However, it appears that *N. ceranae* is an emerging pathogen that has increased its distribution in the past decade by jumping from Asian honey bees, *Apis cerana*, to Europeanhoney bees [24]. *N. ceranae* has now been detected on four continents (Asia, Europe, North America, and South America) and it may be displacing *N. apis* in European honey bees [24]. Originally isolated from Asian honey bees [11], *N. ceranae* is highly pathogenic when experimentally inoculated into European honey bees [17], and is associated with reduced honey production and increased winter mortality [16].

The first honey simple in which *N. ceranae* was detected dates back to the year 2000. In subsequent years, the number of samples containing *N. ceranae* tended to increase, as did the detection of *Nosema spp.* in adult worker bees. The presence of *N. ceranae* as early as

2000, long before generalized bee depopulation and colony losses in 2004 may be consistent with a long incubation period for nosemosis type C or related with other unknown factors.
It is now clear that *N. ceranae* is not a new parasite of the European honeybee. *N. ceranae* was detected in bee samples collected in the USA in 1996 and Europe in 1998. The delay in recognising *N. ceranae* is attributable to the routine use of microscopy as a diagnostic technique for the detection of *Nosema*-like spores. However, the spores of *N. ceranae* and *N. apis* are similar in size, which has resulted in them being diagnosed as the latter. Molecular techniques such as polymerase chain reaction (PCR) have not been commonly used to diagnose nosemosis, but these are necessary to identify each of the two species. The advent of molecular assays for *Nosema* spp has driven the detection of *N. ceranae* worldwide [22].

N. ceranae has been associated with a condition which has killed hundreds of thousands of honeybee colonies in the USA, known there as colony collapse disorder (CCD). This organism has also been reported to be more severe on honeybee colonies than *N. apis* and is having a major impact on honeybee colonies in Spain [7].

N. ceranae appears to be replacing *Nosema apis*, at least in some populations of European honey bees. This replacement is an enigma because the spores of the new parasite are less durable than those of *N. apis*. Virulence data at both the individual bee and at the colony level are conflicting possibly because the impact of this parasite differs in different environments. The recent advancements in *N. ceranae* genetics, with a draft assembly of the *N. ceranae* genome available, are discussed and the need for increased research on the impacts of this parasite on European honey bees is emphasized [8].

A multiplex PCR-based method, in which two small-subunit rRNA regions are simultaneously amplified in a single reaction, was designed for parallel detection of honeybee microsporidians (*Nosema apis* and *Nosema ceranae*). Each of two pairs of primers exclusively amplified the 16S rRNA targeted gene of a specific microsporidian. The multiplex PCR assay was useful for specific detection of the two species of microsporidians related to bee nosemosis, not only in purified spores but also in honeybee homogenates and in naturally infected bees. The multiplex PCR assay was also able to detect coinfections by the two species. Screening of bee samples from Spain, Switzerland, France, and Germany using the PCR technique revealed a greater presence of *N. ceranae* than of *N. apis* in Europe, although both species are widely distributed. From the year 2000 onward, statistically significant differences have been found in the proportions of *Nosema spp*. spore-positive samples collected between and within years. In the first period examined (1999 to 2002), the smallest number of samples diagnosed as *Nosema* positive had been found during the summer months, showing clear seasonality in the diagnosis, which is characteristic of *N. apis*. From 2003 onward a change in the tendency resulted in an increase in *Nosema*-positive samples in all months until 2005, when a total absence of seasonality was detected. A significant causative association between the presence of *N. ceranae* and hive depopulation clearly indicates that the colonization of *Apis mellifera* by *N. ceranae* is related to bee losses [25].

Polymerase chain reaction specific for the rDNA marker for *Nosema ceranae* and *Nosema apis* was conducted on 84 *Apis mellifera* samples collected from 20 provinces in Turkey. *N. ceranae* was detected from three samples from the provinces of Artvin, Hatay, and Muğla. *N. apis* was detected in samples from the provinces of Sivas, Izmir, Bitlis and Gaziantep. All of the positive samples were from honey bees belonging to the ‘C’ lineage of *A. mellifera*. DNA sequencing analysis of the *N. ceranae* samples revealed that there was no intraspecific variation in the 208 bp of the 16S SSU of *N. ceranae* from Turkey. A TCS analysis revealed that the 16S SSU genotype from Turkey is identical to *N. ceranae* DNA sequences from Europe, Australia, and the United States. TCS analysis also revealed that this genotype is the basal ancestral genotype among six *N. ceranae* genotypes. This is the first study to confirm that *N. ceranae* is present in honey bees from Turkey [34].

In Bulgaria totally 98 locations from all over the country were studied for presence of *N. ceranae* and *N. apis* species. *N. ceranae* had been found in 43 of the apiaries studied, which were about 44 %. The *N. apis* had been found as a present species only in two of apiareas – about 2 % of all studied. Different locations from border regions of Bulgaria (near to Turkey, Greece, Macedonia, Serbia and Romania) had been also studied and *N. ceranae* detected in all of them. The *N. ceranae* invasion is characterized as a new for Bulgaria and its presence in the country is considered as a possible reason for increasing of colony losses in 2010 [15, 30].

The worldwide beekeeping sector has been facing a grave threat, with losses up to 100-1,000 times greater than those previously reported. Nosemosis is one of the most widespread of adult bee diseases and causes significant economic losses to beekeepers worldwide. This disease was originally thought to be caused by a single *Nosema* species, *Nosema apis*, a microsporidian which has a range of debilitating effects on honeybee colonies and adult bees. [21].

Nosemosis can induce queen supersedure [33], reduce pollen collection [1], reduced colony build-up and productivity [12], increased winter losses, colony collapse [18, 19] and shorten bee life span [32].

Despite the scale of this honey bee mortality, the causes underlying this phenomenon remain unclear, yet they are thought to be multifactorial processes. *Nosema ceranae*, a microsporidium recently detected in the European bee all over the world, has been implicated in the global phenomenon of colony loss, although its role remains controversial. A review of the current knowledge about this pathogen is presented focussing on discussion related with divergent results, trying to analyse the differences specially based on different methodologies applied and divisive aspects on pathology while considering a biological or veterinarian point of view [27]. For authors, the disease produced by *N. ceranae* infection cannot be considered a regional problem but rather a global one, as indicated by the wide prevalence of this parasite in multiple hosts. Not only does this type of nosemosis causes a clear pathology on honeybees at both the individual and colony levels, but it also has significant effects on the production of honeybee products [21].

In a similar way as the initial detection of *Varroa* in Europe, active scientific work is raising many questions due to the absence of clinical symptoms in infected colonies and a long incubation period of the pathogen. *N. ceranae* presents a different epidemiological pattern and pathology compared to *N. apis*. The disease caused by *N. ceranae* is now named nosemosis type C [3] and is characterized by the ability to detect the disease-causing agent throughout the year. The continuous death of highly infected bees, mostly foragers, has a clear effect on colony population and productivity. Although there has been a huge effort in the last years to increase knowledge about this disease, significant research is still needed on epidemiology, pathology, prophyllaxis and treatment [20].

Infection experiments have demonstrated that samples from across the world now demonstrate that the infection of *N. ceranae* in *A. mellifera* is a world-wide phenomenon [14, 25] and investigations of historic samples of bees infected with microsporidian spores suggest a replacement process, where *N. ceranae* appears to gradually replace *N. apis* [29]. This implies that *N. ceranae* may be a more virulent parasite. Indeed, in one study, experimental infections of *N. ceranae* in the laboratory resulted in a total mortality of 94.1% 1 week post-infection in three infected replicate cages, and by the 8th day post-infection, all infected bees had died [17]. These survival results of infected bees are in sharp contrast to earlier findings in cage experiments using *N. apis* [9] but also to a limited comparative analysis of the two parasites [29]. Other processes such as transmission rates or parasite replication may also influence the relative prevalence of the two parasites over time. In general, there is a positive relationship among parasite reproduction, virulence and increased transmission [4, 5].

The current prevalence of nosemosis, primarily due to *N. ceranae*, has reached epidemic levels in Spain as confirmed by the analysis of worker honey bees and commercial honey [2].

Parasites are depended on their hosts for energy to reproduce and can exert a significant nutritional stress on them. Energetic demand placed on the host is especially high in cases where the parasite-host complex is less co-evolved. The higher virulence of the newly discovered honeybee pathogen, *Nosema ceranae*, which causes a higher mortality in its new host *Apis mellifera*, might be based on a similar mechanism [26].

Since *N. ceranae* is a fairly recently discovered parasite, there is little knowledge of the variation in infection levels among individual workers within a colony. Mulholland [28] examined the levels of infection in individual bees from five colonies over three seasons using both spore counting and quantitative real-time PCR. The results show considerable intra-colony variation in infection intensity among individual workers with a higher percentage of low-level infections detected by PCR than by spore counting. Colonies generally had the highest percentage of infected bees in early summer (June) and the lowest levels in the fall (September). According to Mulholland *(2012) Nosema apis* had been detected in only 16/705 bees (2.3%) and always as a low-level co-infection with *N. ceranae*. The results also indicate that intra-colony variation in infection levels could influence the accuracy of *Nosema* diagnosis.

The way of infection shows that other *Nosema* species, such as *N. apis*, tend to be associated with increased defecation and spread via a fecal-oral pathway, but because *N. ceranae* does not induce defecation, it may instead be spread via an oral-oral pathway. Cages that separated older infected bees from young uninfected bees were used to test whether *N. ceranae* can be spread during food exchange. Although fecal-oral transmission is still possible in this experimental design, oral-oral infectivity could help explain the rapid spread of *N. ceranae* worldwide [31].

Conclusions

In conclusion, Nosema can be serious if not checked and the beekeeper should have an understanding of the nature and spread of the disease and the measures for the control of the disease.

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BP1. THE DARK SIDE OF TOXOPLASMA GONDII

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BP2. ENTAMOEBA HISTOLYTICA

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BP5. ВНИМАНИЕ: ТЕНИИ!

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BP6. SCHISTOSOMA

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Session C.

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

Assoc. Anton Kril, DVM, PhD
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CO1. EFFECTS OF LEAD AND SALINOMYCIN ON SOME ORGAN WEIGHT/BODY WEIGHT INDICES IN FEMALE MICE, SUBJECTED TO SUBACUTE LEAD INTOXICATION

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Abstract

Lead (Pb) is one of the most toxic metal ions. It has been reported that Pb crosses the blood brain barrier and accumulates in the brain, affecting the function of the central nerve system. It has been proven that Pb causes encephalopathy in children and adults as well [1, 2]. Renal functions and immune system are also affected by Pb [4].

For the treatment of Pb-intoxication a therapy with chelating agents has been applied. 2, 3 dimercaptosuccinic acid (DMSA) is a chelating agent of first choice in the therapy of Pb-intoxication. Recently it has been demonstrated that the polyether ionophorous antibiotic monensin improves the effectiveness of DMSA in reducing the concentration of Pb in the brain and bones in rats subjected to Pb-intoxication [3]. To the best of our knowledge there is no information for the possible application of salinomycin (less toxic representative of the polyether ionophorous antibiotics) as an antidote for Pb-intoxication.

Herein we present novel preliminary information regarding the effects of Pb and salinomycin on some organ weight/body weight indices in female mice, subjected to subacute Pb-intoxication. Data demonstrated that Pb induced alterations of kidneys weight/body weight and spleen weight/body weight indices while the liver weight/body weight and brain weight/body weight indices of Pb-intoxicated animals remain unaffected compared to the controls. The treatment with salinomycin of animals, exposed to Pb intoxication, restored the kidney weight/body weight index to normal value, suggesting that the antibiotic improves the renal function of Pb-treated animals. Further studies are needed to understand the effects of Pb and salinomycin on the liver, kidneys, spleen and brain functions of animals subjected to subacute Pb-intoxication.

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CO2. CHANGES IN MICE SPERM COUNT AFTER SUBACUTE INTOXICATION WITH LEAD AND SALINOMYCIN DETOXICATION - PRELIMINARY DATA

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Abstract

Exposure to heavy metals is the most important risk factor in the assessment of spermatogenesis. About 30–40 % cases of infertility are caused by the male factor, and most of them are due to the small quantity of spermatozoa or to impaired sperm morphology or motility [1]. There are sufficient data in literature indicating that exposure to toxic metals as lead (Pb), cadmium (Cd), and arsenic (As) affect germ cell DNA and suggest possible direct testicular toxicity [2]. We used a mouse experimental model to investigate the *in vivo* effects of Pb and the chelating agent salinomycin on sperm count in adulthood. Animals were divided into three groups: normal control (receiving distilled water and food): Pb group, exposed to 60 mg/kg b.w. $Pb(NO_3)_2$ daily for the first 2 weeks of the experimental protocol and Pb+Salinomycin group, receiving salinomycin (20 mg/kg) after Pb-intoxication from 15^{th} to 28^{th} days. Testes and epididymides were sampled and weighed. Spermatozoa were isolated

from both vasa deferentia and counted. Our preliminary data from a limited number of animals demonstrated reduction by almost 50 % of gonado-somatic index (ratio of testicular weight to body weight – TW/BW) after Pb-administration and restoration near to the control value after detoxication with salinomycin. We found a similar tendency in epididymal index (ratio of epididymal weight to BW). Concerning sperm count our results were not very reliable – even after salinomycin treatment spermatozoa remained 3-fold lower compared to the control. Probably this parameter is the most sensitive to exposure to Pb. These preliminary data are encouraging but future studies would elucidate if salinomycin could be a good chelating agent in the therapy of lead intoxications.

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CO3. IN OVO STUDY ON THE GENOTOXIC AND CARCINOGENIC POTENTIAL OF ANTHRACENE-DRIVED SCHIFF BASES AND ANTHRACENE-CONTAINING AMINOPHOSPHONATES

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Abstract

Avian embryos have been proposed as reliable experimental models, alternative to laboratory animals for studies on genotoxicity and carcinogenicity of chemical compounds. Genotoxic and carcinogenic potential of recently synthesized anthracene-derived Schiff bases and novel anthracene-containing aminophosphonates were assessed by the hen's egg test for micronucleus induction (HET-MN) and the *in ovo* carcinogenicity assay (IOCA). The end points measured in these *in ovo* assays are the formation of micronuclei in the erythrocytes of avian embryos and the appearance of preneoplastic lesions in the avian embryonal liver, respectively. N-nitrosodimethylamine (NDMA), a chemical compound with known genotoxic and carcinogenic activity *in ovo* was used as a positive control in the present study. The results from the HET-MN test showed that NDMA induced a statistically significant increase

in the frequencies of the micronucleated erythrocytes, as well as in the total number of erythrocytes with nuclear abnormalities, as compared to vehicle-treated control. In contrast, none of the test compounds induced significant changes in the parameters measured. Histological examination of the embryonal livers of the NDMA-treated embryos revealed the appearance of clear, basophilic and mixed cell foci, regarded as preneoplastic lesions, formation of tubular structures and hyperplasia of cholangiocytes. Similar morphological alterations were not found in the livers of the embryos treated with the tested Schiff bases and aminophosphonates.

Keywords: in ovo models, aminophosphonic acid, Schiff base, carcinogenicity, genotoxicity

Introduction

The *α*-aminophosphonates are organophosphorus compounds, which have found a wide range of applications in the areas of industrial, agricultural, and medicinal chemistry owing to their biological and physical properties as well as their utility as synthetic intermediates. As analogues of natural amino acid, *α*-aminophosphonates constitute an important class of compounds with diverse biological activities, including enzyme inhibitory, antibacterial [4], antifungal [8], antiviral [10] and antitumor effects [5]. Some aminophosphonate derivatives inhibit bone resorbtion, delay the progression of bone metastases, exert direct cytostatic effects on a variety of human tumour cells and have found clinical application in the treatment of bone disorders and cancer [2].

Anthracene-bearing *α*-aminophosphonates might be of particular interest in the design of new antitumor therapeuticals considering the fact that the DNA-intercalating anthracene-derived planar structure is the main pharmacophoric fragment of some cytostatic drugs used for treatment of human cancers [6]. Some of these anthracene-containing substances have been reported to display strong antiproliferative activity against several tumor cell lines, including multidrug resistant phenotypes [7].

In a previous publication [5] we reported the synthesis of a new Schiff base, 9-anthrylidene-furfurylamine and three novel anthracene-containing α-aminophosphonates, [N-methyl(dimethoxyphosphonyl)-1-(9-anthryl)]-p-toluidine, [N-methyl(diethoxyphosphonyl)-1-(9-anthryl)]-p-toluidine and [N-methyl(diethoxyphosphonyl)-1-(9-anthryl)]furfurylamine. In addition, the results from the studies on the *in vitro* safety assessment, the antitumor activity and subcellular distribution of these compounds in normal and tumor cells, and their *in vivo* genotoxicity in ICR mice were also presented [5].

In this study the genotoxic and carcinogenic potential of these novel aminophosphonates and the Schiff bases used as their synthetic precursors, were assessed by implementation of the hen's egg test for micronucleus induction [9] and the *in ovo* carcinogenicity assay [3]. The ability of the test chemicals to induce micronucleated erythrocytes in avian embryos and the appearance of preneoplastic lesions in the avian embryonal liver are the end points measured in the HET-MN and the IOCA assays, respectively. These *in ovo* tests allow a reliable, rapid and inexpensive assessment of the genotoxic and carcinogenic potential of various chemical compounds and are strictly in line with animal protection regulations and ethical aspects of scientific investigations [3, 9].

Materials and methods

Test compounds

- Schiff bases: Anthrylidene-p-toluidine **(1)**; 9-Anthrylidene-furfurylamine **(2)**
- Aminophosphonates: [N-Methyl(diethoxyphosphonyl)-1-(9-anthryl)]furfurylamine **(3)**;
[N-Methyl(dimethoxyphosphonyl)-1-(9-anthryl)]-p-toluidine **(4)**;
[N-Methyl (diethoxyphosphonyl)-1-(9-anthryl)]-p-toluidine **(5)**

- N-nitrosodimethylamine (NDMA), CAS № 62-75-9 (*Sigma Aldrich, Germany*)
- Dimethylsulfoxide (DMSO), CAS № 67-68-5 (*Sigma Aldrich, Germany*)

Avian embryos

White Leghorn chicken, 15 I line embryos were used in all experiments.

Hen's egg test for micronucleus induction (HET-MN)

Eggs were incubated horizontally in an automatically rotating incubator at 37.5±0.5°C; 70±10% relative humidity and candeled before treatment in order to use fertile eggs only. The test substances, dissolved in DMSO were inoculated into the egg albumen on the ninth embryonic day (E9). Blood sampling was performed two days later (E11). The obtained blood was spread out on slides without any anticoagulant treatment. The blood smears were air-dried and May-Gruenwald-Giemsa stained. Scoring of micronucleated erythrocytes was performed according the accepted criteria [10]. Briefly, the principal criterion for classifying a structure as micronucleus is its three-dimensionality and its resemblance to the cell nucleus, especially its similar staining and texture. The size should not exceed two thirds of the size of the erythrocyte nucleus, with a distinct border and round to oval in shape. At least two slides from each embryo were prepared and 3500 erythrocytes per embryo were scored. Mann-Whitney U test was used to determine the significance of the differences in the frequencies of micronucleated erythrocytes.

In ovo carcinogenicity assay (IOCA)

The tested compounds were inoculated into the egg albumen, at a dose of 1 mg/egg during the first hours of incubation. Negative control eggs were treated with equivalent volume of the vehicle. Eggs treated with N-nitrosodimetylamine (0.5 mg/egg) were used as positive controls. The eggs were incubated horizontally in an automatically rotating incubator at 37.5±0.5 °C and 70±10% relative humidity. The incubation was terminated four days before hatching by cooling the eggs for an hour at 4°C. The embryos were examined for macroscopic lesions and the body weight and liver weight were determined for all treatment groups. The embryonal livers were immediately fixed in 10% phosphate-buffered formalin. Tissue samples were dehydrated, paraffin embedded, sectioned at 5 μm and stained with hematoxylin and eosin for light microscopy. The dystrophic, hypertrophic and preneoplastic lesions found in the livers of the treated embryos were classified according the accepted criteria [3]. The statistical analysis was performed by One-way ANOVA (GraphPad Prizm5 Software). $p < 0.05$ was accepted as the lowest level of statistical significance.

Results and discussion

The genotoxic potential of the Schiff bases and aminophosphonates was assessed by the HET-MN test (Table 1). The positive control compound NDMA induced a statistically significant increase in the frequencies of the micronucleated and binucleated erythrocytes, as well as in the total number of erythrocytes with nuclear abnormalities, compared to vehicle-treated controls. As opposed to the NDMA none of the test compounds induced significant alterations in the parameters measured.

Table 1. Genotoxicity testing of the Schiff bases 1, 2 and the aminophosphonates 3, 4 and 5 on White Leghorn chicken embryos

Treatment groups	Number of examined eggs	Embryo Mortality (%)	Number of MNE ‰ mean±S.D.	Number of BNE ‰ mean±S.D.	Total number of ENA ‰ mean±S.D.
Dimethylsulfoxide	6	0	0.3±0.4	0.1±0.5	0.4±0.4
N-nitrosodimethylamine	6	45.5	7.8±1.6**	2.3±0.8*	10.1±1.8**
Schiff base 1	6	50.0	0.2±0.2	0.1±0.2	0.4±0.4
Schiff base 2	6	16.7	0.3±0.2	0.1±0.1	0.5±0.3
Aminophosphonate 3	6	33.3	0.1±0.1	0.1±0.1	0.1±0.1
Aminophosphonate 4	6	0.0	0.2±0.2	0.1±0.1	0.3±0.4
Aminophosphonate 5	6	33.3	0.1±0.2	0.1±0.1	0.2±0.3

Statistics: Mann-Whitney U test; * $p<0.05$; ** $p<0.01$ compared to the vehicle-treatment; MNE-micronucleated erythrocytes; BNE-binucleated erythrocytes; ENA-erythrocytes with nuclear abnormalities.

Gross pathology, after the performance of the IOCA, revealed that the viscera of most of the embryos treated with the Schiff bases and aminophosphonates were indistinguishable from those of control embryos. Only the livers of small number of embryos treated with aminophosphonate 3 (1 embryo) and aminophosphonate 4 (2 embryos) were slightly reduced in size and greenish in color, indicating accumulation and retention of bile. The values of the other measured indicators of embryotoxicity are shown in Table 2.

Table 2. Influence of the *in ovo* treatment of White Leghorn chicken embryos with the Schiff bases 1, 2 and the aminophosphonates 3, 4 and 5 on the embryo weight, liver weight and relative liver weight.

Tratment groups	Number of inoculated eggs	Embryo mortality (%)	Embryo weight (g) mean±SD	Liver weight (g) mean±SD	Relative liver weight (%) mean±SD

Dimethylsulfoxide	10	12.5	18.2±7.1	0.5±0.1	2.7±0.3
N-nitrosodimethylamine	10	45.5	17.2±3.9	0.4±0.1	2.3±0.2
Schiff base 1	15	83.3	15.5±3.2	0.4±0.0	2.6±0.5
Schiff base 2	14	63.6	28.3±6.3	0.7±0.1	2.4±0.9
Aminophosphonate 3	7	14.3	22.0±3.0	0.5±0.1	2.4±0.2
Aminophosphonate 4	15	41.7	14.6±2.8	0.4±0.1	2.7±0.3
Aminophosphonate 5	14	81.8	37.0±1.4	0.6±0.1	1.6±0.3

Statistics: One-way ANOVA; * $p<0.05$; ** $p<0.01$ compared to the vehicle-treatment

Despite the observed macroscopic changes and in the liver size, the mean embryo and liver weights, and the relative liver weight showed no significant differences, as compared to the vehicle-treated controls. In the NDMA-treatment group all measured parameters were decreased, compared to negative control, but the differences were not significant. Light microscopy revealed the appearance of three types of foci of altered hepatocytes (FAHs) – clear, acidophilic and basofhilic cell foci. Similar focal alterations observed in adult rat liver have been regarded as preneoplastic lesions and used as end points in some *in vivo* carcinogenicity tests [1]. The fact that such preneoplastic lesions can be induced *in ovo*, suggests that avian embryos can be used as an alternative model system for studies on chemical carcinogenicity. In addition to the FAHs, tubular structure formation, presence of megalocytes and hyperplasia of cholangiocytes were observed in livers of NDMA-treated embryos. Preneoplastic liver lesions were not found in the embryos treated with the Schiff bases and aminophosphonates.

Conclusion

The application of the recently synthesized anthracene-containing aminophosphonates and their precursor Schiff bases on 15 I line, White Leghorn chicken embryos does not lead to formation of micronucleated erythrocytes and to the induction of preneoplastic liver lesions, which is in concordance with the previously established negligible adverse effects, indicating higher safety of the compounds.

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CO4. CRITERIA FOR MECHANISM-BASED ENZYME INACTIVATION

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Abstract

A large number of therapeutic agents and other xenobiotics have been shown to undergo metabolic enzyme activation to form biologically reactive intermediates. Specific bindings of reactive intermediates to the active enzyme sites may be exploited in the design of irreversible inhibitors oftentimes referred to as "suicide substrates", "catalysis dependent" or "mechanism-based" inactivators.

A number of criteria are routinely used to assess whether a substrate is a mechanism-based inactivator. It must be demonstrated that the reaction:

- *Required metabolic transformation:* the inactivation requires that all of the typical cofactors are present and that metabolism is occurring.
- *Time dependence:* the loss of enzyme activity must exhibit time dependence. Thus, a plot of the logarithm of the enzyme activity remaining versus time should give a straight line.
- *Inactivation rate should be pseudo-first order* and should not exhibit any lag time.
- *Noncompetitive and saturable:* the inactivation exhibits saturation kinetics with respect to the concentration of the inactivator.
- *Substrate protection:* the enzyme should be protected from inactivation by a normal (noninhibitory) substrate and thus shows a slower rate of inactivation.
- *Irreversibility:* the enzyme activity should not return upon dialysis, gel filtration or in the presence of exogenous "scavenger" nucleophiles (glutathione) since the inhibitor should be covalently bound to the apoprotein or the enzyme prosthetic group.
- *Stoichiometry of inactivation:* it should be possible to demonstrate a 1:1 stoichiometry of inactivator to enzyme molecule inactivated.

The fulfillment of these criteria will be demonstrated for the selective cytochrome p450 inactivation (for CYP 2B1/2B6) by series of different derivatives of dithiocarbonic acid (xanthates).

Studies of mechanism-based inactivation reactions of different xenobiotics would help:

- To better determination of different substrate specificity.
- To help QSAR and 3D analysis of enzyme active site.
- In toxicology: to alter the metabolic pro-carcinogens activation and enhanced detoxication process.
- In early drug development: to predict adverse drug reactions and avoid possible future drug-drug interactions.

CO5. ACYLPEPTIDE HYDROLASE AS A BIOMARKER OF EXPOSURE TO ORGANOPHOSPHATES

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Abstract

Organophosphorus (OP) chemicals have been in use for over 50 years and their acute toxicity is relatively well understood. Chronic low-level exposure to OPs has been implicated as a causal factor in a variety of different forms of human ill-health involving the nervous or immune systems. These effects appear to occur at exposure levels too low to be attributable to inhibition of acetylcholinesterase activity. It is well known that the major mode of action of these compounds is by covalent phosphorylation of the active site serine of serine hydrolases. This results in slowly reversible or irreversible inhibition of the target enzyme. The principle

target is acetylcholinesterase, however a whole range of other serine hydrolases are active in the nervous, immune systems and blood and therefore present possible targets. Such possible target is acylpeptide hydrolase (APH). The aim of this presentation is to review data for APH activity after OP exposure as well as some own in vivo and in vitro experiments with OP nerve agents and pesticides.

N-Acylpeptide hydrolase (EC 3.4.19.1) catalyzes the hydrolysis of N-acylated peptide substrates of various sizes and with different types of acyl groups (acetyl, chloroacetyl, formyl, and carbamyl) to generate an acylamino acid and a peptide with a free NH. The enzyme help the post-translational acetylation of intracellular proteins and peptides. It is localized in hepatocytes, brain cells and erythrocytes. It is generally assumed to be an efficient means of protecting these substances from proteolytic degradation in eukaryotic cells, and thus of increasing their half-life. Thus APH and the proteasome act in coordination to clear cytotoxic denatured proteins from cells. APH may be involved in regulation of neuropeptide turnover, which provides a new and plausible mechanism for its proposed cognitive enhancement effect.

APH have been shown to be an essential target for reaction with organophosphates pesticides. Significant inhibition was shown after dichlorvos, naled, and trichlorfon, DFP and only slight inhibition after lethal ip dose of sarin. APH is inhibited by both chlorpyrifos (CPS) and metabolites of tricresyl phosphate (TCP). The purified APH was inhibited by CPO, diazoxon (DZO), paraoxon (PO), PSP, and the classical NTE inhibitor, mipafox.

In in vivo experiments with rats poisoned with high doses of soman and tabun, it was find out that the changes in CHE's activity in blood, liver and brain, were less sensitive than the changes in APH activity. Our data are the first evidence which shown selective, significant and long lasting (up to 80% from the control at day 7th) inhibition of Er-APH activity after tabun poisoning and only slight decreased (15%) after 24h of soman intoxication. APH inhibition was observed also after low non-convulsive tabun doses. In in vitro experiments with human erythrocytes the inhibition potency of different OP pesticides on ACHE and APH activities, were compared

These data show that blood APH activity would be an appropriate marker for OP chronic exposure.

CO6. INCREASED TOXICITY OF NEWLY SYNTHESIZED PEPTIDOMIMETICS BY DIMETHYL SULPHOXIDE AS SOLVENT IN RODENTS

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Intorduction

The effect of solvent on the toxicity of drugs and pharmacological agents is extremely relevant in the medical and pharmaceutical practice [1]. Two newly synthesized peptidomimetics derived from the amino acid L-valine [2] exhibited significant CNS pharmacological activity and low toxicity in rodents (more than 2000 mg/kg b.w.).

Aim

Aim of this study is to compare the effects of some commonly used solvents (water, sunflower oil and Dimethyl sulfoxide (DMSO) on main toxic biochemical and histological parameters of laboratory rodents after 3-days of treatment with effective doses of the newly synthesized compounds.

Methods

The experiments were conducted on mature male Wistar rats. The compounds with codes M6 and P6 (150 mg/kg, intraperitoneally, for 3 days) were dissolved in equal concentration in three different types of solvent - oil solution (sunflower oil), water (gum arabic) and an organic solvent, DMSO. Main biochemical parameters of toxicity in urine and histological samples of liver and kidneys were tested on day 4.

Results

In oil solution and aqueous suspension, the compounds do not cause significant changes in the studied biochemical urine parameters as well as in the hepatic and renal parenchymal histology. In contrast, dissolved in DMSO compounds demonstrated significant hepatic and renal toxicity comparable for both studied compounds, and accompanied by some biochemical changes in the urine. The control group of animals treated only with DMSO had no significant histological and biochemical urinary changes, demonstrating that the negative effect observed is not the result of solvent toxicity.

Conclusion

Increased toxicity of newly synthesized compounds dissolved in DMSO happens under an unknown mechanism. It is probably due to improved solubility and facilitated penetration of M6 and P6 through membranes, as well as to other pharmacokinetic changes produced by DMSO [3]. An important interaction solvent-compound is also possible to have occurred on the level of hepatic drug metabolism. This suggestion is supported by the established significant parenchymal changes in the liver.

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СР1. СИНТЕТИЧНА БИОЛОГИЯ: ПРИЛОЖЕНИЕ В МЕДИЦИНАТА

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Session D.

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DO1. BIOCHEMICAL STUDIES IN RATS WITH EXPERIMENTAL DEFECTS FILLED WITH DIFFERENT HYDROXYAPATITE SUBSTITUTES

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Abstract

Biochemical blood indices (Ca, P, total alkaline phosphatase, bone alkaline phosphatase and osteocalcin) in rats with implanted in tibia hydroxyapatite-gelatine-xanthan gum composite materials were studied. Two types of composites with high temperature treated ceramic powder and additionally high energy treated ceramic powder were applied. The result showed reduction of parameters under study on the 14th d p i and their increased on the 84 d p i. Based on the results obtained, both materials are believed to have the necessary properties for clinical use. Both materials can promote osteoconduction and are biocompatible. The result indicated that both materials behaves like bone-bonding implants but the material with high temperature treated ceramic powder may be have better properties than the additionally high energy treated.

Introduction

Several studies have been performed to develop and improve new biocompatible materials that favor the repair of bone defects in order to provide, restore and maintain bone volume and quality in regions with impaired anatomical structure. Bone substitutes should have characteristics such as biocompatibility, nontoxicity and resistance to deformations. The diagnostic value of cellular and biochemical blood profile in many infectious and non-infectious diseases in different animal species is acknowledged and well documented.

Calcium and inorganic phosphate are closely associated in metabolism. In mammals the major portion of dietary calcium is used for bone formation. Inorganic phosphorous is the second most abundant element in an animal body after calcium, with 80% of inorganic phosphate found in the bones and teeth, with the remainder located in the body fluids and soft tissues. Several types of hydroxyapatite compounds have been tested for osteoconduction, and have yielded similar results to those obtained from autogenous graft [5].

The main objective of the study was to investigate the relationship between blood concentration of some biochemical markers (Ca, P, total alkaline phosphatase, bone alkaline phosphatase and osteocalcin) and an implantation of hydroxyapatite-gelatine-xanthan gum composite materials in rats with experimental bone defects. Two types of composites with high temperature treated ceramic powder and additionally high energy treated ceramic powder were applied.

Materials and methods

Preparation of calcium phosphate ceramic powders

The calcium phosphate powders were prepared by the method of wet precipitation of amorphous precursor, followed by step-wise high temperature treatment. The precipitation was done by a drop-wise method of $(NH_4)_2HPO_4$ (0.3M) and $Ca(NO3)_2$ (0.5M) aqua solutions with pH 12, the phosphate solution was added to calcium one. The precipitate was matured 24 hours and then was subjected to gelling with xanthan gum. Lyophilization, calcination at 300ºC for 3 hours, washing, secondary lyophilization and step-wise (200, 400, 600, 800, and 1000 ºC, each for 3 hours) sintering was next performed (sample W15B). The half of the powder was additionally high energy treated in agate mill for 10h (sample W16B) .

The chemical and phase compositions of the precursor and the ceramic powder were determined chemically (Ca^{2+} - complexometrically with EDTA at pH 10, P-spectrophotometrically by NOVA 60 equipment) and by X-ray diffraction analysis (Bruker D8 advance XRD apparatus).

Preparation of the composite scaffolds

Both powders, the high temperature treated ceramic powder (sample W15B) and the high energy treated ceramic powder (sample W16B), were used for the composite scaffolds preparation. The hydrogels from gelatin (20 mass.%) and xanthan gum (1 mass %) were preliminary prepared and mixed in a volume ratio 1:1. They were homogenized with the powders in ratios 1:0.8 (in the case of sample W15B) and 1:2.1 (in the case of sample W16B). Then, the dense pastes were molded as cylinders (2mm diameter) and lyophilized. The cylinders were hardened by 1% glutaraldehyde.

Animal model.

Male adult rats (Rattus norvegicus albinus, Wistar), weighing on average 300-400 g, randomly were distributed into 3 groups of 8 animals: group 1-controls with control defects which were left empty, group 1 and group 2 with defects filled according to the material inserted into the experimental cavity W16B and W15B. The protocol was approved by the Animal Care and Use Committee of the Institute of Experimental Morphology, Pathology and Anthropology with Museum to the Bulgarian Academy of Sciences. The animals were

submitted to general anesthesia with ketamine chlorhydrate 50 mg.kg-1 (0.05 mL/ 100 g) and xylazine chlorhydrate 5 mg.kg-1 (0.025 mL/100 g), given intramuscularly. After hair removal from the upper region of the head, lidocain chlorhydrate 2% with norepinephrine 1: 50.000 was injected subcutaneously at the site in order to provide hemostasis and additional intraoperative analgesia, in addition to helping with pain management in the immediate postoperative period. Surgical access was obtained by a linear coronal dermoperiosteal incision measuring 1.5 cm in length. A tibia defect was made in the center of each frontal bone with a trephine bar. The periosteum was divulged and pulled apart along with the adjacent soft tissues, exposing the surface of parietal bones. A cavity measuring 4 mm in diameter was made by using a cylindrical multiple blade bur. The experimental materials were inserted into the cavity. The procedure of the operation was according Katthagen and Mittelmeler [3].The defect was completely covered and the skin was closed using 4.0 nylon suture. On day1, 14 and 84 blood samples were taken for biochemical analyses. Blood plasma samples were analyzed for Ca and P, TAP and BAP using biochemical analyzer. Osteocalcin was measured using a commercial ELISA kit (Rat osteocalcin, BGP, ELISA system; GE Healthcare, San Clemente, CA, USA).

Results and discussion

Bi-phase (hydroxyapatite (HA) and tricalcium phosphate (β-TCP)) ceramic powder with a domination of HA and a molar ratio Ca/P = 1.5 was prepared. The high energy treatment leaded to amorphization of β-TCP as it was found also in our previous studies [2].

On the 1st day of the experiment the level of Ca and P was similar in all rats (Fig.1). On the 14th d p i the level of these macroelements was reduced significantly. The reduction of Ca was similar in all rats. The reduction of P was in the biggest extent in the rats with implants W15B. On the 84 d p i the level of Ca and P was elevated significantly compared to those on the 14th d p i. The elevation of Ca was the highest in the controls compared with the groups with implants. The elevation was higher in the rats with implants from gr.1. The elevation of P was higher in rats from gr. 1compared to the controls and gr. 2. The increase of P was the smallest extent in the rats with implants W15B (Fig.1).

Fig.1. Ca and P concentration in blood in rats with bone defects filled with hydroxyapatite

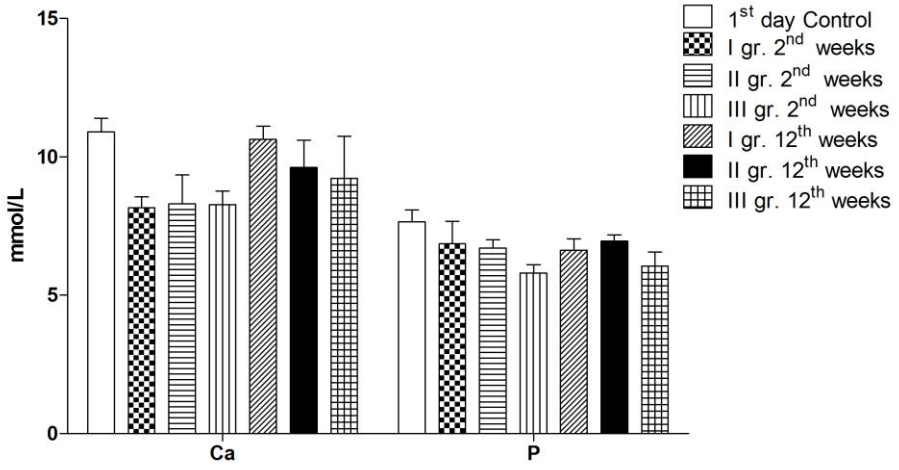
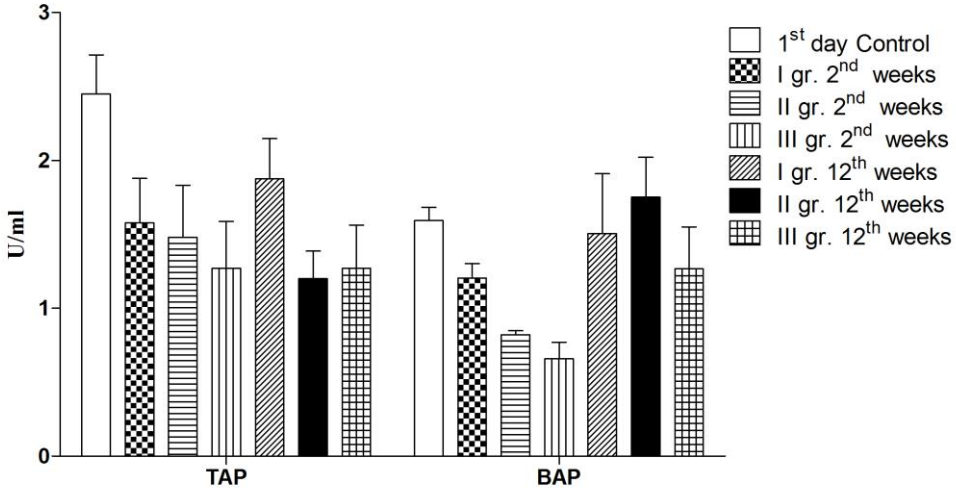


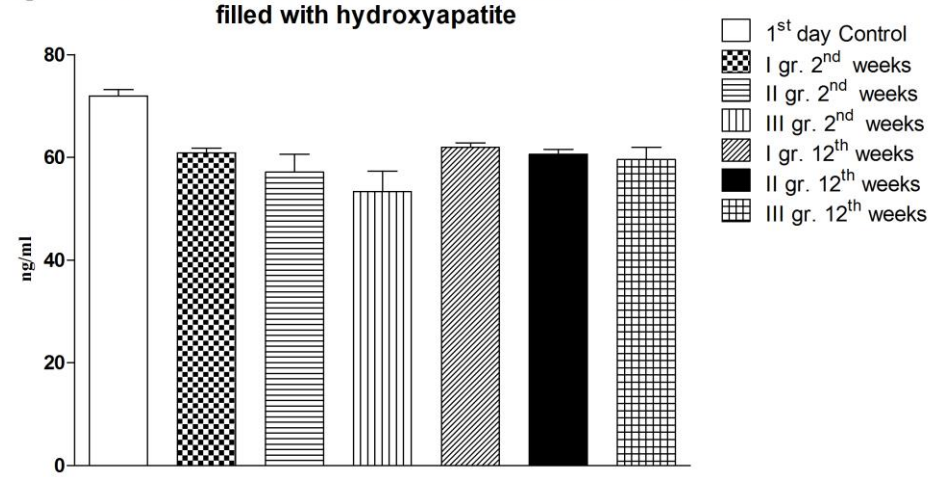
Fig.2. Activity of phosphatases in blood in rats with bone defects filled with hydroxyapatite



On the 1st d p i the activity of the phosphatases was similar (Fig.2). On the 14th d p i their activity was reduced in all rats compared to that at the beginning of the experiment. The reduction of TAF activity was in the biggest extent in the rats from gr. 3. The TAF was the highest in the rats of control group (Fig.2). The BAP activity was the highest in the controls than that in the rest rats with implants. On the 84 d p I there was elevation in the activity of both phosphatases in all rats compared to those from the 14 d p i. TAP activity was the highest in the controls compared to that in the rest groups. BAP activity was the highest in the rats with implant W16B (Fig. 2).

The level of osteocalcin was similar in all rats on the 1st d p I (Fig. 3). On the 14 d p I there was a significant reduction in it in all groups. It was in the smallest extent in the rats from gr. 3. On the 84 d p i the osteocalcin level was increased in equal extent in all rats.

Fig.3. Levels of osteocalcin in blood in rats with bone defects filled with hydroxyapatite



Biochemical bone markers are widely used in human medical practice for control of

therapeutic schedules and monitoring of cell activity in metabolic bone disorders and related diseases [4]. In animals bone markers were used very rear to determine the differences in bone formation and resorption levels in horses, dogs, rats and cats [1]. Osteocalcin is a bone formation marker. Decline in osteocalcin level resulted in low bone formation. Alkaline phosphatase activity was used as an index of osteoblastic activity. BAP activity is consider to be a more sensitive marker of bone formation. These markers are responsible for the routine turnover of bone matrix and maintenance of the bone. As there was significant increase in BAP activity in gr. 2, this may suggest that the rate of bone repair can be increased beyond control levels. So that there was an advantage of implant material W15 leading to its efficiency of filling the bone defect through incorporation of the material into the healing site. The result indicated that hydroxyapatite behaves like bone-bonding implants but the material W16B may be have better properties. Based on the results obtained and on the literature reports, both materials are believed to have the necessary properties for clinical use. Both materials can promote osteoconduction and are biocompatible.

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DO2. MURINE BONE MAROOW CELLS AND CULTURES FROM BONE EXPLANTS IN THE EVALUATION OF BIOCOMPATIBILITY OF CALCIUM PHOSPHATE MATERIALS FOR BONE IMPLANTS

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Abstract

The aim of this study was to evaluate the effect of calcium phosphates materials on viability and proliferation of murine cells obtained from cultured bone explants (BECs) and marrow (BMCs) cells. BECs and BMCs were obtained from 2-month-old ICR mice as it was previously described [1]. All experiments with laboratory animals were performed in accordance to the Veterinary Medical Office in Bulgaria which follows the European Committee Standards concerning the care and use of laboratory animals.

The influence of the compounds tested on cell viability and proliferation was examined after 72 h and 144 h treatment using thiazolyl blue tetrazolium bromide (MTT) test and trypan blue dye exclusion technique (TB).

Acknowledgement: This study was supported by Grant DTK-02-70/2009, National Science Fund, Bulgaria, and by the European Social Fund and Republic of Bulgaria, Operational Programme "Human Resources Development" 2007-2013 framework, Grant № BG051PO001-3.3.06-0048 from 04.10.2012.

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DO3. DEVELOPMENT OF NOVEL *IN VITRO*-CULTIVATION TECHNIQUES FOR DERIVATION OF CORNEAL EPITHELIAL EXPLANTS

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Abstract

Novel techniques for *in vitro*-cultivation and incubation of limbal and oral epithelial stem cells were developed. Isolated in both cases tissue explants were cultivated in growth medium, supplemented with 10% Fetal Calf Serum (FCS), antibiotic mixture (100 IU/ml Penicillin, 100 μg/ml Streptomycin and 25 μg/ml Amphotherycin B), L-Gluthamine (Sigma-Aldrich), 10 ng/ml Epidermal Growth Factor (EGF - Sigma-Aldrich), 5 μg/ml insulin, 0.4 μg/ml hydrocortisone, 24 μg/ml adenine, as well as previously conditioned of feeder cells (3T3 mouse fibroblasts) cultural fluid, in 5% CO_2/95% humidity incubator at 37ºC, and observed on every 24 hours by inverted microscope with CCD camera. All cells were characterized on the basis of their morphological characteristics: shape, appearance, presence of mitotic figures, as well as confluence and adherence of substrate. The isolated from human cadaver limbus tissue explants were cultivated in Petri-dishes, containing glass cover-slips, over-laid with stretched vitelline membranes from fresh hen's eggs. During the period of *in vitro*-cultivation, formation of non-adherent cell sheets, as well as of adherent to the membrane cell layers, composed by differentiated corneal epithelial cells, were noticed. Chemically-modified by treatment with gelatine-glutharaldehyde vitelline membrane indicated characteristics as a suitable substrate, able to maintain the growth of epithelial stem cells with limbal origin, which proved the abilities about its application in construction of implants from "cell-membrane" type for the needs of reparative ophthalmology. In contrast, the non-modified vitelline membrane didn't show these properties. Epithelial stem cells and tissue explants from human oral mucosa were *in vitro*-cultivated and incubated in similar laboratory conditions, because they were also proved to induce expression of limbal epithelial stem cell markers [1-4]. Techniques for substrate adhesion of the isolated tissue explants from human oral mucosa on glass and plastic lamella, previously treated with poly-L-Lysine, with gelatine and with FCS, respectively, were tested. Formation of both adherent and non-adherent cell sheets, consisting of cells with different morphology and maturation degree, was observed. On the other hand, in application of cold trypsinization laboratory technique, primary cultures of human oral mucosa epithelial stem cells were derived, which showed signs of early cell differentiation. For this goal, the isolated tissue explants were treated with trypsine-EDTA at 4ºC for 8 hours. Future experiments in this direction are necessary, which should be connected mainly with proof of limbal stem cell markers in the so cultivated cells and tissue explants, as well as *in vivo* test with experimental animals for final confirmation of the eventual therapeutic success.

Keywords: epithelial stem cells; vitelline membrane; tissue explants; in vitro-cultivation.

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DO4. ПРОУЧВАНЕ ВЪРХУ ВЛИЯНИЕТО НА КРИОКОНСЕРВАЦИЯТА, ПО ОТНОШЕНИЕ ПОДВИЖНОСТТА И ДНК ФРАГМЕНТАЦИОННИЯ ИНДЕКС НА СПЕРМАТОЗОИДИТЕ, ПРИ СВЕЖА И ОБРАБОТЕНА СЕМЕННА ПРОБА

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Резюме

Инфертилитетът е основен клиничен проблем сред двойките, като най-разпространенният фактор за това е нарушение в спермалните показатели. Качеството на сперматозоида определя оплождането и нормалното развитие на ембриона. Задълбоченото познание на спермалните клетки, техния генезиз, хроматин и други параметри, е от основно значение за успешната репродукция – есетствена или асистирана.

Криоконсервацията е широко използван метод за запазване на фертилността при мъжете в случаи на лъче- и химиотерапия, които се налагат при лечението на повечето онкологични заболявания и съответно могат да доведат до трайно увреждане на репродуктивните функции.

Целта на проучването е да се установи какъв ефект оказва криосъхраняването върху прогресивната подвижност и ДНК фрагментационният индекс на сперматозоидите, като се сравнят два различни метода на обработване на семенната проба при криоконсервация – замразяване на необработен еякулат и замразяване на сперматозоиди след изолирането им през двоен плътностен градиент. Бяха анализирани данните получени от спермалния анализ и стойностите на ДНК фрагментационния индекс на 30 мъже, след размразяване, както и след един час инкубиране на стайна температура, като се сравняват двата метода на обработване на семенната проба. Спермалният анализ – концетрация и подвижност на сперматозоидите, бе извършен съгласно препоръките на последната пета редакция на Ръководството на СЗО за извършване на спермален анализ, 2010 г.

ДНК фрагментационният индекс беше определен флоуцитометрично, използвайки метахроматичните свойства на акридин оранж.

Заключенията, до които стигнахме в проучването, са, че обработването на семенният материал чрез двоен плътностен градиент преди замразяване селектира сперматозоиди с добра прогресивна подвижност и с ниски стойности на ДНК фрагментационния индекс. Криоконсервацията не влошава ДНК фрагментационният индекс на замразените сперматозоиди, но култивирането на размразената проба за един час на стайна температура влошава стойностите на този показател. Сперматозоиди, с

високи стойности на прогресивна подвижност (a+b%), се свързват с ниски нива на ДНК фрагментация.

DO5. ASSESSMENT OF FROZEN-THAWED HUMAN SPERM USING AN ANNEXIN V BINDING AND DNA INTEGRITY TEST BY FLOW CYTOMETRY

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Abstract

Apoptosis is physiological process during mature tissue to remove unwanted cells. It is called programmed cell death or cell suicide. In vital cells the phosphatydylserin (PS) are located on inner leaflet of cell membrane; in early phase of apoptosis PS residues are presented on the outer leaflet of the membrane. PS can be detected by fluorochrome-labeled Annexin V, the anticoagulant that reacts with high affinity with of this phospholipid. Annexin V can be used to identify both apoptotic and necrotic cells. To distinguish these two groups the DNA binding propidium iodide (PI) that stains permeable cells should be used. Thus Annexin V+/PI- cells can be considered apoptotic ones, while these binding both Annexin V and PI can be considered dead ones.

49 patients with infertility were studied. All of the procedures were approved by Ethic Committee of Medical University – Pleven. Semen was obtained by masturbation and samples were frozen. Semen analysis was performed according to WHO standards 2010. Annexin V/FITS binding was performed with Annexin V Apoptosis Detection kit (eBioscience). The FACscan (Becton Dickinson, USA) flow cytometer was used for analyzed samples. The DNA integrity test was performed by flow cytometer.

The results obtained revealed that the mean percentage of Annexin V+/PI- cells was 9.41% (SD=6.35). The percentage of Annexin V+/PI+ was 37.87% (SD=16.3). We detected a positive statistically significant correlation between the percentage of Annexin V+/PI- and poor morphology group (t=1.91, p=0.03). The percentage of Annexin V+/PI- positively and significantly correlated with the poor motile sperm group A+B (t=1.99, p=0.02) and sperm group with DNA damage (DFI>25%) – p=0.03.

The study show that normal sperm morphology correlates positively with the sperm cell’s functional competence and Annexin V binding is a good marker of sperm quality in andrological diagnosis. The correlation with DNA fragmentation index could predict the onset of pregnancy by using frozen sperm.

Introduction

Conventional semen analysis includes assessment of sperm concentration, motility and percentage of morphologically normal forms. Normal sperm morphology correlates with

fertility potential. The results of semen analysis are predictive of fertility potential as these parameters show high biological variability.

Sperm DNA damage and sperm apoptosis have been considered as potentially useful indicators of male fertility Apoptosis is a physiological process during in mature tissue to remove unwanted cells. It is called programmed cell death or cell suicide.

A cascade of events characterized programmed cell death – condensation and fragmentation of sperm chromatin, compaction of cytoplasmic membrane organelles, reduced mitochondrial transmembrane potential, production of reactive oxygen species.

Apoptosis is being characterized by changes of the plasma membrane. An early sign of apoptosis is the externalization of phosphatydylserin (PS). In vital cells the phosphatydylserin residues are located on inner leaflet of cell membrane; in early phase of apoptosis PS residues are presented on the outer leaflet of the membrane. PS can be detected by fluorochrome-labeled Annexin V, the anticoagulant that reacts with high affinity with of this phospholipid. Annexin V can be used to identify both apoptotic and necrotic cells. To distinguish these two groups the DNA binding propidium iodide (PI) that stains permeable cells should be used. Thus Annexin V+/PI- cells can be considered apoptotic ones, while these binding both Annexin V and PI can be considered dead ones.

Apoptosis has been associated with male infertility [1–4]. Relatively high rates of apoptosis have been reported in testicular biopsies from infertile men with different degrees of testicular insufficiency [5, 6]. It has been reported that the proportions of apoptotic sperm are higher in ejaculated semen samples from infertile men compared with fertile men [4]. Several studies have explored the relationship between the parameters of a conventional semen analysis and apoptosis in ejaculated semen. These studies concur that there is a significant negative correlation between the proportion of apoptotic cells and sperm viability and motility in the ejaculate [4,7–10]. In contrast, there are only a few studies on the relationship between sperm morphology, applying Tygerberg's strict criteria [1], and the proportion of apoptotic sperm [11, 12].

Material and methods

Semen samples from 49 patients with infertility were studied. The mean age is 35.3 (SD=6.4) All of the procedures were approved by Ethic Committee of Medical University – Pleven. Semen was obtained by masturbation and samples were frozen. Semen analysis (including concentration, motility and evaluation of sperm morphology) was performed according to WHO standards 2010 and the Tygerberg strict criteria.

Annexin V staning: Annexin V/FITS binding was performed with Annexin V Apoptosis Detection kit (eBioscience). Harvested cells were washed once in 1X PBS, then once in 1X Binding Buffer. Resuspended cells in 1X Binding Buffer at 1-5x106/mL and added 5 µL of fluorochrome-conjugated Annexin V to 100 µL of the cell suspension. Cells were incubate 10-15 minutes at room temperature and then were washed in 1X Binding Buffer and resuspended in 200 µL of 1X Binding Buffer. 5 µL of Propidium Iodide Staining Solutionwere added and analyzed by flow cytometry within 4 hours, storing at 2-8 C^0 in the dark. The samples analysis was performed on FACscan (Becton Dickinson, USA).

The DNA integrity test: Sperm DNA damage was determined by acridine orange staining method on flow cytometry On the day of analysis, the samples were thawed and the test was performed as soon as possible. All buffers and samples need to be kept at 4 to 6 C^0 and the test to be run in box with ice. After wash with TNE (0.15M NaCL, 0.01M TrisHCL, 1mM EDTA, pH 7.4) buffer, the sperm concentration was adjusted to approximately 1-2x10^6 cells/ml and 200 µL was placed in 12x75 flow cytometer tubes. 400 µl of low-pH (pH 1.2) detergent solution containing 0.1% Triton X-100, 0.15M NaCl and 0.08M HCl was added for 30 sec. immediately followed by 1.2 ml staining buffer (phosphate-citrate buffer, pH 6.0) with

6 mg/l acridine orange (AO) (chromatographically purified; cat. No. 318337-1G, Sigma-Aldrich, USA). Cells were acquired on FACScan flow cytometer (Becton Dickinson). A total of 5000 events were accumulated for each measurement at a low flow rate. Under these experimental conditions, when excited with a 488 nm light source, AO intercalated with double-stranded DNA emits green fluorescence and AO associated with single-stranded DNA emits red fluorescence. Thus, sperm chromatin damage can be quantified by the flow cytometric measurements of the metachromatic shift from green (native, double-stranded DNA) to red (denatured, single-stranded DNA) fluorescence and displayed as red (fragmented DNA) versus green (DNA stainability) fluorescence intensity cytogram patterns. Data for each acquired specimen was saved and analyzed by FlowJo software (TreeStar, Inc.,Ashland,OR,). Computer gates are used to determine the proportion of spermatozoa with increased levels of red fluorescence (fragmented single stranded DNA) and green fluorescence (native double stranded DNA). The results were presented as a percentage of sperm DNA fragmentation index (DFI).

Statistical analysis: Statistical analysis was performed using Statgraphics plus 2.1 software for Windows. The data were expressed as mean ± standard deviation (mean ±SD). The relationship between percentage of Annexin V+/PI- cells or Annexin V+/PI+ cells and such parameters as concentration, motility, morphology of spermatozoa and DNA integrity test of the same person. Statistical significance was set at $p \leq 0.05$.

Results

The results from apoptosis detection with Annexin V/FITS test showed mean percentage of Annexin V+/PI- cells 9.41% (SD=6.35) and the percentage of Annexin V+/PI+ 37.87% (SD=16.3). The same group of samples, analyzed for DNA damage showed mean of DFI 17.96% (SD=8.1%).

According to the semen analysis results, the samples were subdivided into the following group – good morphology group samples (percentage of normal forms ≥4%) and poor morphology group (percentage of normal forms ≤ 4%). On the other hand the samples were grouped according with their motility into sperm samples with normal motility (a+b≥32%) and sperm samples with poor motility (a+b≤ 32%). The same subdivided has been made in regard to DNA integrity test as a cut off value of 25% DFI has been used.

We detected a positive statistically significant correlation between the percentage of Annexin V+/PI- and poor morphology group ($t=1.91$, $p=0.03$). The percentage of Annexin V+/PI- positively and significantly correlated with the poor motility sperm group ($t=1.99$, $p=0.02$) and the group with increased DNA damage (DFI>25%) – $p=0.03$.

Discussion

AnnexinV binding by particles of phosphatydylserin (PS) translocated from the inner to outer leaflet of cell membrane, is one of the methods important in detection of apoptosis process. The simultaneous labeling by PI allows identifying both apoptotic and necrotic cells [14] The labeled cells can be detected by flow cytometry technique that offers the possibility of rapid and accurate measurement of a multitude of cells attributes in large cell population [15]. In this study we analyzed Annexin V and PI binding with use of flow cytometry method to broaden the knowledge of programmed cell death in human spermatozoa. DNA damage in spermatozoa is a common phenomenon. Membrane changes detected by the Annexin V assay precede the double strand breaks of DNA determined by DNA integrity test. Therefore, the fraction of the spermatozoa, which is positive in the Annexin V assay, represents those spermatozoa that are in the beginning of the apoptotic process. There is a correlation with the semen quality determined by conventional parameters, but the correlation is not as strong as with the outcomes of the DNA integrity test.

In an attempt to elucidate the possible correlation between the normal sperm morphology as assessed with strict criteria [13], and sperm physiological, chromosomal, and genetic properties, in relation to fertilization, embryo quality, and pregnancy, numerous functional parameters as zona binding tests [16], acrosome reaction [17], sperm nuclear DNA normality [18, 19], sperm chromosome complement [21], and sperm nucleus DNA fragmentation. All of these studies have shown that normal sperm morphology correlates positively with the sperm cell's functional competence. In addition, the present study confirms that normal sperm morphology correlates significantly negative with the externalization of PS (P<.0001). Some studies attribute this to the process of apoptosis, as the externalization of PS is an early marker of the programmed cell death and assume that annexin V binding is a good marker of semen quality in andrological diagnosis [14, 22]. On the other hand, Henkel et al. [23] showed that the early parameters of apoptosis annexin V binding and Fas expression are not predictive for fertilization or pregnancy. Only the late parameter of apoptosis, DNA fragmentation, could significantly predict the onset of pregnancy by using ejaculated sperm.

Conclusion

The study shows that normal sperm morphology correlates positively with the sperm cell's functional competence and Annexin V binding is a good marker of sperm quality in andrological diagnosis. The correlation with DNA fragmentation index could predict the onset of pregnancy by using frozen sperm.

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DP1. СТЕРИЛИТЕТЪТ ПРИ МЪЖЕТЕ – ПОГЛЕД ОТ ВСИЧКИ СТРАНИ

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DP2. IN VITRO MATURATION OF HUMAN OOCYTES

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Abstract

In vitro maturation (IVM) of human oocytes could be used to overcome some forms of infertility. IVM is a reproductive technology which helps to avoid the risks associated with hormonal stimulation. After immature oocytes retrieval these cells are matured under controlled laboratory conditions and extrude the first polar body in vitro without gonadotrophins.

The main target group for IVM are women with polycystic ovary syndrome who are at high risk of ovarian hyperstimulation syndrome (OHSS). Other candidates are women who are poor responders to stimulation.

Compared with conventional in vitro fertilization the main advantages of IVM include avoiding potential risks of OHSS, lower cost and simpler treatments. Regrettably the clinical results of IVM treatment are far from satisfactory and the rates of implantation and clinical pregnancy remain disappointingly low.

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DP3. МЕТОДИ ЗА ОПЛОЖДАНЕ IN VITRO

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Биологически факултет, СУ „Св. Климент Охридски”, бул. „Драган Цанков” 8, София, България

Session E.

Chairpersons:

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

Assoc. Prof. Ivayla Pantcheva-Kadreva, PhD
Faculty of Chemistry and Pharmacy, Sofia University "St. Kliment Ohridski"

Secretary: Assoc. Prof. Elena Gardeva, DVM, PhD
Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences

EO1. SYNCHROTRON RADIATION CIRCULAR DICHROISM AND ITS APPLICATION FOR BIOMEDICAL PURPOSES

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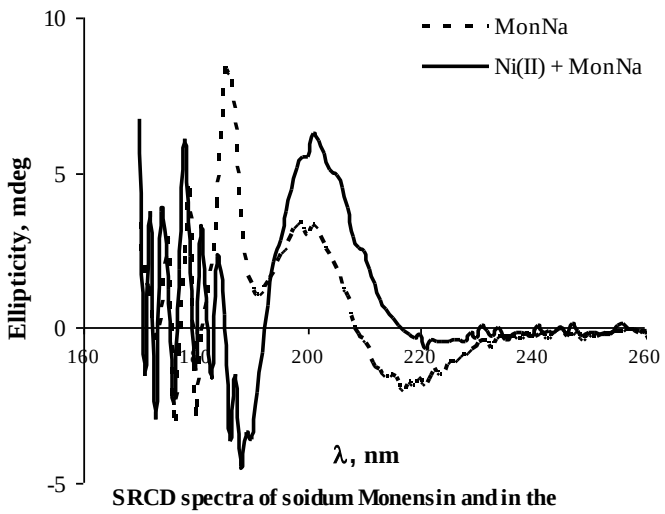
Abstract

Conventional circular dichroism (cCD) spectroscopy is a popular technique studying secondary structure of chiral molecules (far-UV CD), tertiary structure of proteins (near-UV CD) or metal-containing compounds (visible CD).

In recent years it became possible to use synchrotrons as light sources for CD, with the technique being known as Synchrotron Radiation Circular Dichroism (SRCD).

Generally, CD and SRCD spectra are very similar over the wavelength region from 350 to 175 nm, except at the lower wavelength extreme due to the limitations of the light source intensity in the conventional instrument. This additional low wavelength vacuum ultraviolet (VUV) data contains a large amount of extra information, including a number of peaks consistent with previously predicted charge transfer transitions [1].

Recently we applied SRCD spectroscopy to evaluate the complexation ability of ionophorous antibiotic Monensin towards some metal ions in methanolic solutions. Data revealed that absorption of the ligand is highly dependent on the metal ion coordinated and the



SRCD spectra of soidum Monensin and in the presence of Ni(II) ions

conformational changes occurring in solution differ (in some cases significantly) from the crystallo-graphic data obtained in solid state.

Acknowledgement: IP is grateful to the project ISA-12-116 "Application of SRCD in the study of the interaction between metal ions and biologically active molecules - peptides, proteins and drug candidates".

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EO2. COMPLEXATION OF OBIDOXIME WITH RESPECT TO ITS REACTIVATION ABILITY

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Abstract

Acetylcholinesterase (AChE) is an enzyme that hydrolyses acetylcholine in the synaptic cleft and terminates its action. When enzyme does not function normally, the neuro-muscular transmittance is interrupted leading to muscle contractions, paralysis and death. Among the substances that inhibit AChE are organophosphorus compounds (OPC as nerve gases, insecticides) which bind specifically to the enzyme and block its function.

Oxime-containing compounds are family well known to restore inhibited by OPC acetylcholinesterase due to their greater affinity for the organic phosphate residue than the enzyme. The main disadvantages of such a therapy are due to the absence of "universal" reactivators towards various inhibitors, as well as to their fast elimination from the organism.

Here reported are the results obtained on complexation ability of Obidoxime (quaternary pyridinium aldoxime-containing compound) to bind Pd(II) ions in different media (water or buffer with pH 6.3 / 7.4). The coordination was followed spectrophotometrically to find that the presence of Pd(II) affords formation of oximate ions probably placed in different environment than simply deprotonated oxime molecules by inorganic base. Although it was not possible to isolate new Pd(II)-containing species up to now, the conversion of oxime in reaction mixture was calculated by software developed by Antonov et al. [1, 2]. The modified oximate (*c.a.* 80%) accompanied by initial oxime (*c.a.* 20%) was tested for its *in vitro* ability to reactivate rat brain AChE inhibited by paraoxon.

Acknowledgement: This research is supported by the Bulgarian National Science Fund (DDVU-02-78).

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EO3. ANTITUMOR ACTIVITY OF *ARTHRONEMA AFRICANUM* C-PHYCOCYANIN IN *IN VIVO* EXPERIMENTAL MODEL

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Abstract

The aim of the present study was to evaluate the antitumor efficiency of a highly purified C- phycocyanin (C-PC) from *Arthronema africanum* by using Graffi myeloid tumor in hamsters as a model system. Transplantability, mortality, mean survival time and inhibition of tumor growth were determined as markers for *in vivo* antitumor activity. The results showed that C-PC significantly decreased the transplantability and the growth of tumors, as well as had pronounced protective effect on the hamsters with experimental Graffi tumor. These results indicate the antitumor activity of *Arthronema africanum* C- PC and its potential as a natural antitumor agent.

Introduction

C-phycocyanins, a light-harvesting pigment synthesized by cyanoprokaryotes and two eukaryote algal genera (Rhodophyta and Cryptophyta), are of great importance because of their various biological activities and pharmacological properties. C-phycocyanin is a water soluble, non-toxic biliprotein which exert antibacterial [8], antifungal, antiviral [6] and anti-cancer [12, 17] activity, anti-oxidant [4, 11], anti-inflammatory, fibrinolytic [3] and free radical scavenging properties [1]. Since phycocyanin from *Arthronema africanum* has not been investigated for such activities yet, we studied the effects of the cyanobacterial biliprotein on Graffi tumor-bearing hamsters.

Materials and Methods

Source of C-phycocyanin

Arthronema africanum strain Lukavsky 1980/1 was used. The cyanobacterium was grown photoautotrophically for 7 days in mineral nutrient medium, at 32°C and continuous lateral illumination with cool-white fluorescent lamps at a photon flux density of 250 μE m^2/s. A carbon source was provided by bubbling 1% (v⁄v) CO_2 in 10 l air/h through the cultures.

Isolation and purification of C-phycocyanin

Pure C-phycocyanin from *A. africanum* was obtained by the method of Minkova et al. [5]. The equations of Siegelman and Kycia [13] were used to estimate the phycobiliprotein content.

Experimental animals

Golden Syrian hamsters, 2–4 months old, weighing approximately 100 g were purchased from a breeding base of the Oncology Center, Sofia. The animals were kept under standard conditions in individual plastic cages with free access to food and water. All studies were performed in accordance with the Guide for Care and Use of Laboratory Animals, as proposed by the Committee on Care Laboratory Animal Resources, Commission on Life Sciences and National Research Council, and a work permit No. 11130006.

Experimental *Graffi* myeloid tumor in hamsters

An experimental Graffi myeloid tumor was created and maintained monthly *in vivo* by subcutaneous transplantation of live tumor cells (2×10^6/ml PBS) in the interscapular area of hamsters [15]. Between the 10^{th} and the 15^{th} day after tumor transplantation on the back of the hamster a solid subcutaneous tumor appeared, which progressively increased in size and after about 30 days caused death of experimental animals. Spontaneous regression in this experimental tumor model was not observed.

Phycocyanin treatments

A stock solution of 1.0 mg/ml C-PC was freshly prepared in PBS for each experiment. Experimental animals were divided into three experimental groups of eight hamsters and treated as follow: Gr.1 - hamsters treated i.p. with 5.0 mg/kg C-PC, 2 h before transplantation of 5.10^4 viable tumor cells; Gr.2 - hamsters treated twice i.p. with 5.0 mg/kg C-PC, 2 h before and 24 h after transplantation of 5.10^4 viable tumor cells; Gr.3 – hamsters transplanted with 5.10^4 viable tumor cells and treated with the diluent (PBS) alone (control group).

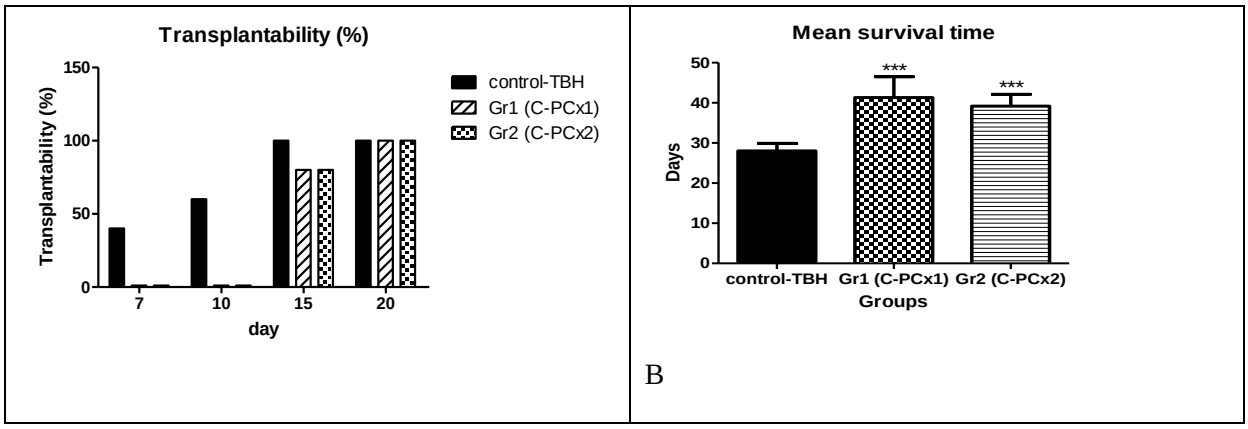
Transplantability (T%) and Mortality (M%), Mean survival time (MST) and Inhibition of tumor growth (ITG) were determined as markers for *in vivo* antitumor activity.

Statistical analysis

All the data were expressed as mean ± standard deviation (SD). The statistical significance between the treatments was evaluated by one-way ANOVA and with Bonferroni's post hoc test using GraphPAD InStat, Software, USA. Values of *** $P < 0.001$ were considered significant.

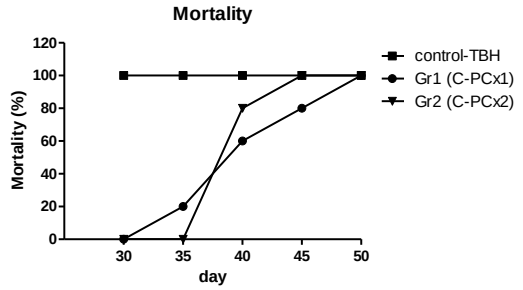
Results and Discussion

The C-PC obtained from *A. africanum* by our improved, simple, non-chromatographic, rivanol-sulfate procedure showed high purity (A_{620}/A_{280} of 4.3) and overall recovery (55%). The effect of C-PC on the transplantability (T) of Graffi tumor in hamsters was monitored at 7, 10, 15 and 20^{th} day after tumor transplantation. It wasn't observed any appearance of



B

A



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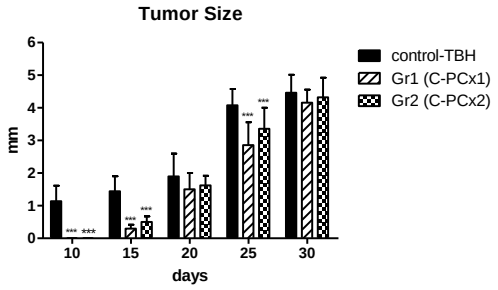

D

Fig. 1. In vivo effects of C-PC on Graffi myeloid tumor in hamsters: A-Transplantability (%); B-Mean survival time; C-Mortality (%); D-Tumor size.

tumors (transplantability 0%) at the 7th and 10th days of the study in both groups treated with C-PC, while in the control group, 40 and 60% of the hamsters, respectively developed tumors (Fig. 1A). At day 15th of the observation, the transplantability in the untreated hamsters reached 100% and tumors appeared in 80% of the hamsters in C-PC- treated groups. On the 20th day, the transplantability was 100% in all (treated and untreated) hamsters (Fig. 1A). It was established a significant elongation of the mean survival time (MST) in both experimental groups treated with C-PC, compared with untreated controls (TBH). Mean survival time in the untreated TBH was 28.2 ± 1.9 days, while in the C-PC-treated groups 1 and 2 it reached 41.33 ± 5.2 and 39.17 ± 2.9 days, respectively (Fig. 1B). As can be seen on the chart presented in Figure 1C, C-PC-treatment significantly decreased the mortality of TBH at all stages of the study. None of the hamsters died in the groups 1 and 2 treated with C-PC, till the 30th and 35th day of the study, respectively. The lowest rate of mortality was observed in the group 1, treated with a single dose of C-PC, and 20% of the hamsters survived more than 45 days (Fig. 1C). C-phycocyanin of *A. africanum* was also able to inhibit tumor growth. The average size of tumors in hamsters treated once or twice with C-PC was significantly lower than in untreated TBH at days 15, 20 and 25th (on the day 10th, the treatments caused 100% inhibition of tumor growth) (Fig. 1D). The most significant inhibition of tumor size was observed in group 1 - hamsters treated with a single dose of C-PC (0.3 ± 0.12; 1.5 ± 0.5 and 2.86 ± 0.7 mm at 15, 20 and 25 days, respectively, controls being 1.44 ± 0.46; 1.9 ± 0.7 and 4.08 ± 0.5 mm, respectively) (Fig. 1D).

The antitumor potential of C-PC from unexplored cyanobacterial source - *A. africanum*, was evaluated in this study in order to support the efforts for fulfilling the tremendous need of new natural compounds, effective against various kind of tumors, as an alternative to chemotherapeutic drugs that often showed side effects. The antitumor effects of largely studied C-PC isolated from *Spirulina* are well known. *Spirulina platensis* C-PC was demonstrated to be a selective inhibitor of cyclo-oxygenase-2, that is upregulated during inflammation and cancer [10], and inducer of apoptosis in chronic myeloid leukaemia cells [14] and rat histiocytoma cell line AK5 [9]. C-phycocyanin of *S. platensis* was also shown to inhibit the growth of human hepatocellular carcinoma cell line SMMC-7721 [2], and three human leukemia cell lines (HL-60, K562 and U937) [16]. The safety of the phycobiliprotein

was found to be at high rates in rats and mice, with no adverse effects on the healthy experimental animals [7].

The present study describes for the first time the antitumor activity of C-PC produced in high amount by *A. africanum.* This natural biliprotein pigment was able to decrease significantly the transplantability of Graffi tumors, tumor growth and mortality of TBH, while the mean survival time of the TBH was substantially prolonged.

In conclusion, our investigations demonstrated that the C-phycocyanin from *Arthronema africanum* has antitumor activity *in vivo* and is a promising natural antitumor agent in experimental conditions. Further investigations are needed to understand the molecular mechanisms of its action.

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EO4. EFFECT OF ISOXICAM AND ITS COPPER COMPLEX ON VIABILITY OF CULTURED HUMAN BREAST CANCER CELLS

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EO5. BIOLOGICAL ACTIVITY OF RGD PEPTIDES

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Abstract

The RGD (L-argininyl-glycyl-L-aspatric acid) sequence (Fig. 1) is present in many extracellular matrix proteins.

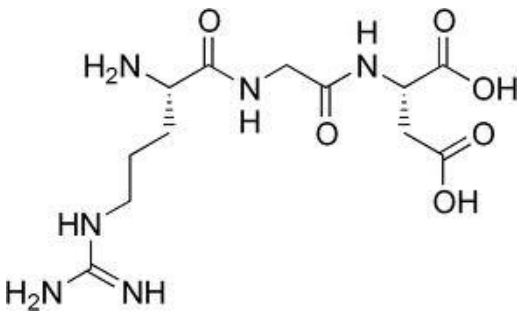

Figure 1. Chemical structure of the original RGD sequence

It was discovered in fibronectin in 1984 [14]. The finding that only three amino acids would form an essential recognition site for cells in a very large protein was surprising. This observation was, however, soon confirmed and it was proven that the RGD sequence is the cell attachment site of many other adhesive proteins. A partial list of adhesive proteins with RGD sites includes fibronectin, vitronectin, fibrinogen, von Willebrand factor, thrombospondin, laminin, entactin, tenascin, osteopontin, bone sialoprotein, and, under some conditions, collagens [16]. These findings and the subsequent discovery of integrins, cell surface receptors that recognize the RGD sequence of various proteins, have given RGD a central role in the cell adhesion biology.

Integrins are heterodimeric cell surface receptors that mediate cell–cell and cell–extracellular matrix (ECM) interactions. The growth of many cells is dependent on their anchorage to the defined substratum. Functions of integrins have been shown to be involved in several important cellular processes, including cell differentiation, angiogenesis, apoptosis, cell migration, and tumor growth [10, 11]. The integrin αvβ3 plays an important role in angiogenesis. It is expressed on tumoral endothelial cells as well as on some tumor cells. [6]. RGD peptides are well-known to bind preferentially to αvβ3 integrin. RGD-binding causes conformational changes of αvβ3 (Fig.2) [6]. Although integrin cytoplasmic tails are much smaller than their extracellular domains, they play roles in integrin signaling events. In this context, targeting tumor cells or tumor vasculature by RGD-based strategies is a promising approach for cancer therapy and diagnosis.

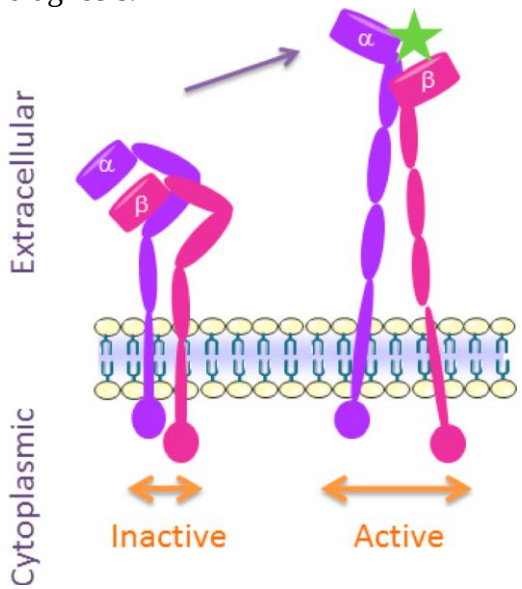

Figure 2. Conformational changes in αvβ3 integrin upon binding with RGD (star). After binding the extracellular domains extend and straighten. The figure is from ref. 6.

It appeared that small synthetic peptides (a few hundred daltons) that contain the amino acid sequence RGD could mediate cell attachment similarly its considerably larger parental molecule (a hundred thousand daltons). These peptides have been used widely in different studies as inhibitors of integrin–ligand interactions, thus affecting cell adhesion, migration, tumor growth and metastasis [21]. Short peptides containing the RGD sequence can mimic cell adhesion proteins in two ways: When coated onto a surface, they promote cell adhesion, whereas in solution they prevent adhesion [16].

Many different RGD peptides have been developed. Linear and cyclic RGD peptides, and chemically designed peptidomimetics are currently being tested by researchers.

RGD peptides are commonly known as antimetastatic agents. RGD and their analogues are able to decrease the number of spontaneous and experimental metastases in *in vivo* models [17, 18, 20]. Moreover, descriptions exist of some of their *in vitro* activities, like the inhibition of tumor cell adhesion to extracellular matrix proteins and the inhibition of migration in a three dimensional gel [9, 13, 22]. The anti-angiogenic activity of RGD peptides was also postulated [6, 19].

Currently, understanding of apoptosis mechanisms has resulted in new strategies for treating certain illnesses, including cancer. Several clinical testing are undergoing. Apoptosis is typically accompanied by the activation of a class of death proteases called caspases. The apoptotic pathway is consisted of several triggers, modulators and effectors. Loss of adhesion of integrins to their ligands is one such trigger**.** Recently, RGD peptides have also been found to induce apoptosis in cells such as MDCK cells [7], glomerular mesangial cells [5] and lymphocytes [3].

There are experimental data showing that RGD-containing peptide may induce an anchorage-independent apoptosis by entering into the cell and directly promoting caspase-3 activation [1, 3, 4]. Buckley et al., 1999 [3] announce that the intracellular protein pro-caspase-3 contains RGD motif, as well as a potential RGD-binding motif. The authors suggest that RGD peptides connect to this RGD-binding motif and induce apoptosis by triggering conformational changes in pro-caspase-3 and respectively its autoprocessing and activation.

The results of Aguzzi et al., 2004 [2] indicate that RGD directly binds and activates caspases 8 and 9 and induces apoptosis of Human umbilical vein endothelial cells (HUVECs) with a mechanism independent from its antiadhesive effect. Buckley et al. (1999) [3] showed that the breast carcinoma cell line MCF-7 with a functional deletion of the caspase-3 gene was not inhibited in cell growth after RGD peptides treatment. However Huang et al. 2007 [8] could cause cell death in MCF-7 cell line induced by cyclic-RGD peptide designed by them. They showed that the cell death had been triggered through blocking integrin signaling to the extracellular matrix and activation of caspase pathway.

RGD-based strategies include antagonist drugs (peptidic or peptidomimetic) of the RGD sequence, RGD-conjugates, polymerisation, and the grafting of the RGD peptide or peptidomimetic, as targeting ligand, at the surface of nanocarriers [6].

The main problem for biomedical application of RGD peptides is the fact that large doses of them are required for significant anti-metastatic effects *in vivo*, probably due to their rapid degradation by various peptidases and their rapid excretion from the blood into the urine. To overcome these problems, the development of an appropriate drug delivery system is required to improve *in vivo* stability and prolong plasma half-lives. Several strategies such as peptide-cyclization and incorporation of D-amino acids have been reported to improve stability in blood by inhibiting enzymatic degradation [24].

The sequence, structure and conformation of the RGD-containing peptide may play a crucial role in the ligand/receptor interaction and/or in the stability of the interaction. Cyclic RGD peptides and peptide templates confer greater stability and selectivity over linear peptides [6]. One of them is cilengitide, the most advanced specific integrin inhibitor in oncology. Cilengitide (Merck KGaA, Darmstadt, Germany) is a synthetic Arg-Gly-Asp (RGD) pentapeptide recognizing the RGD ligand-binding motif on the integrin receptors αvβ3 and αvβ5 and competitively blocks integrin ligand binding [23]. It has been shown to diminish angiogenesis *in vitro* [23]. It has shown antitumor activity against glioma and has entered Phase II of clinical trials [23].

Targeting tumor cells or tumor vasculature by peptides is a promising strategy for delivering cytotoxic drugs for cancer therapy. RGD peptides conjugated with different cytostatic agents are likely to exhibit a tumour-targeting and antiangiogenic synergetic effect. During the last few years, a number of RGD-cytotoxic drugs were developed and showed promising activities *in vitro* and *in vivo* [12, 15, 19].

In conclusion, the RGD peptides and mimics can be used to probe integrin functions in various biological systems. Drug design based on the RGD structure may provide new treatments for diseases such as thrombosis, osteoporosis, and cancer, taking in mind that the integrin-mediated cell attachment influences and regulates cell migration, growth, differentiation, and apoptosis.

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EO6. BIOLOGICAL PROPERTIES OF GLYCOSAMINOGLYCANS

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Abstract

Human milk contains large number of bioactive substances. Among them different nutrients, cytokines, antibodies, enzymes and growth factors are present. Proteoglycans present in milk are recognized as major constituents of the innate immune system. They digest into glycosaminoglycans and a core protein. The fate of glycosaminoglycans in the developing neonate is to interact with a wide range of bioactive molecules, such as growth factors and chemokines, to regulate cell behaviors in normal and in pathological processes. Further studies of milk glycosaminoglycans seem a promising approach toward developing novel agents for the prevention and treatment of different enteric and respiratory diseases.

Keywords: glycosaminoglycans, immunological defense.

Introduction

Human milk contains a large number of sialylated oligosaccharides, many of which are also present in bovine colostrum. They could inhibit viral pathogens. Moreover, human milk oligosaccharides could reduce inflammatory processes in the intestinal mucosa. For that reason oligosaccharides and other glycans in milk are considered the major constituents of an innate immune system. In this way the mother protects her infant from enteric and other pathogens via breastfeeding.

Glycosaminoglycans (GAGs) are group of sulfated linear polysaccharides, constituted by repeating disaccharide units. Differences between GAGs' amounts in human and bovine milk were recently characterized. The total amount of GAGs was seven times higher in human mature milk, compared to bovine milk. It was found that total amount of glycosaminoglycans in human milk was 416 mg/L. Moreover composition of chondroitin sulfate (CS) and heparin sulfate (HS) were predominant in human milk [3]. Further study demonstrated that GAGs concentrations varied between the studied periods. Concentration value was highest at day 4 of lactation and gradually decreased [4]. GAGs are classified in four major groups, as shown on Fig.1.

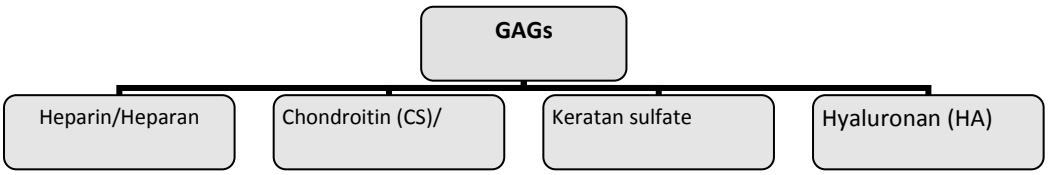

Fig.1. GAGs classification.

As a part of the extracellular matrix (ECM) GAGs play an essential role in organogenesis, and in many human diseases. Therefore, accumulation and turnover of extracellular matrix components are the hallmarks of tissue injury. Communications between glycosaminoglycans depend on their structure. Even minimal modifications within the chains may change the biological properties of the molecules and ECM respectively.

Biological properties of GAGs

Glycosaminoglycans in human milk are synthesized in the alveolar cells of the mammary gland. GAGs synthesized at Golgi cleave a core protein to form proteoglycans (PGs). These GAGs are ingested by breastfed infants. It is supposed that after ingestion of PGs, a digestion of the protein occurs in the small intestine. Released aminoacids are adsorbed. Mucous membrane of the intestine could be passed by molecules of the right size and all other remaining molecules are not digested [8].The remaining free GAGs are either absorbed or transported to the colon where they serve as prebiotics and when passed to colon they are considered dietary fibers. It is believed, that glycosaminoglycans could be absorbed if their size ranges between 1500 to 2500 Da. Apparently enzyme portioning of glycosaminoglycans is very important for later uptake of these agents. The fate of glycosaminoglycans after adsorption is to serve as antioxidant agents. In the infant gut though, where the enzymes are not mature, oligosaccharides are indigestible and therefore are not used as a nutrient. Many studies prove that glycosaminoglycans are capable of inhibiting the binding of different pathogens to their host cell glycans. That binding inhibition is capable of protecting mammals from enteric infections by virulent strains like *Campylobacter, Escherichia coli*, norovirus and rotavirus [16, 22]. Intestinal mucosa is among the most heavily glycosylated tissues in the body. For that reason functions of the cell surface glycans include communication with the extracellular environment, including cell-cell communication, and binding to hormones and other signaling agents. Some GAGs (in particular chondroitin sulfate) are demonstrated to inhibit the binding of the human immunodeficiency virus (HIV) envelop glycoprotein gp120 to the cellular CD4 receptor [17]. This finding proves that human milk GAGs are able to behave as soluble receptors and have therefore the power to inhibit the binding of different pathogens to the intestinal mucosa, thus protecting the infant from severe infections [9, 16]. Interestingly, data exists that GAGs could be mediated by different chemokines. These molecules are crucial to routine immune surveillance and homeostasis. Many soluble GAGs can bind chemokines, competing that way with cell receptors, which leads to abrogation of their chemotactic potential. [14].

Another positive effect that GAGs possess is the stimulation of the chondrocyte metabolism [25]. Thus they are used in osteoarthritis therapy. A question arises whether GAGs are being destroyed in the small intestine or remain unchanged during the digestion process. Few studies are available on this topic. They all prove GAGs as important anti-inflammatory factors for the joints [19] but no precise daily amount for per oral intake are stated.

In lungs hyaluronic acid (HA) is widely distributed as part of the extracellular matrix. Biological role of HA in lungs depends on its molecular weight. The hight molecular weight HA (HMW-HA) functions as a cellular supporter and stabilizes structures. It also regulates cell to cell adhesion and the movement of interstitial fluid and macromolecules in the lung. The low molecular weight HA (LMW-HA; under 500 kDa) functions as an intracellular signaling molecule in inflammation. HA levels in bloodstream may serve as an indicator of the lung damage. It is thought that decreased levels of HA increase the neutrophil elastase activity which leads to formation of pro-inflammatory cytokines [11]. It is also believed that HA may stimulate different cell types and interact with variety of stem cells (mesenchimal, hematopoetic). Hyaluronan is synthesized by membrane-bound synthases on the inner surface of the plasma membrane. The chains are extruded through pore-like structures into the extracellular space. In many diseases hyaluronan synthases (HAS) may have dysregulated expression. *In vitro* experiments showed that expression of HAS isoforms may be regulated by different growth factors and cytokines. For example TNF-apha may stimulate HA production by lung fibroblasts in humans [6]. EGF can induce HAS expression in rat epidermal keratinocytes [18]. Increased levels of HA are considered a signal of injury and are

observed in many autoimmune diseases [7]. During pathological conditions HA fragmentation occurs, as a result of ROS species release from injured tissues.

In blood vessels HA function depends on its molecular weight. HMW-HA reduces angiogenesis whereas LMW-HA induces it [21]. It is believed that HA exists in bound and in soluble form in the ECM. Hyaluronan binds different proteins and influences their function. The free form is highly dynamic in solution. Soluble HA is likely to be a perfect dynamic colonizer of the extracellular space. CD44 is the major cell-surface HA binding protein [1]. Another GAG found in blood is heparin. It can prolong the process of blood clothing and is used as an anticoagulant agent. In recent years heparin was documented to have anti-inflammatory activity. It was used in treatment of inflammatory bowel disease, rheumatoid arthritis and bronchial asthma [5]. An intriguing report reveals the possible fate of heparin in breast milk, after treatment of the mother with anticoagulant agents. Low molecular weight heparin (LMWH) is used in thromboprophylaxis during pregnancy and Caesarean section [20]. In that trial, slight excretion of LMWH into breast milk was seen in some patients, though other results trace no LMWH in milk samples after oral intake [10]. For that reason when heparin is transmitted into the infants gut, thromboprophylaxis is unlikely to have any effect on the nursing infant.

Chondroitin sulfate and HA are involved in the remodeling processes in the brain. Hyaluronan forms fiber-like structures along the migratory pathways in the developing brain of mice. Their possible role is to support guided neural migration [2].

GAGs are able to modulate some proteins by either acting as structural elements controlled by proteinases. When binding occurs, GAGs ensure exposure of the binding regions on the target protein. Other biological functions of glycosaminoglycans are to act as co-receptors for some inhibitors, playing important roles for the acceleration of proteinase inhibition.

Staining of GAGs

Both electron microscopy and light microscopy are applicable methods for visualization of GAGs after appropriate staining. Glycosaminoglycans stain with cationic dyes such as Alcian blue, ruthenium red, and toluidine blue. The mechanism is not well understood but it is thought that binding and precipitation of acid substituted polymeric molecules occurs [15]. Earlier it was shown that glycocalyx and mucous droplets are particularly well stained with osmium-ferrocyanide [12]. Some variations of osmium-ferrocyanide and cationic dye staining were also performed. An example is toluidine blue plus glutaraldehyde procedure, for the staining of cartilage PGs [23]. Fluorescent methods are also available for the detection of sulfated glycosaminoglycans, using antibodies against different GAG structures [24, 13].

In conclusion, glycosaminoglycans should be considered a major component of a human milk immunological defense system. Further studies of milk GAGs seem a promising approach toward developing novel agents for the prevention and treatment of enteric and respiratory diseases. New insight is needed on GAGs communication with different growth factors found in colostrum and milk. Understanding the modulatory effects of GAGs on proteinase activities is expected to lead to new insights in the understanding of some molecular systems present in pathological states, providing new targets for drug therapy.

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EO7. EXPRESSION OF CARBOHYDRATE-BINDING PROTEINS IN CULTURE MEDIUM FROM MCF-7 ADENOCARCINOMA CELLS TREATED WITH LANTHANIUM-CHOLATE COMPLEX

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Abstract

Carbohydrate-binding proteins (CBPs) were upregulated in nontreated and treated with La $(Col)_3\,2H_2O$ MCF-7 adenocarcinoma cell line compared to the blank control. D-mannosamine CBP was downregulated, whereas D-fucose CBP had the same expression in the blank control

and nontreated cells. Treatment with higher doses (100 μg/ml) of $La(Col)_3$ led to up-regulation N-Ac-β-D-mannosamine and D-fucose CBPs. Treatment with higher doses(100 μg/ml) of $La(Col)_3.2H_2O$ led to down-regulation of D-mannosamine and D-glucosamine CBPs, whereas expression of D-mannose, N-Ac-D-mannosamine, galactose and D-mannosamine CBPs were the same in groups treated with 10 μg/ml and 100 μg/ml $La(Col)_3.2H_2O$ Compared to nontreated cells N-Ac-β-D-mannosamine D-galactosamine, mannose D-glucosamine, N-Ac-D-glucosamine and fucose CBPs were up-regulated, whereas galactose and D-mannosamine CBPs were down-regulated.

Keywords: MCF-7 adenocarcinoma, carbohydrate-binding proteins, lanthanium

Introduction

Carbohydrate-binding proteins (CBPs) are receptors that specifically recognize carbohydrate portion of glycoconugates. Neoplastic cell, contrary to the 'normal' ones, have different phenotype in respect to glycosylation of their proteins. Such difference is accompanied also with differential expression of the carbohydrate-binding proteins.

The aim of our study was to evaluate expression and secretion of carbohydrate-binding proteins in culture media from MCF-7 adenocarcinoma cells treated with $La(Col)_3.2H_2O$. To our knowledge there are no data on influence of lanthanium cholate complexes on the expression of tumor agglutinins.

Materials and Methods

Synthesis of $La(Chol)_3 \cdot 2H_2O$: A solution of 0.5 mmol (0.215 g) $La(NO_3).6H_2O$ in 10 ml of distilled water was added to a stoichiometric amount of sodium cholate ($C_{24}H_{39}O_5Na$) 1.5 mmol (0.645 g) dissolved in 15 ml of distilled water. The resulting mixture was stirred and heated for 1 hour. A white precipitate formed was filtered and washed with water and dried over P_4O_{10}.

Culturing and treatment of MCF-7 tumor cells: MCF-7 cells were grown as monolayers in a combination of E-199 and Iscove's modified Dulbecco's medium (IMDM) supplemented with 5 to 10% calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). Adherent cells were detached using a 0,05% trypsin (Gibco) - 0,02% EDTA mixture. At the 24^{th} h monolayers were covered with different concentrations (10 μg/ml and 100 μg/ml) of $La(Col)_3.2H_2O$ and examined after 24 h.

Haemagglutination experiments: Agglutination assays were done in microtitter U plates using serial two-fold dilutions. For sugar inhibition studies, 1M of the corresponding sugars were added in place of the 0.15 M NaCl and preincubated with the lectin source for 30 min at room temperature.

Results

N-Ac-β –D-mannosamine-binding proteins:

We found that carbohydrate-binding proteins (CBP) with specificity towards N-Ac-β –D-mannosamine were elevated in culture media from nontreated MCF-7 breast adenocarcinoma cells and tumor cells treated with 10 μg/ml and 100 μg/ml $La(Col)_3.2H_2O$, see figure 1. Levels of this protein were higher in culture medium from nontreated MCF-7 breast adenocarcinoma cells, compared to its levels in culture media from tumor cells treated with 10 μg/ml $La(Col)_3.2H_2O$.

D-galactosamine-binding proteins:

Higher levels of CBPs with specificity towards D-galactosamine were detected in culture media from nontreated MCF-7 breast adenocarcinoma cells and culture media from tumor

cells treated with 10 μg/ml and 100 μg/ml $La(Col)_2.2H_2O$, see figure 1. Treatment with higher doses of $La(Col)_2$ led to lower levels of the CBP with specificity towards D-galactosamine.

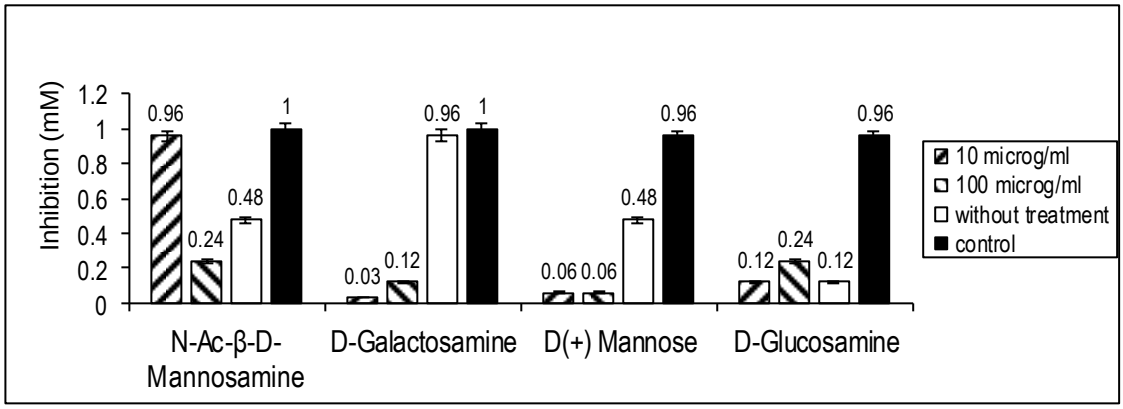

Figure 1: Inhibition of haemagglutination with N-Ac-β-D-mannosamine D-galactosamine, mannose and glucosamine. (Legend: **10 μg/ml** – MCF-7 breast adenocarcinoma cells treated with culture media containing 10 μg/ml $La(Col)_3$; **100 μg/ml** – MCF-7 breast adenocarcinoma cells treated with culture media containing 100 μg/ ml $La(Col)_3$; **MCF-7 without treatment –** culture media from nontreated cells (control I); and **control –** culture media containing 10% fetal calf serum and antibiotics).

D-mannose-binding proteins:

We found higher levels of CBPs with specificity towards D-mannose in culture medium from nontreated MCF-7 breast adenocarcinoma cells and culture medium from tumor cells treated with 10 μg/ml and 100 μg/ml $La(Col)_2.2H_2O$, see figure 1. Culture media from treated tumor cells had the same levels of CBPs with specificity towards D-mannose, see figure 1.

D-glucosamine-binding proteins:

Treatment with higher doses of $La(Col)_2$ led to lower levels of this CBP in the culture medium from treated cells, compared to its levels in culture media from nontreated and treated with 10 μg/ml $La(Col)_2.2H_2O$. The culture medium from nontreated tumor cells and the culture medium from MCF-7 breast adenocarcinoma treated with 10 μg/ ml $La(Col)_2.2H_2O$ had the same levels of the D-glucosamine-binding CBP, see figure 1.

N-Ac-D-glucosamine-binding proteins:

N-Ac-D-glucosamine-binding protein was elevated in culture media from treated tumor cells, as compared to its levels in the culture medium from nontreated MCF-7 breast adenocarcinoma cells. However levels of this CBP were the same in culture media from MCF-7 breast adenocarcinoma cells treated with 10 μg/ml and 100 μg/ml $La(Col)_3.2H_2O$, see figure 2.

Galactose-binding proteins:

Galactose-binding protein levels were lower in culture media from tumor cells treated with 100 μg/ml $La(Col)_3.2H_2O$, as compared to the blank control. Levels of this CBP were the same in culture medium from MCF-7 breast adenocarcinoma cells treated with 10 μg/ml $La(Col)_3.2H_2O$ as to its levels in the blank control, see figure 2.

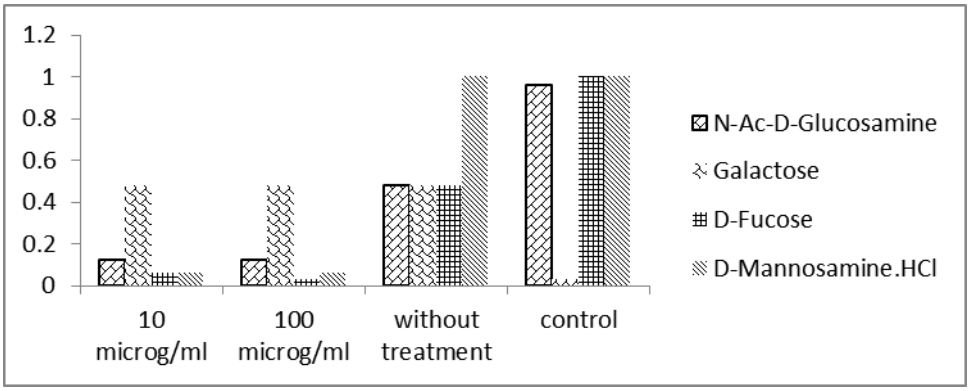

Figure 2: Inhibition of haemagglutination with N-Ac-D-glucosamine, galactose, D-fucose and

D-mannosamine.HCl. (Legend: **10 μg/ml** – MCF-7 breast adenocarcinoma cells treated with culture media containing 10 μg/ml $La(Col)_3$; **100 μg/ml** – MCF-7 breast adenocarcinoma cells treated with culture media containing 100 μg/ml $La(Col)_3$; **MCF-7 without treatment –** culture media from nontreated cells (control I); and **control –** culture media containing 10% fetal calf serum and antibiotics).

Fucose-binding proteins:

We found higher levels of carbohydrate-binding proteins (CBP) with specificity towards D-fucose in culture media from MCF-7 breast adenocarcinoma cells treated with *La*$(Col)_3.2H_2O$, as compared to the levels of this CBP in culture medium from cells without treatment and to its levels in the blank control. Higher levels of fucose-binding proteins were also observed in culture medium from MCF-7 adenocarcinoma cells treated with 100 μg/ml $La(Col)_3.2H_2O$ as compared to the levels of this CBP in culture medium from tumor cells treated with 10 μg/ml $La(Col)_3.2H_2O$, see figure 2.

Mannosamine-binding proteins:

We found higher levels of mannosamine-binding proteins in culture media from treated tumor cells as compared to the levels of this CBP in the culture media from nontreated MCF-7 adenocarcinoma cell. Levels of this CBP was the same in culture media from tumor cells treated with10 and 100 μg/ml $La(Col)_3.2H_2O$, see figure 2.

Discussion

Carbohydrate-binding proteins are often differentially expressed in tumor cells. Galectin-3 expression in the cytoplasm is greatly increased in cell lines propagated from malignant ascites [2]. Expression of galectins correlate directly with aggressive tumor potential in several human breast carcinoma cell lines [3].We also found that galectins are upregulated in Guerin carcinoma [4], Zajdela hepatoma [5] and Ehrlich carcinoma [6]. Serum

levels of galectins in tumor-bearing patients are also found to be elevated. MCF-7 subclones with a high level of galectin-9 expression formed tight clusters during proliferation in vitro, whereas a subclone (K10) with the lowest level of galectin-9 expression did not. However, K10 cells stably transfected with a galectin-9 expression vector aggregated in culture and in nude mice. This points out to the role of galectin-9 in formation of cellular aggregates [1].

Treatment with lanthanium complexes led to lower expression/secretion of these proteins, reflecting the possible antitumor effect of this complex. We found that N-Ac-β-D-mannosamine-, D-galactosamine-, N-Ac-D-glucosamine-, D-fucose- D-mannosamine- and mannose-binding proteins were up-regulated in the tumor cells and their levels in the culture medium were higher than the levels of these proteins in the culture medium from non-treated cells.

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EP1. BRIEFLY ABOUT BRCA GENES AND HEREDITARY BREAST CANCER

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Abstract

Mutations in tumor-suppressor BRCA1 and BRCA2 genes are the most commonly identified germ line gene mutations in patients with hereditary breast cancer. This mini-review summarizes data about physiological role of these genes in normal and pathological conditions as well as about biology of BRCA-connected.

Introduction

Despite advances in diagnosis and treatment of breast cancer, it still remains the most common and deadly malignancy affecting women worldwide [34]. Many factors such as environmental and hormonal factors, and genetic susceptibility play a role in the etiology of breast cancer. Hereditary breast cancer (HBC) represents approximately 5% to 10% of breast cancers and a larger portion of patients with early-onset disease [27].

Recent studies suggest that thousands of genes may contribute to breast cancer pathophysiologies when deregulated by genomic or epigenomic events. Hereditary breast cancer runs in families where several members in different generations are affected. So far, 13 breast cancer susceptibility genes of high or moderate penetrance have been identified including high penetrance genes BRCA1 and BRCA2 accounting for about 5% of all breast cancers, CHEK2, PTEN, TP53, ATM, STK11/LKB1, CDH1, NBS1, RAD50, BRIP1 and PALB2. Hereditary breast cancer can be also part of multiple cancer syndromes [2, 22, 33, 35]. There are different types of mutations listed in Breast Cancer Information Core professional databases and most of them are small insertions or deletions. For example, frameshift mutations are the main type of mutation in BRCA 1 and BRCA2 genes. Missense mutations, intronic variant sequences and unclassified variants with unclear role in breast cancer susceptibility have been also reported [2, 19].

BRCA 1 and 2 genes

BRCA1 (located at locus 17q21) and BRCA2 (located at locus 13q12.3) are the two most important breast cancer susceptibility genes. They have a relatively low mutation rate, and the most frequent sites of mutation are in exon 11 [2]. The mutations are highly penetrant - until the age of 70 women carrying a mutation on BRCA1 or BRCA2 gene have a 45-85% probability of developing breast cancer, and 11-62% probability of developing ovarian cancer [14, 15, 17], with variation related to genetic background [23]. Most of these mutations consist of deletions, insertions, nonsense mutations, and splice variants, however an increasing number of large genomic rearrangements have been identified in these genes [8]. A list of known mutations can be found in the Breast Information Core databases

[http://nhgri.nih.gov/Intramural_research/Lab_transfer/Bic]. Certain BRCA mutations have been identified in some specific ethnic groups (Icelanders, Jews, Russians, etc). In the general population ~0.1% to 0.2% are carriers of BRCA1 or BRCA2 mutations, with specific subgroups of the population (such as Ashkenazi Jewish) population having an increased prevalence of carriers [13, 30]. The overall prevalence of BRCA1/2 mutations is estimated to be from 1 in 400 to 1 in 800 while in Ashkenazi Jewish is found to be 1 in 40 [21].

Biological role

The products of BRCA genes take part in many many critical cellular functions, including repair of DNA double-strand breaks. BRCA1, is essential for homologous recombination (HR), a fundamental process for maintaining genome stability that permits the reactivation of blocked replication forks. BRCA1- and BRCA2-deficient cells display genomic instability due to impaired DNA repair and may subsequently be predisposed to malignant transformation. Namely the role of BRCA1 and BRCA2 in DNA repair is being exploited to develop novel therapies, for example, using the poly-ADP-ribose polymerase (PARP) inhibitors [6, 10].

BRCA related cancers

Mutations in BRCA1 or BRCA2 are not simply associated with increased breast cancer risk. Mutation carriers are also susceptible to cancers of the ovary, prostate and male breast [28, 31]. There are data that these mutations can be also connected with cancers of the stomach and pancreas [24].

Hereditary breast cancer arising in carriers of mutations in the BRCA1 and BRCA2 genes differs from sporadic breast cancer and from non-BRCA1/2 familial breast carcinomas in terms of morphological and immunohistochemical characteristics. BRCA1-associated carcinomas are poorly differentiated infiltrating ductal carcinomas that frequently show morphological features of typical or atypical medullary carcinoma. BRCA2-associated breast carcinomas tend to be of higher grade than sporadic age-matched controls. BRCA1 tumors have been found to be more frequently estrogen receptor- and progesterone receptor-negative, and p53-positive than are age-matched controls, whereas these differences are not usually found in BRCA2-associated tumors. Both genotypes have a low frequency of HER2 expression/amplification [11, 12, 25]. It has been reported that women with triple negative breast cancer diagnosed below 50 years have >10% likelihood of carrying a BRCA1 mutation and may take advantage of such genetic testing (Robertson et al., 2012). The expression of the cell-cycle proteins cyclins A, B1 and E and SKP2 is associated with a BRCA1 phenotype, whereas cyclin D1 and p27 expression is associated with BRCA2 carcinomas. Most BRCA1 breast carcinomas are characterized by the expression of basal (myoepithelial) markers, such as cytokeratin 5/6 and or P-cadherin. These features could be used to distinguish patients who are likely to carry a BRCA1 or BRCA2 germline mutation, thus indicating which gene should be screened for first in families with a high incidence of breast and ovarian cancer [11, 12, 25].

Hereditary breast and ovarian cancer is more likely to appear at younger age than sporadic cancer, and in some cases bilateral breast cancer or both breast and ovarian cancer develop [8, 32]. If a woman bearing a mutation develops cancer in one breast, her risk of developing cancer in the other breast depends on the particular gene that is mutated and on her age at the onset of disease [20].

One of the interesting aspects of BRCA1-linked cancers is the observed specificity for estrogen-responsive tissues such as breast and ovary. Recent studies in this area have revealed a complex relationship between BRCA1 and estrogen receptor alpha (ERalpha) signaling. Estrogen stimulation increases expression of BRCA1 at the mRNA and protein level and conversely BRCA1 functions to both induce ERalpha mRNA expression and act as a negative regulator of ERalpha signaling [9].

Identification of carriers

The discovery of breast cancer-predisposition genes BRCA1 and BRCA2 made it possible to identify carriers accurately, to take measures to reduce the risk of breast and ovarian cancers in carriers, and to develop a new generation of targeted therapies such as PARP inhibitors. Since the identification of BRCA1 and 2, testing for mutations in these genes has been offered to cancer patients and their families by clinical genetics services. The implementation of this diagnostic approach meets some difficulties: i) These services are provided across Europe by a small number of health professionals, and are therefore low volume, and low capacity and patients experience considerable delays, both in seeing a clinician and in laboratory testing [18]; ii) Since mutations in BRCA1 and BRCA2 are distributed throughout the world it is very important the benefits of genetic testing and of targeted therapies to become available also to women who live outside of North America and Western Europe; iii) The sequencing of the genes is expensive and since the information derived may have a profound effect on the individual and family members, it is important that testing is done only when the risk of carrying a mutation is thought to be high [36]. Age at onset, HER2 status, and either ER (estrogene receptor) or PR (progesterone receptor) status (as compared with sporadic or non-BRCA1/BRCA2 cancers), have been suggested to be the most effective, independent predictors of BRCA1 mutations [7].

Mathematical models (such as Gail risk model and Cuzick-Tyrer model) for estimation of breast cancer risk have been developed on the basis of epidemiological studies. It is possible to identify women at high risk for this disease using patient history data and the analysis of various demographic and hereditary factors [16]. Genetic models aiming at calculating individual risk for BRCA1 and BRCA2 mutation carrier-status have also been designed. These models usually take into account the type of tumor and age at occurrence as well as family history. It has been suggested that the incorporation of pathology data into risk assessment models can improve prediction of carrier status and identification of individuals who may benefit from testing [5, 36].

Prophylaxis and new treatment

The role these genes play in DNA repair is thought to explain why tumors associated with them are particularly sensitive to platin derivatives and poly(ADP-ribose) polymerase inhibitors. The role of PARP in single-strand DNA break repair is relevant, leading to replication-associated lesions that cannot be repaired if homologous recombination repair (HRR) is defective, and the synthetic lethality of PARP inhibitors in HRR-defective cancer. Although HRR defects are classically associated with BRCA1 and 2 mutations involved in familial breast and ovarian cancer, many other causes of HRR defects cam exist [4]. In addition, BRCA1-mutation carriers seem to benefit from anthracycline-taxane-containing regimens as much as sporadic triple-negative breast cancers do [1].

In carriers of BRCA1 and BRCA2, prophylactic bilateral mastectomy and adnexectomy has been shown to significantly lower the incidence of breast and ovarian cancer. Prophylactic adnexectomy also decreases the breast-and-ovarian-cancer-specific mortality, as well as the overall mortality [21].

Once a BRCA1 or BRCA2 mutation has been identified in a family, testing of at-risk relatives can identify those family members who are also at risk because of the familial mutation and thus need increased surveillance and early intervention when a cancer is diagnosed [26].

Conclusion

Increasing knowledge about the molecular bases of hereditary breast cancer and improvement of methods for genetic testing will allow the development of new approaches for early identification of high-risk individuals and timely implementation of appropriate treatment preventive care or prophylactic surgeries. Undoubtedly this will be a step forward towards the so-called individualized medicine

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EP2. FEW WORDS ABOUT TRIPLE NEGATIVE BREAST CANCER

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EP3. BREAST CANCER CELL LINES AS EXPERIMENTAL MODELS IN ONCOLOGY

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EP4. APPLICATION OF HUMAN TUMOR CELL LINES FOR EVALUATING ANTITUMOR PROPERTIES OF SALINOMYCIN AND ITS METAL COMPLEXES

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EP5. VIRUS-TRANSFORMED RAT SARCOMA CELLS IN SEARCHING FOR NEW ANTITUMOR AGENTS

Abedulkadir Abudalleh[1,2], Tanya Zhivkova[2], Boyka Andonova-Lilova[2], Lora Dyakova[3], Dimitar Ivanov[2], Marin Alexandrov[2], Pavel Mitrenga[2], Radostina Alexandrova[2]

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EP6. CHLORELLA IS A HEALTH CARE

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EP7. НИЕ НЕ ЖИВЕЕМ, ЗА ДА ЯДЕМ, А ЯДЕМ, ЗА ДА ЖИВЕЕМ

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EP8. КОМПЛИМЕНТИ ЗА КОМПЛЕМЕНТА

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EP9. БИОСИНТЕЗ НА ПЪРВИЧИНИ И ВТОРИЧНИ МЕТАБОЛИТИ ЗА ПРИЛОЖЕНИЕ В МЕДИЦИНА

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Session F.

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Assoc. Prof. Ivan Goshev, PhD
Institute of Organic Chemistry, Bulgarian Academy of Sciences

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FO1. DETERMINATION OF ANTIOXIDATIVE PROPERTIES

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Abstract

It is of a great interest to quantify the antioxidant properties of various biological materials because antioxidants are clearly important to human life. Antioxidant function can be defined as the ability of a compound to reduce pro-oxidant agents. There are many different methods for determining antioxidant function which rely on different generators of free radicals, acting by different mechanisms. In the literature, antioxidant properties are denoted as antioxidant capacity, antioxidant power and antioxidant potential. From the current point of view, the mix of methods should be used for assessing antioxidant activities in vitro to cover all the aspects of antioxidant efficacy.

This present contribution describes three different methods for assessing antioxidant properties - Oxygen Radical Absorbance Capacity (ORAC), Total Radical-trapping Antioxidative Parameter (TRAP) and Hydroxyl (HO) Radical Averting Capacity (HORAC). The first two methods assess the peroxyl radical chain-breaking ability of antioxidants by hydrogen atom transfer pathway. The third method - HORAC measures their metal-chelating radical prevention activity. Despite the methods described in this contribution there are many other approaches to evaluate antioxidant properties of biological materials such as 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) radical assay, diphenylpicrylhydrazyl (DPPH) radical assay, b-Carotene bleaching assay, ferric reducing antioxidant power (FRAP) assay, cupric ion reducing antioxidant capacity (CUPRAC) assay etc.

FO2. LUMINOMETRIC EXAMINATION OF OXIDATIVE BURST OF PHAGOCYTES

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Abstract

As early as within the first minutes of their stimulation, during the so-called respiratory burst, phagocytes produce highly reactive oxygen species (ROS) known to belong among the most efficient microbicidal mechanisms. Phagocytes thus represent the front-line defence cells in protecting the organism against infection and play an irreplaceable role in the proper performance of the immune system. Moreover, excessive ROS production can also damage the body`s own cells and tissues and contribute to the development of a number of serious diseases. All these reasons make analysis of the metabolic activity of phagocytes important in clinical practice as well as in the development and testing pharmaceuticals and in biomedical research. Among the several existing methods, recording the phagocytosing leukocyte chemiluminescence (CL) response is the most convenient method to measure the cells` oxidative burst.

Typical CL responses of human whole blood neutrophils to the activators frequently used in our laboratory (opsonized zymosan, phorbol myristate acetate, N-formyl-Met-Leu-Phe and calcium ionophore A23187) are shown in the contribution. Results obtained by measuring luminol- and iso-luminol enhanced CL of human leukocytes stimulated with Streptococcus mutans are also described.

FO3. ANTIOXIDANT AND *IN VITRO* ANTICANCER ACTIVITY OF NEW ANALOGS OF OCTREOTIDE

Svetlana Ts. Staykova[1], Ivan Goshev[2], Diana W. Wesselinova[3], Liubomir T. Vezenkov[1], Aleksandra D. Kalistratova[1], Emilia D. Naydenova[1]

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Abstract

Oxygen free radicals are products of normal cellular metabolism. The harmful effect of free radicals (oxidative stress) can damage cellular lipids, proteins, or DNA inhibiting their normal function. Somatostatin analogs are antineoplastic agents capable to inhibit tumor growth and tumor angiogenesis. Octreotide (D-Phe-c(Cys-Phe-D-Trp-Lys-Thr-Cys)-Thr-ol) is selective somatostatin analog that have been used in the treatment of cancers.

The aim of the present study was the synthesis, antioxidant and antiproliferative effects of new C-amide analogs of Octreotide modified at positions 5 and 6. In order to elucidate the influence of the length of the side chain on the antitumor activity Lys at position

5 was substituted by Orn, Dab (diaminobutanoic acid) and Dap (diaminopropanoic acid). Thr at position 6 was replaced by steric restricted amino acid Tle (t-leucine). New analogs are C-terminal amides. The peptides have been prepared by solid-phase peptide synthesis - Fmoc-strategy. Fmoc-Rink-Amide MBHA resin was used as a solid-phase carrier, and TBTU was used as a coupling reagent. For direct disulfide bond formation on the solid phase $Tl(CF_3CO_2)_3$ in DMF has been employed. The peptide purity was checked by electrospray ionization massspectrometry. The antioxidant activity was measured by HORAC (Hydroxyl Radical Averting Capacity) and ORAC (Oxygen Radical Antioxidant Capacity) tests. Compound **1 (**D-Phe-c(Cys-Phe-D-Trp-Dab-Tle-Cys)-Thr-NH_2) showed most expressed antioxidant effect.

The *in vitro* antiproliferative activity were evaluated against 4 human tumor cell lines (HeLa, Hep G-2, MDA-MB-231, HT-29) and in the non-tumor Lep-3 cell line.

The compounds showed different activity depending on the cell line and amount applied. The most antiproliferative effect exert the peptides **3** (D-Phe-c(Cys-Phe-D-Trp-Lys-Tle-Cys)-Thr-NH_2), **4** (D-Phe-c(Cys-Phe-D-Trp-Orn-Tle-Cys)-Thr-NH_2), **5** (RC-102) against the HeLa and HepG-2 cells with viability of tested cells about 20% at the higher concentrations of compounds (4 M). The peptides were not cytotoxic to the normal Lep-3 cells. All tested substances express a significantly higher antioxydant capacity by comparison with the standart - gallic acid.

FO4. ANTIOXIDANT ACTIVITY OF SOME BENZIMIDAZOLE DERIVATIVES TO DEFINITE TUMOR CELL LINES

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Abstract

A group of bis(benzimidazol-2-yl)amines have been already evaluated for cytotoxicity in vitro to human colorectal cancer cell line HT-29, breast cancer cells MDA-MB-231 and normal spleen cells and two of them (B1 and B2) have been taken for the purposes of our present investigations. From the second group of compounds representing 1,3-disubstituted-2,3-dihydro-2-iminobenzimidazoles two substances (B3 and B4) have been chosen because of their most pronounced antiproliferative effect to human colorectal cancer cell line HT-29, breast cancer cells MDA-MB-231 and normal spleen cells, using the in vitro proliferative MTS-test. It was important to estimate the cause for this suppressive activity of the compounds. We proposed that this could be due to their antioxidant capacity.

The substances were examined for antioxidant activity against hydroxyl and peroxyl radicals, applying the HORAC and ORAC methods and showed considerable capacity. The scavenging capacity of B2 towards hydroxyl radicals is the highest, followed by B1. It was estimated that B2 has the greatest scavenger capacity of oxygen radicals, emitted by the examined cells followed in descending order by B1, B3 and B4. The observed differences can be considered

as impact of their structure on the Me+2-helating activity and effective H-atom donation. A correlation was observed between the structure of the particular substance and the expressed antioxidant potential. The latter correlated also with the effect on the tested tumor cell lines. This result means that tumor cells are accompanied by a measurable emission of ROS whichit might be regulated by a proper application of antioxidants.

Session G.

Chairpersons:

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GO1. CHROMATIN COMET ASSAY – A POWERFFUL TOOL FOR MONITORING CHROMATIN ORGANIZATION DURING AGEING

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Abstract

The process of cellular ageing is intricate and quite complex. It involves changes in the activity of a large subset of genes and results in the activation of pathways which normally lead cells and organisms to death. All pathways activated during ageing concern the organization of the genetic material. The altered gene expression in ageing cells is a result of altered structure of the underlying chromatin. Therefore, it is of utmost interest to study the way chromatin organization changes during ageing. Exciting and challenging is the possibility to better characterize these processes in order to more precisely govern its outcomes, especially when they are related to premature age-associated diseases like Hutchinson Progeria and Werner Syndromes.

Saccharomyces cerevisiae is an invaluable model organism for studying the processes of cellular ageing. As single-celled eukaryotes, easily handled and genetically familiar these organisms are extensively employed in discovering the molecular mechanisms that underlie ageing in norm and pathology. Two major types of ageing can be differentiated in *Saccharomyces cerevisiae* – replicative and chronological lifespans (RLS and CLS) both serving as models respectively for the ageing of mitotically active cells in humans and for the ageing of cells which have already lost their dividing potential. Most of the human cells are highly differentiated and have stopped to divide retaining their lives for a certain period of time. They follow the ageing pathway of chronologically ageing yeast cells.

In order to study chromatin structure along the timeline of ageing we have followed the potential of chronologically ageing *Saccharomyces cerevisiae* cultures to retain their viability almost to their end. At certain time points we have performed the developed by us method of Chromatin Comet Assay in order to study the way chromatin organization changes with ageing. The method is fast, sensitive and allows evaluation of the global chromatin

organization at a single-cell level. It allowed us to outline the major chromatin reorganizations in the timeline of this process, which was a missing point in the majority of ageing related studies.

Introduction

Cellular ageing is a biological process affecting all cells and organisms which with the time certainly leads to death. The process itself is quite complicated as it incorporates different molecular mechanisms which can distress living organisms differently [11]. Ageing cells lose their dividing potential, reshape some of their organelles, reorganize and alter the expression of certain genes and gradually progress to dying [9]. Though normal the process itself is harmful and debilitating for the organisms, especially when an early onset of these changes becomes a reason for the development of age-related diseases like a Hutchinson Gilford progeria and Werner Syndromes. For that reason age-associated changes in the organisms are a matter of extensive study aiming to reveal and understand these intricate molecular mechanisms in order to handle and control them.

DNA in all living organisms is not naked. It is organized in chromatin with histone proteins, thus allowing the genetic information to be safe and at the same time prepared for necessary activation [6]. Chromatin structure and organization predetermine the activity of the genome and thus take part in all cellular processes, including ageing [1].

Age- related changes in chromatin include unusual redistribution of heterochromatin marks and unspecific activation of certain genes [7]. Global organization of chromatin and the resultant alterations in it with the propagation of ageing are little understood.

Here, we show our results obtained by *Saccharomyces cerevisiae* cells as a model organism for studying the changes in chromatin structure organization during ageing. The elusive and hard for studying higher-order chromatin structures are probed by the developed by us method of Chromatin Comet Assay (ChCA). Yeast cells normally proceeding through their life were studied for changes in chromatin structure at certain points of their lifespan. Results draw a detailed picture of chromatin alterations propagated through the time. Notably, we have observed an increase in the compaction of the genome with ageing. Generally chromatin seems completely reorganized and devoid of hierarchical organization.

Materials and methods

Yeast strains and culturing conditions

Saccharomyces cerevisiae yeast cells were used as model organisms in the current research -

*MAT**a** his4-912δ-ADE2 his4-912δ lys2-128δ can1 trp1 ura3 ACT3*.

Yeast cells were cultured in complete minimal media, supplemented with 2% Dextrose, 1.7% Yeast Nitrogen Base (YNB) and with the appropriate amino acids. The time for cultivation was 15 days at optimal condition (30 ˚C) in a water bath shaker.

Chromatin Comet Assay (ChCA)

Yeast cells grown to logarithmic ($1x10^7$ cells/ml) and stationary ($2x10^7$ cells/ml) phase were subjected to Chromatin Yeast Comet Assay (Georgieva et al., 2008; 2012). All Comet Assay procedures are in neutral conditions as follows: after solidifying of micro gels and subsequent *in situ* nuclease treatment of cells the slides were submerged in neutral lysis solution (146 mM NaCl; 30 mM EDTA; 10 mM Tris-HCl and 0.1% N-lauroylsarcozine, pH 7.5) for 20 min in a cold room at 10 ˚C. Slides were then washed in 0.5 x TBE buffer (89 mM Tris; 89 mM Boric-acid; 5 mM EDTA, pH 8). Electrophoresis followed for 10 min at 0.45

V/cm in the same buffer. After successive dehydration in 75% and 100% ethanol for 5 min each, the slides were left to air-dry.

Comets were observed under a Leitz epi-fluorescence microscope (Orthoplan, VARIO ORTHOMAT 2) using 450-490 nm band-pass filter following the staining of DNA with the fluorescent dye SYBR green I (Molecular Probes Inc, Eugene, OR, USA). Pictures were taken with digital camera, Olympus μ800, at a resolution of 3 mpx.

The obtained results were quantified as described in Georgieva et al. [3].

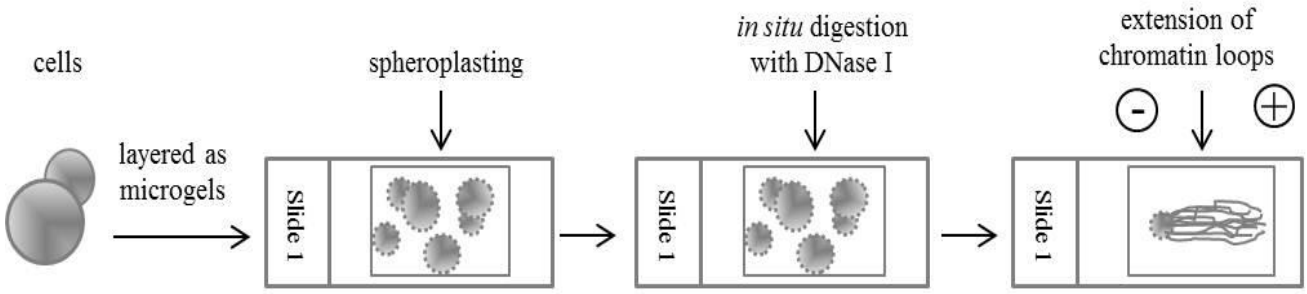

Figure 1. A schematic representation of Chromatin Comet Assay.
Four major steps accompany the execution of the method. The first one is crucial as it includes mild spheroplasting of the studied yeast cells. The second one represents preparation of cell-agarose suspensions and microgels. The third involves digestion of chromatin with nucleases and the forth is electrophoretic extension of chromatin loops [2;3]. Results are visualized under fluorescent microscope after staining of the gels with SYBR green.

A detailed schematic representation of the method is given on Figure 1.

Results and discussion

Saccharomyces cerevisiae cells are being used as a model for ageing research for many years [12]. Scientists accept them as a perfect mirror reflecting the key mechanisms that control ageing in humans. Single eukaryotes with well-studied genetics and molecular biology these organisms offer good opportunities for following and studying ageing. Interestingly, recently these cells have been suggested as a fine molecular clock for following human ageing phenotypes [10]. And though ageing research on *Saccharomyces cerevisiae* cells succeeded in drawing a detailed picture of some of the major alterations in the cellular morphology as well as in gene expression profiles of young and old cells [5], scares are data about the global organization of chromatin and the changes in it during the process of ageing. Higher-order chromatin organization is the most elusive and difficult to be studied and yet it is considered that it controls and guides gene expression and all phenotypic manifestations during cellular processes [8].

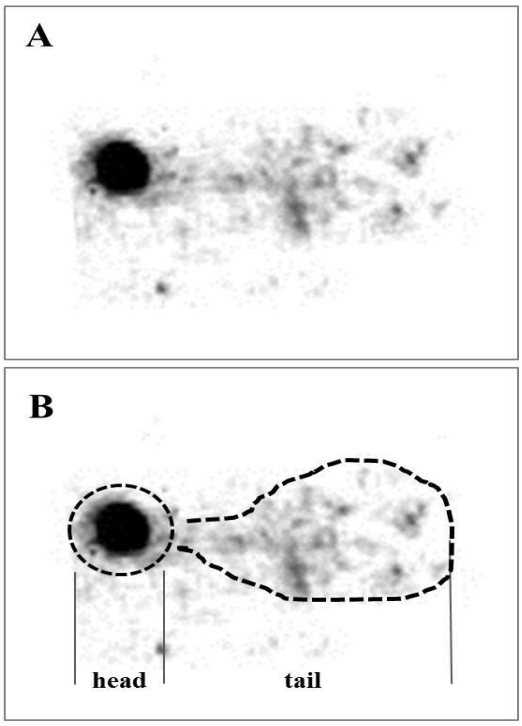

Figure 2. Chromatin Comet Assay on yeast cells:

A: A comet-like image.

Yeast cells were grown to early logarithmic and late stationary phase and were subjected to Chromatin Comet Assay (Figure 1). The method is fast, easy-to-handle and very sensitive as it probes chromatin structure at its higher-order levels of

compaction and at a single-cell level [3]. Cells were mixed with low-gelling agarose and are layered as microgels onto microscopic slides. The agarose-cell suspensions were *in situ* digested with deoxyribonuclease I (DNase I) for relaxation of chromatin loops and were further extended under mild electric field. The obtained images under fluorescent microscope with DNA specific fluorescent dye pretty much resemble astronomical comets and this gives the name of the method itself (Figure 2).

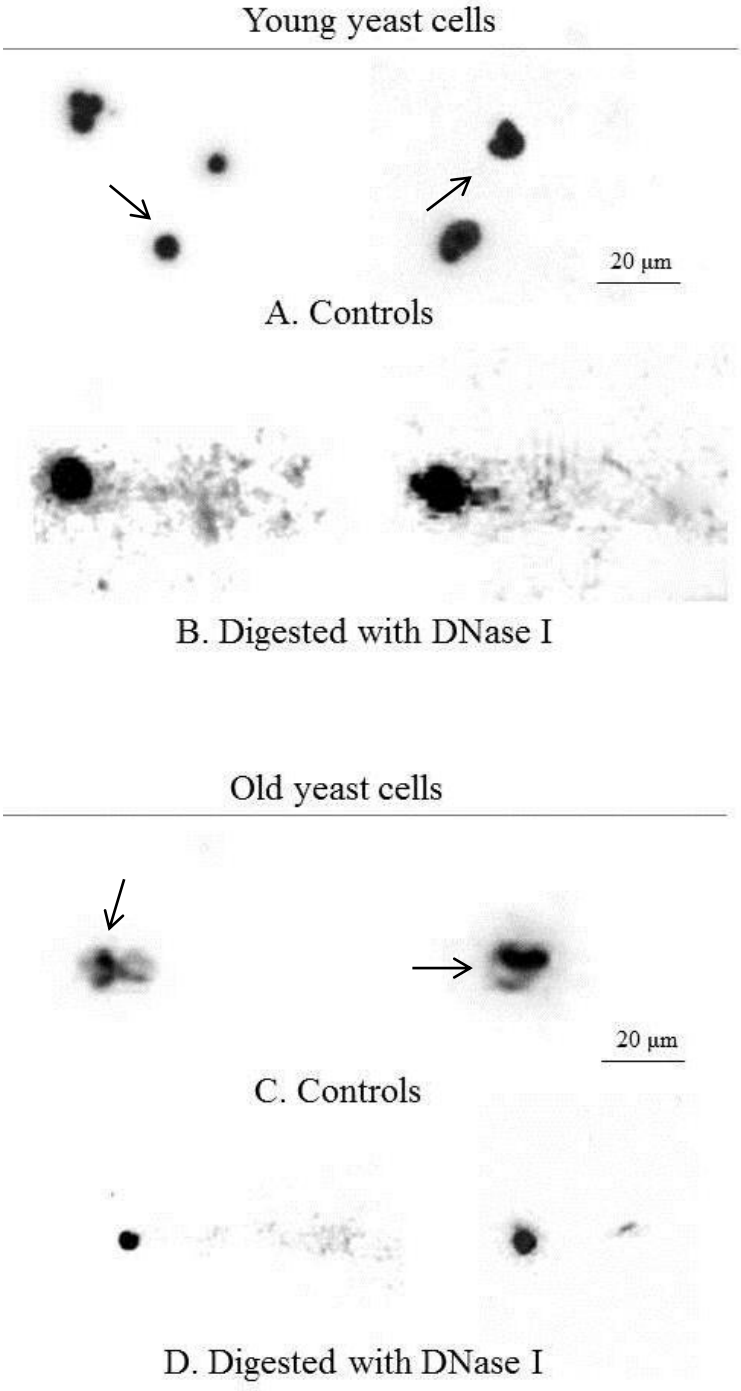

Figure 3. Chromatin Comet Assay on young and old yeast cells:

Cells were layered as microgels on microscopic slides and were *in situ* digested with DNase I and further processed to ChCA.

Young (at early logarithmic phase) and old (at late stationary phase) yeast cells gave differently shaped comet images after execution of the method of Chromatin Comet Assay (Figure 3). As seen on Figure 3 control nuclei of young cells (Figure 3A, arrows) were with regularly structured DNA visible from the rounded nuclear shapes as well as by the intensity of DNA in the residual nuclei. Conversely, nuclei from old cells (Figure 3C arrows) had altered, less intensive and distorted appearance indicative of reshaped and altered genetic material. When *in situ* digested with DNase I, a nuclease routinely used in chromatin research, young cells produced regularly extended chromatin loops (Figure 3B) which nicely formed comet tail-like structures when observed under fluorescent microscope. These results confirmed the existence of regularity in chromatin structure organization in the studied young cells, which was completely omitted in the old cells (Figure 3D). Old yeast cells, i.e. cells in late stationary phase, possessed highly condensed and less accessible to the action of DNase I chromatin as seen from the comet images on Figure 3D. These cells produced poor and less intensive comet tails and possessed highly condensed and shrunk residual nuclei.

This result are the first which show monitoring of higher-order chromatin structure in ageing cells, especially with the method of Chromatin Comet Assay and though yet preliminary they draw an interesting picture of the way chromatin is reshaped during the ageing process.

Scientists in the field of ageing research are being extensively studying the change in gene expression patterns in young and old cells [4] regarding changes in DNA methylation patterns, posttranslational modifications of histones and other epigenetic phenomena. The results strikingly show total reshaping of the epigenetic landscape of ageing cells and together with our results for alterations in the higher-order organization of the genome, detected by the method of ChCA, the overall picture of epigenetic changes that accompany ageing in eukaryotic cells gets more detailed and refined.

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GO2. CHANGES IN THE CHROMATIN STRUCTURE REVEALED BY CHROMATIN COMET ASSAY (CHCA) IN THE FRAGILE X CHROMOSOME SYNDROME MODEL *DROSOPHILA MELANOGASTER*

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Abstract

Fragile X syndrome (FXS), is the most common inherited form of mental retardation which affects approximately 1:4500 males and 1:9000 females. Subjects with FXS display learning difficulties, delayed language acquisition, impairment of fine motor skills, and behavioral deficits reminiscent of autism. The fragile X mental retardation-1 (FMR1) gene is located in the X chromosome and mutations in this gene are primarily responsible for the development of FXS. The syndrome is caused by an abnormal number of a CGG sequence in the 5' untranslated region of the gene. Pathology arises when the repeat number exceeds a critical threshold. In individuals with the full mutation of over 200 repeats, there is extensive methylation, including the CpG islands in the gene's promoter region, which results in a transcriptional silencing of the gene. The mutated gene does not produce the gene product, Fragile X Mental Retardation Protein FMRP. The protein has been shown to bind approximately 4% of the mRNA expressed in the mammalian brain including its own messenger. The fruit fly Drosophila melanogaster has a single FMR1 gene (dfmr1), as opposed to the human tripartite FMR1/FXR1/FXR2 gene family. dFMR1 is required during Drosophila brain development to control the exit from quiescence and the proliferative capacity of neuroblasts as well as the neuron production. As the core mechanisms of complex behaviors such as learning and memory and circadian rhythms appear to be conserved, studies of Fragile X syndrome using Drosophila as a model organism provide good possibilities in this respect. It represents a platform for identifying biological processes that likely underlie the abnormal morphology of dendritic spines and behavioral disturbances observed in Fragile X patients.

In this study we looked over the general organization of chromatin of neuronal cells taken from a Drosophila line overexpressing dFMR1 in the brain. Brain cells from these flies were subjected to Chromatin Comet Assay (ChCA) and compared with the higher-order chromatin organization of the wild type cells. We observed a difference in the chromatin condensation between the two lines. We speculate that it might be due to some disturbance in the interaction of dFMRP with specific mRNAs or with some chromatin proteins or both. The differentiation between these possible functions requires further clarification.

Fragile X syndrome pathobiology

The syndrome of Fragile X chromosome (FXS) is one of the most common forms of inherited mental retardation with an estimated incidence of 1 in 4,000 in men and 1 in 8,000 in women. Patients with FXS display learning difficulties, impairment of fine motor skills, deficits, reminiscent of autism, and behavioral features of mental retardation. The gene responsible for the Fragile X syndrome, FMR, is located in the X chromosome [10]. The syndrome is caused by an abnormal amplification of the CGG repeat in the 5' untranslated region of the gene. Pathology arises when the repeat exceeds a critical threshold of 200 repeats. This expansion causes hypermethylation and transcriptional silencing of the gene

[14]. FMRP is an RNA binding protein with multiple functions in the brain (for a recent review see [8]). The functions of protein product FMRP include binding to specific mRNAs and thus involvement in their transcript stability, trafficking and translation control. In individuals with the full mutation of over 200 repeats there is an extensive methylation of the CpG islands in the gene's promoter region, which results in its transcriptional silencing. The lack of FMRP is accompanied by defects in the synaptic morphology and maturation and by synaptic dysfunction. At the protein level, it is yet not clear how the loss of a single protein, FMRP, leads to mental retardation and behavioral problems. FMRP is implicated in many interesting biological phenomena, spanning from the control of local protein synthesis in neuronal processes to chromatin remodeling. The fragile X-related gene family is well conserved throughout evolution; orthologues of FMR1, FXR1 and FXR2 exist in mouse, chicken ~~and~~ Xenopus and Drosophila melanogaster.

FXS in the model organism - the fruit fly Drosphila melanogaster

Drosophila melanogaster contains a single fragile X related gene, dfmr1 [13]. It encodes a protein (dFMR1) which is required for the translational control of specific neuronal mRNAs [14]. Together with at least six other proteins dFMR1 is involved in the formation of dFMRP-RNA granules, which are believed to be result of translational repression and inhibition of dendritic branching of specialized class neurons [4].

It has also been shown that dFMR1 is involved in the cellular proliferation and may be therefore required for the exit from the quiescence state of the cells [2]. Additionally, dFMRP interacts with components of the neuronal cytoskeleton, thus affecting neuronal architecture.

Chromatin in dFMR mutants

Chromatin is the media in which all vitally important nuclear processes like transcription, replication and repair take place [11]. It is accepted that chromatin consists of several levels of compaction, transiting dynamically between one another during cellular lifetime [1;2]. The best studied is the first level of compaction, the nucleosome and nucleosome arrays. Due to the lack of reliable methods next levels of chromatin structures - namely the 30-nm fiber, chromatin loops and chromosome territories are poorly understood. It is important that changes in the chromatin structure accompany specific gene expression states which are needed for cellular differentiation and tissue development.

How the changes in chromatin are coordinated with the cell division and the cell differentiation is largely unknown. It is assumed that chromatin changes and nuclear processes must be linked in order to ensure accurate propagation of epigenetic information and maintenance of cell fate. This assumption has been recently boldly evidenced by high-throughput experiments coming from van Steensel's lab [9]. By a genome-wide analysis of 53 chromatin components (proteins and protein complexes) they separated Drosophila chromatin

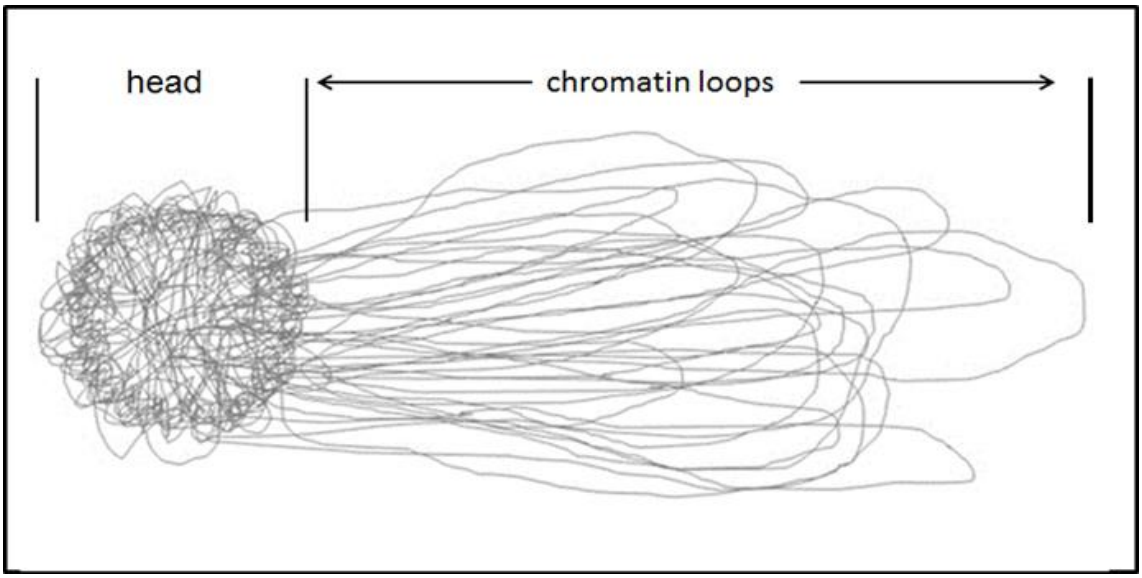

Figure 1. A schematic presentation of the chromatin organization of DNA in the method of ChCA.

into five principal types. Thus they provided evidence that the different chromatin types help to target DNA-binding factors to specific genomic regions. A wealth of information connects the organization of these chromatin domains with the modulation of their activity. For example, a repressive chromatin type that covers about half of the genome lacks classic heterochromatin markers and transcriptionally active euchromatin can be separated in two types that differ in molecular organization and H3K36 methylation and therefore regulate distinct classes of genes (for the review of these experiments see [12].

In this respect some data obtained by Drosophila dFMR1 null mutants revealed interesting phenomena. Deshpande et al. showed that dfmr1 embryos display defects in the rapid nuclear division cycles that precede gastrulation [5]. Importantly, these aberrations in nuclear division correlated with a defect in the assembly of centromeric/centric heterochromatin. The authors followed in details the distribution of the well-known mark for heterochromatin assembly – HP1. They showed that the localization of HP1 in the pole cell nuclei of dfmr1-mutants is more diffuse than in wild type. Furthermore, these authors have found that a reduction in the dose of the dfmr1 gene in the zygote is sufficient to compromise the heterochromatic silencing of a transgene inserted on the fourth chromosome. These results taken together with the physical association between dFMR1 and the components of the RNAi machinery (described in the same paper) support the idea that dFMR1 functions as a cofactor in an RNAi pathway required in the early embryos for the proper execution of the nuclear division cycles and for the assembly of functional centric/centromeric heterochromatin. Thus the conclusion is that dfmr1 is required to maintain silenced states as the fruit fly develops. Therefore, it is clear that Fmrp is capable of affecting the expression of many genes, some of which produce mRNA molecules that are direct binding targets of the FMR1 protein while others may be targets of FMR1 mediated chromatin epigenetic gene regulation.

Notably, these phenotypes have been described by the distribution of an important nuclear protein – HP1 relying on its participation in the heterochromatin formation [7]. The cause of silencing of the white gene mutation, when located to a heterochromatin region in otherwise wild type flies (position effect variegation) is another observed phenotype, affected by the mutant state of the gene dfrm1. The molecular mechanisms and the chromatin structure, underlying the different position effects in the wild type and in the mutant flies, were not investigated in this study. It is of interest therefore to use a method which can directly observe the chromatin structure and to shed more light on its changes per se.

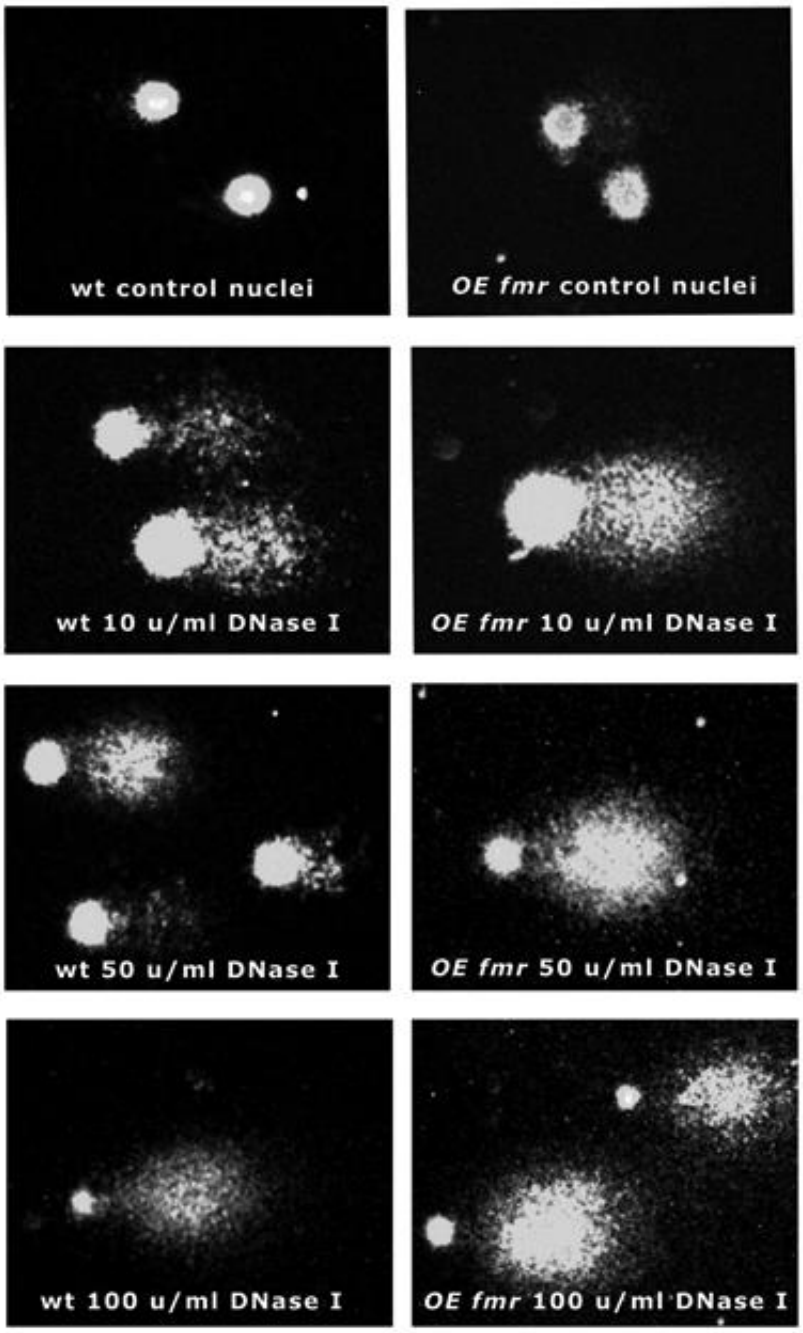

Figure 2. ChCA with *Drosophila* larvae brain cells overexpressing dFMR1.

On the other hand, until now there is no published observation of the chromatin changes in cells overexpressing dFMR1. Using the method of Chromatin Comet Assay (ChCA), developed in our lab, we were able to look at the higher order chromatin structures of Drosophila brain cells overexpressing dFMR1.

Examination of the higher order chromatin structures in Drosophila brain cells overexpressing dFMR1.

The method of Comet assay or Single Cell Gel Electrophoresis is an intricate technique for revealing damages in the DNA molecule, caused by different genotoxic agents. It has endured numerous developments and applications for different purposes starting from genotoxicology, environmental monitoring, health, etc. [6].

We developed a new variant of the Comet assay in order to make it convenient for chromatin studies. Using the potentials of the vastly explored for chromatin research nuclease DNase I, we preformed a Comet assay in which the structure and the size of chromatin loops can be assessed (Fig. 1). Because the execution of the technique has its own advantages and provides valuable information about the genome compaction it is called Chromatin Comet Assay (ChCA) (Georgieva et al., 2007; 2012).

DNase I is an enzyme that can make single-strand cuts in the DNA in the areas of the molecule where it is free from proteins. Therefore, it is very convenient to access the levels of compaction of DNA with histones and other chromatin proteins. Moreover, by means of this enzyme the work of Chromatin remodeling complexes can be followed in details. In the Chromatin Comet Assay on Drosophila cells we treated nuclei from larval brain cells, overexpressing dFMR1, with different concentrations of DNase I. The results showed that notable differences exist between the chromatin organization of these cells and the cells from

the normal (wild type) larval brains. As can be seen from Fig.2, the chromatin loops in the comet tails from cells, overexpressing dFMRP and appear denser and longer.
We argue that the chromatin of such cells is more relaxed and accessible to the nuclease than the chromatin of normal, wild type cells.

Therefore, we speculate that the protein dFMR1 by itself or by regulating the expression of other proteins, participates in the organization of the higher order chromatin structures such as loops. Although it is tempting to assume that the participation of dFMR1 in the chromatin organization is direct, we favor the idea that dFMRP might bind mRNAs , encoding other proteins, and change their metabolism. These proteins, most probably, play roles in the building and maintenance of chromatin. Our results, although preliminary, show that the roles of dFMR1 in chromatin are quite complex and should be studied in details.

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GO3. IAPs, SMAC AND THEIR ROLE IN APOPTOSIS

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Introduction

Apoptotic cell death (form of programmed cell death) is an essential mechanism for maintaining homeostasis and regulation of physiological growth control in multicellular organism. Apoptosis can be triggered by a wide range of stimuli that are encountered during normal and pathophysiological processes. These include death receptor agonist, alteration in temperature and osmolarity, DNA damaging agents, free oxygen radicals, removal of nutrients, oxygen or growth factors, pro-inflammatory cytokines, ischemia and accumulation of misfolded ER proteins (endoplasmic reticulum proteins). There are two well-known apoptotic pathways – extrinsic and intrinsic. Both pathways lead to activation of caspases that represent the main executioner molecules of the apoptotic process [8].

The extrinsic pathway also known as death receptor pathway is mediated by a subgroup of Tumor Necrosis Factor receptors (TNFR) superfamily that includes TNFR, Fas and TRAIL-R. Binding of death ligands (TNFα, FasL, TRAIL) to their receptors results in assembly of death-inducing signaling complex (DISC). Formation of DISC leads to recruitment and activation of initiator caspases -8 and -10. They subsequently cleave and activate downstream effector caspases (caspase-3 or -7) and also cleave the pro-apoptotic Bcl-2 member Bid to its activated tBid form [4, 8]. The effector caspases in their turn cleave different cell substrates (actin, nuclear lamin; DNA-dependant protein kinases; ICAD and DFF45 etc.) and dismantle the cell to apoptotic bodies. Upon cleavage tBid translocates to the mitochondria, where it triggers MOMP (mitochondrial outer membrane permeability) through activation of pro-apoptotic Bax/Bak proteins (other Bcl-2 members). That is one of the cross-talks between the extrinsic and intrinsic apoptotic pathways [4, 8].

The intrinsic pathway also called mitochondrial pathway is largely centered and regulated by mitochondria. Different apoptotic signals induce activation of Bax/Bak proteins which in turn form pores in outer mitochondrial membrane. Via these pores some pro-apoptotic proteins such as cytochrome c and Smac (Second mitochondria-derived activator of caspases) are released simultaneously from intermembrane space into the cytosol. In presence of dATP/ATP, cytochrome c and Apaf-1 (apoptotic protease activating factor-1) interact and form a complex known as apoptosome. Initiar procaspase-9 is recruited to the apoptosome and activated and in turn activates effector caspase-3 and -7. It is widely accepted that activation of effector caspases is another point of convergence of the two apoptotic pathways [8].

Apoptosis is a highly regulated mechanism engaging complex interaction between pro-apoptotic (Smac, Bcl-2 pro-apoptotic member, etc) and anti-apoptotic (anti-apoptotic

members of Bcl-2 family, c-FLIPs proteins, IAPs protein family) molecules and regulation of caspase activity. For example IAPs proteins (Inhibitor of Apoptosis Proteins) are inhibitors of both apoptotic pathways while Smac exerts its pro-apoptotic activity by acting as an endogenous inhibitor of IAPs proteins Dysregulations of apoptotic pathways can lead to severe pathological consequences. Up-regulation of apoptosis contributes to neurodegenerative disorders. Suppression of apoptosis causes autoimmune diseases and is hallmark of cancer [9]. The ability of cancer cells to evade apoptosis is related to various biochemical properties of these cells, and particularly, to the up-regulation of anti-apoptotic genes such as certain members of the Bcl-2 family and members IAP protein family [7, 9].

IAPs proteins (**I**nhibitor of **A**poptosis **P**roteins)

Particularly, three lines of evidence support a role for IAP proteins in cancer: (1) elevated expression levels of IAP proteins (XIAP, c-IAP1 and c-IAP2), in a number of human cancer types correlate with tumor grade; (2) down-regulation of XIAP or c-IAP1 by various agents results in sensitization of cancer cells to chemotherapy- and γ-induced apoptosis; (3) c-IAP1 and c-IAP2 genes are subject to chromosomal amplification in various tumors.

IAPs proteins were first described in baculoviruses that use them to suppress host cell's apoptosis. IAPs are highly evolutionary conserved proteins from Drosophila to vertebrates. Eight mammalian IAPs proteins are known – X chromosome- linked IAP (XIAP), cellular IAP1 and IAP2 (c-IAP1 and c-IAP2), neuronal apoptosis inhibitory protein (NAIP), survivin, BRUCE, livin, and testis-specific IAP (Ts-IAP). IAPs differ extremely in size, ranging from 102 amino acids (survivin) to 4845 (BRUCE). All IAPs are characterized with structural motifs called baculoviral IAP repeat (BIR) domains. BIR domain is 70–80-amino-acid cysteine- and histidine-rich domain that chelates zinc ions. The number of BIR domains in a given IAP varies from one to three. BIR domains are located in the N-terminal region and mediate the interaction with caspases (particularly BIR2 and BIR3 domains). Beyond caspases, BIR domains bind other proteins such as borealin and aurora B kinase (cell division), TRAF2 (TNF-receptor superfamily intracellular signaling) etc. Beyond BIR domains, a C-terminal really interesting new gene (RING) domain and ubiquitin-associated (UBA) domain are found in individual IAPs (XIAP, c-IAP1 and c-IAP2). RING domain exhibits ubiquitin E3 ligase activity so IAPs are capable of promoting ubiquitination and proteasomal degradation of themselves and their binding partners. In addition c-IAP1 and c-IAP2 possess and a CARD (caspase associated recruitment domain). The best studied proteins are c-IAP1, c-IAP2, XIAP and are recognized as regulators of apoptosis. All of these proteins have three BIR domains and RING domains. The corresponding BIR domains in XIAP, c-IAP1 and c-IAP2 are highly conserved in both sequence and function. For example BIR3 of XIAP is more similar to BIR3 of c-IAP1 and c-IAP2 than to BIR2 and BIR1 of XIAP. As far as other IAPs, Bruce and Survivin are considered to be regulators of mitosis [5].

Anti-apoptotic IAPs members inhibit both apoptotic pathways. The best-investigated and most potent inhibitor of the IAP family is XIAP. XIAP directly inhibits both initiator and effector caspases via its BIR2 and BIR3 domains through distinct mechanisms. The XIAP-BIR2 domain as well as linker residues upstream of XIAP-BIR2, contribute to inhibition of caspases-3 and -7. By contrast, the XIAP-BIR3 domain selectively binds and inhibits initiator caspases-9. In addition to its role as a direct caspase inhibitor, XIAP can block apoptosis through its RING domain [9].

Structural analyses have revealed the precise mechanism of XIAP-mediated inhibition of caspase-3 and -7. The linker peptide (18 amino acids residues long) occupies the active site of caspases resulting in a blockade of a substrate entry. Caspase active site has binding pockets for the P4-P3-P2-P1 residues of the substrates named S4-S3-S2-S1, respectively. Asp 148 of XIAP, critical for inhibition of caspase-3, binds the S4 pocket in same manner as P4

residue of covalent peptide inhibitors. Val146 occupies S2 and Gly144 is located close to the S1 pocket. Although linker sequence plays dominant role in inhibition when it is isolated, this fragment is insufficient. Linker peptide needs to be led in an "active" conformation by a surrounding BIR domain [9].

Interaction between XIAP and caspase-9 involves two steps – anchoring interaction with a caspase N-terminal tetrapeptide and heterodimerization of BIR3 with caspase-9. XIAP-BIR3 binds to the Ala-Thr-Pro-Phe tetrapeptide of the small subunit of caspase-9 that is exposed after activation of the caspase. Therefore BIR3 blocks the protease's active site. Mutation of Trp310, Glu314 or His343 in BIR3 entirely abolishes XIAP-mediated inhibition of caspase-9. On the other hand crystal structural analysis of caspase-9 – BIR3 complexes reveal that BIR3 also prevents caspases-9 dimerization by interacting with the caspase's dimerization interface [9].

c-IAP1 and c-IAP2 were originally identified by their association with TNF receptor 1- and 2-associated complexes via TRAFs (TRAF2). c-IAPs and TRAF2 molecules interact through their BIR1 and TRAF-N domains, respectively. Although they might bind to caspases-7 and -9, they cannot directly inhibit caspase proteolytic activity Therefore it has been suggested that they might regulate apoptosis indirectly, by influencing receptor-mediated apoptosis [13]. Functions of c-IAP1 and c-IAP2 were identified by using IAC molecules (IAP antagonist compounds). IACs induce proteasomal degradation of c-IAPs but no detectable loss of XIAP or TRAF2. IAC binds BIR2 and BIR3 domains of c-IAPs, which is speculated to induce conformational changes in c-IAPs molecules. That in turn stimulates cIAPs auto-ubiquitination and rapid proteasome-dependent degradation that occurs in 5 minutes. Degradation depends upon c-IAP's RING domain and cellular E1, E2 and ubiquitin. Mutation of one of the zinc-coordinating residues in the RING domain of c-IAP1 (histidine 588) to alanine prevents its E3 ligase activity. Removal of c-IAP1 leads to activation of canonical and non-canonical NF-κB signaling pathways. cIAP-1 and -2 are recruited to the proximal TNFR1- complexes where they cooperate with TRAF2 to suppress caspase-8 activation. Their degradation likely alters signaling to favor a TNF-R1 complex 2 and caspase-8 activation. On the other hand TNFα is produced in response to IAC-induced NF-kB activation. Thus autocrine increased levels of TNFα kill cells through enhanced apoptotic TNF-R1 signaling [10, 11].

IACs trigger noncanonical NF-kB pathway. Noncanonical NF-kB signaling is largely controlled by NIK (NF-κB-inducing kinase), a highly labile ser/thr kinase. NIK phosphorylates IKKα, leading to phosphorilation of p100 and subsequent processing to p52. It has been reported that c-IAP1 and c-IAP2 are the ubiquitin ligases responsible for NIK proteasomal degradation resulting in blocking that pathway. It is suggested that TRAF2 provides a critical scaffolding link between the c-IAP1 and its substrate NIK. IAP antagonists, promote c-IAPs degradation resulting in NIK stabilization and activation of the noncanonical NF-kB pathway. It has also been demonstrated that cytokine-mediated physiological pathways that trigger the processing of NF-kB2 (p100), involve the degradation of c-IAP1. These findings establish c-IAPs as seminal mediators of signaling pathways that activate NF-kB [10, 11].

Smac (**S**econd **m**itochondria-derived **a**ctivator of **c**aspases)

Natural IAP antagonists include Grim, Reaper, Hid proteins (RHG) in Drosophila and Smac in mammals. Second mitochondria-derived activator of caspases (Smac), also termed DIABLO (direct IAP-binding protein with low pI) is an endogenous inhibitor of IAPs. Initially Smac was defined as a factor that promotes caspase-3 activity, along with other soluble factors (Apaf-1, cyt. c, procaspase-9). However when Smac was administered alone in a cell-free test system, it didn't show any pro-caspase-3 processing activity. Now it is known

that Smac exerts its pro-apoptotic effect through interactions with IAPs, resulting in releasing the inhibitory effect of XIAP upon active caspases (-3, -7 and -9). Smac also antagonizes the ubiquitin ligase activity of IAPs and thereby stimulates their autoubiquitination and proteasomal degradation [2].

Normally Smac is localized in mitochondrial intermembrane space, but it is released into the cytoplasm in response to apoptotic stimuli, similarly to cytochrome c. A recent study shows that overexpression of XIAP can cause Smac mitochondrial retention. That prolonged release refers selectively to the dimeric but not the monomeric Smac molecules [3].

Smac protein is encoded by a nuclear gene and includes 239 amino acids. By Edman degradation and direct sequestration analysis it is established that first 55 amino acids are mitochondrial-targeting signal (MTS). This MTS is subject to proteolytic cleavage during Smac processing in mitochondria. Thus, it is exposed a tetrapeptide sequence of four hydrophobic amino acids, Ala-Val-Pro-Ile, at the N-terminus of the mature Smac. AVPI tetrapeptide itself is the major IAP-binding motif (IBM) in mammals, and fruit flies. Structural analysis revealed that AVPI motif binds to the highly conserved sequence of binding-surface-groove on XIAP-BIR3 and Ala, plays a major role in this interaction. Replacement of Ala to Met or Gly abrogates Smac function [9].

Functionally active Smac protein is a stable homodimeric molecule with a length of 130 Å and rigid, expanded arch-shaped form. Smac is associated with various IAPs (c-IAP1, c-IAP2, XIAP, survivin). Dimeric Smac protein binds to both BIR2 and BIR3 domains of IAPs, but not to BIR1, via its two AVPI motifs. In contrast monomeric Smac mutants bind BIR3, but cannot form a stable complex with BIR2 domain, that makes them weaker or insufficient IAP-inhibitors [5, 9].

AVPI competes Ala-Thr-Pro-Phe tetrapeptide of caspase-9 for XIAP-BIR3 binding groove resulting in release inhibitory effect of XIAP. X-ray structures of AVPI-BIR3 complex reveal amino acids of BIR3 (Gly306, Leu307, Thr308, Asp309, Trp310, Lys297, Lys299, Glu314, Gln319, Trp323, Tyr324) engaged in the interaction. These amino acids form a hydrophilic and two hydrophobic binding pockets for AVPI. For example, hydrophilic region is generated by Glu314, Gln319, Trp323 and Thr308. So, free NH_2 group of AVPI can form strong H-bonds to Glu314 and Gln319 on BIR3. On the other hand there are two hydrophobic regions – larger and smaller ones. The larger one is due to the side chains of Trp323 and Tyr324, whereas the smaller hydrophobic pocket is formed by Leu292, Val298, Lys297 and Lys299. The side chains of Val and Pro of AVPI, lie over the larger hydrophobic region, whereas the side chain of Ile lies over the smaller region. These three regions could be considered as the pharmacophore fundamental for the interaction (Figure 1) [5].

The overall mechanism of binding of BIR2 domains resulting in derepression of caspase-3 and -7 remains unclear. Binding to the BIR2 domain requires not only the AVPI tetrapeptide but also an extensive surface available only in the dimeric Smac protein. Smac-BIR2-caspase-3 complex suggest that steric clashes preclude XIAP-BIR2 from simultaneously binding to caspase-3 and Smac [9].

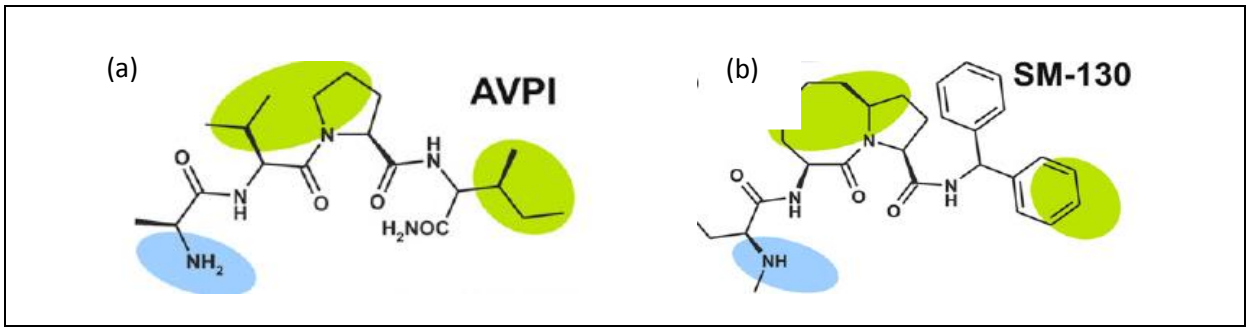

Figure 1. Comparison of the XIAP-BIR3 binding pocket in complex with (a) AVPI and (b) with SM-130 (Smac-mimetic). The figure is from ref. [5].

As a pro-apoptotic molecule, levels of Smac are quite important in determining the sensitivity of cancer cells to apoptosis induced by diverse stimuli. It could be expected that cells with lower Smac expression show higher apoptosis resistance and are selected during cancer progression. Interestingly there are established both inverse correlation between Smac expression levels and cancer progression (Renal Cell Carcinoma (RCC), hepatocellular carcinoma (HCC), lung cancer, testicular germ cancers etc) and a direct correlation (cervical cancer; gastric adenocarcinomas). Therefore expression levels of Smac could be useful as a prognostic or therapeutic marker or to provide new targets for drug design and directed therapy aimed to inhibit IAPs functions [6].

Several studies have shown that overexpression of Smac sensitizes neoplastic cells to apoptotic death. These findings prompted the development of Smac mimetics as therapeutic agents. It is shown that permeable Smac mimetics sensitize cancer cells to apoptosis mediated by B granzyme (Hodgkin lymphoma cells), antineoplastic agents (breast cancer and glioblastoma cell lines), death factors such as TRAIL and TNF-α and are able to induce apoptosis by itself in cells, which have high expression levels of XIAP and c-IAP1. These results show that Smac mimetics could be useful as adyuvant therapy or as stand-alone therapy [6].

In the past decade, a broad spectrum of chemically diverse IAP antagonists has been developed. They are all called IAC (IAP antagonist compounds) and are grouped as follows: BIR3– antagonists (including monovalent Smac mimetics, aminothiazoles and embelins); BIR2– antagonists (polyphenylureas and arylsulfonamides); and BIR2–BIR3-inhibiting bivalent Smac mimetics. Smac mimetics represent by far the largest fraction of IACs. [5].
Monovalent Smac mimetics were designed to mimic the Smac AVPI-binding motif. They exhibit high affinities to XIAP, cIAP1, cIAP2 and ML-IAP proteins. Monovalent Smac mimetics could be subdivided to acyclic and cyclic molecules (bicyclic and tricyclic). Bivalent Smac mimetics contain two modified AVPI-binding motifs connected via appropriate linkers. They bind to XIAP-BIR2–BIR3 with an extremely high affinity, exceeding that of monovalent Smac mimetics and Smac protein itself [5].

It was also established that different IACs target IAPs molecules differently. Varfolomeev et al. designed monovalent (MV1) and bivalent (BV6) Smac mimetics. It is established that BV6 binds intramolecularly to both the BIR2 and BIR3 domains of XIAP. However BV6 binding to c-IAP1 is different. BV6 binds the BIR3 domains of two c-IAP1 molecules inducing c-IAP1 dimerization [10].

Smac mimetics are reported to be very efficient in the induction of apoptosis in tumorigenic cells as single agents and sensitizing cancer cells to different therapeutic agents (cisplatin, doxorubicin, etoposide, TRAIL, etc). A variety of Smac mimetics and peptides are tested in preclinical studies [1, 7]. There are also Smac mimetics (LCL161; GDC-0917; AT-406 – monovalent mimetics; HGS1029 – bivalent mimetic) that have been entered in phase I clinical studies. During experiments it was found that Smac mimetics effects depend on some

factors. The effects are inverse correlated with Bcl-2, c-FLIP$_L$ and caspase-3 protein levels and direct correlated with cellular IAPs levels [1, 7].

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GO4. ESTABLISHMENT OF STABLY TRANSFECTED MDCK CELL LINE EXPRESSING WILD TYPE OF HBEST1 PROTEIN

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Abstract

Best vitelliform macular dystrophy (BVMD) is an autosomal dominant disorder with onset generally in childhood with the striking appearance of a yellow yolklike lesion in the macula. Affected individuals initially have normal vision followed by decreased central visual acuity and normal peripheral vision. BVMD is due to mutations of gene *BEST1* on chromosome 11. *BEST1* is responsible for bestrophin-1 (Best1) protein synthesis. Bestrophin-1 is a transmembrane protein and localizes on the basolateral cell membranes of the retinal pigmented epithelium only. Best1 is thought to be an ion channel or a regulator of ion transport, or both. The exact structure and functions of the protein and the pathogenesis of BVMD are still under discussion. For a better understanding of structure and functions of bestrophin-1 protein it is important to establish a model system with an appropriate bestrophin expression.

The aim of our work was to establish a model system stably expressing hBest1. We transfected MDCK II cells with a vector (p. Receiver) containing wild type of h*BEST1* gene. After transfection a single cell colonies were isolated and analyzed by immunofluoresce staining and Western blot. The positive cells were cultured to complete confluence on Petri-dish.

The expression of Best1 was stable even after 25 passages and after freezing and thawing. We consider that this stably transfected cell line can be used as an appropriate model for clarifying of Best1 functions and analyzes of effects of different mutations of the protein.

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GO5. VISUALIZATION OF SYNAPTIC CIRCUITS

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Abstract

In order to analyze in details the neuronal network architecture different methods, based on genetical labeling of neurons with distinct colors, were developed. The combination

of two fluorescent proteins (FPs) in one animal and their stably expressing in a mosaic manner has been achieved by: generation of chimeric mice mixing stem cells of two colors; selection and crossing of mouse lines, which showed a variegated expression of yellow fluorescent protein and cyan fluorescent protein or crossing over, where FP genes were split. The first two approaches are cumbersome, and all three generate only a limited palette of colors. Livet et al (2007) designed two genetic strategies, called Brainbow, for stochastic expression of multiple fluorescent proteins from a single transgene, using Cre/lox recombination. The differential expression of multiple copies of these constructs generates FP mixtures, allowing the labeling of individual neurons and glia with as many as 90 distinguishable colors. The expression in some lines also allowed the scientists to map glial territories and follow glial cells and neurons over time in vivo. The ability of the Brainbow system to label uniquely many individual cells within a population may facilitate the analysis of neuronal circuitry on a large scale.

GP1. ПРЕЦИЗЕН МЕТОД ЗА ИЗВЛИЧАНЕ НА ДНК ОТ БИОЛОГИЧНИ СЛЕДИ ВЪРХУ АМУНИЦИИ ЗА ЦЕЛИТЕ НА ГЕНЕТИЧНОТО ПРОФИЛИРАНЕ

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GP2. ГЕННА ТЕРАПИЯ И ДИАБЕТ

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Session H.

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HO1. EFFECTS OF LOW SELENIUM INTAKE ON ENDOCRINE SYSTEM

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Abstract

Selenium (Se) is an essential trace element that performs its physiological action through selenoproteins. Three major groups of selenoproteins are described: Glutathione peroxidases (GPx), Thioredoxin reductases (TrxR), and Iodothironine deiodinases (D). The biological role of selenoproteins in mammals includes an optimal endocrine and immune function, prevention of the cardiovascular and neoplastic diseases. Tissue expression of these enzymes depends on daily Se intake. Selenium enters the organism mainly with food. It has been established that a diet containing 0.1μg Se/g of food is enough for normal growth and reproduction. Se- dependant cellular GPx-1 is the most abundant intracellular isoform of the GPx antioxidant enzyme family and plays a major role in the control of reactive oxygen species. TrxR catalyze NAD(P)H-dependant reduction of oxidized thioredoxin and participate in different redox systems. Iodothironine deiodinases regulate normal three-iodothyronin (T_3) plasma level.

A general observation during Se depletion was the retention or redistribution of Se to the brain, the endocrine organs, and the reproductive organs, whereas liver, muscle, skin, and other large tissues rapidly lose their Se.

GPx1, GPx3 and TrxR1 increased expression must occur during synthesis of thyroid hormones because of protective action on thyroid cells against oxidative injury. D1 in rats and D2 in humans also increase to accelerate production of bioactive T_3. Se deficiency decreases expression of these selenoproteins and may induce thyroid destruction.

B-cells of Islets of Langerhans in pancreas are sensitive to oxidative stress while showing a low capacity of antioxidative systems. Se-deficient animals have low serum insulin

levels, and their islet cells show impaired protein secretion that is normalized by Se and vitamin E treatment.

Se deficiency causes a marked decrease in GPx activity in an adrenal cell line associated with decreased steroid hormone production.

Developmental studies in rats showed changes associated with Se deficiency, *e.g.*, changes in the morphology of spermatids and spermatozoa, and finally, complete absence of mature germinal cells.

Se deficiency leads to degeneration of ovaries and atresy of follicles. *In vitro* studies revealed that Se supply and expression of GPx activity, together with other antioxidative enzymes, assist in ovarian function regulation by Foliculo-stimulating hormone.

Se-deficient male rats were shown to develop osteopenia and impaired bone metabolism, growth retardation, and reduction of Growth hormone levels. Reduced activity of deiodinases in the pituitary may be one reason for these findings.

Keywords: *selenium deficiency, endocrine system*

HO2. URINARY BLADDER MOTILITY IN EXPERIMENTALLY INDUCED DIABETES MELLITUS

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Introduction

Diabetes mellitus and insulin deficiency concerns the whole organism and leads to a number of complications particularly in cardiovascular, nervous and excretory systems. Diabetic bladder dysfunction is among the most common and costly consequences of diabetes. Angiotensin II (Ang II) and Arginine - Vasopressin (AVP) are important regulators of the smooth muscle tone, acting by incensement of calcium concentration. As calcium sensitivity of smooth muscles in experimental diabetes is reported to be altered, together with a bladder hyper reactivity, it is interesting how diabetes changes the detrusor response to these peptides.

Aim

To investigate the changes in the contractile response of diabetic urinary bladder due to influences with Angiotensin II, Arginine-vasopressin and calcium excess.

Materials and methods

Wistar rats from both sexes were used. Diabetes was induced by a single intraperitoneal injection of 60 mg/kg Streptozotocine. Only those animals, which had more than 16 mmol/l blood glucose levels on the 72-hour of the injection, were considered to be diabetic. Six weeks after the diabetes induction, smooth muscle preparations of the urinary bladder were prepared and examined by the isolated tissues method. For a control healthy rats of the same age were used. The smooth muscle preparations were influenced by Ang II and AVP in dose of 1 μmol, and also by addition of 10 mmol $CaCl_2$. The following parameters

were analyzed: amplitude, integral force (AUC) and time-parameters of the contraction: T_{hc} (half-contraction time), T_c (contraction time), T_{hr} (half-relaxation time), T_{chr} (contraction plus half-relaxation time).

Results and discussion

Regarding to Ang II-provoked contraction, no changes were observed in the contractile activity parameters of the diabetic bladder, with the exception of T_c, which was significantly prolonged (63.39 ± 9.30g vs 32.25 ± 3.30g). On the other side, the development of AVP-mediated contraction was significantly altered in diabetic detrusor: reduced AUC (257.49 ± 43.39 gs vs 871.54 ± 90.99 gs); shorter T_{hr} (89.11 ± 22.15 vs 255.30 ± 35.13) and T_{chr} (207.94 ± 23.07 vs 382.70 ± 43.09). Most interesting changes were registered in the response to $CaCl_2$, where reduced AUC and T_{chr} in combination with faster T_{hc} (11.94 ± 3.13 vs 41.44 ± 10.89) and T_c (69.33 ± 11.96 vs 166.13 ± 26.43), were established. The divergent results in the response to calcium excess may be due to an interaction of harmful and compensatory processes in the diabetic bladder.

Conclusion

Despite the similar pattern of action of Ang II and AVP and the involvement of calcium in the development of the smooth muscle process, in diabetic detrusor the response to AVP is significantly affected.

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НОЗ. СУБМАКСИМАЛНА ИЗДРЪЖЛИВОСТ И ВРЕМЕ ДО ИЗТОЩЕНИЕ НА ПЛЪХОВЕ, ПОДЛОЖЕНИ НА КОМБИНИРАНА ВИСОКО ЛИПИДНА И ВИСОКО ВЪГЛЕХИДРАТНА ДИЕТА БЕЗ ДОБАВЕН ХОЛЕСТЕРОЛ

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Абстракт

Нарастването на честотата на свръхтеглото и затлъстяването се свързва както с нерационалния хранителен режим така и с намалената физическа активност през последните десетилетия. Проучването имаше за цел да изследва промяната на някои функционални показатели при приложение на четири месечна диетична програма при плъхове. В експеримента се използваха мъжки плъхове Wistar с начална телесна маса 160-180 g. Животните бяха разделени на две групи: контролна група (C, n=24), приемаща стандартна храна за плъхове и експериментална група (D, n=24), която имаше свободен достъп до високо липидна и високо въглехидратна храна, без добавен холестерол. В началото и на всяка 4-та седмица бяха определяни субмаксималната издръжливост и максималното време до изтощение. В началото на проучването максималното време до изтощение при група C (n=24) беше 13,19±0.45 min, а при група D (n=24) бе 14.37±0.36 min, P>0.05. На 4-та седмица животните от група C (n=24) бягаха 14.92±0.38 min, а от група D (n=24) 15.17±0.31 min, P>0.05. Получените резултати от 8-ма седмица бяха 17.71±0.63 min (група C, n=24) и 12.94±0.53 min (група D, n=24), P<0.05. На 12-та седмица животните от група C (n=13) бягаха 14.21±0.69 min, а от група D (n=16)12.21±0.45 min, отново със сигнификантна разлика. Резултатите от 16-та седмица бяха 13.32±0.62 min (група C, n=7) и 09.60±0.52 min (група D, n=9), P<0.001. Резултатите от тестовете за субмаксимална издръжливост не показаха сигнификантни разлики между експерименталните групи. 16-седмичното приложение на комбинирана високо липидна и високо въглехидранта диета без добавен холестерол не промени субмаксималната издръжливост, но намали времето до изтощение при диетично манипулираната група от 8-ма седмица до края на експерименталния период.

Увод

Нарастването на честотата на свръхтеглото и затлъстяването се свързва както с нерационалния хранителен режим така и с намалената физическа активност през последните десетилетия [5, 9, 11, 12]. Успоредно с това расте честотата и на дислипидемията, нарушенията във въглехидратната обмяна, сърдечносъдовите заболявания, захарен диабет и др. социално значими здравни проблеми [4]. Ефектът на диетичния режим върху физическата работоспособност е широко изследван при хора и при плъхове. Влияние оказва състава на приеманата храна и продължителността на нейното приложение [6, 10]. Някои проучвания сочат, че физическата издръжливост при плъхове може да се повиши само чрез продължително прилагане на високо липидна диета, но метода не може да се препоръча за подобрение на издръжливостта

при хора [6]. Кратко приложение на високо липидна диета при плъхове намалява субмаксималната издръжливост и води до когнитивни нарушения [10].

Цел

Проучването имаше за цел да изследва промяната на физическата работоспособност при приложение на четири месечна диетична програма при плъхове. То е част от комплексно изследване на изменението на функционални, метаболитни, клинично-химични и хистологични показатели на различни етапи от диетично индуциране на Метаболитен синдром при плъхове.

Материали и методи

Животни

В експеримента се използваха мъжки плъхове, порода Wistar, с начална телесна маса 160-180 g, които бяха взети от вивариума на Медицински Университет-Пловдив. Плъховете имаха достъп до вода и храна *ad libitum* и бяха настанени в индивидуални метаболитни клетки. Те бяха отглеждани при температура 20 ºC ± 1 ºC, контролирана влажност и 12:12 h светъл-тъмен фото-период. Целият експериментален протокол беше одобрен от Комисията по етично отношение към животните, към Българската агенция по безопасност на храните. Плъховете бяха отглеждани и всички експериментални процедури бяха извършени съобразно препоръките на Европейската комисия за защита и хуманно отношение към лабораторните животни. Всички плъхове начално бяха хранени със стандартна храна за две седмици, докато се адаптират към метаболитните клетки. За провеждане на функционалните тестове беше необходимо животните да бягат на тредмил. Тъй като бягането на тредмил е умение, което плъховете трябва да развият и поддържат, преди началото на експеримента всички плъхове бяха тренирани на тредмил за малки опитни животни (Columbus Instruments, Columbus, Ohio, USA) за 5 min, три пъти седмично. Такова натоварване не предизвиква адаптационни промени, но води до привикване на плъховете към бягането на тредмил и позволява селекция на животните, които бягат спонтанно [3, 7]. Около 14% от плъховете отказаха да бягат на тредмил и бяха изключени от проучването в края на предварителния етап. Останалите животни бяха разделени на две групи: контролна група (C, n=24), приемаща стандартна храна за плъхове и експериментална група (D, n=24), която имаше свободен достъп до високо липидна и високо въглехидратна храна. За поддържане на умението да бягат на тредмил, всички животни бяха тренирани за 5 min, три пъти седмично, със скорост 27 m/min, при наклон на лентата 5º (около 70-75% от VO_{2max}) за периода на експеримента.

Диета

Контролната група приемаше стандартна храна *ad libitum* за лабораторни плъхове (Белтъци: Масти: Въглехидрати – 19.5: 4.0: 61 mg%, - 18.5: 10.5: 71 En%), предоставена от вивариума на МУ-Пловдив, произведена от фабрика за фуражи АМИКО ООД- с. Белозем. Диетично манипулираната група имаше свободен достъп до комбинирана високо липидна и високо въглехидратна храна, произведена от фабрика за фуражи АМИКО ООД- с. Белозем, допълнена с растителни мазнини (Белтъци: Масти: Въглехидрати – 15.2: 15.35: 64 mg%, - 14: 30: 56 En%). Комбинираните високо липидни и високо въглехидратни диети при плъхове водят до развитие на затлъстяване, инсулинова резистентност и нарушения в мастния метаболизъм, което ги прави подходящи за индуциране на МетС [1, 2, 8].

Функционални тестове

В началото на опита и на всяка 4-та седмица от експерименталния период животните бяха подложени на тест за субмаксимална издръжливост по Lambert [7]. Беше използван тредмил за малки опитни животни (Columbus Instruments, Columbus, Ohio, USA).Субмаксималната издръжливост беше определена при бягане на тредмил със скорост 27 m/min и 5º наклон. Отчиташе се времето на достигане до пълно изтощение и невъзможност да се запази по-дълго позицията на плъха върху лентата.

В началото на експеримента, на 4-та, 8-ма, 12-та и 16-та седмица животните бяха подложени на тест за определяне времето до изтощение по. Пиково натоварване се постигна със стъпаловидно покачване на скоростта на движение и наклона на лентата на тредмила [3]. Плъховете бяха премахвани от теста когато не можеха повече да задържат позицията си на тредмила.

Всички тестове се проведоха след два дни възстановителен период от адаптационните тренировки.

Статистическа обработка

Резултатите са представени като средно аритметично ± SEM. Данните от експеримента са анализирани със Student *t*-test, статистическа програма SPSS v. 13.0.

Резултати и обсъждане

В началото на експеримента субмаксималната издръжливост при група C (n=23) беше 52±7.57 min, а при група D (n=23) бе 60.74±4.48 min, P>0.05. На 4-та седмица животните от група C (n=22) бягаха 41.14±3.54 min, а от група D (n=22) 46.81±3.35 min, отново без сигнификантна разлика. Получените резултати от 8-ма седмица бяха 35.83±2.76 min (група C, n=24) и 38.78±2.40 min (група D, n=23), P>0.05. На 12-та седмица животните от група C (n=16) бягаха 22.81±2.47 min, а от група D (n=15) 20.53±1.00 min, отново без сигнификантна разлика. Резултатите от 16-та седмица бяха 17.43±0.92 min (група C, n=7) и 15.00±1.41 min (група D, n=8), P>0.05. Резултатите от тестовете за субмаксимална издръжливост не показаха сигнификантни разлики между експерименталните групи.

Максималното време до изтощение при група D беше по-ниско на 8-ма седмица и се запази така до края на експерименталния период. В началото на експеримента максималното време до изтощение при група C (n=24) беше 13,19±0.45 min, а при група D (n=24) бе 14.37±0.36 min, P>0.05. На 4-та седмица животните от група C (n=24) бягаха 14.92±0.38 min, а от група D (n=24) 15.17±0.31 min, отново без сигнификантна разлика. Получените резултати от 8-ма седмица бяха 17.71±0.63 min (група C, n=24) и 12.94±0.53 min (група D, n=24), P<0.05. На 12-та седмица животните от група C (n=13) бягаха 14.21±0.69 min, а от група D (n=16)12.21±0.45 min, отново със сигнификантна разлика. Резултатите от 16-та седмица бяха 13.32±0.62 min (група C, n=7) и 09.60±0.52 min (група D, n=9), P<0.001.

Данните относно влиянието на хранителния режим върху някои показатели на физическата работоспособност са непълни и противоречиви което дава основание за следващи проучвания в тази област [6, 10]. В това изследване се проследиха промените в субмаксималната издръжливост и времето до изтощение на различни етапи от индуцирането на метаболитен синдром с продължителна комбинирана високо липидна и високо въглехидратна диета без добавен холестерол. Четири месечното приложение на комбинирана високо липидна и високо въглехидратна диета, без добавен холестерол не промени съществено субмаксималната издръжливост на плъховете. Максималното време до изтощение беше сигнификантно намалено при диетично манипулираните експериментални животни от осма седмица до края на проучването.

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HO4. EXPERIMENTAL MODEL OF PARKINSON'S DISEASE – PROTECTIVE ROLE OF VASOACTIVE INTESTINAL PEPTIDE

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Abstract

Parkinson's disease is an age-related neurodegenerative disease that is pathologically characterized by the selective loss of dopaminergic neurons in the substantia nigra. Neurons of substantia nigra are particularly vulnerable to oxidative stress, because the oxidative metabolism of dopamine has the potent to generate cytotoxic free radicals. The aim of the present study was to measure *in vivo* the level of glutathione reductase activity, lipid peroxidation and their lateralization in different brain regions (cortex, striatum, hippocampus) in Parkinson's disease model in the presence or absence of vasoactive intestinal peptide. Our results demonstrated that vasoactive intestinal peptide decreases the activity of the enzyme glutathione reductase and inhibits lipid peroxidation in the experimental model of Parkinson's disease counteracting in such way against membrane damage and ameliorating the cell viability.

Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder whose neuropathological basis primarily involves a progressive degeneration of dopaminergic neurons of the substantia nigra pars compacta and the subsequent loss of their projecting nerve fibres. PD is a serious motor disease including disorders of mobility, fine tremor, rigidity and posture caused by a relentless deterioration of dopaminergic cells in substantia nigra. However it also includes disorders of affect, cognitive dysfunction and mental confusion, sleep disorder and addiction suggesting pathology of frontal lobe and other central nervous system sites. Neurons of substantia nigra are particularly vulnerable to oxidative stress, because the oxidative metabolism of dopamine has the potent to generate cytotoxic free radicals. The team of Illiana Gozes [6] demonstrated that vasoactive intestinal peptide (VIP) and its superactive analogue, stearyl-Nle17-VIP are highly protective against dopamine and 6-hydroxydopamine (6-OHDA). The aim of this study was to measure *in vivo* the level of glutathione reductase activity, lipid peroxidation and their lateralization in different brain regions (cortex, striatum, hippocampus) in Parkinson's disease model in the presence or absence of VIP.

Materials and Methods

The experiments have been performed according to the "Principles of laboratory animal care" (NIH publication No. 85-23), and the rules of the Ethics Committee of the Institute of Neurobiology, Bulgarian Academy of Sciences (registration FWA 00003059 by the US Department of Health and Human Services).

Surgical Procedures

A total of 12 male Wistar rats, weighing 130-180 g at the time of surgery, were randomly divided in groups and housed in cages with free access to rat chow and water. The rats were anesthetized with chloral hydrate (400 mg/kg, i.p.), had their heads shaved, and placed in a stereotaxic apparatus. The scalp was cleaned with a jodine solution, incised on the

midline and a burr hole was drilled through the skull at the appropriate location. The target coordinates were: AP = +0.2; LR = -3.0; H = -5.6 according to the stereotaxic atlas [7]. The experimental group received an injection of 20 μg/2 μl of 6-OHDA (Sigma-Aldrich, St. Louis, MO, USA; calculated as free base, dissolved in ice-cold saline with 0.02 % ascorbic acid) while the control group received an injection of 2 μl saline. All injections were made into the right striatum area by a Hamilton microsyringe at a rate of 1 μl/min. The needle was left in place an additional 2 min before being slowly withdrawn. The wound was closed with stainless steel clips and the rat was allowed to recover before being returned to its cage. VIP (13 μg/2□μl) was injected in the striatum twice - 15 min before 6-OHDA lesion and at the 20st day after surgery.

Biochemical Procedures

Protein content was measured by the method of Lowry et al. [4]. Lipid peroxidation in the absence and in the presence of an inducer (5.10–5 M Fe^{2+}) was determined by the amount of the thiobarbituric acid-reactive substances, formed in fresh preparations for 60 min at 37°C [2]. The absorbance was read at 532 nm against appropriate blanks; the absorbance at 600 nm was considered to be a non-specific baseline and was, therefore, subtracted from A532. Glutathione reductase activity was measured by the method of Pinto & Bartley [8].

Data Analysis

Results were expressed as mean ± S.E.M. Statistical analysis of the data was performed by Student's *t*-test for unpaired data or by one-way analysis of variance (ANOVA) followed by Newman-Keuls post-test. *P*-values < 0.05 were considered significant.

Results and Discussion

The experimental model of Parkinson's disease was proved by the rotational behavior of rats induced by apomorphine (0.5 mg/kg, s.c.) two weeks after surgery [9, 3]. Levels of lipid peroxidation and glutathione reductase in the left and right striatum, hippocampus and cortex of control (saline 2 μl into the right striatum) and Parkinsonian rats (6-OHDA 20 μg/2 μl into the right striatum) in VIP (13 μg/2□μl) presence or absence were evaluated at the 21st day after surgery, and are shown on the Table 1 and Table 2, respectively.

Table 1. Levels of Fe-ascorbat induced lipid peroxidation in the left and right cortex, striatum and hippocampus of control and Parkinsonian rats

BRAIN STRUCTURE	LEFT			RIGHT		
	CONTROLS	PD	PD + VIP	CONTROLS	PD	PD + VIP
Cortex	10.49 ± 2.25	23.91 ± 4.73*	14.41 ± 2.04#	9.71 ± 2.25	20.07 ± 4.94*	17.48 ± 4.12#
Striatum	15.01 ± 3.16	23.76 ± 2.25*	16.84 ± 5.98	13.75 ± 3.16	19.26 ± 2.09	12.86 ± 2.89#
Hippocampus	11.34 ± 3.71	18.79 ± 6.61	13.65 ± 1.76	11.50 ± 3.89	19.48 ± 6.15	18.32 ± 5.51

*P < 0.05 vs. Control; #P < 0.05 vs. Parkinson's disease group without VIP; n = 5 rat

Table 2. Levels of glutathione reductase in the left and right cortex, striatum and hippocampus of control and Parkinsonian rats

BRAIN STRUCTURE	LEFT			RIGHT		
	CONTROLS	PD	PD + VIP	CONTROLS	PD	PD + VIP
Cortex	8.17 ± 0.84	15.60 ± 4.41*	10.35 ± 3.24	9.28 ± 1.36	11.64 ± 2.43	8.21 ± 3.10
Striatum	8.85 ± 1.75	10.01± 0.42	10.87 ± 2.64	10.56 ± 0.32	13.78 ± 1.04	12.95 ± 2.47
Hippocampus	9.67 ± 1.18	12.51 ± 3.57	10.61 ± 3.13	9.09 ± 0.28	16.05 ± 3.77*	10.61 ± 1.83#

*$P < 0.05$ vs. Control; #$P < 0.05$ vs. Parkinson's disease group without VIP; $n = 4$ rats

Parkinson's disease is more common in the elderly, with most cases occurring after the age of 50. Unfortunately up to now no cure is available. Treatment in the initial stage aims for an optimal tradeoff between good symptom control and side-effects resulting from improvement of dopaminergic function. The start of levodopa (or L-DOPA) treatment may be delayed by using other medications such as MAO-B inhibitors and dopamine agonists, in the hope of delaying the onset of dyskinesias [10]. In the late stage the aim is to reduce symptoms while controlling fluctuations of the response to medication. Although current treatments alleviate some symptoms of the disease, chronic use of these drugs is not effective in deterring the progression of Parkinson's disease and has been associated with highly undesired debilitating side effects. In addition, the etiology of the Parkinson's disease remains unknown, which has impeded the development of effective therapies.

Neuroprotective effect of vasoactive intestinal peptide in a mouse model of Parkinson's disease by blocking microglial activation was shown [1]. Moreover, Offen *et* al. [6] reported neuroprotection by stearyl-Nle17-VIP, vasoactive intestinal peptide, and NAP (8aa) against the buthionine sulfoximine, a selective inhibitor of glutathione synthesis, suggesting that the mechanism may involve the glutathione antioxidant system. Our results are in accordance with the above-mentioned hypothesis, showing that vasoactive intestinal peptide decreased the activity of the enzyme glutathione reductase in a Parkinson's disease model.

In recent years it has become apparent that the oxidation of lipids, or lipid peroxidation, is a crucial step in the pathogenesis of several disease states in adult and infant patients. The reactive oxygen species (hydroxyl radical, hydrogen peroxide etc.) readily attack the polyunsaturated fatty acids of the fatty acid membrane, initiating a self-propagating chain reaction. The destruction of membrane lipids and the end-products of such lipid peroxidation reactions are especially dangerous for the viability of cells, even tissues. Since lipid peroxidation is a self-propagating chain-reaction, the initial oxidation of only a few lipid molecules can result in significant tissue damage [5]. In our experiments we demonstrated that vasoactive intestinal peptide inhibits lipid peroxidation in the experimental model of Parkinson's disease counteracting in such way against membrane damage and ameliorating the cell viability.

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HO5. ANIMAL MODELS OF DEPRESSION

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Abstract

A survey of World Health Organization (2007) of over 200,000 patients across the world shows that in comparison to chronic diseases such as diabetes and arthritis, the depression has the most harmful effect on human health. Despite the wide variety of antidepressants and alternative therapies, the world-wide treatment of depression needs new medicines and methods, since nowadays many patients can not be successfully cured. Even in cases of perfect diagnosis and treatment with standard antidepressants, the remission rate is

only 30-40%. The treatment disadvantages and lack of effective drugs for this grate social problem focus the efforts of scientists to clarify the molecular mechanisms leading to this disease and to search for new and more effective antidepressants. The current understanding of the depression pathogenesis and the development of drugs is based mostly on animal models. Indeed the unique and complex features of depression in humans complicate the symptom's prediction of this disease in animal models. The animals lack self-consciousness, self-reflection and the opinion of others. In addition some conditions such as depressing mood, low self-esteem or suicidality are hardly accessible in non-humans. However, the depression, as other mental disorders, consists of endophenotypes that can be reproduced and evaluated in animals on physiological, endocrinological and neuroanatomical, as well as behavioral level. This presentation offers an overview on the animal models of depression, including acute stress, models of prolonged physical or social stress, models of secondary depression, genetic models and experiments, designed to elucidate the mechanisms of antidepressant action. These models are critically evaluated in relation to their accessibility, validity and repetitiveness, ability to correct interpretation of molecular interactions, as well as to their potential to be used for invention of new remedies against depression.

HO6. PHARMACOLOGICAL MODULATION OF COGNITION WITH NEW PEPTIDOMIMETICS IN SOCIALLY ISOLATED RATS

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Abstract

Newly synthesized peptidomimetics were synthesized on the basis of essential aminoacid L-valine and nicotinic acid. Previous data have shown that some of them (positional isomers with codes M6 and P6) can significantly improve cognitive functions in rodents (mice and rats). Provoking aggressive behaviour in rodents via social isolation can be used as model for study some changes in cognitive functions and brain plasticity.
Purpose of the study was to evaluate the modulating effect of the tested compounds on cognition in socially isolated and aggressive rats and the accompanying biochemical changes induced by M6 and P6 in hippocampus of rats.

Methods

Experimental model of aggression induced by social isolation (over 6 weeks) in male Wistar rats was used to study changes in cognitive functions.Wistar rats were treated with effective doses of compounds (100 mg/kg bw, i.p., 3 days). The cognitive functions (learning and memory - Step-through test and exploratory activity – Hole-board test) were studied, as well as changes in the serotoninergic neurotransmission in hippocampus (synaptosomal serotonin uptake and serotonin release) using liquid scintillation methods.

Results

Cognitive dysfunctions were found coupled with some changes in the aggressive rats' hippocampal serotonin content. After 3-day administration of the compounds, significant depressive effects upon central nervous system of aggressive rats were observed. It was found out that the two isomeric peptidomimetics modulated some memory functions (long-term memory and exploratory behavior). The two compounds altered significantly serotonin release and especially serotonin uptake in the hippocampal tissue of aggressive animals in comparison to both groups of collectively reared and aggressive control rats.
The observed variation in effects of M6 and P6 can be explained with their positional isomery and some physicochemical differences.

Conclusion

The newly synthesized peptidomimetics are effective modulators of aggressive behavior due to their possible affinity for serotonin receptors in hippocampus. Their influence on serotonin levels in the hippocampus of aggressive animals deserves further studies and the compounds promise future development as potential pharmacological agents.

HO7. BEHAVIORAL EFFECTS OF CHRONIC LOSARTAN TREATMENT AFTER KAINIC ACID STATUS EPILEPTICUS

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Abstract

Depression is frequent comorbidity in epilepsy. However the link between epilepsy and behavioral deficit are unclear. Angiotensin AT_1 receptors participate in a range of biologically detrimental actions while the selective AT_1 receptor antagonists has demonstrated a broad spectrum of activities associated with anxiety, locomotion and depression. The aim of the present study was to characterize the effect of losartan treatment during epileptogenesis on diurnal rhythms of behavioral changes of Wistar rats in kainate (KA) model of temporal lobe of epilepsy (TLE). Behavioral tests (open field, elevated plus-maze, sucrose consumption test and forced swimming test) were conducted a month after status epilepticus during the losartan treatment. The AT_1 receptor antagonist (10 mg/kg/day, diluted in drinking water, 4 weeks) potentiated the increased locomotion in KA-treated rats during the light phase and unlike KA-vehicle group increased motor activity also during the dark phase in KA-losartan group. Losrtan alleviated the low anxiety level without diurnal variations in KA-treated rats in open field and the elevated plus maze tests. The drug evoked anhedonia in sucrose consumption test during the light phase both in intact rats and KA-treated rats. Taken together, long-term losartan treatment showed a potential to reduce some of the deleterious behavioral alterations developing after SE in a diurnal phase-dependent mode.

Introduction

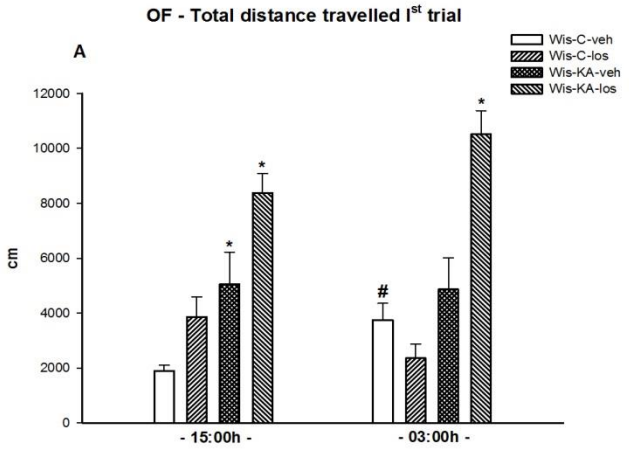
Clinical data has demonstrated that psychiatric disorders, including anxiety and depression, are the most common comorbid psychiatric condition in temporal lobe epilepsy

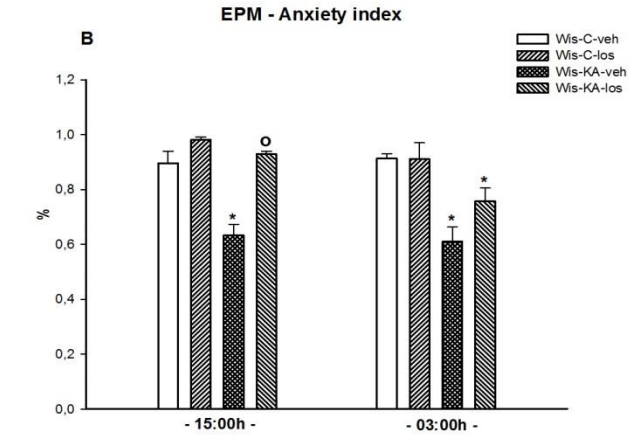
(TLE) [2]. Recently, our research group examined behavioral and neurochemical markers of depression in kainate (KA) model of TLE in two rat strains, normotensive Wistar and spontaneously hypertensive rats (SHRs) [5].

The putative role of renin-angiotensin system (RAS) in regulation of certain behavioral functions including stress responses, cognitive processes and depression has been described in details in several recent reviews by Wright and Harding [6, 7, 8]. Recently presented data revealing an up-regulation of AT_1 receptors and their mRNA expression in the hippocampus of Wistar audiogenic rat strain in a model of TLE as well as in patients diagnosed with TLE [1, 4]. supports the presumption that AT_1 receptor subtype should exert a putative role in limbic epilepsy. In view of the fact that AT_1 receptor antagonists are broadly used in clinic and the accumulating experimental evidence in support of the suggestion that these drugs can be effective in epileptic patients, the major rationale of this study was further to evaluate and compare the efficacy of the selective AT_1 receptor antagonist losartan on disturbed diurnal behavioral changes during chronic epileptic state in KA model of TLE in Wistar rats.

Methods

Male Wistar rats (n=15) were kept under standardized conditions (21±2 ºC, 12/12-h light/dark cycle) in individual cages and fed ad libitum. Losartan treatment started three hours after the beginning of SE at a dose of 10 mg/kg diluted in the drinking water for four weeks. The protocol of KA-induced SE was executed according to Hellier et al. [3] with little modification aiming to decrease the mortality of rats during the *status*. Video monitoring (24 h) was performed under controlled conditions (12 h normal light/dark cycles, 20±3 ºC, 40–50 % relative humidity) to detect spontaneous motor seizures. Taste preference behavior was evaluated by a sucrose consumption test (SCT). Open field test (OF), elevated plus-maze test (EPM) and forced swimming test (FST) were performed at two time points i.e., at 15:00 p.m. and 03:00 a.m., respectively. The above mentioned tests are previously described in details elsewhere (Tchekalarova et al., 2011). All data were analyzed by three-way ANOVA followed by Bonferroni post hoc t-test.





Results and Discussion

To our knowledge, this is the first work focused on the efficacy of long-term treatment with the selective AT1 receptor antagonist losartan during epileptogenesis on behavioral alterations in normotensive Wistar rats.

Fig. 1. Values are means±SEM; *p<0.05 vs controls, ^{o}p<0.05 vs epileptic rats, $^{\#}$p < 0.05 within a group (15:00 h vs 03:00 h). **A)** Total distance moved in open field test. Analysis of data by three-way ANOVA indicated main Epilepsy effect [$F_{1,5}$= 48,686, p<0.05] as well as Epilepsy x Drug treatment effect [$F_{1,5}$= 7,792, p<0.05]. **B)** Anxiety index in elevated plus-maze test. ANOVA indicated main Epilepsy effect [$F_{1,8}$= 38,692, p<0.05], Drug effect [$F_{1,8}$= 17,916, p<0.05] as well as Epilepsy x Drug treatment effect [$F_{1,8}$= 8,343, p<0.05].

Only control rats exhibited diurnal variations ($^{\#}$p<0.05) of the total distance in OF test (Fig. 1, **A**). The chronic treatment with losartan showed a tendency to enhance KA-induced increase in locomotion during the light phase. Moreover, losartan increased motor activity also during the dark phase in epileptic rats (*p<0.05) (Fig. 1, **A**). The low anxiety level was alleviated by losartan in KA-treated rats in the EPM tests and the significance was reached during the light phase (^{o}p<0.05) (Fig. 1, **B)**.

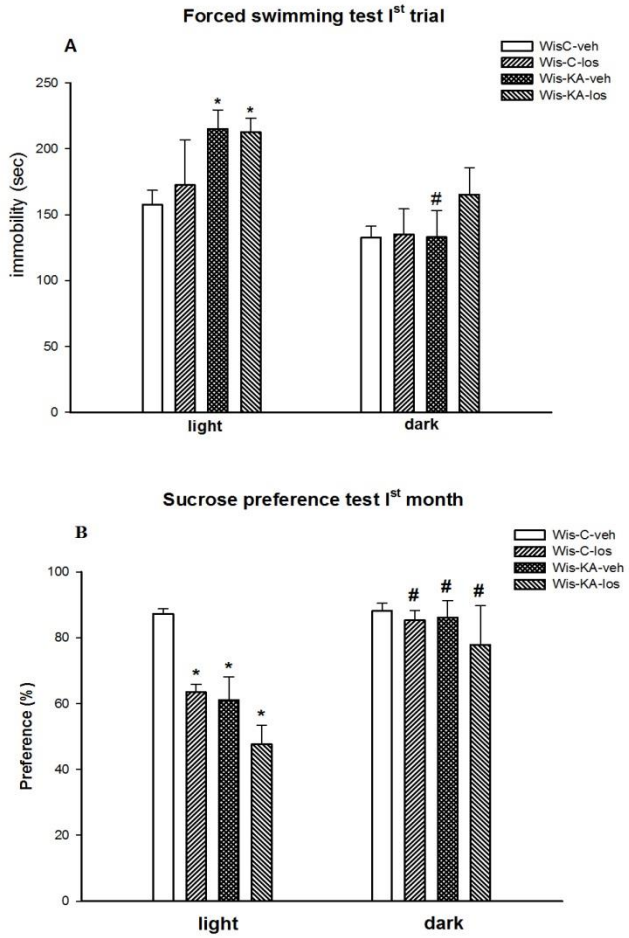


Fig. 2. Values are means ± SEM; *p < 0.05 vs controls, ^{o}p<0.05 vs epileptic rats, #p < 0.05 within a group (15:00 h versus 03:00 h). **A)** Dynamics of immobility time in forced swimming test. Three-way ANOVA indicated main Epilepsy effect [F1,67= 7.910, p<0.05] and Phase effect [F1,67= 24.437, p<0.05]. **B)** Dynamics of sucrose preference. ANOVA indicated main Epilepsy effect [F1,7= 12,199, p<0.05], Drug effect [F1,7= 10,873, p<0.05] and Phase effect [F1,7= 28,077, p<0.05].

In the forced swimming test immobility time was increased (*p<0.05) (Fig. 2, **A**) in KA- vehicle and KA-losartan treated group during the light phase. Diurnal variations in depressive-like behavior was detected only in KA vehicle group ($^{\#}$p<0.05) (Fig. 2, **A,B**). Losartan evoked depressive-like responses in both intact rats and KA-treated rats with decreased consumption of sucrose significantly during the light phase (*p<0.05). Our results provided evidence that losartan provoked differences in diurnal rhythms and behavioral

changes. Taken together, long-term losartan treatment showed that could have a place in the therapy and reduce some of the deleterious behavioral alterations developing after SE in a phase-dependent mode.

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HO8. INTERSTRAIN DIFFERENCES IN THE EFFECT OF LONG-TERM TREATMENT WITH MELATONIN ON KAINIC ACID-INDUCED STATUS EPILEPTICUS,OXIDATIVE STRESS AND THE EXPRESSION OF HEAT SHOCK PROTEINS

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Abstract

The present study compared the effects of subchronic treatment with melatonin administered via s.c. osmotic minipumps for 14 days (10 mg/kg per day) on kainic acivd (KA)-induced status epilepticus, oxidative stress and expression of heat shock protein (HSP) 70 in the frontal cortex and hippocampus between Wistar rats and spontaneously hypertensive rats (SHRs). SHRs showed increased lipid peroxidation (LP) in the cortex and hippocampus and decreased cytosolic superoxide dismutase (SOD/CuZn) production in the cortex compared to Wistar rats. Long-lasting seizures induced by KA (12 mg/kg, i.p.) were accompanied by increased LP and expression of HSP 70 in the hippocampus of the two strains and increased SOD/CuZn production in the frontal cortex of SHRs. Pretreatment with melatonin failed to supress the KA-induced SE in the two strains though the latency for seizure onset was significantly increased in SHRs. The increased LP induced by KA in the hippocampus was attenuated by melatonin pretreatment both in Wistar rats and SHRs. The increase of SOD/CuZn and mitochondrial SOD/Mn production was strain- and area-specific in melatonin- KA treated groups. Melatonin prevented the KA-induced increased expression of HSP 70 in the hippocampus of KA-treated Wistar rats.

Introduction

The neurotoxin kainic acid (KA) triggers neuropathologic cellular changes in the hippocampus characterized by an overloading of intracellular calcium, activation of intracellular enzyme cascades and increased levels of free radicals/reactive oxygen species (ROS) [7]. The formation of free radicals results in an extensive lipid peroxidation, which damage cellular organelles and membranes, and finally leads to cell death. The disturbance in the levels of the antioxidant enzymes is a crucial step involved in dysregulation of physiological processes implicated in the pathogenesis of arterial hypertension [4]. Experimental data demonstrated that single injection of melatonin in rats before and during the KA- or pilocarpine-induced status epilepticus (SE) has neuroprotective effect by reducing the neuronal death, supragranular mossy fiber sprouting, lipid peroxidation (LP), and microglial activation [1]. The aim of the present investigation was to check out and compare the efficacy of subchronic melatonin exposure on KA-induced seizure severity and changes in the LP, enzymatic antioxidant defense systems and heat shock protein (HSP) 70 expression in the frontal cortex and hippocampus at 4 hour (h) following SE in normotensive Wistar rats and SHRs.

Materials and methods

The experiments were performed on adult male normotensive Wistar rats and spontaneously hypertensive rats (SHRs). The rats were adapted for one week under

standardized laboratory conditions. Melatonin was applied for a period of two weeks via osmotic minipumps at a dose of 10 mg/kg/day. Alzet osmotic minipumps, pumping rate 0.5 μl/h. On the 14th day vehicle/melatonin infused animals received i.p. injection of KA at a dose of 12 mg/kg. After decapitation the frontal cortex and the hippocampi were bilaterally removed. The extent of lipid peroxidation, cytosolic and mitochondrial SOD were expressed as U/ml. HSP 70 was analyzed by Western Blotting.

Results and Discussion

The latency for onset of the first clonic seizure induced by KA injection was significantly lower in SHRs-KA-veh group compared to Wistar-KA-veh group ($^{o}p=0.02$). Melatonin pretreatment significantly increased the latency for the onset of the first clonic seizure in SHRs (*p=0.034). We can suggest that the higher efficacy of melatonin treatment on seizure activity in SHRs than in Wistar rats might be related to simultaneous decrease of the blood pressure detected in epileptic SHRs. Our results are in support of previous finding that single melatonin injection is able to prevent the KA-induced LP augmentation in mice [5].

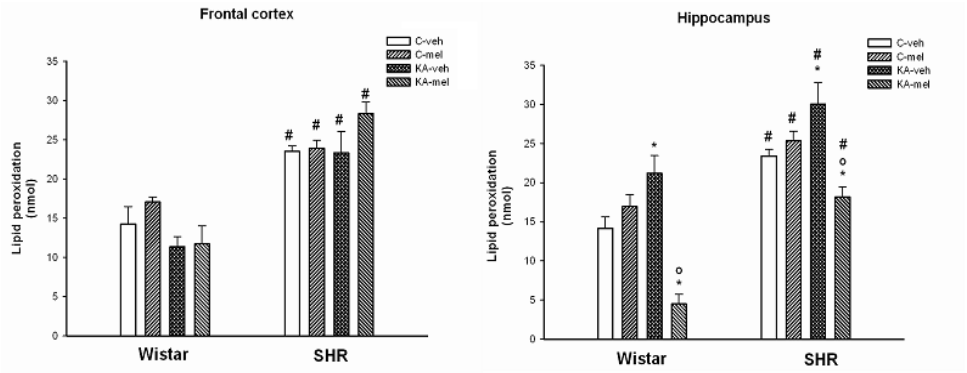


Fig.1

SHRs showed higher level of LP in naive and melatonin treated controls (#p<0.001), as well in KA- vehicle (#p<0.001) and KA- melatonin groups (#p=0.066) compared to Wistar rats in the frontal cortex. KA-treatment provoked a significant increase in the hippocampal LP in Wistar rats (*p=0.028), which level was dramatically decreased even below the control level after melatonin pretreatment ($^{o}p<0.001$).

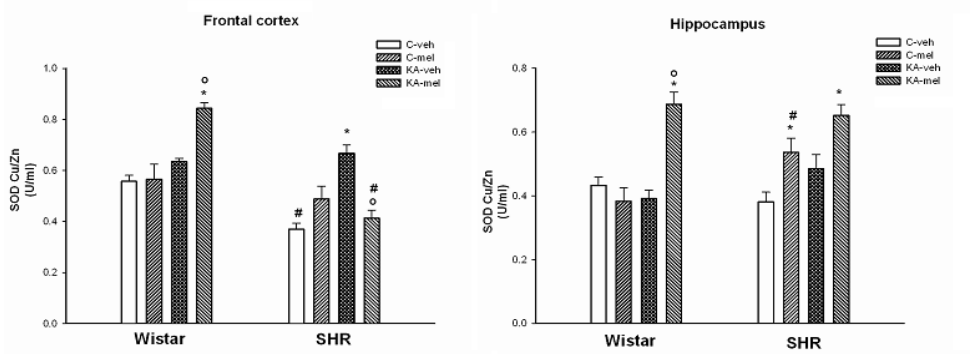


Fig. 2

KA-treated SHRs also displayed an increased oxidative stress in the hipocampi (*p=0.008), which was abolished by melatonin pretreatment ($^{o}p=<0.001$) (Fig.1). Naive SHRs showed lower SOD Cu/Zn level in the frontal cortex compared to Wistar controls (#p<0.001).

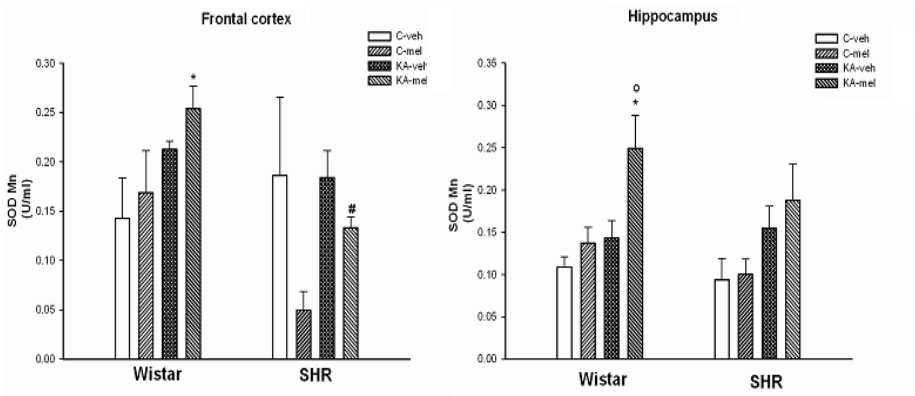
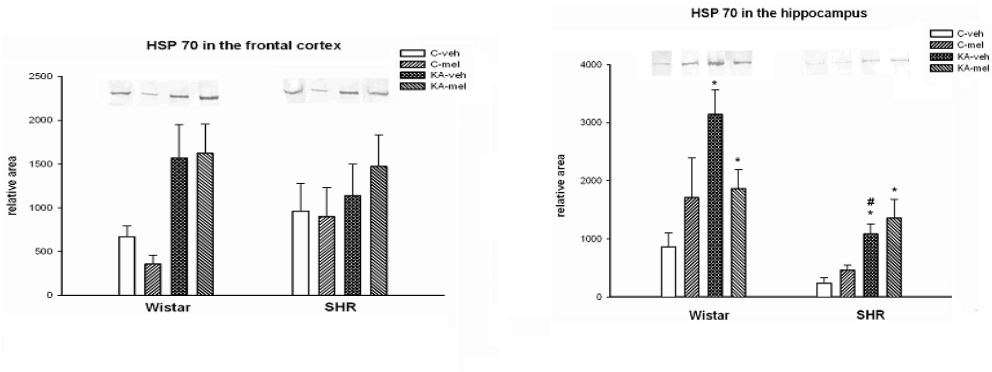


Fig. 3

KA-treatment increased the enzyme level only in SHR's frontal cortex (*p<0.001). Although melatonin pretreatment abolished the KA-induced SOD Cu/Zn increase in the frontal cortex of SHRs (^{o}p<0.001). On the other hand, the hormone treatment raised SOD Cu/Zn cortical level in KA-treated Wistar (^{o}p<0.001), and hippocampal (^{o}p<0.001) enzyme level in both strains as well as in naive SHRs (*p= 0.016) (Fig 2). Melatonin pretreatment showed dual effect – it decreased cortical SOD Mn level both in naive (#p=0.024) and KA-treated SHRs (#p<0.001) but increased the enzyme level in the hippocampi of KA-treated Wistar rats (^{o}p= 0.020) (Fig. 3).

In our study, the markers of oxidative stress, which showed an increased LP and decreased SOD Cu/Zn activity in a model of essential hypertension compared to Wistar rats indicates an enhanced oxidative stress in SHRs. These results confirm previous findings that naive SHRs are characterized by a disturbed oxidative defence system compared to Wistar Kyoto rats in physiological conditions [6]. We found that KA provoked an enhancement of the cytosolic SOD Cu/Zn 4 hours after SE only in the frontal cortex of SHRs. Literature data showed that seizures and SE could alter oxidative stress by either activation or suppression of free radicals scavenging enzymes such as SOD in different brain areas [2]. KA-treatment increased HSP70 level in hippocampus of Wistar rats (*p<0.001) and in the hippocampi of SHRs (*p= 0.003) but this effect was weaker in SHRs (#p=0.001). The frontal cortex of SHRs remais unaffected by the neurotoxin. Melatonin pretreatment was able to abolish only the KA-induced increase in HSP70 level in the hippocampi of Wistar rats (^{o}p=0.05) (Fig. 4).



A tendency for increased expression in the frontal cortex and a significant upregulation in the hippocampi of HSP 70 was detected both in Wistar rats and SHRs after the KA-induced SE. These results agree with previous findings that SE produced by systemic KA induced an expression of HSP 70 in neurons known to be susceptible to this neurotoxin and in the hippocampus, in particular [3]. In conclusion, SHRs showed increased seizure susceptibility and disturbed defence antioxidant system compared to normotensive Wistar

rats. Subchronic systemic melatonin treatment exerted a mild anticonvulsant effect following KA injection in SHRs but not in Wistar rats. However, the efficacy of melatonin in preventing the KA-induced changes in the markers of oxidative stress and neurotoxicity was more pronounced in Wistar rats than in SHRs suggesting a lack of a direct link between the seizure activity and these markers.

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