PROCEEDINGS

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

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Поради епидемиологичната обстановка в страната вследствие на пандемията COVID-19, Работната среща беше проведена дистанционно

THE PROGRAM OF THE WORKSHOP

In vitro investigation on citocompatibility and osteoinductivity of new materials for bone implants

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Bone damage repair is a multifaceted, coordinated physiological process that involves new bone formation and resorption, ultimately aimed at restoring broken bone to its original state. Various approaches are currently being applied to accelerate the recovery of bone defects, which include the use of 'gold standard'- autologous bone grafts as well as allografts. These grafts may not be readily available and procedural complications may lead to undesirable results. The search for new materials for bone regeneration is a challenge in biomedical research. For bone substitutes are used calcium phosphates, metal alloys, composite materials, etc. Bone substitutes based on calcium phosphates require a special interest because of the following advantages: (1) these materials are part of the mineral phase in the bone tissue; (2) they are bioactive, biodegradable and bioresorbable; (3) support bone healing.

The aim of our study was to investigate the cytocompatibility and osteoinductive activity of newly synthesized calcium phosphate materials for bone implants modified with metals [Mg(II), Zn(II), Sr(II)]. The materials (a total number of 21) are divided into three groups as follows: Group 1 - Powder samples of metal-modified [Mg(II), Zn(II), Sr(II)] calcium phosphates; Group 2 - Composite materials; Group 3 - Cements - Amorphous calcium phosphates with acids [tartaric, tartaric, lactic] and metals [Mg2+ or Na1+]. The experiments were performed with: non-tumor cell lines established from human embryonic (Lep3, MRC-5) and newborn (BJ) tissues; embryonic mouse fibroblasts (BALB /c 3T3); bovine kidney cells (MDBK); primary cell cultures from mouse and rat bone marrow as well as primary cell cultures and permalent cell line derived from mouse bone explants; human tumor cells (MCF-7 breast cancer, HeLa cervical carcinoma, SAOS-2 osteosarcoma); human mesenchymal stem cells from adipose tissue.

The studies were carried out by methods with different cellular / molecular targets and mechanisms of action, such as: cytotoxicity assays (MTT / MTS test, neutral red uptake cytotoxicity assay, crystal violet staining, trypan blue dye exclusion technique); immunocytochemical tests to evaluate cell proliferative activity (detection of Ki-63), methods to evaluate the presence of cytopathological changes (H&E staining, double staining with acridine orange and propidium iodide, light microscopy and SEM), genotoxicity assays (alkaline variant of Comet assay); methods to evaluate

osteogenic activity (Alizarin red staining; evaluation of alkaline phosphatase) as well as statistical analysis.

The investigations were performed by two groups of experiments: i) indirect experiments - the cells were cultured in calcium phosphate modified medium (CPM), in which the materials were pre-incubated for different periods of time (1 - 21 days) or in the presence of the materials themselves; ii) direct experiments (in which the cells were seeded on / in the materials) for a period of 1-6 days.

Our results revealed that: 1) With few exceptions (Zn-7, ACP-TA-Na, ACP-LA) all examined materials show high cytocompatibility - cell survival / proliferative activity found in indirect (for the three groups of materials) and direct (for the materials from Group III) experiments is $\geq$ 70% compared to the control. Data were obtained using a wide range of cell models (human, mouse, rat and bovine constant cell lines and primary cultures) and methods with different cell targets (molecules, organelles) and mechanisms of action. Manifestations of genotoxicity (double-stranded breaks in DNA molecules) and cytopathological changes were not detected. 2) With an increase in the initial seeding density of the cells, elevation in the percentage of living cells compared to the control has been observed. This dependence is most likely due to the ability of cells to secrete biologically active substances that stimulate both their own proliferation and that of the surrounding cells. The percentage of viable cells relative to the control increases with prolonging time of culturing as well as with increasing amount of the material. 3) A cell-specific response has been found - the relatively higher sensitivity of mouse and rat bone marrow cells to the cytotoxic action of the tested materials can be explained at least partially by the lack or low expression of the P-glycoprotein - ATP-dependent efflux pump, which is involved in the expulsion of xenobiotics from the cell. 4) ACP-TA administered for 21 days in the form of a conventional culture medium and differentiating osteogenic medium, pre-incubated in its presence for 3 and 6 days, shows no cytotoxic effects and stimulates the deposition of calcium crystals and production of alkaline phosphatase in human mesenchymal stem adipose tissue cells - evidence of targeting osteogenic differentiation of these cells.

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Cytotoxic activity of metal [Cu(II), Co(II)] complexes with Schiff bases and Disulfiram in human and animal tumor cells

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Introduction. Cancer is the second leading cause of death globally, and is responsible for an estimated 9.6 million deaths in 2018. Globally, about 1 in 6 deaths is due to cancer**.** Despite undoubted success in early diagnosis and treatment of breast cancer, it remains one of the most common and lethal neoplastic diseases in the world among women. Modern treatment of cancer includes surgery, drug therapy and / or radiation therapy, administered alone or most often in combination. Two groups of newly synthesized metal [Cu(II), Co(II)] complexes with Schiff bases and their anticancer properties were the topic of our investigations. Disulfiram (DS), an FDA (Food and Drug administration, USA) approved drug for the treatment of alcoholism has been also reported to express promising antitumor activity in cellular and animal models as well as in humans. According to the literature available, in this study we report for the first time data about inhibitory effect of disulfiram on viability and 2D / 3D growth of cultured LSR-SF-SR rat sarcoma cells transformed by *Rous sarcoma virus* strain *Schmidt-Ruppin* (SR-RSV).

Aim. To investigate the influence of newly synthesized metal [Cu(II), Co(II)] complexes with Schiff bases and disulfiram on cell viability and proliferative activity of laboratory-cultured human and animal tumor cells as well as their ability to induce cytomorphological changes.

Materials and Methods. Our study was focused on the cytoxic activity of the following compounds: i) two groups of metal compounds – complexes of Co(II) and Cu(II) with the same Schiff bases obtained by a condensation reaction between o-vanillin (Val) and the amino acids tyrosine (Tyr), threonine (Thr), Trpt (tryptophan) or serine (Ser). The metal complexes were administered in concentrations of 10, 50, 100, 200 and 400 μg / ml; ii) Disulfitam applied at a concentration range of 1-100 μg / ml.
The following cell cultures were used as model systems in our investigations: i) cell lines derived from two of the most common malignancies in women – cancers of the mammary gland (MCF-7, MDA-MB-231) and the cervix (HeLa); cell lines

established from transplantable tumors induced by avian leukemia and sarcoma retroviruses - LSCC-SF-Ms29 clone E7 (transplantable chicken hepatoma caused by the myelocytomatous virus Ms29) and LSR-SF-SR (transplantable rat sarcoma caused by SR-RSV); non-tumor human embryonic fibroblastoid cells (Lep-3); primary cell cultures from transplantable myeloid tumor in hamster as well as spleen lymphocytes and peritoneal macrophages from healthy and tumor-bearing hamsters.

The investigations included **short-term experiments** (24-96 h, with monolayer cell cultures) performed by cytotoxicity assays (MTT test, neutral red uptake assay, crystal violet staining; trypan blye dye exclusion technique), cytological and immunocytological methods to evaluate proliferative potential, cytopathological changes and cell death (haematoxylin and eosin staining, double staining with acridine orange and propidium iodide), molecularbiological methods to estimate genotoxicity (Comet assay at alkaline pH) and methods to detect autophagy (immunocytochemical detection of LC3B protein and Confocal microscopy).

Long-term experiments (16-32 days) were performed by 3D colony-forming method.

Results. Our results revealed that:
1) The investigated complexes of Cu(II) and Co(II) with Schiff bases cause cytopathological changes and reduce to various degrees the survival and 2D / 3D growth of the cell cultures used as experimental models. The observed effect is enhanced with increasing concentration and treatment time.

2) Cu(II) complexes with Schiff bases show higher cytotoxic activity (especially the CuVanSer complex) compared to Co(II) complexes with the same ligands. It is possible that cells deactivate the applied Co(II) complexes by forming autophagosomes, whereas during treatment with Cu(II) complexes the initiated autophagy concludes with cell death.

3) The most pronounced cytotoxic effect in both groups of investigated metal complexes is expressed by the VanSer ligand complexes - CuVanSer and CoVanSer.

4) A cell-specific response was observed, which most likely reflects the differences in the origin, biology and behavior of the malignancies from which the cell cultures used as model systems were derived, as well as the uniqueness of each tumor / tumor cell line.

5) The CC_{50} (μM, MTT, 72 h) determined in Co(II) complexes was higher in non-tumor human embryonic Lep-3 cells than in HeLa, MCF-7 and MDA-MB-231 tumor cells. According to the CC_{50} calculated under the same conditions, in the majority of cases the sensitivity of non-tumor human cells to the cytotoxic action of Cu(II) complexes exceeds that of cancer cells.

6) Disulfiram administered at concentrations of 1-100μg / ml significantly inhibits the survival rate and proliferative activity of cultured in its presence rat sarcoma cells transformed with *Rous sarcoma virus* strain *Schmidt-Ruppin* (LSR-SF-SR).

7) With a few exceptions, all investigated compounds (metal complexes with Schiff bases, disulfiram) show a weaker cytotoxic effect compared to conventionally used in clinical practice anticancer agents cisplatin, oxaliplatin and epirubicin.

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Zn(II)/Au(I) and Zn(II)Ag(I) complexes with Schiff bases significantly reduce 3D growth of osteosarcoma and pancreatic cancer cells in vitro

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Compared to the traditional monolayer (2D) cell cultures, 3D cultures often behave like cells in living organisms. Numerous studies have shown that the differential gene expression between cells cultured in 2D and 3D plays a major role in the differences in morphology, proliferation, and drug sensitivity observed in cells cultured in 2D and 3D.

The aim of our study was to evaluate the long-term ability of six newly synthesized complexes of Zn(II)/Au(I) and Zn(II)/Ag(I) with Schiff bases Salen, Salampy and Saldmen to inhibit the 3D growth of human tumor cell colonies in a semi-solid medium.

As a model systems we used permanent cell lines established from tumors characterized by extreme aggressiveness and high mortality rate - osteosarcoma (Saos-2) and pancreatic cancer (MIA PaCa-2) in human.

Tumor cells (10^3 cells per well) suspended in 0.45% purified agarose in D-MEM medium containing different concentrations of the compounds examined (ranging from 0.05 to 20 μg/ml) were layered in 24 well microplates. Non-treated cells served as a control. The presence/absence of colonies was registered using an inverted light microscope during a period of 16 - 32 days. Colony inhibitory concentration (CIC, μg/ml), at which the compounds tested completely inhibit the ability of tumor cells to grow in semi-solid medium, was determined.

It has been found in our investigations that the metal complexes completely inhibit 3D growth of human osteosarcoma and pancreatic cancer cells in a semi-solid medium administered at concentrations ≥ 0.1 μg/ml for Zn(II)/Au(I) with Salen, Salampy or Saldmen, ≥ and 5 μg/ml for Zn(II)Ag(I) with tke same ligands. Applied independently, the ligand Salen does not inhibit 3D colony-forming ability of tumor cells.

3D growing colonies have a number of advantages over conventional and widely used monolayer cultures such as the time of cultivation. The long-term period of 3D cell colonies cultivation (32 days in the study presented) allows us to assess the clonogenic survival of cancer cells and to obtain valuable information about stability of cytotoxic effect of the compounds investigated.

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Complexes of Zn(II), Zn(II)/Au(I) and Zn(II)/Ag(I) with Schiff bases derived from 2,6-diformyl cresol (diald) as potential antitumor agents

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Despite advances in diagnosis and treatment, morbidity and mortality from malignancies continue to represent a major health burden. Malignant neoplasms are the second cause of death globally, after heart diseases, expecting to be ahead of them in the very near future (Press release №263 of the World Health Organization, 12 September 2018, https://www.who.int/news-room/fact-sheets/detail/cancer). Among the main obstacles preventing successful treatment of malignancies are (multi) drug resistance of cancer cells as well as side and toxic effects of currently available conventional medications in clinical oncology.

The search for new, highly effective antitumor drugs with good biological tolerance is one of the major challenges for modern biomedical science.

The potential antineoplastic activity of different metals and metal compounds has been under special interest during the recent years. Zinc, gold, silver and their compounds are attractive for the scientists because as an essential element, zinc is expected to be less toxic than platinum, for example; gold and silver compounds also show relatively low toxicity (Drake, Hazelwood, 2005). On the other hand Schiff bases and especially their derivatives/metal complexes have been reported to express a wide range of biological activities, including antitumor properties (Qin et al, 2013; Gaikwad et al, 2016).

That is why our aim is to evaluate the cytotoxic/ potential antitumor effect of newly synthesized complexes of Zn(II), Zn(II)/Au(I) and Zn(II)/Ag(I) with Schiff bases derived from 2,6-diformyl cresol (diald) - Aepy, Ampy and Dmen in cell lines obtained from some of the most common and highly aggressive neoplasms in humans (cancers of the breast, lung, colon/rectum, liver, pancreas, osteosarcoma and glioblastoma multiforme) - for most of them, the currently available therapeutic options are extremely limited.

In the study, a complex approach involving methods with different molecular targets and mechanisms of action will be conducted. Short-term experiments (up to 72 hours, with monolayer cultures) and long-term experiments (≥15-20 days with 3D colonies of cancer cells in a semi-solid medium) will be carried out - this will allow the assessment of rapid cytotoxic effect of the compounds, as well as the duration and irreversibility of their action.

The results obtained by us will give us information about antiproliferative and / or proapoptotic activity of the tested compounds in a wide range of tumor cell lines and will contribute to clarifying relationship between the chemical structure, chemical and physicochemical characteristics of the metal complexes with potential antitumor activity on one side and their biological activity on the other. Such information will facilitate the development of new antitumor agents with improved in the desired direction properties. The compounds with the most promising cytotoxic / antitumor activity in cell cultures will be directed to future studies on their mechanism of action, as well as their potential antineoplastic effect and biological tolerance in model systems in vivo.

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Ehrlich Ascites Carcinoma – in vivo model of breast cancer

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Ehrlich ascites carcinoma is a spontaneous murine mammary adenocarcinoma adapted to ascites form and carried in outbred mice by serial intraperitoneal passages. EAC is used as ascites or a solid form, that is, if ascites fluid contains the tumor cell that injects i.p., the ascites form is obtained, but if it contains s.c., a solid form is obtained. The advantage of EAC is that the same tumor cell line can be used in *in vivo* experiments with ascites and solid tumor form. Ehrlich solid carcinoma allows the volume of tumor formation to be determined on a daily basis without the need to kill experimental animals. This allows us to measure the development of the tumor over time. Tumor volume, indicative of tumor growth, can be determined by measuring two perpendiculars diameters with calipers and converting to cm^3 using the following formula:

Tumor volume = (width)2 × length/2. This gives a great advantage in the research of new anti-cancer drugs. In addition, Ehrlich solid carcinoma allows to be examined by histological methods to determine the interaction of the tumor with the surrounding healthy tissue, presence of necrotic and apoptotic areas in the tumor formation, changes in enzyme activity in tumor cells and the intercellular matrix. Immunohistochemistry can be used to determine and change the expression of specific tumor antigens.

Key words: Ehrlich Ascites Carcinoma, breast cancer

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Study of the cytomorphological alterations induced by mollusk hemocyanins in Ehrlich ascites carcinoma cells

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Abstract

Neoplastic diseases are one of the greatest challenges for pharmacology and medicine due to their high incidence and mortality and significant health and social impact. The search for novel more safe and effective antitumor therapeutics with natural origin is one of the main trends of contemporary oncology research. In this respect, hemocyanins - oligomeric copper-containing glycoproteins that function as oxygen carriers in the hemolymph of mollusks and arthropods species represent significant interest because they combine strong immunostimulating activity and direct anticancer effects. In the present study, the antitumor activity of total hemocyanins isolated from *Helix aspersa*, *Helix lucorum* (HaH-total and HlH-total) and their subunits (α–HaH; βc-HaH; α-HlH; βc-HlH); subunits of *Rapana venosa* hemocyanin (RvH I and RvH II) and *Helix aspersa* mucus was examined in Ehrlich Ascites Carcinoma (EAC) cell line by MTT assay. The tested bioactive substances that showed higher antiproliferative activity in the MTT assay – α-HaH, βc-HlH and Helix aspersa mucus were further used in morphological studies aiming to analyze the mechanisms that mediate their anticancer action and the nature of the cell death induced in EAC cells. The proapoptotic activity of the tested bioactive substances was analyzed by double fluorochroming with acridine orange and ethidum bromide (AO/EB) and the alterations in the nuclear morphology of the tumor cells was studied by DAPI staining. The fluorescent microscopy analysis of AO/EB stained cell revealed normal morphology and monolayer growth of the control untreated EAC cells with homogenous green staining that is characteristic for the viable cells. A large number of EAC cells in different stages of mitosis were found. In contrast, mitotic cells were not observed in the cell cultures treated with the mollusk hemocyanins and mucus, which is an indication for their antiproliferative effects. Moreover, numerous early apoptotic cells with intensive green staining due to the chromatin condensation and late apoptotic cells with orange fluorescence resulting from the increased membrane permeability allowing the passage of ethidium bromide were observed in the treated EAC cultures. The ability of the tested bioactive compounds to induce apoptosis was further confirmed by fluorescent microscopy analysis of the nuclear morphology of DAPI stained EAC tumor cells. The control EAC tumor cells showed normal morphology of the nucleus with oval shape, smooth outlines and homogenous blue staining. The nuclei of the cells treated with α-HaH, βc-HlH and *Helix aspersa* mucus were irregular in shape, more brightly colored and with intense condensation of chromatin. Some treated cell showed nuclear fragmentation and formation of apoptotic bodies. The results of the presented cytomorphological study indicate that

the mechanism of the antitumor activity of the tested bioactive substances isolated from *Helix aspersa*, *Helix lucorum* includes the induction of apoptosis and suggests their potential use in anticancer therapy.

Key words: hemocyanins, Ehrlich ascites carcinoma, antiproliferative activity, apoptosis

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Effect of hyperglycemia on apoptotic protein expression in pubertal rat testes – an immunoblotting approach

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Problem: Complications due to diabetes mellitus (DM) include impaired proceeding of spermatogenesis and male infertility. Investigation of apoptotic protein expression in conditions of hyperglycemia would contribute to elucidate the mechanisms underlying reproductive disorders in diabetic males. Bax is a multidomain, proapoptotic member of the Bcl-2 family that is required for normal spermatogenesis in different mammalian species. The ratio of Bax/Bcl-2 family members is a critical determinant of cell fate: elevated Bcl-2 favors extended cell survival and increased levels of Bax expression accelerate cell death.

Aim: The ***aim*** of our study was to investigate the Bax expression in pubertal rat testes in order to explore the possible mechanisms underlying germ cell apoptosis induced by experimental hyperglycemia.

Method of study: DM was induced by single intraperitoneal injection of streptozotocin at dose of 100 mg/kg b.w. on day 1 (neonatally, NDM) or day 10 (prepubertally, PDM) in rats. Treated animals were sacrificed on day 18 (early puberty). To detect changes in expression of the proapoptotic protein Bax by SDS-PAGE and Western blot testis lysates were used. As a loading control housekeeping protein α-actin was applied.

Results: Our results revealed strong but not constant reaction of antibody against proapoptotic Bax protein in pubertal rat testes. We detected bands on the membrane corresponding to 21kDa Bax protein in most of the NDM and PDM samples. In the control group Bax expression was not detected. Bax protein levels in both NDM and PDM testes increased after hyperglycemia, however Bax expression in NDM testes were higher than in PDM experimental samples.

Conclusion: The experimentally induced diabetes mellitus (NDM and PDM) leads to higher Bax protein expression that favors apoptosis in pubertal rat testes. Expression of pro-apoptotic factor Bax is probably involved in suppression of spermatogenesis caused by hyperglycemia.

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Key words: Diabetes mellitus, testis, apoptosis, Bax

The muscle phase of trichinellosis in mice is associated with increased ST3Gal-6 sialyltransferase activity in skeletal muscle fibers

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Abstract

In our previous work we showed that the de-differentiation of the occupied portion of muscle fibers toward a Nurse cell after invasion by *Trichinella spiralis* is associated with increased intracellular accumulation of α-2,3-sialylated glycoproteins. Our current investigation demonstrated up-regulated protein expression of the enzyme ST3Gal-6 in mouse skeletal muscles invaded by *T. spiralis*. Muscle samples were collected at certain time points after *per os* invasion. Immunohistochemistry was performed using a rabbit polyclonal antibody against ST3Gal-6 sialyltransferase. We found up-regulation of this enzyme shortly after the occupation of the muscle fibers, which persisted also in the mature Nurse cell. The ST3Gal-6 sialyltransferase is responsible for the transfer of sialic acid to galactose preferably on Gal-β-1,4-GlcNAc lactosamine structure and, to a lesser extent, on Gal-β-1,3-GlcNAc, as acceptors. The substrate specificity of this sialyltransferase and its up-regulation during the muscle phase of trichinellosis are particularly interesting since the lactosamine is one of the anticipated oligosaccharide structures of the α-dystroglycan. Alpha-dystroglycan is the only muscle protein known so far to be sialylated and plays a crucial role in the maintenance of the structural integrity of the skeletal muscle tissue. One of the enigmatic features of the Nurse cell of *T. spiralis* is its ability to stay integrated within the surrounding unaffected tissue even when the contractile properties of it muscle fiber are irreversibly lost. Our finding raises two important questions:

1. Could be ST3Gal-6 the sialyltransferase that is responsible for the sialylation of the α-dystroglycan?
2. Does the biosynthesis of α-dystroglycan continue also during the de-differentiation of skeletal muscle fiber toward a Nurse cell?

Our instant efforts are currently dedicated to elucidate these two matters.

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Petri nets representation of mucin type O-glycan biosynthesis

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Abstract

Mucin-type O-glycosylation is a widespread post-translational modification of proteins. Representing mucin O-type biosynthesis with Petri nets formalism is indispensable for analysis of this pathway. Our analysis show that synthesis of Tn antigen could be a bottleneck for production of Core 1 to Core 8 antigens. Either downregulation of polypeptide N-acetylgalactosaminyltransferase or availability of UDP-GalNAc and/or Ser/Thr peptide could affect such outcome. Another bootleneck is glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase. Modified expression, as observed in cancer, will affect synthesis of sialyl-T antigen and Core1/Core 2 antigens. Other points of interest are acetylgalactosaminy l-O-glycosyl-glycoprotein beta-1,3-N-acetylglucosaminyltransferase, which affects synthesis of Core 3/Core 4 antigens. Keratan sulfate pathway can also be influenced by down/upregulation of polypeptide N-acetylgalactosaminyltransferase, glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase and beta -1,3-galactosyl-O-glycosyl-glycoprotein beta-1,6-N-acetylglucosaminyltransferase.

Key words: mucin type O-glycan biosynthesis, Petri nets.

Introduction

Mucin-type O-glycosylation is a widespread post-translational modification of proteins. Mucin-type O-glycans are found on many glycoproteins and are highly diverse in their structures. Such type of glycans are also found on many cell surface and secreted proteins, and consequently are uniquely positioned to modulate cell recognition, adhesion, and communication events. [1]

O-glycans are assembled by the addition of individual sugar residues by specific glycosyltransferases.Mucin-type glycosylation is characterized by the initial addition of a GalNAc sugar to the hydroxyl group of serine or threonine residues. The initiation of this process by peptidyl GalNAc-transferases is ruled by the sequence context of putative O-glycosylation sites. The process of this type of O-glycosylation is confined to the cis and trans Golgi compartments. [2].

Mucins are major components in mucus secretions and cell membranes on wet-surfaced epithelia. O-glycans contribute to maintaining the highly extended and rigid structure of mucins, conferring to them specific physical and biological properties essential for their protective functions. Secreted mucins in the tear have been involved in the clearance of debris and pathogens from the surface of the eye. Mucin O-glycans on the cell-surface glycocalyx protects corneal and conjunctival epithelia, and as such prevent bacterial adhesion. Mucins play role in sorting and secretion of glycoproteins and influence protein conformation. [3].

Dramatic alterations of the glycosylation machinery are observed in cancer cells, resulting in aberrantly O-glycosylated proteins that expose previously masked peptide motifs and new antigenic targets. In disease the biosynthesis of O-glycans is

abnormal, stemming from the abnormal expression and activities of the glycosyltransferases involved. [4].

Material and Methods

We used KEGG/ENZYME database as a source of pathway information. Bipartite Petri graphs G = (V1, V2; E) were constructed from two disjoint sets of nodes, called places (V = P) and transitions (V = T), V1, ∪ V2 =V, which are connected by edges e ∈ E ⊆ V. The input range I (x) of an element x ∈ P ∪ T of a Petri net is given by I (x) ={y|(y, x) ∈ E}, the output range as O(x) ={y|(x, y) ∈ E} [9] and [5].

Results

The first product of mucin type O-glycan biosynthesis, Tn antigen (see Fig. 2), is obtained by action of GALNT enzyme (see Table 2) on Protein L-serine and UDP - N - acetylgalactosamine (see Table 1). GALNT adds GalNAc to L-serine of the protein substrate.

Tn antigen is a crossroad substrate, which through action of differen enzymes can give different products, such as sialyl-Tn antigen and Core 1 to Core 8 glycans. Action of ST6GalNAc I on Tn antigen produces sialyl-Tn antigen (see Fig. 2), which has terminal sialic acid. However action of C1GALT1 on Tn give a range of final products, namely sialyl-T antigen, and Core 2. Direct product of C1GALT1 is T antigen (see Fig. 2), a glycan with terminal galactose. We can obtain sialyl-T antigen, containing terminal sialic acid, through action of SIAT4A on T antigen. Sialyl-T antigen is converted to disialyl-T antigen by action of SIAT7A.

From Core 1 glycans we can obtain Core 2 glycans through action of GCNT1. Core 2 glycans are characterized by two terminal sugars - GlcNAc and Gal (see Fig. 2). Next, Core 2 products could be precursors for the Keratane sulfate biosynthesis.

From Tn antigen we may go to Core 4 glycans by action of several enzymes. Core 3 product, containing two terminal GlcNAc (see Fig. 2), is obtained by action of B3GNT6 on Tn antigen. Next Core 3 is converted to Core 4 by GCNT3.

Core 6 glycans, containing terminal galactose attached to 4 GlcNAc and 6 GalNAc (see Fig. 2), can be syntheiszed from Tn antigen through action of acetylgalactosaminyl - O - glycosyl - glycoprotein beta - 1,6 - N-acetylglucosaminyltransferase. Core 6 glycans are substrates of B4GALT5 enzyme for synthesis of F1 α.

There are spin-off pathways of mucin type O-glycan biosynthesis pathway. For example Cores 5, 7 and 8 can be obtained from Tn antigen. Core 5 antigen contain terminal galactose attached to 3 GalNAcs, whereas Core 7 is composed of terminal galactose linked to 6 GalNAcs (see Fig. 2). Core 8 also has terminal galactose linked to 3 GalNAcs (see Fig. 2).

Discussion

As we can see from Fig. 1, Tn anigen can be a bottleneck for synthesis of a range of products. Tn antigen is syntheised from protein L-serine and UDP-N-acetylgalactosamine by action of polypeptide N-acetylgalactosaminyltransferase (GALNT). The GALNTs are a family of conserved enzymes that initiate the mucin-type O-glycosylation in cells. The family of GALNTs are responsible for the altered glycosylation in cancer. Thus GALNT-T6 and -T3 expressions correlate significantly with tumour differentiation. [6]. Consistent up- or down-regulation of GALNTs expression during cancer development has been

frequently reported. For example GALNT shows a stage-dependent expression at the different stages of colorectal cancer [7]. Pancreatic ductal adenocarcinomas produce higher levels of truncated O-glycan structures (such as Tn and sTn) than normal pancreata. Dysregulated activity of GALNT leads to increased expression of these truncated O-glycans. Knockout of GALNT leads to increased tumorigenicity in KPC mice [8].

Another point of interest (see Fig. 1) is T antigen, or Core 1 glycans. T antigen is precursor of sialyl-T antigen and Core 2 glycans. The expression of sialyl-3T antigen, which is the product of SIAT4A, is substantially increased in prostate cancer [9]. However higher sialyl-T antigen production may be influenced not only by upregulation of SIAT4A, but also from altered expression of C1GALT1 and GALNT. Indirect evidence of C1GALT1 upregulation, by increased levels of C1GALT1 autoantibodies, is presented for head and neck cancer [10]. As reported above consistent up-regulation of GALNTs expression during cancer development has been frequently reported. Similar to sialyl-T antigen Core 2 glycans can amass due to upregulation of either GCNT1,C1GALT1 and/or GALNT enzymes. We have evidence that expression of C1GALT1 and GALNT is upregulated in cancer (see above). As for GCNT1, this enzyme is significantly up-regulated in clinical prostate carcinoma [11]. Overexpression of Core 2 glycans was found in human prostate cancer cell line C4-2B(4) [12]. However it is not clear, as suggested by our Petri Net presentation of mucin type O-glycan biosynthesis, upregulation of which of the three transitions (enzymes) is responsible for this overexpression of Core 2 glycans.

Next point of interest are Core 3 and Core 4 glycans (see Fig.1). We couldn't find any information for enchanced expression of Core 3, Core 4 and B3GNT6. However in mouse pancreatic cancer tumors, GCNT3 upregulation was correlated with increased expression of mucins. [13]. Also, GCNT3 is overexpressed in highly metastatic melanomas. [14]. We couldn't find data for enhanced expression of Core 6 glycans in tumors. No data of upregulation of acetylgalactosaminyl-O-glycosyl-glycoprotein beta - 1,6 - N - acetylglucosaminyl transferase is available, but B4GALT5 is highly expressed in MDR cells [15].

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Table 1 : Representation of places (substrates and products) in fig. 1.

	Name	Structure
1	Protein L-serine	C4H6N2O3R2
1.1	UDP-N-acetylgalactosamine	(UDP-GalNAc)1
Tn	Tn antigen	(GalNAc)1 (Ser/Thr)1
2	Sialyl-Tn antigen	(GalNAc)1 (Neu5Ac)1 (Ser/Thr)1
3	T antigen (Core 1)	(Gal)1 (GalNAc)1 (Ser/Thr)1
3.1	sialyl-T antigen	(Gal)1 (GalNAc)1 (Neu5Ac)1 (Ser/Thr)1
3.1.1	disialyl-T antigen	(Gal)1 (GalNAc)1 (Neu5Ac)2 (Ser/Thr)1
3.2	Core 2	(Gal)1 (GalNAc)1 (GlcNAc)1 (Ser/Thr)1
3.3	Core 3	(GalNAc)1 (GlcNAc)1 (Ser/Thr)1
3.3.1	Core 4	(GalNAc)1 (GlcNAc)2 (Ser/Thr)1
3.4	Core 5	(GalNAc)2 (Ser/Thr)1
3.5	Core 6	(GalNAc)1 (GlcNAc)1 (Ser/Thr)1
3.5.1	F1 α	(Gal)1 (GalNAc)1 (GlcNAc)1 (Ser/Thr)1
3.6	Core 7	(GalNAc)2 (Ser/Thr)1
3.7	Core 8	(Gal)1 (GalNAc)1 (Ser/Thr)1

Table 2: Representation of transitions (enzymes) in fig.1.

	Name	Definition	EC Number
1’	GALNT	polypeptide N-acetylgalactosaminyltransferase	2.4.1.41
2’	ST6GalNAc I	alpha-N-acetylgalactosaminide alpha-2,6-sialyltransferase (sialyltransferase 7A)	2.4.99.3
3’	C1GALT1	glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase	2.4.1.122
3.1’	SIAT4A	beta-galactoside alpha-2,3-sialyltransferase (sialyltransferase 4A)	2.4.99.4
3.1.1’	SIAT7A	alpha-N-acetylgalactosaminide alpha-2,6-sialyltransferase (sialyltransferase 7A)	2.4.99.3
3.2’	GCNT1	beta-1,3-galactosyl-O-glycosyl-glycoprotein beta-1,6-N-acetylglucosaminyltransferase	2.4.1.102
3.3’	B3GNT6	acetylgalactosaminyl-O-glycosyl-glycoprotein beta-1,3-N-acetylglucosaminyltransferase	2.4.1.147
3.3.1’	GCNT3	N-acetylglucosaminyltransferase 3, mucin type	2.4.1.-
3.5’		acetylgalactosaminyl-O-glycosyl-glycoprotein beta-1,6-N-acetylglucosaminyltransferase	2.4.1.148
3.5.1’	B4GALT5	beta-1,4-galactosyltransferase 5	2.4.1.-

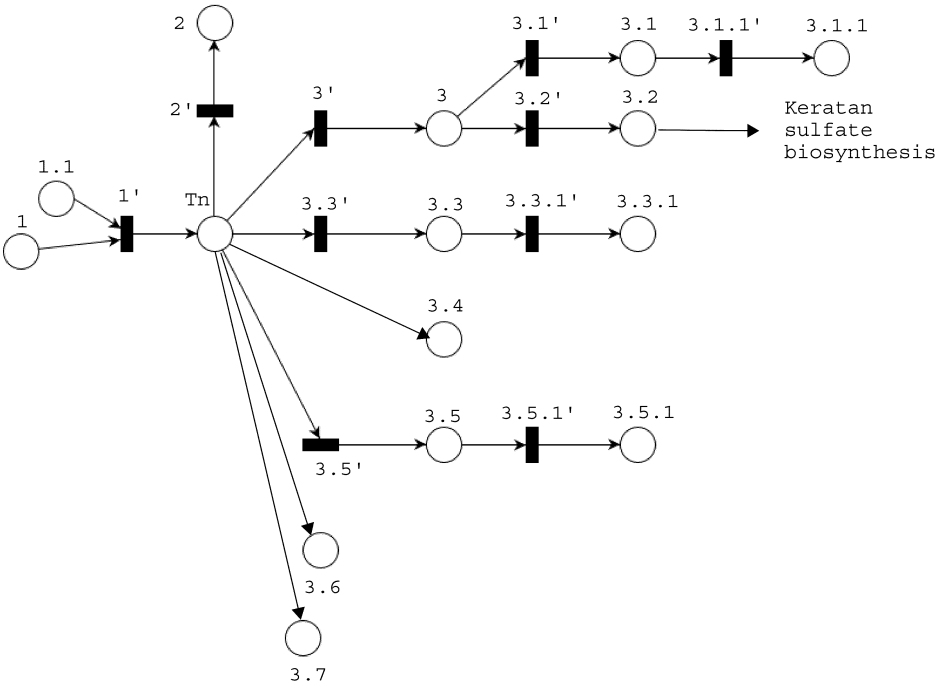

Fig. 1: Petri nets representation of mucin type O-glycan biosynthesis.

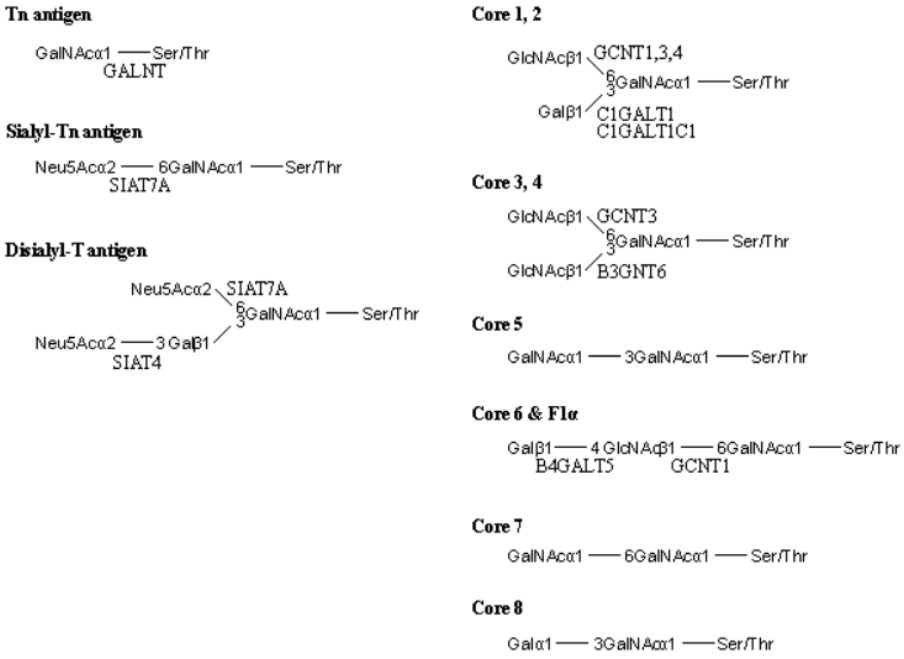

Fig. 2: Schematic presentation of mucin type O-glycans.

Паразити и противопаразитно третиране на лабораторни зайци, отглеждани в клетка

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Резюме
Целта на настоящото проучване беше да установим евентуалната опаразитеност на лабораторни зайци и ефективността на приложеното им впоследствие лечение. Бяха установени хелминти от два нематодни рода - *Passalurus*, с екстензивност на инвазията (ЕИ) 10,5%и *Nematodirus* с ЕИ 5,3%, както и протозои от род *Eimeria* (ЕИ 57,9 %), представен от видовете *E.magna,E. media*и *E.perforans*. От еймериите при най-много животни беше установен вида *E. media.* Три от зайците, опаразитени с еймерии, бяха с изявени клинични признаци на заболяването. Опаразитените животни бяха третирани индивидуално според установения паразитологичен статус с Fenbendazole в доза 25mg/kg, Sulfachlorpyrazi n30% в доза 1г на литър вода за 10 дни и поливитаминни препарати. Паразитологичният статус на зайците беше изследван отново на 10 ден и 40 ден след третирането, като при установяване на паразити отново беше извършвано третиране с подходящото за съответния етиологичен агент противопаразитно средство. Резултатите от проведеното изследване показаха следното: Лечението на лабораторни зайци с признаци на еймериоза със Sulfachlorpyrazinи поливитаминни препарати води до тяхното клинично възстановяване, но не спира носителството и отделянето на ооцисти. Приложението на фенбендазол води до преустановяване на отделянето на нематодни яйца от родовете *Passalurus*и *Nematodirus*, но 40 дни след третирането отново се наблюдава излъчителство на пасалурусни яйца, което сочи, че еднократното третиране с фенбендазол не е достатъчно за окончателното излекуване на зайците от пасалуроза.

Ключови думи: лабораторни зайци, *Eimeria, Passalurus,Nematodirus,* противопаразитно третиране

Увод

Експерименталната научната работа включва редица етапи, един от които е “in vivo” или провеждане на изпитания върху живи организми. Най-често за тази цел се използват лабораторни животни - мишки, плъхове, зайци и други. За провеждане на достоверни изследвания опитните животни трябва да са здрави. Наличието на инфекции, явни или скрити, могат да увеличат биологичната вариабилност на лабораторните животни (Eslampanah et al., 2014), което да се отрази върху резултатите от провежданите експерименти. Паразитните инфекции са често срещани сред лабораторните животни. Въпреки предприемането на профилактични мерки заразяването с паразити може да се случи още във фермите, където се отглеждат животни за експериментални цели или по-късно във вивариумите на научните центрове. Ето защо е необходимо преди започване на експерименти, в които са включени лабораторни животни, те да бъдат

подложени на паразитологични изследвания и при нужда да бъдат обезпаразитени с подходящите срещу установените паразити лекарствени средства. Имайки предвид това си поставихме за цел да изследваме паразитологичния статус на лабораторни зайци, отглеждани в индивидуални клетки, и да проведем последващо противопаразитно третиране.

Материали и методи

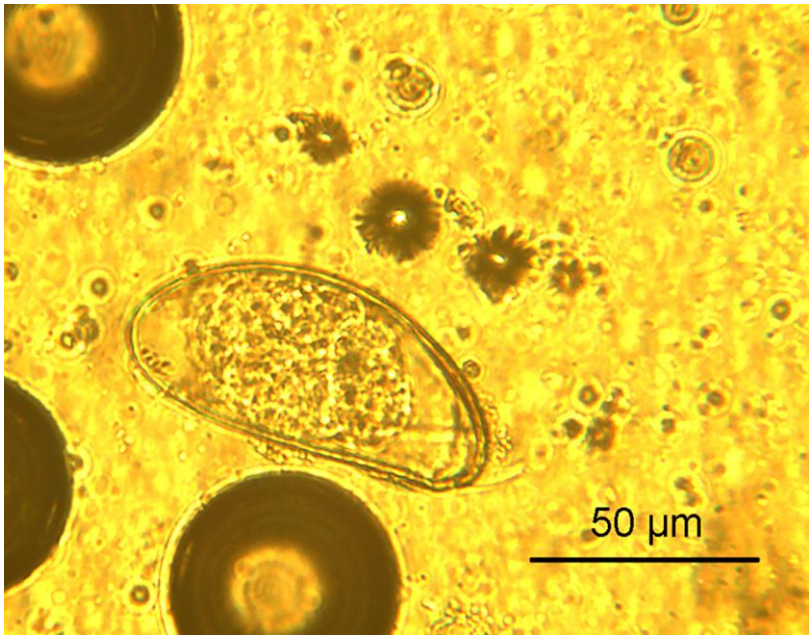
В изследването бяха включени 19 животни от породата Бял новозеландски заек, всички от мъжки пол. Те бяха доставени от Института по животновъдни науки – Костинброд на 3 месечна възраст. Във вивариума на ИЕМПАМ-БАН бяха настанени в индивидуални клетки. За храна получаваха концентриран фураж за зайци за угояване без добавка на кокцидиостатици, сено за зайци-домашни любимци и моркови. По време на отглеждането им 3 от животните проявиха признаци на заболяване - отслабване, разрошена козина, диария и дехидратация. Това наложи извършването на паразитологични изследвания на всички животни от групата. Бяха изследвани индивидуални копропроби по овоскопските методи на Фюлеборн и последователните утаявания, ларвоскопския метод на Вайда и метода на Мак Мастер за количествена оводиагностика (Койнарски и кол., 2009). Таксономичната идентификация на установените ооцисти се извърши с помощта на ключа от определителя на Eckert et al. (1995), а на нематодните яйца по Foreyt (2001). Опаразитените животни бяха третирани индивидуално според установения паразитологичен статус и както е предписано в указанията на производителя с Panacur 10% susp. BG® (активно вещество – Fenbendazole), Sulfachlorpyrazin 30%, Вигамин С, VeyFo Jecuplex® (съдържащ L-карнитин, бетаин, витамини от трупа В и есенциални аминокиселини) и Catosal®(съдържащ витамин В12). Изследвахме паразитологичния статус на зайците на 10 ден и 40 ден след третирането, като при установяване на паразити отново беше извършвано третиране с подходящото за съответния етиологичен агент противопаразитно средство. През целия период клетките на животните бяха почиствани с вода, сапун и прибавен към тях 96% етилов спирт.

Резултати и обсъждане

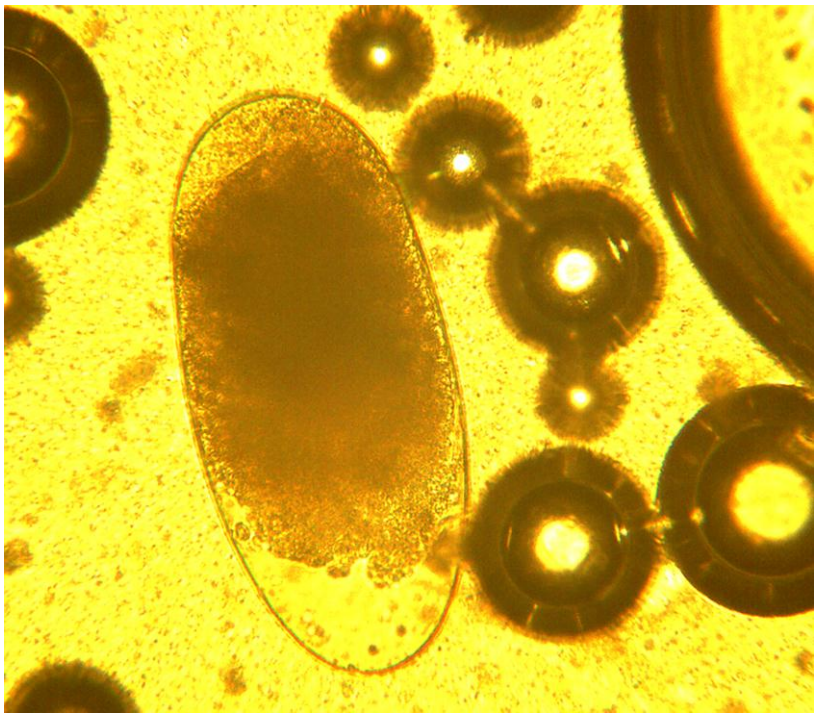
Резултатите от изследването са представени в Таблица 1. Бяха установени хелминти от два нематодни рода – *Passalurus* (Фиг. 1) и *Nematodirus* (Фиг. 2) и протозои от род *Eimeria*, представен от видовете *E. magna* (Фиг. 3), *E. media* (Фиг. 4) и *E. perforans* (Фиг. 5).

Таблица 1. Екстензивност на инвазията при лабораторни зайци преди и след третиране с противопаразитни средства

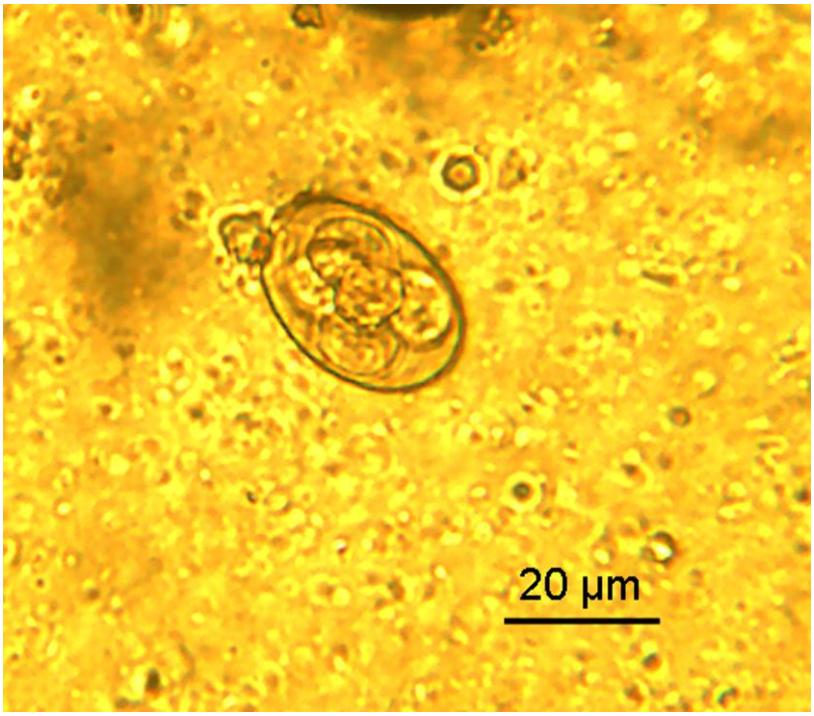
	Eimeria	*Passalurus*	*Nematodirus*
Преди третиране	57,9 %	10,5 %	5,3 %
След 10 дни	37,5 %	-	-
След 40 дни	42,9 %	35,7 %	-



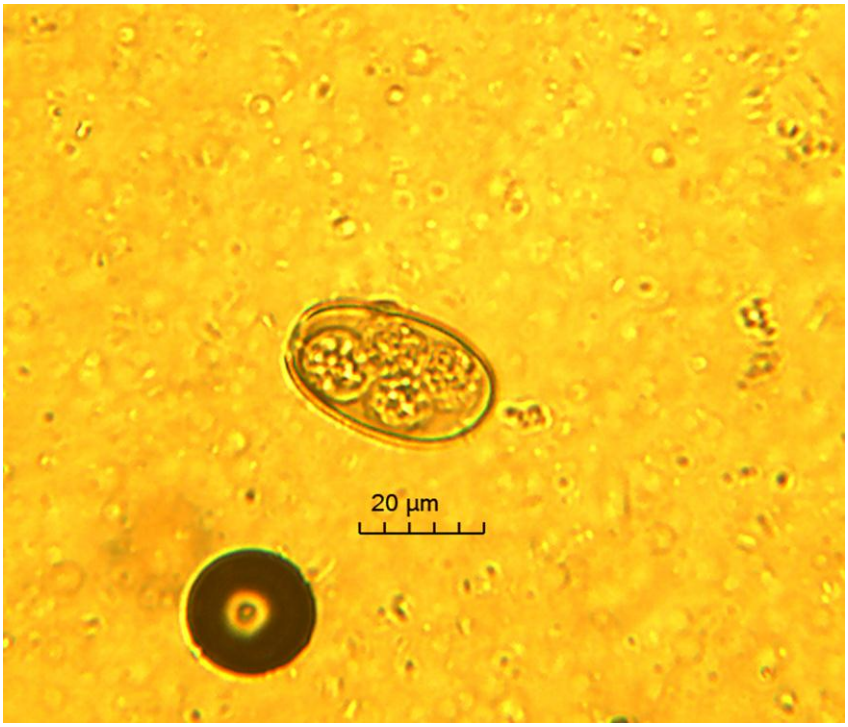
Фиг. 1. Яйце на нематод от род *Passalurus*; копропроба от лабораторен заек (ориг.)



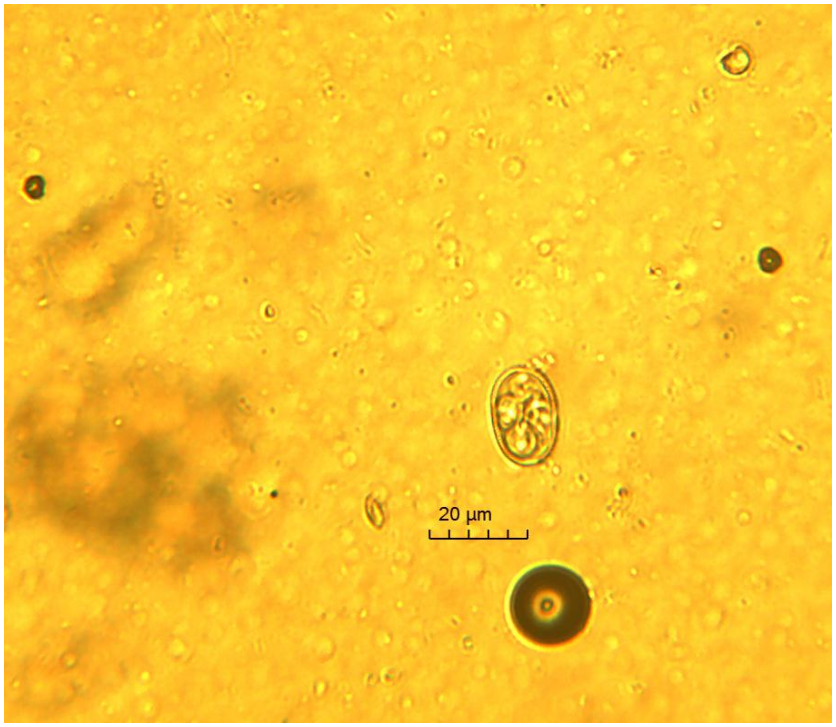
Фиг. 2. Яйце на нематод от род *Nematodirus*; копропроба от лабораторен заек (ориг.)



Фиг. 3. *Eimeria magna*; копропроба от лабораторен заек (ориг.)



Фиг. 4. *Eimeria media*; копропроба от лабораторен заек (ориг.)



Фиг. 5. *Eimeria perforance*; копропроба от лабораторен заек (ориг.)

Както се вижда от Таблица 1, в началото на изследването род *Eimeria* беше с най-висока есктензивност на инвазията, последван от род *Passalurus* и род *Nematodirus*. Родовете *Eimeria* и *Passalurus* са били с най-висока екстензивност при изследванията върху домашни зайци и от други автори (Nosal et al., 2006; Eslampanah et al., 2014; Kornas et al., 2015; Mäkitaipale et al., 2017). При нашето изследване от еймериите най-често наблюдавахме вида *E. media*. Този вид е бил най-често срещан и при изследванията на Kornas et al. (2015).

Десет дни след третирането в копропробите от всички зайци не бяха открити яйца от двата рода нематоди. Установихме обаче, че отделянето на еймерийни ооцисти продължава. Въпреки че екстензивността на инвазията с тези паразити беше понижена – от 57,9 % на 37,5 %, след това изследване отново извършихме третиране на животните със Sulfachlorpyrazin. Четиридесет дни след началото на изследването екстензивността на инвазията с кокцидии отново беше повишена, но нивата ѝ бяха по-ниски в сравнение с тези преди първото третиране. На този етап не открихме яйца от род *Nematodirus*, но находката показа наличие на яйца от род *Passalurus*, и то с почти трикратно по–висока екстензивност на инвазията в сравнение с тази преди третирането с Panacur (Табл. 1).

По време на изследването проследихме трите животни, които показаха изявени клинични признаци на заболяване - отслабване, разрошена козина, диария и дехидратация. Беше установено, че те са опаразитени с еймерии. Доминантен вид и при трите животни беше *E. media*, а при преброяването на ооцистите установихме средно 270 000 ооцисти в 1 гр. фекална маса. След приложеното лечение болните зайци се възстановиха клинично, но продължиха да бъдат носители и да отделят ооцисти.

Заключение

Лечението на лабораторни зайци с признаци на еймериоза със Sulfachlorpyrazin 30% в доза 1г на литър вода за 10 дни и поливитаминни препарати води до тяхното клинично възстановяване, но не спира носителството и отделянето на ооцисти.

Приложението на Fenbendazole в доза 25 mg/kg вози до преустановяване на отделянето на нематодни яйца от родовете *Passalurus* и *Nematodirus*, но 40 дни след третирането отново се наблюдава излъчителство на пасалурусни яйца, което сочи, че еднократното третиране с фенбендазол не е достатъчно за окончателното излекуване на зайците от пасалуроза.

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Resistance of *varroa destructor* mite to some acaricides

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Abstract

Varroa destructor is an ectoparasitic mite on the honey bee *Apis mellifera*, that is causing enormous damage to beekeeping worldwide.

Synthetic varroacides have been used the longest time and most commonly by beekeepers worldwide. One of the most important reasons for the development of high resistance is the improper use of acaricide by the beekeepers themselves. The main mistakes that beekeepers most often make are applying lower or higher doses than necessary, frequent use of the same product or those of one synthetic group. This is the main reason of the low efficacy of the applied acaricides than expected efficacy according to the label. This involves risk of Varroa destructor mite developing acaricides resistance. Therefore it is necessary to monitor the effectiveness of acaricides treatments to ensure appropriate protection of the honeybee colonies. Recently in many honey bee apiaries, high infestation levels of *V. destructor* mite are being detected in colonies of *Apis mellifera* after treatment with some synthetic acaricide like as organophosphate coumaphos, formamidine amitraz, pyrethroids tau-fluvalinate, etc.

The purpose of this work is to presents short review of recent studies to estimate the acaricide resistance of *Varroa destructor* mite populations to some common used varroacides in honey bee colonies worldwide.

Keywords: *Apis mellifera, Varroa destructor* mite, synthetic acaricides, resistance

Introduction

The parasitic mite *Varroa destructor* is a major damaging pest of the western honey bee *Apis mellifera* worldwide. This necessitates the use of a number of means by beekeepers to combat it. Without treatment, colonies generally collapse within a few years. Most commonly used varroacides are synthetic and organic acaricides, because they are easy to apply and cheap. The most popular commercially available ones include amitraz, coumaphos, flumethrin, fluvalinate, formic acid, oxalic acid and thymol. Prolonged use of conventional acaricides causes Varroa mites to rapidly develop resistance and bee products can become contaminated. Residues of acaricides are present in high concentrations throughout the hive and bees are exposed to them all year around and can affect the bee health and the human health as a consumer of bee products.

In many cases as wax combs are contaminated with high concentrations of synthetic acaricide residues and Varroa mites are chronically exposed to them, the parasite may develop resistance faster. The development of acaricide resistance in *Varroa* populations is a global issue. Numerous commercially available acaricides and their active substances have been shown to have negative effects on honey bee brood development, queen, worker bees and drone reproductive health, learning,

longevity and colony strength [16]. Most of synthetic acaricides do not only act alone, but also in synergic combinations to affect bee health.

At present, high infestation levels of *V. destructor* are being detected in colonies of *Apis mellifera* after treatment with coumaphos. Maggi et al. [9] have been reported for the first time the resistance to coumaphos in *V. destructor* in Argentina. They have studied the LC50 of coumaphos in *V. destructor* from four apiaries with high mite density after treatment with coumaphos and have found that the LC_{50} have been 112, 319, 127 and 133 μg/Petri dish for mites from the four apiaries. Significant LC_{50} differences have been detected between resistant and susceptible mites. LC_{50} have increased 197–559-fold when compared to the corresponding baseline, suggesting the development of resistance. The next year the same authors [10] have been detected high infestation levels of *V. destructor* in colonies of *Apis mellifera* after treatment with amitraz. They have determined the LC50 of amitraz in *V. destructor* from three apiaries with high mite density after treatment with the acaricide. The LC50 values have been 3.9, 3.5, and 3.7 μg/Petri dish for mites from three different apiaries. Significant LC50 differences have been detected between resistant and susceptible mites. LC50 increased 35–39-fold when compared to the corresponding baseline and they are suggesting the development of resistance.

Maggi et al. [11] have been studed the susceptibility of *V. destructor* populations from Uruguay to fluvalinate, flumethrin, coumaphos, and amitraz. Such populations had never been exposed to synthetic acaricides. They have been also determined whether acaricide resistance to coumaphos occurred in apiaries in which miticide rotation had been applied. In their study, they have been found that high mite mortality at lower pesticide concentrations could be observed in susceptible populations of *V. destructor*. According them the detoxification mechanisms of *V. destructor* have been inefficient to counteract the toxic effect of the substances tested. With regard to the active toxicants tested in this study, only LC_{50} of coumaphos could be estimated (0.15 μ/dish). For amitraz, fluvalinate, and flumethrin, the estimated LC50 has been below 0.3 μ/dish and could not have been calculated given the high mite mortality registered at all concentrations assayed. These results have been proved that all acaricides featured higher miticide effects when susceptible mite populations have been considered. The finding that resistance is detected in apiaries subjected to acaricide rotation schemes is fairly new and alarming at the same time and demonstrates the potential of *V. destructor* to continually adapt to the selection pressures imposed by beekeepers.

The occurrence of resistant mites is not associated with the misuse of treatments as has often been claimed, since the same end result will occur irrespective if the treatment has been used correctly or not. Pyrethroid resistant mites have been first detected in the Lombarby region in the north-west of Italy around 1991 [12]. Resistant mites quickly spread via bee movement into the neighbouring regions of southern Switzerland, Slovenia and southern France. From there it continued its spread throughout Europe following established colony trade routes in France. finally reaching Germany in 1997, Finland via possible bee movement from Italy in 1998 and UK in August 2001 [12]. This has been caused mainly by beekeepers moving infested colonies.

Semkiw et al. [15] have been studied the effectiveness of amitraz used as the contact varroacide (Biowar 500 formulation) to control *V. destructor* in honeybee colonies by Polish beekeepers for a long time. They have been found that the efficacy of the strips has been lower in colonies with larger amount of brood, but this rule is valid for all synthetic acaricides.

Bąk et al. [4] have examined Varroa mites from 79 apiaries from north-eastern Poland. Varroa mites resistant to fluvalinate and flumethrin have been found in one apiary

situated near Olsztyn. These parasites have showed an unusual ability to survive as compared to parasites from the other apiaries. In this apiary LC95 for fluvalinate has been 5000 ppm, and for flumethrin, 100 ppm. Mites with a high risk of developing resistance to fluvalinate and flumethrin have been discovered in two apiaries situated near Kwidzyn.

Kamler et al. [8] have developed and tested a polypropylene vial bioassay on tau-fluvalinate-, acrinathrin-, and amitraz-resistant mite populations from three apiaries in Czechia. The results that they have found indicated that polyproplyne vial tests can be used to determine discriminating concentrations for the early detection of acaricide resistant Varroa. They have been concluded that multiple-resistance in some apiaries may indicate metabolic resistance.

Due to the facts of widespread of the developed resistance to the miticides of fluvalinate and coumaphos Rinkevich [13] investigated the resistance of amitraz was evaluated in commercial beekeeping operations in Louisiana. Higes et al. [7] have reported that in Spain, there is great concern because there are many therapeutic failures after acaricide treatments intended to control varroosis outbreaks. In their work, a simple bioassay methodology has been used to test whether some reports on low efficacy in different regions of Spain were in fact related to reduced Varroa sensitivity to the most used acaricides. This biological test has proven to be very effective in assessing the presence of mites, which are assessed after exposure to acaricides. In the tested samples, the mortality caused by coumaphos ranged from 2 to 89%; for tau fluvalinate, it varies from 5 to 96%. On the other hand, amitraz caused 100% mortality in all cases. These results suggest the presence of Varroa, resistance to coumaphos and fluvalinate in apiary samples, even in those where these active ingredients cannot be used in recent years.

According to Rinkevich et al. [14] the loss of effective amitraz treatments to control Varroa mites is a disconcerting prospect due to the low rate of product development to specifically and effectively control Varroa mites. For practical purposes concerning managing mites with chemical means, the beekeeper may have to balance taking a honey crop with colony survival.

The appearance of coumaphos resistant mites is a worrying trend, and it needs to be established quickly if a similar mechanism to that used by pyrethroid resistant mites is been employed. Molecular biology will help us understand the resistance mechanisms and the costs associated with resistance, which in turn will help in the development of the most effective control strategy [12]. According to Martin [12] if the resistance mechanisms employed by varroa do in fact have no or a small cost then rotational use of different acaricides, i.e. using a pyrethroid one year then coumaphos in the next year followed by a pyrethroid in the next and so on, will be ineffective.

In Bulgaria have been made several field experiments about the effectiveness and resistance of flumethrin and fluvalinate. Gurgulova et al. [5] have been investigated the effectiveness of Varostop strips (as active ingredient it contains 3.6 mg flumethrin per one wood strip) conducting three field experiments in intervals of ten years. The results have been showed high average effectiveness of Varostop of 98,60% (from 96,8% to 99,27%), and 99,15%, respectively for 1997 and 2002 and average effectiveness of 98,95% (from 98,12% to 99,47%) for 2007.

Gurgulova et al. [1] have been studied the acaricide effectiveness of the both products Varostop (3.6 mg flumethrin per stnp) and Ecostop (5.0 g thymol and 2.0 g mint oil per plate) developed for Varroa control from "Primavet-Sofia" Ltd. The study has been carried out on 30 bee colonies at a test apiary in the region of the city of Stara Zagota a comparative examination during spring treatment. They have found that Ecostop

manifested its effect (90.59 ±1.83 %) agains Varroa in a dose of 2 piates to colonies with strength of 1.72±0.04 kg and parasite infestation rate of 4.98±1.40 % among the bees and 46.80±21.17 % among the brood. Varostop in a dose of 2 strips applied in colonies with strength 1.82±0.05 kg has manifested 99.15±0.39 % effectiveness. In a study conducted in 2011 Gurgulova et al. [2] compare the action of flumethrin (Varostop - strips) and fluvalinat (Varotom- strips) against *Varroa destructor* mite. They have found higher effectiveness for Varostop (98.17±0.62%) than Varotom (88.97± 2.71%). The high efficiency of Varostop-strips was confirmed in a study conducted in the fall of 2017 [3].
Based on the conducted experiments we can conclude that there is no established resistance of *Varroa* mite to flumethrin in Bulgaria yet.

Conclusions

Different hypotheses could be postulated to explain the Varroa mite resistance to some synthetic acaricides:
- The presence of nearby apiaries with resistant mite populations from which high rates of parasites re-infestation are produced.
- A high amount of residues of syntetic acaricides in wax, favoring resistance development. Sublethal concentrations of acaricides in this matrix would lead to a continuous change in mite susceptibility to coumaphos, tau fluvalinate and amitraz in individuals increasingly resistant. As wax combs are contaminated with high concentrations of acaricide residues and Varroa mites are chronically exposed to them, the parasite may develop resistance faster.
- Synthetic acaricides must be used judiciously so the long term effectiveness of these compounds can be maximized.
- The consistent use of acaricides from the same chemical group should be avoided. Every year it is necessary to be applied miticide rotation.
- A resistance monitoring network should be established to ensure the sustainability of miticide use for Varroa control.

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Toxicity of the most popular acaricides against *varroa destructor* mite

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Abstract

Every year worldwide, honey bees are responsible for over 90% of pollination in almost 85% of crops that need pollination. Without a sufficient number of healthy and viable bees, this process would be impossible and losses to agriculture would amount to billions of euros a year.

In recent years, there has been a sharp decline in the number of bee colonies worldwide, a phenomenon known as colonies collapse disorder (CCD).

The reasons for this phenomenon are numerous and often complex - from adverse climate change, lack of sufficient quality grazing, improper operation to obtain more production, the use of banned or highly toxic pesticides to treat crops, parasitic, viral and bacterial infections to improper use of preparations used in bee colonies to control bee diseases. One of the reasons for the high mortality in bee colonies is the mite *Varroa destructor*.

The lack of a 100% effective and safe mite control agent leads to continuous and severe prevention using various chemicals, which when applied incorrectly often lead to the creation of resistant mite offspring, the presence of residues in bee products - honey, pollen, wax, as well as and intoxication of the bees, brood, or queen themselves.

This work presents a short review on the recent research on the toxicity of the most commonly used acaricides against the ectoparasitic mite *Varroa destructor*.

Key words: honeybees *Apis mellifera, Varroa destructor* mite, acaricides, toxicity

Introduction

Today, synthetic acaricides (specifically coumaphos, amitraz, and tau-fluvalinate) are the most common methods to control *Varroa* infestations. These compounds, however, are frequently related to a wide range of side effects in the host, as well as a long half-life inside the hive matrices as wax and honey [8].

The mite *Varroa destructor*, a parasite of honey bees (*Apis mellifera* L.) can attack both adult bees and brood, and beekeepers are frequently compelled to use acaricides to avoid colony death. Acaricides should be minimally harmful to the bees, whilst maintaining toxicity to mites. The problems conecting with the use of chemical acrirides used in apiculture are, on the one hand, a negative effect on the bees themselves and, on the other hand, the presence of residues in bee products, which reflect on human health.

The role that pesticides and their residues in hive products may play in colony collapse disorder and other colony problems is discussed. Although no single pesticide has been shown to cause colony collapse disorder, the additive and synergistic effects of multiple pesticide exposures may contribute to declining honey bee health. There are many studies that confirm the negative effects of chemical varroacides if they are used incorrectly. Some acaricides may affect the synthesis, transport, action, or elimination

of natural molecules, such as hormones or enzymes, that are responsible for maintaining development, immune mechanisms, and behavior of treated bee colonies.

The aim of the present work is to perform a short overview on the studies conducted with different acaricides in bee colonies.

Pettis at al. [20] have studied the effect of organophosphate pesticide coumaphos on young honey bee larvae transferred into the queen cups in known concentrations of coumaphos in wax (0 to 1000 mg/kg). When they have placed these larvae in queenless colonies and examined ten days later to determine the rate of rejection or acceptance as indicated by a mature sealed queen cell. They have found that all queens failed to develop at 1000 mg/kg, and greater than 50% of the queen cells were rejected at the 100 mg/kg concentration. Additionally, queens that survived exposure to 100 mg/kg coumaphos weighed significantly less than control queens.

Dahlgren [5] in her dissertation has been quantified differences between queen and worker tolerance of five acaricides and clarifies the honey bee mechanism of tolerance for coumaphos. Selected acaricides have been topically applied to adult queen and worker bees to generate dose-response curves and LD50s. She has been found that twenty-four hours after treatment, queens were 3-times more tolerant of tau-fluvalinate and 6-times more tolerant of thymol than workers when adjusted for body weight differences between workers (108 mg) and queens (180 mg). Queens survived the highest administered doses of fenpyroximate (1620 μg/g) and coumaphos (2700 μg/g) indicating that queens have been at least 12-fold more tolerant of coumaphos and at least 40-fold more tolerant of fenpyroximate than workers. However, queens treated with as little as 54 μg/g of fenpyroximate exhibited have reduced survival over 6 weeks following treatment. Amitraz has been the only acaricide tested for which queens have been not more tolerant than workers. According Dahlgren caste differences have been likely due to metabolism, but further studies are needed to determine specific mechanisms.

Dahlgren et al. [4] have been applied five acaricides, each with a different mode of action, to young adult queen and worker bees to generate dose–response curves and LD_{50}. Twenty-four hours after treatment, queens have been found to be three-times more tolerant of tau-fluvalinate and six-times more tolerant of thymol than workers when adjusted for body weight differences between workers (108 mg) and queens (180 mg). Queens have survived the highest administered doses of fenpyroximate (1620 μg/g) and coumaphos (2700 μg/g) indicating that queens have been at least 11-fold more tolerant of coumaphos and at least 54-fold more tolerant of fenpyroximate than workers. They have found that, queens treated with as little as 54 μg/g of fenpyroximate exhibited reduced survival over 6 wk after treatment. According their results amitraz has been the only acaricide tested for which queens were not more tolerant than workers. They have been concluded that the striking difference in acaricide tolerance of queen and worker honey bees suggests physiological differences in how the two castes have been affected by xenobiotics**.**

Smodis Skerl et al. [26] have been studied honey bee colonies, which have been exposed to pesticides used in agriculture or within bee hives by beekeepers: coumaphos; diazinon; amitraz or fluvalinate. On the sixth day after treatment, coumaphos has been found in the royal jelly (250 ng/g) secreted by nurse workers and fluvalinate whas been found in both bee heads (105 ng/g, 8 days after treatment) and in larvae (110 ng/g, 4 days after treatment). Amitraz residues in all sampled material have been below the level of detection of 10 ng/g. Diazinon has been not detected in any of the analysed samples. They have found large quantities of fluvalinate in bee heads and larvae, and coumaphos residues in royal jelly. They have been observed that sub-lethal

doses of acaricides applied to bees in a colony can also induce elevated cell death in the larval midgut, salivary glands of larvae and hypopharingeal glands of adult bees.
Gregorc [10] has been observed problems four hours after the insertion of coumaphos strips (CheckMite+; Bayer) in one apiary populated with *A. m. carnica* colonies a commercial beekeeper in the Gorenjska region, Slovenia, with approximately 60 hives in the summer 2010. Colonies in this apiary have never been treated with coumaphos before. The previous control against *V. destructor* have been performed by trickling 2.9% (w/w) oxalic acid in sugar solution in the previous autumn. Four hours after the coumaphos treatment, the beekeeper have observed abnormal worker bee behaviour. Bees have been started to leave the hives, fly extensively around them, cluster on the front hive wall, and drop down in the grass in front of the hives. Workers also gathered into small clusters with 10 to 40 bees, and have been dying around the treated hives with extended wings, and curved, shortened and tremoring abdomens. Bees have been also clustered on the back side of the hives, on the hive entrance, and the inside walls. Brood combs were not adequately covered by workers and dead workers were found on the hive bottom board. Dead workers were collected from the hive bottom boards, a second sample of live workers have been collected from the honey combs from the upper hive compartments and a third group of workers have been collected whilst crawling on the grass in front of the hives. A fourth sample of workers was collected from the neighbouring untreated colonies. Quantities of coumaphos in workers sampled from the brood chambers, honey compartments, and in front of the hives have been 1,771; 606 and 514 µg/kg respectively. Workers from honey compartments from untreated colonies have been coumaphos negative. The treated colony populations have been reduced by approximately one third of their previous adult bee population. Two months after the coumaphos application, however, colony development in the treated hives have been normal, and the worker population in the brood chambers have been comparable to the untreated colonies.

According to Chauzat et al., [3] honey bee exposure to some pesticides may have an adverse effect on colony health. These chemicals may affect the synthesis, transport, action, or elimination of natural molecules, such as hormones or enzymes, that are responsible for maintaining development, immune mechanisms, and behavior. They have been examined the possible relationship between low levels of pesticide residues in apicultural matrices (honey, pollen collected by honey bees, beeswax) and colony health as measured by colony mortality and adult and brood population abundance. They have been found no association between the presence of pesticide residues and with variation in populations of adult or broods or with colony mortality.

Gregorc et al. [11] have been studied also the influence of one of nine pesticides and/or challenged with the parasitic mite, *Varroa destructor* honey bee (*Apis mellifera*) larvae reared *in vitro.* Total RNA has been extracted from individual larvae and first strand cDNAs have been generated. They have been found that, Varroa-parasitized brood have been higher transcripts of Deformed Wing Virus than did control larvae (0.001) and have been not changed in pesticide treated larvae. As they have been expected, Varroa-parasitized brood has been higher transcripts of Deformed Wing Virus than did control larvae (< 0.001). Varroa parasitism, arguably coupled with virus infection, resulted in significantly higher transcript abundances for the antimicrobial peptides abaecin, hymenoptaecin, and defensin1. They have found that transcript levels for Hexameric storage protein (Hsp70) have been significantly upregulated in imidacloprid, fluvalinate, coumaphos, myclobutanil, and amitraz treated larvae.

Matías D. Maggi et al., [18] have been estimated the LC50 baseline levels for amitraz, coumaphos, fluvalinate, and flumethrin in susceptible *Varroa destructor* populations in Argentina. Concentration response bioassays have been conducted with each acaricide. According their study laboratory results of lethal concentrations (LC50) have been: 0.1 μg/dish for amitraz; 0.29 μg/dish for fluvalinate; 0.34 μg/dish for flumethrin; and 0.57 μg/dish for coumaphos, respectively.

Boncristiani et al., [1] present data for hives treated with five different acaricides; Apiguard (thymol), Apistan (tau-fluvalinate), Checkmite (coumaphos), Miteaway (formic acid) and ApiVar (amitraz). According their results thymol, coumaphos and formic acid are able to alter some metabolic responses. These include detoxification gene expression pathways, components of the immune system responsible for cellular response and the c-Jun amino-terminal kinase (JNK) pathway, and developmental genes. These have been could potentially interfere with the health of individual honey bees and entire colonies.

Johnson et al., [16] have been made laboratory bioassays based on mortality rates in adult worker bees demonstrated interactive effects among acaricides, as well as between acaricides and antimicrobial drugs and between acaricides and fungicides. Toxicity of the acaricide tau-fluvalinate increased in combination with other acaricides and most other compounds tested (15 of 17) while amitraz toxicity has been mostly unchanged (1 of 15). The sterol biosynthesis inhibiting (SBI) fungicide prochloraz elevated the toxicity of the acaricides tau-fluvalinate, coumaphos and fenpyroximate, likely through inhibition of detoxicative cytochrome P450 monooxygenase activity. Four other SBI fungicides increased the toxicity of tau-fluvalinate in a dose-dependent manner, although possible evidence of P450 induction has been observed at the lowest fungicide doses. Non-transitive interactions between some acaricides have been observed. Sublethal amitraz pre-treatment increased the toxicity of the three P450-detoxified acaricides, but amitraz toxicity has been not changed by sublethal treatment with the same three acaricides. A two-fold change in the toxicity of tau-fluvalinate has been observed between years, suggesting a possible change in the genetic composition of the bees tested. In the other study Jonson et al., [17] have been investigated the influence of some kinds of pesticides on the viability of sperm produced by drones with which the queen mates. In this study they have been applied a range of sublethal doses of six miticides (tau-fluvalinate, coumaphos, fenpyroximate, amitraz, thymol and oxalic acid) to 1–4 day old adult drones. Sperm viability has been not affected by any of the six miticide treatments, which suggests that acute miticide exposure in adult drones has little effect on drones' reproductive potential. However, two of the six miticides tested had an effect on drone recapture rate: fenpyroximate reduced and thymol increased the likelihood of drone recapture. These results have been suggest that future research on the effect of pesticides to drone sperm viability should focus on exposure in the immature stages and that beekeepers concerned with drone reproductive health may be able to safely apply miticides when only adult drones are present in bee colonies.

Rinkevich et al., [24] have been investigated if there are differences in insecticide sensitivity between honey bees of different genetic backgrounds (Carniolan, Italian, and Russian stocks) and assess if insecticide sensitivity varies with age. They have been found that Italian bees have been the most sensitive of these stocks to insecticides, but variation has largely dependent on the class of insecticide tested. There have been almost no differences in organophosphate bioassays between honey bee stocks (<1-fold), moderate differences in pyrethroid bioassays (1.5 to 3-fold), and dramatic differences in neonicotinoid bioassays (3.4 to 33.3-fold). Synergism bioassays with

piperonyl butoxide, amitraz, and coumaphos showed increased phenothrin sensitivity in all stocks and also demonstrated further physiological differences between stocks. In addition, as bees aged, the sensitivity to phenothrin significantly decreased, but the sensitivity to naled significantly increased.

Residues of the organophosphate acaricide coumaphos and the neonicotinoid insecticide imidacloprid, widely used to combat Varroa mites and for crop protection in agriculture, respectively, have been detected in wax, pollen and comb samples. Chaimanee et al. (2016) have been assessed the effects of these compounds at different doses on the viability of sperm stored in the honey bee queens' spermatheca. They have been found that sub-lethal doses of imidacloprid (0. 02 ppm) decreased sperm viability by 50%, 7 days after treatment. Sperm viability has been a downward trend (about 33%) in queens treated with high doses of coumaphos (100 ppm), but there has been not significant difference. The expression of genes that are involved in development, immune responses and detoxification in honey bee queens and workers exposed to chemicals have been measured by qPCR analysis. The data has been showed that expression levels of specific genes were triggered 1 day after treatment. The expression levels of P450 subfamily genes, CYP306A1, CYP4G11 and CYP6AS14 have been decreased in honey bee queens treated with low doses of coumaphos (5 ppm) and imidacloprid (0.02 ppm). Moreover, these two compounds suppressed the expression of genes related to antioxidation, immunity and development in queens at day 1. Up-regulation of antioxidants by these compounds in worker bees have been observed at day 1. Coumaphos also has been caused a repression of CYP306A1 and CYP4G11 in workers. Antioxidants appear to prevent chemical damage to honey bees. They also have been found that DWV replication increased in workers treated with imidacloprid. This research clearly has been demonstrates that chemical exposure can affect sperm viability in queen honey bees.

Premrov et al. [21] have been found accumulation of coumaphos in bee brood and beeswax. In beeswax, the accumulation of coumaphos has been determined not only in hives where it was used but also in hives nearby in which coumaphos was not used. Due to the coumaphos accumulation this drug has been should be used only in strongly affected bee colonies.

According to Dai et al. [6] the acute toxicity of five insecticides to honey bee larvae using an improved *in vitro* rearing method from most to least toxic has been follow: chlorpyrifos > fluvalinate > coumaphos = imidacloprid > amitraz, as the LC_{50} values (mg L^{-1}) for each tested pesticide have been as follows: amitraz, 494.27; chlorpyrifos, 15.39; coumaphos, 90.01; fluvalinate, 27.69; and imidacloprid, 138.84. The LD_{50} values in μg per larva were 14.83 (amitraz), 0.46 (chlorpyrifos), 2.70 (coumaphos), 0.83 (fluvalinate) and 4.17 (imidacloprid).

Rinkevich et al. [25] have been treated colonies with the miticide amitraz (Apivar®), used IPM practices, or left some colonies untreated, and then have been measured the effect of different levels of mite infestations on the sensitivity of bees to phenothrin, amitraz, and clothianidin. Sensitivity to all insecticides varied throughout the year among and within treatment groups. They have been found in-hive amitraz treatment according to the labeled use did not synergize sensitivity to the pesticides tested and this should alleviate concern over potential synergistic effects.

Tihelka [27] has been summarized that to keep the population of the Varroa mites low, beekeepers relay on the use of synthetic and organic acaricides, the most popular commercially available ones include amitraz, coumaphos, flumethrin, fluvalinate, formic acid, oxalic acid and thymol. Numerous commercially available acaricides and their active substances have been shown to have negative effects on

honey bee brood development, queen and drone reproductive health, learning, longevity and colony strength. Acaricides do not only act alone, but also in synergic combinations to affect bee health. In combination with other stressors, acaricides could be a contributing factor to colony collapses.

Gregorc et al. [12] have been evaluated the acaricides: coumaphos, tau-fluvalinate, amitraz, thymol, and natural plant compounds (hop acids), which were the active ingredients. The acaricide efficacies in the colonies have been evaluated in conjunction with the final coumaphos applications. All of the tested acaricides significantly increased the overall Varroa mortality in the laboratory experiment. The acaricide toxicity to the Varroa mites has been consistent in both the caged adult honey bees and workers in the queen-right colonies, although, two of these acaricides, coumaphos at the highest doses and hop acids, have been comparatively more toxic to the worker bees.

Gregorc et al. [13] have been tested also coumaphos at 92,600 ppb concentration, in combination with 5 and 20 ppb imidacloprid. They have been found that coumaphos induced significantly higher bee mortality, which has been associated with down regulation of catalase compared to coumaphos and imidacloprid (5/20 ppb) mixtures, whereas, both imidacloprid concentrations independently had no effect on bee mortality. Mixture of coumaphos and imidacloprid have been reduced daily bee consumption of a control food patty to 10 mg from a coumaphos intake of 14.3 mg and 18.4 and 13.7 mg for imidacloprid (5 and 20) ppb, respectively. While coumaphos and imidacloprid mixtures have been induced down-regulation of antioxidant genes with noticeable midgut tissue damage, imidacloprid has been induced intensive gene up-regulations with less midgut apoptosis.

Gashout et al. [9] have been studied honey bees (*Apis mellifera* L.) which have been exposed to LD05 and LD50 doses of five commonly used acaricides for controlling the parasitic mite, *Varroa destructor*. LD50 values at 48 h post-treatment have been showed that tau-fluvalinate has been the most toxic, followed by amitraz, coumaphos, thymol, and formic acid. Among the acaricides tested, coumaphos, amitraz, and thymol have been appear to be the safest acaricides based on their hazard ratios, and a good marker to detect differences between the effects of sub-lethal doses of acaricides is monitoring changes in acetylcholinesterase gene expression.

Dai et al. [7] have been studied the effects of chronic exposure to common acaricides on *Apis mellifera* survival, developmental rate and larval weight have been tested in the laboratory in vitro and fed a diet containing amitraz: 1.5, 11, 25 and 46 mg/L; coumaphos: 1.8, 6, 8 and 25 mg/L; or fluvalinate: 0.1, 1, 2.4 and 6 mg/L. They have been concluded that if larvae have exposed to acaricides at concentrations similar to maximum residue in pollen and honey/nectar had been no detectable change in survival or developmental rate. Dai et al. [7] have been suggested that residues of amitraz, coumaphos and fluvalinate at the levels they have tested are unlikely to impact immature worker bee survival in the field bee colonies.

Haber et al. [14] have been focused on seven varroacide products (amitraz, coumaphos, fluvalinate, hop oil, oxalic acid, formic acid, and thymol) and six nonchemical practices (drone brood removal, small-cell comb, screened bottom boards, powdered sugar, mite-resistant bees, and splitting colonies) suggested to aid in Varroa control. They have been found that nearly all large-scale beekeepers used at least one varroacide, whereas small-scale beekeepers were more likely to use only nonchemical practices or not use any Varroa control. They have found also that use of amitraz was associated with the lowest winter losses, than any other varroacide product.

Mitton et al. [19] have been explored the effect of *p*-coumaric (600 μM) and indole-3-acetic acid (2, 20 or 200 μM) supplementation on the survival and activity of key detoxification enzymes of honey bees exposed to *tau*-fluvalinate. Their results have been showed that dietary *p*-coumaric acid increased the levels of cytochrome P450 and glutathione reductase activity in bees treated with *tau*-fluvalinate, as well as in the untreated controls, while glutathione-S-transferase activity was lower in treated bees than in untreated. According to authors the enzymatic changes have been related to the detoxification mechanisms observed in bees that have been fed with *p*-coumaric and indole-3-acetic acids could be responsible for the increased survival of bees treated with *tau*-fluvalinate compared to those that received a control diet.

Qi et al. [22] have been studied, the effects of flumethrin, a pyrethroid miticide used on bee colonies, was evaluated using bee larvae reared *in vitro*. They have been found that flumethrin induced significant mortality during larval metamorphosis and adult emergence. After continuous exposure during the larval stage, significant changes have been observed in antioxidative enzymes (SOD and CAT), lipid peroxidation (MDA, LPO, and POD), and detoxification enzymes (GSH, GST, and GR) in the late instar larvae before pupation. Qi et al. have been believed that these findings indicate that flumethrin itself has been toxic to bee larvae and has been potential risks during colony development.

Hudson et al. [15] have been used an in vitro rearing method to characterize the effects of seven pesticides (amitraz, coumaphos, fluvalinate, chlorpyrifos, imidacloprid, chlorothalonil, glyphosate) on developing brood subjected dietary exposure at worse-case environmental concentrations have been detected in wax and pollen. Their findings suggest that pesticide exposure during larvae development may affect the survival and health of immature honey bees, thus contributing to overall colony stress or loss. Additionally, they have been concluded that pesticide exposure altered gene expression of detoxification enzymes.

According to Zikic et al. [28] coumaphos had a negligible effect on bee survival, but it significantly affected their oxidative status: superoxide dismutase (SOD), catalase (CAT) and glutathione S-transferase (GST) activities, and the concentrations of malonyl dialdehyde (MDA). Coumaphos significantly ($p<0.0001$) have been decreased SOD activity in non-infested bees, but increased it in those infested with *Varroa destructor* mite. They have been found that coumaphos in non-infested hives caused oxidative stress per se, not unlike varroa in infested colonies. However, in infested colonies it has been decreased oxidative stress, owing to its efficacy against Varroa mites and contributed to the recovery of bee colonies. In spite of its certain downsides, coumaphos remains an effective anti-varroa substance, but should be used with precaution, not to add to the effects of environmental factors which may cause red-ox misbalance.

Conclusions

The application of a some chemical acaricides, especially when it is for the first time, must be done very carefully, in accordance with the conditions - temperature, humidity, grazing, distance between bee colonies. Acaricide treatment in closely placed bee hives with strong colonies should be performed with great caution and also considered as a potential source of acute toxicity.

When choosing a treatment for varroosis, the impact on neighboring apiaries (especially if they are biologically certified) should be taken into account, due to the effect of some acaricides, such as coumaphos, which is also found in beeswax of untreated hives nearby. The involvement of coumaphos strips has to be further

investigated in order to minimize the potential negative effects to colonies. In spite of its certain side effects, coumaphos remains an effective anti-varroa substance, but should be used with precaution.

Different bee castes (queen, worker honey bee, drone, honey bee larvae and brood) have different tolerances to the chemical acaricides used. There are differences in insecticide sensitivity between honey bees of different genetic backgrounds (Carniolan, Italian, and Russian stocks).

The chemical exposure of synthetic acaricides can affect sperm viability in queen honey bees.

Acaricides have a different mode of action, depending on whether they are applied to infested whit *Varroa destructor* or not infested colonies. Some acaricides as a flumethrin can induced significant mortality during larval metamorphosis and adult emergence. Chemical compounds like coumaphos (5 ppm) and imidacloprid (0.02 ppm suppressed the expression of genes related to antioxidation, immunity and development in queens.

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The molds of the genus Aspergillus and their toxins- health hazards in food

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Abstract
Micheli, an Italian priest and naturalist, first described and named in 1729 the fungi Aspergillus (aspergillum from the Latin term aspergere, "to scatter"- a device used for sprinkling holy water), because of their shape. These microscopic fungi are widespread saprophytes or parasites, especially in warmer regions. However, our most frequent contacts with Aspergillus remain plants and products of plant origin. In immunocompromised patients the fungus is causative agent of the disease aspergillosis, affecting the brain and participates in the formation of mycetomas in the pulmonary upper airways, alveoli, the maxillary sinuses or sabotages bone marrow transplants. Also, in the period of 1960-1970, were identified highly toxic metabolites of Aspergillus molds– aflatoxins. They were grouped in four main groups- aflatoxins B_1, B_2, G_1 and G_2 and along with more than 17 derivatives of their compounds are pro causative agents of mycotoxicoses and cancer.

Key words: fungi Aspergillus, aspergillosis, mycetomas, aflatoxins, mycotoxicoses and cancer.

Brief overview

The molds of the genus Aspergillus were first described in 1729 by Pierre Micheli, an Italian priest and naturalist who observed the fungus under a microscope. He was the first who started to study fungi and proved their growth on a medium in opposite of the theory of the "spontaneous generation". Micheli named the fungi Aspergillus (aspergillum from the Latin aspergere, "to scatter"- a device used for sprinkling holy water), because of their shape (Dorland, 2003). These microscopic fungi are widespread in the upper soil layers, especially in warmer regions. Most of the molds of this genus are saprophytes, but there are also representatives that parasitize on humans and animals. However, our most frequent contacts with *Aspergillus* remain plants and products of plant origin. There the fungus forms colonies most often with green, blue or blue-green color, rarely in another color. Such colonies can be observed on bread and pasta when stored in damp rooms, as well as on leather goods.
Interestingly, of the known 300–350 species, sexual reproduction has been found in about 64%. The reproduction of these moulds can be done in two ways - combining nuclear material from two different individuals in heterothallic fungi or self-fertilization in homothallic aspergillus (Varga et al., 2014). *Aspergillus fumigatus* (*A. fumigatus*) is a leading fungal pathogen and the best known species, parasitizing in immunocompromised patients - humans and animals (Cimon et al., 2001; Bakare et al., 2003). The fungus is causative agent of the disease aspergillosis, often affecting the brain (Fig. 1) and participates in the formation of biofilm and mycetomas in the pulmonary upper airways, alveoli, or in the maxillary sinuses (Mensi et al., 2004; Filler

and Sheppard, 2006; Mowat et al., 2009). *A. fumigatus* accomplish angioinvasion and damage endothelium leading to proinflammatory response and activation of coagulation factors (Ben-Ami et al., 2010).

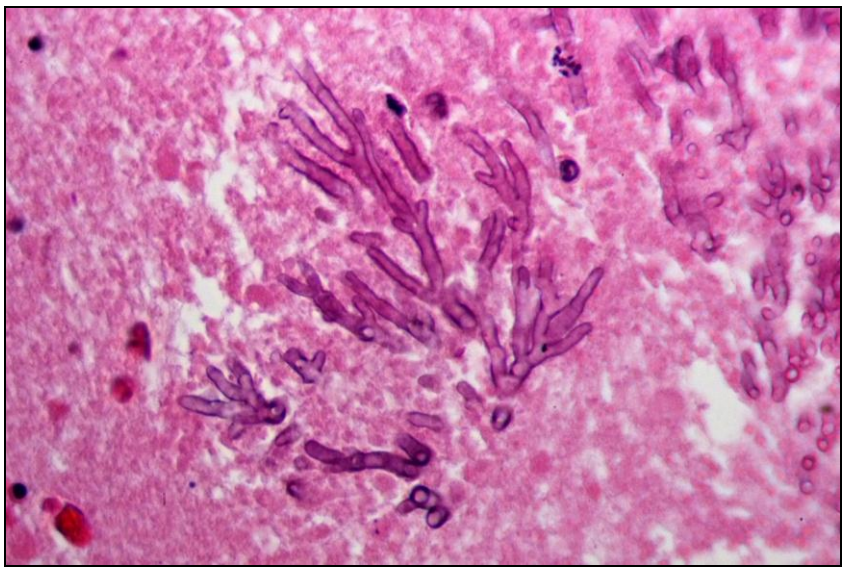

Fig.1. Cerebral aspergillosis in an immunocompromised child, HE
Source:https://commons.wikimedia.org/wiki/File:Cerebral_aspergillosis_in_an_immunocompromised_child,_HE.jpg#filehistory

This species is one of the most significant opportunistic fungi in bone marrow transplant patients (Singh, 2005). Other known representatives pathogenic to humans and animals are *A. flavus, A. parasiticus, A. nomius* and *A. niger*, which produce highly toxic carcinogenic difuranocoumarin derivatives– aflatoxins (Chen et al., 2016; Mahato et al., 2019). *A. flavus* is a mesophilic fungus, that grows at a temperature of 6-7 ° C to 45-46 ° C and can synthesize aflatoxins. Important factors that potentiates the fungus for the synthesis of aflatoxins are the humidity of the substrate and the atmospheric air.

Aflatoxins are compounds with a similar structure, forming a specific group of highly oxygenated heterocyclic compounds present naturally in fodder. Four main groups of aflatoxins B_1, B_2, G_1 and G_2 and about 17 derivatives of their compounds have been identified since now. The first report of aflatoxins was made in Britain in 1961, when severe intoxication was observed in turkeys, killing 100,000 birds (Smith et al., 2019). Numerous publications from around the world on poisonings in calves and pigs fed peanut meal contaminated with microscopic Aspergillus fungi followed, and later the causative toxins- aflatoxins were identified as products of these molds. Aflatoxins as contaminants of peanut meal imported from India were also described in Bulgaria (Станкушев и Спесивцева, 1971)

These are some of the most studied mycotoxins due to their association with a number of diseases, including cancer in humans and domestic animals (Shank, 1976; 1977). The great attention paid to them was based on the fact that they have a strong carcinogenic effect in laboratory animals and cause fatal aflatoxicosis in many species.

The synthesis of mycotoxins from Aspergillus fungi is generally influenced by geographical features and environmental conditions, by agronomic and agricultural practices during the cultivation of crops, the collection, transport and storage of raw materials. A number of authors point out that the main factors for the development of

microscopic fungi producing aflatoxins are the presence of amino acids, high humidity and temperature, aeration and the way the substrate is treated (Борисова, 2009)

These mycotoxins have low molecular weight and are resistant to many processing processes. They are sensitive to ultraviolet rays, but are stable during heat treatment and it does not affect their concentration durin cooking. This is the reason why they are often found in peanuts or waffles and other peanut cream products that are being withdrawn from market.

Sensitivity to aflatoxins in different animals varies by species and age, but the LD_{50} (lethal dose for 50% of animals) is in the range of 0.3 to 18 mg/kg body weight. The main difference in the species sensitivity is primarily due to variations in metabolic rates of toxic substance metabolism. Turkeys and waterfowl are the most affected among birds, while hens, quails, pheasants and hens are more resilient. In mammals, monogastric animals and young ruminants are more sensitive (Peles et al., 2019).

Humans are exposed to aflatoxins through food contaminated with the fungi that produce them. Symptoms that occur in acute poisoning are characterized by: vomiting, abdominal pain, pulmonary edema, convulsions, coma and death from cerebral edema, fatty degeneration of the liver, kidneys and heart. The main route of entry of aflatoxins into the body is alimentary. Once in the digestive tract, aflatoxins are distributed to all organs, including the brain, through the blood and lymph. The incoming toxins accumulate rapidly mainly in the liver, where they are metabolized and excreted or by bile and faeces, or through the kidneys and urine. An important fact is the possibility of several isoenzymes belonging to the cytochromes P450 supergene family to metabolize aflatoxins through oxidative reactions to other metabolites with different carcinogenic potential. (Peles et al., 2019). While some aflatoxins may enhance their action by oxidation, reduction and hydrolysis and the formation of polarity and activity-enhancing functional groups (metabolic toxicity), others may conjugate with glucuronic acid and block their functional groups. by reducing their toxic effects.

The emergence of new compounds with strong toxic properties in metabolic conversion is the case with the bio-transformation of aflatoxins B_1 and B_2 and their conversion into aflatoxins M_1 and M_2, respectively. For example, aflatoxins M_1 and M_2 were the first to be isolated from the milk of lactating animals fed with feed containing with aflatoxins. In cow's milk, metabolites M_1 and M_2 can be detected at a lower concentration than the initial concentration of aflatoxins B_1 and B_2 (Fink-Gremmels, 2008). Also other aflatoxin metabolites could be found in milk, originating from hepatic-biotransformation reactions (Fink-Gremmels, 2008). Recent data show that aflatoxicol- the major metabolite of aflatoxin B_1 produced by the rumen microflora is also excreted with milk (Carvajal et al., 2003).

The main target organ after aflatoxicosis is the liver (Fig. 2) and it is also accompanied by the development of liver cancer in some cases. Although aflatoxicosis is primarily a liver disease and causes liver damage (Serck-Hanssen, 1970), it also affects the growth, development, health status, productivity and reproduction of animals and humans.

Fig.2. Acute liver damage after consumption of aflatoxins
Source:http://vet.uga.edu/ivcvm/courses/vpat5215/digestive/week03/liverdiseases/hepatotoxins.htm

For example, consumption of high levels of these toxins with food causes gastrointestinal dysfunction and decreased digestion, anemia and jaundice- symptoms which can be mistaken with different diseases. Pregnant women can miscarry due to the embryotoxic effect of aflatoxins, and breastfeeding mothers can poison their babies with milk.

The mechanism of toxic action of aflatoxins is associated with inhibition of protein synthesis by DNA modeling and inhibition of messenger RNA synthesis. Impaired protein synthesis is a prerequisite for disorders in the formation of enzymes and energy starvation for the cell. The synthesis of triglycerides, phospholipids and cholesterol is also impaired. Aflatoxins have a pathological effect on mitochondria, the endoplasmic reticulum and lysosomes, releasing hydrolytic enzymes that further damage cells.

Aflatoxin B_1, aflatoxin M_1, and aflatoxin G_1 have been shown to cause various cancers in many laboratory animals, but the International Agency for Research on Cancer (IARC) has so far only identified aflatoxin B_1 as a human carcinogen. based on numerous epidemiological studies from Africa and Asia for the development of liver cancer.

In conclusion, various aflatoxins pass through food and feed and converted into metabolites that retain biological activity and impair health and productivity. According to us and other authors the assessment of undesirable effects of Aspergillus fungi should include also the antimicrobial activity on microbiome, which role in macroorganism is crucial.

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Development of a novel approach for comet assay staining applicable for visible light microscopy

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Abstract
The Single-Cell Gel Electrophoresis (SCGE or Comet assay) is a technique for detecting and evaluating DNA damage, which allows genotoxicity evaluation of chemical compounds or physical agents in both laboratory conditions and the environment. Since its development, SCGE was introduced as a standard method for evaluation of DNA damage, biomonitoring and genotoxicity testing. Later on, the development of many modifications of the Comet assay enabled it to be used as a tool for studying a plethora of phenomena including pollution, food safety and even chromatin structure. Notably, one of the major disadvantages of the Comet assay is the necessity of the expensive fluorescent microscope for results visualization.
Here, we review our recent endeavors in making the Comet Assay more versatile through accommodation of the observation of the results under visible light microscopy.

Keywords: Comet assay, DNA stains, genotoxicity, fluorescent microscopy, chromatin structure

Observation of DNA in the cell nucleus

One of the doyens in the cell nuclear architecture, Peter Cook, has said: "The cellular nucleus is probably the most suspicious structure in the cell" [1]. Indeed, the nucleus was determined to be the most important structure soon after its discovery and therefore, was a subject of interest for many decades and to many scientists. The nucleus is transparent for the visible light microscopy. However, many natural and synthetic stains were developed, that give the nucleus colour. By the invention of the electron microscopy, a possibility emerged to observe the cell in resolution and detail far surpassing those of the conventional light microscope allowing investigation of the cell nucleus as well. For decades, the electron microscope was one of the most powerful tools and the golden standard in the repertoire of biologists, working to elucidate the structure and functions of cellular compartments. However, mostly due to its cost and the specific requirements for sample preparation, the electron microscopy was partially replaced by the fluorescent microscope. Similar to the conventional light microscope, the fluorescent microscope utilizes light, however, the

light that is observed is a product of the excitation of fluorescing molecules. A lot of methods for fluorescent observation of biological objects, including cellular compartments such as the nucleus have been developed. Some of those methods employ simple fluorescent chemical compounds, while others involve complex bioengineered molecules, that possess both fluorescent properties and specific affinity for certain cellular molecules or structures, thus allowing targeted observation of the cellular structure of interest.

Today, the observation of the cell remains a valuable method for both research and medical diagnostic purposes. Conventional light microscopy and fluorescent microscopy are the two techniques that are most commonly used for this.

Staining of cellular structures for visible light microscopy

Due to their different chemical composition and chemical and physical properties, the parts of a cell are stained by diverse types of stains or dyes. Depending on the electric charge of the dye, there are three main groups of dyes: anionic or acidic; cationic or basic; and neutral or amphoteric. Fourth additional groups of compounds that provide colour to cellular components are the pigments that stain hydrophobic structures like lipids. The stains used in biology can be divided also by the composition – simple or complex stains. For example, eosin, an anionic stain, reacts with positively charged cellular structures, and thus stains the cytoplasm and the extracellular matrix in pink. Hematoxylin, a cationic dye, binds to negatively charged cellular structures and stains the nucleus in blue. Applied together, eosin and hematoxylin are the most commonly used tissue stains in histological and medical diagnosis.

Widely used in cytology, histology, microbiology, haematology and medical diagnosis are also the stains called Romanowsky type. The Romanowsky type stains represent a large family of compound stains used mainly in histological observations. They usually consist of one anionic and one cationic dye. The anionic molecule is typically eosin or similar derivative of fluorescein and the cationic molecule most often is a thiazine dye. Throughout the years, because of the separate work of many biologists, physicians and parasitologists, a vast number of Romanowsky type stains have been developed, differing in composition and the details that they reveal in the stained tissues or cells. A main characteristic and advantage of Romanowsky type stains over the other stains is the polychromatic reaction that they produce with the stained sample itself or simply polychromasia. Polychromasia and the closely related but separate phenomenon metachromasia allow the Romanovsky type stain to colour the biological sample in a wide palette of distinguishable tones, usually from pale pink to red and from pale blue to almost black or purple. The superior staining ability of Romanowsky type stains is due to the independent reaction of the two dyes present with the suitable cellular substrate on one hand and the ability of the dyes to form complexes between themselves and together with the substrate on the other [3]. Additionally, the metachromatic reaction causes a colour shift in some of the stained structures, so that the final stained object possesses far more colour shades that one could expect from the two simple dyes alone. Unfortunately, up until now, the exact mechanisms of the metachromatic reaction remain unexplained. However, some authors have made efforts for its elucidation [5, 10, 11].

Comet assay

Based on the study done by Cook et al. in 1967, who analyzed the nuclear structure, the Single-Cell Gel Electrophoresis (SCGE) was developed in 1984 by Ostling and Johanson [1, 8]. SCGE relies on the mobility of DNA under an electric field. If cells are in the suitable matrix – agarose gel and lysed by detergents, the lysis results in nuclei immobilization in the space of the matrix. This allows performing electrophoresis on them – the gel containing the immobilized nuclei is placed in a constant external electric field. If DNA in the nuclei has been damaged by genotoxic action under these conditions, it is migrating from the remaining nucleus towards the positive pole. The electrophoretic movement of DNA loops away from the nuclei results in particular structures resembling astronomical comet, hence the widely used name of the method is the Comet assay. The cell comet has a head – the remainder of the cell nucleus and a tail, which is made of DNA strands and loops. The form, length and size of the tail, as well as other parameters, depending on the amount and quality of DNA damage. Therefore, using the SCGE, the amount and traits of DNA damaging agents can be assessed, observed, measured and analyzed. To that order, the comets resulting from the Comet assay are observed, photographed and analyzed, which allows reliable detection and evaluation of presence and levels of genotoxic effects on the cells.

Since the creation of the method of Comet assay, the results have been observed using a fluorescent microscope, by several fluorescent dyes, including acridine orange, ethidium bromide, DAPI and others. Later on, a new fluorescent dye was created - SYBR Green, which provides far superior results and to this day, it is the most sensitive fluorescent dye for Comet assay. Despite the many advantages of Comet assay over other DNA damage and genotoxicity assays, the requirement of fluorescent microscopes for performing the method is still an obstacle in many laboratories or certain places where they do not have one. For example, the protection of the environment is an important issue and serious biomonitoring is required on-place for the timely response to pollutions. The requirement of a fluorescent microscope for SCGA means that the detection of genotoxic environmental pollution is delayed in time and therefore it is not performed on-site. Therefore, removing this requirement and substituting it with the utilization of the much cheaper and also portable solution of conventional light microscopy is a task with major importance for many fields.

For the visible light microscopy observation of comets in Comet assay a silver staining method was also developed [6]. However, the silver staining is time-consuming (two to three days), laborious, hard to perform and worse - highly sensitive to various internal and external factors. Adding to those negatives, the results often are less than satisfactory, as the background is heavily contaminated with non-specific adhered silver; the method also has an overall loss of sensitivity compared to the fluorescent methods. Thus, although some Comet assay techniques suitable for conventional visible light microscopy do exist, they do not provide any improvement in portability and applicability of the method.

The first real progress toward resolving the requirement of the fluorescent microscope for performing the method of comet assay was made in 2014. Osipov et al. evaluate the possibility of utilization of Romanowsky type stains for conventional light microscopy visualization of comets [7]. Despite being a proof of principle, the method developed by Osipov et al., by our experience, appeared to be difficult if not impossible to be reproduced. This is most probably due to some specificities of the Romanovsky type stains that the authors may have overlooked.

We have focused our efforts on the development of convenient staining to make a possible observation of comets in Comet assay under visible light (Fig. 1). The method of staining developed by us shows that the Comet assay can be used in portable laboratories and can be performed directly on the site of pollution [9]. Besides, this method of staining turned out to be sensitive enough to be applied for studying of chromatin structure. This was done by us in a method called Chromatin Comet Assay (ChCA), as is shown in Figure 1 [4].

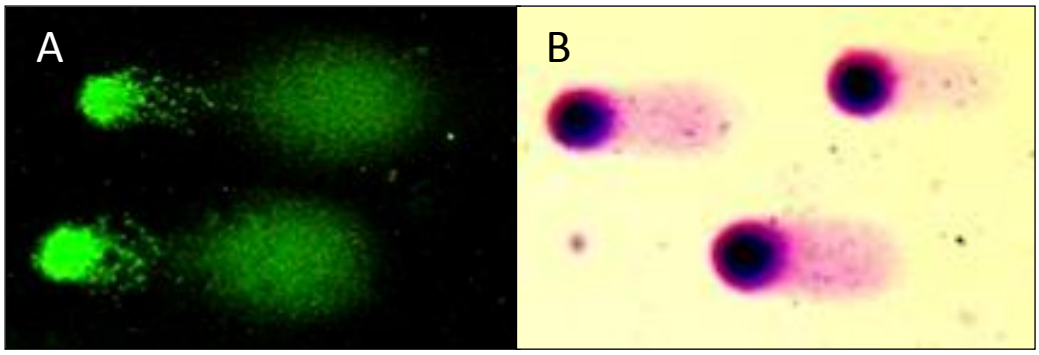

Figure 1. Chromatin Comet assay (ChCA) on mussel cells: A. Comets stained with SYBR Green and visualized by fluorescent microscopy; B. Comets stained for visible light microscopy (P.I. & G.M., unpublished results)

In conclusion, the results obtained by the methodology developed by us proved that comets can be visualized for observation under visible light microspore. Thus, the method of Comet assay can be made a lot less expensive and simpler.

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The monoamine oxidase family and its role in psychology and neuropathology

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Abstract

The monoamine oxidase family consists of two isoenzymes MAO-A and MAO-B, which are mitochondrial enzymes and take a central part in the metabolism of amine neurotransmitters, and therefore are important in many neurological functions. Their genes are X chromosome located and share high similarities. Both MAO-A and MAO-B are flavoprotein oxidases and the present FAD-binding domain in their molecules is essential for the catalytic function.

Here, we discuss in detail the importance of MAO-A, the different applications of its inhibitors and the role of the enzyme in the pathology of certain neurodegenerative diseases and psychological disorders.

Keywords: monoamine oxidase, MAO-A, MAO-B, amine neurotransmitters, MAO-A inhibitors, neurodegenerative diseases

The monoamine oxidase family: structure and functions

The enzyme monoamine oxidase (MAO) has first been isolated in 1928 from a rabbit liver by Mary Hare and later two forms of the enzyme have been established and grouped in the monoamine oxidase family. It consists of two isoenzymes – the monoamine oxidase A (MAO-A) and B (MAO-B) which have an incredibly important role in the metabolism of monoamine neurotransmitters [2]. The genes for both MAO-A and MAO-B are located on the X-chromosome and in humans specifically they are located on the short arm (Xp11.23) and span over 60kb. It has been hypothesised that the two genes are derived from one ancestral gene since they both consist of 15 exons with the exact organisation of exons and introns [15]. The structure of MAO genes, especially MAOA is of great significance when it comes to certain disorders and neurological conditions. For example, the Brunner syndrome is caused by a nonsense mutation in exon 8 of the MAOA gene, which leads to complete deficiency of the enzyme, and results in severe behavioural changes and mental retardation. Moreover, the varying polymorphisms of MAOA can be biomarkers of

some psychiatric disorders [2, 3]. In the promoter region of the MAOA gene, a functional polymorphism of a variable number tandem repeat (VNTR) has been widely recognised as important since the number of repeats proved responsible for more or less efficient transcription. The MAOA-uVNTR repeat sequence can be present in 2, 3, 3.5, 4, 5 or 6 copies where 3.5 and 4 are associated with more efficient transcription. Taken altogether it is clearly proved that polymorphism in MAOA gene is of great significance correlating with psychiatric disorders and certain behavioural traits [14].

Molecular structure of MAO-A and MAO-B

MAO-A and MAO-B proteins share 70% amino acid identity. The two enzymes are flavoprotein oxidases which are characterised with a flavin adenine dinucleotide (FAD)-binding domain as part of their structure. Additionally, they have a substrate-binding and a membrane-binding domains (**Figure 1**). The substrate-binding site of these enzymes is mainly hydrophobic and consists of a cavity that is larger in MAO-A compared to MAO-B. Other structural changes, which are due to a difference in the amino acid sequence between MAO-A and MAO-B, are considered responsible for a varying preference of substrates for the two enzymes [8]. The FAD-binding domain of the two enzymes allows substrate oxidation via two half-reactions. First, the flavin cofactor (FAD) is reduced to $FADH_2$ by accepting two hydrogen atoms while the substrate amine is converted into an imine. Second, $FADH_2$ is re-oxidised by molecular oxygen back to FAD with H_2O_2 as a product, which then goes on to hydrolyse the imine into a corresponding aldehyde and ammonia [2, 8].

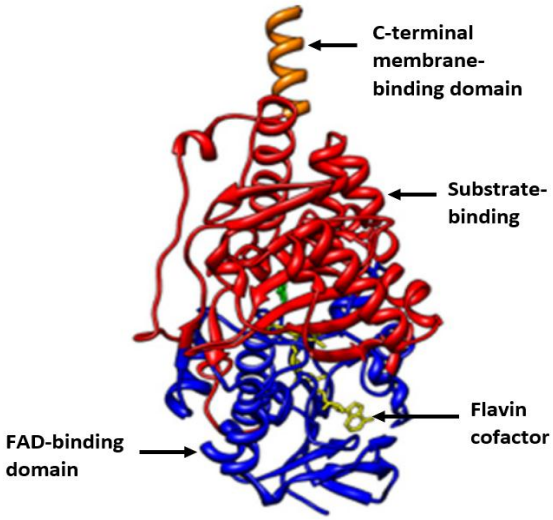

Figure 1. Ribbon representation of human MAO.
The image is generated on the program Chimera, using the Protein Data Bank. Important structural parts of the enzyme are indicated with arrows. (Image adapted from Gaweska, H., Fitzpatrick, P.F., 2011 [8]).

The functions of MAO-A and MAO-B

As mentioned above the main function of the MAO enzymes is in the metabolism of amine neurotransmitters as they catalyse their degradation and therefore inactivation. MAO-A and MAO-B catalyse the degradation of key neurotransmitters like serotonin, dopamine, adrenaline, noradrenaline and others. Therefore, they play an important role in the regulation of the central and peripheral nervous system. Irregularities in the expression or function of these enzymes are connected to various psychological conditions and neurodegenerative diseases, which will be discussed in more details. The two isoforms of MAO, MAO-A and MAO-B, possess some differences in their enzymatic activity, as they seem to have variations in the neurotransmitters they preferentially metabolise. **Table 1** shows the preferential neurotransmitters for both enzymes. Noteworthy, even though some neurotransmitters

are preferentially metabolised by either MAO-A or MAO-B, there are many which are shared between the two (**Table 1**) [20].

MAO-A and MAO-B protein expression

MAO-A and MAO-B are bound to the outer mitochondrial membrane and are expressed in most cells. However, there is a distinction in the overall distribution of the two enzymes in both the central nervous system and peripheral tissues. Some of the patterns of expression of each enzyme explain the preference in certain neurotransmitter degradation but also display some discrepancies [2]. In addition, **Table 1** indicates the places of the highest expression of MAO-A and MAO-B in human tissues.

Table 1. MAO-A and MAO-B substrates (neurotransmitters) and predominant expression sites in human tissues.

Both differences in substrate preferences and common substrates are shown [2, 6, 16, 20]

	MAO-A	***MAO-B***
Preferred neurotransmitters	Serotonin Noradrenalin	Benzylamine Beta-phenylethylamine
Common neurotransmitters	Dopamine Adrenalin Tryptamine Tyramine	
Sites of high levels of expression	Catecholaminergic neurons Liver Lung Placenta Small intestine	Serotonergic neurons Histaminergic Cells Small intestine Liver Brain

MAO-A inhibitors

MAO inhibitors are widely used to treat both, psychological disorders and some neurodegenerative diseases. A majority of the inhibitors are classified as anti-depressants as their mode of function is through the inhibition of MAO-A rather than MAO-B. However, since the inhibitors are selective, it is sometimes difficult to distinguish which isoenzyme has been inhibited [6].

Irreversible MAO-A inhibitors

The MAO-A inhibitors can be classified into irreversible and reversible inhibitors. One highly selective irreversible inhibitor for MAO-A is clorgyline. Studies have shown that clorgyline increases serotonin and noradrenaline levels in different brain regions and thereby alleviates some depression symptoms. While proven to be effective, irreversible MAO-A inhibitors are capable of causing an unwanted side effect called the “cheese reaction”. Tyramine is an amine present in food and beverages, specifically some fermented foods like cheese (the reason for the effect name). Tyramine is metabolised by MAO-A and when an irreversible inhibitor is used to block the function of the enzyme, tyramine is not metabolised and can gain access to the circulatory system. This can lead to a release of noradrenaline from the sympathetic neurons thus resulting in a hypertensive reaction [22].

Reversible MAO-A inhibitors

To avoid the side effect of irreversible inhibitors, the use of reversible MAO-A inhibitors has been demonstrated as a suitable alternative. Reversible inhibitors of MAO-A (RIMAs) manage to inhibit the enzyme but if any tyramine is ingested and therefore substrate levels increase, the inhibitory property of the drug is reversed and tyramine can be metabolised. The major RIMA used clinically is moclobemide and some other well studied RIMAs are toloxatone, befloxatone and brofaromine [7, 22]. There have been studies on other RIMAs since this is the more successful and less harmful type of inhibitor, like CX157 (3-fluoro-7-(2,2,2-trifluoromethoxy) phenoxathiin-10,10-dioxide) which was shown to have a potent inhibitory effect on MAO-A [7].

Biomedical applications of MAO-A inhibitors

MAO-A inhibitors as already mentioned are widely recognised for treatment of depressive disorders by increasing the levels of serotonin, however, that is not their only possible use. Options to use them as part of the treatment of attention deficit hyperactivity disorder (ADHD) in combination with stimulant medications have been explored [10]. As MAO-A is known to play a major role in some neurodegenerative disorders, MAO-A inhibitors could be used as part of the treatment. For instance, MAO inhibitors have shown a neuroprotective effect in Alzheimer's disease (AD). The inhibitors play their protective role in AD by correcting the chemical imbalances in the brain thus influencing the modulation of Aβ accumulation [4]. Another major neurodegenerative disorder, Parkinson's disease (PD), can potentially include MAO-A inhibitors as part of the treatment. Until recently there has been little attention to the use of MAO-A in the treatment for Parkinson's disease due to the undesirable effect of irreversible MAO-A inhibitors and also since MAO-B inhibitors have shown to be effective in increasing dopamine levels in the brain. However, since RIMAs remove the problem of irreversible inhibitors, they have shown potential in both treating depression in Parkinson's patients and alleviating the PD symptoms when used in tandem with MAO-B inhibitors [21].

MAO-A and neuropathology

As mentioned above MAO-A can play a major role in the pathology of various disorders and conditions, as it is an essential enzyme regulating the levels of important neurotransmitters. MAO-A has been connected to multiple neuropsychiatric disorders and behavioural traits such as increased aggression, antisocial behaviour and panic disorders [12]. Here, we are discussing in further detail how MAO-A can influence the neuropathology of a few neurological pathologies.

MAO-A and depression

Depressive disorders are known to be associated with imbalances in neurotransmitters and impaired function of neurons in the brain. The first association of MAO-A and the major depressive disorder (MDD) was made after MAO inhibitors were presented as a successful antidepressant therapy. It has been proposed that MAO-A and serotonin play a significant role in the neuropathology of MDD. The main idea was that the enzymatic activity of the monoamine oxidase suppresses the activity of the serotonin pathway. The levels and signalling of serotonin are essential in the early prenatal brain development as it is involved in neuronal proliferation, migration and differentiation. Moreover, serotonin is very important in adult neurogenesis and new neurons are necessary for proper mood control and in regulating

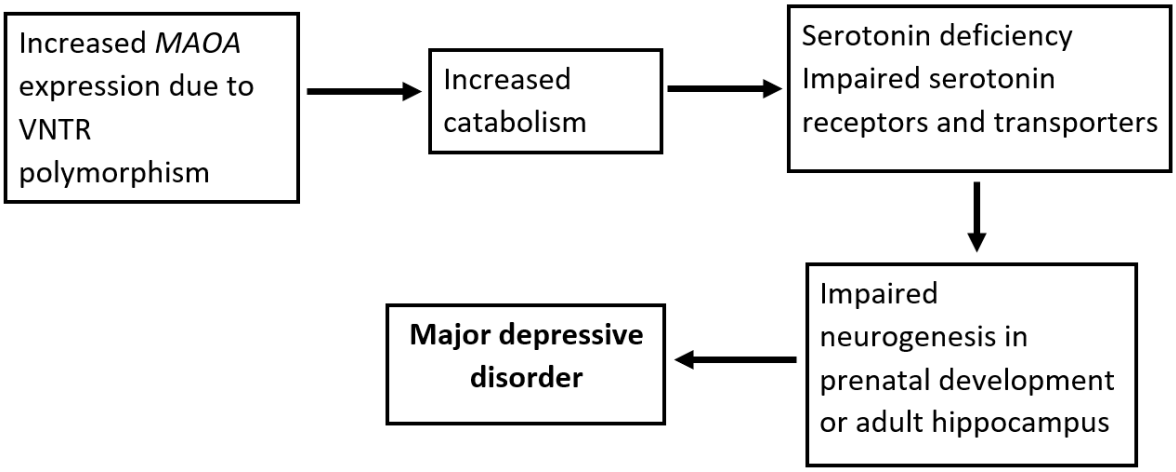

Figure 2. A schematic representation of the role increased expression of *MAOA* in the neuropathology of MDD.
The increased expression of the *MAOA* gene is often due to the MAOA-uVNTR where the number of copies is most likely either 3.5 or 4.

depressive tendencies. Higher levels of MAO-A due to increased expression of the MAOA gene can occur because of environmental stressors or due to the polymorphism of VNTR, which was above-mentioned. In **Figure 2** the results of the increased expression of MAOA due to the VNTR polymorphism are depicted as a scheme [12].

Antisocial behaviour and social anxiety

The appropriate balance of MAO-A activity is essential since reduced levels of MAO-A enzyme and enzymatic activity are also markers of undesirable psychological traits. A strong association between aggressive behaviour and highly reduced MAO-A activity has been recorded, especially in males. Reduced activity and availability of MAO-A has also been shown to play a role in antisocial behaviour. It has been observed in mice with reduced MAO-A enzymatic activity which is very similar to that of humans that harbour the VNTR polymorphisms associated with lower expression of MAOA. These males did not exhibit aggressive behaviour but rather demonstrated antisocial behaviours similar to some behavioural disorders and social anxiety. This was linked to an apparent decrease of serotonin in some brain

regions and differed from knockout mice that usually presented aggressive behaviour along with overall higher levels of noradrenaline [1]. Another study concludes that MAOA-uVNTR polymorphisms, which result in low levels of MAO-A alter neural functions, which results in numerous antisocial behaviours [19].

Migraines and pain

Pain can occur when there is some imbalance or impairment in neurological pathways and therefore it can be deduced that MAO-A could play a role in its mechanisms. The MAOA-uVNTR polymorphisms associated with low levels of activity of the enzyme have been shown to correlate with migraine patients [5]. The relation between MAO-A activity and migraines might once again possibly be due to the effect the enzyme has on serotonin levels. The serotonergic system is known to play an important role in the pathology of migraines and migraines are often considered a syndrome of chronically low levels of serotonin [9]. Drastically lowering the levels of serotonin through degradation catalysed by MAO-A could also be associated with other types of pain as this neurotransmitter is part of other neurological pathways of ache. A correlation between the activity of MAO-A and neuropathic pain has been shown by the use of MAO-A inhibitors as a successful treatment of the condition. An increase of MAO-A by chronic constriction of the static nerve leads to a decrease in the availability of serotonin, which is important for the descending pain modulation pathways [18].

Parkinson's and Alzheimer's diseases

Even though MAO-A inhibitors have been shown to have a positive effect on reducing the symptoms of neurodegenerative diseases such as Parkinson's (PD) and Alzheimer's (AD), it is yet unclear what the exact involvement of the enzyme in the pathology of these diseases is. Both MAO isoenzymes are considered to participate in PD neuropathology. There are some recordings of increased availability of MAO-A in dopaminergic neurons leading to decrease of dopamine levels which is the central neurotransmitter in the pathology of PD. However, it is not entirely clear if this is sufficient to consider MAO-A as a central player in the disease [17]. The case for AD is also similar to that of PD when it comes to the role of MAO-A. Again, both MAO-A and MAO-B are considered as crucial for the pathology of AD, however, the mechanisms are unclear. Increased levels of MAO-A have been correlated to neurodegeneration which is a prominent part of both PD and AD. The generation of hydrogen peroxide in the enzymatic reaction of MAO could potentially be a mechanism of the neurodegeneration due to extreme oxidative stress and damage observed in these diseases [11, 13].

Conclusion

MAO-A is an enzyme of great importance for the proper neurological functions as it is essential for the metabolism of key neurotransmitters. An appropriate level of the enzyme is of the essence as either higher or lower deviations can lead to severe neurological conditions. However, this is an indication that MAO-A can be a prominent target of treatment for various psychological and neurodegenerative disorders.

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