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ANIMAL BIOTECHNOLOGY 2015

Institute of Genetics and Reproduction of Farm Animals
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Dear Participants and Colleagues,

We are very pleased to welcome you on the 3rd International Scientific Conference **„Animal Biotechnology 2015“**. 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 actual research from the field of animal biotechnology, with a special focus on embryo manipulations. The topic areas of the conference will involve transgenesis, cloning, *in vitro* fertilization, cryopreservation as well as genetic and epigenetic effects on gametes and embryos.

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 10th, 2015

Peter Chrenek

NATIONAL AGRICULTURAL AND FOOD CENTRE
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SIRT1 AS A KEY FACTOR FOR HISTONE CODE ESTABLISHMENT IN EARLY EMBRYO, FROM A PERSPECTIVE OF ASSISTED REPRODUCTION

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ABSTRACT

Both the paternal and the maternal pronuclear chromatin undergo the erasure and re-establishment of epigenetic marks during mammalian zygotic development. These epigenetic changes regulate the totipotency, self-renewal and eventually cell differentiation within the preimplantation embryo. The demethylation of DNA and establishment of adequate post-translational histone modifications, called histone code within the zygote, are required for successful development and reflects the male or female origin of chromatin.
Further epigenetic changes are necessary for developmentally regulated transcription and determination of embryonic cell lineage as the embryo blastomeres become transcriptionally active during major zygotic genome activation (MZGA). In addition to DNA methylation, histone code modifications and their regulation are intensively studied. Sirtuin SIRT1, a member of the $NADP^+$-dependent histone deacetylase family, modifies histones via direct deacetylation as well as indirectly through non-histone substrate regulation. Positive effects of SIRT1 activation on cell viability and embryonic development have been described, and correct histone code modulation is the proposed mode of SIRT1 action. Understanding SIRT1-dependent signalling will provide new tools for assisted reproductive technology in animals and therapy in humans, wherein the inadequate epigenetic modification is a possible explanation for the failure of embryo development *in vitro*.

Key words: zygote; embryonic development; DNA methylation; histone code; deacetylase; sirtuin; SIRT1

INTRODUCTION

The oocyte, a terminally differentiated haploid female germ cell, becomes a totipotent zygote after fusion with a spermatozoon during the precisely orchestrated process of fertilisation. Thereafter, second oocyte meiosis is complete, second polar body is extruded, and the paternal (male) and maternal (female) pronucleus formation takes place. At the onset of pronuclear development, male chromatin tightly packed within the sperm head undergoes rapid decondensation, protamine-histone exchange and male pronucleus formation. The zygote containing female and male pronuclei enters first mitosis, termed embryo cleavage, and produces two nearly identical diploid blastomeres. Subsequent cell cycles follow and further milestones of pre-embryo

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development are reached, such as the major zygotic genome activation (MZGA), formation of morula and blastocyst differentiation and hatching. Chromatin consisting of DNA and histones is dynamically regulated during this period (Yanagimachi, 1988).

Nucleosome, the functional unit of eukaryotic chromatin, consists of ~ 147 base pairs (bp) of DNA wrapped around a histone core formed by an octamer of four different core histone variants (H2A, H2B, H3 and H4), and strung together by linker histone H1. The DNA within this complex is modified by the ligation of methyl groups onto developmentally pre-programmed CpG sites, termed DNA methylation. Together with post-translational modifications of core histones, such epigenetic modifications play a key role in both gametogenesis and early embryonic development (reviewed by Shi and Wu, 2009).

A number of core histone splicing variants are known in somatic cells as well as in gametes, zygotes and embryos. In addition to alternative splicing, histones' post-translational modifications, e.g. methylation and acetylation, affect the structure and function of chromatin (reviewed by Yuan and Zhu, 2013). Adequate epigenetic changes determine the transcriptional activity/chromatin status of a zygote; they are essential for gene imprinting and transition of the totipotent zygote to the differentiated embryo expressing its own genome during MZGA (Patrat *et al.*, 2009; Dahl *et al.*, 2010; Latham and Schultz, 2001). Various upstream factors regulate epigenetic changes, resulting in embryonic chromatin remodelling observed during development. Correct epigenetic changes affecting zygotic pronuclei determine both the zygote quality and the subsequent embryo development. In their sum, these epigenetic changes endow the nearly transcriptionally silent embryonic genome with only minor gene expression activity. As such, maternal storage and inheritance of mRNAs and proteins plays a key role in the regulation of early epigenetic changes that essentially rely on the existing, oocyte-stored pool of RNAs and proteins. Epigenetic changes are subsequently required for modulation of transcriptional activity through genome reprogramming, setting the stage for ensuing cellular differentiation (Latham *et al*., 1991; Latham and Sapienza, 1998; Segev *et al.*, 2001; Yan, 2014; Uysal *et al*., 2015).

In vitro fertilisation (IVF) or intracytoplasmic sperm injection (ICSI), common methods utilised in assisted reproductive therapy (ART), allow for the continuous observation of early embryonic development including pronuclear biogenesis and subsequent embryo cleavage all the way up to, and including blastocyst formation. On the other hand, IVF- and ICSI-derived embryos show lower efficiency in development success, lowering the success rate of human ART *in vitro* embryo production, as well as in livestock and rodents. Differences in epigenetic modifications are likely contributors to such developmental failures (Peat and Reik, 2012; Farifteh *et al.*, 2014; Matoba *et al.*, 2014; Mao *et al.*, 2015). Therefore, the study of the epigenetic mechanism offers possibilities to improve ART and *in vitro* embryo production.

Epigenetic regulation through DNA methylation

Epigenetic changes of the embryo start immediately after fertilisation when the pronuclear development takes place. These changes include DNA methylation based on 5'-methylcytosine (5mC) appearance, associated with gene imprinting and DNA stabilisation (Wigler, 1981; Stein *et al*., 1982). In addition to DNA methylation, post-translational modifications of histones, generally called histone code, occur and predetermine transcriptional activity and chromatin stability (Dimitrov *et al*., 1993; Aoki *et al*., 1997).

The DNA methyl transferases (DNMTs) are responsible for 5'-methylcytosine formation, thus determining gene expression, gene imprinting and predisposition to DNA strand breakage. The DNMT1 protein binds to a hemi-methylated double-stranded DNA during replication (Bestor, 2000; Giraldo *et al*., 2013) and is responsible for the maintenance of methylation patterns (Hirasawa *et al*., 2008). Enzyme DNMT3 is able to *de novo* methylate existing double-stranded DNA (Okano *et al.*, 1999). Both DNMT1 and DNMT3 are involved in gene imprinting during gametogenesis and embryonic cell differentiation, as well as in the maintenance of specific methylation patterns during preimplantation development (Kato *et al*., 2007; Hirasawa *et al*., 2008; Smallwood *et al.*, 2011).

Before MZGA, the ooplasm-stored proteins and proteins translated from maternally inherited mRNAs after fertilization control epigenetic modifications, assuring that the embryonic DNA undergoes demethylation for the maintenance of totipotency. Such pre-MZGA modifications prepare the embryo for *de novo* DNA methylation and cell differentiation via heterochromatin formation, gene silencing and X-chromosome inactivation (Mayer *et al*., 2000; Dahl *et al*., 2011). Therefore, DNA demethylation of a highly methylated zygotic pronucleus is a key event immediately after fertilisation (Mayer *et al*., 2000; Dean and Ferguson-Smith, 2001; Reik *et al*., 2001). Asymmetric parent-of-origin dynamics of chromatin and DNA demethylation patterning of maternal and paternal pronuclei have previously been described (Guo *et al*., 2014). Demethylation of DNA in the paternal pronucleus occurs earlier than in the maternal pronucleus. Whereas the paternal pronucleus is demethylated within four hours after fertilisation, the maternal DNA

methylation persists until blastocyst stage (Mayer *et al*., 2000; Dean and Ferguson-Smith, 2001; Reik and Walter, 2001; Guo *et al.*, 2014). The major wave of genome-wide demethylation occurs at the 2-cell stage of the human embryo development (Guo *et al*., 2014). Rapid paternal DNA demethylation appears to be an active TET3 dioxygenase-dependent process, resulting in the creation of oxidised 5mC forms, already detectable prior to first round of zygotic DNA replication (Mayer *et al*., 2000; Dean and Ferguson-Smith, 2001; Gu *et al*., 2011; Wossidlo *et al*., 2011). Contrary to the general assumption of passive maternal DNA demethylation over consecutive embryo cleavages until late morula stage, recent studies have identified a basal level of active demethylation process in the maternal DNA through detection of oxidised 5mC forms in both parental pronuclei (Guo *et al*., 2014; Shen *et al*., 2014).

Altogether, correct zygotic DNA demethylation is essential for embryonic cell totipotency and re-methylation of DNA during subsequent embryonic cell differentiation. Besides DNA methylation, adequate post-translational modifications of histones determine zygotic genome stability, inheritance/maintenance of parent-specific gene expression and proper formation of the zygotic pronuclei and blastomere nuclei.

Epigenetic regulation by histone code

Histone variants (H1, H2A, H2B, H3, H4), their splicing forms (*e.g*. H2A.Z, MacroH2A, H2A-Bbd and H2A.X for H2A), and post-translational modifications, such as acetylation, methylation, phosphorylation, ubiquitination and sumoylation together termed histone code, are responsible for structural and functional modifications of the nucleosome (Kamakaka and Biggins, 2005). Zygote formation represents a dynamic phase of early development encompassing rapid protamine-histone exchange and immediate pronucleus biogenesis including histone code modification. Histone modifications in the zygote are associated with specific nucleosomal features. Whereas histone acetylation and methylation on lysine residues are markers of transcriptional activity, phosphorylation (*e.g*. that of H2A.X, abbreviated as γH2A.X) or ubiquitination (*e.g*. that of H2A.Z) determine histone recycling and DNA breaks (Chen *et al*., 1998; Kuo and Yang, 2008).

Among aforementioned histones, splicing variants and post-translational modifications histone H3 are well known. Histone H3 variants in differentiated somatic cells and embryonic stem cells comprise H3.1, H3.2 and H3.3 (Yuan and Zhu, 2013; Zhou and Dean, 2015). Pronuclear asymmetry is manifested at the onset of development wherein H3.1 and H3.2 variants are absent from the paternal pronucleus of early mouse zygotes, and H3.3 is the predominant H3 variant within paternal chromatin (van der Heijden *et al*., 2005; Torres – Padilla *et al*., 2006).

Histone H3 acetylation is denoted as a marker of transcriptional activity (Hebbes *et al.*, 1988, 1994), facilitating the binding of transcription factors to chromatin (Lee *et al*., 1993; Vettese – Dadey *et al*., 1996). However, H3 acetylation is also frequently associated with DNA damage (Khobta *et al*., 2010). Lysine residues K9 and K14 are critical sites for the acetylation of histone H3 (Bjerling *et al.*, 2002). Despite the transcriptional silence inherent to meiosis, the histone acetylation pattern plays a role in oocyte maturation (Kim *et al*., 2003; Endo *et al*., 2005). In the embryo, histone acetylation predicates the oncoming major wave of transcription at MZGA (Adenot *et al.*, 1997).

Histone methylation is considered as an opposite to histone acetylation. Histone methylation is crucial for genome stabilisation, epigenetic inheritance and cellular memory maintenance (Grunstein, 1997; Zhang and Reinberg, 2001; Grewal and Jia, 2007; Muramatsu *et al*., 2013). In the zygote, while the maternal pronucleus is typically di- and tri-methylated (me2/3) on lysine residues K4, K9, and K27 of histone H3, the paternal pronucleus displays lesser histone methylation (Figure 1). Paternal pronucleus is restricted to monomethylation of H3 on K4, K9 and K27, which, however, is also present in the maternal pronucleus (Lepikhov and Walter, 2004; Santos *et al*., 2005; van der Heijden *et al*., 2005). In addition to the pronucleus, H3K9me2/3 is fundamental for epigenetic changes resulting in DNA stabilisation, gene silencing, heterochromatin establishment and X-chromosome inactivation during inner cell mass (ICM) formation (Bannister and Miska, 2000; Rea *et al*., 2000; Cao *et al*., 2002; Plath *et al*., 2004). Although the above-mentioned patterns of histone methylation are associated with gene silencing, the methylation of H3K4 coincides with active transcription sites (Heintzman *et al*., 2007; Eissenberg and Shilatifard, 2010) and appears essential for genome reprogramming, increasing around the time of MZGA in the mouse (Shao *et al*., 2014).

Regulation of the histone code

Histone acetylation is specifically catalysed by histone acetyltransferases (HATs) capable of removing the acetyl group (Brownell and Allis, 1996). Alternatively, non-HATs enzymes with histone acetyltransferase activity, such as transcription initiation factors TFIID and ELP3, are subunits of elongator/RNA polymerase II (Mizzen *et al*., 1996; Wittschieben *et al*., 1999). Among them, HAT1 is responsible for acetylation of newly synthesised histones including H3, as well as the maintenance of acetylation during mammalian embryo development

(Nagarajan *et al*., 2013). On the other hand, histone deacetylases (HDACs), discussed in more detail below, are responsible for acetyl group removal and thus enact under-acetylation of their substrate histones (Tauton *et al*., 1996; Dangond *et al*., 2001). Early embryonic development is regulated by HDACs through deacetylation of both histones and non-histone substrates including α-tubulin, especially until fertilisation when HDACs activity is naturally reduced (Matsubara *et al.*, 2013). Interestingly, overall inhibition of HDACs improves the quality of somatic cell nuclear transfer (SCNT)-derived embryos by an increase of histone acetylation and down-regulation of DNMT1 (Hou *et al*., 2014; Mao *et al*., 2015).

After the HDACs release acetyl group, methyltransferase activity increases following the exposure of binding sites for the methyl group (Dangond *et al*., 2001). A wide spectrum of enzymes with methyltransferase activity appears to be essential for the zygote and early embryo where they are responsible for histone methylation. Among histone methyltransferases, the suppressor of variegation 3-9 homologue 1 and 2 (SUV39H1, SUV39H2, also known as KMT1A, KMT1B), euchromatic histone-lysine

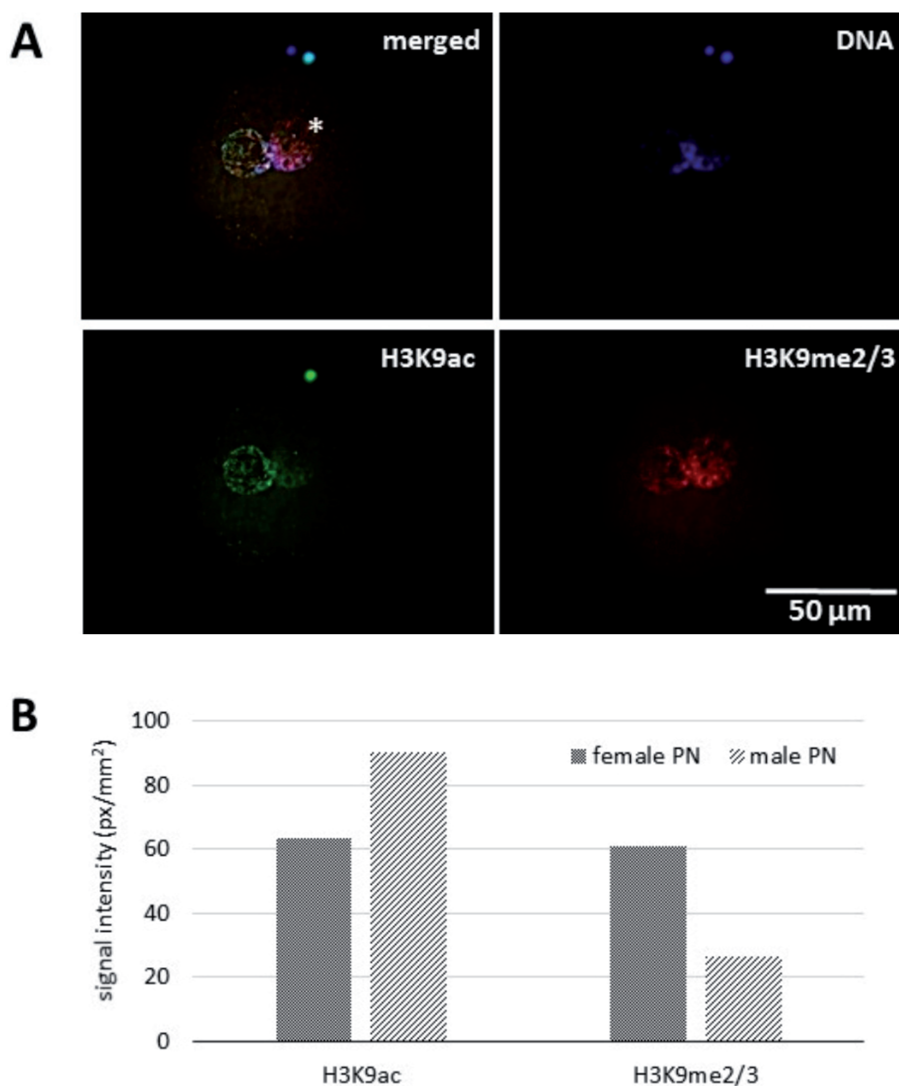

Fig. 1: Asymmetry of the histone code in the porcine zygote pronuclei. Different intensities of acetylated (green) and methylated (red) histone H3 labeling, representing paternal and maternal female pronucleus (PN), respectively, is present (A). The paternal pronucleus was identified by the presence of pre-labeled sperm mitochondria, indicated by asterisk. Signal intensity profile shows higher H3K9 acetylation and lower H3K9 methylation in the paternal pronucleus, in contrast with maternal pronucleus (B).

N-methyltransferase 1 and 2 (EHMT1, EHMT2, also GLP and G9A, respectively), SET domain bifurcated 1 and 2 (SETDB1, SETDB2) and mixed lineage leukemia family (MLL/SET) enzymes transfer the methyl group to lysine in the N-terminal tail of histones and establish heterochromatin marked by H3K9me2/3 modification (Rea *et al.*, 2000; Tachibana *et al.*, 2001; Völkel and Andgrad, 2007; Park *et al.*, 2011; Shao *et al.*, 2014; Golding *et al.*, 2015).

The above mentioned SUV39H1 is an established key factor for facultative heterochromatin formation, genome stability and regulation of gene expression by transcription factors (Firestein *et al.*, 2000; Nielsen *et al.*, 2001; Peters *et al.*, 2001; Vaquero *et al.*, 2004). The activity of this enzyme is important for embryogenesis and determination of embryonic cell lineage (Park *et al.*, 2011; Shao *et al.*, 2014). Heterochromatin formation mediated by SUV39H1 involves the linkage of multiple proteins, such as heterochomatin proteins HP1α and HP1β. Cross-linking of SUV39H1 and HP1 is associated with centromeric regions. The constitution of an SUV39H1-HP1 methylation system is important for chromosome segregation (Aagaard *et al.*, 2000) and H3K9 methylation (Bannister *et al.*, 2001; Lachner *et al.*, 2001; Nakayama *et al.*, 2001; Jacobs and Khorasanizadeh, 2002; Maison and Almouzni, 2004; Park *et al.*, 2011).

The formation of the SUV39H1-HP1-H3K9me2/3 complex is associated with other marks of genome stability, such as DNA methylation (Johnson *et al.*, 2002; Lehnertz *et al.*, 2003; Peters *et al.*, 2003; Peters and Schubeler, 2005; Yeo *et al.*, 2005). Therefore, DNMT1 and DNMT3B seem to be strictly downstream factors of SUV39H1 on pericentromeric chromosome loci in embryonic stem cells, where DNMTs form complexes with HP1 isoforms (Lehnertz *et al.*, 2003). Moreover, DNMT1 interacts directly with histone H3 methyl transferase G9A at the replication fork, resulting in H3K9 methylation (Cheedipudi *et al.*, 2014; Esteve *et al.*, 2005), and a positive feedback loop is indicated. Methylated H3K9 also recruits co-factors of other DNA methyltransferases (Karagianni *et al.*, 2008).

In summary, SUV39H1 exerts a positive effect on early embryonic development. In accordance with this assumption, understanding molecular mechanisms leading to SUV39H1 activation will facilitate further progress in ART. Recent studies point to non-histone substrates of NAD$^+$-dependent histone deacetylates, sirtuins, targeting a wide spectrum of factors with cumulative effects resulting in histone methylation following their direct deacetylation (Vaquero *et al.*, 2007a; Li *et al.*, 2009; Bosch – Presegue *et al.*, 2011).

Sirtuins: the favourite histone deacetylase

The family of histone deacetylases (HDACs) is responsible for histone deacetylation on lysine residues (Allfrey, 1964; Fujimoto, 1972). Based on the original description, the HDACs are divided into three classes: Rpd3p (class I), Hda1p (class II) and Sir2p (class III). An important group within this family is the NAD$^+$-dependent class III of HDACs, together called the sirtuins. The sirtuin family comprises 7 members (SIRT1 - 7), collectively identified as key regulators of lifespan and longevity in various organisms. Sirtuin activity has been linked to protection against DNA damage and repair of DNA strand breaks (Haigis and Guarente, 2006; Kim and Um, 2008; Canto and Auwerx, 2009; Milner, 2009; Herranz *et al.*, 2010). Beneficial effects of sirtuins during gametogenesis and early embryo development have been described (Coussens *et al.*, 2008; Kawamura *et al.*, 2010; Kwak *et al.*, 2012a, 2012b; Bell *et al.*, 2014; Di Emidio *et al.*, 2014; Zhang *et al.*, 2014). One possible explanation of sirtuins‘ protective role is their ability to deacetylate histone H1 on K26, H3 on K9, K14, K26 and K56, and H4 on K8, K12 and K16 (Vaquero *et al.*, 2004; Vaquero *et al.*, 2007b; Oberdoerffer *et al.*, 2008; Das *et al.*, 2009; Chen *et al.*, 2010). These deacetylations lead to a greater abundance of methylated histones, acting as heterochromatin marks. Histone methylation requires lysine residue release and activation of multiple methyltransferases (Vaquero *et al.*, 2004; Yuan and Zhu, 2013).

The above mentioned SUV39H1 methyltransferase is activated by deacetylation of K266 within its catalytic SET domain by SIRT1 (Rea *et al.*, 2000; Vaquero *et al.*, 2007a), which accumulates in the zygotic pronuclei (Figure 2). In addition to induction of deacetylating activity, SIRT1 may protect and prolong the half-life of SUV39H1 by suppressing its proteasomal degradation promoted by polyubiquitination via MDM2 E3-type ubiquitin ligase (Bosch – Presegue *et al.*, 2011). Therefore, H3K9me2/3 increases in the presence of activated SIRT1 (Peters *et al.*, 2003; Vaquero *et al.*, 2004; Vaquero *et al.*, 2007a). The H3K9me2/3 is able to protect H3 against proteasomal degradation due to HP1α recognition followed by ICBP90 binding (Karagianni *et al.*, 2008). This complex enables heterochromatin establishment and maintenance, relevant for epigenetic regulation of mammalian development (Peters *et al.*, 2003; Matoba *et al.*, 2014).

In addition to histone code modification, SIRT1 is capable of affecting signalling mediated by transcriptional factors, such as p53, proteins of the Forkhead box O-class family (e. g. FOXO1, FOXO3A), and p65, a subunit of NF–κB (Kawahara *et al.*, 2009; Kawamura *et al.*, 2010; Wang *et al.*, 2012; Shinozaki *et al.*, 2014). Expression of p53 negatively determines the blastocyst quality and plays a role

in response to DNA damage during embryogenesis. The aforementioned ubiquitin ligase MDM2 is involved in proteasomal degradation of p53 (O'Neill *et al*., 2012; Tollini et al., 2014) and cross-talk between MDM2 and p53 regulates proteasomal degradation of FOXO3A (Fu *et al*., 2009). Regulation by MDM2 and/or the marking-up of deacetylated lysine residues in FOXO for ubiquitination are two possible ways of SIRT1 signalling leading to improved embryonic development due to FOXO regulation (Chen *et al*., 2010; Wang *et al*., 2012, 2014; Chao *et al*., 2014; Sparks *et al*., 2014; Tseng *et al*., 2014).

In addition to MDM2 signalling, SIRT1 affects various cell survival-related functions, including mitochondrial metabolism, apoptosis and maintenance of telomere length (Palacios *et al*., 2010; Wang *et al*., 2013; Zhang *et al*., 2015). The extensive spectrum of SIRT1 targets indicates its complex effect, with the prospect of utilisation for improvement of *in vitro* embryo production. However, many non-histone targets and exact SIRT1 molecular mechanisms in early embryonic development remain undefined.

Significance of SIRT1 understanding for assisted reproduction and *in vitro* embryo production

The multiplicity of cellular pathways involving SIRT1 signalling (Figure 3) accounts for the well-known pro-survival effect of resveratrol, a strong activator of sirtuin favouring SIRT1 (Hubbard *et al*., 2013; Lakshminarasimhan *et al*., 2013). The positive effect of SIRT1 activation on oocyte maturation, early embryonic development and blastocyst rate has been described in numerous studies (Lee *et al*., 2010; Kwak *et al*., 2012a; Giaretta *et al*., 2013; Sato *et al*., 2014; Takeo *et al*., 2014;

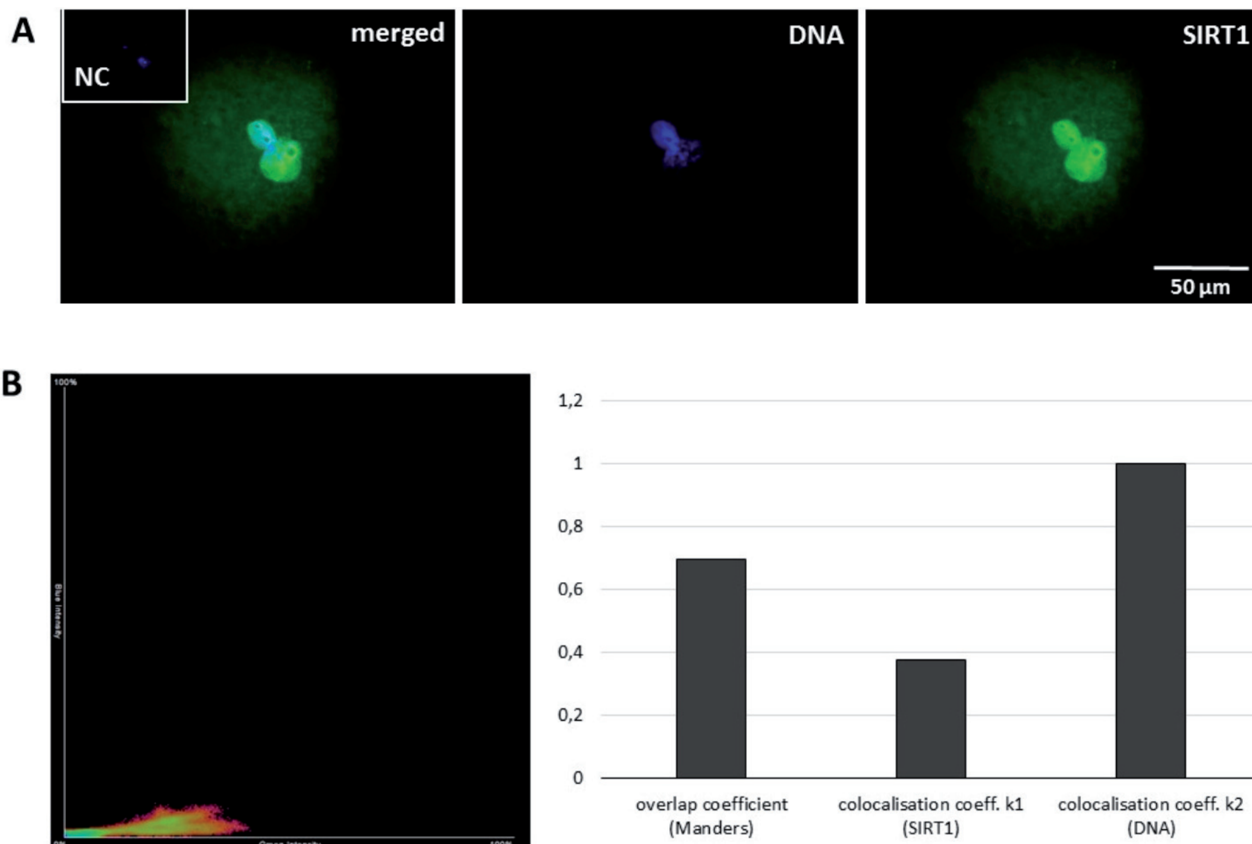

Fig. 2: The SIRT1 protein (green) in porcine zygote. Co-localisation of SIRT1 and DNA indicates SIRT1 accumulation in the pronuclei. Weak SIRT1 signal in cytoplasm is in accordance with existence of SIRT1 non-histone targets (A). Manders' overlap coefficient shows 70 % total signal of SIRT1 in the pronucleus wherein 99 % of chromatin is co-localised with SIRT1 (B). NC: negative control for SIRT1 immunolocalization (anti-SIRT1 antibody was replaced with a non-immune serum during sample processing).

Itami *et al*., 2015). Although SIRT1-improved embryonic development is well known, SIRT1 signalling in embryos is not understood, and research focused on its targets and determinants is still insufficient.

Epigenetic changes and histone code dynamics are potential subjects of SIRT1 and thus possible targets for further improvement of *in vitro* embryo production, which is inferior to *in vivo* development. In addition to IVF, epigenetic modifications play a key role in assisted reproductive technologies, such as ICSI and SCNT, where SIRT1 activity may be altered (Kwak *et al*., 2012b; Peat and Reik, 2014; Mao *et al*., 2015). Subsequently, varied modifications of the DNA and histone code during zygotic and embryonic development could be responsible for the high failure rates of these techniques.

The involvement of SIRT1 in epigenetic inheritance provides an opportunity for the utilisation of new knowledge based on SIRT1 study. However, comprehensive research needs to be undertaken before its application to *in vitro* techniques and methods of both assisted reproduction in farm animals and human reproduction therapy. Particular efforts in our laboratories will focus on the cross-section of SIRT1 and HDAC-mediated epigenetic regulation with the ubiquitin-proteasome system, which plays important

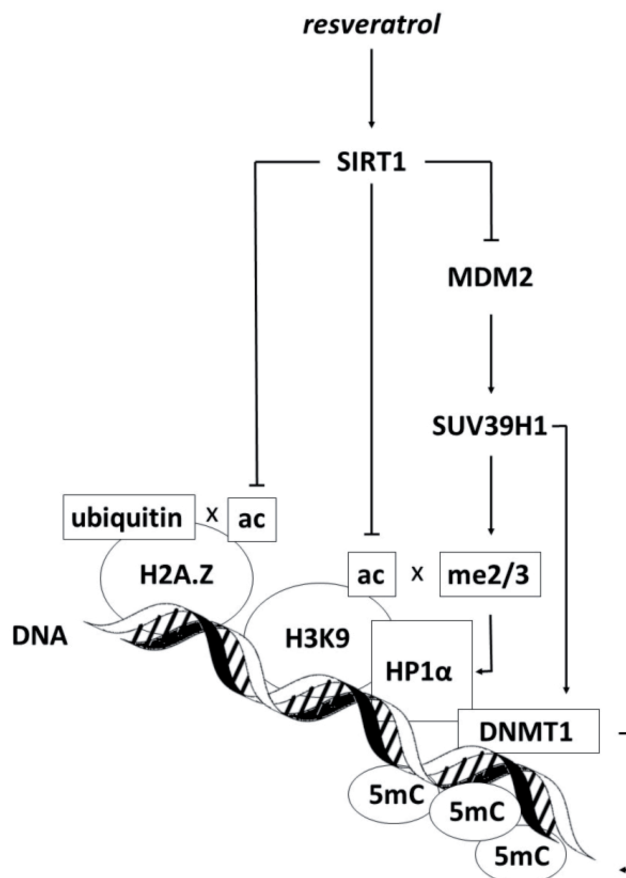

Fig. 3: The involvement of SIRT1 in histone code modifications and heterochromatin establishment. The complex of H3K9me2/3 - HP1α - DNMT1 causes DNA methylation accompanied by the presence of aforementioned histone heterochromatin markers. The SIRT1 protein is able to suppress MDM2 - mediated proteolysis of SUV39H1 and thus increase the presence of heterochromatin markers. Direct deacetylation of histones enables the methylation of H3 as well as ubiquitination and proteolytic degradation of H2A.Z. Altogether, these genome changes can be beneficial for genome stabilisation in zygotic pronuclei and thus improvement of further embryonic development *in vitro*. MDM2: Mouse Double Minute 2 homolog, E3 - ubiquitin ligase; SUV39H1: Suppressor of Variegation 3 - 9 Drosophila, homolog 1, the histone methyl transferase; ac: acetyl group; me2/3: di – or trimethyl group; HP1α: Heterochromatin Protein 1α; DNMT1: DNA Methyl Transferase 1; 5mC: 5'-methylcytosine.

roles in gametogenesis, fertilization and pre-embryo development (Sutovsky, 2003; Mtango *et al*., 2014; Nevoral and Sutovsky, 2015).

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Review

RELATIONSHIP BETWEEN THE OCCURRENCE OF APOPTOSIS AND DISORDERS IN PREIMPLANTATION EMBRYO ENVIRONMENT

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ABSTRACT

Increased incidence of apoptosis in preimplantation embryos is considered to be an indicator of inadequate developmental environment and is used as a sensitive marker of embryo quality in both experimental and clinical conditions. This review summarizes data on physiological characteristics of apoptotic cell death in mouse, rabbit, cow and pig preimplantation embryos. Furthermore, it shows how apoptosis incidence can be regulated by various physiological and non-physiological external factors present during various stages of embryonic development *in vitro* and *in vivo*.

Key words: preimplantation embryo; apoptosis; *in vitro*; maternal environment

INTRODUCTION

Apoptosis (cell death by suicide) is a physiological process occurring spontaneously in the majority of cell populations including embryonic cells (Pampfer and Donnay, 1999). The typical signs of apoptosis are the final results of a complex cascade of biochemical events. It has been shown, that apoptosis plays an important role in cellular response to suboptimal developmental conditions and stress. Thus, increased incidence of cell death is considered to be an indicator of inadequate environment for the development of preimplantation embryos *in vivo* or *in vitro* (Betts and King, 2001; Huppertz and Herrler, 2005).

During preimplantation period, apoptosis is usually triggered to fulfill one of two functions: morphogenetic or reparatory. Morphogenetic function includes elimination of incorrectly differentiated cells, e.g. cells of inner cell mass (ICM) line translocated to trophectoderm (TE) line or cells with superfluous potential. Therefore, apoptosis can act as control mechanism for the regulation of total cell number in embryo. Reparatory function includes elimination of damaged cells. Cell damage can be caused by internal (intracellular) factors, such as non-reparable disorders of DNA replication or cell division, or by external (extracellular) factors. Specific external factors represent the presence of environmental ligands triggering apoptotic process after binding to appropriate receptor (e.g. cytokines, glucocorticoids, etc.); non-specific external factors represent various physical and chemical impacts, which can cause irreversible damage of essential cellular systems (oxidative stress, radiation, chemotherapy, etc.), or non-invasive defects, such as absence of growth factors or other important (e.g. nutritional) substances in the environment of developing embryo (Levy *et al.*, 2001; Fabian *et al.*, 2005b; Penaloza *et al.*, 2006).

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Physiological characteristics of apoptosis in preimplantation embryos

We have shown that blastocysts of four evaluated mammalian species (mouse, rabbit, cow and pig) spontaneously display the presence of apoptotic cell death in relatively high number of obtained embryos (see Table 1). Incidence of spontaneous apoptosis differs among species and it always reaches higher values in blastocysts produced *in vitro*, when compared to *in vivo* delivered ones (Fabian *et al.*, 2005a,b; Gjørret *et al*., 2007; Fabian *et al*., 2007b, 2009b).

Apoptotic cells are located in the both early cell lineages – in the embryoblast and in the trophoblast (Figure 1F), and after reaching terminal stages of apoptotic process they are phagocyted by neighboring cells or extruded to the blastocoele or the perivitelline space. The extruded cells usually undergo secondary necrosis (Fabian *et al*., 2005b). Besides, mammalian blastocysts show high sensitivity to apoptosis induction by chemical treatment, i.e. supplementation of TNFα, actinomycin D or staurosporine during *in vitro* culture (Gjørret *et al*., 2007; Fabian *et al*., 2007a, 2007b).

Early embryos at stages following the embryonic genome activation (EGA) display sporadic incidence of apoptotic cell death (Figure 1A-E). They are able to respond to apoptotic induction; however, the effect is lower when compared to blastocysts. Embryos at stages preceding EGA do not show the presence of spontaneous apoptosis and their ability to response to apoptosis induction is extremely low (Gjørret *et al*., 2003; Fabian *et al.*, 2007b). Interestingly, the timing of the onset of spontaneous apoptotic wave overlaps with the onset of passive DNA cytosine demethylation in embryonic genome, even when embryonic development is arrested (Fabian *et al*., 2009a).

Our results proved that initiated apoptosis can be regulated by specific external impacts. For example, the addition of growth factor IGF-I into culture media had significant anti-apoptotic effect on both spontaneous and induced apoptotic processes in mouse embryos developing *in vitro* (Fabian *et al*., 2004).

Based on our *in vitro* experiments, we can conclude that inappropriate environmental conditions might affect the occurrence and the incidence of apoptosis and subsequently the quality of produced embryos more significantly at the later stages of preimplantation development than at the earlier ones.

Relationship between apoptosis incidence and maternal environment

In the following *in vivo* studies, performed on mouse animal model, we used apoptosis incidence as one of the main markers of embryo quality during the evaluation of the effect of various disorders of maternal homeostasis on preimplantation development.

Toxicological study on mouse females showed that maternal sub-chronic intoxication by relatively low doses of herbicide BASTA 15 might strongly affect developmental abilities and quality of preimplantation embryos, represented by a significant reduction in cell numbers in blastocysts and a significant elevation of the percentage of apoptotic cells per blastocysts (Fabian *et al*., 2011).

The other experimental study has shown that the presence of acute non-specific inflammation (hapten-induced colitis or paw *oedema*), accompanied by systemic biochemical changes (but not hyperthermia) in maternal organism during the preimplantation period would probably not affect the development and growth of embryo. Significant increase in the apoptosis

Table 1: Average incidence of apoptosis in blastocysts of four animal species produced *in vivo* or *in vitro*

		Embryonic day blastocyst	Average cell number per blastocysts	Average incidence of apoptotic cells in evaluated apoptotic cell	Average percentage of blastocyst with at least 1
Pig	*in vivo*	ED 5	± 100	0.8 %	50 %
Rabbit	*in vivo*	ED 4.5	± 300	0.2 %	50 %
	in vitro	ED 4.5	± 200	1.4 %	80 %
Mouse	*in vivo*	ED 4	± 50	1.2 %	25 %
	in vivo	ED 5	± 120	4.5 %	70 %
	in vitro	ED 5	± 90	6.0 %	85 %
Cow	*in vitro*	ED 7	± 175	8.9 %	100 %

Values were extrapolated from results of several previously published studies (Fabian *et al.*, 2005a, 2005b; Gjørret *et al*., 2007; Fabian *et al*., 2007b, 2009b).

incidence was recorded only in expanded blastocysts obtained from additional culture *in vitro*. Thus, we hypothesized that the presence of inflammatory mediators in the environment of developing preimplantation embryos might negatively affect physiology of cellular processes at sub-morphological level, however, embryos are able to manage with these changes using standard reparatory mechanism (Fabian *et al*., 2010).

Results obtained on mouse model simulating natural development of obesity and leanness in mammals showed that alterations in maternal body condition might have impact on reproductive process even at the time of ovulation, fertilization and early preimplantation development *in vivo* (Fabian *et al.*, 2015). Both elevated and decreased body fat deposits were accompanied by increased incidence of blastocysts containing at least one apoptotic cell and increased percentage of apoptotic cells per blastocyst. In case of obesity, the effect was dependent on the stage of its development (Kubandová *et al*., 2014).

Based on our *in vitro* and *in vivo* experiments, we can conclude that apoptosis is a sensitive marker of the quality of naturally produced preimplantation embryos, and it can be used in both experimental and clinical conditions, involving the improvement of biotechnological techniques.

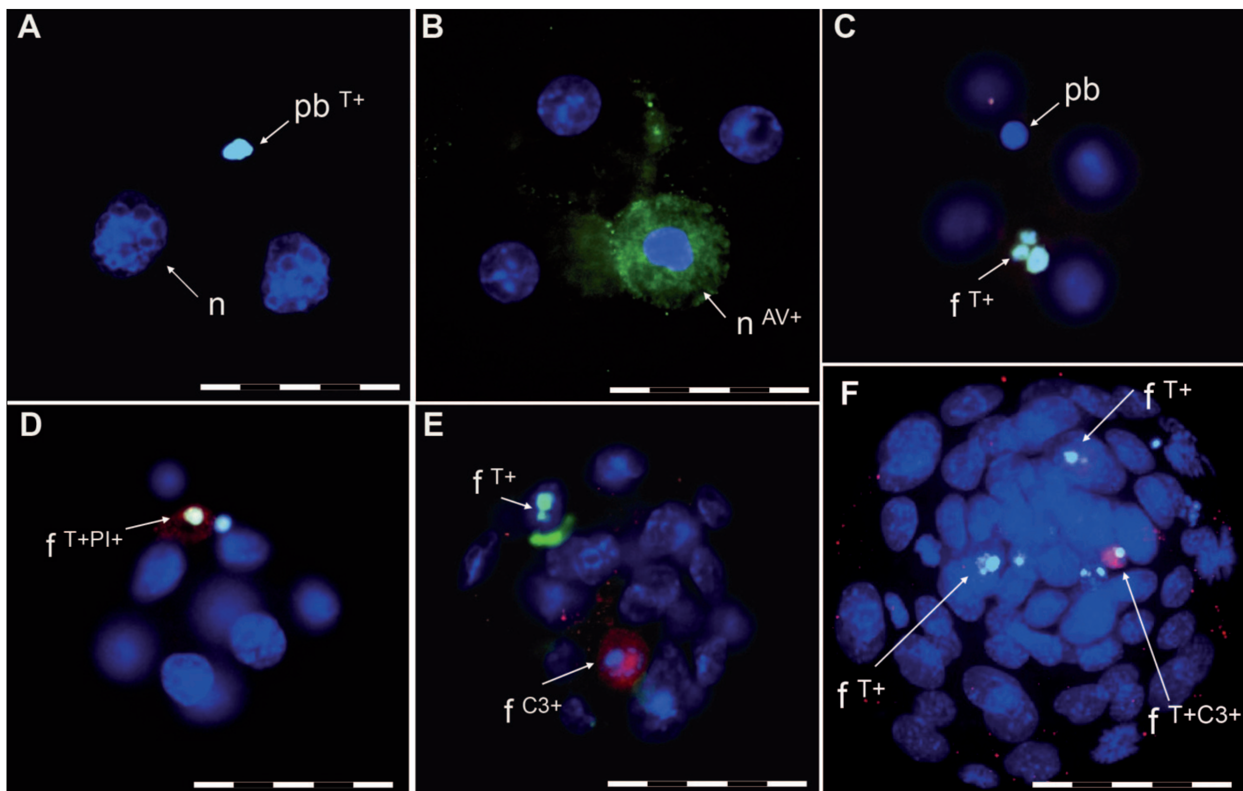

Fig. 1: Illustrative microphotographs of preimplantation embryos with dead cells displaying various features of apoptosis: A, 2-cell; B, 4-cell; C, 5-cell; D, 9-cell embryo; E, morula; F, blastocyst; pb, polar body; n, cell with normal nuclear morphology; f, cell with fragmented nuclear morphology. AV+, presence of phosphatidylserine flip on cell membrane (annexin V staining); T+, presence of specific DNA degradation in the nucleoplasm (TUNEL assay); C3+, presence of active caspase-3 in the nucleoplasm (immunohistochemistry); PI+, positive vital staining with propidium iodide illustrating corruption of nuclear membrane (Fluorescence microscopy, scale bar 50 µm.)

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BOVINE OOCYTE *IN VITRO* MATURATION AND CRYOPRESERVATION: MIRAGE OR REALITY

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ABSTRACT

Selected historical and current aspects of bovine oocyte maturation *in vitro* and cryopreservation were described and discussed in this paper. We have been working on both subjects for years with a rather moderate success, having however good opportunity to observe slow but constant progress being achieved by many research teams worldwide.

Key words: bovine oocyte; oocyte maturation; *in vitro* maturation; cryopreservation; vitrification

INTRODUCTION

An efficient using of oocytes in bovine reproductive biotechnology requires high quality *in vitro* maturation procedures and effective, reliable cryopreservation techniques. Problems related to bovine oocyte *in vitro* maturation and cryopreservation have been focusing attention of researchers for years, and still is difficult to say that all these problems have already been solved. We discuss here some key factors limiting developmental competence of *in vitro* matured oocytes and factors affecting success level of current vitrification methods.

Complex problems with cryopreservation

An efficient, innocuous method of bovine oocyte cryopreservation has been an ambition of cryobiologists for years. Neither numerous experiments on traditional controlled slow freezing, nor more promising vitrification approach was satisfactory. Initially, only a low ratio of blastocyst development (2 to 10 %) was achieved and 2 or 3 calves were born in few occasions, but a final efficiency of those methods was too low to become applicable for practical use. First analyses of biological background of observed vulnerability of bovine oocytes indicated particular sensitivity of certain cellular structures of these cells (cytoskeleton, cortical granules, lipid droplets) on destruction resulting from low temperature and/or cryoprotective agents exposition (Agca *et al*., 1998; Hyttel *et al*., 2000; Rho *et al*., 2002; for review see: Gajda and Smorag, 2009; Prentice and Anzar, 2011; Saragusty and Arav, 2011). Attention was paid also to a spatial mitochondria distribution in cells and on possible compromising a mitochondrial membrane potential (Rho *et al*., 2002; Jones *et al*., 2003)

In 1996 Martino *et al*. presented their results of vitrification, which were through-breaking due to a novel approach. Very small volume of vitrification solutions (few microliters) containing a number

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of bovine cumulus-oocyte complexes (COCs) were mounted on a small electron microscope grid and plunged into liquid nitrogen. As a result, a significant acceleration of cooling rate was achieved, and due to this - much better survival rate of vitrified oocytes enabling development *in vitro* of higher than before ratio of blastocysts (15 %). Idea of such an approach derived from 5 years earlier experiments on vitrification of fruit fly embryos was referred to as Minimum Sample Size (MSS) vitrification system. In 1998 Vajta and his colleagues described even higher blastocysts ratio (25 %) obtained after *in vitro* fertilization (IVF) of oocytes vitrified according to MSS approach. They vitrified oocytes loaded into the tip of narrow plastic capillary made of warmed up and pulled off plastic insemination straws. This method was referred to as an open pulled straw (OPS) vitrification. Many similar methods employing different embryo holders, as for example glass capillaries (Hochi *et al*., 2000), nylon loops (cryoloop) (Lane *et al*., 1999; Begin *et al*., 2003) and many others (Dinnyes *et al*., 2000; Kuwayama *et al*., 2005) were described after that.

In all these MSS vitrification experiments improvement of efficiency was originally attributed mainly to an increase in the cooing rate due to extreme limitation of volume of vitrified sample. Instead of 2,000 to 2,500 ºC/min cooling rate obtainable for insemination straw, such microvolumetric conditions allow to achieve cooling rates of 10,000 ºC to even about 100,000 ºC/min (Martino *et al*., 1996; Arav and Zeron, 1997; Vajta *et al*., 1998; Vajta *et al*., 2000). Such cooling acceleration provides substantial shortening of the time period required for transfer of cells through deleterious range of temperatures, between +20 and -20 ºC. Transition of cells across this dangerous range of temperatures may lead to changes in oolemma bi-layer structure and functionality, dependent on phase transition temperature. This particular temperature point differs depending on maturation stage of oocytes affecting fluidity of membranes (Arav *et al*., 1996). Extremely fast transition across mentioned temperature range should be necessarily considered if innocuous cryopreservation is taken into account. In response to this requirement, microvolumetric vitrification devices (such as CryoTop, CryoLeaf etc.) have been designed, produced and commercially offered to practitioners and researchers.

More recently a significance of high warming rate, easily obtainable in majority of MSS vitrification systems was indicated (Seki and Mazur, 2009). The majority of currently developed vitrification devices allow an obtaining of very high warming rates, enabling high survival rate of eggs or embryos, to some extent independently of their individual cooling rate. This is particularly important in closed vitrification systems recommended from sanitary reasons in human assisted reproduction. On the other hand, microvolumetric vitrification allowed considering a possible decrease in cryoprotectant(s) (CPs) concentration (Arav and Zeron, 1997; Dinnyes *et al*., 2000; Criado *et al*., 2011). In 2005, Kuwayama *et al*. demonstrated a very efficient vitrification device (CryoTop) enabling use of a lower concentration (30 %) of membrane permeating CPs, ethylene glycol and dimethylsulphoxide (DMSO), instead of 40 % used in OPS system. Till now several reports have been published on using MSS vitrification approach for cryopreservation of oocytes and early embryos from species that were extremely difficult to cryopreserve using former methods e.g. bovine oocytes (Dinnyes *et al*., 2000; Hochi *et al*., 2000; Li *et al*., 2002; Chian *et al*., 2004; Anchamparuthy *et al*., 2009), oocytes and cleavage stage embryos of goat (Begin *et al*., 2003) pig (Berthelot *et al*., 2000; Missumi *et al*., 2003), and of many other species (see for reviews Gajda and Smorag, 2009; Saragusty and Arav, 2011; Mullen and Fahy 2012). Due to its efficiency, this approach was also employed in human oocyte and embryo cryopreservation (Liebermann *et al*., 2003; Cobo *et al*., 2008; Kuwayama *et al*., 2005; Papis *et al*., 2011).

Unfortunately, in spite of several attempts of many researchers to improve bovine oocyte vitrification performed during last decade (Hochi *et al.*, 2000; Diez *et al*., 2005; Horvath and Seidel, 2008; Magnusson *et al*., 2008; Yang *et al*., 2008; Sripunya *et al*., 2010; Zhou *et al*., 2010), the obtained results (Checura and Seidel, 2007; Chankitisakul *et al*., 2013) only exceptionally approached the level of efficiency, described more than 15 years earlier (Vajta *et al.*, 1998; Papis *et al.*, 2000; Dinnyes *et al.*, 2000).

The real reason of permanent, still existing problems with bovine oocyte cryopreservation is not fully elucidated yet. Successful oocyte cryopreservation requires not only "physical" survival, but also undisturbed further performance of this very complex germ cell. One of important oocyte function affected by cryopreservation, regardless of the method used, is fertilization process, which, if improper, decreases significantly a general efficiency of a whole procedure. Difficult beginnings of human oocyte cryopreservation due to the low efficiency of fertilization were finally overcame by the introducing ISCI - intracytoplasmic sperm injection, which allows to by-pass side effects of cryopreservation, such as cortical granule premature release and/or *zona pellucida* hardening. Unfortunately, in case of bovine cryopreserved oocytes, ICSI procedure is usually much less efficient (Rho *et al*., 2004; Liang *et al*., 2011), even if additional activation is applied.

As mentioned above, low efficiency of embryonic development after IVF of cryopreserved oocytes can

be caused either by problems with successful sperm-egg fusion, or by a hindered pre-term activation that normally occurs after fertilization. Sperm-egg fusion requires a proper cytoskeleton and microvilli structure, as well as expression of proteins that will bind to sperm and mediate the fusion (Le Naour *et al.*, 2000; Saunders *et al*., 2002; Stein *et al*., 2004; Runge *et al.*, 2007). Proper oocyte activation depends on Ca^{2+} oscillations induced by a fertilizing sperm, which lead to the degradation of cyclin B and inactivation of MPF complex (reviewed in: Motlik *et al*., 1998; Ducibella and Fissore, 2008). Cyclin B degradation requires also a proper alignment of the chromosomes in the metaphase II spindle (Stein *et al.*, 2004). Ability of the oocyte to produce Ca^{2+} oscillations has been previously shown to be affected by prolonged *in vitro* culture (aging) and oxidative stress (Jones and Whittingham, 1996; Igarashi *et al*., 1997; Takahashi *et al*., 2003). Therefore, it is likely, that this process is perturbed in *in vitro* matured and vitrified oocytes as well.

Key feature of successful fertilization of mammalian eggs seems to be an intracellular Ca^{2+} wave oscillations triggered by sperm cytosolic factor(s) such as PLC zeta, leading to an appropriate activation of the oocyte and several downstream effects triggering subsequent developmental features (Ajduk *et al*., 2008). On the other hand, wave of calcium ions released from endoplasmic reticulum causes a cortical reaction of the oocyte, resulting in CG exocytose and subsequent changes caused mainly by ovastacin in the *zona pellucida* ZP2 glycoprotein structure conformation (Ducibella and Fissore, 2008; Machaty, 2013; Burkart *et al*., 2014). In this way, subzonal entry of additional number of sperm is blocked, being at least one of two mechanisms preventing polyspermic fertilization of mammalian oocytes. From papers describing such mechanisms in mouse, rat, sheep, pig and human oocytes it is quite obvious that cryoprotective agents, such as ethylene glycol (EG) or dimethylsulphoxide (DMSO), typically used in slow freezing or vitrification solution formulations, may adversely affect basic mechanisms underlying normal fertilization processes, such as a sperm-egg fusion, exocytose of cortical granules, generation of intracellular calcium oscillations, etc. (Ruppert-Lingham *et al*., 2003; Takahashi *et al*., 2004; Tian *et al*., 2007; Fujiwara *et al*., 2010; Gualtieri *et al*., 2011). Similar adverse effects may be also exerted by freezing procedures *per se*.

Taken all above considerations together, it seems reasonable to limit time of exposure and/or concentration of vitrification solution used for vitrification of particularly sensitive bovine oocytes. In 1999 we presented the first effects of a microdroplet vitrification system (Papis *et al*., 1999a; Papis *et al*., 1999b), technically based on earlier methods (Landa and Tepla, 1990; Riha *et al*., (1992). For some variants of equilibration, the generation of 29.6 % blastocyst from mature oocytes subjected to IVF after vitrification was described in these papers. Such a high ratio of blastocyst stage embryos, two pregnancies after transfer of 4 embryos to 4 recipients and a healthy calf delivered in February 1999 occurred possibly in part due to the microvolumetric vitrification, but mainly due to a gentle pre-equilibration of oocytes in diluted (3 %) solution of ethylene glycol. In this approach, necessary intracellular concentration of cryoprotective agent was achieved mainly due to a transient dehydration of cells after short (30 s) equilibration in solution of 5.5 M ethylene glycol and 1.0 M sucrose - main components of vitrification medium. Described here pre-equilibration system turned out to be very efficient for bovine Day 3 embryo vitrification (Papis *et al*., 1999b) and for Day 2 embryos as well (Papis, unpublished) elevating significantly an efficacy of such embryo vitrification according to MSSV approach comparing with OPS method (Vajta *et al*., 1998; Vajta, 2000). Unfortunately, the same method of vitrification of immature bovine germinal vesicle stage oocytes was less successful (Papis *et al*., 2013). The whole procedure has been patented in Japan (Method of Cryopreservation of Cells; no. 3044323) in March 2000.

Other authors attempted to employ the pre-equilibration system with different level of success for vitrification of oocytes intended for enucleation and cloning (Dinnyes *et al*., 2000; Chang *et al*., 2004; Yang *et al*., 2008), for banking of oocytes obtained from endangered bovine breeds (Li *et al*., 2002), buffalo (Liang *et al*., 2011) or for vitrification of goat oocytes and cleaving embryos (Begin *et al*., 2003). Compromised results reported by those authors may be caused by methodological modifications applied, as for instance different components of vitrification media and/or higher concentration of pre-equilibration solution resulting in the need of using a sucrose solution during warming leading to undesirable, in our opinion, shrinkage of warmed cells.

From the above-shown data we can conclude that our best results of bovine oocyte vitrification, presented 15 years ago, might have been obtained due to an accidental beneficial interaction between efficient vitrification procedure and sperm source and/or preparation method (Papis *et al*., 1999a; Papis *et al*., 2000). Our own attempts to adapt the method developed in Japan to local laboratory conditions in Poland, failed to be equally successful, as we were able to get less than a half of the previously reported blastocyst ratio (Papis *et al.,* 2003, unpublished). The other results show that development of embryos obtained after chemical activation and nuclear transfer is usually much better than after standard

IVF procedure (Dinnyes *et al*., 2000; Yang *et al*., 2008), which supports the notion that cryopreserved bovine oocytes have a decreased capability of fertilization.

Nevertheless, despite of using extremely fast procedures, the recent success of bovine oocyte vitrification usually remains disappointing (Hochi *et al*., 2000; Diez *et al*., 2005; Yang *et al*., 2008; Sripunya *et al*., 2010; Zhou *et al*., 2010). The serious hope recently came from Tamas Samfai laboratory in Japan, where after many years of intense research on lipid droplet destructive influence on cryopreservation effects (Nagashima *et al*., 1999; Romek *et al*., 2009; Fu *et al*., 2011) a positive effects of L-carnitine supplemented maturation media was described (Chankitisakul *et al*., 2013). L-carnitine, which increases metabolism of lipids in cells, is capable of decreasing lipid contents in oocytes. Lipid droplets abundant in certain cells, such as bovine and pig oocytes or embryos, had been recognized as the other key factor decreasing the efficiency cryopreservation of these cells. It remains unclear, how decreasing of lipid content in the oocyte may overcome all above mentioned problems with the proper fertilization mechanisms after vitrification. However, from practical point of view, it would be significant that smart combination of metabolic digestion of lipids with precisely tailored vitrification procedures may give soon an efficient and reliable effects allowing for wider practical use of bovine oocyte cryopreservation.

In vitro **maturation**

The other, extremely important molecular/ cellular factor possibly affecting cryoresistance of oocytes and further developmental capability of embryos is lower level of developmental competence of oocytes obtained from ovarian follicles and subjected to *in vitro* maturation (IVM). Comparison of the effects of IVM, in terms of good quality embryo development and offspring health, both in animal and human reproduction revealed several impairments of *in vitro* mature oocytes in comparison with *in vivo* maturation (Nagai, 2001; Rizos *et al*., 2002; Eppig *et al*., 2009). Bovine oocytes acquire the developmental competence during follicle growth and become fully competent in a dominant follicle, having a final diameter of about 115 µm (Hyttel *et al*., 1997; Hendricksen *et al*., 2000). The process of acquisition of optimum developmental competence, allowing oocyte for a proper maturation, fertilization and early embryonic development, is known as capacitation (Hyttel *et al*., 1997). It is believed that one of crucial aspects of oocyte capacitation is a finalizing of transcription and completing full amount of maternal RNA, which will decide on proper development of an early embryo, until the activation of the embryonic genome. Other factors, as for example a proper level of heterogenous RNA polyadenylation (Brevini-Gandolfi and Gandolfi, 2001) and accumulation of cAMP (Luciano *et al*., 1999), were also considered as important aspects of oocyte capacitation. It was noticed that prolonged (up to 4 h) storage of ovaries *post-mortem* (Sirard and Blondin, 1996; Blondin *et al*., 1997) or inhibition of the maturation of oocytes released from follicles with specific cell cycle inhibitors may be beneficial for achieving higher level of the oocyte competence. Whilst mechanisms of the first effect remain unclear, it was assumed that temporal inhibition of maturation progression enables oocytes to finalize maternal RNA synthesis, stopped otherwise prematurely by an increase in the maturation promoting factor (MPF) activity (Lonergan *et al*., 1997; Ponderato *et al*., 2001; Hashimoto *et al*., 2002).

So far, only a number of cell cycle inhibitors are known. Some of them are able to prevent resumption of meiosis by protein synthesis inhibition (cycloheximide) (Lonergan *et al*., 1997) or by protein phosphorylation, thus maintaining inactive form of p34cdc2/cyclin B complex (MPF) (6-dimethylaminopurine) (Avery *et al.*, 1998). However, efficiency of cycle inhibition mediated via these drugs was not complete and/or full reversibility was questionable. More recently, an effective using of more specific and apparently harmless inhibitors was described. Roscovitine (Mermillod *et al*., 2000; Ponderato *et al*., 2001) or butyrolactone I (Lonergan *et al*., 2000; Ponderato *et al.*, 2001) are specific cdc2- and cdk2-kinase inhibitors, able to arrest the cell cycle in the transition between G1/S or G2/M stages. Mermillod *et al*. (2000) reported 80 % of bovine oocytes arrested at the GV stage after 24 h incubation with roscovitine and 89 % of oocytes that progressed to metaphase II after additional 24 h maturation without this drug. Finally almost 40 % of these oocytes developed to the blastocysts after IVF.

Our own recent attempts to improve oocyte quality using roscovitine cycle inhibition brought moderate success. In the best experimental variants blastocyst ratio obtained from inhibited oocytes was comparable (but not higher) to those obtained after classical IVM. However, our subsequent experiments using type 3-specific phosphodiesterase (PDE3) inhibitor - cilostamide combined with roscovitine, gave slightly higher blastocyst yield (Stachowiak, *et al*., 2013) but did not solve the problem. From this point of view a new concept based on more detailed insights into oocyte-follicle interactions seems very promising. Oocyte-secreted factors (OFS), such as growth differentiation factor 9 (GDF9), to some extent bone morphogenetic protein 15 (BMP15) and perhaps many others, were found out to be essential for folliculogenesis and female fertility (Gilchrist, 2011). These factors function in a paracrine manner on granulosa and cumulus cells, mainly during the antral phase of follicular

growth, inducing, probably through the morphogenic gradient derived from the oocyte (Hussein *et al*., 2005; Gilchrist *et al*., 2008). Although numerous functions of OFS signaling still remain under investigation, it seems clear that capability of the oocyte to control some functions of the cumulus cells may be crucial for acquisition of its own developmental competence (Gilchrist *et al.*, 2008). To take a practical advantage of these recent findings, a new system of oocyte IVM called Simulated Physiological Oocyte Maturation (SPOM) has been recently proposed (Albuz *et al*., 2010; Gilchrist, 2011). SPOM is an integrated IVM system that includes a short pre-IVM phase (1-2 h) and an extended IVM phase that synergize to generate high embryo and fetal yields following embryo transfer. Forskolin and IBMX, a cAMP- modulating agents, included to the oocyte pick-up medium generate a rapid and large increase in cAMP level, which resembles the increase occurring in COCs after the pre-ovulatory gonadotropin surge *in vivo* (Albuz *et al.*, 2010). An increase in both COC and oocyte cAMP levels prevents rapid loss of oocyte-cumulus gap-junctional communication at the pick-up time (Hussein *et al*., 2005; Albuz *et al*., 2010) and simultaneously loads the oocyte with cAMP, preventing precocious spontaneous resumption of meiosis. The extended IVM phase of SPOM slows down meiotic resumption, which is, however, overridden or induced by FSH. Oocytes subjected to SPOM system are exposed throughout maturation to a low concentration of a type 3-specific PDE3 inhibitor. The PDE3 inhibitor (cilostamide) concentration is too low to completely inhibit meiosis, but sufficient to impair meiotic maturation in the absence of the meiosis-inducing hormone. In SPOM, a relatively high concentration of FSH (100 mIU/mL) is needed to induce oocyte maturation in the presence of the PDE3 inhibitor. Taken together, SPOM method mimics some of important newly described molecular mechanisms that occur during oocyte maturation *in vivo*. Its application should lead to an increase in IVM efficiency, oocyte developmental competence and embryo *in vitro* production.

We assume that it may also affect cryoresistance, fertilization susceptibility and subsequent embryo development of bovine oocytes subjected to vitrification. Our preliminary trials using SPOM of bovine oocytes resulted however in decrease of the oocyte cleaving ratio. Interestingly, a good quality blastocyst rate developed in those cleaved embryos exceeded ratio obtained in control, traditionally matured oocytes (Duda, 2013). This result needs confirmation as it may eventually offer a method for increasing an efficiency of bovine *in vitro* embryo production. From the research performed very recently in Germany, it seems obvious that SPOM may interfere with proper embryo development on the oocyte DNA/histon methylation or acetylation level, affecting epigenetic characteristics of an embryo (Heiligentag *et al.*, 2015). Unfortunately, we found quite difficult to get financial support for this kind of research in Poland, and therefore continuation of our research is problematic. Nevertheless, it seems desirable to continue a work on SPOM and related maturation methods (including the use of active recombinant OSFs) to increase quantity and quality of bovine oocytes available for reproduction or cloning.

CONCLUSION

Reviewing historical and contemporary aspects of bovine oocyte cryopreservation and *in vitro* maturation, we found quite obvious that long lasting efforts of many distinguished researchers from many countries gave much worse effects than it was expected by scientific community and practitioners. There are, however, some evidence leading to the conclusion that recently a significant progress has though been made. Taken together, an answer to the title question seems now to be much closer to “reality” than ever.

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Review

SEVERAL KEY ASPECTS OF THE RESEARCH ON BOVINE PREIMPLANTATION EMBRYOS AT THE RESEARCH INSTITUTE FOR ANIMAL PRODUCTION (RIAP) IN NITRA

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ABSTRACT

This short review attempts to describe several important aspects of research at the RIAP Nitra in the area of embryo manipulation with *in vivo*-derived or *in vitro* produced cattle embryos performed from the beginning of an embryo transfer era and up to date. The paper summarizes results of more important studies including: manipulations on embryos (embryo transfer, embryo bisection for creation of identical calf twins, embryo cryopreservation), *in vitro* embryo production (IVP), the assessment of embryo quality according to morphology, nucleic acid synthetic activity in the blastomeres or the fine ultrastructure of embryo organelles, the use of bovine embryos as a model for testing bovine virus transmission, gamma-irradiation at the Chernobyl´s disaster and other influences, with referring to the articles, written by the employees of the RIAP during this period and published in the national and international literature. All obtained results as well as accumulated knowledge and methodical experience in this area provide a strong basis, which can be used at creating the gene bank of animal genetic resources.

Key words: cattle; embryo transfer; manipulation; *in vitro* fertilization

INTRODUCTION

Over a period of approximately forty years, commercial bovine embryo transfer and its associated technologies have become a large international business. The bovine embryo transfer industry, as it is known today, arose in North America in the early 1970s, and the dissemination of modern reproductive technologies over the world was significantly influenced by the fact, that the intercontinental transportation of live animals was very expensive and time-consuming. Reproductive technologies have gradually expanded around the word also to other continents, and in several countries workplaces within research institutes, universities or centres were established, which began to focus on research in the field of preimplantation embryo manipulations.

Also, in the former Czechoslovakia, the research in the area of collection and transfer of bovine embryos began intensively since 1973, when the International Embryo Transfer Research Team from the former Eastern bloc countries (Czechoslovakia, German Democratic Republic, Poland, USSR, Romania, Bulgaria, Hungary) and Mongolia was formed by the scientists from institutions of Academies of the Sciences, research institutes, high schools, universities and breeding services with the purpose to elaborate validated approaches of superovulation and synchronization of donor animals, embryo recovery and embryo transfer.

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On the basis of experimental research the implementation outputs were subsequently processed. In 1978 - the manual for surgical embryo transfer, and in 1982 - the completed protocol of non-surgical isolation and transfer of cattle embryos were implemented to practice. Also, biotechnical approaches for superovulation and synchronization of the sexual cycle in farm animals were elaborated.

After the validation of non-surgical isolation and transfer of cattle embryos in 1980-1984 in Slovakia the realization teams with centres in Nitra, Bernolakovo, Banská Bystrica and Košice were established. Sixty experts, involved into this activity, were trained at the RIAP Nitra and then acted according to the instruction under the RIAP Nitra coordination. Since 1985 the members of realization teams began to apply embryo transfer into practice under systematic and planned schedule. The aim was to obtain offspring from mothers of bulls and mothers with high milk production, as well as to produce calves for fattening. In the former Czechoslovakia, in 1987 about 16000 embryo transfers were performed with 55 % success rate, and in 1988 - even 22000 embryo transfers with 60 % success rate, of which about 5000 transfers were done in Slovakia. About a third transfer was designed to produce fattening calves. On the basis of the management and success in embryo transfer, the former Czechoslovakia was ranked among the most advanced countries using these biotechniques.

The work in field of assisted reproduction in cattle with a focus on bovine preimplantation embryos, which was initiated at the RIAP in Nitra already in 80-s years of the last century, continues to date but with the more emphasis on production of bovine embryos *in vitro* using *in vitro* fertilization (IVF) technique.

***In vivo* recovery of embryos and their quality**

Initial investigations on isolation and transfer of embryos at the RIAP Nitra were carried out on laboratory animals and rabbits. In 1970-s, pigs as first farm animal model were used for the study of oestrus synchronization in donors and recipients, superovulation, surgical recovery and transfer of embryos, their evaluation and culture *in vitro* (Pivko *et al*., 1973). First offspring after surgical isolation and transfer of porcine embryos was obtained in 1975.

Embryo quality and fertility rate mostly depends on quality of oocytes, therefore, this task was studied by autoradiography at the level of light and electron microscopy. It was found that during oocyte maturation at the germinal vesicle stage, viable oocytes, being either connected with cumulus cells or after finishing this connection, intensively synthesize RNA (Motlík *et al.*, 1978) and glycoproteins (Pivko *et al*., 1982a; Fléchon *et al.*, 1986). Newly synthesized proteins and RNA are localized in the germinal vesicle. Synthesized material, accumulated under the *zona pellucida*, is represented by hyaluronic acid containing glycosaminoglycans formed by cumulus cell secretion activity under influence of gonadotropins. Therefore, in the peri-ovulation period synthesis of proteoglycans by oocytes and cumulus cells takes place, and extracellular envelopes are formed with relative tight binding to the *zona pellucida*. Such a proper synthesis of RNA, specific proteins and glycosaminoglycans significantly affects processes of fertilization and early embryo development.

First experiments (beginning in 1980-s) on application of cattle embryos in embryo transfer at the RIAP were focused on the generation of calf twins. It was induced by the fact that such techniques as selection of animals for superovulation and exogenous application of gonadotropins did not bring satisfactory results. On the other hand, generation of twins by embryo transfer showed to be promising idea. In the experiment by Pivko *et al.* (1982b), 74 embryos were transferred to 37 recipients and 70 % of cows were pregnant (26), of them 65 % (17) gave birth to twins.

The next research on bovine embryos about non-surgical isolation of bovine embryos from superovulated cows by flushing of uterine horns at 7-9 days following insemination was reported by Kubovičova *et al.* (1991). This study was focused on the quality evaluation of *in vivo* -derived embryos on the basis of morphological criteria. Totally, 3337 preimplantation embryos were collected within the period of 1986-1988, of them 56.7 % (1892 embryos) were suitable for embryo transfer or cryopreservation and about 40 % (1327 embryos, including infertile eggs) were classified as inappropriate ones from the standpoint of their quality.

Quality of embryos recovered from cows on farms is associated with strict selection of donor cows with special focus on general health status of sexual organs accompanied with a temporal vitamin supplementation and nutrition improvement. Among the causes of high occurrence of improper embryos and unfertilized eggs collected at flushing there are an environment (animal housing, nutrition, zoohygienic conditions, donor cows itself), disturbed homeostasis of the organism, spermiotoxicity of vaginal mucus, inadequate response to the hormonal treatment, endometritides as well as low viability and motility of spermatozoa. However, technical and material facilities at the embryo transfer (embryotoxicity of flushing solutions, catheters and culture media) should not also be ignored.

Since embryo flushings in our experiments were performed on the 7th - 9th days after inseminations, the collected embryos were at different stages of development: compact morula, early blastocyst, blastocyst and expanded blastocyst. For embryo transfer more suitable embryos were morula, blastocyst and

expanded blastocyst stage, whilst early blastocysts were also suitable for micromanipulations, and in this research they were used for the generation of identical twins.

At the definition of qualitative criteria for embryos the main emphasis was put on regular formation of embryoblastic and trophoblastic cells, regular-shaped and intact *zona pellucida*, the perivitelline (PV) space and blastocoele cavity. Destruction, fragmentation and disintegration of embryonal cells were evaluated under a stereomicroscope using the following scale:

Excellent – an ideal embryo, round-shaped, symmetrical, with the cells homogenous in size, colour and structure;

Good – the rare occurrence of blastomere fragments in the PV space, irregular shape of blastomeres, and slight incidence of vesicles;

Fair – slight occurrence of scraps of excluded blastomeres and a few degenerated cells in the PV space, the vesicle formation progresses;

Poor – serious developmental disorders: numerous blastomeres excluded into the PV space, degenerated and differently-sized cells, numerous large vesicles, fragment formation.

According to this classification, from total number of flushed embryos (3337) 45.55 % was of excellent, 11.15 - good, 3.54 - fair and 39.77 % of poor quality. After flushing of superovulated cows a high number of fragmented embryos were yielded. This high rate of unfertilized eggs and fragmented embryos may be caused by abnormalities related to superovulation treatment. In practical conditions it is not possible to define exactly a margin between embryos of fair or poor quality.

Since morphological criteria of embryo evaluation are often insufficient, techniques of vital staining using various fluorochromes, for instance FDA (fluorescein diacetate) acquire special importance. Pivko *et al.* (1986) after FDA staining observed clear fluorescence in bovine embryos of preimplantation stages, in particular, fragmented or abnormal embryos accumulated less FDA signal than normal viable embryos. The association between integration/disintegration of blastomeres and FDA accumulating ability was supported also by the experiment with the intended destruction of the embryo by deep freezing. Following thawing it was possible to determine the extent of disintegration using FDA test. It was found that FDA-negative blastomeres have extensive degenerative alterations in the cytoplasm and no longer synthesize RNA (Pivko *et al*., 1986).

For evaluation of early embryo quality the processes related to initiation of the embryonal transcription and running of nucleogenesis were investigated. Definition of nucleologenesis stages enables using the morphology of nucleus and nucleolus as relevant morpho-functional marker of embryo viability evaluation. Using autoradiographical and immunocytochemical analyses of ultrastructural morphology and dynamics of nucleic acids Pivko *et al.* (1996) studied differential and functional status of early cattle embryos.

Manipulation on bovine embryos

At the beginning of the era of embryo transfer a main application of ET was generation of twin calves. Three common approaches to produce calf twins with the use of embryo transfer were known:

a) Transfer of two embryos into one recipient cow,
b) Transfer of two embryo halves after micromanipulations into one recipient cow (Holy *et al*., 1985),
c) Transfer of one embryo into contra-lateral uterine horn of previously inseminated recipient (Říha *et al*., 1986).

Using embryo transfer of fresh and frozen-thawed embryos into contra-lateral uterine horn of recipient cows Grafenau *et al.* (1992) obtained 59.2 and 55.8 % pregnancy rates, resp., and the birth of 1.44 calves per one pregnant recipient.

One of embryo manipulations commonly used on bovine embryos during 80s years of last century was microsurgical embryo bisection in order to generate identical calf twins. At the Research Institute of Animal Production two genetically identical calves were generated in 1987, and chimerical mice were created in 1989 by the team of Dr. P. Babušík (unpublished results).

The survivability of bovine embryo halves two hours after bisection is relatively high (65-90 %; Picard *et al*., 1986). Microsurgical bisection of the embryo leads to mechanical damage of about 1/5 of cells from total number of blastocyst cells. Embryo halves are retarded in the development but have a great regenerating ability resulting in the compaction of halves and the formation of blastocoelic cavity (Picard *et al*., 1986).

Pivko *et al*. (1995) analysed proliferating activity of early embryos recovered non-surgically (flushing of the uterine horn) from cows of Slovak Pinzgau breed. Early embryos at the blastocyst stage were bisected into two equal halves, cultured in TCM medium with 20 % fetal calf serum for two hours and then analyzed autoradiographically with the use of 3H-labelled thymidine (Pivko *et al*., 1995). Blastocyst cells actively proliferated (especially inner cells and those undamaged) already two hours following bisection, what was proved by the DNA synthetic activity indicating on running cell cycles.

Microsurgical bisection of bovine embryos in combination with embryo transfer techniques was applied to obtain non-identical calf twins for improving

the efficiency of beef cattle production (Sreenan and Danagh, 1984; Říha and Polášek, 1987; Holy *et al*., 1985).

Among embryo manipulations, the team headed by prof. J. Pivko performed intracytoplasmic sperm injection (ICSI) (Pivko *et al.*, 2003) and parthenogenetic activation by electric AC/DC pulse (Pivko *et al*., 2004) to produce bovine embryos *in vitro*. The volume of the Golgi apparatus was significantly increased in the ICSI-derived embryos compared to parthenogenetic embryos. Expansion of the Golgi apparatus was probably caused by the ICSI technique used. In early 8-blastomere embryos following AC/DC activation and ICSI a high relative volume of vacuoles and lipids was observed what led to decrease in the volume of organelles participating in the proteosynthesis.

Bovine embryos as a model for testing bovine viral infections

Epidemiological aspects of embryo transfer in farm animals remain currently highly actual task. Exchange of genetic material (oocytes, embryos, and sperm) within the country and between countries necessitated devoting more attention to security and reduction of health risk at the embryo transfer. Health risk of viral infection associated with embryo transfer is not dependent only from animals - donors of sperm and embryos, but also from environmental conditions to which gametes and embryos are exposed. More often of them are flushing and culture media and conditions of recovery, manipulations, storage and transfer.

We studied development and viability characteristics of bovine embryos after their experimental infection with bovine herpesvirus-1 (BHV-1) (Makarevich *et al*., 2007). In this study we examined whether: (1) the *in vitro* exposure of embryos to the BHV-1 virus can compromise their further development and alter the ultrastructural morphology of cellular organelles; (2) whether the *zona pellucida* can be a barrier protecting embryos against infection; and (3) whether trypsin washing after viral exposure can prevent virus penetration inside the embryo and subsequent virus induced damages. It was found that BHV-1 exposure impairs embryo development independent of the presence of ZP or the trypsin treatment step, since most of experimentally infected embryos were arrested at the morula stage. Therefore, *zona pellucida* itself may not be an enough barrier to prevent virus-induced damages, unless it is accompanied by trypsin washing (Makarevich *et al*., 2007).

In the further study, Kubovicova *et al.* (2008) performed experimental infection of bovine *in vivo* isolated embryos with bovine viral diarrhoea virus (BVDV). BVDV viral suspension was introduced under the ZP by the microinjection procedure and ultrastructural morphology of cell organelles was examined. About 83 % of embryos were arrested at the morula stage and this arrest was associated with irreversible alterations in the ultrastructure of organelles like disintegrated nuclei, the loss of the nucleoli, intercellular gap junctions and dilatation of intercellular space.

Several studies proved that *in vivo* and *in vitro* produced embryos are different. Also health risk using *in vitro* culture may be related to contamination of serum-supplemented medium, bovine serum albumin and other biological additives. Viral contamination at the stage of *in vitro* fertilization can be caused by the BVDV virus adhered to the membrane of sperm, which enters into the oocyte following the penetration of the *zona pellucida*. Effective sanitary procedures preventing transmission of infections by embryos of farm animals were elaborated by the International Embryo Transfer Society (IETS) and International Office of Epizootology (OIE). These procedures represent methodical instruction for washing of embryos before the transfer in order to eliminate pathogens, eventually cells or cellular fragment adhered to the *zona pellucida*. Application of these simple washing steps on embryos before their *in vitro* culture or transfer diminishes the risk of infection transmission to minimum, if not completely.

Influence of gamma-irradiation on quality of bovine embryos

After the accident at the Chernobyl´ nuclear power station we determined autoradiographically the RNA synthetic activity in early embryos derived from cows reared in the irradiated zones, which had chronic exposure to gamma-irradiation. The nuclei and nucleoli of such embryos have revealed no deviations from physiologically normal nucleogenesis (Pivko *et al.*, 1997). On the contrary, in the embryos which were experimentally exposed to gama-irradiation at certain doses (1, 2 and 4 Gy gamma) using a cobalt bomb 60Co, at dose of 4 Gy we have found termination of RNA synthesis and increased incidence of various forms of chromatin segregation in blastomere nuclei, particularly in the form of marginalization of chromatin (Pivko *et al*., 2002).

***In vitro* production of bovine embryos for research purposes**

In vivo isolated embryos, derived from cows of defined breeds and genotypes, are intensively used in practice for breeding purposes, e.g. in embryo transfer, cryopreservation for the purpose of storage of animal gene resource a.o. Oppositely, biological material for creation of *in vitro* produced embryos (oocytes) is mostly available from local slaughterhouses (excepting ovum pick-up technique), their origin is rather undefined and such embryos cannot be used for breeding purposes.

Nevertheless, the application of *in vitro* produced cattle embryos represents good model for their use to test different external effects or conditions. *In vitro* fertilization expands possibilities of cattle reproduction by the creation of transferable embryos originated from cows *post-mortem*. This technique also provides a source of embryos for research in assisted reproduction and biotechnology.

During 1980-1990s embryo transfer in cattle in Slovakia had 50-60 % success rate. Following transfer of 5-6 transferable (1st and 2nd quality grades) embryos at average 2-3 calves were born. This fact induced more intensive application of *in vitro* embryo production (IVP) technique involving maturation of oocytes (*in vitro* maturation, IVM), their fertilization (*in vitro* fertilization, IVF) and culture of embryos up to higher preimplantation stages (*in vitro* culture, IVC). Sustainable results over the world indicate that average success rate was about 80-90 % for IVM, 75-85 % for IVF and 30-40 % for IVC, of them about 10-12 liveborn calves per 100 oocytes can be born. However, problems of variable success rate are often occurred, what is caused by genetic origin of gametes (oocytes, sperm) and by using different baths of serum or serum derivatives, which contain a number of unknown growth-stimulating substances. It was also showed that prolonged *in vitro* culture period may have unfavourable long-term consequences, like prolonged pregnancy length, large size of foetus and certain morphological abnormalities. It is assumed that these alterations may be caused by changes in the control of the gene expression.

RIAP started dealing with bovine *in vitro* embryo production in the end of 1990s. First studies were done in cooperation with Agricultural centre of Finland and the reports described about proliferation potential and apoptosis of bovine *in vitro* produced embryos (Makarevich and Markkula, 2002), which were cultured in SOF– medium in the atmosphere of incubator with suppressed oxygen tension. During the 2000s the experiments on bovine *in vitro* embryo production at the RIAP were intensively continued. Kubovičová *et al*. (2003) reported about bovine *in vitro* fertilization using ICSI, where 42.2 % of oocytes developed to embryos compared to 24.5 % obtained in classic IVF procedure. However, ICSI-derived embryos showed altered ultrastructural morphology of organelles (Pivko *et al.*, 2003).

The experiments on comparison of *in vivo* or *in vitro* produced embryos were focused on the ultrastructure of pre-compacted embryos at 1 to 8-cell stage (Pivko *et al*., 2003; 2004). Comparison of *in vivo* and *in vitro* embryos showed that IVP embryos had an altered ultrastructural morphology. Early embryos produced *in vitro*, either by AC/DC pulse activation or using ICSI, had a decreased relative volume of the cytoplasm in comparison with *in vivo* embryos, which can be explained by a higher occurrence of vacuoles and lipids in the blastomere cytoplasm.

Limiting factor at these techniques is a low success of embryo production of good quality and developmental capacity. The quality of IVP embryos depends on conditions of cultural milieu, particularly temperature, light regime, composition and properties of a culture medium, gas composition in the incubator, sperm quality and others.

As an *in vitro* maturation medium, TCM 199 medium added with fetal calf serum and gonadotropic hormones more often is used. There are known several embryo culture media commonly used for bovine *in vitro* produced embryos: TCM199 (Shamsuddin *et al*., 1994), KSOM (Liu and Foote, 1995), CR1α (Rosenkranz *et al*., 1990), SOF (synthetic oviduct fluid; Tervit *et al*., 1972), ISM1, B2 INRA (Camous *et al*., 1984), G1.2/G2.2 (Lane *et al*., 2003), etc.

Olexikova *et al.* (2009) tested three culture systems for production of bovine embryos *in vitro*: 1) SOF- medium without O_2 suppression, 2) SOF-medium with O_2 suppression in the atmosphere to 5 %, and 3) B2-INRA medium in co-culture with BRL-(Buffalo Rat Liver) cell monolayer. Embryo development was assessed on the basis of embryo cleavage rate and blastocyst rate. It was found that more successful culture system in given laboratory conditions was a combination of B2-INRA medium and BRL cell co-culture, which allowed obtaining higher cleavage and blastocyst rates compared to other systems tested in this study.

The ability of BRL cells to promote preimplantation embryo development is perhaps based on the secretion of different growth factors by these cells, like insulin-like growth factor I (IGF-I), epidermal growth factor (EGF), leukaemia inhibitory factor (LIF) and others (Duszewska *et al.*, 2000). Makarevich and Markkula (2002) have found that the addition of insulin-like growth factor I into maturation (IVM) and/or culture (IVC) medium (SOF-medium) stimulates cell proliferation (PCNA) and reduces apoptosis in fresh bovine IVP embryos, therefore, improving the quality of blastocysts.

Later, Makarevich *et al.* (2012a) tested the addition of IGF-I into post-thaw medium on bovine *in vivo* isolated embryos following long-term cryostorage. Survival effect of IGF-I was confirmed, as the addition of IGF-I during post-thaw culture (48 h) improved the quality of bovine cryopreserved embryos. In particular, IGF-I advanced development of thawed embryos to the blastocyst stage, elevated total cell number, decreased percentage of apoptosis and improved actin cytoskeleton quality.

Quality of IVP embryos may be estimated also on the basis of tolerance against cryopreservation. IVP

embryos are more sensitive to cryopreservation than their *in vivo* counterparts. Thus, Markkula *et al*. (2001) analyzed proliferating cell nuclear antigen (PCNA) in fresh and vitrified IVP blastocysts and have found that the blastomeres with missing PCNA reaction indicated on the disorders that were not observed under morphological evaluation. Generally, Day 7 blastocysts tolerated the cryopreservation better than Day 8 blastocysts.

Pivko *et al*. (2003b) analysed ultrastructure of bovine IVP embryos following cryopreservation in open pull straws (OPS vitrification, Vajta *et al*., 1997). They observed that vitrification and warming of IVP embryos resulted in immediate injuries at the cellular and sub-cellular levels, however the most of them were normalised following 24 hours of post-thaw culture.

Embryo yield in relation to the cow´ body condition and the season

In recent years experiments on bovine IVP embryos were focused on interrelations of cow body condition and quality of embryos. Bovine oocytes were recovered from cows of BCS 1, 2 or 3, then *in vitro* fertilized with bull semen and cultured until preimplantation stage embryos. Body condition of cows affected initial quality of oocytes, but did not affect embryo cleavage, blastocyst rate and actin quality of subsequent IVP embryos (Kubovičová *et al*., 2012; Chrenek *et al*., 2015).

Different situation was observed on embryos *in vivo* recovered from superovulated cows (Kubovičová *et al*., 2013; Makarevich *et al*., 2015). The cow ´s body condition affected the overall embryo recovery rate (proportion of collected embryos to palpated corpora lutea). The significantly higher number of embryos was collected from cows with average body condition (BCS3 - 65.81 % embryo recovery rate) compared to the cows with low (BCS2 - 50.6 %) or high (BCS4 - 21.43 %) condition. Also the season significantly affected embryo recovery rate. The significantly higher percentage of embryos was recovered during spring months (59.6 % recovery rate) compared to summer months (37.0 %) and slightly increased again during the autumn (48.3 %). On the contrary, the quality (yield of transferable embryos) was better during the autumn months (78.9 %) compared to spring (58.4 %) or summer (60.0 %) months.

The use of bovine IVF for analysis of sperm penetrating/fertilizing ability

In recent several years bovine IVF system is intensively used at the RIAP for evaluation of sperm penetrating or fertilizing ability *in vitro*. Before using a male for breeding purposes, is it important to know that he is fertile. Testing individual male fertility by artificial insemination is expensive and labour intensive procedure. The most adequate method to assess the sperm fertility may be in vitro fertilization, since this procedure evaluates the spermatozoa-oocyte interactions occurring during fertilization process, allowing determination of different endpoints in early stages of the embryo development. In our research we used bovine pre-matured oocytes in heterologous system to examining ram sperm penetrating/fertilizing ability. Fertilizing ability of ram spermatozoa was tested following 48 h of cooling storage in the presence of growth factors, either EGF (Makarevich *et al*., 2011) or IGF-I (Makarevich *et al*., 2012b). Penetration ability was measured basing on the number of oocytes with at least one sperm inside the *zona pellucida*, and fertilizing ability was measured by counting the number of divided embryos. In these experiments it was found that IGF-I improved the penetration and cleavage rate of embryos compared to control, whilst EGF did not improve these characteristics. However, these studies validated the use of bovine IVF system for evaluation of fertilizing capacity of ram sperm. At present at the RIAP, the examinations of fertilizing ability of bovine sperm doses from Pinzgau bulls are intensively carried out using bovine IVF system.

CONCLUSION

This review demonstrates that the RIAP was and to date is still a key institution in cattle assisted reproduction, which has a long-term tradition of research with bovine embryos both *in vivo* and *in vitro*. However, in recent decades interest in embryo transfer and other assisted reproduction techniques in cattle was rapidly dropped. It is a result of the current agricultural policy and situation in the branch of animal production in Slovakia. During last decade, cattle population in Slovakia rapidly decreased. Moreover, interest of breeding centres or individual farmers in embryo transfer and other assisted techniques dropped to minimum, and the main reason of this situation is an absence of state support in this field of animal production. Nevertheless, for more than 40 years of this research activity, in the laboratories and farms of the RIAP several key techniques and methods of animal superovulation, embryo recovery, embryo manipulations and transfer, quality evaluation, cryopreservation and *in vitro* embryo production were elaborated and implemented to practice. Important findings were published in international and domestic literature. Moreover, the RIAP during many years was a holder of a special accreditation (registered under the code ETTSR01) permitting embryo transfer procedures for whole the Slovak Republic. In order to restore

activities of the embryo transfer workplace at the RIAP with the competence to export embryos to the Europe Union it would be necessary to equip additionally the workplace with material and personnel facilities in order to meet requirements not only for experimentation but also for trading (exchange) with embryos of genetically significant and endangered animal species within the EU.

At present, investigations on bovine embryos at the RIAP are focused mostly on *in vitro* embryo production, optimization of cryopreservation techniques for long-term storage of embryos, oocytes and ovarian tissues in order to elaborate methodological approaches for creating embryos with improved cryotolerance. Therefore, altogether these events demonstrate that the RIAP possess with a proper basis for implementation of methodical, equipment and personal facilities for the establishing gene bank of animal genetic resources in Slovakia.

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REACTION PATTERN OF MONOCLONAL ANTIBODY IVA-50 (CD9) DURING CAPACITATION OF BULL SPERM

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Ejaculated mammalian spermatozoa must undergo several biochemical and morphological changes in the female reproductive tract to acquire the ability to fertilize the egg. These changes, collectively called capacitation, include also the rearrangement of sperm surface proteins acquired from seminal plasma as well as epididymal secretion. One of the proteins recently detected exclusively on plasma membrane of bovine sperm is CD9 molecule, tetraspanin, whose essential role for gamete fusion was previously confirmed on oocytes, but no data are available regarding the dynamics of this molecule during capacitation in cattle.

It is well known that process of cryopreservation influences the sperm plasma membrane permeability and changes its protein composition; frozen-thawed bovine spermatozoa were referred to as capacitated or able to capacitate very easily (in 30 min). The aim of the present study was to monitor the pattern of CD9 on freshly ejaculated, frozen-thawed (capacitated-like) sperm and also on sperm during *in vitro* capacitation process using anti-CD9 antibody (mAb IVA-50). The chlortetracycline fluorescence analysis, based on the assessment of Ca-related changes during the capacitation of spermatozoa, was applied to detect the portion of capacitated sperm. When frozen-thawed or freshly ejaculated sperm (capacitated for 30 min or 4 h in TL medium for sperm cell capacitation at 39 °C with 5 % CO_2 in humidified atmosphere) were analysed, comparable results were obtained. The capacitation process did not change the pattern of CD9 molecule on freshly ejaculated and frozen-thawed sperm. IVA-50 reactivity exceeded 77 % in all tested samples and the positive immunofluorescent signal remained unchanged in form of fine grains either on the apical part or through the entire anterior region of the sperm head. When CD9 study was carried out in mice, only 10 % of capacitated or freshly recovered cauda epididymal sperm have been stained and fluorescent signal appeared mainly as a thin line in the acrosomal region. It seems that despite the fact, that CD9 is highly conserved molecule in mammals, species-dependent differences in gamete protein organization are obvious and distinct mechanisms of involvement of CD9 in the fertilization process are assumed.

Key words: spermatozoa; capacitation; CD9 molecule

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THE USE OF MUTANT *LOX66* AND *LOX71* SITES TO TARGET TRANSGENE INTEGRATION AT A PRE-CHARACTERIZED GENOMIC LOCUS

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Genetic engineering strategies usually require permanent modification of the target genome. There are many techniques available for stable integration of transgenes into mammalian cells. However, these methods mostly result in integration at random chromosomal locations of an uncontrolled number of transgene copies that express at levels which generally cannot be predicted or reproduced because of the position effect. The inability to control the site of integration, the number of integrated copies and the expression level has impeded progress in studies of both gene expression and the physiological effects of transgenes. To change this situation, several methods have been developed that enable a targeted integration into stable and consistently expressed genomic loci. Targeting transgenes into pre-characterized loci yields predictable expression patterns due to the invariable transcriptional control exerted by the given endogenous regulatory sequences. While homologous recombination can provide great specificity to the integration process, its efficiency for most biotechnologically relevant cell lines is much too low. In this situation, site-specific integration systems are of increasing relevance, as they provide targeting frequencies at least three orders of magnitude higher than those resulting from homologous recombination.

Cre-mediated site-specific recombination in mammalian cells represents a useful tool for genome engineering, allowing precise and repeated site-specific integration. The strategies are based either on heterospecific *lox* sites carrying mutation(s) in the central 8-bp spacer region (RMCE strategy) or on *lox* sites mutated in the left (L) and right (R) inverted repeat region, allowing integrative recombination. Recombination between an L mutant *lox* and R mutant *lox* sequences results in the generation of a double mutant *lox* site having mutations in both repeat regions and a wild type *lox*P site. The double mutant *lox* site is not an effective substrate for Cre recombinase, therefore the recombination reaction proceeds exclusively in one direction. Integrative recombination is useful tool for the production of transgenic cells or animals because any DNA of interest can be introduced into a chromosomally located *lox* site.

In this work we have demonstrated successful integrative recombination of DsRed gene joined with *lox*71 into pre-characterized genomic locus containing *lox66*-EGFP gene in stable transformed mouse NIH 3T3 fibroblasts. Although the integrative recombination efficiency using *lox71*/*lox66* sites without any selection of recombinants is low, the advantage of this strategy is its simplicity.

Key words: integrative recombination; mouse fibroblasts; Cre recombinase; *lox71*/*lox66*

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THE EFFECT OF ANTLEROGENIC STEM CELLS ON THE QUALITY OF CRYOPRESERVED BOAR SEMEN

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The aim of this study was to assess the effect of nutritional supplementation of a homogenate derived from the antlerogenic stem cells (HOMASC) of *Cervus elaphus* on sperm cell characteristics in frozen-thawed boar semen. The study was carried out on 49 ejaculates collected from 7 boars of Polish Line 990 breed from the experimental group and the same number of animals, ejaculates and the breed of boars in the control group. Only in the experimental group each male received in the ration homogenate formulation of the antlerogenic stem cells at the amount of 1 ml/30 kg body weight, every day for 90 days boars. After supplementation of HOMASC the semen was collected and frozen. The sperm characteristics were assessed using CASA analyzer and flow cytometry. The motility parameters obtained by CASA were: VAP, VSL, VCL, ALH, BCF, LIN, MOT, PMOT and RAPID. Sperm membranes and acrosome integrity (SYBR-14/PI and PNA/PI), assessment of mitochondria activity (JC-1), rupture of DNA (TUNEL), chromatin status (SCSA), membrane fluidity and apoptosis (YO-PRO-1/M540) and membrane lipid peroxidation (C11 BODIPY581/591) were evaluated. The addition of the HOMASC of Cervus elaphus into the boars' diet significantly decreased ($P \leq 0.01$) the percentage of dead sperm in the frozen-thawed semen but also caused an increase in the percentage of dying sperm. Furthermore, the addition the homogenate significantly increased ($P \leq 0.01$) the percentage of live sperm with intact acrosome and decreased the percentage of dead sperm with a damaged acrosome. There was no effect of boars' diet supplementation on apoptotic and capacitation changes in frozen-thawed sperm. After thawing a significant reduction ($P \leq 0.01$) in lipid peroxidation in spermatozoa of boars fed with homogenate was observed. Nutritional intake of homogenate significantly ($P \leq 0.01$) decreased the percentage of sperm with damaged chromatin in frozen-thawed sperm, while no changes were observed in the proportion of immature spermatozoa. The use of the HOMASC for boar feeding significantly ($P \leq 0.01$) increased the percentage of motile sperm, sperm with progressive and rapid movement and significantly ($P\leq 0.01$) increased speed, straightness and linearity of frozen-thawed sperm.

Key words: boar; spermatozoa; cryopreservation; motility; membrane

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MYCOTOXINS AS EPIGENETIC FACTOR

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Mycotoxins are secondary mould metabolites and generally ubiquitous contaminants of food and feed. Epigenetic factors modulate the structure of the chromatin, thereby affecting the transcription of genes in the genome. Changes in chromatin structure are associated with the activation and silencing of gene transcription, and reversible post-translational modifications of histones are related to chromatin structure transitions. Mycotoxins and organic chemicals can interfere in the cascade of cell machinery and thus affect cellular function. Mycotoxins with carcinogenic potency include aflatoxins, sterigmatocystin, ochratoxin, fumonisins, zearalenone, and some *Penicillium* toxins. Most of these carcinogenic mycotoxins are genotoxic agents. Mycotoxin toxicity is exerted via multiple pathways, such as DNA and RNA synthesis inhibition, inhibition of microtubule assembly and of tubulin polymerization, alteration of mitochondrial functionality with consequent increase in reactive oxygen species (ROS) production, inactivation of the heat shock protein and activation of the signal transduction pathway and the caspase-cascade system that results in apoptotic cell death. Based on this, the aim of our study was to determine the activity of superoxide dismutase (SOD), glutathione peroxidase (GPx), and accumulation of Hsp70 in porcine ovarian granulosa cells after deoxynivalenol (DON) and zearalenone (ZEA) exposure *in vitro*. Porcine ovarian granulosa cells were incubated with DON/ZEA administrations for 48 h as follows: group A (10/10 ng/ml), group B (100/100 ng/ml), group C (1000/1000 ng/ml). We found that both mycotoxins induced stress reaction in porcine ovarian granulosa cells and promoted accumulation of Hsp70, what resulted in decreasing activities of SOD and GPx. These results contribute towards the understanding of cellular stress and its response to mycotoxin exposure. Mycotoxin exposure can lead to formation of reactive oxygen species in the body that could activate and deactivate various epigenetic mechanisms leading to the emergency of various diseases.

Key words: mycotoxins; capacitation; epigenetic factors

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ADIPONECTIN MAY PLAY A COMPLEMENTARY ROLE TO INSULIN IN STIMULATED GLUCOSE UPTAKE IN MOUSE BLASTOCYST

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Experimental data indicate that insulin stimulates glucose uptake in mouse blastocysts via IGF-I receptor-mediated

translocation of GLUT8 glucose transporter. In rabbit blastocyst, adiponectin, but not insulin, stimulated glucose uptake, and the increase in glucose transport was associated with translocation of GLUT4 glucose transporter to the cell membrane. The aim of this study was to find out whether adiponectin can stimulate glucose uptake in mouse blastocyst and to examine the expression of GLUT4 in mouse blastocysts. Mouse blastocysts were cultured in the medium supplemented with full-length or globular adiponectin for 2 h, afterwards the blastocysts were transferred to pulse droplet containing 0.3 mM of 3-O-methyl-D-[1-3H] glucose and the radioactivity of embryos was determined in liquid scintillation analyser. We found significantly higher uptake of 3-OMG in blastocysts treated with full-length adiponectin when compared to the control group. Dubious effects were observed after globular adiponectin treatment; therefore, additional measurements are necessary to obtain conclusive results. Using RT-PCR with specific oligonucleotide primers, we detected GLUT4 and GLUT8 transcripts in ICR mouse blastocysts. Moreover, our immunohistochemical study showed the presence of GLUT4 protein in mouse blastocysts. In summary, our results indicate that adiponectin can stimulate glucose uptake in mouse blastocysts. We also confirmed expression of GLUT4 in mouse blastocysts, what suggests possible involvement of this transporter in the adiponectin-stimulated glucose uptake.

Key words: mouse; embryo; glucose uptake; adiponectin

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EMBRYO TRANSFER AND ENDANGERED BREEDS

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Preservation of livestock breeds that may be in danger of extinction is very important. Of the 3831 breeds or breed varieties of donkeys, buffaloes, cattle, goats, horses, pigs and sheep believed to exist or to have existed in the past century, 618 (16 %) have apparently extinct. A few years ago, the Food and Agriculture Organisation (FAO)s Global Data Bank for Domestic Livestock, which carried 2047 entries, showed 221 cattle breeds to be at risk, and most of them (60 %) in the developed countries. In developing countries threats to genetic diversity usually take the form of increased use of AI and indiscriminate crossbreeding of indigenous breeds. The intensification of farming in these countries can mean that indigenous breeds are in danger of being pushed to extinction because native farmers, aiming at greater productivity, employ exotic breeds such as Holsteins and Friesians. A thousand species have been lost during the last centuries and today it is estimated that one-third of breeding animals are threatened to extinction. The disappearance of many breeds has usually been in the name of progress, driven by intensification of food production methods, which has favoured the most productive breeds. Embryo transfer technology is now regarded as a vital tool for genetic preservation of endangered species and breeds. It enables the establishment of embryo banks and embryo to be transferred into populations with decreased biodiversity. In native breeds of cattle, embryo transfer can be used to preserve genetic lines with good maternal characteristics, fertility, adaptation to extreme climatic or nutritional conditions, and natural resistance to disease.

Key words: embryo transfer; livestock breeds; preservation

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EXPRESSION OF ADRENERGIC RECEPTOR TRANSCRIPTS IN MOUSE EMBRYONIC STEM CELLS AND PREIMPLANTATION EMBRYOS

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Adrenaline and noradrenaline were detected in the female reproductive tract, and experimental results indicate that these catecholamines can influence preimplantation embryo development. Moreover, accumulating evidence indicates that catecholamines can influence cell proliferation and differentiation in mouse embryonic stem cells (derived from preimplantation embryos) as well. Using RT-PCR we examined expression of adrenergic receptor subtypes in undifferentiated and spontaneously differentiating mouse embryonic stem (ES) cells, and compared their expression with the expression profiles found in mouse preimplantation embryos. We detected eight adrenergic receptor subtypes in undifferentiated mouse ES cells, but only three subtypes were found in mouse blastocysts. In three adrenergic receptors (α1D, α2B, β1), we found higher expression in the spontaneously differentiating ES cells than in undifferentiated ES cells, and the α1B adrenoceptor was not even detectable in the undifferentiated cells. These results indicate that genes encoding all types of adrenergic receptors are transcribed in mouse embryonic stem cells, and some of them are differentially expressed during ES cell differentiation. In addition, our results showed significant differences in the expression of adrenoreceptor transcripts between embryonic stem cells and mouse blastocysts (from which ES cells are derived).

Key words: catecholamines; embryonic cells; G-protein-coupled receptors

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CHANGES IN THE REACTION PATTERN OF MONOCLONAL ANTIBODY IVA-50 (CD9) ON BULL SPERM AFTER ACROSOME REACTION

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Acrosome reaction is a necessary part of gamete interaction leading to successful fertilization of mammals. This process is initiated by the binding of sperm to *zona pellucida* proteins and helps the sperm get through the *zona pellucida* to enter oocyte plasma membrane. In this study, changes in CD9 distribution during the acrosome reaction of bull spermatozoa were evaluated using mAb IVA-50 that recognizes the CD9 molecule. Tetraspanin molecule CD9 is located in plasma membrane in the acrosomal region of ejaculated as well as capacitated bovine sperm. In our analysis, frozen-thawed spermatozoa were treated and acrosome reaction was stimulated *in vitro* either physiologically (by *zona pellucida*-intact oocytes) or artificially (by calcium ionophore). We can summarize that the ratio of sperm reactive with mAb IVA-50 decreased simultaneously with prolonging the time of induction of an acrosome reaction. Significant differences in number of IVA-50 stained sperm were observed after 40 or 60 minutes of treatment. Obtained results were independent of the way of acrosome exocytosis stimulation. Based on these results, the role of sperm CD9 molecule in the events preceding the sperm-oocyte fusion can be supposed.

Key words: CD9; tetraspanin; bull; sperm; acrosome reaction

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THE ROLE OF ADIPOSE TISSUE IN METABOLISM

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Adipose tissue plays an important role in an active body as it produces many bioactive substances. Adipose tissue is a reservoir of energy that we need. If we have a reasonable amount of fat, fat cells actually produce health-promoting substances. Abundance of fat in the cells results in the production of substances that are involved in the development of serious cardiovascular and cancer diseases or *diabetes mellitus* 2. Adipocytes play an important role in energy and glucose metabolism. In addition to serving as a site for energy storage (in the form of triglycerides), adipocytes act as endocrine cells, secreting molecules that regulate energy expenditure, food intake, and glucose metabolism. Recent findings suggest that the size of adipocytes is a major modulator of their endocrine function. For example, hypertrophic adipocytes secrete greater amounts of tumour necrosis factor α and free fatty acids than normal adipocytes, and this excess secretion has been hypothesized to cause insulin resistance. Obesity is associated with inflammation in adipose tissue, namely an infiltration and expansion of macrophages, which produce inflammatory cytokines that interfere with insulin signalling, and a loss of protective cells that promote adipose homeostasis. Thus, it is now clear that inflammation is an underlying cause or contributor to diabetes II, as well as many other obesity-induced diseases, including atherosclerosis and cancer. Inflammatory pathways contribute to impaired glucose handling by adipocytes, hepatocytes, and muscle cells and interfere with insulin production and insulin signalling. Further investigations are necessary to clarify the contribution of individual cellular components of adipose tissue in order to determine the function of these components as a cohesive unit.

Key words: adipocytes; metabolism; insulin resistance; obesity

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CHANGES IN LIPID PARAMETERS OF RABBIT´S BLOOD FOLLOWING APPLICATION OF PATULIN

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Excessive energy load, in combination with inflammation-impaired *de novo* adipogenesis, results in the hypertrophy of existing adipocytes that eventually fail to store fatty acids, resulting in their leakage into circulation and infiltration into other organs. The aim of the submitted abstract was to find out an impact of intramuscular application of patulin in concentration 10 $\mu g.kg^{-1}$ of body weight on the parameters of lipid metabolism (cholesterol, triacylglycerol - TAG, high density lipoproteins - HDL, low density lipoproteins - LDL) of rabbit's blood. Patulin was administered two times per week. Female rabbits of maternal albinotic line and paternal acromalitic line were used. The animals were divided into experimental group E1 and the control group C. The water and feed were available *ad libitum*. After 30 days of feeding rabbits were slaughtered and blood samples (n = 5 in each group) were obtained. The average value of cholesterol (2.51 ± 0.69 $mmol.l^{-1}$), serum concentration of TAG (1.08 ± 0.40 $mmol.l^{-1}$), value of HDL (0.86 ± 0.20 $mmol.l^{-1}$) and LDL (0.72 ± 0.33 $mmol.l^{-1}$) in the experimental group did not differ significantly ($P > 0.05$) compared to the control group (2.20 ± 0.62 $mmol.l^{-1}$, 0.71 ± 0.26 $mmol.l^{-1}$, 0.51 ± 0.40 $mmol.l^{-1}$ and 0.39 ± 0.28 $mmol.l^{-1}$, resp.). We assume that the particular dose of patulin used in our study was probably not enough to change all the investigated parameters.

Key words: patulin; cholesterol; TAG; HDL; LDL; rabbit

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POLYMORPHISM OF BOLA-DRB3 GENE AND ITS ASSOCIATION WITH SPONTANEOUS EMBRYONIC LOSS AFTER *IN VITRO* FERTILIZATION IN HOLSTEIN-FRESIAN CATTLE

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The primary function of the major histocompatibility complex (MHC) is the induction of the immune response. It has been also suggested that the MHC genes may play a role in mate selection. The main hypothesis about their role assumes that animal sexual preferences stem from and are driven by the pursuit of the greatest genetic diversity at MHC loci among mates which should result in heterozygous offspring. It has been confirmed by studies on rodents, lemurs, human etc. BoLA- DRB3 is the most polymorphic gene of the major histocompatibility complex in cattle. Many studies showed that polymorphism at BoLA- DRB3 locus was associated with susceptibility or resistance to some infectious diseases in cattle (mainly with susceptibility to clinical and subclinical mastitis in dairy cattle). Furthermore, in the study of Kovalyuk *et al*. (2012) it was observed that in case of using semen of bulls with the rare BoLA- DRB3 alleles, the number of semen doses per effective insemination was at least 15 % lower than in case of using semen of bulls carrying common alleles. The aim of our study was to analyse the association between polymorphism at the BoLA-DRB3 locus and embryonic loss after *in vitro* fertilization. Bovine oocytes recovered from bovine ovaries at slaughtering were matured in E199 medium with 10 % FCS and 1IU FSH and LH and fertilized *in vitro* with sperm of Holstein-Fresian bull during 20 h. Presumptive zygotes were cultured in ISM1 culture medium for three days, afterwards the embryos were evaluated and selected into two groups: 1) cleavage (2-, 4-, 6- or 8- cell stage) and 2) undeveloped embryos (no cell cleavage). The genotypes at BoLA-DRB3 locus of 72 developed and 119 undeveloped embryos were identified by PCR-RFLP method using RsaI, HaeIII, BstUI and PsuI endonucleases. From among 24 identified alleles the most frequent was DRB3.2*24 which is typical for Holstein- Fresian cattle. It was found that embryonic mortality was significantly associated with homozygosity at BoLA-DRB3 locus.

Key words: cattle; embryo; *in vitro*; MHC; BoLA gene

MORPHOLOGICAL CHANGES OF MITOCHONDRIAL-ENDOPLASMIC RETICULUM ASSOCIATION DURING *IN VITRO* FERTILIZATION OF BOVINE OOCYTES

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Bovine oocytes used for *in vitro* fertilization (IVF) are characterized by the level of both nuclear and cytoplasmic maturation. One of the most important parameters of cytoplasmic maturation is mitochondrial status of oocytes. Mitochondrial clusters are formed as large circular agglomerates around the peripheral network of endoplasmic reticulum (ER) during oocyte *in vitro* maturation (IVM). The association of mitochondrial clusters with ATP reserves has been verified. The aim of this study was to characterize the morphological changes of mitochondrial-ER association in bovine oocytes during maturation and fertilization. The oocytes were collected from the ovaries of slaughtered cows and selected according to the ooplasm and cumulus morphology. Only the oocytes suitable for IVM-IVF procedures were matured for 24 hours using a standard protocol. Subsequently, the oocytes were transferred into IVF-Talp medium and either inseminated with spermatozoa of a proven bull or cultured without spermatozoa in the same medium. Adequate number of oocytes was collected at 24 h after maturation and at 6, 12 and 18 h after insemination or culture. They were stained with Sytox-Green for both chromatin and mitochondria visualization and with Calnexin antibody for ER network morphology, and assessed by confocal microscopy. About one half of mature oocytes (49.2 %) showed mitochondrial clusters after maturation. The proportion of oocytes with mitochondrial clusters decreased significantly ($P \leq 0.01$) in the fertilized oocytes (12.5 %) but, on the other hand, it was not changed in the unfertilized oocytes (47.5 %) or oocytes cultured without spermatozoa (47.1 %) at the 6h interval. The proportion of the oocytes with clusters even decreased ($P \leq 0.05$) in the fertilized oocytes (1.1 %) or in the oocytes cultured without spermatozoa (16.7 %), but it increased to 62.1 % in the unfertilized oocytes at the 12h interval. The proportion of oocytes with clusters was not changed in the fertilized oocytes and decreased in the unfertilized oocytes (35.1) or the oocytes cultured without spermatozoa (21.1 %) at the 18h interval. It can be concluded that mitochondrial status of bovine oocytes changes during IVM-IVF. While in the fertilized oocytes the mitochondrial-ER association is fast disintegrating due to the fertilization process, in the unfertilized oocytes it is maintained for 12 hours following the oocyte contact with spermatozoa.

Key words: bovine; oocyte; *in vitro* fertilization; mitochondrial morphology

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CAPACITATION – INDUCED BULL SPERM PROTEIN CHANGES DETECTED BY A SET OF MONOCLONAL ANTIBODIES

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The process of fertilization is characterized by complex set of events. Before spermatozoa can fertilize an oocyte, it must undergo a cascade of biochemical and physiological changes that facilitate its binding and penetration into the oocyte. The sperm acquire ability to fertilize oocyte in a female

genital tract in a time-dependent process called capacitation. This process involves reorganization of membrane proteins, an increase in membrane fluidity, cholesterol efflux, ion fluxes resulting in alteration of sperm membrane potential, increased tyrosine phosphorylation of proteins and induction of hyperactivation.
In our study a set of 34 anti-sperm monoclonal antibodies (mAbs) was used to detect changes of sperm surface protein reaction patterns after capacitation. MAbs were produced using hybridoma cell lines obtained after intrasplenic immunization of BALB/c mice with intact bull sperm. Capacitation was induced by TL Sperm capacitation medium. The changes in the reaction patterns were evaluated by indirect immunofluorescence, PAGE-SDS and two-dimensional gel electrophoresis followed by detection with anti-sperm mAbs and anti-phosphotyrosine α-PY antibody to detect changes in phosphorylated protein spectra. The changes were observed in the reaction patterns of mAbs and in the percentage of reactive sperm. In the indirect immunofluorescence the percentage of reactive sperm after treating with mAbs IVA 508-1, IVA 513-16, IVA 519-19, IVA 520-4, IVA 526-7, IVA 527-10 and IVA 582 increased in comparison with control. The percentage of reactive sperm after treating with mAbs IVA 517-1, IVA 520-41 and IVA 520-42 decreased in comparison with control. Western blot analysis showed changes in molecular weight of proteins detected by mAbs IVA 519-3, IVA 520-4, IVA 520-41, IVA 520-42 and IVA 527-10. Two-dimensional electrophoresis of proteins detected by mAbs IVA 520-4, IVA 520-41, IVA 520-42 and IVA 527-10 showed that reactive region changed to less acidic region after capacitation.

Key words: bull; sperm; capacitation; mAb

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EFFECTS OF THE HPA LECTIN ON RABBIT SPERM MOTILITY AND FERTILITY

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The aim of this study was to test the effect of the HPA-lectin (the membrane biomarker with affinity to the epitope N-acetyl-α-D-galactosamine) added to the insemination dose on motility and conception rate of broiler rabbits.
The experiments were performed on males (18) and females (403) of rabbit broiler lines M91 and P91 bred at VÚŽV-NPPC Nitra and kept in a partly air-conditioned experimental hall. The CASA (Computer Assisted Sperm Analysis) system SpermVision (Minitüb, Tiefenbach, SRN) was used for the evaluation of sperm motility. To evaluate the conception rate, the control and experimental (with HPA-lectin) females were artificially inseminated (AI) with fresh heterospermic doses (0.5 ml per female). 48 hours before A.I. each female was treated with 25 I.U. PMSG (Sergon, Bioveta, Czech Republic). Immediately after the A.I. all females were intramuscularly injected with 2.5 ug of synthetic GnRH (Supergestran, Ferring Pharmaceuticals, Czech Republic). All insemination doses (ID) were diluted with a commercial diluent Minitüb (Verdünnungsmischung for Kaninchensperma with antibiotics, Germany). The sperm concentration was not less than 15.0 x 10^6 cells per insemination dose. The insemination doses were supplemented with HPA - *Helix pomatia* (garden snail, HPA) lectin (20 µl per 0.5 ml ID) and then incubated at room temperature for 0, 30 and 60 min. The differences between the experimental (with HPA lectin) and control group in the parameter VSL – straight-line velocity (µm/s) - were highly significant at 0 min ($p = 0.0015$), and a significant at 30 min ($p = 0.0497$), whilst non-significant at 60 minutes (0.4914). The motility of spermatozoa was increasing at 0 and 30 min and decreasing at 60 min. Therefore, for female insemination sperm samples at 30 min incubation with HPA lectin were chosen. Average number of live-born kits (8.93 pcs) and conception rate (56.35 %) in the group with HPA lectin were not significantly different compared to the control group (8.21 pcs; 53.79 %, resp.). The obtained results suggest a positive trend of HPA-lectin effect on sperm motility. However, HPA-lectin did not improve female fertility compared to control. In conclusion, although HPA-lectin seems to be suitable additive to improve rabbit sperm motility, however motility parameters may not be in direct association with rabbit female fertility.

Key words: HPA-lectine; rabbit; sperm; motility; conception rate; fertility

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PRESENCE OF INTEGRIN MOLECULE CD18 IN THE CATTLE REPRODUCTIVE SYSTEM

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Integrins are transmembrane receptors that are the bridges for cell-cell and cell-extracellular matrix (ECM) interactions. Integrins have two different chains, α (alpha) and β (beta) subunits. In mammals, there are eighteen α and eight β subunits. A given chain may combine with multiple partners resulting in different integrins. In molecule CD18 the β chain may be paired with four different α subunit (11a, 11b, 11c, 11d). CD18 is important in adhesive interactions of leukocytes and endothelial cells. In cattle the mutation in CD18 gene is leading to BLAD (Bovine Leukocyte Adhesion Deficiency) which causes extreme susceptibility to infection of diseased animals. Participation of CD18 in cell-cell adhesion suggested the possible role of this molecule in spermatozoa – egg fusion or in other steps of reproduction processes. The possible role of CD18 (integrins) in reproduction was studied in man and some animal species. Several experimental results support the involvement of egg integrins during sperm - egg interaction.

It was found by immunofluorescence studies that both hamster and human oocytes expressed CD11b/CD18 (MAC/1) a β2 class antigen, which could mediate the sperm egg binding (Fénichel and Durand-Clément, 1998). The aim of this study was to identify the distribution of the CD18 molecule on bovine gametes and reproductive tissues.

Tissues for the histochemical staining: bovine testis, epididymis, ovary and accessory glands have been obtained at local slaughterhouses. Ejaculated spermatozoa were received from Slovak Breeding Services Inc. The oocytes at different developmental stages were obtained after the culture in *in vitro* maturation medium. The CD18 molecule was detected by indirect immunoperoxidase test (tissue sections and oocytes) and immunofluorescence test (spermatozoa) using IVA-35 the anti-CD18 monoclonal antibody. The presence of CD18 on spermatozoa and male reproductive tissues was analysed during the spermatocytogenesis in the seminiferous tubules of the bull testis and different parts of epididymis. In the cross-sections of the testes and epididymis tested in immunoperoxidase assay no positive reaction was found either in the tested tissue or in the developmental forms of spermatozoa. Positive reaction was found in luminal secrets of the epididymis, probably with soluble form of CD18. In the immunofluorescence assay only the minor population of ejaculated spermatozoa was slightly reactive. In the cow reproductive tract the reaction with CD18 molecule was found in the horn of uterus; the follicular and *in vitro* cultured oocytes were also positive.

The study demonstrates the presence of CD18 in some organs of the reproductive tract of cattle, but the expression of CD18 molecules on gametes was not detected unambiguously, although weak reaction of cow oocytes with CD18 antibody was noted.

Key words: cattle; reproductive tract; CD18; integrins

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NUCLEOLOGENESIS IN EARLY PORCINE EMBRYOS

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The initial development of mammalian pre-implantation embryos is governed by gene transcripts and polypeptides produced by and stored in the oocyte during its development. The transition from maternal to embryonic control is accompanied by a series of morphological and physiological changes. The nucleolus is the organelle that morphologically reflects changes in the metabolism and physiology of the cell. Our study investigated nucleolar dynamics (as one viability marker) in porcine embryos developed *in vivo* (IVD) and compared this physiological standard to that of embryos produced by *in vitro* fertilization (IVF), parthenogenetic activation (PA), or somatic cell nuclear transfer (SCNT). At the ultrastructural level porcine IVP zygotes and embryos display a well-synchronized pattern of chromatin dynamics compatible with genome activation and regular nucleolar formation at the four-cell stage. Production of porcine embryos under *in vitro* conditions (IVP) by IVF, PA, or SCNT is associated with altered chromatin remodeling, delayed nucleolar formation, and poorly defined lineage segregation at the blastocyst stage. The intranuclear localization of nuclear proteins is observed towards the end of the third cell cycle in IVD. IVP embryos lack labelling for topoisomerase I, and the allocation of remaining nuclear proteins is delayed by one cell cycle. Nucleologenesis in porcine IVP embryos is markedly disturbed and this may be one reason for the high rate of embryonic and fetal mortality.

Key words: nucleolus; porcine pre-implantation embryos; viability

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EFFECT OF SELECTED PLANTS ON RABBIT OVARIAN FUNCTIONS

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Rooibos, chia and yucca are plants widely used in folk medicine, food and drink production, but their influence on reproductive processes has been studied insufficiently. The aim of this study was to examine possible action of rooibos, chia and yucca on the release of hormonal regulators of reproduction (progesterone, P4, testosterone, T, estradiol, E and insulin-like growth factor I, IGF-I) and on fecundity in rabbits. Fragments of rabbit´s ovaries were incubated with rooibos, chia and yucca for 48 hours. Hormones were determined by RIA. It was observed that rooibos addition to the culture medium inhibited P4 and T release. IGF-I release was significantly stimulated by rooibos at all doses added. Moreover, chia addition had inhibitory effect on P4 and T release, but not on IGF-I release. P4 release was stimulated after yucca treatment at all doses added. Yucca addition did not affect testosterone or estradiol release. Preliminary *in vivo* experiment showed that feeding of rabbits with yucca significantly increased their conception and kindling rate. The differences between control and yucca-treated groups in the number of liveborn, stillborn and weaned pups per doe

were not statistically significant. Results of this study suggest the multiple sites of action (release of hormones, conception and kindling rate) of these medical plants on rabbit reproductive functions and their potential applicability for improvement of reproductive efficiency.

Key words: rabbit; ovary; plant extract; reproduction

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THE EFFECT OF CROSSING TSIGAI EWES AND IMPROVED VALACHIAN WITH LACAUNE EWES ON MILKABILITY

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Sheep farming has a long tradition in Slovakia. In the 11th-12th centuries, sheep constituted 5 % of the total number of bred animals. Their original use was in the production of milk, meat, wool and fur. However, besides the production of lamb meat, only the production of milk is economically interesting today. Dual-purpose breeds Tsigai (TS) and Improved Valachian (IV) are the most-common sheep breeds in Slovakia. They have lower milk production than typical milk breeds (e.g. Lacaune-LC), but they are more robust what is important for our breeding conditions. At present, in order to improve their milk yield and milkability TS and IV breeds are crossed with Lacaune. The aim of this work was to summarise knowledge from our laboratory related to the evaluation of milkability of main sheep crossbreds used in Slovakia (project MLIEKO No. 26220220196, KEGA 006SPU-4/2014). It was demonstrated that crossbreds have higher machine milk yield and total milk yield than purebred TS and IV, but there is a tendency of higher machine stripping yield compared to the purebred animals. The effect of breed is also evident in different occurrence of milk flow types indicating different physiological response of ewes to milking stimulation. From three types of milk flow curves (1 peak - no response, 2 peak - positive response, and plateau - indicating high milk production and possible positive response) classified in sheep during machine milking, the lowest occurrence of 1 peak milk flow was noted in crossbred animals as compared to purebred animals. This can indicate a possibly higher sensitivity to udder stimulation by machine and better adaptation of both crossbred ewes to machine milking than purebred TS and IV. However, the crossing with LC negatively influenced the teat position in TS × LC and IV × LC crossbreds causing above mentioned higher stripping yield of milk. This should be taken into consideration for future breeding programmes. With the crossing, some parameters of milkability had been improved, but some parameters of udder morphology had been worsted, what could negatively affect milk out of the udder without stripping. When machine stripping yield is high, the intervention of the milker during machine milking is necessary. This may lengthen the time of milking and reduce labour efficiency during milking.

Key words: dairy ewes; crossing; milkability; udder morphology

IMMUNOPHENOTYPING OF THE RABBIT ADULT STEM CELLS

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There are three criteria that cells require to meet in order to be qualified as a stem cell: 1) they need to be capable of self-renewal, i.e. undergoing symmetric or asymmetric divisions; 2) a single cell must be capable of multilineage differentiation and 3) capacity for *in vivo* re-population and functional reconstitution of a given tissue. Stem cells are able to differentiate into cell types beyond the tissues in which they normally reside. This is often referred to as stem cell plasticity. Stem cells are also believed to be slow cycling but highly clonogenic and generally represent a small percentage of the total cellular make-up of a particular organ. Adult stem cells (ASCs) are multipotent stem cells that can differentiate into a range of cell types related to the tissue of origin of these cells, so that their differentiation potential is limited compared to pluripotent stem cells. They constitute a pool of cells that proliferate and differentiate into cells required to maintain the integrity of the tissue, especially in case of injury. Most common ASCs used in biomedicine are hematopoietic (HSCs), mesenchymal (MSCs) and amniotic fluid stem cells (AFSCs). HSCs generate all the blood cells and can thus be considered as being multipotent and capable of regenerating the complex hematopoietic system. Although the phenotype of human and mouse HSCs is well-known, the phenotypic expression of the rabbit HSCs is still unclear. Regarding the MSCs, according to the International Society for Cellular Therapy, the human MSCs are defined as cells that are (i) plastic adherent; (ii) express typical surface molecules e.g. CD105, CD73 and CD90 etc.; and (iii) can differentiate into osteogenic, chondrogenic and adipogenic lineages. Rabbit MSCs share some markers with human MSCs. AFSCs clearly display a unique phenotype that is mostly multipotent but borders on pluripotency. Their phenotype is very similar to that of MSCs. Adult stem cells can be isolated using different methods, e.g. magnetic-activated cells sorting (HSCs, MSCs) or plastic adherence (MSCs, AFSCs). These cells could be then identified and recognized through:

(i) their morphology observed by phase contrast microscopy; (ii) ultrastructural analysis using transmission electron microscopy (TEM); (iii) CD marker expression, detected using immunostaining, reverse transcription-polymerase chain reaction (RT-PCR) and flow cytometry analysis; and (iv) differentiation ability using a commercial assay (e.g. osteogenic, adipogenic and chondrogenic differentiation assay; Tan *et al*., 2013). The importance of the animal ASCs for the agricultural applications lies in the preservation of the animal gene resources via these cells.

Key words: rabbit; adult stem cells; FACS; PCR; TEM

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DIETARY EFFECT OF *LIPPIA CITRIODORA* EXTRACT ON SEMEN QUALITY CHARACTERISTICS IN MALE HARES (*Lepus europaeus Pallas*, 1778)

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Natural antioxidants have been widely reported to have potent antioxidant, anti-inflammatory, and antimicrobic activities related especially to their phenolic content.

The aim of the present study was to evaluate the effect of dietary supplementation with a natural extract of *Lippia citriodora*, titrated in verbascoside, on some quality traits of semen in male hare. Welfare status of animals was also monitored.

Hares were randomly divided into four groups of 3 animals each, homogeneous by age and body weight, and fed *ad libitum* and free access to water until the end of the trial. Animals were fed for 240 days a commercial diet assigned to four dietary treatments: control diet (CON) and diet supplemented with 1 g.kg^{-1} of natural extract (low natural extract - LNE) or 1.5 g.kg^{-1} of natural extract (medium natural extract - MNE) or 2 g.kg^{-1} of natural extract (high natural extract - HNE). All hares were subjected to the following experimental measurements: weekly pattern of feed intake, body weight and blood samples at 0 days and a 240 days of trial, and semen collection at 180 days, 210 days and 240 days of trial.

The body weight and feed intake of the hares were not affected by the experimental treatment. At the end of the trial, sperm volume, pH and sperm concentration values were not affected by *Lippia citriodora* extract treatment, and the mean values recorded were 0.543 ml, 7.4 and 263.25x10^{6} per ejaculate, respectively. The dietary treatment negatively affected ($P < 0.05$) the sperm motility values in LNE, MNE and HNE groups.

In conclusion, the results of the present work underline a possible negative effect of the *Lippia citriodora* extract on the semen quality characteristics, besides the improvement in welfare status of the treated hares, expressed by a better lipid profile and improved plasma oxidative markers.

Key words: antioxidant supplement; biochemical parameters; hare spermatozoa

CONSERVATION OF ANIMAL GENETIC RESOURCES IN THE REPUBLIC OF SERBIA

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Organized work on the conservation of animal genetic resources in Serbia started in late 1994 by identification and creation of inventory of existing domestic animal breeds and strains. There were a total of 35 identified and described breeds of cattle during this period, and data on them are forwarded to the central database of FAO, Rome. Conservation of indigenous breeds and strains started at the same time. For each breed included in the program of conservation it is anticipated to form three herds in accordance with the FAO program.

Agricultural Strategy of the Republic of Serbia (2005) contains elements related to the conservation and sustainable use of biodiversity, i.e. animal genetic resources. The main elements of this strategy are related to: identification and monitoring of biodiversity and processes, i.e. activities that have a significant negative impact on the state of biodiversity *in situ* and *ex situ* conservation of biodiversity, access to genetic resources, access to biotechnologies and their transfer, exchange of information, technical and scientific cooperation in the field of conservation and sustainable use of biodiversity, distribution of benefits and others.

Criteria of breed selection for conservation must be multiple and well and reasonably chosen. Criteria must respect the potential value of the breed, that is, the genetic constitution and eventually useful genes for future research at breeder discretion. The possibility of losing a breed is also one of the important criteria, because once lost genes or gene combinations can never be brought back in any way. In addition to these criteria, we should take into account the economical, social, cultural and other aspects of conservation. When it comes to population size, analysis of domestic animals species and breeds encountered in the Republic of Serbia shows that many of them are endangered and disappearing: Podolian cow, Busha cattle, Domestic buffalo, Mountain horse, Nonius, Balkan donkey, Mangalitsa, Morava pig, Resava pig, Pirot sheep, Bardoka sheep, Krivi Vir sheep, Karakachan sheep, Lipa sheep, Valachian sheep, Choka Tsigai sheep, Balkan goat, Svrlig chicken, Banat naked-neck chicken and Sombor capor chicken.

Key words: animal biodiversity; genetic resources; conservation

YUCCA SCHIDIGERA PLANT EXTRACT NEGATIVELY AFFECTS RABBIT EMBRYO DEVELOPMENT *IN VITRO*

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The goal of this study was to examine the effect of *Yucca*

schidigera plant extract (YSE) on the rabbit embryo development *in vitro*. Zygotes at the pronuclear stage were flushed from the oviducts of hormonally stimulated rabbit females and subsequently cultured *in vitro* in k-DMEM medium supplemented with 10 % of fetal bovine serum and different concentrations of YSE (E1 - 0,1 $mg.ml^{-1}$; E2 - 0,01 $mg.ml^{-1}$; E3 - 0,001 $mg.ml^{-1}$) up to the blastocyst stage (120h). At the end of culture period the blastocysts were stained with DAPI fluorochrome for the total cell number determination.
Although there were no significant differences between the experimental (E1 - 38.7 ± 23.6; E2 - 56.7 ± 36.6; E3 - 44.6 ± 32.1) and the control (C - 59.4 ± 30.0) groups in the blastocyst rate, our results suggest that increasing of YSE concentration by 0.1 $mg.ml^{-1}$ negativelly affected developmental potency of embryos. Negative effect of the highest concentration of YSE (0.1 $mg.ml^{-1}$) in the culture medium was manifested also in the decrease ($P < 0.05$) of blastocyst total cells number (E1 - 68.4±14.2), when compared to the control (C - 90.6 ± 21.0). Lower concentrations of YSE in culture medium had no effect on the blastocyst total cell number (E2 - 80.2 ± 25.6; E3 - 81.8 ± 16.1) compared to the control (C - 90.6 ± 21.0). According to our results we can conclude, that higher concentration of *Yucca schidigera* plant extract added to culture media negatively affected embryo total cell number and blastocyst rate.

Key words: rabbit; embryo; *Yucca schidigera* extract; *in vitro* development

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THE REPRODUCTION AND GROWTH CHARACTERISTIC OF ORAVKA CHICKEN

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The reproduction and growth characteristics of two inbred (OR 1 and OR 2) and one outbred (OR 3) lines of Oravka chicken were compared. Oravka is a dual-purpose chicken breed. There are important not only egg production but also the meat production. The *in situ* conserved flock kept at the National Agriculture and Food Centre - Research Institute for Animal Production (RIAP) Nitra was involved in the experiment. All three lines were of the same origin. The males and females were progeny of the same cock.
The females were randomly divided into three lines. In two lines the males from the same lines – half-sibling were used as father. In the third line the non-relative cock from the private breeder was used as a father. The reproduction (fecundity, embryonic mortality, hatchability) and growth characteristics were examined.
We observed the highest hatchability and lowest embryonic and postembryonic mortality in the outbred line. In inbred lines we also observed more chicken that were culled due to degenerative changes of limbs, substandard color etc. In the outbred line this fact was observed rarely.
The fecundity in lines OR 1, OR 2 and OR 3 was 89.62 %, 84.21 % and 85.71 % respectively. The hatchability from fertilized egg was 80.50 %, 80.26 % and 91.67 % respectively. The chicken of outbred line had higher live weight in all categories when compared to inbred lines. Significant difference was recorded between outbred line OR 3 (450.47 ± 64,45 g) and inbred line OR 1 (491.43 ± 100.30 g) at 5 weeks age ($P = 0.0027$).
According to our results we can conclude that outbred line had better reproduction characteristics than inbred lines. The outbred line chickens had higher live weight than inbred ones.

Key words: Oravka breed; reproduction; growth

MOLECULAR SCREENING FOR SINE INSERTION IN THE MITF GENE IN DOGS

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SINEs (Short Interspersed Elements) are mobile transposons of approximately 100-400 bp. SINEC_Cf is the most abundant canine -specific SINE in dog genome and it is estimated to be present in half of all genes.
Most SINE insertions induce no damage to the host genome, because only a small part of canine genome is functional. The results of many genetic studies suggest that SINE insertions play a significant and major role in mammalian evolution and the phenotypic diversity of dog breeds. Occasionally, SINEs insertion in coding regions can disrupt ORFs, modulate gene expression, alter splicing, create genomic deletions or have a post-insertional impact through unequal homologous recombination.
A SINEC_Cf insertion located 3.5 kb upstream of the MITF 1M promoter of the MITF gene causes piebald spotting in numerous canine breeds due to the predicted altered transcription of the MITF-gene (Schmutz *et al*., 2009). Here we present a newly developed efficient strategy for targeted rapid SINEC_Cf identification by RFLP analysis of the MITF gene.

Key words: MITF; SINE; coat color; canid

EFFECT OF AMYGDALIN AND APRICOT KERNELS ON DEVELOPMENTAL RATE AND QUALITY OF RABBIT EMBRYOS

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The aim of our study was to examine the effect of amygdalin and apricot kernels on developmental rate and quality of rabbit embryos. In our experiments 12 rabbit females (Californian rabbit) were used. The animals were divided into three experimental groups (IM, D1, D2) and the control group. The IM group was intramuscularly injected with amygdalin isolated from apricot kernels (≥ 99 % purity; 0.6 $mg.kg^{-1}$ of live weight); D1 and D2 groups were fed by a mixture of commercial diet and apricot seeds (D1: 60 $mg.kg^{-1}$ of live weight, D2: 300 $mg.kg^{-1}$ of live weight) during 4 weeks period. A total of 135 pronuclear stage zygotes were collected from superovulated rabbit females 19 h *post coitum* and cultured

under *in vitro* conditions (38.5 °C, 5 % CO_2) for 72 h to reach blastocyst stage. Afterwards, these embryos were analyzed for the developmental rate, embryo cell number (DAPI) and incidence of the dead (propidium iodide, PI) or apoptotic (Yo-Pro-1) cells using fluorescence labelling. A one-way ANOVA were used to analyze differences between groups, and data shown are least squares means ± standard error of the mean. No significant differences were found in the blastocyst stage between control (C; 77.14 ± 19.04) and experimental (IM – 60.6 ± 22.8; D1 – 73.53 ± 30.12; D2 - 66.97 ± 20.91) groups. In regards to the embryo quality, average total cell number in the experimental groups (IM – 100.42 ± 16.16; D1 - 56.07 ± 15.65; D2 - 102.02 ± 29.11) did not differ significantly from control group (83.3 ± 13.15). Also, no any differences were noticed in the incidence of dead or apoptotic cells between these groups. In conclusion, amygdalin had no effect on developmental potential and quality of rabbit embryos.

Key words: rabbit; embryo; amygdalin; apoptosis

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CHARACTERIZATION OF MACROPHAGES IN RABBIT SEMEN

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We aimed at evaluating the occurrence of macrophages in rabbit semen and investigating their impact on the spermatozoa quality. We compared the detection methods using Neutral Red dye, fluorescently conjugated acetylated low-density lipoprotein (AcLDL) and monoclonal antibody CD14. Fresh semen samples collected from broiler rabbit lines M91 and P91 (n = 30) were used. Subsequently, the rabbits were divided into two groups according to semen macrophage concentration, and semen quality was compared in two heterospermic samples. We applied Computer Assisted Semen Analysis (CASA) system to determine motility parameters. Sperm viability parameters, such as occurrence of apoptotic (Yo-Pro-1) and dead/necrotic (propidium iodide) sperm and damage to sperm plasma membrane integrity (PNA) were determined using flow cytometry. Obtained results were evaluated statistically by t-test using SigmaPlot software and expressed as the means ± SEM. The concentration of macrophages in the control group was $0.24 \pm 0.06 \times 10^6.mL^{-1}$ (n = 16), whilst in the experimental group it was $9.02 \pm 0.29 \times 10^6.mL^{-1}$ (n = 4). Concerning the used methods, no significant differences in the number of identified macrophages either by Neutral Red, AcLDL or CD14 methods were found: 0.21 ± 0.11 vs. 0.25 ± 0.07 vs. 0.23 ± 0.02 in the control, and 8.82 ± 0.38 vs. 9.15 ± 0.32 vs. $9.08 \pm 0.19 \times 10^6.mL^{-1}$ macrophages in the experimental groups, respectively. The total motility and progressive movement were decreased in the experimental group ($P < 0.001$). Significantly increased proportions of the apoptotic and necrotic spermatozoa and spermatozoa with disruptions of the plasma membrane integrity in the experimental group were noticed. In conclusion, staining of semen macrophages using either of the methods (Neutral Red, AcLDL dyes or CD14 monoclonal antibody) is similarly reliable. Based on our results, higher presence of macrophages in rabbit semen may have negative effect on some parameters of spermatozoa evaluated *in vitro*.

Key words: macrophages; rabbit; spermatozoa; CASA; flow cytometry

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QUANTIFICATION OF BOVINE GENES INVOLVED IN LIPID METABOLISM USING REAL-TIME RT-PCR

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Molecular genetic markers underlying the complex traits of meat production in cattle were subjected to extensive scientific research in the last few decades because the genes involved in regulation of growth and quality of carcass are important in terms of profitability for farmers as well as for beef industry. The aim of this preliminary study was to analyze the developmental change in the relative gene expression of both genes involved in fatty acid metabolism (*ACACA, DGAT1, FABP4*) and myogenic regulatory factors *(MYF5, MYOD1* and *MYOG*) in the longissimus lumborum muscle (MLL) of Czech Fleckvieh Cattle throughout its life cycle. Acetyl-CoA carboxylase alpha (*ACACA*), Diacylglycerol O-acyltransferase 1 (*DGAT1*) and Fatty acid binding protein 4 (*FABP4*) take part in triglyceride synthesis and play an important role in lipid metabolism. They are associated mainly with variations in the fatty acid (FA) contents in muscles, intramuscular fat deposition and beef marbling. Myogenic factor 5 (*MYF5*), Myogenic differentiation factor 1 (*MYOD1*) and Myogenin (*MYOG*) belong to the myogenic regulatory factor family of transcription factors that regulate myogenesis, skeletal muscle differentiation and fibre development in Vertebrata. Their genes are suitable candidates for molecular markers of growth, economically relevant body measurement traits and meat production traits in cattle. In this study the biopsy samples of the MLL were collected at age of six or twelve months from three bulls and three heifers of Czech Fleckvieh cattle. The animals were sampled also immediately after slaughtering at the age of 18 mo. Relative levels of target and reference gene mRNA were determined using two-step real-time reverse transcription qPCR with gene specific Taqman hydrolyses probes. Analysis of qPCR experimental data was carried out automatically with qBase+ Premium software (Biogazelle, Belgium). The relative expression was calculated for each sample as a ratio of the target

gene mean *Cq* (threshold cycle) to the 3 reference genes mean *Cq* using the Pfaffl formula. The relationship between the particular gene expressions was evaluated by means of Pearson´s correlation coefficient. Results pointed out the differences among relative levels of mRNA transcripts of selected genes with regard to age and sex of animals. A higher relative gene expression was measured in the biopsy samples from heifers (i.e. at age 6 or 12 mo) compared to their slaughter samples (i.e. at age 18 mo). In addition, the expression levels found out in the 18 mo heifers were the lowest and simultaneously the most balanced throughout the study. Gene expressions ascertained in bull samples were the highest in their early age and at once in the 18 mo of age and lowest in the 2nd biopsy samples at the age of 12 mo. The RNA transcripts of the myogenic regulatory factor genes were expressed at a higher level than genes of fatty acid metabolism (except of *FABP4*) in the first taken biopsy samples. Expression of the FA metabolism genes was consistent in the biopsy samples of heifers but their considerable decrease was apparent in bulls of 12 mo. Then the oldest bulls showed again the elevated expression of the FA metabolism genes. Pearson´s correlation test revealed a strong positive correlation among all myogenic regulatory factor genes, ranging from 0.795 to 0.953, which indicates their synchronized action in the bovine muscle metabolism. Correlations between the relative gene expressions of *ACACA* and *DGAT1* or *FABP4* were positive and reached rather the middle level around 0.5. Although the experimental design of the study was proved as suitable, a further research is needed to expand these preliminary findings.

Key words: biopsy; cattle; fatty acid; genetic expression; muscle; myogenic factor

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VIABILITY OF RABBIT MESENCHYMAL STEM CELLS DURING EARLY PASSAGING

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Rabbit is a suitable biological model for stem cell experiments due to cellular and tissue physiology which closely resembling human mesenchymal stem cells (MSCs). However, human MSCs are well defined by surface markers; the expression of specific markers is the matter of many studies of rabbit stem cells. The aim of our preliminary study was to evaluate viability and apoptosis of MSCs from early passages (P1-P3) and to detect surface marker proteins that identify MSCs. Briefly, we harvested rabbit mononuclear bone marrow cells of three New Zealand White line rabbits. Cells were separated by gradient density centrifugation. Cells from the suspension were counted, mixed with α-MEM medium supplemented with 20 % FBS and seeded into tissue culture flasks. Medium exchanges were performed every 3-4 days. Upon reaching about 70 % confluence MSCs were trypsinized. MSCs from passages P1, P2, P3 were stained by Annexin-V, Yo-Pro-1 and PI to test the viability and apoptosis by fluorescent microscopy and flow cytometry. Cells from P3 were used for detection of surface markers. Our MSCs were positive to CD44 and CD29 surface markers, commonly used to profile MSCs. We also used CD90 and CD45 markers as a negative control. Fluorescent microscopy revealed that early apoptotic cells had the trend of decreasing incidence from P1 to P3. The proportion of early apoptotic cells stained by Annexin-V was lower in P2 than in P1 ($P < 0.05$) and also decreased from P2 to P3 ($P < 0.01$). Using Yo-Pro-1, we revealed that apoptotic incidence was lower in P3 compared to P1 ($P < 0.05$). On the other hand, using flow cytometry wet observed statistically significant decrease in Annexin-V positive cells in P3 compared to P1 ($P < 0.01$). Fluorescent microscopy revealed a decreasing trend in apoptosis rates related to passaging with more statistically significant results. But this trend was not observed using flow cytometry, which is considered to be more reliable and accurate method, compared to fluorescent microscopy. These observations suggest that the passaging of stem cells does not affect their viability.

Key words: rabbit; MSC; viability; passaging

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TLR4 GENE POLYMORPHISM PRESERVED IN TWO CATTLE BREEDS OF GENETIC RESOURCES

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Allelic variants of disease resistance genes in the historical animal breeds are supposed to reflect local infection pressure. They represent a reservoir for breeding programmes and help to counteract gene pool erosion. Therefore, screening for the diversity of innate immunity receptors belonging to the Toll-like receptors (*TLR*) family was carried out in two cattle breeds. The survey covered limited herds of Czech Red and Czech Red Pied included in the conservation programme. The polymorphism has been discovered with high-throughput sequencing of pooled PCR amplicons from coding regions of all bovine *TLRs* using the PacBio platform. The detected SNPs were subsequently validated with Sanger sequencing and appropriate genotyping techniques (PCR-AFLP, ARMS). In the case of *TLR4*, whose product participates in the early recognition of G-negative bacterial pathogens, eight SNPs were validated in the coding and adjacent regions. In spite of long reads from 700 to 1200 nt, which are characteristic for the PacBio platform, the phasing of SNPs was mostly based on the calculation by the PHASE programme. The probabilistic approach predicted 18 haplotypes, what is a significantly higher diversity than reported for the European production breeds. Although three haplotypes were shared by the two breeds at similar frequencies, nine haplotypes showed preference for the Czech Red and six for the Czech Red Pied cattle. Haplotype B1, common in European breeds, was greatly reduced in Czech Red. Although the haplotype frequencies might have been distorted by the bottleneck in the history of both populations, the ancient breed Czech Red appears to harbour more haplotype diversity than the Czech Red Pied as a Simmental variant. This difference could correspond to the phenotypic features of the local breeds and the speculative association of the Czech Red breed with short-horn aurochs.

A picture will be clearer after inclusion of validated SNPs from the remaining members of the *TLR* family. In view of the presence of production herds of Czech Red Pied in parallel to the conserved nucleus herd, the effect of intensive breeding on the *TLR* diversity can be evaluated in this case. The processing of the results also confirmed the advantages of using the PacBio technology for resequencing of genes of interest. Limited capacity of runs is outweighed by the length of reads, single-strand sequencing and a low error rate, which are helpful in subsequent SNP discovery and direct phasing.

Key words: cattle; genetic resources; innate immunity; Toll-like receptors; haplotypes

EFFECT OF CRYOSTORAGE LENGTH ON FERTILIZING ABILITY OF PINZGAU BULL SEMEN *IN VITRO*

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The aim of the study was to evaluate fertilizing ability of Pinzgau bulls semen after different periods of storage in liquid nitrogen. The semen samples of 15 bulls were divided according to cryostorage length into the three groups: less than 7 years (group 1), 7 to 13 (group 2) and 14 or more years (group 3). Straws with frozen semen were thawed in a water bath and the motile fraction of sperm was isolated by modified swim-up method. Motile sperm ($2x10^6$.ml^{-1}) were co-incubated with matured bovine oocytes in a fertilization drops (IVF-TALP) under mineral oil at 38.5 °C in presence of 25 µg.ml^{-1} heparin for 20 h. Totally, 739 oocytes were used for *in vitro* fertilization test. Following fertilization the presumptive zygotes were stained with DAPI nuclear stain and status of chromatin was examined under fluorescence microscope. Penetration of sperm heads into the ooplasm of oocytes and formation of pronuclei were evaluated. No significant difference in fertilizing ability among the experimental groups was found. The total rates of penetrated/fertilized eggs for group 1, 2 and 3 were 79.31, 76.40 and 76.14 %, respectively. Significantly higher pronuclear formation was observed in the group 1, where 72.9 % of eggs have two visible pronuclei at 20 hours post-insemination compared to 65.72 and 47.21 % in groups 2 and 3, respectively. Also, syngamy of both pronuclei was observed in 4 % of fertilized eggs from group 1 (20 hpi), probably due to faster pronuclear formation, whilst no syngamy was observed in groups 2 and 3. In conclusion, length of cryostorage had no direct influence on penetrating and fertilizing ability of long-term cryostored Pinzgau bull semen. However, long-term cryostorage can affect speed of pronuclear formation.

Key words: Pinzgau; bull; sperm; fertility; cryopreservation

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EFFECT OF PUNICALAGIN AND FSH ON PORCINE OVARIAN GRANULOSA CELLS *IN VITRO*

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Punica granatum (Pomegranate) is one of the oldest edible fruits in the world, which contains number of bioactive compounds like ellagitannins, polyphenols etc. Punicalagin is the predominant ellagitannin of pomegranate and is presented in form of two anomers - punicalagin α and β. Punicalagins are contained in husk, pulp or seed. Punicalagin is metabolised to ellagic acid (antioxidant), and microorganisms of colon can metabolize ellagic acid to urolithins. Punicalagin or its metabolites are able to induce changes in intracellular mechanism of ovarian cells. These compounds can be protective agents; however at higher concentrations it can have opposite action on viability of animal cells. The aim of our *in vitro* study was to examine effect of punicalagins or their metabolites in combination with follicle-stimulating hormone (FSH) on the secretion of steroids hormones (progesterone and 17β-estradiol) by porcine ovarian granulosa cells. Granulosa cells from the ovaries of pre-pubertal pigs were cultured at various doses of punicalagin (0.01, 0.1, 1, 10 and 100 µg.ml^{-1}) and FSH (10 ng.ml^{-1}) during 24 h. Steroid hormones of female reproductive system - progesterone and 17β-estradiol were determined by Enzyme-linked immunosorbent assay (ELISA, Multiscan FC, ThermoFisher Scientific, Vantaa, Finland). Secretions of progesterone and 17β-estradiol by granulosa cells were insignificantly ($P \geq 0.05$) affected by punicalagin and FSH treatments at all used doses. In this preliminary study punicalagin in combination with FSH at tested doses did not affect secretion of progesterone or 17β-estradiol by porcine ovarian granulosa cells *in vitro*.

Key words: 17β-estradiol; follicle-stimulating hormone; granulosa cells; progesterone; punicalagin

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INFLUENCE OF POLYMORPHISM G.9476869G>A IN MYELOPEROXIDASE (MPO) GENE ON THE ANTIOXIDANT ACTIVITY IN MILK OF POLISH HOLSTEIN-FRIESIAN COWS

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The milk is a good source of proteins, fat and vitamins, as well as anti-oxidant compounds. The major antioxidants present in milk are vitamins A, C and E, carotenoids, coenzyme Q10, conjugated linoleic acid (CLA), whey protein, peptides and enzymes: catalase, lactoperoxidase, glutathione peroxidase and superoxide dismutase. The enzyme with antioxidant activity is myeloperoxidase (MPO).

The aim of this study was an identification of polymorphism in the myeloperoxidase gene and determination of its influence on the antioxidant activity in milk of Polish Holstein-Friesian cows. In addition, we attempted to associate the MPO gene polymorphism with milk production traits. For this purpose, the antioxidant activity of milk was measured using TEAC (Trolox Equivalent Antioxidant Capacity) method. To identify polymorphism in the MPO gene a PCR-RFLP method was used. g.9476869G>A polymorphism of the MPO gene was identified in the analyzed herd of cows. Statistically significant associations were found between genotype and antioxidant activity of milk and average daily milk yield of cows.

Key words: cattle; Polish Holstein-Friesian; MPO gene; milk; antioxidant activity

CONTAMINATION OF FLUSHING MEDIUM BY STENOTROPHOMONAS MALTOPHILIA AFFECTS THE QUALITY OF SHEEP EMBRYOS

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Increasing use of gametes, progress in micromanipulations with oocytes and embryos as well as advances in gamete cryopreservation and long-term storage in liquid nitrogen represents an increased risk of contamination of germ cells by bacteria *Stenotrophomonas maltophilia*. The main goal of our work was to verify the influence of possible bacterial contamination of flushing facilities and medium in the MOET (multiple ovulation and embryo transfer) system on the yield and quality of sheep embryos. Influence of a possible contamination on a quality and quantity of ovine embryos was monitored on a group of 16 ewes. For our analysis we used the primocultivation method using MacConkey agar and XLD agar. We evaluated the colony growth and microscopic findings by Gram staining. The presence of bacterial contamination was proved by biochemical test NEFERMtest 24. Contamination of flushing medium by *Stenotrophomonas maltophilia* markedly affected the yield as well as quality of embryos. In the group of intact ewes we observed an average number 4.625 of total embryos and 4.125 of transferable embryos. In the group of *Stenotrophomonas maltophilia* infected sheep we observed the average number 1.625 embryos and only 0.625 were transferable.

Key words: bacteria; embryos; flashing medium; sheep

Acknowledgment: VEGA 1/0366/15.

EVALUATION OF PINZGAU BULL SPERM FOLLOWING LONG-TERM STORAGE IN LIQUID NITROGEN

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Cryopreservation of livestock semen has been used to improve the breeding of animals of genetic importance, and has contributed to the conservation of endangered species. For the cryobank establishment and operation it is reasonable to control long-term storage effects on post-thaw survival of mammalian sperm. The aim of the study was to examine sperm viability of Pinzgau bull frozen-thawed sperm following long-term storage in liquid nitrogen. Insemination doses, provided by Slovak Biological Services Inc. (Lužianky, Slovak Republic), were slow-frozen and stored in containers with liquid nitrogen for 1 -18 years. The sperm samples were divided into three groups according to the length of storage: A ($\leq$ 7 years), B (8-13 years) and C ($\geq$ 14 years). Post-thaw sperm assessment of total motility (TM; CASA analysis), apoptotic and dead/necrotic sperm occurrence (fluorescent probe YoPro-1 and propidium iodide) and sperm morphology (light microscopy) was performed immediately after thawing in water bath at 37 ± 1 °C for 1 min. No significant influence of storage length on the sperm motility of Pinzgau bulls was noted. The post-thaw total motility in all the groups was about 40 % i.e. in accordance to the commercial insemination dose standards (post-thaw TM $\geq$ 30 %). Proportion of apoptotic sperm ranged from 21 to 23 % and occurrence of necrotic/dead sperm was at the level of 27-30 % with no significant differences among the groups. In terms of morphological changes, most of the individuals examined demonstrated morphology in accordance to the commercial insemination dose standards (malformation rate $\leq$ 20 %) with no significant differences between groups. The significant differences noted were rather due to inter-male variability in susceptibility of sperm to the same freezing-thawing protocol used, than due to storage itself. Therefore, it can be suggested that individual differences are an important factor that should be taken into account when semen from individual bulls is to be stored for a long period as a genetic resource.

Key words: Pinzgau cattle; sperm viability; long-term storage

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