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ANIMAL BIOTECHNOLOGY 2018

NATIONAL AGRICULTURAL AND FOOD CENTRE
RESEARCH INSTITUTE FOR ANIMAL PRODUCTION NITRA

Department of Genetics and Reproduction of Farm Animals
NPPC – Research Institute for Animal Production Nitra
Slovak Republic

Faculty of Biotechnology and Food Sciences
Slovak University of Agriculture in Nitra
Slovak Republic

Slovak Academy of Agricultural Sciences

Main topic of the conference:

Laboratory, farm and wild animals as a biological model

December 6th, 2018
Nitra, Slovak Republic

Slovak University of Agriculture in Nitra
NPPC – Research Institute for Animal Production Nitra

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Dear Participants and Dear Colleagues,

We are very pleased to welcome you to the 6th International Scientific Conference "Animal Biotechnology 2018". The conference is organized by the Research Institute for Animal Production Nitra, National Agricultural and Food Centre in cooperation with the Faculty of Biotechnology and Food Science, Slovak University of Agriculture Nitra.

The aim of the conference is presentation of an actual research from the field of animal biotechnology, with a special focus on *Laboratory, farm and wild animals as a biological model*.

Moreover, the conference will provide an opportunity to gather researchers engaged in this and adjacent fields of research in order to exchange their skill and experience as well as to establish potential collaboration in a given task. We would appreciate attendance and participation on this conference of colleagues from various research institutions and universities.

We wish you cordial and warm atmosphere at our conference for presentation, creative and fruitful discussion and inspiring ideas for future research.

Nitra, December 6th, 2018

Peter Chrenek

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BIOTECHNOLOGY IN THE CONTEXT OF THE 100-YEAR HISTORY OF CZECHOSLOVAKIA: A REVIEW

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ABSTRACT

In the context of Czechoslovak history, permanent institutional and state attention is evident in the possibilities of using biotechnology in industry, agriculture and pharmacy. In the hierarchy of time already in 1924 (28.12.1924) at the founding of the Czechoslovak Academy of Agriculture. In the 1950s of 20th century, an utmost importance in animal breeding was attributed to artificial insemination, as a biotechnique of intensification of the reproductive potential of livestock (cattle, pigs, horses and sheep). The issue of biotechnology in animal production is a permanent part of the research of the Department of Farm Animal Genetics and Reproduction at NPPC-RIAP Nitra. Laboratories of the department are focused on the research in fields of *in vitro* fertilization, molecular genetic analysis, transgenesis, generation of cloned (genetically identical) and chimeric individuals, stem cells and cryopreservation of reproductive cells to create a gene bank and preserve animal genetic diversity.

Key words: Czechoslovakia; biotechnology; history

INTRODUCTION

Biotechnology belongs to the scientific and technological phenomenon of the 21st century, but their roots are connected with human history, especially with the fermentation processes (bread, beer, wine) already 4000-2500 years before Christ.

Biotechnology has a very wide range of definitions, mainly related to the development of knowledge. According to the Organization of United Nations – the "Biological Diversity Agreement", biotechnology means any technology, which uses living organisms or their components to produce or modify products, to breed microorganisms, plants and animals for specific use. This is an official and generally accepted legislative directive.

What is crucial is, that biotechnology is the organic link of life (living organisms and functions; BIO) with technology (level of knowledge in genetics, physics, chemistry and biology in general), with methodology (their practical application) in the creation, change of the use of living organisms for intensive and targeted production of useful resources of the existence of a growing human population.

In the context of our common Czechoslovak history, permanent institutional and state attention is evident in the possibilities of using biotechnology in industry, agriculture and pharmacy. In the hierarchy of time already in 1924 (28.12.1924) at the founding of the Czechoslovak Academy of Agriculture undertaking by Dr. Milan Hodža – later President and Minister of Agriculture of the Czechoslovak Republic and the future Prime Minister of the Czechoslovak Socialist Republic, the 4th CHA Agricultural and industrial department was established, which involved the sugar industry, the starch industry, the distillery,

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the dairy, the miller and others, including the use of waste etc., as a department "Learning and application of biotechnology of agricultural production for general use." Since 1948, this department has resisted and still justified the status of "bio-technique" and "technology" mainly in agriculture. In 1928, Dr. Ing. Jaroslav Kříženecký issued the publication "Animal Farming", in which he considers agriculture as a biological production and as a biotechnique (Bulla *et al*., 2014).

Dr. Kříženecký has done a lot of useful activity for genetics and biotechnology in the Czechoslovakia, particularly within RIAP activity in Viglaš. Moreover, in 1963, he was greatly deserving to build an exhibition of J. G. Mendel in the Moravian Museum in Brno on the occasion of the 150th anniversary of his birth. After the "Lysenko Period" in Czech genetics, Czechoslovakia became the country of its gradual renaissance in the genetic science.

In 1946, the High School of Agricultural and Forestry Engineering in Košice (VŠPLI) was established, which also included the Department of Zootechnics and Biotechnology of Animal Products (mainly feed processing, milk, etc.). In the 1950s of 20th century, an utmost importance in animal breeding was attributed to **artificial insemination**, as a biotechnique of intensification of the reproductive potential of livestock (cattle, pigs, horses and sheep). At the same time, it is a method of preventing some invasive diseases and, in particular, a method that opens up new possibilities in the breeding process. An important role in this process was played by prof. Dr. Ing. Karel Koubek (Professor of the Czech Agricultural University in Prague) as the creator of the concept, its application in science, research and practice.

In Slovakia, an important role in the implementation process was played by Ing. Ján Mozoľa – the director of State breeding companies in the creation of insemination stations and system of training of technicians for the application of this method also among private breeders. The development of biotechnology and, consequently, insemination techniques for individual livestock species has led to improvements in breeding estimation methods, which has enabled the development and implementation of modern long-term selection programs from performance tests, CC methods, BLUP (MOET), AM-model, etc. up to current **genomic selection**.

Significant acceleration in the development of biotechnology occurred after the economic consultation of the RVHP Member States in June 1984, where it was required to prepare a proposal for a comprehensive program of Science and Technology Progress for 15-20 years. The program was approved in December 1985. It consisted of 5 priority directions and one was - the development and application of biotechnology. VÚŽV (RIAP) in Nitra was responsible (for whole ČSSR) for "Development of bio-engineering methods in livestock production to improve the utility and technological characteristics of farm animals". In other areas the coordination was realized by Czech Academy of Sciences. The Research Institute of Animal Production (RIAP) in Nitra was appointed as the Head of Scientific and Technological Development and coordinator of ŠVTP POG "Selected problems of development of the agro-industrial complex" and of the state task POG 529 820 "Biological and technical intensification of livestock production". The whole program, entitled "Long-term Comprehensive Program for the Development and Implementation of Biotechnologies in the Czechoslovak Socialist Republic", was approved by the Government of the Czechoslovak Socialist Republic. Jaromir Obzina the Deputy Prime Minister and Chairman of the State Commission for Scientific, Technical and Investment Development was a coordinator of this program.

One of the implemented steps was the creation of the Soviet-Czechoslovakian Biotechnology Laboratory at the RIAP in Nitra (1986-1995), where 17 scientists from the USSR and three from Poland were co-operating with the staff of the ÚFGŽ CSAV in Liběchov, VÚŽV in Uhříněves, VŠV in Brno, VŠV in Košice and the BAV Institute in Kostinbrod (Bulgaria). In spite of a relative short period of its activity this laboratory has proved its eligibility and relevance mainly in the area of reproductive biotechnology of farm animals. Most of its members have applied to the positions of leading scientists at home and abroad. Research activities continued at the Department of Genetics and Experimental Biology and today at the Department of Genetics and Reproduction of Farm Animals (NPPC–VÚŽV Nitra) in the direction of mapping of milk protein polymorphism in animal genomes,

micromanipulations with embryos, creation of identical individuals, experimental transgenesis and biodiversity research (1994-2002) (Bulla and Chrenek, 2007; Chrenek *et al*., 2011, 2016).

In 2002, the Faculty of Biotechnology and Food Sciences was established at the Slovak Agricultural University in Nitra, which continues the tradition of using modern biological and chemical methods in research and pedagogy for realization in social practice and application on the international scientific scale in close cooperation with RIAP in Nitra.

The issue of biotechnology in animal production is a permanent part of the research of the Department of Farm Animal Genetics and Reproduction at NPPC-RIAP Nitra. Laboratories of the department are focused on the research in fields of *in vitro* fertilization, molecular genetic analysis, transgenesis, generation of cloned (genetically identical) and chimeric individuals, stem cells and cryopreservation of reproductive cells to create a gene bank and preserve animal genetic diversity. An important activity at the NPPC–RIAP Nitra since 2013 is the annual organization of the international scientific conference "Animal Biotechnology", where the latest results from the field of animal biotechnology are presented.

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CYTOGENETIC STUDIES OF MESENCHYMAL STEM CELLS IN RABBIT: A MINI-REVIEW

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ABSTRACT

The aim of the present review is to summarize current knowledges of *in vitro* studies, focused on the determination of rabbit stem cells of different origin, based on their cytogenetic examination. Stem cells represent valuable model to study the biological traits or processes of health and targeted tissues, affected by various internal or external detrimental factors. Furthermore, these cells provide a promising mechanism of treatment of existing human or animal diseases. Although recent knowledges based on serious *in vitro* studies bring positive promises, there are still remained a lot of issues focused to the safety of stem cell usage in the context of their clinical application. In this way, the stability of the genome across individual generations of passaged cells plays an important role, evaluated on the basis of chromosomal profile, including aneuploidy and structural studies. In the given context, various culture conditions and manipulations among the studies play a crucial role in the definition of the final chromosomal status. Up to date, there are numbers of reliable animal models used as donors of embryonic or somatic stem cells. In this way, the rabbit represents an available source with numerous advantages for cytogenetic analysis.

Key words: stem cell; chromosome; rabbit

INTRODUCTION

Stem cells are undifferentiated cells capable of self-renewal and of differentiation into specific terminally-differentiated cell types. Based on the source of derivation, they can be divided into somatic stem cells (SSCs) or embryonic stem cells (ESCs) (Rebuzzini *et al*., 2015). Somatic stem cells exert a crucial role in the maintenance of tissue homeostasis and participate in the repairing processes within their specific tissue. The ESCs are able to differentiate into almost all mature foetal and adult cell types, and thus they are defined as pluripotent cells (Cockburn and Rossant, 2010). While in the embryonic stem cells various chromosomal disorder has been widely reported, the mesenchymal stem cells are characterized as genetically stable during culture (Borgonovo *et al*., 2014). Mesenchymal stem cells (MSCs) are present in many adult tissues (Kang *et al*., 2012), capable of high proliferation and multi-lineage differentiation (Jin *et al*., 2013). Bone marrow was the first tissue, where the MSCs (BM-MSCs) were identified. Stem cells with this kind of origin possess various advantages as are: high osteogenic differentiation capacity, well investigated properties applied in use with biomaterials and not ethically controversial background (Till and McCullough, 1961; Kang *et al*., 2012). Invasivity of the BM-MSCs harvesting initiates the interest in finding more accessible sources of MSCs (Pontikoglou *et al*., 2011). SSCs have been identified in many different organs (i.e. skeletal muscle, heart, liver, fat, umbilical cord blood or placenta) (Rebuzzini *et al*.,

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2015). The potential use of stem cells (SCs) for tissue engineering (Katari *et al*., 2015), regenerative medicine (Grompe, 2012), disease modelling (Merkle and Eggan, 2013), toxicological studies (Seiler and Spielmann, 2011), drug delivery (Li *et al*., 2008) and as *in vitro* model for the study of basic developmental processes implies large-scale *in vitro* culture (Rebuzzini *et al*., 2015). *In vitro*, SSCs display greater plasticity, showing higher differentiation potential than *in vivo*. *In vivo*, SSCs can differentiate either in one, few or multiple cell lineages and, thus, are classified as unipotent (e.g. spermatogonia, oogonia), oligopotent (e.g. neural stem cells, NSCs) or multipotent (e.g. hematopoietic SCs) (Jiang *et al*., 2002; Franco-Lambert *et al*., 2009). The perspectives of stem cell clinical use is coupled with a serious issue about potential risk of forming tumours, the migration far away from the site of infusion and colonization of other tissues, the dedifferentiation of SC-derived differentiated cells, the establishment of an incorrect epigenetic and genetic status and an abnormal chromosome complement (Rebuzzini *et al*., 2015). On the basis of above mentioned facts, it is necessary to provide studies deeply focused on the determination of cytogenetic traits across generations of cultured stem cells. This advice is in accordance to several papers with evidence of stem cells difficulty to maintain a correct chromosome complement during prolonged expansion (Rebuzzini *et al*., 2011; Oliveira *et al*., 2014). As a promising animal model and the donor of stem cells for such studies, the rabbit has several advantages not only due to physiological manipulations – more easily carried out than those in mice, but also it is phylogenetically closer to primates than are rodents (Wang *et al*., 2007). For example, several authors have focused their force to study the rabbit embryonic stem cell behaviour under *in vitro* conditions (Fang *et al*., 2006; Wang *et al*., 2007).

Culture Conditions and Chromosomes

The effect of *in vitro* conditions on genomic stability of cells attracts the attention during the last years. However, the variability among culture protocols applied in different laboratories for derivation and culture of SCs complicates the identification of the source of such variations. The techniques used for cell detachment and disaggregation seem to be a major factor affecting the maintenance of genome integrity during long culture. Mechanical or manual methods (pipetting, flushing until the colonies are detached and disaggregated) are more gentle – less aggressive passaging techniques for subculturing and preserve better genome integrity than the use of enzymes (trypsin, collagenase) (Buzzard *et al*., 2004; Mitalipova *et al*., 2005; Lefort *et al*., 2008). To accelerate steps focused to disaggregation a modified enzymatic dissociation solution (0.25 % trypsin, 0.1 % collagenase IV, 20 % KSR, and 1 m M $CaCl_2$ in PBS), in combination with manual dissection for bulk passaging, has been proposed for hESC dissociation, demonstrating the maintenance of a normal chromosome complement even after more than 100 passages (Suemori *et al*., 2006). Several studies were focused on oxygen concentration during culture, but with contrasting results. Some studies suggested to use the O_2 concentrations between 1 to 7 % to significantly reduce the incidence of aneuploidies in hMSCs (Holzwarth *et al*., 2010; Li *et al*., 2011; Tsai *et al*., 2011; Estrada *et al*., 2012); whereas, the others recorded increased risk of aneuploidies and microsatellite instability in mouse NSCs, human bone marrow MSCs and human adipose SCs under the O_2 concentration between 1 and 5 % of chromosomes, even at early passages (Oliveira *et al*., 2012; Ueyama *et al*., 2012). High rates of aneuploidy, gaps and breaks were reported in hESC lines cultured under 21 % concentration of the O_2, in comparison with lower concentrations (Forsyth *et al*., 2006; Lim *et al*., 2011). A fundamental component of the SC medium is the serum. However, its animal (calf or bovine) or artificial (knockout serum only used for ESC culture) origin does not seem to play a role in the maintenance of genome stability (Inzunza *et al*., 2005; Ludwig *et al*., 2006). Similarly, the choice of a cell feeder layer (mouse embryonic or immortalized fibroblasts) or of supportive matrixes (gelatine, fibronectin, etc.) during the derivation and maintenance of stem cell lines does not seem to influence either the onset or the restraint of aberrations in stem cells genome (Cowan *et al*., 2004; Draper *et al*., 2004; Guo *et al*., 2005; Maitra *et al*., 2005; Mitalipova *et al*., 2005; Imreh *et al*., 2006; Sugawara *et al*., 2006; Rebuzzini *et al*., 2008).

Techniques for Karyotype Analyses

Several techniques are currently available to investigate the integrity of the chromosome complement of a cell line. Each method has advantages and disadvantages in terms of sensitivity, resolution and final costs (Catalina *et al*., 2007). Conventional banding techniques (G-, Q- or DAPI) allow a snapshot of the entire chromosome complement and the ordinary gross karyotype control of a cell line. These techniques, providing 300–400 stained bands, facilitate the detection of incorrect chromosome numbers (aneuploidies), mosaicism and large structural chromosome abnormalities, such as translocations, deletions or insertions.

Chromosomal status of cultured cells

Number of studies on chromosome variability have been performed on human mesenchymal stromal cells (MSCs) derived from the bone marrow. Independent laboratories reported contrasting results on the accumulation of chromosomal aberrations during *in vitro* culture (Ben-David *et al*., 2012; Sensebé *et al*., 2012). Some authors reported that human bone marrow-derived MSCs remain chromosomally stable throughout long-term culture, whereas others claimed the occurrence of numerical and structural chromosome aberrations within passages of *in vitro* culture (Pittenger and Martin, 2004; Soukup *et al*., 2006; Bernardo *et al*., 2007; Zhang *et al*., 2007; Sensebé *et al*., 2012). The study of Tomkova *et al*. (2017), focused on the aneuploidy determination of G-stained rabbit endothelial (peripheral blood) and mesenchymal (bone marrow, 3rd passage) metaphase stem cells, shows 73.3 % and 66.6 % diploidy or 26.6 % and 33.5 % aneuploidy, respectively. The results are partly similar to those of Kovacik *et al*. (2017), who focused to the chromosomal status monitoring of cultured rabbit stem cells isolated from the amniotic fluid and detected aneuploidy in the first three passages as follows: 18.18 %; 25.81 % and 23.53 %, respectively. Mentioned authors, on the basis of statistical analysis outputs, found no significant difference in the aneuploidy presence between stem cell cultures evaluated at various passages. These findings are in accordance to the results of Asadi-Yousefabad *et al*. (2015).

CONCLUSION

Current science provides wide options and promises in the use of stem cells with an expected success. This statement is supported by many scientific studies performed on a wide spectrum of stem cells isolated from and applied to different animal models. However, there are still remained studies with inconsistent results, which reveal not only positive, but also negative issues coupled with isolation, culture or determination of evaluated samples. Based on this fact, it seems reasonable to perform *in vitro* experimental studies in order to bring more detailed and clear answers, how to extract benefits from stem cell features in human and animal field. In this way, cytogenetic studies are useful tool to acquire early information about the genetic background of growing cells in the context of their future clinical use, basing on the chromosomal number and structure.

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OOPLASM CRYOPRESERVATION: OVARIAN FRAGMENTS VERSUS OOCYTES ALONE

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ABSTRACT

The cryopreservation of the farm animal ooplasm is very useful in the conservation of female gametes. Since the use of histological assay alone is insufficient to assess the freezing-thawing process, the future oocyte developmental competence need to be validated by *in vitro* fertilization and embryo production. The aim of the study was to find optimal method of cryostorage of bovine oocytes with preserving their viability and fertility. In the first experimental series we vitrified ovarian fragments isolated from the cortical area of the cow ovary, which contained antral follicles, using two vitrification techniques: solid-surface vitrification (SSV) and liquid vitrification (LV). After warming we isolated the oocytes by follicle aspiration and placed them into the *in vitro* maturation (IVM). At maximum, only 8.3 % of vitrified/warmed (LV) oocytes were matured, whilst none of the oocytes were matured following SSV technique. In all frozen ovarian fragments, regardless of vitrification technique, serious damages in the oocytes were detected at the histological and ultrastructural levels, what prevented the oocyte development. In the second series of experiments, cumulus-oocyte complexes without surrounding ovarian tissue were vitrified following IVM procedure. Using this scheme we obtained more than 50 % embryo cleavage rate and 4.5 % of embryos reached the blastocyst stage, which proves that the cumulus-oocyte complexes after vitrification can retain their developmental ability. Our preliminary results show that cryopreservation of previously matured oocytes is more promising than the vitrification of ovarian tissue fragments.

Key words: bovine; ovary; oocyte; embryo; vitrification

INTRODUCTION

Cryopreservation of ovaries, their surface tissues or ovarian follicles represents a possible source of female gametes in future. In case of serious damage of the animal (limb fractures and others), when it is necessary to slaughter the animal, the ovarian tissue, ovarian follicle or the entire ovary can be collected and frozen. After thawing, the biological material may be transplanted or cultured *in vitro* as a source of oocytes for *in vitro* fertilization (IVF) and *in vitro* embryo production (IVP).

Cryopreservation of ovarian tissue offers many advantages over mature oocytes or embryos to preserve female germline of endangered animals. Firstly, the ovary contains a large pool of oocytes enclosed in follicles. Secondly, ovarian tissue can be collected from animals of almost all developmental age (adult, prepubertal and foetus) and status (alive or dead) (Cleary *et al*., 2001).

The cryopreservation of ovarian cortex containing the antral follicles would certainly be very useful in the conservation of female gametes. There are two possibilities: the excision of slices of the ovarian cortex after the puberty onset without disturbing the female reproductive system. Another way is to remove the whole ovaries after the animal's death, to be used later in techniques such as grafting or *in vitro* maturation. The attempt to *in vitro* mature oocytes from primordial follicles

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has not yielded satisfactory results (Lunardi *et al*., 2017). However, there are reports that small antral follicles frozen as part of vitrified ovarian tissues could be the source of oocytes for *in vitro* fertilization and successful embryo production (Faheem *et al*., 2011). Nevertheless, cryopreservation of ovarian tissue is still problematic and should be optimized to handle the diversity of cell types and tissue components (oocytes, granulosa cells, extracellular matrix) (Hovatta, 2005). The use of histology alone is insufficient to assess the freezing-thawing process as morphological analysis, and it is not often correlated with the ability or developmental competence of the oocytes (Celestino *et al*., 2008; Santos *et al*., 2007). The results need to be validated by *in vitro* oocyte development and by fertilization *in vitro*.

The aim of the study was to find optimal method of cryostorage of bovine oocytes with preserving their viability and fertility.

MATERIAL AND METHODS

Cryopreservation of ovarian fragments

Ovarian fragments (n = 451; approximate size of 4 x 4 mm), containing antral follicles (2-4 mm), were isolated from undefined cows at a local abattoir, and frozen by two methods previously used for freezing of ovarian primordial follicles.

For solid surface vitrification (SSV), ovarian fragments were exposed to 4 % ethylene glycol (EG) in DPBS + 10 % FBS for 15 min and then rinsed in a vitrification solution composed of 35 % EG and 0.4 M trehalose in DPBS + 10 % FBS. After 5 min equilibration in an ice bath, fragments were placed in a minimum volume of vitrification solution onto the surface of a metal plate pre-cooled by an immersion into a liquid nitrogen (LN).

For liquid vitrification (LV), ovarian fragments were equilibrated in a vitrification medium containing 40 % EG, 30 % Ficoll 70, 1M sucrose and 4 $mg.ml^{-1}$ of BSA at room temperature for 5 min. Then the tissues in 1.8 ml cryovials were placed into LN. After thawing the fragments were processed for histology (toluidine blue, haematoxylin-eosin) and ultrastructure (transmission electron microscopy) analyses.

Part of the oocytes were tested for the ability to mature *in vitro* (IVM). After 24 h of IVM in maturation medium (TCM 199 (Gibco), sodium pyruvate (0.25 $mmol.l^{-1}$), gentamycin (0.05 $mg.ml^{-1}$), foetal bovine serum (FBS) 10 % and FSH/LH (1/1 I.U., Pluset) at 38.5 °C and 5 % CO_2, the oocytes were fixed in formalin, stained with a DAPI dye and fluorescently evaluated.

Cryopreservation of oocytes

For cryopreservation of matured oocytes alone, ultra-rapid cooling technique in minimum volume was used. Matured oocytes with cumulus cell layers were placed into equilibration solution (ES: 7.5 % ethylene glycol +7.5 % DMSO in M199-HEPES, supplemented 20 % foetal calf serum and 50 $ng.ml^{-1}$ gentamycin) for 5 min. Following equilibration the oocytes were transferred to vitrification solution (15 % EG + 15 % DMSO + 0.5M sucrose in M199-HEPES) at room temperature for 45-60 sec. The oocytes (10-15) in a small drop were placed with a glass micropipette onto 300 mesh nickel grids (electron microscopy grade), an excessive medium was removed by a filtration paper and then the oocytes were immediately plunged into liquid nitrogen for storage (several weeks). For warming, nickel grids were directly transferred into thawing solution (1M sucrose in M199-Hepes, at 37 °C) for 1 min. The warmed oocytes were transferred to the diluent solution (0.5M sucrose in M199-HEPES) for 3 min, and then washed twice in M199-HEPES with FCS for 5 min. Oocyte survival was evaluated on the basis of the integrity of the oocyte membrane and the zona pellucida after 20 h culture post-thawing.

***In vitro* fertilization (IVF) of vitrified-warmed oocytes and embryo culture**

Warmed oocytes were washed in IVF-TALP medium (TALP solution, 10 $\mu g.mL^{-1}$ heparin, 50 $ng.ml^{-1}$ gentamycin) and put into 100-µl droplets of IVF medium under a mineral oil, where the sperm (at 2×10^6 per ml) and PHE solution (20 µM penicylamine, 10 µM hypotaurine, 1 µM epinephrine) was previously added, and incubated for 18 h at 39 °C in 5 % CO_2. Following insemination, presumptive zygotes were vortexed in centrifuge tubes containing 0.5 ml holding medium for 45 s to remove residual cumulus cells. Denuded zygotes were transferred to the dish with the granulosa cell (about 10 %) layer for 24 h. Afterwards, the embryos were transferred to a new dish with the granulosa cells (about 40 % of confluence) in B2 medium with 10 % FBS.

On the Day 2 since insemination, the cleavage, and on Day 7, 8 and 9 – the number of blastocysts were counted.

RESULTS

When ovarian fragments were cut off and cryopreserved by an SSV vitrification technique, none of the recovered oocytes (n = 47) were matured *in vitro*. When an LV technique was applied, only 8.4 % of oocytes showed the signs of nuclear maturation in contrast to 60.6 % in the control (IVM) group. Most of degenerated oocytes were found in the SSV group after thawing or thawing/IVM (Table 1.).

In all frozen ovarian fragments, regardless of vitrification technique, serious damages of oocytes at the light or electron microscopy levels were detected like extensive vacuolization and disintegration of the ooplasm and organelle dislocation. Visible *zona pellucida* cracks and deformities of oocytes were caused likely by the mechanical action of ice crystals formed in the follicle cavity. The granulosa cell nuclei were largely pyknotic. Germinal vesicles showed disintegrated nuclear envelope. Microvilli of cytoplasmic membrane were disrupted. The damaged *zona pellucida* acquired layer-like structure, and cells of *corona radiata* showed extensive damages. In conclusion, our experiments did not confirm that the oocytes, frozen in small antral follicles from ovarian tissue fragments using SSV or LV, are able to mature *in vitro*, due to extensive cellular damages revealed by histological and ultrastructural analyses.

In the second series of experiments we chose another approach where we tried to freeze cumulus-oocyte complexes without surrounding ovarian tissue. The oocytes were matured by the incubation in maturation medium and afterwards immediately frozen by a liquid vitrification. The results of oocyte vitrification and *in vitro* fertilization are presented in Table 2.

From a total of 184 oocytes vitrified oocytes, 116 were warmed and afterwards only 66 oocytes were selected for IVF procedure. As a control group, 175 freshly isolated oocytes were matured

Table 1. Developmental status of oocytes after cryopreservation/*in vitro* maturation (IVM)

Groups	Total no. oocytes (N)	GV-stage, n (%)	Metaphase-stage, n (%)	Degenerated oocytes, n (%)
Control (fresh)	98	65 (66.21)	32 (32.7)	1 (1)
Control (IVM)	71	27 (38.0)	43 (60.6)	1 (1.4)
LV–thawed	29	21 (72.4)	4 (13.8)	4 (13.8)
LV–thawed/IVM	48	38 (79.2)	4 (8.3)	6 (12.5)
SSV–thawed	32	22 (68.8)	2 (6.3)	8 (25.0)
SSV–thawed/IVM	47	31 (66.0)	0 (0)	16 (34)
Totally oocytes	325	204	85	36

Table 2. Development of fresh or vitrified-warmed oocytes after IVF

Groups	Oocytes totally	Oocytes vitrified	Oocytes warmed	Oocytes in IVF	Embryo cleavage	Blastocyst rate
Vitrified	197	184	116	66	34 (51.50 %)[a]	3 (4.55 %)[a]
Control	175	-	-	175	141 (80.57 %)[b]	34 (19.43 %)[b]

[a] versus [b] – difference is significant at $p < 0.05$ (Chi-square)

in vitro and immediately subjected to the IVF procedure. Cleavage rate of vitrified oocytes was significantly lower (51.5 %; $p < 0.05$) than that of a fresh control oocytes (80.57 %). In the vitrified group only 3 expanded blastocysts were developed (4.55 %) in comparison with 19.43 % in the fresh control group oocytes ($p < 0.05$).

DISCUSSION

The oocyte has always been the most challenging specimen to cryopreserve because of its high sensitivity and intolerance to cryopreservation due to large cytoplasmic volume and intricate cellular structure (Kim, 2006).

An alternative approach to harvesting and vitrifying oocytes would be to cryopreserve ovarian tissues instead of follicles. Dissection of the ovary into ovarian fragments followed by puncture of the follicles is considered as an effective technique to get a high number of oocytes in excellent conditions (Faheem *et al*., 2011). Therefore, attention must be given to preserve this yield of good oocytes. Faheem *et al*. (2011) isolated bovine oocytes for *in vitro* culture using the procedure of dissection of ovarian cortex into small fragments followed by puncture of the follicle from frozen-thawed ovarian tissue. They recovered a great number of good quality oocytes, which were successfully *in vitro* matured (maturation rate 73-80 %), fertilized and developed into *in vitro* produced embryos of morula and blastocyst stage.

In our study we performed two steps of the experiments. In the first step we tried to vitrify ovarian fragments isolated from the cortical area of the ovary. Following vitrification/warming we isolated the oocytes from antral follicles by a punction or aspiration and then placed them into *in vitro* maturation (IVM). Two vitrification techniques (solid-surface vitrification – SSV and liquid vitrification – LV) were applied for cryopreservation of ovarian fragments. At maximum, only 8.3 % of vitrified (LV) oocytes were matured during IVM, whilst none of the oocytes were matured following SSV technique. This poor oocyte development was caused by substantial injuries in the oocyte structure, which was also confirmed by histological and ultrastructural analyses. These injuries were probably arisen as a consequence of thawing process. Therefore, our first step experiments did not confirm that the oocytes, frozen in small antral follicles from ovarian tissue fragments using SSV or LV, are able to mature *in vitro*, due to the loss of developmental competence.

In the second step, the cumulus-oocyte complexes were isolated from the follicles and put into the maturation *in vitro*. Afterward, matured oocytes enclosed by cumulus cells, were vitrified by ultra-rapid cooling technique in minimal volume and stored in liquid nitrogen for several weeks. After warming the oocytes were *in vitro* fertilized and put into embryo culture medium to develop until higher embryonal stages. Using this scheme we obtained more than 50 % cleavage rate and 4.5 % reached the expanded blastocyst stage, which proves that the cumulus-oocyte complexes after vitrification can retain their developmental ability. Similar results were published also by Chian *et al*. 2004 (7.4 %) and Zhou *et al*. 2010 (5.4 %).

A success in bovine oocytes cryopreservation was achieved firstly when an approach of minimizing the vitrified sample was used in order to obtain a much faster cooling rate. Martino *et al*. (1996), using the minimum volume technique, reported development to the blastocyst stage or high cleavage rate of vitrified *in vitro* matured bovine oocytes. Better results (more than 10 % of blastocyst) after bovine oocytes cryopreservation were achieved by Vajta *et al*. (1998), Papis *et al*. (2001) and Ishi *et al*. (2018).

Our preliminary experiments show that cryopreservation of matured cumulus-oocyte complexes is more promising than the vitrification of ovarian tissue fragments. Although, in our experiments, we obtained only few blastocysts (4.5 %) following IVF of vitrified oocytes, blastocyst development exceeding 10 % is really possible. Due to the high variability of results, a standard method for bovine oocyte cryopreservation remains to be optimized.

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THE INTERPLAY OF MOUSE AND PORCINE BIOMODELS FOR ENDOCRINE DISRUPTOR REPRODUCTIVE IMPAIRMENT

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ABSTRACT

Individual mammalian organisms, used as a biomedical model, represent partial benefits and drawbacks for transfer of knowledge into human medicine. Whereas some models, such as rhesus monkey, are more similar to human, its usage is difficult and controversial. In contrast, well-designed combination of several mammalian models shows effective way how to verify a hypothesis and, based on conservativeness of observed phenomenon, implicate it into the human medicine. The aim of this overview is to compare individual mammalian biomodels, with respect to reproductive biology, elucidation of reproductive toxicology and, in particular, to an effect of endocrine disruptors. The literature search is supplemented with own experimental designs and observations obtained using mouse and porcine models. Our findings point out advantages of *in vivo* exposure of oocyte, sperm or embryos of outbred mice to endocrine disruptors followed by verification using porcine *in vitro* treatment of cumulus-oocyte complexes with identical endocrine disrupting compound. In summary, the association between *in vivo* and *in vitro* exposure suggests about highly-relevant and available model for testing of endocrine disruptors and risk assessment for human reproductive health.

Key words: oocyte; embryonic development; endocrine disruptor; bisphenol; reproductive health

INTRODUCTION

Plastic compounds (bisphenols) (Žalmanová *et al.*, 2016), pesticides (DDT, vinclozoline, pyrethroids) (Petr *et al.*, 2013; AL-Hussaini *et al.*, 2018), flame retardants (organophosphates) (Carignan *et al.*, 2018) and others are continuously introduced into the environment during industrial production and plastic usage. These compounds are considered to be environmental pollutants and, in many cases, represent dangerous agents with endocrine disrupting effect (Gingrich *et al.*, 2018). Endocrine disruptors, firstly defined by Colborn *et al.*, (1993) are generally described as being: i) permanent exposure to humankind due to their ubiquitousness, ii) very low exposure doses that do not achieve toxic effects in a dose-dependent manner, iii) non-monotonic curve of endocrine disruptor effect, often results in more deleterious effects of lower doses than higher ones (Daston *et al.*, 2003; Vandenberg *et al.*, 2012) and iv) temporal window of sensitivity to effects of endocrine disruptors is often observed during specific stages of ontogenetic development, when targets for ED are temporarily present in an organism.

Without doubt, human health is under intensive spatiotemporal pressure of sub-toxic doses of several endocrine disruptors. These agents

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are carried to human organism through various paths of exposure, such as dermal contact, inhalation and, so often, via consumption of contaminated beverages and/or foodstuffs. Although endocrine disruptors are circulating in the bloodstream immediately after the exposure, it remains unexceptionally without clinical manifestations. These circumstances should be taken into account for the appropriate selection of an adequate biomodel for testing individual endocrine disruptors, particularly with concern to which signal pathways are expectedly disrupted.

In contrast to apparent harmlessness, human and animal reproductive health is significantly affected through three different mechanisms for endocrine disruptor action: the first, genetic or genotoxic effect (Smith-Oliver and Butterworth, 1987; Tiwari *et al.*, 2012), is mostly unapplied because of sub-toxic doses. Others, non-genetic (Viñas and Watson, 2013) and epigenetic (Skinner, 2014) molecular action frequently occur. There are major manifestations of these modes of action: hormone imbalance with many physiological consequences and inadequate epigenome changes, respectively. In addition to systemic organism-wide response to hormone imbalance (i. e. non-genetic effect), transgenerational inheritance of epigenetic endocrine disruptor-driven effect personates the risk for further generations (Nilsson *et al.*, 2012; Rodgers *et al.*, 2015).

Poor health from endocrine disruption-induced hazards, abundant in human and animal health, is obvious. Accordingly, the risk assessment is inevitable. Biomonitoring data have been systematically collected through many countries, however, models serve experimental simulation of endocrine disruptor exposure followed by comprehensive analyses. In general: there are several different models, such as *in vivo*, *in vitro* and *in silico*. Although the *in silico* model means detailed computational simulation with many advantages, *in vivo* and *in vitro* models are based on real live elements (i.e. the animal and the cell, respectively). Both biomodels have individual benefits and drawbacks and, therefore, the selection of the model represents an essential step in the risk assessment of individual endocrine disruptors.

Biomodels

Many well established simple invertebrate models, such as nematodes (roundworm *Caenorhabditis elegans*), insects (fruit fly *Drosophila melanogaster*) and echinoderms (sea urchins), have been historically used for a description of biological processes (Kuo *et al.*, 2000; Wang *et al.*, 2004; Al Rawi *et al.*, 2011), and more recently for pollutant assessment (Bošnjak *et al.*, 2014; Quesada-Calderón *et al.*, 2017; Zhou, 2018). Due to the sensitivity, more often water living organisms (i. e. snails, molluscs), invertebrate species represent easy available models for pollutant screening for vertebrates (deFur, 2004). Similarly, non-mammalian vertebrate species (zebrafish *Danio rerio*, african clawed frog *Xenopus laevis*) are frequently used in toxicological and similar studies (Iwamuro *et al.*, 2003; Barros *et al.*, 2018). There are several specific possibilities, such as neurohormonal study (molluscs) (Shomrat *et al.*, 2010), juvenile or pheromone investigation (insects) (Bomtorin *et al.*, 2014), availability of gametes (*Xenopus* spp.) (Gelaude *et al.*, 2015), that favour the utilization of these lower-class animal species as a bioindicator of environmental pollution (Bouchard *et al.*, 2009; Marquis *et al.*, 2009; Blahova *et al.*, 2018). The presence of the endocrine system and the conservativeness of some hormonal signalling offer the possibility to test endocrine disrupting effect (Keay *et al.*, 2006). However, the complex signal pathways in mammals possibly affected by endocrine disruptors remains largely unknown and the studying molecular action of such compounds requires mammalian biomodels. Presumable targeted pathways in these mammalian models are considered to be more similar to human than non-mammalian species.

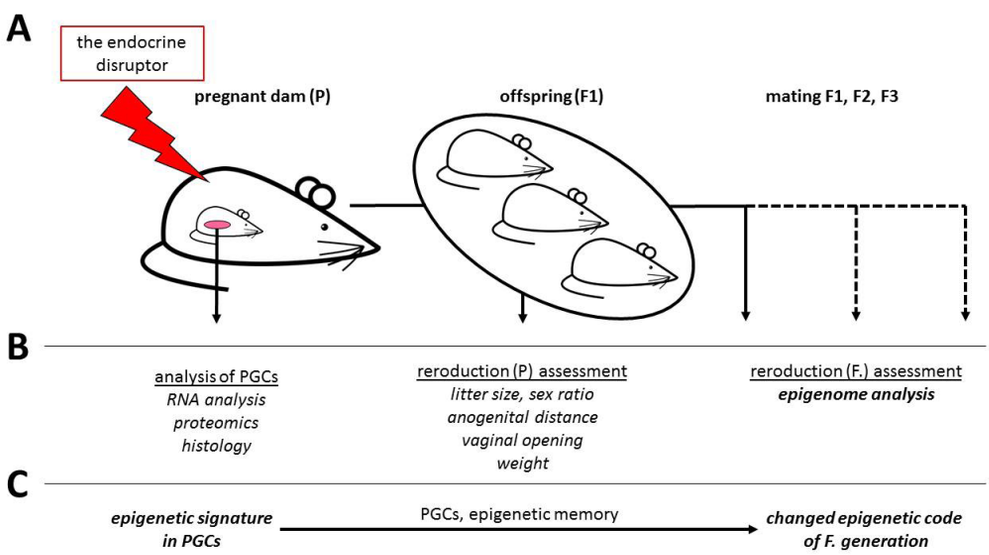
Rodent models for *in vivo* exposure

Laboratory rats and mice have many advantages and are commonly used for *in vivo* testing the effects of endocrine disruptors. The possibility of *in vivo* treatment of primordial germ cells, oocytes, spermatozoa and/or embryos allows the major benefit for evaluating endocrine disruptor effects on such reproductive functions. There are several ways of administrating compounds of interest,

with respect to considered path of endocrine disruptor exposure. Oral administration through drinking water, feed, dosage in corn oil, or more invasive subcutaneous and intraperitoneal administration of tested compound dissolved in a vehicle (saline, corn oil, etc.) are available and commonly used. Although injections can provide exact dosage of the compound [expressed as ng (µg, mg) g (kg) $bw^{-1}.day^{-1}$], the invasiveness makes these approaches more laborious and stressful for the animal facility and animals, respectively. Moreover, oral intake of endocrine disruptors via water or feed more precisely simulate the real exposure of human population, where systemic organism response, as well as pharmacokinetics of tested compound, are expected.

Rat and mouse model are desirable for a few different experimental schemes, combining each other. Individually, the acute (days) or chronic (weeks) exposure can be used as simple assessment of tested compounds on reproductive functions. More sophisticated approaches are available, such as *in utero* exposure or breastfeeding exposure, treating the pregnant and nursing dam, respectively.

In utero exposure length differs with respect to subsequent physiological features: embryo transport through fallopian tube occurring at embryonic day E0.5 – E3.5, blastocyst hatching at E3.5-4.5, embryo implantation and placental development at E6.5 – 9.5 (Slevin *et al.*, 2006), primordial germ cell (PGC) migration and epigenetic reprogramming at E7.5 – 14.5 (Doyle *et al.*, 2013), including progressive mitosis of PGCs, followed by organogenesis until delivery (Chen *et al.*, 2013). The epigenetic reprogramming window can cause transgenerational inheritance of endocrine disruptor-affected epigenome (Figure 1) and the exposure between E7 – E14 allows the study of this effect separately (Rahman *et al.*, 2017). On the contrast, whole-gestation exposure mimics the real endocrine disruptor impact. However, the beginning of exposure at E7.5, when the strict placental exposure of the foetus is considered, seems



(A) Environmental influence of the endocrine disruptor is capable of modifying the epigenome of foetuses (F1 generation) *in utero* of directly exposed dams (P generation). When the disruptor affects primordial germ cells (PGcs), the 3rd generation of offspring (F2) is affected. Although the rewriting of genetic information (mutation) does not occur, the impact of environment to parental (P) generation is inherited.
(B) Thorough molecular analyses of PGCs, gametes, and embryos (RNA analysis, proteomics and histology), as well as non-invasively achieved data of clinical reproduction (litter size, sex ratio, weight) are required. Oestrogenic or androgenic effects of endocrine disruptors are trackable through selected features (anogenital distance and puberty onset via vaginal opening).
(C) The further transmission of environmental factor-modulated epigenome to F3 and other generations through the epigenetic memory is assumed.

Figure 1. Presumed transgenerational effect of endocrine disruptors

to be more proper and the treatment is equivalent to the transplacental exposure.

In addition to *in utero* exposure, the dosage of milking dams offers the exposure through breastfeeding. This approach serves as a unique model for bisphenol evaluation, for reasons as follows: i) general sign of breastfeeding is exclusive milk intake by pups, ii) liposolubility in high-fatty diet, iii) extreme sensitivity of juvenile organisms to endocrine disruption, iv) breastfeeding as susceptible exposure window for gametogenesis at early stage of development (Sunyer *et al.*, 2006), and v) model the potential risk for babies exposed to polycarbonate milk bottles and plastic toys (Quitmeyer and Roberts, 2007; Andaluri *et al.*, 2018).

Both *in utero* and lactation exposure does not allow the exact dosage for treatment of the F1 generation through pregnant or milking dams. There are two possibilities for indirect estimation of genuine exposure by the compound, with different drawbacks of routine usage – firstly, analytical methods for tracking of endocrine disruptor level in blood plasma (Argmann and Auwerx, 2006) or breast milk (DePeters and Hovey, 2009; Muranishi *et al.*, 2016). In the second way, the ability to calculate the exposure using a physiologically based pharmacokinetic (PBPK) model (Karrer *et al.*, 2018), or even improved pregnancy PBPK model (Sharma *et al.*, 2018). Although this modelling requires comprehensive biomonitoring data, cohort studies and a highly sophisticated mathematical approach, PBPK modelling seems to be potent for experimental designing and, in particular, for implications of experimental data in human or veterinary medicine.

The choice of appropriate genetic background is the general advantage of the rat and mouse as a biomodel. With respect to the major features of an experiment, i. e. risk assessment of tested compound for human population (Chemek *et al.*, 2016) or the molecular action and endocrine disruptor-affected signalling pathways (Dolinoy *et al.*, 2007), outbred and inbred strains are available for use (Table 1). Additionally, genetically modified inbred strains can be used for precise study of molecular mechanism of endocrine disrupting effects (Liu *et al.*, 2015).

Besides the advantages of the aforementioned *in vivo* exposure, several weaknesses are obvious: inter-species differences in organs (uterus, placenta), tissues (placental barriers) and cells (molecular regulation of oocyte maturation). These reasons support the usage of advanced biomodels like pigs (*Sus scrofa*) and cattle (*Bos taurus*), in particular for the explanation of molecular mechanism and conservativeness of the mode of action of endocrine disruptors.

Mammalian i*n vitro* models for elucidation

Abovementioned disadvantages of lower mammalian species establishes higher mammals as more appropriate for *in vivo* study. Historically, laboratory cats and dogs were sporadically used, however, they have not become widely utilized. Rhesus monkey (*Maccaca mulatta*) or other non-hominin primates are most appropriate, mainly for the similarity of macaque genome to human. Nevertheless, unavailability, expensiveness or ethic problems arise resulting in rare utilization, mostly for basic study (Sutovsky *et al.*, 1999; He *et al.*, 2014; Song *et al.*, 2016), cancer research (Lertpiriyapong *et al.*, 2014; Dray *et al.*, 2018) and regenerative medicine (Higginbotham *et al.*, 2015; Kim *et al.*, 2018) due to the similarity of macaque genome to human. Alternatively, farm animals are exploited for similar studies, however, the *in vivo* treatment-based experiments are poor applicable

Table 1. Overview of frequently used strains of mouse and rat

	Mouse	Rat
Inbred strains	C57BL/6 BALB/c C3H	F334 LEW SHR
Outbred strains	ICR (CD1) NMRI (HsdWin:NMRI) MF1 (HsdoLA:MF1)	Sprague Dawley Wistar Han Lister Hooded

alike. Therefore, the utilization of farm animals destined for breeding (males) or meat industry (females) is widespread. Porcine and bovine models are the most available and allow *in vitro* culture of gametes.

In vitro maturation (IVM) of animal oocytes represents a relevant tool for endocrine disruptor testing as the highly-similar cell to human. Oftentimes, no interpolation is necessary for knowledge transfer to veterinary sciences being an identically used biomodel. Moreover, the presence of surrounding cumulus cells, creating cumulus-oocyte complex, provide the measurement of cumulus expansion (Zámostná *et al.*, 2016), other biological phenomenon accompanying oocyte maturation (Procházka *et al.*, 1998) and a biomarker of oocyte quality (Nevoral *et al.*, 2015; Blaha *et al.*, 2017). Generally, *in vitro* culture does not simulate the systemic response of the organism to endocrine disruption. On the other hand, oocyte IVM combined with biomonitoring obtains the relevant model for human oocyte exposure *in vivo* (unpublished). Specifically, quantified concentration of tracked endocrine disruptors in human follicular fluid is easily usable for *in vitro* treatment via the supplementation of culture media at equal concentration (Žalmanová *et al.*, 2017a). This IVM model simulates the oocyte enclosed in the ovarian follicle and surrounded with a matrix containing defined amounts of the endocrine disruptor.

In vitro fertilization (IVF), the technique familiar for human reproductive medicine (Balaban *et al.*, 2014) and biotechnology for farm animals (Pavlok *et al.*, 1989; Abeydeera *et al.*, 1998) follows both *in vivo* and *in vitro* maturation of oocytes. These oocytes acquire developmental competence during meiotic maturation and, therefore, the maturation presupposes the success of the embryonic development (Kim *et al.*, 2008; Nevoral *et al.*, 2014). Accordingly, the exposure of *in vitro* mature oocytes offers relevant observations of the endocrine disruptor impact on embryonic development through the oocyte quality (unpublished). A possible effect of endocrine disruptors on embryos in the fallopian tube and uterus is eliminated in this experimental scheme. Thus, observed phenotypes of oocytes exposed to endocrine disruptors after IVF directly represent the effect of an endocrine disruptor on the oocyte developmental competence acquired during IVM.

In vitro maturation and early embryonic development show several inter-species differences. There is physiologically prolonged nuclear envelope breakdown in prophase-arrested porcine oocyte when the maturation is physiologically initiated (Fulka *et al.*, 1986; Motlik *et al.*, 1998). Therefore, this phase provides an endocrine disruptor-sensitive window specific for porcine oocytes. Strongly affected chromatin changes induced by the endocrine disruptor *in vitro*, such as epigenetic modifications (Wang *et al.*, 2016) and/or aneuploidy incidence (Žalmanová *et al.*, 2017a), can be considered in porcine oocytes. This model extends the possibility of molecular study of candidate compounds during oocyte maturation. Chromatin is highly error-prone during oocyte maturation (Hornak *et al.*, 2011) and, therefore, molecular analyses after endocrine disruptor exposure are highly relevant. Aneuploidy disorders (Down syndrome, Patau syndrome), genetic disorders (haemophilia, cystic fibrosis) and epigenetic imprinting failures (Prader-Willi syndrome, Angelman syndrome) are clinical manifestations of oocyte aneuploidy-derived consequences and present serious issues for human health. Endocrine disruptors are capable to increase the incidence of oocyte aneuploidy (Hunt *et al.*, 2003; Žalmanová *et al.*, 2017b; Nevoral *et al.*, 2018) and, thus, the risk of aneuploidy-derived diseases.

Evaluation of early embryonic development represents another way to assess endocrine disruptor impact on the oocyte and embryo quality. The genetic analysis provides the comprehensive approach, including advanced methods of epigenome analysis. For *in vitro* embryo production, the porcine model seems to be less appropriate due to high incidence of polyspermic fertilization and polyploidy of embryos. Comparing to other farm animal models, the bovine model provides highly reliable methods for IVF and *in vitro* embryo production, based on protocols often used intensively for commercial purpose. Overall, the mouse embryo offers the unique model of embryonic development beyond the gastrula stage (Sozen *et al.*, 2018), incompatible with embryos of other species. Taken together, the combination of individual *in vitro* models meets the requirement for a suitable model.

CONCLUSIONS

Many animal models for biological study have been well established through the decades, followed by biomedical applications and bioindicators. The interplay of models offers the observation of exposure-dependent phenotype, followed by the study of the molecular action of tested endocrine disruptor. There is the potent scheme for risk assessment of an endocrine disruptor for human health, combining a few approaches in the following order: i) simultaneous biomonitoring on human body fluids, followed by simultaneous ii) PBPK usage for *in vivo* exposure of the rodent model and iii) the *in vitro* exposure of oocytes and embryos of farm animals. This approach provides studying the effect and molecular mechanisms of endocrine disruptors acting at environmentally exposed doses, based on human biomonitoring. Additionally, the elucidation of attained observations and the test of conservativeness are available using individual *in vivo* and *in vitro* models. The results obtained from the model interplay, represent highly relevant outputs of the endocrine disruptor testing towards the elimination of environmental pollutants and the human health protection.

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PARTHENOGENETIC ACTIVATION OF PORCINE OOCYTES IN *IN VITRO* CONDITIONS

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Effective oocyte activation is a key point in an embryo technology. Activation of *in vitro* matured (IVM) oocytes is essential for successful production of transgenic animals, parthenogenetic production of embryonic stem cells and nuclear transfer. Knowledge of parthenogenetic activation also contributes to better understanding of the mechanisms of fertilization, early embryonic development and general principle of cell signalling pathways.

Present methods for parthenogenetic embryo production depend on the use of *in vitro* maturated oocytes with full meiotic competence and combination of different chemical, physical and enzymatic stimuli. The goal of our study was to compare the success of electrical and chemical activation. This study compared the rates of blastocysts following parthenogenetic activation with electrical pulse with the use of chemical activation (ionomycin). IVM oocytes with a first polar body and high quality were randomly allocated into two groups. The first oocyte group (electrical) was activated by combination of electrical pulse (38 V and 100 μs) and 6-dimethylaminopurine (6-DMAP). The second oocyte group (chemical) was activated by combination of ionomycin (10 mM) and 6-DMAP (2 mM). Activated oocytes were cultured for 7 days in PZM3 medium at 38.5 °C in 5 % CO_2. In both types of activation we reached a high level of cleavage (electrical activation - 77.5 % vs chemical activation - 80 %), which represents successful oocyte activation. Among two types of activation, significant differences ($p < 0.01$) in the blastocyst formation (electrical activation - 15 % vs chemical activation - 21 %) were found. In conclusion, our results demonstrated that the optimal activation protocol of *in vitro* matured porcine oocytes was chemical activation consisted of combination of 10mM ionomycin and 2mM 6-DMAP.

Key words: oocyte; embryo; parthenogenetic activation

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MEIOTIC COMPETENCE OF BOVINE OOCYTES INFLUENCES MITOCHONDRIAL GENE EXPRESSION DURING *IN VITRO* MATURATION

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For the *in vitro* studies, oocytes are usually collected from antral follicles independently of the follicular development stage. Such heterogeneous population of the oocytes differs in meiotic and developmental potential. It is generally accepted that the meiotic and developmental competencies of bovine oocytes can be expressed by both size and health of follicles from which the oocytes are recovered. The objectives of the present study were to assess the changes in expression of mRNA transcripts playing an important role in mitochondrial DNA metabolism during *in vitro* maturation of bovine oocytes with different meiotic competence. Meiotically more and less competent oocytes were obtained separately either from medium (MF) or small (SF) follicles, categorized into healthy and light-atretic categories according to oocyte morphology and matured using a standard protocol. They were examined at 0 and 24 hours of maturation. Either total RNA or poly(A) RNA was extracted from oocytes and the evaluation of gene expression of three mitochondrial factors (TFAM, TFB1M, and TFB2M), MATER, and Luciferase, as external standard, was performed by an RT-PCR. The level of TFAM, TFB1M and MATER poly(A) RNA transcripts significantly decreased in both MF and SF oocytes after maturation compared with that before maturation. The healthy MF and SF oocytes contained significantly higher amounts of TFB1M at the GV stage than light-atretic oocytes. However, no differences in the transcript abundance were found among these categories at the MII stage. While in healthy and light-atretic MF oocytes TFB2M abundance revealed no differences among the GV and MII stage, in SF oocytes TFB2M was increased at the MII stage. In light-atretic SF oocytes the increase was significant ($p < 0.05$) The level of TFAM total RNA significantly increased after maturation compared with that before maturation in all oocyte categories. On the other hand, no significant differences in the transcript abundance were found for TFB1M, TFB2M and MATER total RNA between the GV and MII stages in any oocyte category. The significantly higher amount of MATER total RNA was detected only in healthy MF oocytes in comparison with that in light-atretic MF oocytes after maturation. It can be concluded that differences in mitochondrial genes, MATER poly(A) and total RNA expression were revealed among the maturing healthy and light-atretic meiotically more and less competent bovine oocytes.

Key words: bovine oocytes; meiotic competence; *in vitro* maturation; gene expression

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RECOMBINANT HUMAN LACTOFERRIN PRODUCED IN MILK OF TRANSGENIC GOATS

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Technology for the production of human recombinant proteins from milk of animals allows to obtain various proteins at large amount. On their basis this provides a possibility (including economical reason) to involve into commercial production not only medical products, but also nutritional ingredients to special food products that will open a new area of application of recombinant proteins in human life.

It is known, that lactoferrin is the protein with many major properties and functions, such as anti-viral, antibacterial, antiphlogistic, antineoplastic, immunomodulatory, anticancerogenic, bacteriostatic and antipathogenic. This protein will probably become one of the main and affordable preventive mean for various illnesses of the century. Therefore, human lactoferrin gets a special value in amount that will satisfy all-age people needs. The technology has been implemented with the recombinant human lactoferrin (rhLF) produced in goat milk. As a result, the herd of goats producing active recombinant human lactoferrin in milk was created. Integration of human lactoferrin construct into the goat genome had no negative effect on health and reproductive traits of animals. Human lactoferrin gene, ensuring synthesis of protein in the mammary gland of producing animals, is transmitted steadily from generation to generation. The identical main physical and chemical characteristics of human recombinant lactoferrin and natural lactoferrin in woman's milk were proved, and biological activity of the protein was shown.

The obtained data will contribute to the development of nutritional supplements and drug forms of rhLF with various functional application.

Key words: transgenesis; goats; milk; protein; lactoferrin

BOVINE MODEL FOR THE STUDY OF MOLECULES IMPORTANT FOR REPRODUCTION

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The presence and specific distribution of membrane proteins in gametes are crucial for the process of fertilization. Our main objective was to study these molecules both in male and female reproductive tract, as well as in early embryogenesis, preferentially using the bovine model. Although, currently we concentrate on detecting members of tetraspanin superfamily proteins (CD81, CD9), in our previous papers we characterised complement regulatory proteins (CD46, CD52) in bull sperm and reproductive system. The changes in expression and localisation of molecules of interest are studied in gametes at different stages of their development (testicular, epididymal and ejaculated sperm, germinal vesicle oocytes, metaphase I- or metaphase II- oocytes). The physiological role of targeted molecules is examined during processes of sperm capacitation and acrosome reaction, oocyte maturation, fertilization and early embryogenesis using different methodological approaches. By comparing our results with other findings on murine and porcine models, we provide the knowledge related to mammalian fertilization.

Key words: reproduction; fertilization; gametes; sperm; oocyte

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QUAIL CAM AS A TOOL FOR DIAGNOSIS OF MICROBIOLOGICAL AND ONCOLOGICAL DISEASES

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Avian chorioallantoic membrane (CAM) is an excellent *in vivo* experimental model. It is affordable, easily accessible and it develops quickly, which can be an advantage in many kinds of research. CAM serves as a respiratory organ during development of avian embryo; it is richly vascularized and immunodeficient. During *ex ovo* cultivation CAM is easily observable, with good access for applying experimental substances topically or internally. Quail CAM, in comparison with more commonly used chick embryos, has an advantage in the shorter development time, their sexual maturation is quicker, their egg production is higher and less breeding space is needed. CAM structure is similar to many tissues like lung, bladder, retina or tissue of hemato-encephalic barrier. These options open the possibilities for research in various fields. In our research we used quail CAM to study angiogenesis, pharmacokinetics of drugs and nanoparticles. We also used it for photodynamic diagnosis and therapy of tumours and microbial diseases.

On the third day of embryonal development (ED3), fertilized eggs were disinfected, opened and the embryos were transferred into six-well tissue culture plates and incubated at 37 °C and 60-70 % humidity. On ED7 the studied substances, tumour cells or microorganisms were applied onto CAM surface, depending on the type of experiment. On ED9 the photosensitizer was added and after 1, 3, 5 and 24 hours CAM was illuminated using near-UV light and induced fluorescence was recorded by a digital camera. CAM tissue was evaluated by fractal analysis, histological techniques, real time PCR and other methods.

Our results indicate that Japanese quail CAM model is a useful tool for the study of anti-vascular therapy, tumour angiogenesis, development of new biophotonic techniques as well as novel drug testing.

Key words: Japanese quail; chorioallantoic membrane; angiogenesis; photodynamic diagnosis

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EXPRESSION OF HUMAN MESENCHYMAL STEM CELL MARKERS (CD73, CD90 AND CD105) IN RABBIT ADIPOSE-DERIVED STEM CELLS

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Mesenchymal stem cells were for the first time identified in the adipose tissue (ADSCs) in 2001. There are many studies that tried to characterize ADSCs using surface marker expression analysis by flow cytometry. Studies point out to differences in the expression of individual markers among species. According to the literature, human ADSCs express typical mesenchymal CD markers, such as CD13, CD29, CD44, CD63, CD73, CD90 and CD105, while are negative for hematopoietic markers CD14, CD34, CD45. In our previous studies, we confirmed positive expression of CD29 and CD44 and negative expression of CD34 and CD45 markers on rabbit ADSCs using flow cytometry. In this study, rabbit ADSCs were isolated and cultured until the third passage as described in our previous study. Then, cells were stained with following monoclonal antibodies: anti-mouse CD73 in PE-Cy^7 (clone TY/11.8; eBioscience, Austria), anti-rat CD90 in PE-Cy^7 (clone OX-7; BD Biosciences, USA) and anti-rabbit CD105 in FITC (clone SN6; GeneTex, USA). Samples were assessed using FACS Calibur (BD Biosciences, USA) and CellQuest Pro software (BD Biosciences, USA). At least 50 000 events were analyzed for each sample. The experiment was replicated three times. Obtained data were statistically evaluated using a SigmaPlot software (Systat Software Inc., Germany) and expressed as the mean ± SEM. We observed low expression of all selected markers: CD73 (3.62 % ± 1.91 %), CD90 (9.15 % ± 8.01) and CD105 (4.8 % ± 0.93). Our results are comparable to those of other authors, who examined the expression of these surface markers using flow cytometry on other sources of rabbit stem cells, such as the bone marrow or amniotic fluid. Low expression of these markers could be due to the low affinity of the antibodies. Therefore, it is recommended to use PCR as another method for detection of gene expression, since these markers are probably characteristic also for rabbit mesenchymal stem cells.

Key words: rabbit; adipose-derived stem cells; CD markers; flow cytometry

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ANALYSIS OF THE ESTROGEN RECEPTOR (ESR) GENOTYPES IN WILD BOAR

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The wild boar (*Sus scrofa*) is the main ancestor of domestic pigs, with which it is in a close genetic affinity. The commercial pig breeds were assessed for the polymorphism of the ESR gene, associated with the increased litter size. Our study aimed at investigating the existence of the ESR candidate gene polymorphism in a population of wild boar in Slovakia. Genomic DNA was isolated from the muscle tissue 23 of unrelated wild boars by phenol-chloroform extraction and ethanol precipitation. Polymorphism was detected by a PCR-RFLP. Primers were designed from published porcine ESR sequence and were used to amplify 120 bp fragment. PCR products were digested with a restriction endonuclease PvuII. Restriction fragments were separated by electrophoresis on a 4 % agarose gel. Testing the ESR genes, we found the presence of the AA genotype in all tested individuals. This indicates the absence of PvuII polymorphism in a population of wild boar. On the other hand, in our previous study on domestic breeds of pigs, mainly in Large White and Landrace, the polymorphism of the ESR gene was demonstrated. The frequency of the B allele was in the range of 0.25 – 0.33.

Key words: wild boar; polymorphism; ESR; PvuII

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DEVELOPMENT OF *DIABETES MELLITUS* IN ZUCKER DIABETIC FATTY RATS FED BY HIGH-ENERGY DIET

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The incompletely controlled *diabetes mellitus* (DM), especially concerning the diet, can cause serious complications. Insulin resistance, relative insulin deficiency and elevated glucose level are major symptoms of the type II diabetes (T2DM), considered as one of the most common diseases. The suitable animal model of T2DM is Zucker diabetic fatty (ZDF) rats, which are deficient in the leptin receptors. These animals show fa/fa genotype, they are obese with glucose intolerance, hyperlipidemia, hyperglycaemia and hyperinsulinemia. The goal of this study was to analyse the effect of chronic high-energy diet (live weight, intake of feed and water, glucose and insulin metabolism) on DM complications in ZDF rats. Male ZDF rats (n = 20) and their lean controls (non-diabetic, n = 10) at the age of 3 months were used in the experiment. Animals were supplied with water and diet on *ad libitum* base. Rats were divided into three groups as follows: lean untreated rats (C) fed by KKZ-P/M (10 $MJ.kg^{-1}$), obese rats fed by KKZ-P/M (10 $MJ.kg^{-1}$, E1) and obese rats fed by enriched high-energy diet (E2, enriched

KKZ-P/M, 20 MJ.kg^{-1}). The consumption of feed and water, the live weight, the glucose and insulin levels were determined. ZDF rats in both experimental groups (E1 and E2) showed hyperphagia, obesity, insulin resistance and high hyperglycaemia. High-energy diet induced hyperglycaemia followed by earlier onset of other diabetic symptoms and complications.

Key words: *diabetes mellitus*; Zucker diabetic fatty rats; high-energy diet

Acknowledgements: This study was supported by the APVV grant no 15/0229.

CELL RECEPTOR-MEDIATING COMMUNICATION BETWEEN A PREIMPLANTATION EMBRYO AND SURROUNDING ENVIRONMENT: CLUES FROM MOUSE AND RABBIT MODELS

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Preimplantation period of embryo development is one of the most sensitive phases in mammalian ontogeny, and disturbances at this developmental stage can result in poor pregnancy outcomes (resulting both from natural conception and from biotechnology procedures). Experimental studies have shown that maternal physiological condition and external environmental factors can significantly influence preimplantation embryo development, indicating a communication between the early embryo and its environment. The study of communication between the early embryo and surrounding environment has been mainly focused on protein signaling molecules, such as growth factors and cytokines. However, small-molecule ligands, such as biogenic monoamines, have been shown to influence preimplantation embryo development as well, and results obtained in mouse and rabbit models indicate that biogenic monoamine receptors are expressed in preimplantation embryos. Several adrenergic, dopamine, serotonin and histamine receptors were detected in mouse and rabbit ovulated oocytes and preimplantation embryos, as well as in mouse embryonic stem cells. Although the physiological role of biogenic monoamine receptors in early embryonic cells is not fully understood, experimental data indicate their involvement in the regulation of cell proliferation, differentiation and survival under physiological as well as unfavorable or pathological conditions (e.g. during maternal stress).

Key words: enviroment; embryo; development; receptor; proliferation

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POTENTIAL EFFECTS OF PLANT ALKALOID BERBERINE ON OVARIAN CELLS *IN VITRO*

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Berberine (BBR) is a plant isoquinoline alkaloid with a long history in Chinese medicine, present in root and shoot systems of clinically important medicinal plants. This phytochemical compound is found in different plant families such as *Berberidaceae*, *Ranunculaceae* and *Papaveraceae* families. BBR is a widely used as a natural product that showed pharmacological activities in modern and traditional medicine with non-toxic effect on humans. Current clinical research on BBR points out several medical applications, beneficial for therapies of chronic diseases, such as diabetes, hypertension and hypercholesterolemia. However, BBR effects on human reproduction remain unknown. The aim of the *in vitro* study was to examine the effect of BBR treatment (at the concentrations: 5; 10; 25; 50; 100 µg.ml^{-1}) for 24 h on viability of ovarian cells *in vitro*. Cultures of human ovarian granulosa-lutein cells (HGL5) and human ovarian carcinoma cells (OVCAR-3) were used, and the metabolic activity was determined by *AlamarBlue™* cell viability assay. The viability of HGL5 was not influenced by the berberine treatment at all concentrations used in the study. On the other hand, BBR addition significantly inhibited the viability of OVCAR-3 cells at all doses tested ($P \leq 0.05$). In conclusion, our study suggests potential anti-cancer effect of BBR on OVCAR-3 cell line. BBR might be one of such potential agents for ovary cancer therapy. However, further studies are necessary to define a therapeutic potential of BBR.

Key words: berberine; ovarian cells; viability; cancer

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PRESERVATION AND PRODUCTIVITY CONTROL OF MANGALITSA, AUTOCHTHONOUS PIG BREED IN NORTHERN SERBIA, VOJVODINA

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Mangalitsa is an autochthonous breed of pigs in Serbia, which is mostly raised in the northern parts of the country. Like many other autochthonous breeds of animals, Mangalitsa was on the verge of extinction. Although in the past this was a widespread breed in Serbia, its number was significantly reduced in the 20th century, because the breeders accepted an intensive form of raising of pigs, for which the Mangalitsa was not adequate. Also, many meadows and forests, in which the Mangalitsas are fed, have been destroyed. Lately, the number of Mangalitsa was increased and stabilized but its current status is still defined as endangered. The reason for the increased interest in the raising of Mangalitsa lies in the fact, that consumers recognized the quality of the Mangalitsa meat products. Mangalitsa meat contains more monounsaturated and less saturated fatty acids in

comparison with meat of commercial pig breeds. Since the Mangalitsa pigs are adapted to an extensive way of keeping and have a high disease resistance, this makes them ideal for organic production. A good example of the organic raising of Mangalitsa pigs is applied in the special nature reserve "Zasavica". Currently, only *in vivo* conservation is carried out in Serbia. Another reason for increasing the number of Mangalitsa are subsidies provided to the farmers by the Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia. According to the data from the Main Breeding Organization for Vojvodina, the number of Mangalitsa pigs under control during the period 2013-2017 is on the rise, both for males and females. Implemented selection measures include the control of the productivity of sows and boars. The main goal of the breeding is to preserve and increase the population as well as to improve the genetic basis of the Mangalitsa population in the Republic of Serbia. In the future, new modern methods of conservation and biotechnological methods should be introduced in order to preserve the purity of the breed and increase the number of Mangalitsa pigs.

Key words: mangalitsa; autochthonous pig breed; *in vivo* conservation; productivity control

RAPID GENOTYPING OF THE SHORT AND HIGHLY VARIABLE REGION IN MTDNA OF HERBIVORES

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Mitochondrial DNA is found in nearly all eukaryotic organisms. mtDNA is a short, circular and relatively conserved DNA molecule transmitted maternally. It is one of the most useful tools in population genetics or phylogenetics for maternal inheritance. Screening of polymorphisms of mtDNA is suitable for comparisons among individuals from the same population as well as among distantly related species. mtDNA has an extremely variable region acquired by 2 billion years of mtDNA evolution, and is more variable than the nuclear genome itself. Furthermore, it is an ideal genetic marker for analysis of problematic, low quality and low quantity materials i.e. faeces. Here we present the application of universal primers for rapid genotyping of the highly variable region of mtDNA in cattle, chamois, deer, goat and sheep. DNAs of cattle, deer, chamois, goat and sheep from our DNA bank were used for validation of the method. DNA was extracted from hair roots or blood using Wizard Genomic DNA purification Kit (Promega). PCR reactions were performed with 20-40 ng DNA, 35 cycles and 53°C annealing temperature (Thermo-Start PCR Master Mix, Thermo Scientific). PCR fragments were cycle-sequenced using BigDye Terminator Cycle sequencing Kit version 1.1 and were run on an Avant 3100 Genetic Analyser (Applied Biosystems). Sequences were aligned using Geneious software (Biomatters) and BLAST (NCBI). In summary, we have validated a method for rapid genetic screening of the highly variable region in mtDNA of cattle, chamois, deer, goat and sheep, which can be used for species determination and population studies among even wider variety of herbivore species.

Key words: mtDNA; genotyping; herbivores

ANTIBIOTIC RESISTANCE OF ENTEROBACTERIACEAE SPECIES ISOLATED FROM RECTAL SWABS OF SHEEP

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The main objective of this study was to determine antibiotic resistance od *Enterobacteriaceae* species isolated from sheep rectal swabs and identification of isolated species as potential resistant gene vectors. Sixty nine sheep rectal swabs were obtained from different farms of Slovakia. Isolation of *Enterobacteriaceae* species was done on MacConkey agar during 24 h at 37 ± 1 °C under air condition. Cultures were purified by four-way streak plate method. Pure cultures were identified by MALDI TOF Mass Spectrometry using MALDI Biotyper 3.1 software (Bruker Daltonics, Germany). Antibiotic susceptibility testing was performed by disk diffusion methodology according to EUCAST. Ampicillin resistance was confirmed by indirect method using MALDI TOF MS, where beta-lactamase hydrolysis of ampicillin was assessed and resistance mechanism was detected. The following antibiotics were used in this study: streptomycin (10 µg/disc), tetracycline (30 µg.disc), chloramphenicol (30 µg/disc), oxacillin (1 µg/disc), ampicillin (10 µg/disc), nalixidic acid (30 µg/disc), amikacin (30 µg/disc), gentamicin (30 µg/disc), levofloxacin (5 µg/disc), piperacillin (30 µg/disc) and tigecycline (15 µg/disc). Overall, from 69 samples the resistance was revealed in following: 5 strains against tetracycline, 2 strains against tetracycline, 4 strains against chloramphenicol, 14 strains against oxacillin, 7 strains against ampicillin and 9 strains against levofloxacin. Antibiotic resistances against nalixidic acid, amikacin, gentamicin, piperacillin and tigecycline were not found in this study. Seven strains were purified by four-way streak plate method and identified by MALDI TOF MS as *Escherichia coli*, *Serratia odorifera* bv.1, *Enterobacter aerogenes*, *Citrobacter farmeri*, *Proteus vulrgaris*, *Klebsiella* spp. and *Yersinia* spp. Disk diffusion method showed resistance of *Escherichia coli* against three antibiotics: chloramphenicol, ampicillin and levofloxacine. *Serrattia odorifera* bv. 1 was resistant against streptomycin and tetracycline. Oxacillin resistance was detected in *Enterobacter areogenes* and resistance against chloramphenicol in *Klebsiella* spp. Other identified bacteria isolated from rectal swabs of sheep were identified as susceptible to antibiotics, which were used in this study. MALDI TOF MS analysis showed that ampicillin was hydrolysed by beta-lactamases produced by *Escherichia coli*, and its decay products as ampicillin with disrupted amide bound (366 ± 0.6 m/z), its monosodium salt (389 ± 0.6 m/z),

its disodium salt (412 ± 0.6 m/z), spontaneous decarboxyled ampicillin (323 ± 0.6 m/z) and decarboxyled ampicillin sodium salt (344 ± 0.6 m/z) were detected. Therefore, enzyme hydrolysis strategy was confirmed as main strategy of antibiotic resistance in *E. coli*. In conclusion, we determined that the resistance against several antibiotics was found in rectal swabs of sheep and it is spread within sheep breeding. Also, enzymatic resistance mechanism is a main resistant strategy within *Enterobacteriaceae* genera in sheep breeding.

Key words: antibiotic resistance; sheep; *Enterobacteriaceae*; MALDI TOF MS

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CHICKEN STEM CELLS AS A POTENTIAL SOURCE FOR ANIMAL GENE BANK

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Biosecurity and sustainability in chicken production requires reliable germplasm conservation. Germplasm conservation in chicken is more challenging in comparison to other livestock species. Embryo cryopreservation is not feasible for egg-laying animals, and chicken semen conservation in different chicken breeds has variable success. A potential solution is the cryopreservation of the committed diploid stem cell, blastodermal cells (BCs), the precursor of gametes and primordial germ cells (PGCs). BCs and PGCs are the lineage-restricted cells found at early embryonic stages in birds. This research dealt with isolation, characterisation and cryopreservation of chicken stem cells for the animal gene bank purposes. Trypan blue, fluorescence microscopy, flow cytometry and transmission electron microscopy were used for the viability assessment and characterisation of fresh and frozen/thawed chicken stem cells. Our results showed that BCs contain lipid granules, which prevent successful freezing even though different methods of cryopreservation were used. However, in contrast, PGCs contain a smaller amount of lipid granules, and, therefore, PGCs are more suitable for cryopreservation. The present study suggest that PGCs should be considered as more preferable source for animal biobanking, and the choice of proper cell source should be done carefully.

Key words: chicken; blastodermal cells; primordial germ cells; cryopreservation; viability

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INBREDISATION – THE WAY FOR CREATION OF GENETICALLY UNIFORM RABBIT GROUPS

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Utilization of rabbits as experimental animals is oriented to specific populations because of user goals. Human diseases, organ transplantations, physiological experiments, feeding trials or genetic experiments are most frequent examples for exploration of rabbits. These utilizations are associated with homogenous genetic background, which can be reached by inbredisation process. Depending on the type of observed and applied traits, homozygotation can be an effective way to create appropriate populations with unique characteristics.

We used 3 panmictic rabbit populations (New Zealand White – NZW, Californian – C and Nitra rabbit – Ni) for directed selection during 5 generations. In the course of inbredisation, the animals were mated *inter se* (full siblings with each other). Initial (founder) animals were selected from a wider population and they formed the original parental groups with 20 does and 5 males of each breed. At average, the same number of selected animals was mated for next generation. Selection criteria were following: for NZW it were – increase of live weight, for C – high level of untroubled behaviour, and for Ni – long ear shell with clear blood vessel. NZW animals were selected on the basis of regular weekly measuring of live weight. Californian rabbits were tested in open field equipment for peaceful habitus as a number of movements per time unit. Animals in Nitra breed population were selected basing on the ear length and good visualisation of central ear blood vessel. In addition to the selected traits, in all three populations the standard breed characteristics were maintained.

After the 5 year selection process the results are following: in the 5th generation of NZW rabbit population an average initial live weight was increased daily from 25.4 ± 6.2 g to 30.2 ± 3.2 g. It represents difference in 411.6 g at slaughter age in favour of the inbred population. Panmictic Californian rabbits had 25.6 ± 7.5 movements in contrast to 14.5 ± 5.7 units for animals in inbred population. The length of ear in the initial Nitra rabbits was 11.5 ± 1.6 cm in comparison to the inbred population (13.5 ± 1.8 cm). According to these results, inbredisation is an effective process for creating relevant populations.

Key words: rabbit; inbredisation; selected traits; live weight; ear length

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DIFFERENCES BETWEEN EWES AND MOUFLONS IN SELECTED METABOLIC PARAMETERS IN RELATION TO THE YIELD AND EMBRYO QUALITY

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The aim of our research was to study the effect of prevalence the serum cholesterol value, urea and total protein before and after superovulation on the yield and quality of embryos in ewes and mouflons. In ewes, positive correlation was found between the level of cholesterol and superovulatory response (r = 0.54), the total number of embryos (r = 0.01) and number of transferable embryos (r = 0.39). Levels of urea were in negative correlation with the superovulatory response (r = -0.42), the total number of flushed embryos (r = -0.49) or transferable embryos (r = -0.58). In mouflons, positive correlation was found between the level of cholesterol and superovulatory response (r = 0.68), total number of embryos (r = 0.50) and transferable embryos (r = 0.48). Levels of urea were in negative correlation with the superovulatory response (r = -0.37), the total number of flushed embryos (r = -0.56) and transferable embryos (r = -0.64). The influence of total proteins in blood serum of donor ewes and mouflons on effectiveness of embryo transfer was not proved in our research. No statistically significant differences between ewes and mouflons in terms of the evaluated parameters were observed.

Key words: blood; embryos; ewes; metabolic parameters; mouflon females

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EVALUATION OF NOVEL ANTIBODIES RAISED AGAINST THE RABBIT CD34 SYNTHETIC PEPTIDE (G1SJT2)

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The aim of this preliminary study was to evaluate the antigen specificity of anti-CD34 antibodies raised in mice against the specific immunogenic oligopeptide synthesized according to the predicted rabbit CD34 protein (G1SJT2). Experimental procedure was performed as described previously (Vasicek *et al.*, 2017 and 2018). Briefly, peripheral blood mononuclear cells (PBMCs) from three young (four months-old) and clinically health rabbits of New Zealand White (NZW) line were used in the experiment. Isolated cells were single stained with 28 different mouse anti-rabbit CD34 monoclonal antibodies: IgG1 isotype sub-clones (4/118, 4/131, 8/80, 8/98, 8/109, 59/1, 59/2, 59/3, 171/123, 171/124, 171/125, 172/2, 172/47, 391/145, 391/157, 391/186, 398/115, 398/117, 398/124, 740/247, 740/252, 873/193, 873/197, 945/68 and 945/95), IgG2a isotype sub-clone (473/174) and IgG2b isotype sub-clones (369/1 and 369/33). Goat anti-mouse Ig-APC (BD Biosciences, USA) was used as a secondary antibody. According to obtained results from flow cytometry, three anti-CD34 antibodies with higher detectable number of $CD34^+$ cells (369/1 and 369/33 – both IgG2b and 473/174 – IgG2a) were chosen for the double staining with CD45 antibody (clone L12/201, IgG1; Bio-Rad Antibodies, USA). As secondary antibodies, antimouse IgG2a-FITC (Thermo Fisher Scientific, USA) or IgG2b-FITC (BioLegend, USA) and IgG1-PE (Miltenyi Biotec, Germany) were used. To exclude dead cells from the analysis 7-AAD (Bioscience, Austria) was used. Cells (at least 50,000 events) were analyzed using a flow cytometer FACSCalibur (BD Biosciences, USA). Modified flow cytometry method based on ISHAGE society was used to determine $CD34^+$ and $CD34^+CD45^-$ cell counts. Observed results were evaluated statistically (Holm-Sidak method in SigmaPlot software), and expressed as the means ± SEM.
We observed significantly higher ($P < 0.001$) expression (%) of CD34 antigen within PBMCs using antibodies: 369/1 and 369/33 (1.27 ± 0.32 and 1.37 ± 0.47, respectively). Slightly increased expression was also found by using of 473/174 antibody (0.50 ± 0.12) compared to the others. Those three antibodies were used to determine undifferentiated $CD34^+CD45^-$ cells within PBMCs. Antibody 473/174 revealed significantly higher ($P < 0.01$) percentage of $CD34^+CD45^-$ cells (0.25 ± 0.02) in comparison to other antibodies 369/1 and 369/33 (0.18 ± 0.06 and 0.11 ± 0.03, respectively). In conclusion, clone 473/174 seems to be another promising antibody for the detection of CD34 antigen within the rabbit PBMCs. However, further analyses are required in order to determine the true CD34 antigen specificity of all newly prepared monoclonal antibodies.

Key words: rabbit; HSCs; CD34 antibodies; flow cytometry

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EFFECT OF DIFFERENT EGGSHELL COLOUR ON EXTERNAL AND INTERNAL EGG QUALITY OF JAPANESE QUAIL (*COTURNIX JAPONICA*)

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Totally 300 egg from Japanese quail breed were used to analyze the effect of eggshell colour on internal and external egg quality. The birds were housed as 1 male and 3 females per cage of 0.12 m^2 area at the Research Institute for Animal Production Nitra and fed with a mixture of 11.7 MJ metabolic energy and 200.0 g of crude protein during the experiment. Feed and water were given *ad libitum*. Analysis of external and internal characteristics of Japanese quail eggs was performed in the laboratory of the Department of Poultry Science and Small Farm Animals of the Slovak University of Agriculture in Nitra. The eggs were divided into five groups according to the colour (each n = 60) based up on pigment

found on the surface of the eggshell: brown (brown pigmentation), black (greyish white eggshell pigmented with various sizes of black pigments), white (non-pigmented eggshell), spotted (small black pin dots on greyish brown eggshell) and blue (slightly blue pigments). This research was conducted to investigate the effects of eggshell colour on the egg weight, egg shape index, eggshell weight, eggshell percentage, eggshell thickness, eggshell strength, albumen weight, albumen percentage, albumen index, Haugh unit, yolk weight, yolk percentage, yolk index and yolk colour. Results revealed that eggshell colour had significant ($P < 0.05$) effect on the egg weight (from 11.66 g in spotted group to 12.98 g in black group). The egg shape index did not express any significant ($P > 0.05$) differences between different eggshell colours. Non-significant ($P > 0.05$) effects were recorded for eggshell weight, eggshell percentage, eggshell thickness and eggshell strength among different eggshell colours. Results also showed that eggshell colour had significant ($P < 0.05$) effect on albumen index (from 9.32 % in brown group to 9.63 % in white group), Haugh unit (from 87.51 in brown group to 89.85 in white group), yolk weight (from 4.11 g in spotted group to 4.36 g in black group) and yolk percentage (from 32.22 % in spotted group to 34.89 % in black group). For albumen weight, albumen percentage, yolk index and yolk colour no significant ($P > 0.05$) differences among eggshell colours were observed.

Key words: Japanese quail; egg; eggshell colour; external quality; internal quality

COMPOSITION OF ALVEOLAR AND CISTERNAL MILK OF TSIGAI AND IMPROVED VALACHIAN BREEDS

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The aim of this study was to investigate the composition of the milk in the cisternal and alveolar compartments of the udder in the most bred breeds in Slovakia [Tsigai (TS), n = 9 and Improved Valachian (IV), n = 9]. Measurements took place at the middle stage of lactation. Cisternal milk was milked out after i.v. administration of atosiban (10 $\mu g.kg^{-1}$ body weight) and alveolar milk after i.v. oxytocin administration (4 IU/animal). Milk composition was analysed for percentage of fat, protein, lactose, solids and solids-not-fat with MilkoScan FT120 (Foss, Hillerød, Denmark). The ratios between cisternal and alveolar milk were 55:45 in TS and 65:35 in IV. No significant differences ($P > 0.05$) in milk composition were found in both milk fractions between breeds in protein content of alveolar milk (5.63 ± 0.57 vs. 5.17 ± 0.37 % in TS and IV, resp.) and milk fat concentration in the cisternal as well as in the alveolar fraction (7.94 ± 1.49 vs. 7.55 ± 0.97 % and 9.38 ± 2.02 vs. 9.27 ± 1.39 %, resp.). In conclusion, in both breeds the large amount of milk is present in the alveolar compartments of the udder, indicating the need of milk ejection reflex occurrence for complete milk removal during milking.

Key words: alveolar milk; cisternal milk; ewes; Tsigai; Improved Valachian

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ASSOCIATION BETWEEN SELECTED TRACE ELEMENTS AND HEPATIC PROFILE IN COMMON CARP (*CYPRINUS CARPIO*)

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Chronic exposure to heavy metals and other trace elements causes several injuries in organism. Biomarkers, such as hepatic enzymes, are good indicators of liver injury. The aim of the present study was to investigate the associations between responses to selected trace elements (aluminium – Al, barium – Ba, lithium – Li, molybdenum – Mo) and hepatic enzymes (aspartate aminotransferase – AST, alanine aminotransferase – ALT, alkaline phosphatase – ALP, bilirubin (Bili) and creatine kinase – CK) in Common Carp blood serum.

Totally, 42 freshwater fishes (*Cyprinus carpio*) were caught by seine net. The blood samples, taken by a cardiac puncture method, were allowed to coagulate and then centrifuged for 20 min at 3000 rpm. Blood serum concentrations of AST, ALT, ALP, Bili and CK were measured using DiaSys (Diagnostic Systems GmbH, Holzheim, Germany) commercial kits and the semi-automated clinical chemistry analyzer Randox RX Monza (Randox Laboratories, Crumlin, UK). The content of selected trace elements (Al, Ba, Li, Mo) in blood serum was determined by inductively-coupled plasma optical emission spectrometry (ICP-OES, Agilent Technologies Australia (M) Pty Ltd.). Statistical analyses were performed using STATGRAPHICS Centurion software (©StatPoint Technologies,Inc., USA). The following scheme of descending concentrations of trace elements in blood serum was used: Al (0.61 $mg.L^{-1}$) > Ba (0.17 $mg.L^{-1}$) > Mo (7.13 $\mu g.L^{-1}$) > Li (5.24 $\mu g.L^{-1}$). Levels of serum markers were comparable with other authors, except higher ALP (6.36 $\mu kat.L^{-1}$). Correlation analysis showed significant positive relationship between ALT and

Mo (r = 0.4032; P < 0.01) and significant negative association between CK and Ba (r = -0.3780; P < 0.05) or Li (r = -0.3925; P < 0.05). Insignificant Pearson correlations were detected between other trace elements and hepatic profile markers. Al and Li were in positive association with AST and Bili (n.s.). Mo and Ba insignificantly positively correlated with ALP and negatively with cholesterol and bilirubin. On the other hand, the analysis showed insignificant positive correlation between Al and cholesterol or bilirubin.
In conclusion, obtained data indicate that trace elements affect hepatic profile markers of Common Carp. However, there were no serious damages observed in the health status except for ALP, which may indicate the bile duct epithelial damage. The correlation analysis confirmed statistically significant interactions.

Key words: trace elements; hepatic profile; Common Carp

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ADDITIVES IN JAPANESE QUAIL NUTRITION

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The aim of this study was to analyse the effect of humic substances (HS) on the quality traits (colour, water-holding capacity and pH), serum minerals (calcium, phosphorus, sodium, potassium and chlorides) and the serum metabolites (glucose, total protein, triglycerides and cholesterols) in breast and thigh meats of Japanese quails. A total of 60 animals (30 males and 30 females) were involved in the experiment. The birds, fed by the standard basal diet, were divided into four experimental groups as follows: probiotic females (PF, n = 10) and males (PM, n = 10) received probiotic preparation at the single dose of 1 g.kg^{-1} of feed mixture, humic acids females (HF, n = 10) and males (HM, n = 10) received humic acids at the single dose 3 g.kg^{-1} of feed mixture. The groups fed basal diet without any additive served as the control groups (CF; n = 10, CM, n = 10). After 210 days the quails were slaughtered and the blood samples and samples of muscles (breast and thigh) were collected. The treatments by probiotic and humic acids caused significant increase in serum calcium levels in the female groups when compared to the male groups. Serum phosphorus was significantly increased in the PF group in comparison to the PM group. Both treatments significantly decreased amount of HDL cholesterol in the female groups in comparison to the female control. 24 hours after slaughter, the meat pH in different muscles showed significant differences. In conclusion, the effect of the treatment with probiotics and humic acids was dose dependent. This suggests that the estimation of an effective dose of additives used in poultry feeding plays an important role.

Key words: Japanese quails; probiotics; humic acids; blood biochemical parameters; meat quality

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CYTOTOXIC EVALUATION OF NONYLPHENOL IN TM3 LEYDIG CELL LINE

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Current widespread research of health effects potentially associated with endocrine disruptors has a high priority nowadays. Alkylphenol ethoxylates, a class of non-ionic surfactants, are degraded into alkylphenol diethoxylates and alkylphenol monoethoxylates. These are subsequently degraded into other sub-products and persist in the environment for a long time. Alkylphenols are common environmental contaminants originating from industrial processes, which are widely used as a component of paints, pesticides and herbicides. The most studied alkylphenols are nonylphenols and octylphenols. They have the ability to mimic effects of reproductive hormones and can interfere with the endocrine system leading to reproductive disorders at different levels of cellular system. A significant body of evidence, based upon laboratory experiments and meta-analysis, indicates that exposure to alkylphenols is associated with male reproductive malfunctions and impairment of spermatogenesis followed by irreversible changes in steroidogenesis. The primary objective of our *in vitro* study is to provide a knowledge about the cytotoxic effect of nonylphenols on TM3 cell line. In our study, the effect of 4-nonylphenol on the Leydig cell functions at lower doses (0.04-5.0 µg.mL^{-1}) was evaluated. Cytotoxicity was assessed by measuring inhibition of metabolic activity (AlamarBlueTM) and loss of membrane integrity (CFDA-AM) in order to identify the mode of toxic action after 24 h of culture. Significant (P < 0.001) increase in metabolic inhibition at 2.5 and 5.0 µg.mL^{-1} was revealed, whereas significant (P < 0.001) loss of membrane integrity was occurred at the highest dose (5.0 µg.mL^{-1}) of 4-nonylphenol. Additional *in vivo* and *in vitro* studies are required to better understand the nature of the effects of alkylphenols and their mechanisms of action in altering male reproductive functions.

Key words: nonylphenol; cytotoxicity; Leydig cells

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VALIDATION OF BOVINE 50K SNP CHIP TRANSFER ABILITY INTO NON-MODEL WILD ANIMALS

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The aim of this study was to apply bovine 50k genotyping array in cross-species genotyping of red deer (*Cervus elaphus*) and to validate its suitability for the studies of genetic diversity in non-model wild animal species. The analysed population of red deer consisted of 27 biological samples of farmed animals, which were male progeny of sires from New Zealand and dams from Hungary and free living trophy animals from legal hunting in Slovakia. All of animals included in this study were genotyped using the Illumina BovineSNP50 BeadChip v2 in a commercial lab. The first scan of obtained genotyping data showed significant decrease in the genotyping quality compared to the species from the *Bovidae* family. However, the observed call rate at the level of 61.66 % was in accordance to previous studies, which clearly confirmed that the cross-species application of commercially developed genotyping array on evolutionary related species can lead to 1.5 % decrease of call rate per each million year divergence between species. The subsequent quality control of genotyping data was performed to retain in database only autosomal SNPs with lower than 10 % of missing genotypes and minor allele frequency greater than 0.01 %. Applied quality control resulted in 53.98 % of SNPs that were successfully genotyped in at least 90 % of animals and up to 1579 markers that could be regarded as polymorphic and informative for the subsequent study of genetic diversity. The analysis of the level of genetic diversity conserved within species as well as potential admixture with non-native deer populations clearly validated that the obtained set of polymorphic markers is very suitable mainly to identify the genetic background of analysed animals. For this purpose we used previously published genomic data for four species from the genus *Cervus*: Axis deer (*A. Axis*), Fallow deer (*D. dama*), Sika deer (*C. nippon*) and Wapiti deer (*C. canadensis*). Our results showed that the allele frequencies varied continuously across the three main regions composed from *Cervus*, *Dama* and *Axis* genera. As expected, due to the phylogeny of analysed species, the Wright's FST index and Nei's genetic distances revealed the closest genetic affinity between the species from *Cervus* genus, whereas the highest genetic distance was found between *Axis* and *Dama* genera. The discriminant analysis of principal components, as well as Bayesian cluster analysis, demonstrated the presence of admixed individuals only between the species Red deer and Wapiti deer, probably as a consequence of the introduction of non-native red deer populations into some parts of Slovakia during the 19th century. Any level of admixture between the Slovak Red deer and Sika was not confirmed. Based on this it can be concluded that alongside with other Central European Red deer populations the Slovak population can provide valuable gene pool. In practical point of view our results can be beneficial not only for the future improvement of deer farming in Slovakia, but also for other local populations, mainly in respect to the conservation of its genetic resources, sustainable management and prevention of genetic diversity loss due to the hybridisation with non-native populations.

Key words: cross-species genotyping; deer; diversity; non-model species

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QUALITY OF CRYOPRESERVED SHEEP SEMEN: COMPARISON OF TWO SPERM CONCENTRATIONS

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Artificial insemination in sheep is a challenge because of particularities of the ovine cervix. Laparoscopic insemination is practised in large sheep-holding countries but not in the countries with small-scaled agriculture because of high costs and time consumption. Sperm concentration of 150 x 10^6 spermatozoa is sufficient for laparoscopic insemination, but too low for pre-cervical insemination. The influence of two different sperm concentrations (300 x 10^6 sperm.ml^{-1} and 600 x 10^6 sperm.ml^{-1}) of cryopreserved semen on the quality parameters was examined. After thawing the acrosomes of the sperm cells were evaluated using fluorescent staining. Density, motility, progressive movement and viability were analysed using CASA. The significance of differences was assessed by using general linear models procedures (IBM Statistics 22). Significant differences in the motility and progressive movement between the two concentrations were recorded. In the vitality, incidence of pathological forms and faulty acrosomes only numerical differences were seen. In all examinations better results were seen in the samples with 300 x 10^6 sperm.ml^{-1}. However, in order to recommend an optimal sperm concentration for production of sperm doses, further examinations of pregnancy rates and fertility parameters under field conditions are required.

Key words: sheep; semen; concentration; cryopreservation; CASA

INFLUENCE OF GRAPEFRUIT SEED-EXTRACT ON THE EXSHEATHMENT RATE OF PARASITIC INFECTIOUS THIRD LARVAE

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Frequent deworming during the last years resulted in higher rates of resistances. Usually, secondary plant ingredients are tested *in vitro* prior to the use in the field. The aim of this trial was to examine the influence of grapefruit seed extract on the exsheathment of parasitic third stage larvae. The third stage larvae were obtained from faeces of naturally infected

goats. The main proportion was represented by *Haemonchus contortus* (66 %) followed by *Trichostrongylus* spp. (28 %). The remainder was composed of *Teladorsagia* spp. (2 %), *Trichochstonrgylus* spp. (3 %) and *Strongyloides* spp. (1 %). The larvae were incubated with different dilutions of grapefruit seed extract (1.6 and 3.2 mg.ml^{-1}), tetramisol hydrochloride [positive control (600 µg.ml^{-1})] and PBS (negative control), respectively. Exsheathment was observed 20, 40 and 60 min after adding the exsheathment fluid. Statistical differences were assessed by using general linear models procedures (IBM Statistics 22).

Grapefruit seed extract showed significant influence on the exsheathment of parasitic third stage larvae *in vitro*. 100 % exsheathment rate was reached in the negative control; positive control showed larval exsheathment under 5 %. Grapefruit seed extract showed low exsheathment rates of 6 % (1.6 mg.ml^{-1}) and 15 % (3.2 mg.ml^{-1}). Statistically significant differences were seen between all groups except the two grapefruit seed groups. Although this trial was conducted with a mixture of parasitic larvae, further examinations should be done with monocultures of parasitic larvae to evaluate the effect in different parasitic species.

Grapefruit seed extract seems to be a candidate tool used for a parasite control. In order to determine an appropriate concentration, the type and duration of the feeding, further studies under field conditions are required.

Key words: grapefruit seed; larvae; *Haemonchus contortus*; goats; parasite control

THE SENSITIVITY OF MOUSE PREIMPLANTATION EMBRYOS TO FIPRONIL MIGHT DEPEND ON MATERNAL BODY CONDITION

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It was previously documented that insecticide fipronil have the potential to affect *in vitro* and *in vivo* embryonic development.

The aim of our study was, therefore, to evaluate whether fipronil, applied during preimplantation period, can influence the developmental capacity and basic qualitative parameters (embryo growth and the incidence of dead cells) of mouse embryos obtained from dams with different fat deposition.

A two-generation dietary model, based on mice overfeeding during intrauterine and early postnatal development, was used to produce two groups of mice: CN – Normal control females with physiological (7 % – 8 %) amounts of body fat and EXF – Fat mice with elevated (> 11 %) amounts of body fat. Under *in vitro* conditions, 2-cell stage embryos were isolated from spontaneously ovulated control and fat mouse dams and cultured in media with or without addition of fipronil at 1 µM concentration until the blastocyst formation. Stereomicroscopic evaluation of *in vitro* produced embryos showed that fipronil at 1 µM concentration decreased developmental capacity of two-cell embryos isolated from normal (CN) as well as from fat (EXF) mice. Fluorescence staining revealed decreased cell numbers in blastocysts derived from the fipronil-treated EXF group. In contrast, quality of blastocysts collected from the fipronil-treated CN group did not show any significance differences in comparison with non-treated CN group.

The obtained preliminary results show the increased sensitivity to insecticide fipronil in preimplantation embryos isolated from obese mice.

Key words: preimplantation embryo; mouse; *in vitro*; fipronil; obesity

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SOMATIC CELL COUNT IN MASTITIS DIAGNOSED EWES

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This study deals with the diagnostics of subclinical mastitis based on the somatic cell count (SCC) in the ewe's milk. The aim was to determine whether high SCC is related to the presence of a pathogen in the milk. The trials were carried out on two farms of Lacaune sheep breeding. Milk sampling on each farm took place at two dates – March and June 2018 during evening milking. A total of 202 milk samples (101 ewes) were taken from udder halves for analysis of SCC and bacteriological examination for the presence of mastitis pathogens. Based on SCC, the ewes were divided into five groups: up to 0.2 x 10^6 ml^{-1}; 0.2-0.4 x 10^6 ml^{-1}; 0.4-0.6 x 10^6 ml^{-1}; 0.6 x 10^6 ml^{-1}; over 0.6 x 10^6 ml^{-1}. In the first group of SCC there were 61.88 % of milk samples, in the second – 5.45 %, third – 3.46 %, fourth – 2.97 % and in the fifth – 26.24 %. The impact of the farm was marked, where in the first farm there were 81 % of samples in the first SCC group; in the last farm – 9 %, while in the second farm the first and the last groups represented 43.14 % of samples. Higher SCC values were found in infected samples (logx 5.78 ± 0.10 ml^{-1}) compared to uninfected (logx 4.86 ± 0.07 ml^{-1}, $P < 0.001$). Two important infectious mastitis pathogens with very low mastitis rates were isolated: *Staphylococcus* (*S*) *aureus* (3.70 %) and *Streptococcus* (*Str.*) *agalactiae* (1.23 %). More frequently were isolated Coagulase negative staphylococci (CoNS), *S. chromogenes* (44.44 %), *S. epidermidis* (39.51 %), *S. xylosus* (22.22 %), followed by *Candida* sp. (2.47 %), *Klebsiella oxytosa* (2.47 %) and *Str. dysagalactiae* (1.23 %). We conclude that the monitoring of the somatic cell counts in milk is a suitable zootechnical tool in an efficient dairy sheep breeding.

Key words: ewes; milk; somatic cells; mastitis pathogens

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EFFECT OF TRACE ELEMENTS DETECTED IN CARP SEMEN ON SPERMATOZOA QUALITY

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Male reproductive system may be generally considered the barometer of environmental contamination. There are numerous studies investigating effects of individual chemical elements at environmentally related concentrations, however available literature lacks the information about effects of substance mixtures. The objective of the present study was to determine the effect of trace elements in seminal plasma on spermatozoa quality, taking in consideration motility parameters, RedOx status and DNA fragmentation. Reproductively mature male carps were subjected to the study. Following the milt collection, the trace elements were determined using inductively-coupled plasma optical emission spectrometry (ICP-OES) and cold-vapor atomic absorption spectroscopy (CV-AAS). Sperm motility traits were assessed using computer assisted semen analyzer (CASA). RedOX status was determined by following markers: reactive oxygen species production (ROS), total antioxidant capacity status (TAC), malondialdehyde production (MDA) and protein carbonyl production (PC). DNA fragmentation was evaluated using the APO-DIRECT™ fluorescent kit. Detected chemical elements were put in ascending order according to their concentrations: Hg < Cd < Cr < Pb < Se < Mn < Ni < Sr < As < Cu < Fe < Zn. Positive significant correlations were found between Mn, Se, Sr and Zn and velocity and distance sperm parameters. Cu and Hg showed negative associations with progressive motility. Hg also affected malondialdehyde production. In conclusion, the present study suggests the use of multi-component mixtures of environmentally related trace element concentrations when examining the potential reproductive risk.

Key words: semen quality; trace elements; oxidative stress; DNA fragmentation; bio-monitoring

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THE EFFECT OF HEAVY METALS ON ICHTHYOLOGIC INDICES OF FRESHWATER FISH

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Heavy metals from human activities are considered to be one of the most dangerous pollutants of water ecosystems. Water pollution affects various developmental processes of fish, what can result in deformations, small body size and reduced viability. Different fish tissues are widely used as indicators for determination of water pollution level. Moreover, higher concentrations of heavy metals in water can lead to serious health risks, due to bioaccumulation of these elements in fish body and transmission into a food chain.
In the present study, samples collected from 42 freshwater fish (*Cyprinus carpio*) were analyzed for the presence of heavy metals and their correlations with ichthyologic parameters (standard length – mm, total length – mm, weight – g). The blood samples were taken by cardiac puncture, centrifuged for 20 min at 3,000 rpm and blood serum was collected and stored at -20 °C until analyses. Concentrations of heavy metals (Pb, Cd, Hg, Cr, As) were measured using inductively-coupled plasma optical emission spectrometry (ICP-OES), a modern technique for routine determination of heavy metal concentrations in different matrices. Statistical analyses were performed using STATGRAPHICS Centurion (©StatPoint Technologies, Inc., USA). Average values for ichthyology parameters were 397.5 mm for standard length, 470.6 mm for total length and 2053.86 g for weight. The concentrations of lead, cadmium, mercury, arsenic and chrome varied in ranges 0.0 – 0.302, 0.0 – 0.017, 0.0 – 0.004, 0.0 – 0.330 and 0.148 – 1.362 mg.L^{-1}, respectively. Obtained results showed weak positive linear relationship between arsenic concentration and total length parameter ($r = 0.334$; $P < 0.05$) and between mercury concentration and body weight parameter ($r = 0.316$; $P < 0.05$). At last, weak positive linear relationship ($r = 0.300$) was calculated for arsenic concentration and standard length, but it was slightly over the limit of significance ($p = 0.054$). Other correlations between heavy metal concentrations and ichthyologic indices were not observed. Further analyses including more fish species and more elements are necessary for better understanding of relations between heavy metal pollution and qualitative and quantitative parameters of fish.

Key words: heavy metals; freshwater fish; ICP-OES; ichthyology

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CONTENT OF SELECTED METALS IN FEED AND SHEEP'S MILK FROM DIFFERRENT PARTS OF SLOVAKIA

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Milk and milk products represent an important source of macro and micronutrients including minerals. However, milk and dairy products can also contain chemical hazards and contaminants, which represent a technological risk factor for dairy products, for the related commercial image and, above all, for the health consumer. The aim of this study was to describe the content of selected essential elements and toxic metals in feed and sheep's milk from areas of Slovakia with different character of environment. In the regions of Slovakia -Novoť (undisturbed environment; North Slovakia) and Klátova Nová Ves (widely disturbed environment; Western Slovakia), 11 metals have been analyzed (essential elements - calcium, zinc, selenium, iron, magnesium, copper; toxic elements – arsenic, mercury, lead, cadmium, nickel). Analyses of samples were performed by certified testing laboratory Eurofins Bel/Novamann (Nové Zámky, Slovak Republic). The results showed significantly higher content of selected essential elements in feed in spring season from the area with widely disturbed environment (Klátova Nová Ves). Significantly higher content of essential elements in milk was recorded on the farm of Novoť (undisturbed environment). Occurrence of toxic metals in feed from the area with widely disturbed environment during the spring season did not affect their content in milk. It can be concluded, that the use of milk of sheep from these areas for direct use or for dairy product processing is appropriate, safe and poses no health risk for the consumers.

Key words: sheep's milk; toxic metal; essential elements; feed; environment

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PHYSIOLOGICAL LEVELS OF SOMATIC CELL COUNT IN RAW EWE MILK

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Somatic cell count (SCC) in milk is used as a gold indicator of udder health and could be used for detection of subclinical mastitis in herds. Despite negative impact of high SCC on milk yield and milk components, the physiological values of SCC in raw ewe's milk are still under discussion. The aim of this study was to describe the distribution of ewes of two breeds (Slovak dairy ewe – SD and Lacaune – LC) into SCC groups based on their individual SCC. The ewes were divided into five SCC groups (G1= SCC < 200 × 10^3 cells.ml^{-1}, G2 = SCC between 200-400 × 10^3 cells.ml^{-1}, G3 = SCC between 400-600 × 10^3 cells.ml^{-1}, G4 = SCC between 600-1000 × 10^3 cells.ml^{-1} and G5 = SCC > 1000 × 10^3 cells.ml^{-1}). In total, 771 samples were collected from 90 ewes throughout both milking periods from one experimental herd belonging to NPPC – Research Institute for Animal Production Nitra, Slovakia with minimum four milking records per year in 2016 and 2017. Throughout lactation the most ewes were found out in the first two SCC groups (below 400 × 10^3 cells.ml^{-1}) in 2016 and 2017 (78.89 % and 83.33 %, respectively). Thirteen animals (8SD, 5LC) were in SCC groups over 600 × 10^3 cells.ml^{-1} in 2016, however in next lactation only 6 animals of them did not improve SCC during dry period in following lactation in 2017 (5 of them were LC). Twelve animals (4SD, 8LC) were in SCC groups over 600 × 10^3 cells.ml^{-1} in 2017. Our results indicate that the possible physiological level for raw sheep milk might be considered as the value less than 400 × 10^3 cells.ml^{-1} because of the highest percentage of ewes recorded.

Key words: ewe; milk; somatic cell counts

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DIETARY SUPPLEMENTATION OF VERBASCOSIDE: IMPLICATION ON REPRODUCTIVE ASPECTS

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There is an internationally growing interest concerning the use of natural extract sources in animal production area in order to improve the farming welfare and the animal performance. Nowadays, the application of rabbit as an animal model is widely accepted. In addition, the largely expanding rabbit production is mainly attributable to rabbit's high rate of reproduction, high potential of genetic selection, rapid growth rate, early maturation, efficient feed utilization and high quality of meat. The reproductive performance of male livestock is of economic importance, and improving semen quantity and quality, especially for artificial insemination, additionally helps to avoid the loss of valuable genotypes. The spermatozoa of vertebrates, including rabbits, display high rates of metabolic activity and are also rich in polyunsaturated fatty acids, making them particularly susceptible to oxidation by reactive oxygen species (ROS), especially under stress conditions. Within the endogenous antioxidant system of spermatozoa, many substances extracted from plants are able to improve plasma stability of lipid profile and to monitor ROS production. Natural antioxidants have been widely reported to have potent antioxidant, anti-inflammatory and antimicrobic activities related especially to their phenolic content. *Lippia citriodora*, a plant species in the *Verbenaceae* family, is characterised by the presence of several phenolic compounds, including avonoids, phenolic acids, luteolin derivatives and phenylpropanoids. Phenylpropanoid, particularly verbascoside [2-(3',4'-dihydroxyphenyl)ethyl-O-α-L-rhamnopyranosyl-(l-3)-β-D-(4-O-caffeoyl)-glucopyranoside], is the most abundant compound in *Lippia* extract. Verbascoside contains a rhamnose unit bound to glucose, which acts as a bridge, and exhibits a number of biological activities including anti-inflammatory and antioxidant. The protective

activity may be attributed either to the caffeoyl residue in the molecule acting by direct scavenging of reactive oxygen and nitrogen species, or as chain-breaking peroxyl radical scavengers. Based on the information present in bibliography, our research group tested this natural compound on several animal species, through the inclusion in animal feed. In addition, we evaluated verbascoside with the application on *in vitro* spermatozoa cell model, since to date only few findings are known. Based on our research experiences it can be stated that possible negative effect of verbascoside supplementation into feed mixture on semen quality parameters in rabbit bucks and adult brown hares as well as *in vitro*, obviously considering that target organs of antioxidant activities of phenylpropanoid glycosides are various. In addition, it has to be emphasized that the extract showed a reversible action, since the semen traits of treated animals returned to the normality after the dietary administration period. Due to growing interest in dietary application of natural extract, further research is needed to assess the effect of different doses.

Key words: feed additives; phenylpropanoid glycoside; livestock farming

INTERACTIONS BETWEEN THE CONTENT OF SELECTED TRACE ELEMENTS AND SPERM QUALITY IN COMMON CARP

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The reproductive performance of common carp can be influenced by several factors, such as mineral imbalance and aquatic contamination. Molybdenum (Mo) and other essential elements are correlated with semen quality parameters including sperm density and motility. Less attention is given to minor, but potentially dangerous elements, such as lithium (Li) and barium (Ba). The aim of the present study was to investigate the association between the content of selected trace elements and sperm quality in common carp.
Reproductively mature common carps (n = 16) (*Cyprinus carpio*) were used in our study. The milt was collected from the sperm duct post-mortem. Motility parameters (MOT, PRO, DAP, VAP, STR, BCF, concentration) of the semen were determined by the CASA assay. The samples of biological material were stored in a freezer at -20 °C until the processing. Quantification of the elements (Ba, Li, Mo) present in the milt was done using inductively coupled plasma–optical emission spectrophotometer (ICP OES 720, Agilent Technologies Australia (M) Pty Ltd.). Statistical analyses as well as Pearson's correlations were performed using STATGRAPHICS Centurion. Positive correlations were found between spermatozoa concentration and motility ($r = 0.577$; $P < 0.05$), as well as progressive motility ($r = 0.637$; $P < 0.01$). The analysis revealed significant associations between Ba and sperm quality parameters (DAP, $r = 0.530$; $P < 0.05$; VAP, $r = 0.544$; $P < 0.05$; BCF, $r = 0.590$; $P < 0.05$). Li content was correlated with progressive motility ($r = 0.557$; $P < 0.05$). In conclusion, our findings suggest that evaluated elements affected sperm quality in common carp. In further studies is necessary to test broader spectrum of elements, which may influence motility parameters of common carp semen.

Key words: trace elements; carp semen; CASA; ICP-OES; motility

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TESTING OF NUTRIENT REQUIREMENTS OF RED DEER

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Slovakia has experienced a rapid increase in the number of red deer farms in the recent years and according to the amendment to the Act no. 184/2018 Coll. on veterinary care, red deer is treated as a farm animal. In such a situation, precise feeding of red deer is required, as in the case with other farm animals. We carried out feeding and balance experiments with Slovak origin red deer over several years in the laboratory of ruminant physiology of nutrition at the Department of nutrition. The results of these experiments have been used to prepare tables of nutrient requirements for fawns, females and males evaluated for particular seasons of the year. Daily intake of milk replacer for fawns was markedly increased in the first days of life. At 21 days of age, it was 3.750 ml per animal with body weight (BW) 16.9 kg. In the categories of older animals, without milk replacer, at 95 days of age (BW 42.5 kg) / 195 days (BW 90 kg), the daily requirement of dry matter is (DM) 1550 / 2350 g, crude protein (CP) 301 / 400 g, Ca 15 / 24 g, P 10/15 g, metabolizable energy (ME) 16 / 26 MJ. Nutrient requirements were calculated for females weighing 150 kg. From the point of view of physiological burden on females, we can distinguish two periods during the year. In January – March the nutrient requirements are decreasing and in the period of summer during lactation and autumn the nutrient requirements are increasing: DM 2550 / 3450 g, CP 360 / 560 g, Ca 20 / 29 g, P 12 / 18 g, ME 25 / 37 MJ. Males have the greatest body weight before the rut in summer (275 kg) and during the rut in September – October. With significantly reduced food intake and increased activity the weight can fall to 30 %. Daily nutrient intake for males in summer and during rut is following: DM 5010 / 1490 g, CP 830 / 202 g, Ca 54 / 16 g, P 31 / 9 g, ME 56 / 14 MJ.

In this paper we present the first table values of the nutrient requirements tested on Slovak origin red deer.

Key words: red deer; nutrient; requirements

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BROWN HARE (*LEPUS EUROPAEUS*) AS A BIOINDICATOR IN AGRICULTURAL LANDSCAPE

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Brown hare is a bioindicator game animal, whose health status is endangered by anthropic factors. It lives in agricultural landscape affected by foreign substances introduced by industrial fertilisers, pesticides and industrial emissions. Today, it is essentially impossible to find a hare without pathological changes.

The aim of the study was the research of a xenobiotic presence in the agrarian landscape. The samples (parenchymatic organs, fat tissue) were collected by an autopsies of hares harvested during the hunting season (November – December). Organic pollutants were determined by gas chromatography, heavy metals – by an atom spectrophotometry and aflatoxins by a RIA method. Statistical analysis was performed using the Excel software. DDT content in old hares was significantly higher than in the young hare category (three-times; P = 0.000+++). HCB content in old hares was also significantly higher than in the young hare category (25 times; p = 0.0017++). For α+β-HCH no difference was determined between the young and old hares (P = 0.191) or between the sexes (p = 0.1767). Content of γ-HCH in the fat of old hares was significantly higher than in that of young ones (three-times; P = 0.0123+). The effect of sex on the organic pollutant content was essentially non-significant. PCB content was approximately 2.4 times higher in old hares compared to young and the difference was highly significant (P = 0.000+++). The contents of heavy metals (Pb, Hg, Cd) were also determined. Seasonal differences in the content of Pb and Hg in hares were significant; therefore, a season in which the experimental samples were collected was taken into account. No significant seasonal differences were determined for the Cd content, but significant differences were confirmed based on the age of the hares, which is necessary to consider at the result interpretation. Pb and Hg content in the organs of our hares was relatively low. The examined hares probably consumed a feed contaminated by various concentrations of fungal metabolites-mycotoxins. Feeds displayed no noticeable organoleptic differences. Feeding an infected feeds can lead to hidden uncontrolled intake of mycotoxins and even to subclinical and clinical symptoms of aflatoxicosis. In hares, aflatoxicosis can be the cause of numerous undetermined steatoses of the liver in winter or spring season.

Toxicological examinations to determine residues of the selected organic pollutants point towards the fact that not all locations of the monitored territory are affected at the same level, but the levels of organic pollutant, we determined in the hares, did not exceed the limits set by hygienic norms.

Key words: brown hare; bioindicator; organic pollutants; heavy metals; mycotoxins

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POTENTIAL EFFECTS OF GRAPE PHYTOCHEMICALS ON OVARIAN CELLS *IN VITRO*

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Phytochemicals may play an important role in decreasing the risk of chronic disease including certain types of cancer disease. Grapes, one of the most popular and widely cultivated and consumed fruits in the world, are rich in phytochemicals. Human ovarian granulosa cell line (HGL5) and human ovarian carcinoma cell line (OVCAR-3) represent model systems for understanding the molecular mechanisms of phytocompound action in healthy and pathological cells. The aim of our study was to evaluate the effect of grape pomace extract *(Vitis vinifera* L., cultivar *Pinot gris*) at the doses of 0; 6.25; 12,5; 25; 50 and 100 $\mu g.ml^{-1}$ on the viability of HGL-5 cells, as well as on the secretion of steroid hormones and the viability of human ovarian carcinoma cell line (OVCAR-3). The metabolic activity was evaluated by alamarBlueTM cell viability assay and the release of hormones was assayed by ELISA methods. The viability of HGL5 cells was not significantly influenced ($P \geq 0.05$) by grape pomace extract at all concentrations. The secretion of 17ß-estradiol and progesterone was significantly ($P \leq 0.05$) decreased at the highest concentration – 100 $\mu g.ml^{-1}$. On the other hand, the viability of OVCAR-3 cells was significantly ($P \leq 0.05$) decreased after addition of extract at the concentrations of 12.5; 25; 50 and 100 $\mu g.ml^{-1}$ compared to the control.

Our results suggest that phytochemicals of grape pomace extract could be potential regulators of steroidogenenis and viability of human ovarian cells *in vitro*.

Key words: ovarian granulosa cells; grape; viability; steroid hormones

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ALTERATION OF SOME HAEMATOLOGICAL PARAMETERS OF RABIT BLOOD AFTER MYCOTOXIN EXPOSURE

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The goal of the present study was to analyze the effect of a single dose of mycotoxins (deoxynivalenol DON and T-2 toxin) on selected hematological parameters of rabbit's blood. Experimental group of rabbits received mycotoxins intramuscularly (Romer Labs Division Holding GmbH, Tulln, Austria) at the dose of 0.08 mg per kg of body weight 72 hours before slaughter. Whole experiment lasted 90 days. Selected haematological parameters (WBC – total white blood cell count, MID – medium size cell count, GRA – granulocyte count, RBC – red blood cell count, HGB – haemoglobin, HCT – haematocrit, PLT – platelet count, MPV – mean platelet volume, and PDWc – platelet distribution width) were measured using haematology analyser Abacus junior VET (Diatron®, Austria). Neither DON nor T-2 toxin given at a single dose of 0.08 mg had significant effect on rabbit blood. However, our previous studies revealed that various secondary metabolites exhibit a wide range of immunomodulating activity. High doses of deoxynivalenol influenced the lymphocyte count in porcine blood *in vitro*. Results of this study provide the basis for further research of a mycotoxin impact on blood cells.

Key words: mycotoxins; haematological parameters; immunomodulating activity

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UTILIZATION OF BEE BREAD IN THE MODULATION OF AN INTERNAL MILIEU OF ZDF RATS

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Male ZDF rats (a fatty fa/fa mutation; n = 40) and their lean non-diabetic counterparts (controls; +/+ or +/-, with no expression of the fa phenotype; n = 10) at the age of 3 months were involved in the experiment. The animals were divided into 5 groups as follows: the control group (K), the group of obese rats fed with a high-energy diet (P1), the group of obese rats fed a high energy diet and a daily dose of bee bread (P2), the group of obese rats fed with a normal diet and a daily dose of bee bread (P3), and the group of obese rats fed with normal diet (P4). For experimental groups P2 and P3, bee bread was administered daily orally via a probe at the amount of 500 mg per kilogram of live weight. All rats had an access to water and food *ad libitum*. After 3 months of experiment the blood was taken and selected haematological and lipid parameters were determined. The mean values of total cholesterol (TC), triacylglycerides (TAG), HDL and LDL cholesterol were measured using Biolis 24i Premium Biochemical Analyzer (Tokyo Boeki Medi Sys Inc., Japan). We found significant change ($P < 0.05$) in the total number of erythrocytes (RBC). These changes have been attributed to developing diabetes. We observed a significant increase ($P < 0.001$) of all monitored parameters in the all experimental groups when compared to the control group. The contents of TC, TAG, HDL and LDL cholesterol were without significant changes ($P > 0.05$).
The hypolipidemic effect was not statistically significant in the groups with orally administered bee bread. Further experiments with various doses are needed.

Key words: bee bread; high-energy diet; *diabetes mellitus* type 2; Zucker diabetic fatty (ZDF) rats; haematological parameters; lipid profile

Acknowledgements: This study was supported from the APVV grant no. APVV-15-0229.

GLUCOCORTICOID RECEPTOR SPLICE VARIANTS IN MOUSE PREIMPLANTATION EMBRYOS

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We examined whether glucocorticoids (endogenously produced during stress reactions or therapeutically administered) can directly affect preimplantation embryo. Because of alternative processing of glucocorticoid receptor (GR) five trancripts exist in humans, but only three of them (GRα, GRβ, GRγ) were found in mouse adult tissues. Using RT-PCR we detected GRα, GRβ, GRγ splice variants in mouse oocytes and preimplantation embryos in our study. To ascertain whether truncated mouse glucocorticoid receptor, which is orthologous to the human GR-P splice variant (i.e., "mouse GR-P"), is expressed in preimplantation embryos, we designed specific primers using information on the structure of human "canonical" (GR alpha) and GR-P transcripts. Forward primer was located in the "canonical" GR sequence. Two GR-P reverse primers ("RP1" and "RP2") were located in the GR-P-specific sequence. The RP1 reverse primer was used for analysis of GR-P expression in mouse oocytes and embryos and RP2 reverse primer was used to obtain the mRNA sequence of the mouse GR-P ranging beyond the first in-frame STOP codon. We showed the presence of GR-P splice variant in mouse brain tissue, as well as in the blastocyst for the first time. Our results indicated that the first part of mouse GR-P transcript

sequence is located in the end of exon 6 and in exon 7 and the next part is located in the beginning of original intron 7. Our comparison of human GR-P transcript with the mouse orthologue, identified in our study, revealed significant differences within the coding region of transcripts suggesting possible interspecies differences in GR-P functioning.

Key words: preimplantation embryos; glucocorticoid receptor; splice variants

Acknowledgements: This work was supported by the Slovak Academy of Sciences from the project VEGA 2/0039/15.

BREEDING OF MINIPIGS AS LABORATORY ANIMALS

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Minipigs are one of the most important laboratory animals because of their similarity to human. They are small, thus, much easier for handling. Also, requirements of food, space and pharmacologic products are significantly reduced. There are at least 45 breeds of minipigs available worldwide (Smith & Swindle, 2006). While conventional farm pig breeds are extensively used in pork industry, smaller pigs, named minipigs or miniature pigs, are produced by cross-breeding of various purebred or wild species for special needs (Vodicka *et al.*, 2005). The aim of our study was to describe growth intensity and reproduction parameters of minipigs bred in the Institute of Animal Science Prague. The herd was established in 2009. The minipigs of Minnesota type were the basis for a new herd. The stabilization of a phenotype, especially in body weight, was realised during last years. Only natural breeding is used in minipigs. Piglets are weaned at two months of age. The special feed mixture is used for minipigs to fed them.

The data from 89 litters were collected in years 2009-2017. The number of piglets born, born alive and weaned was monitored and percentage of piglet losses was calculated. The growth parameters were measured in 100 piglets-birth weight, weight at weaning and every month until one year of age. The average birth weight of piglets was 352 ± 85 g, weight at weaning – 3520 ± 743 g and live weight at 12 months of age was 49.4 ± 6.3 kg. Reproduction parameters of monitored litters were as follows: number of total born piglets – 8.6 ± 2.9, number of live born piglets – 7.1 ± 2.3, number of weaned piglets – 6.7 ± 2.4 and calculated piglet losses were 6.1 %.

Farm breeding of minipigs is not common in the Czech Republic; breeding as pet animal prevails. There are no available data for comparison. In comparison with original Minnesota minipigs, more piglets born/litter and lower birth weight were monitored in our study. Minnesota breed reaches at average 6 piglets per litter, birth weight of piglets is 590 g and body weight of adult minipig is 55-70 kg (McAnulty *et al.*, 2005).

Key words: minipig; reproduction; growth

GENETIC MARKERS AS A TOOL FOR TRACEABILITY OF ORAVKA PRODUCTS

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The aim of present study was to assess the level of genetic diversity within three autochthonous chicken breeds, as well as to identify the genetic relationships between them based on microsatellite data. In this study, overall 78 animals belonging to the yellow-brownish Oravka (Ob), the white Oravka (Ow) and the Sumavanka (S) breeds were included. The genomic DNA was extracted from feather samples by using commercial kit according to Wizard® Genomic DNA Purification Kit Protocol (Promega Corporation). A panel of eight microsatellite markers (Lei166, Lei192, Lei228, Lei229, Lei234, Lei254, MCW34, and MCW69) were used for animals' genotyping. The level of genetic diversity within each breed was determined based on the calculation of mean number of alleles (MNA), observed heterozygosity (H_o), gene diversity expressed as expected heterozygosity (H_e), effective allele number (N_a) and Shannon's information index (I) using Genalex v6.1. The amount of inbreeding-like effect within breeds, expressed by the Wright's F_{IS} index, was calculated using FSTAT v2.9.3.2. The genetic relationships among breeds were estimated based on the Nei's genetic distance and Wright's F_{ST} index. In addition, the discriminant analysis of principal components (DAPC) was used to examine the genetic structure of breeds under consideration. The mean number of alleles across breeds ranged from 2.50 ± 0.33 (Ow) to 8.00 ± 1.43 (Ob), which signalized a certain decrease of genetic variability mainly in case of the White Oravka and the Sumavanka breeds. This decrease also confirmed the obtained values of effective number of alleles (2.13 to 3.46) and Shannon's information index (0.75 to 1.36). Similarly, the gene diversity indicated higher proportion of heterozygosity within the yellow-brownish Oravka (0.63 ± 0.08) compared to the white Oravka (0.46 ± 0.08) and Sumavanka (0.44 ± 0.10) breeds. However, despite the higher proportion of heterozygotes within the yellow-brownish Oravka, the Wright's F_{IS} index showed that this population can be affected by a relatively strong impact of inbreeding (F_{IS} = 0.17). The lowest value of F_{IS} index (0.03) pointing to low effect of relatives mating was found in the Sumavanka breed (F_{IS} = 0.03). The average values of Nei's distance (0.93 ± 0.23) and F_{ST} index (0.33 ± 0.08) showed that the populations were genetically differentiated. As expected, due to the genetic background of analysed breeds, the highest genetic similarity was found between the white and yellow-brownish Oravka, while the highest genetic distance showed the white Oravka and the Sumavanka breeds. Moreover, the DAPC analysis indicated that between the white and yellow-brownish Oravka some level of admixture can be found. The obtained three discriminant functions corresponded to 67.4 % of total genetic variance conserved in the analysed dataset. Based on the study

results it can be concluded that the state of genetic diversity within analysed autochthonous chicken breeds should be monitored constantly. Moreover, the increase in a sample size could be beneficial to describe their gene pool in a more detailed level, as well as to identify genetically most important individuals for the sustainable management of animal genetic resources in future.

Key words: diversity; chicken; local breeds; microsatellites; traceability

BIOTECHNOLOGY APPLICATION – ARTIFICIAL INSEMINATION AND EMBRYO TRANSFER IN CONTROLLED BREEDING OF WILD ANIMALS

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Mouflon (*Ovis aries musimon*) is the only wild form of sheep living in Europe. Most of the introduced population into continental Europe come from the islands of Sardinia and Corsica. The aim of the work was to create a gene pool of the highest quality donors from the point of view of genetic variability, the quality of the horns and to create a gene pool of frozen embryos of mouflon. Hormonal synchronization was used to induce a superovulation reaction by combining an intravaginal CIDR G device in combination with the hormonal preparation, Pluset® (FSH / LH), which was administered on day 11 to 14 of progesterone treatment at 12 hour intervals. Artificial insemination was performed by 48 hours after removal of the intravaginal tampon. Embryo gain was performed on the 6th day of ovulation at the compact morula stage, eventually early blastula. The embryos obtained were then frozen by cryopreservation techniques and archived in a liquid nitrogen. Experimental embryo transfer of frozen mouflon embryos was performed on the domestic sheep recipient (cross valaška and lacaune). On the 157th day of pregnancy a mouflon male was born. Application of the MOET (Multiple Ovulation and Embryo Transfer) can help to vital rescue programs for endangered animal species.

Key words: mouflon; embryo; embryo transfer; cryopreservation

MOUSE EMBRYOS FROM OBESE DAMS SHOW HIGHER SENSITIVITY TO OXIDATIVE STRESS *IN VITRO*

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Aim of study was to test hypothesis that maternal obesity can affect sensitivity of preimplantation embryos to oxidative stress.

To produce mice with obesity-like phenotype, a trans-generation model based on the over-nutrition of experimental animals during intrauterine and early postnatal development was used. To assess the sensitivity of preimplantation embryos to oxidative stress, an *in vitro* experiment was performed: the 2-cell stage embryos isolated from control and obese females were cultured in the presence of three different chemicals till blastocyst formation. Following oxidative stress inductors were used: AAPH [2,2'-Azobis (2-methylpropionamidine) dihydrochloride] at 0.01 mM, SNP (sodium nitroprusside) at 0.01 mM and BSO (buthionine-sulfoximine) at 5 mM.

Stereomicroscopic evaluation showed that the presence of SNP and BSO negatively affected *in vitro* development of embryos isolated from obese mothers. In control embryos, similar tendency was recorded only after exposition to BSO. In blastocysts originating from obese dams, decreased cell numbers after treatment by AAPH and BSO, and increased incidence of cell death after treatment by SNP and BSO were revealed. Assessment was performed by means of fluorescence imaging. In control group, negative effect on blastocyst quality was observed only after BSO treatment. In conclusion, the results show that preimplantation embryos isolated from obese mice display higher sensitivity to oxidative stress *in vitro* than embryos isolated from control females.

Key words: preimplantation embryo, mouse, *in vitro*, obesity, oxidative stress

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Contents

BULLA, J. – KUBOVIČOVÁ, E. – MAKAREVICH, A. V. – CHRENEK, P.: 147
Biotechnology in the context of the 100-year history of Czechoslovakia: A review

CURLEJ, J. – TOMKOVA, M. – VASICEK, J. – CHRENEK, P.: 150
Cytogenetic studies of mesenchymal stem cells in rabbit: A mini-review

MAKAREVICH, A. V. – OLEXIKOVA, L. – FÖLDEŠIOVÁ, M. – PIVKO, J. – KUBOVIČOVÁ, E.: 156
Ooplasm cryopreservation: ovarian fragments versus oocytes alone

NEVORAL, J. – RÚČKA, V. – KLEIN, P. – ŽALMANOVÁ, T. – HOŠKOVÁ, K. – PROKEŠOVÁ, Š. – ŠTIAVNICKÁ, M. – PETR, J. – KRALICKOVA, M.: 161
The interplay of mouse and porcine biomodels for endocrine disruptor reproductive impairment

Abstracts 171

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