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ASSESSMENT OF SELECTED ANTIOXIDANT PARAMETERS IN RABBIT BLOOD EXPOSED TO EPICATECHIN *IN VIVO* – FOUR WEEKS EXPOSURE

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ABSTRACT

The aim of present study was to analyse selected antioxidant parameters in blood of rabbits after epicatechin administration during four weeks. Animals (adult female rabbits, body weight 4 ± 0.5 kg) were divided into four groups: control group (C) and experimental groups (E1 – E3). Experimental groups received epicatechin in injectable form in doses 10 $\mu g.kg^{-1}$ in E1, 100 $\mu g.kg^{-1}$ in E2 and 1000 $\mu g.kg^{-1}$ in E3 for four weeks three times a week. At the end of experiment the blood was collected, selected antioxidant parameters (catalase - CAT, glutathione peroxidase - GPx, superoxide dismutase - SOD, uric acid - UA, bilirubine and albumin) were analysed by Genesys 10 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) using the commercial kits. The present study has shown that the activity of SOD and activity of CAT was lower in all experimental groups when compare with the control group after four week exposure of epicatechin but without significant differences. Activity of GPx was higher in all experimental groups against the control group but also without significant differences. The highest concentration of UA in rabbit serum was observed in E1 experimental group with the lowest concentration of epicatechin when compared with the other experimental groups and with the control group but without significant differences. Concentration of bilirubine in rabbit serum after administration of epicatechin was insignificantly lowest in all experimental groups in comparison with the control group. Content of albumin was not affected by epicatechin. Further research needs to be focused on the generation of data dealing with antioxidant effects, in both human and animals.

Keywords: Antioxidant parameters, epicatechin, rabbit blood

INTRODUCTION

Oxidative damage to important biomolecules, including lipoprotein and DNA, is considered to accompany arteriosclerosis, carcinogenesis and acceleration of aging. This oxidative damage may be inhibited by daily intake of antioxidants **(Ames, 1983)**. Natural sources for these compounds include fruits and vegetables such as grapes, raspberries, onions, tomatoes, red wine, tea, etc. **(Kähkönen *et al.*, 1999)**. Their antioxidant properties are well defined by *in vitro* experiments **(Rice-Evans *et al.*, 1997; Nastume *et al.*, 2004)** and there are indications of their beneficial effects in the prevention of diseases, when they are part of the diet **(Ortega, 2006)**. The antioxidant activity of dietary polyphenols is considered to be much greater than that of the essential vitamins, therefore contributing significantly to the health benefits of fruit **(Tsao and Yang, 2003)**. The term antioxidant refers to free radical scavengers, inhibitor of lipid peroxidation and chelating agents **(Lee *et al.*, 2003)**. Epicatechin is a member of a group of polyphenolic compounds collectively known as catechins, belonging to flavonoid family. It is a constituent of grape seeds and grape skin tannins, tea tannins, cocoa flavonoids, cola nuts, strawberries and red wine **(Quine and Raghu, 2005)**. Several epidemiological investigations and dietary interventions in humans using flavanol-containing foods indicate an inverse relationship between flavanol intake and the improvement of immune responses and antioxidant defense system **(Sies et al., 2005)**. Specifically, in the last 10 years, a strong interest has been raised in the use of flavonoids and their derivatives for the therapeutic use, such as anti-inflammatory, anticancer, anti-ischemic, and antithrombotic components. Besides from presenting potent antioxidant properties *in vitro*, these compounds have also the ability to modulate the activity of the antioxidant defense enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) **(Chan *et al.*, 2002; Ying *et al.*, 2004; Li *et al.*, 2007)**. SOD converts superoxide to hydrogen peroxide, which is then removed by GPx and CAT **(Afonso *et al.*, 2007)**. Against oxidative stress, the human organism deploys an interactive network of antioxidants. The first line of defense consists of preventive antioxidants, which suppress the formation of free radicals (e.g. antioxidant enzymes). The another line of defense consists of antioxidants that scavenge free radicals by suppressing chain initiation and/or stopping multiple chain reactions (e.g. uric acid, albumin, bilirubin) **(Simos *et al*, 2012)**. Uric acid is a powerful antioxidant and is a scavenger of singlet oxygen and radicals **(Ames *et al.*, 1981)**. Bilirubin is a bile pigment and has an important role as an antioxidant. Bilirubin, through a hydrogen donation mechanism, participates as a scavenger of secondary oxidants formed in the oxidative process and thereby might alleviate oxidant stress in the blood. It might primarily protect cells against lipid peroxidation **(Sedlak and Snyder, 2004)**. Albumin may represent the major and predominant circulating antioxidant in plasma **(Cha and Kim, 1996)**. Albumin represents the quantitatively most important source of thiol in plasma, and this circulating store may be altered in situations where antioxidants become limiting, resulting in changes in the redox status **(Durand *et al.*, 1997)**. However, little is known about the molecular mechanisms of flavanol-mediated bioactivities in both humans and animals.
The aim of present study was to analyse selected antioxidant parameters in blood of rabbits after epicatechin administration during four weeks.

MATERIAL AND METHODS

Animals

Adult female rabbits (n = 16), maternal albinotic line (crossbreed Newzealand white, Buskat rabbit, French silver) and paternal acromalictic line (crossbreed Nitra's rabbit, Californian rabbit, Big light silver) were used in experiment. Rabbits were healthy and their condition was judged as good at the commencement of the experiment. Water was available at any time from automatic drinking troughs. Groups of adult animals were balanced for age (150 days) and body weight (4 ± 0.5 kg) at the beginning of the experiment. Adult rabbits were fed diet of a 12.35 $MJ.kg^{-1}$ of metabolizable diet composed of a pelleted concentrate (table 1).

Animals were divided into four groups: control group (C) and experimental groups (E1 – E3). Experimental groups received epicatechin in injectable form at 10 μg.kg^{-1} in E1, 100 μg.kg^{-1} in E2 and 1000 μg.kg^{-1} in E3 for 4 weeks three times a week.
In this animal study, institutional and national guidelines for the care and use of animals were followed, and all experimental procedures involving animals were approved by ethical committee.

Blood sampling and analyses

Blood samples from *vena auricularis* were taken from all animals by macromethod after four weeks of epicatechin administration. Catalase - CAT, glutathione peroxidase - GPx, superoxide dismutase - SOD, uric acid - UA, bilirubine and albumin were measured using the Genesys 10 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Activity of CAT was performed according to **Beers and Sizer (1952)** monitoring the decrease of H_2O_2 at 240 nm in blood plasma. The calculation of CAT activity was based on the rate of decomposition of H_2O_2, which was proportional to the reduction of the absorbance during 1 min. **(Tvrda *et al.*, 2013)**. SOD and GPx activity and UA content were analyzed with the RANDOX assay kits (Randox Laboratories, Crumlin, UK) according to the manufacturer´s instructions. Albumin concentration was measured using the ALB BioLa Test (PLIVA-Lachema, Brno, Czech Republic) commercial kit. Concentration of bilirubine was measured by photometric test using the commercial kit DiaSys (Diagnostic Systems GmbH, Germany).

Table 1 Chemical composition (g.kg^{-1}) of the experimental diet.

Component	Content (g.kg^{-1})
Dry matter	926.26
Crude protein	192.06
Fat	36.08
Fibre	135.79
Non-nitrogen compounds	483.56
Ash	78.78
Organic matter	847.49
Calcium	9.73
Phosphorus	6.84
Magnesium	2.77
Sodium	1.81
Potassium	10.94
Metabolizable energy	12.35 MJ.kg^{-1}

Statistical analyses

The data used for statistical analyses represent means of values obtained in blood collection. To compare the results, one-way ANOVA test was applied to calculate basic statistic characteristics and to determine significant differences among the experimental and control groups. Statistical software SIGMA PLOT 12.0 (Jandel, Corte Madera, CA, USA) was used. Differences were compared for statistical significance at the level $P< 0.05$.

RESULTS AND DISCUSSION

Polyphenols have various important biological properties in both plants and animals that can be divided into two main categories, with antioxidant and nonantioxidant function **(Shay *et al,* 2015).** Regarding antioxidant action, it is noteworthy that polyphenols are the most abundant antioxidants in the diet with a total daily intake as high as 1 gram, exceeding the intake of vitamin C by about 10-fold and that of vitamin E and carotenoids by about 100-fold **(Scalbert *et al,* 2008)**. In our study, we used 10, 100 and 1000 μg per kg of body weight of epicatechin and analysed selected antioxidant parameters in rabbit blood. The results are presented in Table (2). Epicatechin in these concentrations had no significant influence on the observed parameters ($P > 0.05$). The present study has shown that the activity of SOD and activity of CAT was lower in all experimental groups when compare with the control group after four week exposure of epicatechin but without significant differences ($P > 0.05$). Activity of GPx was higher in all experimental groups against the control group but also without significant differences ($P > 0.05$). It has been reported that catechins has a strong anti-superoxide formation effect, by scavenging superoxide anion **(Ho *et al.* 1999; Reddy *et al*. 2004).** Catalase, present in phagocytes is effective only at high concentration of hydrogen peroxide **(Halliwell and Gutteridge 1989).** Base on this, we can suppose that in this case if there has been an increase of oxidative stress, epicatechin was able to scavenge reactive oxygen species and concentrations of antioxidant enzymes in blood did not rise. In the other hand, the decrease in the activity of SOD may be attributed to the saturation of SOD during the process of converting O_2• to H_2O_2 (**Eraslan *et al*., 2007**). The major function of GPx, which uses glutathione (GSH) as a substrate, is to reduce soluble H_2O_2 and alkyl peroxides (**Bebe and Panemangalore, 2003**). GPx also can decompose H_2O_2 to water (**Tian *et al.*, 1998**). It may be reason why the activity of GPx was slightly increased.

Table 2 Selected antioxidant parameters in rabbit blood after four weeks of exposure of epicatechin

Parameter	SOD	CAT	GPx	UA	Bilirubine	Albumin
Group	[U.ml^{-1}]	[U.mg protein^{-1}]	[U.l^{-1}]	[μmol.l^{-1}]	[μmol.l^{-1}]	[g.l^{-1}]
C	94.75±6.24	0.35±0.12	1205.02±189.49	82.78±11.79	1.85±0.48	35.56±1.23
E1	86.25±7.81	0.28±0.06	1447.92±301.20	109.33±16.46	3.13±0.97	33.58±0.53
E2	89.50±4.20	0.22±0.07	1302.79±41.00	76.53±13.53	2.82±0.34	33.06±1.58
E3	90.00±3.60	0.28±0.07	1235.51±85.19	78.49±9.66	2.2±0.70	36.37±0.38

C - control group without addition of epicatechin. E1 - E3 – experimental groups with addition of epicatechin (10 μg.kg^{-1} in E1, 100 μg.kg^{-1} in E2 and 1000 μg.kg^{-1} in E3 group). The values shown are the mean ± SD (standard deviation).

Uric acid (UA) is involved in a complex reaction with several oxidants and may have some protective effects under certain conditions. On the other hand, uric acid cannot scavenge all radicals, with superoxide as an example. Uric acid is an antioxidant only in the hydrophilic
environment, which is probably a major limitation of the antioxidant function of uric acid **(Sautin and Johnson, 2008)**. In our study, the highest concentration of UA in rabbit serum was observed in E1 experimental group with the lowest concentration of epicatechin (10 μg.kg^{-1}) when compared with the other experimental groups and with the control group but without significant differences ($P > 0.05$). Administration of (+)- catechin hydrate increased levels of uric acid which may be one of the reasons for protection against diet induced oxidative stress **(Mehra *et al.*, 2007)**. Administration of EGCG in healthy human individuals increased plasma antioxidant activity which was not due to changes in EGCG concentration but due to changes in plasma urate concentrations, which might have interfered with the effect of EGCG to promote antioxidant activity **(Susanne *et al.*, 2005)**. In the other hand, high plasma uric acid (UA) is a prerequisite for gout and is also associated with the Metabolic Syndrome and risk factors for cardiovascular diseases **(Kim *et al.*, 2009)**. In our study, the concentration of UA in blood serum of rabbits was lower in groups with higher concentration of epicatechin (100 μg.kg^{-1} in E2 and 1000 μg.kg^{-1} in E3 group) against the control group however without significant differences ($P > 0.05$). There is still no consensus if UA is a protective or a risk factor, however, it seems that the quantity and the duration of the concentration of the uric acid in the blood is essential for this answer **(Oliveira and Burini, 2012)**.
Bilirubin has been reported as a member of the antioxidant family and is even known to have toxic effects at high concentration. The combined evidence from animal and human studies indicates that bilirubin is a major physiologic cytoprotectant and might alleviate oxidative stress in the blood **(Sedlak and Synder, 2004)**. Our results showed that concentration of bilirubine in rabbit serum after administration of epicatechin was insignificantly ($P > 0.05$) lowest in all experimental groups in comparison with the control group. In the study of authors **Petruška *et al.* (2013)** that long-term application of quercetin caused the increase of concentration of bilirubine in rabbit serum. Findings of **Loprinzi and Mahoney (2015)** suggested an association between flavonoid-rich fruit and vegetable consumption and bilirubin levels. If confirmed by prospective and experimental studies, then regular consumption of flavonoid-rich fruits and vegetables should be promoted to increase levels of bilirubin.
Albumin represents a very abundant and important circulating antioxidant **(Roche *et al*., 2008)**. Study of **Bourdon *et al*. (1999)** confirmed and extended the idea that serum albumin is an important protein that presents direct protective

effects. In our study, concentration of serum albumin was very similar among all groups. Thus, we can say that any used concentration of epicatechin have effect on this parameter in rabbits. In study of **Petruška *et al.* (2013)** they observed slight decrease in the content of serum albumin in quercetin groups vs. control group of rabbits. Several lines of evidence strongly suggest that a reduced serum albumin concentration, although within the normal range, is associated with mortality risk **(Bourdon *et al*., 1999).**

CONCLUSION

Selected antioxidant parameters in rabbit blood after four weeks exposure of epicatechin in this study were assessed. Four weeks of intramuscular application of epicatechin at various doses resulted in slight changes in selected antioxidant parameters of rabbits without significant differences. Catechins when compared with other classes of flavonoids are found to be very active in reducing the amount of strand breakage and residual base damage by mechanism other than directly scavenging of hydroxyl radicals before they react with DNA. To determine whether epicatechin act as effective antioxidant *in vivo*, future studies in animals and humans should employ sensitive and specific biomarkers of oxidative damage to DNA, proteins and lipids.

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TOXICOLOGICAL PROPERTIES OF MYCOTOXIN CITRININ

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ABSTRACT

Citrinin (CTN) is a secondary product of fungal metabolism and contaminant of various food and feed materials. This mycotoxin is produced by several fungal strains belonging to the genera *Penicillium*, *Aspergillus* and *Monascus*. *In vitro* and *in vivo* studies have shown clear evidence for reproductive toxicity and teratogenic, nephrotoxic, hepatotoxic and embryotoxic effects of citrinin. Mycotoxins can interfere in the cascade of cell machinery and thus affect cellular function. Citrinin is known mycotoxin that is spread over the world, however the mechanism of its action and other functions are still not known properly. Thus, the aim of this review paper is to summarize knowledge about mycotoxin citrinin, to describe its properties and effects on animal organism. In particular, known mechanism of toxicity is shown. In addition levels of exposure and bioaccessibility of citrinin is discussed. In the future, strategies for preventing the contamination by citrinin and the possibilities of its elimination should be investigated.

Keywords: Mycotoxins, citrinin, toxicity

INTRODUCTION

Mycotoxins are a group of structurally diverse secondary metabolites produced by various fungal species. These toxic compounds can contaminate foodstuffs, crops or human foods. The ingestion of these contaminated materials may be pathogenic in animals and humans as they may lead to serious health problems, such as liver, kidney or nervous system damage, immunosuppression and carcinogenesis. Mycotoxins can be classified as hepatotoxins, nephrotoxins, neurotoxins, immunotoxins and so forth. Cell biologists put them into generic groups such as teratogens, mutagens, carcinogens and allergens **(Bennett and Klich, 2003)**.
The amount of mycotoxins needed to produce adverse health effects varies widely among toxins, as well as for each animal or person's immune system. Two concepts are needed to understand the negative effects of mycotoxins on human health: *acute toxicity*, what is the rapid onset of an adverse effect from a single exposure. The second one- *chronic toxicity*, that means the slow or delayed onset of an adverse effect, usually from multiple, long-term exposures. Mycotoxins can be acutely or chronically toxic, or both, depending on the kind of toxin and the dose. Membrane-active properties of various mycotoxins determine their toxicity. Incorporation of mycotoxins into membrane structures lead to alterations in membrane functions. In general, mycotoxins effects on DNA, RNA, protein synthesis and the pro-apoptotic action causing changes in physiological functions including growth, development and reproduction **(Surai *et al.*, 2008)**.

General characteristics of citrinin

Citrinin [C13H14O5, IUPAC: (3R,4S)-4,6-dihydro-8-hydroxy-3,4,5-trimethyl-6-oxo-3H-2-benzopyran-7- carboxylic acid] (Figure 1) is an acidic lemon-yellow crystal with maximal UV absorption at 250 nm and 333 nm (in methanol), melting at 172 °C. It is sparingly soluble in water but soluble in dilute sodium hydroxide, sodium carbonate, or sodium acetate; in methanol, acetonitrile, ethanol and most of other polar organic solvents **(Deshpande, 2002)**. It is capable of forming chelate complexes and can be degraded in acidic or alkaline solution, or by heating. It is a quinone methide with two intramolecular hydrogen bonds. Citrinin crystallizes in a disordered structure, with the p-quinone and o-quinone two tautomeric forms in a dynamic equilibrium in the solid state. In methanol or methanol/methylene chloride mixtures, citrinin undergoes a Michael-type nucleophilic addition reaction. This reaction is reversible and the equilibrium shifts toward the normal citrinin if temperature is increased in methylene chloride **(Poupko *et al.*, 1997)**.

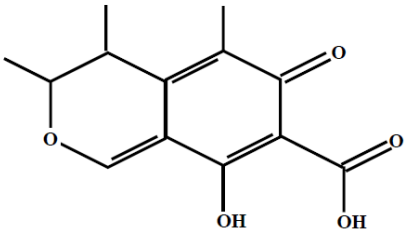

Figure 1 Chemical structure of citrinin

Toxicity of citrinin

Citrinin (CTN) is a secondary metabolite generally produced by various fungi, including *Penicillium*, *Monascus* and *Aspergillus* **(Bennett and Klich, 2003).** CTN is the one of the well-known mycotoxins, which is possibly spread all over the world and it is a natural contaminant of various types of feed and food, occurs mainly in stored grains, corn, wheat, rice, barley and nuts **(CAST, 2003)**. Red chilli, black pepper and dry ginger are the most contaminated spices in which aflatoxins, ochratoxin A and citrinin were present in high concentration. Fennel, caraway and cumin are the spices which can be considered a bit resistant to mycotoxigenic fungi and mycotoxin contamination (**Punam and Kumar, 2015**).
Although citrinin is one of the well-characterized mycotoxins, information on its mechanism of toxic action is limited. Clinically, citrinin was shown to cause renal disease in poultry, pigs, dogs and rats **(Reiss, 1977; Yu *et al.*, 2006)**. CTN has antibiotic properties against grampositive bacteria, but it has never been used as a drug due to its high nephrotoxicity. The kidney is the major target organ of CTN toxicity, but other target organs such as liver and bone marrow have also been reported **(Gupta *et al.*, 1983)**. Historically, CTN is one of the first isolated mycotoxins; however, the data on the mechanism of its toxicity are still controversial and most have been obtained *in vitro*. Like other mycotoxins, CTN could be implicated in porcine nephropathy **(Krogh *et al.*, 1973)**.
The effect of CTN on cell viability was tested with the MTT assay on Vero cells from the green monkey kidney treated with increasing CTN concentrations from 0 $\mu mol.l^{-1}$ to 250 $\mu mol.l^{-1}$ **(Bouslimi *et al.*, 2008)**. Up to the concentration of 60 $\mu mol.l^{-1}$ no significant change in cell viability was observed, and the estimated half maximal inhibitory concentration (IC_{50}) of citrinin was about 220 $\mu mol.l^{-1}$ after 48 hours of exposure. With the same exposure time, the IC_{50} of CTN for human embryonic cell line was 120 $\mu mol.l^{-1}$. At 24 hours of exposure, the IC_{50} of

CTN for human promyelocytic leukaemia (HL-60) cells and porcine kidney PK15 cells was 50 µmol.l^{-1} and 68 µmol.l^{-1}, respectively **(Yu *et al.*, 2006; Šegvić Klarić *et al.*, 2007)**.
In chronic tests, CTN (i) inhibits one of the key enzymes of cholesterol synthesis, leading to a reduced concentration of serum testosterone and hypocholesterolaemia (**Endo and Kuroda, 1976**); (ii) induces multiple immune modulator effects (**Quingqing *et al.*, 2012**); and (iii) triggers nephropathy, hepato- and foetotoxicity and renal adenoma formation in various animal models (**Carlton *et al.*, 1974; Xu *et al.*, 2006**).

Toxicity, carcinogenicity, and teratogenicity

Acute LD_{50} (median lethal dose) of CTN varies with the route of administration, physiological conditions and animal species. Oral LD_{50} for rats is 50 mg.kg^{-1} b.w. **(Sakai, 1955)**, while subcutaneous LD_{50} is 67 mg.kg^{-1} b.w. **(Ambrose and DeEds, 1945)**. The subcutaneous treatment of pregnant rats with 35 mg.kg^{-1} on days 6, 9, and 10 of pregnancy resulted in 50 % or higher maternal mortality **(Reddy *et al.*, 1982)**. Acute lethal doses administered to rabbits, guinea pigs, rats and swine caused swelling of the kidneys and acute tubular necrosis **(Ambrose and DeEds, 1946; Friis *et al.*, 1969; Krogh *et al.*, 1970)**.
Subchronical oral treatment of rats with water suspension isolated from a strain of *Penicillium viridicatum* Westling caused CTN-induced kidney damage characterized by enlarged kidney, hydropic degeneration, loss of brush border and pyknotic nuclei in the proximal tubules **(Friis *et al.*, 1969)**. Treatment of mice with weekly injections of CTN (20 mg.kg^{-1}) for six weeks resulted in a significant decrease in total bone marrow cells, red blood cell precursors, white blood cell precursors, megakaryocytes, decrease in spleen weight and decrease in the total spleen cell count **(Gupta *et al.*, 1983)**. The electron transport system of the kidney and liver mitochondria were considered as the target of the toxic action of citrinin **(Da Lozzo *et al.*, 1998)**.
CTN is embryocidal and foetotoxic in mice **(Hood *et al.*, 1976)**. In pregnant Sprague-Dawley rats, CTN given subcutaneously (35 mg.kg^{-1} b.w.) on gestation day 3 to 15 did not decrease the number of implants and no gross or skeletal malformations were found, but the foetuses were about 22 % smaller than control **(Reddy *et al.*, 1982)**. CTN injected to pregnant rats of the same strain at a dose of 30 mg.kg^{-1} on gestation days 5 to 14 resulted in a few foetal resorptions and minimal malformations **(Mayura *et al.*, 1984)**.

Genotoxic and nephrotoxic effects of citrinin

Genotoxicity of CTN has not been unequivocally established because various test systems gave both positive and negative results. An increase in DNA damage was detected using single cell gel electrophoresis (comet test) in Vero cells exposed 24 h to CTN **(Bouslimi *et al.*, 2008).** However, the same method gave negative results in human-derived liver cells (HepG2) **(Knasmüller *et al.*, 2004)** and human embryonic kidney cells (HEK293) **(Liu *et al.*, 2003)** no matter if Fpg was present or not. This suggests that CTN-induced oxidative stress did not affect DNA. In contrast to negative results, various cell cultures exposed to CTN showed a significant increase in micronucleus frequency **(Šegvić Klarić *et al.*, 2007)**.
Citrinin has been shown to be nephrotoxic when pigs were fed doses of 200-400 mg citrinin/kg for 1 to 2 months **(Harwig and Munro, 1975)**. Another characteristic of citrinin toxicity is polyuria resulting in injury and cell death in the proximal renal tubule **(Lurá *et al.*, 2001)**. *In vitro* studies with renal cortical explants from swine synthesis indicated that DNA, RNA and protein synthesis were inhibited at 0.01 mM citrinin, inhibition of respiration was seen at 1 mM citrinin and organic iron transport was inhibited at 0.01 mM citrinin **(Braunberg *et al.*, 1992)**. CTN along with ochratoxin A (OTA) has been implicated as a potential causative agent in human endemic Balkan nephropathy **(Vrabcheva *et al.*, 2000).** It is revealed that OTA-induced cytotoxicity is mediated by direct DNA damage whereas CTN caused ROS-mediated DNA damage. Cells suffering from DNA damage directly or through ROS take to intrinsic pathway of apoptotic cell death **(Gayathri *et al.*, 2015).**

Mechanism of toxicity

In vertebrate, mycotoxin is metabolized by cytochrome P450 enzymes to metabolite-guanine-N7 adduct (Figure 2). The carcinogenic potency is highly correlated with the extent of total DNA adducts formed *in vivo* **(Eaton and Groopman, 1994)**.
Cytotoxicity and ROS generation are mechanisms of mycotoxins mediated toxicity. ROS (reactive oxygen species) are chemically reactive molecules containing oxygen. They are highly reactive due to the presence of unpaired electrons. ROS formed as a natural byproduct of the normal metabolism of oxygen have important roles in cell signaling and homeostasis. However, during times of environmental stress, ROS levels can increase dramatically as a result of oxidative stress **(Devasagayam *et al.*, 2004)**. Oxidative stress occurs when the concentration of ROS generated exceeds the antioxidant capability of the cell. In other words, oxidative stress describes various deleterious processes resulting from an imbalance between the excessive formation of ROS and limited antioxidant defenses **(Sies, 1991)**. Under normal conditions, ROS are cleared from the cell by the action of superoxide dismutase (SOD), catalase (CAT) or glutathione peroxidase (GPx). The main damage to cells results from the ROS-induced alteration of macromolecules such as polyunsaturated fatty acids in membrane lipids, proteins and DNA. Additionally, oxidative stress and ROS can originate from xenobiotic bioactivation by prostaglandin H synthase (PHS) and lipoxygenases (LPOs) or microsomal P450s which can oxidize xenobiotics to free radical intermediates that react directly or indirectly with oxygen to produce ROS and oxidative stress **(Tafazoli, 2008)**.

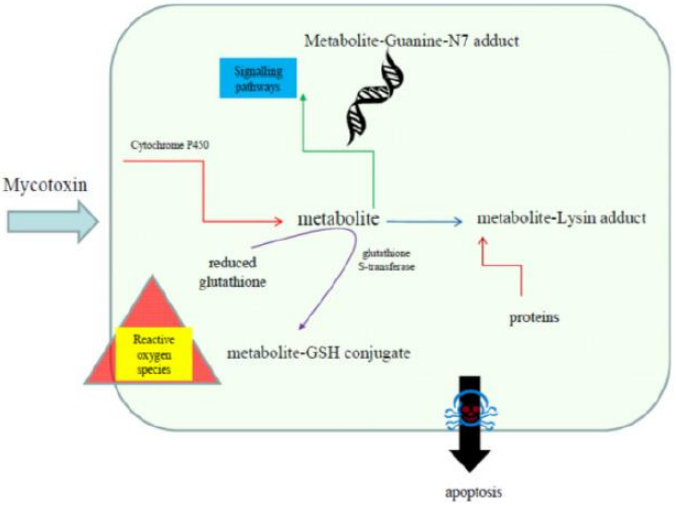

Figure 2 Mycotoxin metabolism in vertebrates **(Vasatkova *et al.*, 2009)**.

CTN under *in vivo* condition has the ability to cause oxidative stress and ROS-mediated DNA damage in mouse skin upon topical exposure leading to enhanced expression of p53, p21/waf1 and Bax proteins that causes cell cycle arrest at the G0/G1 as well as G2/M phases and caused apoptosis through the mitochondria-mediated pathway **(Kumar *et al.*, 2011)**. The p53 protein plays a key role in the DNA damage response pathway by transmitting a variety of stress signals associated with antiproliferative cellular responses that lead to apoptosis **(Jiang *et al.*, 2010)**. In response to CTN-induced DNA damage, overexpression of p53 protein leading to upregulation of p21/waf1 was also observed, which results in arrest of the cell cycle progression at the G0/G1 or G2/M phases **(Abbas and Dutta, 2009)**. CTN exposure could lead to toxicity by enhancing apoptosis of normal skin cells which involves a cascade of events including cell cycle arrest at G0/G1 as well as G2/M phases. The cell cycle arrest by CTN may permit DNA repair, but if it is faulty may allow proliferation of mutated cells, which is generally observed in case of tumorigenesis **(Das *et al.*, 2005b).**
The cytotoxic effects of several mycotoxins including CTN on target tissues and cultured cells are thought to correlate with their apoptosis-inducing ability **(Chan, 2007; Yu *et al.*, 2006)**.
The mycotoxin citrinin triggers an immediate and general antioxidant response in yeast cells. Induction of harmful ROS levels might therefore be the prevalent toxicity mechanism of this toxin. In yeast cells, citrinin activates the expression of antioxidant encoding genes and oxidative stress specific reporters. The ROS activated transcription factor Yap1 is critically involved in the adaptive response to citrinin. Additionally, the mutation of specific toxin exporters such as Pdr5, identifies physiologically important citrinin defense systems. Yeast is an efficient model to unravel toxicity and detoxification mechanisms of mycotoxins **(Pascual-Ahuir *et al.*, 2014).**
Factors such as breeding, sex, environment, nutritional status, as well as other toxic entities can affect the symptoms of intoxication and may contribute to the significance of mycotoxin damage on economic output and animal health **(Binder *et al.*, 2007)**.
CTN causes cell injury, including apoptosis. However, its precise regulatory mechanisms of action, particularly in stem cells and embryos, are currently unclear. Recent studies show that CTN has cytotoxic effects on mouse embryonic stem cells and blastocysts, and is associated with defects in their subsequent development, both *in vitro* and *in vivo*. Experiments with the embryonic stem cell line, ESC-B5, disclose that CTN induces apoptosis via several mechanisms, including ROS generation, increased cytoplasmic free calcium levels, intracellular nitric oxide production, enhanced Bax/Bcl-2 ratio, loss of mitochondrial membrane potential, cytochrome C release, activation of caspase-9 and caspase-3, and p21-activated protein kinase 2 and c-Jun N-terminal protein kinase activation. Additional studies show that CTN promotes cell death via inactivation of the HSP90/multi-chaperone complex and subsequent degradation of Ras and Raf-1, further inhibiting anti-apoptotic processes such as the Ras→ERK signal transduction pathway **(Chan, 2008)**.
A recent study from the European Food Safety Authority **(EFSA, 2012)** preliminarily set the maximal citrinin dose of no concern for nephrotoxicity in humans at an exposure level of 0.2 µg/kg body weight per day. For high

consuming individuals, especially children, the critical citrinin concentration ranges between 9 and 53 µg/kg grain-based products and for average consumers between 19 and 100 µg/kg grain-based products. However, the same study concluded that the impact of uncertainties on the risk assessment of citrinin is large, and that more data regarding both the occurrence of citrinin in food and feed in Europe and the toxicity mechanisms of this mycotoxin are needed.

CONCLUSION

The mechanism of CTN toxicity is not fully understood, especially not whether CTN toxicity and genotoxicity are the consequence of oxidative stress or of increased permeability of mitochondrial membranes. There is also a need for toxicological studies in laboratory animal species to further explore the toxicological potential of citrinin and to characterize the dose-response relationships.

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IN VITRO EFFECT OF 4-NONYLPHENOL ON cAMP STIMULATED ANDROSTENEDIONE PRODUCTION AND VIABILITY OF MICE LEYDIG CELLS

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ABSTRACT

The toxic effect of various environmental contaminants such as heavy metals or industrial chemicals such as alkylphenolson male reproductive systemwasfound. 4-nonylphenol (4-NP), one of the environmental contaminants used in the manufacture of plastics and other products, is released largely into the environment. The majority of studies on NP have focused on their endocrine disrupting and potential adverse effects on reproductive system. The target of our *in vitro* study was to determine the potential impact of 4–nonylphenol as an endocrine disruptor on the hormonal secretion and viability of mice Leydig cells. Cells were cultured with addition of 0.04; 0.2; 1.0; 2.5; and 5.0 µg/mL of 4–nonylphenol for 44h and compared with the control. Cells destined for the determination of androstenedione production were cultured in the presence of cyclic AMP solution. Quantification of cAMP stimulated androstenedion production directly from aliquots of the medium was performed by enzyme linked immunosorbent assay (ELISA). The viability of Leydig cells was detected by the metabolic activity (MTT) assay. The cAMP stimulated androstenedione production significantly *(P<0.05)* decreased at 2.5 and 5.0 µg/mL of 4-nonylphenol concentration. The viability of mice Leydig cells was decreased at the doses of 0.2; 1.0; 2.5 and 5.0 µg/mL of 4-nonylphenol, but this decrease was not significant. Further investigations are essential to clarify the mechanism of action of endocrine disruptor on male mice, as well as to establish the biological significance of the observed phenomena.

Keywords: Leydig cells, nonylphenol, androstenedione, viability

INTRODUCTION

In present is increasing evidence that various chemicals such as pesticides, heavy metals and endocrine disrupters, introduced into the environment have the potential to damage endocrine system in wildlife and humans. The endocrine disrupting chemicals (EDCs) are an exogenous substances or mixture that alters functions of the endocrine system and consequently causes adverse health effects in an intact organisms **(Sanderson, 2006).** These chemicals interfere with the synthesis, metabolism or action of hormones. The list of chemical compound affecting the action of sex hormones is growing. EDCs are able to mimic, block or modulate natural hormones and other chemical messengers. In addition the important effect of EDCs is in change in the ratio of estrogen to testosterone (either directly, through an increase in levels of estrogen mimics, or indirectly through metabolic changes resulting in changed 17 β-estradiol levels). This ratio is crucial for normal sexual development. Besides continuous impact of EDCs increase the risk of development of certain cancers **(Nimrod and Benson, 1996)**. Alkylphenolsethoxylate (APEO) are potential endocrine disrupting chemicals. This group consists of approximately 80% nonylphenolethoxylate (NPEO). NPEO are highly cost effective surfactants with exceptional performance and consequently used widely in industrial, commercial and household applications. The primary degradation product of NPEO is nonylphenol (NP). The term nonylphenol represents a large number of isomeric compounds varying in the point of attachment of the nonyl group to the phenol molecule and in the degree of branching in the nonyl moiety. It is common environmental contaminant widely used as part of detergents, paints and many other synthetic products. With development of industry, large amounts of nonylphenol were discharged into water, nature and environment **(Wang and Shen, 1999).** Many studies have classified NP as hazardous to the health of human and animals, especially to male reproduction. This metabolite has a structure mimicking estradiol and has been reported to have xenoestrogenic effects. NP exposure inhibits the growth of testes, affects the production and survival of sperms and also may cause significantly on hormone concentration **(Blom*et al*., 1998; Han et *al*., 2004, Junk *et al*., 1974)**. Somatic testicular Leydig cells (LC) are an important part development of male reproductive organs and male reproduction. Moreover, LC are the primary and the main place of hormone biosynthesis. These cells are located in the testicular interstitium, between seminiferous tubules. They have spherical shape and contain a lot of amounts smooth endoplasmic reticulum and mitochondria. In the mice are classified two generations of Leydig cells namely fetalLeydig cells and adult Leydig cells. FetalLeydig cells start to appear in the mesenchyme of the developing in prenatal testis at 14.5 weeks. Adult type of Leydig cells originate within the mice testis by day about 56. postnatally. Their formation is the product of active proliferation and differentiation of undifferentiated stem cells. Despite their differences in biochemical and morphological parameters, fetal and adult Leydig cells share the same principal function to produce androgens **(Steinberger and Fischer, 1969)**.
The level of testosterone and other sex hormones are responsible for the maintenance of spermatogenesis and secondary sexual characteristics in the male. We have to emphasize thought that NP affect important mechanism called steroidogenesis. The mechanism by which nonylphenol modulates this process has not been well defined, but it can be partially explained by the inhibition or activation of the key enzymes required for the biosynthesis of hormones in Leydig cells, including CYP11A, 3β-HSD or 17β-HSD **(Laurenza *et al*., 2002; Payne and Sha, 1991)**. In addition, NP administration increased reactive oxygen species (ROS) level and depressed the activity of antioxidant enzymes in testis. Higher level of ROS may initiate a series of reactions that damage cellular components resulting in cell death. Moreover, nonylphenol has been identified that can bind to the estrogen receptor and initiate transcription of the estrogen receptor – regulated genes *in vitro* **(Bolger *et al*., 1998; Danzo, 1997; Gould *et al*., 1998)**. Although a lot of chemicals has the potential to disrupt reproductive function, their actual impact on reproductive health has not been defined thoroughly. The present study was conducted to evaluate the possible effect of 4-nonylphenol at different concentrations on cAMP stimulated androstenedione production and viability in mice Leydig cells.

MATERIAL AND METHODS

Mice Leydig cells can be isolated without enzyme treatment or by trypsinization **(Bilinska and Stoklosowa, 1977)**. In our *in vitro* study interstitial cells were

isolated by mechanical dissociation without enzyme treatment as previously described by **Stoklosowa (1982)** with slight modification. NMRI mice (Institute of Experimental Pharmacology, Slovak Academy of Sciences, Dobra Voda, Slovak Republic), 30-40g of weight and 8-9 week old were kept in a room and maintained on a 12h light:dark cycle and 20-25°C. In brief, twenty-six decapsulated testicles were placed on a grid or a sieve (of about 300 µm opening size) over a beaker. All next steps were performed under sterile conditions. With the help of a 10 mL syringe and needle (Luer 22G), interstitial cells were rinsed out with a vigorous stream of minimum essential medium (MEM, Live Technologies, Bratislava, Slovak Republic) without serum to beaker placed on ice. The cell suspension was subsequently collected and centrifuged. After centrifugation (300 x g, for 10 min, 4°C) the cells were washed twice and resuspended. Subsequently the cell suspension was adjusted with culture medium (MEM) supplemented with 10% fetal bovine serum (FBS; BiochromAG, Berlin, Germany), 100 IU/mL penicillin and 100 µg/mL streptomycin (Sigma – Aldrich, Bratislava, Slovak Republic) to a final concentration of 10^6 cells/mL. The cells were plated into sterile 24-well plates (Nunclon, Denmark). Then were treated for 44h in MEM medium that contained various concentration of 4-nonylphenol (4-n-NP; Fluka, Buchs, Switzerland), 0.04; 0.2; 1.0; 2.5 and 5.0 µg/mL. Cells destined for the determination of androstenedione production were cultured in the presence of cyclic AMP solution (db-cAMP, Sigma – Aldrich, Bratislava, Slovak Republic). All incubations were carried out at 34°C under a humidified atmosphere of 95% air and 5% CO_2. After cell treatment the media were removed and frozen at -20°C until androstenedione determination. The resulting cell suspension was used for cell viability assessment.

Quantification of cAMP-stimulated androstenedione production

Determination of the androstenedione directly from aliquots of the culture media was performed by enzyme linked immunosorbent assay (ELISA). The ELISA kits were purchased from Dialab (androstenedione Cat. # K00197, Austria). These kits were used for quantitative determination steroid hormones and the samples were measured at wavelength 450 nm.

Cytotoxicity evaluation

The viability of the cells exposed to 4-nonylphenol *in vitro* was evaluated by the metabolic activity (MTT) assay **(Mosmann *et al.*, 1983)**. This colorimetric assay measures the conversion of a yellow water-soluble tetrazolium salt (3-(4,5 – dimetylthiazol – 2- yl)-2, 5 – diphenyl tetrazolium bromide) to purple formazan particles by mitochondrial succinate dehydrogenase of intact mitochondria of living cells. The amount of formazan was measured spectrophotometrically. In brief, interstitial cells were stained with a tetrazolium salt - MTT (Sigma, St. Louis, USA) and 24–well plates were inserted into a CO_2 incubator. After 3h of incubation (34°C, humidified atmosphere of 95% air and 5% CO_2) the reaction was stopped with 1 mL/well of isopropanol (2-propanol, p. a. CentralChem, Bratislava, Slovak Republic). The optical density was determined at a measuring wavelength of 570 – 620 nm by an ELISA reader (Multiscan FC, ThermoFisher Scientific, Vantaa, Finland). The data were expressed in percentage of control.

Statistical Analysis

Obtained data were statistically analyzed using PC program GraphPad Prism 3.02 (GraphPad Software Incorporated, San Diego, California, USA). One way analysis of variance (ANOVA) and the Dunnett´s multiple comparison test were used for statistical evaluations. The level of significance was set at ***$(P<0.001)$ ** $(P<0.01)$ and * $(P<0.05)$.

RESULTS AND DISCUSSION

In our *in vitro* study, Leydig cells were exposed to various concentrations of 4-nonylphenol (0.04; 0.2; 1.0; 2.5 and 5.0 µg/mL) for 44h. Different doses of this endocrine disruptor showed a possible effect on cAMP stimulated androstenedione production. As seen in Figure 1, the androstenedione production was significantly $(P<0.05)$ decreased at the 2.5 µg/mL concentration of 4-nonylphenol. CAMP stimulated hormone production also significantly $(P<0.001)$ decreased at the highest doses (5.0 µg/mL) of 4-nonylphenol in comparison with CTRL group (without 4-NP treatment). Results of study indicated dose - dependent decreases in cAMP androstenedione production of Leydig cells following a 44h *in vitro* 4-nonylphenol exposure.

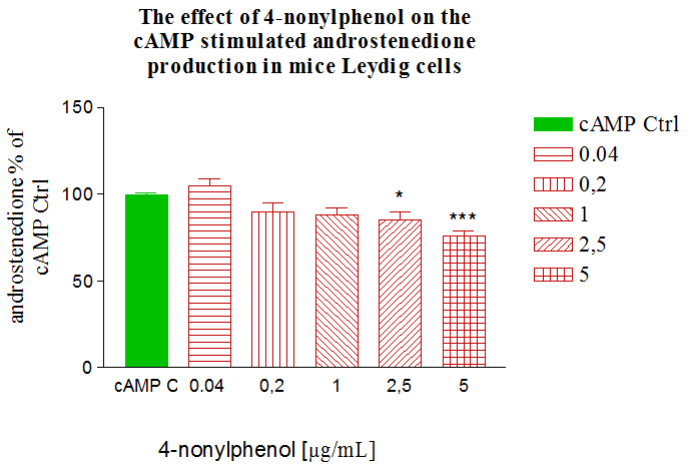

Figure 1 The effects of 4-nonylphenol on the cAMP stimulated androstenedione production after 44h of *in vitro* cultivation.
Legend: Ctrl – control group. Each bar represents the mean (± SEM) androstenedione % of control (untreated) and treated group. The level of significance was set at ***$(P<0.001)$ ** $(P<0.01)$ and * $(P<0.05)$.

The viability of mice Leydig cells was detected by the MTT cytotoxicity assay. The MTT assay measures the reduction of MTT to blue formazan in viable cells. Figure 2 shows potential impact of 4-NP on the Leydig cell viability after a 44h of *in vitro* cultivation. Viability of Leydig cells was slightly decreased at the lowest doses of 4–nonylphenol (0.04 and 0.2 µg/mL). Lower cell viability was recorded in the experimental groups supplemented with 2.5 and 5.0 µg/mL. However, this decrease was not significant in comparison with CTRL group (without 4-NP treatment). The results of this study suggested that lower concentration of 4 – nonylphenol do not damage the mitochondrial activity of mice Leydig cells.

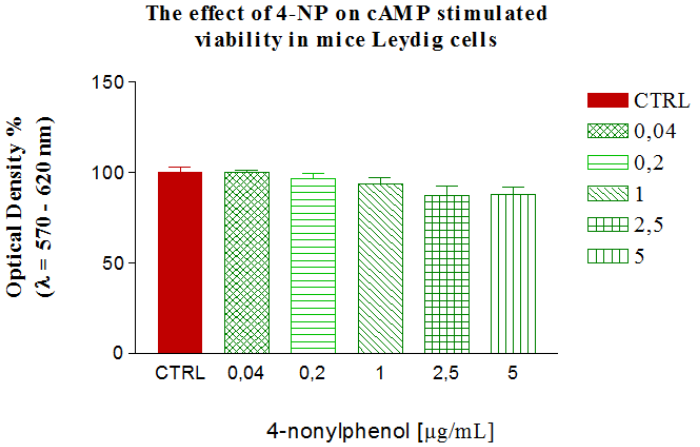

Figure 2 The effects of 4-nonylphenol on the cAMP stimulated Leydig cells viability after 44h of *in vitro* cultivation.
Legend: Ctrl – control group. Each bar represents the mean (± SEM) viability % of control (untreated) and treated group. The level of significance wa set at ***$(P<0.001)$ ** $(P<0.01)$ and * $(P<0.05)$.

Various chemicals found in the human and wildlife environments have the potential to disrupt endocrine functions in exposed organisms. A lot of *in vivo* and *in vitro* experiments on 4-nonylphenol have focused on their endocrine disrupting and potential adverse effects on developing reproductive system **(Sanderson, 2006)**. Previous studies were usually performed using commercial nonylphenol, which is a mixture of many isomers. **Kim *et al.* (2004)** demonstrated that nonylphenol isomers had differential effects on the release of hormones in Leydig cells. The results of our study indicate dose-dependent decrease in cAMP stimulated androstenedione production of Leydig cells following *in vitro* 4-nonylphenol treatment after 44h of cultivation. Androstenedione production was significantly $(P<0.05)$ decreased at the 2.5 µg/mL of 4-nonylphenol. Higher dose (5.0 µg/mL) of 4-nonylphenol also significantly $(P<0.001)$ decreased hormonal production. **Diemer *et al.* (2003)** stimulated the MA-10 cell line using 8-Br-cAMP. In addition, the experimental groups were treated with H_2O_2. The result pointed out a decrease in the progesterone production when applying 100, 250 and 500 µM H_2O_2 in comparison with cells treated with cAMP alone.
In the other *in vitro* study, MA -10 cells were treated with different doses of mono-(2-etylhexyl) phthalate (MEHP) for 24h and then maximally stimulated by luteinizing hormone (LH) for 2h. The ability of the cells to produce cAMP

(essential for steroidogenesis) in response to LH was reduced significantly by exposing the cells to increasing MEHP concentrations. Consequently, it was observed dose-dependent reductions in cAMP stimulated progesterone production without effect on the ability of the cells to reduce MTT. These results suggested that in addition to its effects on cAMP production, MEHP exposure might also affect downstream in the steroidogenic pathway **(Zhou *et al*., 2013)**. We hypothesize that increased oxidative stress in response to 4-nonylphenol might be responsible for the reduced intracellular cAMP and androstenedione production.

Mitochondria are one of the most important organelle of cells. Mitochondria are also target of many toxic compounds since damage to mitochondria which synthesize ATP gives rise to corresponding cells damage **(Higgins and Rogers, 1974; Bragadin *et al*., 1998)**. **Bragadin *et al*. (1999)** found out that a low level dose of nonylphenol inhibits ATP synthesis in mitochondria. **Yao *et al*. (2007)** investigated the effects of nonylphenol on the mitochondrial membrane potential and confirmed that the mitochondrial permeability transition was an important step in the induction of cellular apoptosis. In our *in vitro* study we found that different doses of 4-nonylphenol affected the Leydig cells viability after 44h of cultivation. Some concentration (0.2; 1.0; 2.5 and 5.0 µg/mL) of 4-nonylphenol reduce cells viability. On the other hand, significant decrease was not demonstrated. **Wu *et al*. (2010)** treated rat Leydig cells with different concentrations (42.5; 127.5 and 425.0 µM) of 4-nonylphenol. This highest dose exhibited a cytotoxic effect. **Gong *et al*. (2009)** examined the effect of nonylphenol (0.1; 1.0; 10.0; 20.0 and 30.0 µM) during time periods 6h, 12h and 24h on viability of rat testicular Sertoli cells. In this case a significant decrease was reported after 24h cultivation. Therefore the exposure to nonylphenol in animals and humans must be seriously observed.

CONCLUSION

Recent studies have hypothesized that environmental exposure to nonylphenol poses adverse effects on reproductive system of humans and wildlife species. Therefore the exposure to nonylphenol and other endocrine disruptor in animals and humans must be seriously observed. Many authors investigated the toxic effect of nonylphenol on various types of cells. The data presented in our *in vitro* study describe the impact of endocrine disruptor on the Leydig cell biosynthesis of androstenedione and viability of cells after 44h treatment. We may conclude that some experimental doses (2.5 and 5.0 µg/mL) may significantly decrease cAMP stimulated androstenedione production, however in the case of cell viability an insignificant decrease was observed.

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TOTAL CHOLESTEROL AND CORTICOSTERONE CONCENTRATION RELATIONSHIP IN BLOOD PLASMA OF LAYING HENS

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ABSTRACT

The aim of this study was to analyze the effect of laying hens housing condition on the plasma total cholesterol and corticosterone concentrations relationship at the beginning, middle and end of the laying period. The experiments were performed in ISA BROWN egg production pullets, kept in a poultry house for rearing with deep litter technology. Blood samples (2 ml) of all hens in experimental groups were collected from brachial vein at 22, 28, 35, 41, 47, 52, 58 and 66 weeks of age. Plasma corticosterone levels were analyzed by radioimmunoassay and plasma total cholesterol by spectrometry. One-way ANOVA showed significant differences in cholesterol as well as corticosterone levels of blood plasma of laying hens during the experimental period. A very low relationship was found between individual total plasma cholesterol and corticosterone concentrations during the monitored period.

Keywords: Laying hens, furnished cage technology, blood plasma, cholesterol, corticosterone

INTRODUCTION

Cholesterol is a lipid with a unique structure consisting of four linked hydrocarbon rings forming the bulky steroid structure. Its levels in tissues reflect a balance among dietary uptake, endogenous de novo synthesis, efflux, and utilization to bile acids (**Faust and Kovacs, 2014**). With respect to cholesterol uptake, the low density lipoprotein receptors are well-known for their important roles in regulating plasma and intracellular cholesterol homeostasis (**Soto-Acosta *et al.*, 2013**), they are primarily modulated by intracellular cholesterol levels (**Liu *et al.*, 2012**). As an essential component of cell structure and the precursor of steroid hormones, the amount of cholesterol in chicken muscle will affect avian well-being, and may ultimately influence human health through dietary intake. Glucocorticoids are the counter regulatory hormones with broad effects on carbohydrate, lipid and protein metabolism (**Bamberger *et al.*, 1996**). Glucocorticoids participate in the control of whole body homeostasis and the response of the organism to stress by stimulating there lease of energy stores via promoting glucose mobilization and lipolysis (**Harvey *et al.*, 1986**). Glucocorticoid treatment is associated with increased levels of circulating free fatty acids in humans (**Macfarlane *et al.*, 2008**) and rodents (**Novelli *et al.*, 2008**). In birds it has been reported that corticosterone and insulin interact to regulate triglyceride and cholesterol levels during stress (**Remage-Healey and Romero, 2001**). Glucocorticoids, especially corticosterone, are involved in the control of appetite in poultry (**Yuan *et al.*, 2008**). **El-Letheye *et al.* (2001)** have shown that corticosterone can increase feed intake of chickens. Furthermore, there are reports of augmentation in plasma corticosterone after 24h of feed deprivation in immature chickens (**Geris *et al.*, 1999**). Hyperlipidemia and metabolic abnormalities caused by endogenous glucocorticoid excess have been well documented in mammals and birds. In mammals, increased circulating glucocorticoids together with the altered insulin sensitivity are suggested to be responsible for enhanced visceral fat deposition and hyperlipidemia (**Geraert *et al.*, 1996**). Chronic corticosterone administration did not change plasma lipid profile, except for a moderate increase of high density lipoprotein cholesterol levels in broiler chickens (**Wang *et al.*, 2013**). Chronic endogenous glucocorticoid excess in mammals is associated with metabolic dysfunction and dyslipidemia that are characterized by increased plasma triglyceride and total cholesterol levels (**Duan *et al.*, 2014**).
The objective of our study was to analyze the effect of laying hens housing condition on the plasma total cholesterol and corticosterone concentrations relationship at the beginning, middle and end of the laying period.

MATERIAL AND METHODS

Animals and experimental conditions

The experiments were performed in ISA BROWN egg production pullets, kept in a poultry house for rearing with deep litter technology. The available area, complete feeding mixture, light-dark (L:D) cycle, temperature of housing, relative humidity of air changed according to technological instructions for ISA BROWN pullets. During rearing period standard vaccinations were provided. At the age of 15 weeks, they were transferred into o furnished cage technology according to Council Directive 74/99/EC – three-tier, total area 945 cm^2/bird (8 birds kept on an area of 7560 cm^2 – 180x42x45cm), available area 643 cm^2/bird, 6 nipple drinkers, belt feeder 20 cm/bird, nest (30x35x45cm), perching area 15 cm/bird, devices for dustbathing and scratching, device for claw shortening. Experimental group consisting of 12 birds was established with the mean body weight of 1300 ± 50 g. Throughout the study, the hens were fed with balanced layer feeds that contained 875 $g.kg^{-1}$ dry matter, energy content ME_N 11.1 $MJ.kg^{-1}$, content of nitrogen substances 170.7 $g.kg^{-1}$, Ca 35.9 $g.kg^{-1}$ and P 6.3 $g.kg^{-1}$. A constant light-dark (L:D) cycle (15:9, switching on at 4.00 AM, switching off at 19.00 PM) was maintained in all three technologies as recommended in technological instructions for ISA BROWN pullets. The temperature of housing was in the range from 18 to 20 °C, relative humidity of air was ranging from 65 to 70 %. No red mite and other parasite or viral infection was presented during experimental period.

Blood sampling

Blood samples (2 ml) of all hens in experimental groups were collected from brachial vein at 22, 28, 35, 41, 47, 52, 58 and 66 weeks of age, always between 7.00 and 8.30 am. EDTA was used as anticoagulant. Blood samples were centrifuged (20 min, 4 °C, 2500 g) and the separated plasma was stored at -20 °C until analyzed. Blood sampling was performed randomly in hens kept in standard, enriched and deep litter technology. Utmost care was given to keep the time within 2 minutes between catch and conclusion of blood sampling because this small gap is known to have little or no effect on corticosterone secretion in layers (Craig and Craig, 1985).

Plasma corticosterone and cholesterol analysis

Plasma corticosterone levels were analyzed by radioimmunoassay following dichloromethane (Merck, Darmstadt, Germany) extraction of the steroids from 100 µl aliquots of plasma and using ^{3}H-corticosterone (Amersham, UK) as described previously (**Jezova *et al*., 1994**). Radioactivity of free corticosterone was counted using a liquid scintillation counter (Beckman LS-6500; Beckman-Coulter, USA). Corticosterone (Sigma, Steinheim, Germany, minimal purity 92%, C2505) was used as a standard. Standard curve was performed using 31.5, 62.5, 125, 250, 500, 1000, 2000, 4000, 8000 pg of corticosterone per tube. The sensitivity of corticosterone assay was 0.5 ng.ml^{-1} plasma. The intra- and interassay coefficients of variations were 6 and 8 %, respectively. All hormone measurements were performed in duplicates. Quality of the assay was controlled by repeated analysis of corticosterone in plasma pools with low and high corticosterone concentrations. Measurements of corticosterone in the same control samples were included in all assays performed. Specific antibodies were kindly provided by Prof. C. Oliver, Laboratory of Experimental Neuroendocrinology (Marseille, France). The antibody cross-reacted 100% with cortisone and cortisol, 24% with 11-deoxycorticosterone, 22% with aldosterone, 16% with 17-OH-progesterone, 4% with androstendione, progesterone and testosterone and less than 0.1% with estradiol and estrone.
Total cholesterol concentrations in blood plasma were analysed by spectrometry on the KONELAB T20xt automatic analyser (Thermo Fisher Scientific, Finland) and currently available commercial kits (Biovendor-Laboratorni medicina, Czech Republic).

Statistical analysis

Statistical analysis of the obtained data was performed using the STATISTICA 8.0 programme by single-factor analysis of variance for factor animal age. ANOVA was followed by post-hoc Fischer LSD test for pair-wise comparisons, when appropriate. Evaluation of the interdependence between the cholesterol and corticosterone concentrations was conducted using a correlation coefficient at the level of probability ($P < 0.01$).

RESULTS AND DISCUSSION

One-way ANOVA showed significant differences in blood plasma cholesterol levels of laying hens during the experimental period $F(7, 88)=3.5808$, $p=0.00195$). The Fisher post hoc testing showed a significant increase ($p < 0.01$) of the cholesterol level at the age of 28 weeks (3.81 ± 0.340 mmol.l^{-1}). Subsequently the cholesterol concentrations changed during the experimental period without any significance (figure 1). The highest concentration of cholesterol was recorded in week 47 (4.68 ± 0.918 mmol.l^{-1}). As with cholesterol, the corticosterone concentrations changed during determined period (figure 1). The age of hens significantly influenced the level of plasma corticosterone, as shown by one-way ANOVA ($F(7, 88)=7.2110$, $p=0.00035$). The Fisher post hoc testing showed a significant increase ($p < 0.01$) of the corticosterone level at the age of 52 weeks (1.38 ± 0.067 ng.ml^{-1}). A similar concentration was found at the age of 58 weeks. A significant decrease ($p < 0.01$) was recorded at the end of the experimental period (0.98 ± 0.078 ng.ml^{-1}). A very low relationship was found between the individual total plasma cholesterol and corticosterone concentrations during the monitored period, with a correlation coefficient $r = 0.178$ ($p < 0.05$).

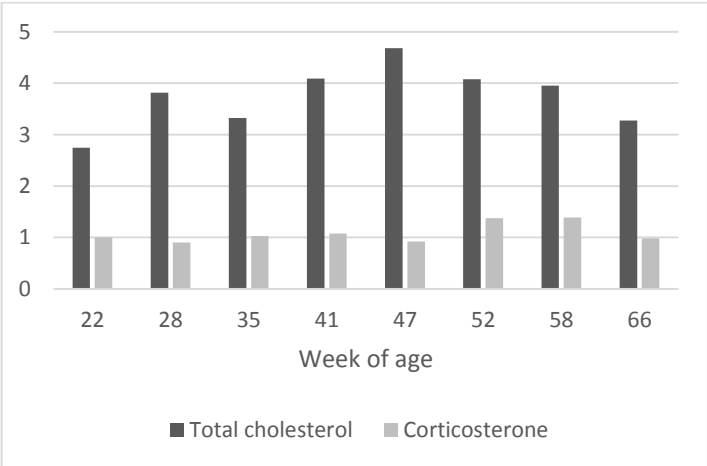

Figure 1 Total cholesterol and corticosterone concentrations in blood plasma of laying hens during experimental period. Total cholesterol is presented in mmol.l^{-1}, corticosterone in ng.ml^{-1}. Data in columns represent mean.

In our experiment, plasma corticosterone levels increased with age, showing some variation in the middle of the trial and a slight decrease at the week 58 up to week 66 of age. Changes in corticosterone levels in the laying period have been attributed to the seasonal effects during breeding (**Koelkebeck *et al*., 1984**). Also, animals are exposed to many external factors. Factors that may induce stress responses include stocking density, temperature, transport, feed restriction, feed contamination, fear and diseases (**Nicol *et al*., 2006**), which can activate the hypothalamic-pituitary-adrenal axis to enhance the release of glucocorticoids from the adrenal gland (**Carsia and Harvey, 2000**). Elevated release of corticosterone from the adrenal cortex in response to stressful stimuli has been proposed to promote gluconeogenesis and lipolysis to break down fat tissues to provide the bird with more energy (**Scanes, 2009**). In our experiment, the concentration of total cholesterol in the blood plasma of laying hens increased in all groups from week 22 to 47 of age, with a subsequent decrease to the end of the monitored period. Intracellular cholesterol content depends on three major factors: cholesterol uptake into cells, de novo cholesterol synthesis within cells, and efflux of cholesterol out of cells (**Feeney *et al*., 2013**). With respect to de novo cholesterol synthesis, multiple mechanisms for the feedback control of cholesterol biosynthesis converge at the rate-limiting enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) (**Garevik et al., 2012**). Glucocorticoids elevate the HMGCR activity in cells (**Cavenee and Melnykovych, 1979**). The chicken HMGCR gene is present in many tissues, including the brain, liver, intestine and skeletal muscle (**Burtea *et al*., 1991**). The liver is a key organ involved in the control of plasma cholesterol, which is largely determined by the rate of removal of low density lipoproteins from the circulation via hepatic low density lipoprotein receptor (**Myant *et al*., 1991**). The number of hepatic low density lipoprotein receptors directly governs plasma low density lipoprotein cholesterol (**Rudling *et al*., 2002**). Glucocorticoids stimulates a concentration- and time-dependent increase of low density lipoprotein receptor biosynthesis in cultured fibroblasts (**Filipovic and Buddecke, 1985**). Receptor-mediated cholesterol uptake is suggested to play a role in maintaining the intracellular free cholesterol pool. **Duan *et al.* (2014)** found that muscular low density lipoprotein receptors levels in corticosterone treated chickens were significantly increased compared with controls. High glucocorticoids levels enhance lipid accumulation into macrophages cultured in vitro by increased cholesterol ester synthesis and decreased cholesterol ester breakdown without altering cholesterol influx or efflux (**Cheng *et al*., 1995**). **Duan *et al.* (2014)** recorded, that chronic corticosterone administration induces cholesterol and triglyceride accumulation in chicken muscle by upregulating their intracellular synthesis and uptake. **Yeon-Hwa Kim *et al.* (2015)** determined significantly higher plasma corticosterone concentrations in corticosterone-treated group, compared with control. Increased concentrations were found also for cholesterol.

CONCLUSION

In this study we analyzed the effect of laying hens housing condition on the plasma total cholesterol and corticosterone concentrations at the beginning, middle and end of the laying period. In conclusion, our experiment revealed no relationship between the blood plasma corticosterone and cholesterol concentrations.

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UTILIZATION OD DIFFERENT MARKERS FOR *HEDERA HELIX*, L. GERMPLASM EVALUATION

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ABSTRACT

Hedera helix is a plant that is valued for its medicinal properties and on the other hand criticized for its invading potential. Beside its medicinal use, a wide utilization as decorative plant is typical for this specie. Up-to date only a few molecular data are available for Hedera helix genome, thus the aim of the study was to evaluate the utilization of different DNA markers for the its further population studies purposes. In total, eight different DNA marker techniques were used for the PCR analysis – iPBS, BARE1 IRAP, Cassandra IRAP, Tst1 IRAP, HACRE 1 IRAP, RAPD, ISSR and miRNA. In total, 38 different iPBS primers were tested with the best results for primers 2152, 2270, 2079, 1899 and 2033. In the case of RAPD primers, all of them worked in the PCR excellent. For ISSR, only a 42 % of the used primers provided results that vere evaluated as very good or excellent ISSR profile. The most successful results were obtained by microsatelite repetitions (GA)8T, (CTG)3GC and (TG)10. A novel type of DNA markers – miRNA provided results that are promising for *Hedera helix*, L. population studies for their further utilization.

Keywords: Hedera helix, L.; iPBS; IRAP; RAPD; ISSR; miRNA; DNA markers

INTRODUCTION

Hedera spp. (*Araliaceae*) is a complex of invasive plant pests that are separated into several distinct taxa. It was introduces mainly as an ornamental plant and up-to-date, several similar forms are sold under the general common name of English ivy (**Reichard, 2000**). Nowadays it is cultivated about 500 different cultures of ivy. Normaly, ivy is popular as decorative plant and has many available cultivars including non-climbing kinds used to cover the soil and compact forms in plots. Thanks to the evergreen and shade-loving qualities, ivy is ideal for conservatories and can create attractive packaging for garden structure.
Hedera helix, L. possess more variants in its morphology and habitat characteristics, it can be found as perennial, herbaceous vine, climber, a herb, a woody subshrub, or very rarely a tree. The stems that are woody, grow in a variable manner as vertically up tree trunks, cliffs or walls, or grow horizontally on the woodland floor (**Grime *et al*., 1988**). In Europe, ivy is distributed from north Africa and the Mediterranean to Norway and eastwards to Latvia and Ukraine, Armenia, Georgia and Iran (**Ellenberg, 1988**). **Laroque (1998)** has reported i tas to be absent in northern and eastern Russia, eastern Poland, the Faroes, Finland, Iceland and Svalbard. The ssp. *Hibernica* is most common along the Atlantic coast of Europe from Ireland to south-west Spain, while ssp. *helix* occupies central and eastern Europe as far as the Ukraine (**Grivet and Petit 2002**). *Hedera helix*, L. is a groundcover that have been used in urban areas for its pros such as appearance, shade-tolerance and easy propagation. Is was reported previously to be usable for the purpose of erosion control and slope stabilization (**Parker, 1996**). Mainly for its extensive utilization in Urban areas landscape formation, now it is regarded as the invador (**Reichard, 2000**).
From the molecular markers point of view, *Hedera*, spp. is one of the species where only a limited information exist. English ivy, is designed as an example of a genomic plasticity which occurs during typical developmental changes from juvenile to adult phase (**Obermayer, 2000**). *Hedera helix* ssp. *helix* $2n = 48$; ssp. *hibernica* $2n = 96$ (**Vargas *et al.* 1999**). Diploid cell DNA content of juvenile leaves is 3.6 pg, and of adult phase leaves 6.2 pg (**Schäffner and Nagl, 1979**). The chromosome number is $2n = 48$ for *Hedera helix* ssp. *Helix* and $2n = 96$ for ssp. *Hibernica* (**Vargas et al., 1999**). **Midori et al. (2006)** identified in their study Hedera species and cultivars most responsible for the invasion of forests in the Pacific Northwest. They have used the RAPD markers as to be universal through the plant kingdom and compared in total 58 selected populations in British Columbia, Oregon, and Washington. The comparison against nine *Hedera* taxa resulted in genetic and morphological identification wehere eighty-five percent of the accessions were attributed to *Hedera hibernica* and fifteen percent to the *Hedera helix* L.
The aim of the performed analysis reported here is the first insight to the utilization of different DNA markers for the *Hedera helix*, L. population studies purposes.

MATERIAL AND METHODS

Plant Material

Juvenile healthy leaves of *Hedera helix*, L. were collected *in situ* during the spring 2015 in the area of Zobor, Nitra. Immediatelly after the collection they were treated with etanol for the purpose of the surface desinfection. When transporting them to the laboratory, they were stored under the -20°C until the further processing. Genomic DNA extraction was performed by GeneJET Plant Genomic DNA kit (ThermoScientific) according the manufacturer instructions. Quantity and quality of extracted DNA was checked by NanoPhotometer P-Class (Implen).

DNA marker terchniques and PCR protocols

In total, eight different DNA marker techniques were used for the PCR analysis. After the optimization procedure individual techniques follow the final thermal profiles as summarized in table 1.

Table 1 Marker techniques and their PCR profiles used in the study.

Marker technique	No. of tested primers	Thermal profile of PCR	Reference	Note for technique
iPBS	38	95°C - 3 min; 35 cycles of: 95°C 30 s; 55°C 40 s; 72°C 120 s; final 72°C 5 min	*	universal/specific
BARE1 IRAP	4	95°C - 3 min; 35 cycles of: 95°C 45 s; 58°C 60 s; 72°C 120 s; final 72°C 10 min	*	specific for BARE 1 retrotransposon
Cassandra IRAP	3	95°C - 3 min; 35 cycles of: 95°C 30 s; 55°C 40 s; 72°C 120 s; final 72°C 5 min	*	specific for Cassandra retrotransposon
Tst1 IRAP	6	94°C - 2 min; 35 cycles of: 94°C 60 s; 55 °C 60 s; 72°C 180 s; final 72°C 10 min	**Bežo et al. (2007)**	specific for Tst 1 retrotransposon
HACRE 1 IRAP	2	95°C - 3 min; 35 cycles of: 95°C 30 s; 62°C 40 s; 72°C 120 s; final 72°C 10 min	**Žiarovská and Bežo (2013)**	specific for HACRE 1 retrotransposon
RAPD	6	94°C - 5 min; 42 cycles of: 94°C 60 s; 38°C 60 s; 72°C 60 s; final 72°C 5 min	**Vivodík et al. (2014)**	universal
ISSR	24	95°C - 3 min; 32 cycles of: 95°C 15 s; 47°C 40 s; 72°C 120 s; final 72°C 7 min	**Batovská et al. (2010)**	universal
miRNA	7	94°C for 5 min; 5 cycles of 94°C 30 s, 64°C 45 s (temp. decreasing by 1°C/cycle), 72°C 60 s; 30 cycles 94°C 30 s, 60°C 45 s, 72°C 60 s; final a72°C 10 min.	**Hlavačková and Ražná (2015)**	universal/species specific

Legend: * modified from the basic PCR protocol in this study

All the amplified products were surveyed for polymorphism using 2% agarose gel electrophoresis.

RESULTS AND DISCUSSION

iPBS technique is used for both – isolation of long terminal repeats retrotransposons as well as an efficient and a general marker system. **Kalendar et al. (2010)** has reported it as suitable for the universal use for both retroviruses and LTR retrotransposons. The method is applicable to any organism with retrotransposons containing primer binding sites that are complementar to the tRNA.

Ones the specific iPBS primers are selected from the universal set for the particular organism, the methoid becames an effective and reproducible one. This is its main pros when comparing it to the unspecific length polymorphism techniques sucha s RAPD or ISSR.

Here, 38 different iPBS primers were tested for the *Hedera helix*, L. in total. Twelve of them provided no amplification pattern in the ivy genome (figure 1). The best results were obtained for primers 2152, 2270, 2079, 1899 and 2033.

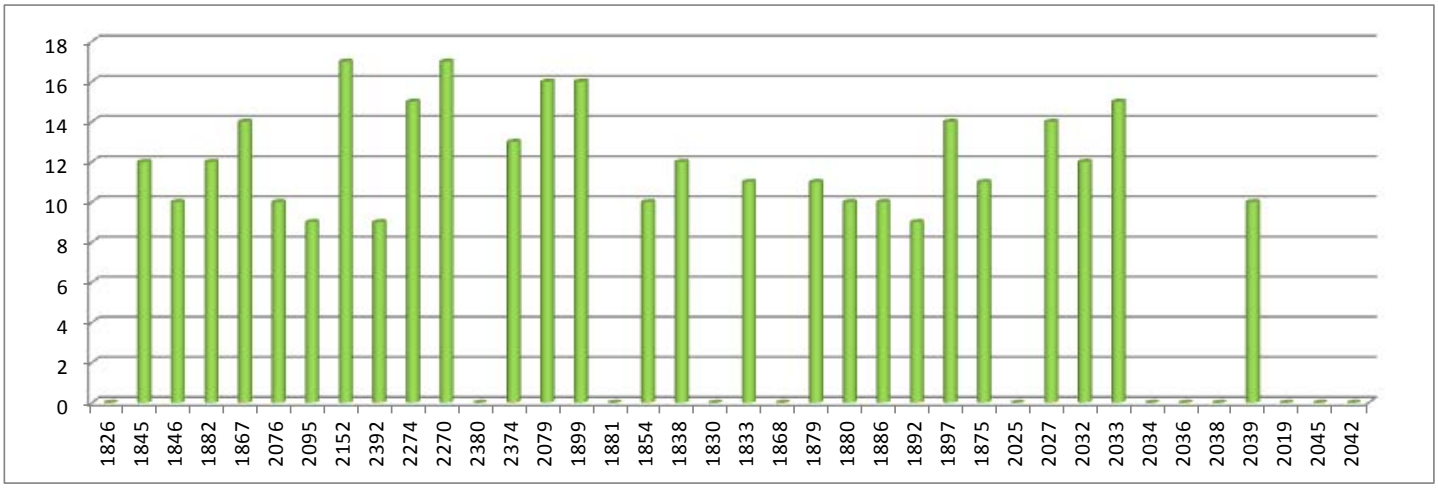

Figure 1 Variability in number of iPBS loci amplification of used primers for the analysed accession of *Hedera helix*, L. Names (number codes) of iPBS primers are as in the **Kalendar et al. (2010)**

The method of iPBS was succesfully used for analysis in many different plant species such *Linum ussitatisimum* (**Smýkal *et al*., 2011**), *Saussurea esthonica* (**Gailīte *et al*., 2011**), *Liparis loeselii* (**Belogrudova *et al*., 2012**) or *Prunus armeniaca* (**Baránek *et al*., 2012**) where was prooved as reliable DNA marker system. Along with the iPBS, another retrotransposon based DNA markers such as IRAP, REMAP, RBIP or SSAP are stil used widely for the analysis of genetic relationships (**Trebichalský *et al*., 2013; Balážová *et al*., 2014; Guo *et al*., 2014**).

Andeden *et al*. 2012 analyzed the genetic diversity within the species of wild growing chickpeas using iPBS retrotransposons and ISSR markers. In total, 136 eventual bands using 10 ISSR primers among 71 entries belonging to class 6 were detected, out of which 135 were polymorphic (99,3%), an average was 13,5 polymorphic fragment per primer, whereas iPBS detected 130 bands of 100% polymorphism on average 13,0 bands per primer. The average of polymorphic information content value was 0,91 for the both of markers system. The clustering of additions and species within the group was almost the same in preparing iPBS and ISSR planar graph NeghorNet (Nnet).

Further, a set of miRNA markers were tested, using the primers and procedure as reported by **Hlavačková and Ražná (2015)** with a successful amplification pattern for ivy in the case of the most primer combinations, that were used. The most amplified fragments were achieved for the primer combination mirR – 156F and 414F – 414R. microRNA length polymorphism marker system was developed by **Fu *et al*. (2013)** and is reported as a technique that possess high reproducibility, sufficient polymorphism and high efficiency of production.

MicroRNA are endogenous non-coding RNAs of the genomes of many organisms. The high conservation of miRNA sequences provides an opportunity for developing specific type of a molecular marker.

Although RAPD is a little overcomed today for the polymorphism analysis of genomic well known plant species it still provides a suitable starting point for those of plant, where limited or none genomic data exist (**Milella *et al*., 2005; Vivodík *et al*., 2015**). The basic difficulty about RAPD is the reproducibility that make it not suitable for the transfer among the laboratories (**Kumar *et al*., 2009**). It is still used for the purposes of rapid sample authentication, but it is not always possible to replicate the fingerprint because even slight instrumentation-dependent variations of PCR can result in variant fingerprints even when the samples of the same genomic DNA are used (**Sucher, Carles, 2008**).

Here, only a five RAPD primers were tested, as the RAPD polymorphism was evaluated by **Midori *et al*. (2006)** in the only DNA marker based study that is actual for *Hedera helix*, L.. Among the used primers (RAPD1 - 5´gatacgttgtc 3´; RAPD2 - 5´cagaagcgga 3´; RAPD3 - 5´aggcccgatg 3´; RAPD4 - 5´cctaaccgtgg 3´; RAPD5 - 5´ctggcgtgtc 3´; RAPD6 - 5´acaccgatgg 3´), all of them provided clear and reproducible banding pattern (figure 2)

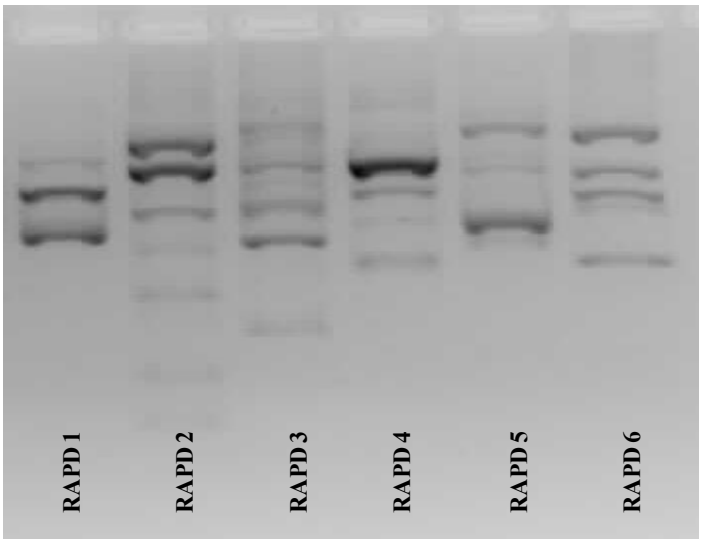

Figure 2 Banding pattern of the RAPD primers used in the study

A different results was obtained in the case of used ISSR markers. Out of the 24 primers used for the testing analysis, 10 of them provided reproducible and clear banding pattern. Eight primers provided no banding pattern and vere evaluated as nonsuitable for further analysis (table 2). The size of the amplified fragments ranged from 3200 bp up to the 2 kb. A range from 5 to the 19 fragments were amplified by the positive tested primers for the analysed Hedera helix, L. accession. The highest number of bands was obtained by $(CTG)_3GC$ microsatellite repeat and the least number by $(CT)_8$.

Table 2 Banding patterns profiles for tested ISSR primers.

Microsatellite repeat	Amplification pattern in *Hedera helix*., L	Microsatellite repeat	Amplification pattern in *Hedera helix*., L
$(GA)_8T$	Excellent	$(ATG)_7$	Very good
$(CA)_8G$	Very good	$(GA)_7G$	Very good
$(AG)_{10}G$	Poor	$CAG(AC)_4AGT$	None
$(AC)_{10}C$	Poor	$(AT)_8$	Very good
$(CTC)_6$ T	Very good	$(GCT)_7$	Poor
$(CAC)_6C$	None	$(TG)_{10}$	Excellent
$(GATA)_2$ $(GACA)_2$	Very good	$(GC)_{13}$	None
$(CTG)_3GC$	Excellent	$(GCA)_5(CCA)_5$	None
$(CA)_6GG$	None	$(TTT)A_5$	None
$(AT)_8T$	None	$AG(TA)_8$	None
$(CT)_8$	None	$(AG)_{13}$	Very good
$AGT(TG)_6$	Poor	$GT(CA)_4$	None

Zhuravlev *et al*. 2003 investigated genetic relationship within the species of *Araliaceae* of Far East. By preliminary sreening of 120 commercial primers, 84 primers effective in PCR reaction were selected. Some of them were used in RAPD analysis of 11 species. By comparing of RAPD pattern obtained, variability of 595 loci was estimated.

Morales *et al*. 2011 used RAPD and ISSR methods in accessing genetic similarity of strawberries. DNA was obtained from 11 cultivars grown under controlled conditions using 40 RAPD and 16 ISSR markers. DNA fragments isolated in agarose gel were used for RAPD method and in polyakrylamid gel for ISSR method. Matrix of genetic similarity was estimated with Jaccard coeficient of similarity. The dendrogram generated by RAPD markers distributed cultivars into three groups, while ISSR markers generated two groups. From 40 RAPD markers only 11 were polymorphic, the others showed either no effect or only a low amplification quality. By the ISSR markers, 6 from 16 evaluated primers showed good amplification quality. There was no direct relationship detected when both types of markers were compared. The grouping designed by ISSR markers was more in line with the origin and genealogy of cultivars than RAPD markers suggested, which can be considered the most effective method for studying the genetic diversity of strawberries.

Kebour *et al*. 2012 used ISSR markers in the study of genetic polymorphism in the pistachio, *Pistacia vera.* During ISSR skreening based on GA (guanin-adenin), CA a GAA, good amplifications products were obtained. A total of 111 band, 60 bands were polymorphic (54,04%), amplified with 6 primers, average 7 bands per primer. Total number of amplified products were in the range from 5 to 10 and the number of polymorphic fragments in the range of 4 to 7. The range of genetic similarities reached the level of 0,84 to 1. The ISSR analysis showed a sufficient polymorphism of a large-scale DNA fingerprinting.

For IRAP analysis test, four different retrotransposons were selected, based on their abundance in plant genomes. All of them, Bare1 retrotransposon, Tst1 retrotransposon, Hacre1 retrotransposon and Cassandra retrotransposon were previously reported as efficient DNA markers for different plant species (**Kalendar *et al*., 2000; Kalendar *et al*., 2008; Buti *et al*., 2009**). All of them were confirmed as presented in the genome of *Hedera helix*, L. by performed analysis. In the cases of BARE1, Tst1 and HACRE 1 retrotransposons, the IRAP profile was with low number of amplified bands ranged from 4 up to the 7 bands with the only exception of Barley P04 primer that provided 11 scorable fragments. A much more bands were obtained for Cassandra retrotransposon primers, that were used in the study. They ranged from 17 up to the 24 fragments what is in concordance with the findings, that Cassandra is present up to date in all plants that were analysed for Cassandra IRAP polymorphism (**Kalendar *et al*., 2008**).

CONCLUSION

For the purposes of molecular analysis of *Hedera helix*, L., different DNA marker techniques were evaluated in this study. Based on the type of the used technique, different results were obtained in their ability to amplified length polymorphism amplicons by PCR. The most stable techniques that have the potential for the purpose of *Hedera helix*, L. population studies were iPBS and IRAP technique for Cassandra retrotransposon. A novel type of DNA markers – miRNA markers were proved as suitable for the molecular studies of *Hedera helix*, L.

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DO PROGESTERONE, IGF-I, IGFBP-3 AND IGFBP-4 RELATE TO SEXUAL MATURATION?

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ABSTRACT

Hormones and binding proteins can regulate reproduction, but their involvement in sexual maturation remains to be elucidated. This study describes possible hormonal regulators of female sexual maturation. For this purpose, the release of steroid hormone progesterone (P_4), insulin-like growth factor I (IGF-I) and IGF-binding proteins (IGFBP-3, IGFBP-4) were shown in this study. Sexual maturation in gilts was found to be associated with a significant increase in the release of P_4, IGF-I and IGFBP-3 *in vitro*. Furthermore, sexual maturation was associated with significant increase in the expression of IGFBP-3 but not in IGFBP-4. The present data obtained from *in vitro* study indicate that sexual maturation in females is influenced by puberty-related changes in porcine ovarian signalling substances: increase in P_4, IGF-I, IGFBP-3 but not IGFBP-4. It suggests that these signalling molecules could be potential regulators of porcine sexual maturation.

Keywords: Sexual maturation, porcine granulosa cells, progesterone, insulin-like growth factor I and IGF-binding proteins

INTRODUCTION

Sexual maturation is associated with ovarian follicular growth and differentiation (**Onagbesan *et al.*, 2009; Palma *et al.*, 2012**). These processes are governed by hormones, growth factors and their binding proteins (**Kolesarova *et al.*, 2008; Sirotkin, 2013**). There is indirect evidence for involvement of several candidate signalling substances in control of sexual maturation and/or related ovarian follicle development. Steroid hormone progesterone (P_4) is essential for normal ovarian cycles (**Arnhold *et al.*, 2009; Hagan *et al.*, 2009**) and contributes to regulation of ovarian follicular development and remodelling (**Astiz, 2013; Mahajan, 2008**). Progesterone produced by porcine ovarian granulosa cells (**Duda *et al.*, 2012; Kolesarova *et al.*, 2009b, 2010**) and the *corpus luteum* (**Gregoraszczuk, 1992,1997; Mahajan, 2008; Shah and Nagarajan, 2013**) is a local paracrine or autocrine promoter of ovarian cell luteinization (**Gregoraszczuk, 1994**). In cyclic animals, when the early follicular growth is initiated, a high amount of P_4 is secreted by secondary, tertiary and luteinized ovarian follicles and active corpora lutea, into the peripheral blood (**Mahajan, 2008**).
Insulin-like growth factor I (IGF-I) is known to stimulate ovarian follicular growth (**Lucy, 2008**) and development (**Carter *et al.*, 2006**) by promoting granulosa cell proliferation, follicular antrum formation (**Mao *et al.*, 2004**), hyperplasia of ovarian surface epithelium (**King *et al.*, 2013**), releasing ovarian hormones (**Kolesarova *et al.*, 2008**) and decreasing ovarian cell apoptosis (**Mao *et al.*, 2004**). IGF-I has been found to be produced by porcine (**Kolesarova *et al.*, 2008, 2009b, 2010**), chicken (**Sirotkin *et al.*, 2006**) and human (**Karamouti *et al.*, 2008**) ovarian cells. The effects of IGF-I on the ovary may be modified by the local production of IGF binding proteins (IGFBPs) (**Sandhu *et al.*, 2002; Yi *et al.*, 2001**). In the ovary, IGFBP-3 appears to neutralize the actions of IGF-I (**Bicsak *et al.*, 1990,1991; Ui *et al.*, 1989**). IGFBP-3 not bound to IGF also affects cells via mechanisms involving binding to specific cell surface receptors and/or transport into the cell (**Xi *et al.*, 2007**). IGFBP-4 modulates autocrine/paracrine action of IGF in both follicular growth and differentiation in the porcine ovary (**Zhou *et al.*, 1996**). The secretion of IGFBP-4 is higher in immature granulosa cells as compared to mature porcine ovarian follicles (**Grimes *et al.*, 1994**).
The general aim of the *in-vitro* experiments with porcine ovarian granulosa cells was to identify possible hormonal regulators of female sexual maturation. For this purpose, these signaling molecules were evaluated in granulosa cells collected from sexually mature and immature gilts.

MATERIALS AND METHODS

Animals

Healthy gilts of Slovakian White breed were reared under standard conditions at the Experimental Station of the Slovak University of Agriculture in Nitra, Slovakia. Conditions of their care and handling corresponded to the instructions of the European Commission (EC) no. 178/2002 and related EC documents and as approved by local ethics committee. Animals (n=35) were assigned at slaughter into two groups: sexually immature (n=18) and animals of the same age having reached sexual maturity (n=17) according to visual characteristics of ovaries (presence of follicles larger than 5 mm).

Preparation, culture and processing of granulosa cells

Ovaries were transported to the laboratory at 4ºC and washed in sterile physiological solution. Ovaries from immature and mature gilts were processed separately. Follicular fluid was aspirated from 3-5 mm follicles, granulosa cells were isolated by centrifugation for 10 min at 200xg followed by washing in sterile DMEM/F12 1:1 medium (BioWhittaker™, Verviers, Belgium) and resuspended in the same medium supplemented with 10% fetal calf serum (BioWhittaker™) and 1% antibotic-antimycotic solution (Sigma, St. Louis, Mo, USA) at a final concentration of 10^6 cells/mL of medium. Portions of the cell suspension were dispensed to 24-welled culture plates (Nunc™, Roskilde, Denmark, 1 ml/well; for RIA) or Lab-Tek 16-welled chamber slides (Nunc Inc., International, Naperville, USA, 100 µl/well; for immunocytochemistry). Both the plate wells and chamber slides were incubated at 37.5ºC and 5% CO_2 in humidified air until a 75 % confluent monolayer was formed (5-7 days), at which point the medium was replaced with fresh medium. Further culture was performed in 300 µl medium in 16-welled chamber slide cells or 1 ml of culture plate. After 2 days of culture the media from wells were removed, wells from chamber slides were washed in ice-cold PBS (ph 7.5). Cells were fixed for 1 h at room temperature in 4% paraformaldehyde, dehydrated in alcohols (70, 80, 96%; 10 min each) and stored in 96% alcohol at -4ºC to await immunocytochemical analysis. Media from plate wells were aspirated and kept at -70 ºC to await RIA.

Immunocytochemistry

Immunocytochemistry was used to detect IGFBP-3, IGFBP-4 in granulosa cells plated on chamber slides. Primary mouse monoclonal antibodies to each petide IGFBP-3, IGFBP-4 (cross-reacting with corresponding rat, human, porcine and chicken substances; all from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) were used as directed by the manufacturer at a dilution of 1:100. Visualisation of the primary antibody binding sites was done with a secondary rabbit polyclonal antibody against mouse IGs, labelled with horseradish peroxidase (Sevac, Prague, Czech Republic; dilution 1:500) and diaminobenzidine (DAB) reagent (Roche Diagnostics Corporation, IN, USA, 10%). The presence of each peptide was determined by light microscopy. To verify these data, in some selected cases primary antibodies were visualised by secondary rabbit or goat monoclonal antibodies against mouse IGs labelled with FITC (Sevac, Prague, Czech Republic) and fluorescent microscopy. Negative control was presented by stained cells omitting primary antibody. During microscopic inspection, the percentage of cells containing visible antigen was determined.

Immunoassay

Levels of progesterone and IGFBP-3 were determined in duplicate in 20-100 µl samples by radioimmunoassay (RIA). Progesterone and IGFBP-3 were evaluated after ethanol extraction using RIA kits from DSL (Webster, USA) according to manufacturer's instructions while IGF-I was assayed as described previously. All RIA were validated for use in samples of culture medium. RIA assay for P_4: the antiserum cross-reacted was <0.001%, the sensitivity was 0.12 ng/mL. RIA assay for IGFBP-3: the antiserum cross-reacted was <0.03%, the sensitivity was 0.5 ng/mL. RIA assay for IGF-I: Inter- and intra-assay coefficients of variation did not exceed 10% and 16%, respectively. The sensitivity of the assay as determined by the dilution method was 0.3 ng/mL.

Statistics

Each experimental group was represented by four culture wells with granulosa cells. Assays of hormonal substances in incubation medium were performed in duplicate. The data presented concerning the effects of each substance are means of values obtained in three separate experiments performed on separate days using separate ovaries. The values of blank controls were subtracted from the values determined by RIA in cell-conditioned medium to exclude any non-specific background (less than 13% of total values). The rates of hormone secretion were calculated per 10^6 cells per day. The proportion of cells containing each analysed substance was calculated following immunocytochemical analysis by counting at least 1000 cells per chamber slide well. Firstly, the data obtained in each experiment were processed by ANOVA. Thereafter, significant differences between the immature groups and mature gilts were evaluated by paired t-test or chi-square (χ^2) test by using statistical software Sigma Plot 9.0 (Jandel, Corte Madera, USA). Differences from controls (P<0.05) were considered as significant.

RESULTS

Release of progesterone by ovarian granulosa cells was significantly higher (p<0.05) in sexually mature gilts (5.3±0.1 ng/mL vs 4.3±0.3 ng/mL) in comparison to sexually immature animals (Fig. 1). Release of IGF-I also followed the same pattern (7.8±0.6 ng/mL vs 6.2±0.2 ng/mL) (Fig. 1). Percentage of ovarian granulosa cells expressing IGFBP-3 was also significantly higher (p<0.05) in sexually mature gilts (53.1±0.4%) than the immature animals (36.5±2.3%) (Fig. 2). Although the IGFBP-4 expression by granulosa cells did not change significantly with sexual maturity (41.9±02.3% in mature gilts vs. 37.2±3.1% in immature ones) (Fig. 2).

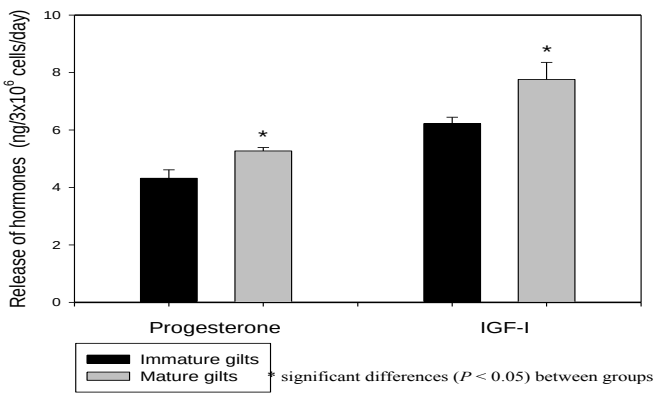

Figure 1 Release of progesterone and IGF-I by ovarian granulosa cells of sexually immature and mature gilts. Values are means ± SD, *significant difference ($P < 0.05$) between corresponding groups of sexually immature (n=18) and mature (n=17) gilts were evaluated by paired t-test and chi-square (χ^2) test.

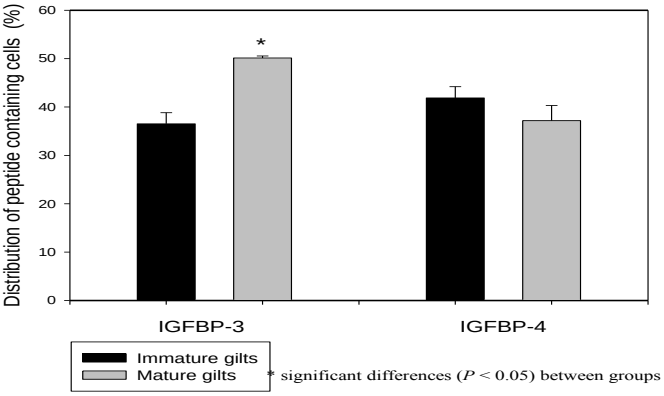

Figure 2 Expression of IGFBP-3 and IGFBP-4 in ovarian granulosa cells of sexually immature and mature gilts. Values are means ± SD, *significant difference ($P < 0.05$) between corresponding groups of sexually immature (n=18) and mature (n=17) gilts were evaluated by paired t-test and chi-square (χ^2) test.

DISCUSSION

Do progesterone, IGF-I, IGFBP-3 and IGFBP-4 relate to sexual maturation?

The previous observation in primates, rats, cattle (**Prunier and Louveau, 1997**) and pig (**Kolesarova *et al.*, 2010; Kolesarova *et al.*, 2008**) make it clear that sexual maturation was associated with the increase in blood concentrations of IGF-I. *In vivo* results concerning IGF-I levels in blood plasma in the study of **Kolesarova *et al.* (2008)** were also confirmed by *in vitro* results from IGF-I release by cultured ovarian granulosa cells indicating the sexual maturation-dependent increase in gilts . We report that increase in IGF-I release *in vitro* in gilts was associated with sexual maturation, and that therefore IGF-I may be involved in control of this process.

In our previous study (**Kolesarova *et al.*, 2008**), we also noted lower (p<0.05) levels of IGFBP-3 in blood plasma and granulosa cells of sexually immature gilts in comparison to mature animals. These results confirm our previous *in vivo* study (**Kolesarova *et al.*, 2010**). Plasma 43-39 kDa IGFBP levels were found to increase whereas plasma 34 kDa IGFBP decreased with age (p<0.01) (**Prunier and Louveau, 1997**).

Expression of IGFBP-4 in granulosa cells did not change with sexual maturity (**Kolesarova *et al.*, 2008**). **Grimes *et al.* (1994)** reported that the secretion of IGFBP-4 was higher in granulosa cells from immature porcine ovarian follicles (**Grimes *et al.*, 1994**). Low molecular weight IGFBPs, especially IGFBP-4, was the highest in small immature follicles that are predominantly atretic in pigs (**Howard *et al.*, 1991; Mondschein *et al.*, 1991; Ryan, 1981**). Our observations, together with previous reports (**Liu *et al.*, 1993; Sirotkin *et al.*, 2001**) suggest that IGFBPs could be important regulators of follicular growth and differentiation.

Possible interrelationships between studied substances

Certain changes observed in our investigations could be primary; others could be secondary, i.e. mediated by upstream regulators. For example, changes in P_4 release may be due to changes in IGF-I output. At least a positive relationship between P_4 and IGF-I concentrations in porcine blood (**Langendijk *et al.*, 2008**) and the ability of IGF-I to activate porcine ovarian steroid hormone release (**Sirotkin *et al.*, 2004**) has been reported. The opposite action of steroids on IGF-I is less probable because previous study showed that gonad steroids are not involved or play only a minor role in the control of IGF-I and IGFBP plasma levels during pubertal development in gilts (**Prunier and Louveau, 1997**). Previous authors showed stimulatory action of IGF-I on granulosa cell steroidogenesis which increased with follicular development, whereas its mitogenic action on granulosa cells decreased with follicular phase progression (**Kolodziejczyk *et al.*, 2003**). Interrelationships between IGF-I and IGFBPs are well known. A strong positive correlation between IGF-I and IGFBP-3 concentration was apparent with increasing age of the animals suggesting functional interrelations between the substances during sexual maturation (**Lee *et al.*, 2002**). Cooperation between IGF-I and IGFBP-4 in control of porcine ovarian folliculogenesis, follicular selection and luteinization was outlined earlier (**Grimes *et al.*, 1994**), wherein increased expression of both IGF-I and IGFBP-4 mRNAs during follicular selection and luteinisation was reported. Furthermore, it was observed that the action of IGFBP-4 on the ovary can be mediated by modulation (**Zhou *et al.*, 1996**) or stimulation (**Sirotkin *et al.*, 2001**) of IGF-I release and/or by inhibition of P_4 output (**Sirotkin *et al.*, 2001**). Therefore, ovarian follicular growth, selection, luteinization and related increase in progesterone release during porcine sexual maturation can be regulated by members of ovarian IGF-I/IGFBP system. In our experiments, the puberty-related changes in IGF-I, IGFBP-3 and P_4, but not in IGFBP4 were observed (**Kolesarova *et al.*, 2008**).

CONCLUSION

The present data obtained from *in vitro* study indicate that sexual maturation in females is influenced by puberty-related changes in porcine ovarian signaling substances: increase in P_4, IGF-I, IGFBP-3 but not IGFBP-4. It suggests that these signaling molecules could be potential regulators of porcine sexual maturation. Therefore, it may be suggested that porcine sexual maturation can be regulated by IGF-I-IGFBP3-P_4, but not by IGF-I-IGFBP4-P_4 system. Although the puberty-related changes don't provide direct evidence of the involvement and physiological role of these signaling molecules in control of sexual maturation, our study enables to identify extracellular signaling substances, which could be potential candidates for induction of porcine puberty and sexual maturation.

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STIMULATORY EFFECT OF AMYGDALIN ON THE VIABILITY AND STEROID HORMONE SECRETION BY PORCINE OVARIAN GRANULOSA CELLS *IN VITRO*

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ABSTRACT

Amygdalin has been one of the most popular "alternative cancer cures" in many European and South American countries. Its anticancer, anti-inflammatory activity and other medicinal benefits have been known for many years. The objective of this in vitro study was to examine the potential impact of amygdalin on the cell viability and production of steroid hormone testosterone by porcine ovarian granulosa cells. Granulosa cells were isolated from porcine ovaries and subsequently cultured without (control) or with amygdalin at various doses (1; 10; 100; 1000 and 10 000 µg/mL) for 24 h. The cell viability was determined by alamarBlue™ reagent and release of testosterone was assayed by ELISA. Obtained results showed a significant ($P<0.05$) increase of testosterone secretion only at the highest dose of amygdalin (10 000 µg/mL). Other experimental doses of amygdalin did not affect the testosterone production. Moreover, amygdalin treatment strongly enhanced the viability of ovarian granulosa cells. The viability was significantly ($P<0.05$) stimulated after amygdalin treatment at all used doses, except the highest concentration (10 000 µg/mL). To conclude, application of amygdalin to culture media positively affected cell viability, but not highest dose (10 000 µg/mL), and stimulated testosterone release by porcine ovarian cell. Present results could help to reveal the potential impact of amygdalin on cellular growth, as well as its mechanism of action in processes of ovarian steroidogenesis.

Keywords: Amygdalin, cell viability, testosterone, ovarian granulosa cells

INTRODUCTION

Natural plant origin products like amygdalin are still a major part of traditional medicine More than 50% of cancer patients in Europe use complementary/ alternative medicine (CAM) instead of, or combined with, conventional therapy (**Nabavizadeh *et al*., 2011; Huebner *et al.*, 2014**). Amygdalin has been one of the most popular "alternative cancer cures" in many European and South American countries for long period (**Chang *et al*., 2006; Hwang *et al*., 2008; Makarević *et al*., 2014**).
This natural substance is occurring in the seeds of various plant species, belonging to the *Rosaceae* family, such as bitter almonds, apricots, apples and other. Its anticancer, anti-inflammatory activity and other medicinal benefits have been known for many years. This bioactive compound is composed of glucose, benzaldehyde, which induces an analgesic action, and hydrocyanic acid, which is an anti-neoplastic compound (**Fukuda *et al*., 2003; Chang *et al*., 2006**). β-glucosidase, one of the enzymes that catalyzes the release of cyanide from amygdalin, is present in the human small intestine and is also found in a variety of common foods (**Strugala *et al*., 1995; Deng *et al*., 2002**). In the late 1970s and early 1980s, amygdalin was reported to selectively kill cancer cells at the tumor site without systemic toxicity and to effectively relieve pain in cancer patients (**Ellison *et al*., 1978**). Nowadays, there are statistics described that by 1978 more than 70,000 patients with cancers in the United States have been treated with amygdalin (**Moss, 2005; Makarević *et al*., 2014; Qian *et al*., 2015**). However, the use of the drug was discouraged when it was demonstrated that amygdalin is metabolized in the body to release significant amount of cyanide thus leading to cyanide poisoning (**Chandler *et al*., 1984; Bromley *et al*., 2005**). Numerous studies have demonstrated the beneficial properties of amygdalin and its ability to effectively induce cell death (**Zhou *et al*., 2012; Chen e*t al*., 2013**). Nevertheless, proponents consider amygdalin a natural cancer cure, whereas opponents warn that amygdalin is ineffective and even toxic (**Makarević *et al*., 2014**).
The endocrine signaling molecules represent source of communication between several organs, as well as specific cell populations. Testosterone, one of the steroid hormones, plays a key role in ovarian cycle, folliculogenesis, cell proliferation, and also in programmed cell death (**Graham *et al*., 1997; Sirotkin, 2014**). Unfortunately, there is still no scientific evidence related to the potential effect of amygdalin on the healthy, non-pathologic cells.
This *in vitro* study demonstrates the potential effect of amygdalin on the cell viability and production of steroid hormone by porcine ovarian granulosa cells (GCs).

MATERIAL AND METHODS

Preparation, culture and processing of granulosa cells from ovaries

Ovaries from non-cyclic pigs were obtained from healthy Slovakian White gilts without obvious reproductive abnormalities. The ovaries were transported to the laboratory in containers at 4 ºC and washed in sterile physiological solution. The follicular fluid was aspirated from 3-5 mm follicles. The granulosa cells (GCs) were isolated by centrifugation for 10 min at 200xg followed by washing in sterile DMEM/F12 1:1 medium (BioWhittaker™, Verviers, Belgium) and resuspended in the same medium supplemented with 10% fetal calf serum (BioWhittaker™, Verviers, Belgium) and 1% antibiotic-antimycotic solution (Sigma, St. Louis, Mo, USA) at the final concentration of 10^6 cells/mL (as detected by a haemocytometer). Portions of the cell suspension were dispensed to 24-welled culture plates (Nunc™, Roskilde, Denmark, 1ml/well; for Enzyme Linked Immuno Sorbent Assay, ELISA). The well plates were incubated at 37 °C and 5% CO_2 in humidified air until a 75% confluent monolayer was formed (4-5 days), at this point, the medium was renewed and ovarian granulosa cells were incubated with the same supplements (DMEM/F12 1:1 medium, 10% fetal calf serum, without 1% antibiotic-antimycotic solution) and without (control) or with amygdalin (1, 10, 100, 1000, 10 000 µg/mL) (≥99 % purity, from apricot kernels, Sigma-Aldrich, St. Louis, Mo, USA) for 24h. After 24h of incubation the culture media from well plates were aspirated and kept at –80°C for subsequent assay. The concentrations of steroid hormones progesterone and estradiol-17β were

assayed using ELISA (Dialab, Wiener Neudorf, Austria) according to the manufacturer´s instructions.

Cell viability test

The cell viability was determined after treatment of amygdalin by alamarBlue™ reagent (BioSource International, Nivelles, Belgium) (**Bannerman *et al*., 2001; Nynca *et al*., 2009**). This assay is based on the ability of living and metabolically active cells to convert the oxidized indigo blue state of alamarBlue dye into the reduced pink state. Isolated granulosa cells were cultured in 96-well plates/100 µL at the concentration 0.1 x 10^5 cells per well (37 °C, 5% CO_2). After pre-incubation (48/72 h), the monolayer of granulosa cells was cultured for 24 hours with or without amygdalin (1, 10, 100, 1000, 10 000 µg/mL) or DMSO (as a positive control). Twenty four hours before the end of cell culture, alamarBlue™ dye was added to all wells. Thereafter, alamarBlue™ reduction was measured spectrophotometrically at 565 and 595 nm and expressed as a percentage according to the manufacturer calculations. All analyses were performed in quadruplicates.

Statistical Analysis

Each experimental group was represented by four culture wells of granulosa cells (each dose = 4 replicates, biological parallels). Assessments of hormone concentrations in the incubation media were performed in duplicates. The data are presented as means of values obtained from one experiment using separate pools of ovaries from 10–12 animals. The significance of differences between the control and experimental groups was evaluated by One-Way ANOVA (Dunnett´s multiple comparison test) using the statistical software GraphPad Prism 3.01 (GraphPad Software Inc., San Diego, CA, USA). The data are expressed as means ± SEM. Differences were compared for statistical significance at the *p*-level less than 0.05 ($P<0.05$).

RESULTS

The effect of amygdalin on viability of porcine ovarian granulosa cells in vitro

The viability of granulosa cells isolated from porcine ovaries was assayed after amygdalin application (Fig.1). Amygdalin aplication led to a significant ($P<0.05$) stimulation in viability of ovarian granulosa cells, compared to control cells. Viability was stimulated by the increasing doses of amygdalin (1, 10, 100, 1000 µg/mL). Whereas, the highest metabolic activity was detected after exposure of amygdalin at dose 1000 µg/mL. However, the highest dose of amygdalin (10 000 µg/mL) did not affect the viability of porcine ovarian granulosa cells, in comparison to untreated control cells.

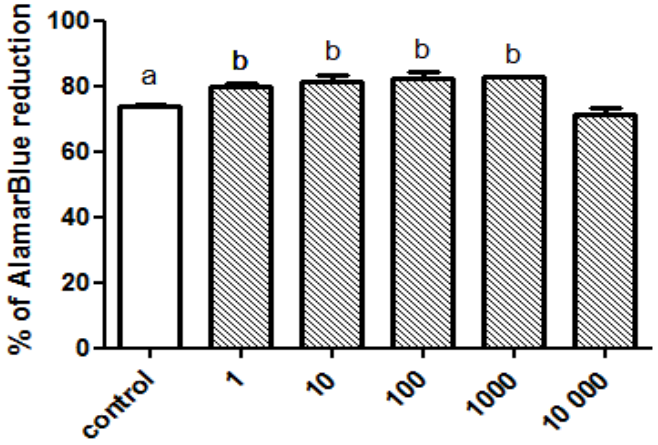

Figure 1 The viability of porcine ovarian granulosa cells incubated for 24 h without (control) or with amygdalin treatment (1, 10, 100, 1000, 10 000 µg/mL). Signs *a, b* denote value significantly ($P<0.05$) different from control group. Significance of differences between the groups was evaluated by One-way ANOVA (Dunnett´s multiple comparison test). The data are expressed as means ± SEM. AlamarBlue™.

The effect of amygdalin on testosterone release by porcine ovarian granulosa cells

The release of steroid hormone testosterone by ovarian granulosa cells after amygdalin addition is shown in Figure 2. Significant ($P<0.05$) stimulation of testosterone production was observed after amygdalin treatment at highest used dose (10 000 µg/mL), in comparison to control group without addition of the substance. However, other experimental doses of amygdalin did not affect the testosterone release by granulosa cells.

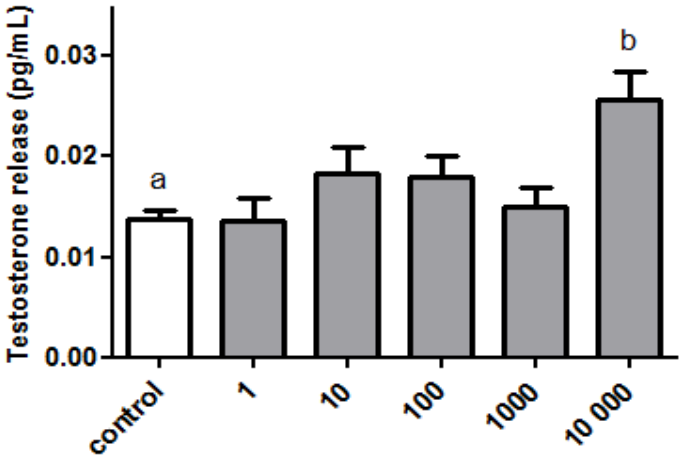

Figure 2 The effect of amygdalin on testosterone release by porcine ovarian granulosa cells. The control represents culture media without amygdalin addition; the experimental groups represent culture media supplemented with amygdalin (1, 10, 100, 1000, 10 000 µg/mL) addition. Signs *a, b* denote value significantly ($P<0.05$) different from control group. Significance of differences between the groups was evaluated by One-way ANOVA (Dunnett´s multiple comparison test). The data are expressed as means ± SEM. ELISA.

DISCUSSION

The present investigation suggests stimulatory impact of amygdalin on a viability of ovarian granulosa cells, as well as the production of steroid hormone testosterone *in vitro*. Granulosa cells, isolated from porcine ovaries, were able to survive, grow in culture and release the steroid hormone after the experimental addition of natural compound amygdalin. Numerous studies have reported the inhibitory action of amygdalin on the proliferation and growth of different cancer cells (**Syrigos *et al*., 1998; Chen *et al*., 2013; Qian *et al*., 2015**). However, possible impact of amygdalin on the cellular growth, differentiation and death of normal, non-pathological cells remains unknown.

In our study, amygdalin strongly enhanced the viability of ovarian granulosa cells at all doses, except the highest used dose (10 000 µg/mL). Viability of ovarian cells was stimulated by the increasing doses of amygdalin (1, 10, 100, 1000 µg/mL). Previously, cytotoxic effect of amygdalin on human prostate cancer cells was evidenced by **Chang *et al*. (2006)**. Amygdalin exhibited a dose-dependent suppression of cell viability at higher concentrations. Moreover, amygdalin is able to block the bladder cancer cell growth *in vitro* by diminishing of cellular regulators (**Makarevic *et al*., 2014**). Recent study carried out by **Nynca *et al*. 2009** demonstrated the effect of a natural phytoestrogen-daidzein on the viability of porcine ovarian granulosa cells. They found that the cell viability was not affected by daidzein application at various doses (0.5-50 µM).

Previous studies described the effect of various natural substances with protective (**Kolesárova *et al*., 2012; Halenár *et al*., 2013**) or toxic (**Ranzenigo *et al*., 2008; Medveďová *et al*., 2011; Maruniaková *et al*., 2013**) potential on the cellular processes in ovarian cells. In addition, the release of steroid hormone testosterone by ovarian granulosa cells after amygdalin application was observed in this examination. Increased secretion ($P<0.05$) of testosterone by granulosa cells was detected only in experimental group with the highest dose of amygdalin (10 000 µg/mL). The present results are in accordance with our recent examination, where a stimulatory effect of amygdalin on the release of 17β-estradiol by ovarian granulosa cells was observed, too. Amygdalin treatment (10 000 µg/mL) resulted in significant ($P<0.05$) increase of the hormone production by porcine ovarian granulosa cells (**Halenár *et al*., 2015**). Interestingly, the presence of amygdalin at selected doses did not affect the progesterone secretion by porcine ovarian GCs.

Similarly, exposure of deoxynivalenol, resveratrol and their combination on the release of progesterone by porcine ovarian granulosa cells was studied by **Kolesárová *et al*. (2012)**. Progesterone release was significantly ($P<0.05$) stimulated by resveratrol treatment at the dose 50 µg/mL, but not at 30 and 10 µg/mL. **Kádasi *et al*. (2012)** also reported a stimulatory effect of curcumin, a natural plant molecule, on the release of testosterone by porcine ovarian granulosa cells.

CONCLUSION

This study helps to reveal the potential impact of amygdalin on cellular growth, mechanism of action in processes of ovarian steroidogenesis. Application of amygdalin to culture media affected cell viability, but not the highest dose (10 000 µg/mL), and stimulated testosterone release by porcine ovarian granulosa cells.

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COMPARISON OF TWO COLORIMETRIC ANTIOXIDANT CAPACITY ASSESSMENT METHODS IN BOVINE SEMEN FRACTIONS

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ABSTRACT

The antioxidant capacity represents the concentration and activity of diverse components which prevent oxidative damage to biomolecules. This study compares the effectiveness two routinely available protocols (Total antioxidant status - TAS and Ferric reducing ability of plasma - FRAP) with respect to the relationship between the antioxidant capacity and semen quality in bovine seminal plasma and spermatozoa. Computer assisted semen analysis was used to evaluate selected spermatozoa motion parameters; TAS and FRAP were assessed using UV/VIS spectrophotometry. Higher antioxidant activity ($P<0.01$) measured by both techniques was recorded in the seminal plasma. At the same time, the antioxidant properties evaluated by both techniques significantly ($P<0.01$) reflected on the semen quality in both seminal plasma as well as spermatozoa. Our results suggest that both methodologies used to assess the antioxidant capacity of semen are equally suitable to be routinely used in veterinary medicine.

Keywords: Bulls, seminal plasma, spermatozoa, antioxidant capacity, total antioxidant status, ferric reducing ability of plasma

INTRODUCTION

Literally every ejaculate is ought to be contaminated with potential sources of reactive oxygen species (ROS) (**Aitken, 1995**), high concentration of which has been associated with a decreased motility (**Eskenazi *et al.*, 2003**) and efficacy of sperm-oocyte fusion (**Agarwal *et al.*, 2007**), increased DNA damage (**Armstrong *et al.*, 1999**) as well as cellular membrane lipid peroxidation (**Aitken, 1995**) in the male gamete.

Because ROS have both physiological and pathological properties, the organism has developed specific defence mechanisms to maintain the ROS levels within a physiological range. Antioxidants are therefore compounds that dispose, scavenge and inhibit the formation of ROS, or oppose their actions (**Sikka *et al.*, 1995**). Due to the small volume of cytoplasm and low concentrations of scavenging enzymes, spermatozoa have limited antioxidant protection. Meanwhile, the seminal plasma has been repeatedly defined as an important protector against possible ROS formation or distribution, due to a vast array of both enzymatic as well as non-enzymatic antioxidants (**Agarwal *et al.*, 2006**).

The chemical diversity of antioxidants makes it difficult to separate, detect or quantify individual antioxidants from a complex biological sample. Therefore, the total antioxidant capacity is often useful to evaluate the general beneficial properties of the sample based on the cooperative action of individual antioxidant compounds (**Apak *et al.*, 2013**).

Several assays have been proposed to estimate the antioxidant capacity in animal reproductive cells and tissues, including 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) (**Badarinath *et al.*, 2010**), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (**Alam *et al.*, 2002**), ferric reducing antioxidant power (FRAP) (**Benzie and Strain, 1999**) or the oxygen radical absorption capacity (ORAC) (**Prior *et al.*, 2003**). According to **Alam *et al.* (2013)**, ABTS-based colorimetric assays and FRAP are the most common methods applied in animal research.

Therefore the aim of this study was to compare the effectiveness of antioxidant capacity determination in bovine seminal plasma and spermatozoa by two popular spectrophotometric tests: ABTS and FRAP. Both methods were validated statistically, and their effectiveness was compared with regard to the most significant spermatozoa motility characteristics.

MATERIAL AND METHODS

Semen samples were collected in duplicates from 37 Simmental-Fleckvieh bulls kept in the Breeding Centre of the Slovak Biological Services, Nitra, Slovakia. The animals were 4–6 years old and fed a standard diet consisting of green and cereal fodder, berseem, straw and commercial mixtures for beef cattle. Water was provided regularly.

The samples were acquired on a regular collection schedule using an artificial vagina and immediately transferred to the laboratory. Basic semen assessment was performed in each sample, including volume (mL), pH and spermatozoa concentration ($x10^6$/mL).

Spermatozoa motility (MOT; percentage of motile spermatozoa; motility > 5 µm/s; %), progressive motility (PROG; percentage of progressive motile spermatozoa; motility > 20 µm/s; %), distance average path (DAP, µm), velocity average path (VAP, µm/s) and amplitude of lateral head displacement (ALH, µm) were determined using the Computer Assisted Semen Analysis (CASA) system based on the SpermVision™ program (Minitube, Tiefenbach, Germany) and Olympus BX 51 phase contrast microscope (Olympus, Tokyo, Japan). The samples were placed into the Makler Counting Chamber (depth 10 µm, 37°C; Sefi Medical Instruments, Haifa, Israel) and assessed. At least 1000 cells were evaluated in each sample (**Massányi *et al.*, 2008**).

The samples were centrifuged (15 min, 10 090 × g, 4°C), seminal plasma was transferred into 1.5 mL tubes and kept frozen (−80 °C). The cell sediments were moved into tubes containing 1.5 mL distilled water and subsequently lysed on ice using sonication (28 kHz, 30 sec). The lysates were centrifuged (15 min, 11 828 × g, 4 °C) and the supernatants containing the intracellular content were transferred into 1.5 mL tubes and stored at −80 °C until further analysis (**Tvrdá *et al.*, 2013a**).

The assessment of TAS originates from the ability of all antioxidants in the sample to neutralize a prooxidant compound. The TAS Randox (Randox Laboratories, Crumlin, Great Britain) assay follows an incubation of ABTS (2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate]) with a peroxidase (metmyoglobin) and H2O2 to produce the ABTS+ radical. This has a relatively stable blue-green color, which may be measured at 600 nm. Antioxidants present in the sample supress this color production to a degree, which is proportional to their concentration. TAS was assessed using the Genesys 10 spectrophotometer

(Thermo Fisher Scientific Inc., Waltham, USA) and is expressed as µmol/mg protein (**Tvrda *et al.*, 2012**).

The FRAP assessment followed the original procedure described by **Benzie and Strain (1996)**. It is a simple test to determine the total antioxidant power, based on the reduction of a ferric-tripyridyl triazine complex to its ferrous colored form in the presence of antioxidants. The FRAP reagent contains 10 mmol/L TPTZ (2, 4 ,6- tripyridyl- s- triazine) solution in 40 mmol/L HCl (Centralchem, Bratislava, Slovak Republic) plus 5 mL of 20 mmol/L FeCl3 (Centralchem, Bratislava, Slovak Republic) and 50 mL of 0.3 mol/L acetate buffer (pH=3.6; Centralchem, Bratislava, Slovak Republic). Aliquots of 100 µL sample were mixed with 3 mL FRAP reagent and the absorbance of reaction mixture was measured at 593 nm for 4 min using the Genesys 10 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA) and is expressed as mmol/mg protein.

Protein concentration was quantified using the DiaSys Total Protein (DiaSys, Holzheim, Germany) commercial kit, based on the Biuret method: copper sulphate reacts with proteins to create a violet blue color complex in alkaline solution, whose intensity is directly proportional to the protein content at 540 nm using a semi-automated clinical chemistry photometric analyzer Microlab 300 (Merck®, Darmstadt, Germany) (**Tvrdá *et al.*, 2011**).

All data were subjected to statistical analysis using the GraphPad Prism program (version 3.02 for Windows, GraphPad Software incorporated, San Diego, California, USA, http://www.graphpad.com/). Results are quoted as arithmetic mean ± standard error (S.E.). Pearson product-moment correlation coefficient analysis for paired samples was used to assess correlations between all examined parameters. Additionally, the samples were categorized in three quality groups according to their motility rates. Comparative analysis of selected parameters in the seminal fractions as well as in the quality groups was carried out by one-way ANOVA with the Bonferroni multiple comparison test. The level of significance for the comparative as well as correlation analysis was set at ***(P<0.001); **(P<0.01); *(P<0.05).

RESULTS AND DISCUSSION

Results from the seminal examination are shown in Table 1. Animal donors presented with no signs of disease or pathology. At the same time, the final values met the criteria established for the Simmental-Fleckvieh bovine breed, which is why a possible health impact on the outcomes from the biochemical assessment was ruled out.

Table 1 Basic seminal and motility characteristics of the samples (n = 37)

PARAMETER	Mean±S.E.
Volume [mL]	7.33±0.50
pH	6.70±0.38
Concentration [×10^6 cells/mL]	3 005±51.91
Motility [%]	87.36±4.55
Progressive motility [%]	83.77±4.99
Distance average path [µm]	35.19±4.05
Velocity average path [µm/s]	82.33±5.01
Amplitude of lateral head displacement [µm]	4.76±0.29

S.E.: standard error

Results of the biochemical quantifications are presented in Table 2. Both antioxidant markers were higher in the seminal plasma when compared to the cell lysates (P<0.001 with respect to TAS; P<0.01 in case of FRAP).

Table 2 Antioxidant parameters of bovine seminal plasma and spermatozoa examined by UV/VIS spectrophotometry (n = 37)

PARAMETER	SEMINAL PLASMA	CELL LYSATES
Total antioxidant status (TAS) [mmol/mg prot]	25.87 ± 2.29	48.22 ± 4.23***
Ferric reducing ability of plasma (FRAP) [µmol/g prot]	151.05 ± 9.56	253.73 ± 14.57**

Mean±S.E. *** - P<0.001.

Table 3 displays the results of the correlation analysis between the motility characteristics, and antioxidant characteristics assessed in bovine seminal plasma and spermatozoa. All spermatozoa motion parameters were significantly (P<0.01) positively correlated with both TAS and FRAP.

Table 3 Correlations between the spermatozoa motility parameters and antioxidant capacity in bovine seminal plasma and cell lysates evaluated by the Pearson's correlation coefficient test (n = 37)

	MOT	PROG	DAP	VAP	ALH	TAS [SP]	FRAP [SP]	TAS [CL]	FRAP [CL]
MOT	1								
PROG	0.972***	1							
DAP	0.950***	0.959***	1						
VAP	0.935***	0.937***	0.936***	1					
ALH	0.933***	0.935***	0.938***	0.945***	1				
TAS [SP]	0.460**	0.478**	0.444**	0.449**	0.450**	1			
FRAP [SP]	0.422**	0.433**	0.412**	0.410**	0.411**	0.451**	1		
TAS [CL]	0.468**	0.485**	0.449**	0.453**	0.459**	0.779***	0.337*	1	
FRAP [CL]	0.452**	0.444**	0.417**	0.421**	0.418**	0.343*	0.758***	0.501**	1

The correlation analysis was based on the value of the correlation coefficient: ±0.111 - ±0.333: low correlation; ±0.334 – ±0.666: moderate correlation; ±0.667 - ±0.999: high correlation. * - P<0.05; ** - P<0.01; *** - P<0.001. MOT - spermatozoa motility [%], PROG - spermatozoa progressive motility [%], DAP – distance average path [µm], VAP – velocity average path [µm/s], ALH – amplitude of lateral head displacement [µm], TAS – total antioxidant status [mmol/mg of protein], FRAP – ferric reducing ability of plasma [µmol/g of protein]. SP – seminal plasma, CL – cell lysates.

To have a better understanding of the results, the samples were categorized in three groups of excellent (Ex; >90% motile; n=13), good (Go; 80–90% motile; n=14) and moderate (Mo; <80% motile; n=10) quality according to their motility rates (Table 4). Mean values for MOT, PROG, DAP, VAP as well as ALH were significantly different between the groups (P<0.001 Ex vs. Go and Ex vs. Mo). The highest TAS and FRAP concentrations were recorded in the Ex group of both seminal fractions. Inversely, the lowest antioxidant activity was detected in the Mo group. Significant differences (P<0.01) were observed when comparing the antioxidant parameters between the Ex and the Mo groups.

Table 4 Average values of seminal motility parameters and antioxidant capacity in the quality groups (Mean ± S.E.) and Bonferroni multiple comparison test results (n=37)

PARAMETER	Ex (n=13)	Go (n=14)	Mo (n=10)
MOT [%]	93.99±0.59	85.64±0.87^{*a}	63.22±0.65$^{***b, ***c}$
PROG [%]	89.67±0.62	79.51±1.33^{*a}	56.94±2.01$^{***b, ***c}$
DAP [µm]	42.12±1.57	36.22±2.05^{*a}	22.31±2.12$^{***b, ***c}$
VAP [µm/s]	90.16±2.22	80.34±1.91^{*a}	60.64±2.03$^{***b, ***c}$
ALH [µm]	5.35±0.12	4.22±0.44^{*a}	2.29±0.19$^{***b, ***c}$
TAS [SP] [mmol/mg prot]	30.11±3.02	26.22±3.10	19.23±2.22^{**b}
FRAP [SP] [µmol/g prot]	189.47±3.99	145.11±5.02^{*a}	120.65±9.55$^{**b, *c}$
TAS [CL] [mmol/mg prot]	56.09±3.02	49.13±2.98	40.04±2.11^{**b}
FRAP [CL] [µmol/g prot]	277.09±9.87	255.18±8.67	228.33±5.55^{**b}

^{a} –Ex vs. Go; ^{b} –Ex vs. Me; ^{c} –Go vs. Me. * - P<0.05; ** - P<0.01; *** - P<0.001. Quality groups are based on the motility values: Ex – Excellent quality (> 90 % motile), Good (80–89 % motile), Moderate quality (< 79 % motile). MOT – motility, PROG – progressive motility, DAP – distance average path [µm], VAP – velocity average path [µm/s], ALH – amplitude of lateral head displacement [µm], TAS – total antioxidant status [mmol/mg of protein], FRAP – ferric reducing ability of plasma [µmol/g of protein]. SP – seminal plasma, CL – cell lysates.

Although conventional semen indicators may meet the conditions of applicability for artificial insemination, traditional assessment of semen immediately after collection may not completely capture the overall spermatozoa fertility, as shown by differences e.g. in spermatozoa viability assessment (**Colebrandner *et al.*, 2003**; **Härtlová *et al.*, 2013**). The reason may lie in changes of biochemical properties of seminal plasma or spermatozoa (**Podstawski *et al.*, 2007**).

The seminal plasma is the central reservoir of antioxidants protecting the ejaculate against oxidative damage (**Agarwal *et al.*, 2006**). Our records agree showing that the TAS as well as FRAP, both techniques routinely used to assess the antioxidant capacity of the sample, were significantly higher (Table 2) in the seminal plasma, suggesting that this semen fraction may be protected by a complex antioxidant system (**Selley *et al.*, 1991**).

It has been reported on different occasions that a proper ROS detoxification, thus the balance between individual components of the antioxidant system in semen is important to maintain the spermatozoa motility (**Khosrowbeygi *et al.*, 2004; Eghbali *et al.*, 2008; Tvrda *et al.*, 2013a,b**). Positive correlations between enzymatic or non-enzymatic antioxidants and sperm motility have been related to lower oxidative insults and cytotoxicity to spermatozoa. Measurements of antioxidant components and characteristics have also shown significant differences between case and control groups and correlated with sperm motility (**Khosrowbeygi *et al.*, 2004; Pahune *et al.*, 2013**).

One advantage of our study was centrifugation of semen samples at high speed to precisely separate the seminal plasma from spermatozoa, as the membrane-bound oxidases or antioxidants associated with cellular debris and/or organelles may have an impact on the seminal plasma antioxidants (**Zini *et al.*, 2000**), and vice versa, as seen in the correlation analysis (Table 3).

Diverse techniques have been developed to assess the antioxidant capacity of a variety of molecules, nevertheless as many variables are taken into account when focusing on the antioxidant properties of a complex sample, the outcomes have to be treated cautiously (**Alam *et al.*, 2002**). There is no universal system able to provide information about the 'true' antioxidant power of a single antioxidant or a complex mixture of antioxidant substances. At the same time, a comparative evaluation of antioxidant capacity is difficult to perform as the activity depends on the substrate, reaction medium, oxidation conditions, interfacial phenomena or the antioxidant partitioning properties between separate phases (**Litescu *et al.*, 2010**). Here we used two very frequently applied methods to estimate the antioxidant capacity of the seminal plasma and lysates.

While the FRAP assay is based on reduction of a ferric-tripyridyl triazine complex to its ferrous colored form in the presence of antioxidants, the TAS is an ABTS related decolorization assay. Although the principles of the assays may be different, both are based on assessing the capacity of all the hydrophilic as well as lipophilic molecules able to metabolize ROS. Independently of the assay applied, the total antioxidant capacity has been found to be associated with semen quality along with sperm viability and functional activity (**Khosrowbeygi *et al.*, 2004; Tvrda *et al.*, 2012; Pahune *et al.*, 2013**). Furthermore, positive correlations between the parameters in both seminal fractions (Table 3), as well as similar associations between the spermatozoa motility characteristics and biochemical markers (Table 4) suggest that both assays are suitable for the measurement of the in vivo antioxidant status of semen.

CONCLUSION

This study sheds more light on the complexity of interactions between semen quality and antioxidant profile of bovine ejaculates. At the same time our results indicate that both methodologies used to assess the antioxidant capacity of biological material are equally suitable to be used in veterinary andrology. Lastly, we recommend that routine evaluation of the seminal antioxidant capacity should be considered during male fertility assessment in animal production.

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ENERGETIC PROFILE OF RABBITS AFTER AMYGDALIN ADMINISTRATION

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ABSTRACT

Amygdalin is a cyanogenic glucoside initially obtained from the seeds of bitter almonds (*Prunus dulcis*). It is a natural product that owns antitumor activity and has also been used for the treatment of asthma, bronchitis, emphysema, leprosy and diabetes. It is composed of one molecule of benzaldehyde, two molecules of glucose and one molecule of hydrocyanic acid. Cyanide is not cancerocidal as long as glucose is available. The present *in vivo* study was designed to reveal whether amygdalin is able to cause changes in the energetic profile of rabbit as a biological model. A 12 adult male rabbits were randomly divided into three groups: the control group without any amygdalin administration and two experimental groups receiving daily intramuscular injections (IM) of amygdalin at 0.6 and 3.0 mg/kg b.w. respectively over the period of 28 days. The body weight of each experimental animal was recorded weekly during the whole study. Serum levels of energetic profile (glucose, triglycerides and cholesterol) were determined. Intramuscular application of amygdalin did not affect ($P \geq 0.05$) the serum levels of none of selected biochemical parameters significantly. In this *in vivo* study, no obvious beneficial or negative effects of amygdalin on energetic profile of male rabbits were demonstrated.

Keywords: Amygdalin, rabbits, glucose, triglycerides, cholesterol

INTRODUCTION

Alternative cancer therapy represents a variety of treatments used by cancer patients for cancer prevention, treatment or management of symptoms caused by the malignancy or cancer therapies (**Balmer, 1998**). Amygdalin is a cyanogenic glucoside initially obtained from the seeds of bitter almonds (*Prunus dulcis*) (**Chwalek and Plé, 2004**). It is a major component of the seeds of prunasin family plants, such as apricots, almonds, peaches, apples, and other rosaceous plants (**Fukuda *et al.*, 2003**). Amygdalin, when pure, is almost entirely harmless (**Sollmann, 1949**).
Amygdalin (D-mandelonitrile-β-D-gentiobioside), $C_{20}H_{27}NO_{11}$, is composed of one molecule of benzaldehyde, two molecules of glucose and one molecule of hydrocyanic acid, which is an anti-neoplastic compound (**Chang *et al.*, 2006**).
Amygdalin is a natural product that owns antitumor activity, less side effects and relatively low priced (**Song and Xu, 2014**). Besides the antitumor activity, amygdalin has also been used for the treatment of asthma, bronchitis, emphysema, leprosy and diabetes (**Zhou *et al.*, 2012**). It is also decomposed by the action of β-D-glucosidase to yield hydrocyanic acid which stimulates the respiratory center reflexively and produces a kind of antitussive and antiasthmatic effects (**Badr and Tawfik, 2010; Lv *et al.*, 2005**).
As reported by **Levi *et al.* (1965)** cyanide is not cancerocidal as long as glucose is available. Therefore it is obvious, the energy profile of patients plays important role in clinical research of amygdalin effect.
The present *in vivo* study was designed to reveal whether amygdalin is able to cause changes in the energetic profile of rabbit as a biological model.

MATERIAL AND METHODS

Chemicals

Amygdalin from apricot kernels (≥99% purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Amygdalin was freshly dissolved in sterile saline and 0.5 ml were applied intramuscularly (IM) to *musculus biceps femoris* on a daily basis.

Animals

Meat line P91 Californian rabbit males (n=12) from the experimental farm of the Animal Production Research Centre Nitra (Slovak Republic) were used in the experiments. The rabbits were 150 days old, weighing 4.00 ± 0.5 kg, and were housed in individual flat-deck wire cages under a constant photoperiod of 12 h of daylight, temperature 20-24 °C and humidity 55 % ± 10 %. The rabbits were fed a standard commercially available feed based on a pelleted concentrate. Animals had free access to feed and water during the study period and no toxic or side effects or death were observed throughout the study. The animals were randomly divided into the three groups, leading to 4 male rabbits in each group. The control group received no amygdalin while the two experimental groups P1 and P2 received a daily intramuscular injection of amygdalin at a dose 0.6 and 3.0 mg/kg b.w. respectively during 28 days. The body weight of each experimental animal was recorded weekly during the whole study. Institutional and national guidelines for the care and use of animals were followed appropriately, and all experimental procedures were approved by the State Veterinary and Food Institute of Slovak Republic, no. 3398/11-221/3 and Ethic Committee.

Blood Sample Collection.

During the experiment, three blood collections were carried out (at the beginning of the experiment 0; after 14 days and 28 days) to control the health of animals. Venous blood from *vena auricularis* was collected into tubes. Blood serum was separated from whole blood by centrifugation at 3000 rpm for 10 min. at 20 °C. The clear supernatant (serum) was then separated from the pellet and kept frozen until analysis.

Analysis

Quantification of glucose, triglycerides and cholesterol after amygdalin supplementation was performed using photometry. Analyses were provided in biochemical and hematological laboratory at the Department of Animal Physiology of SUA through commercial sets DiaSys (Diagnostic Systems GmbH, Germany) on device Rx Monza (Randox Laboratories Ltd., United Kingdom).

Intra-assay, inter-assay coefficients and sensitiveness for selected parameters are shown in Table 1.

Table 1 Intra-assay, inter-assay coefficients and sensitivity for selected parameters

Parameter	Intra-assay coefficient (%)	Inter-assay coefficient (%)	Sensitivity
Glucose	≤1.05	≤3.8	0.22 mmol.l^{-1}
Triglycerides	≤1.6	≤1.23	0.01 mmol.l^{-1}
Cholesterol	≤0.95	≤1.09	0.08 mmol.l^{-1}

Statistical analysis

The significance of differences between the control and experimental groups was evaluated by one-way analysis of variance (ANOVA), with the Dunnett´s multiple comparison test using statistical software GraphPad Prism 3.01 (GraphPad Software Inc., San Diego, CA, USA). The data are expressed as means ± SD. Differences were compared for statistical significance at the *p*-level less than 0.05 ($P<0.05$).

RESULTS AND DISCUSSION

The effect of short-term amygdalin administration on the serum levels of glucose

Serum levels of glucose after short-term IM injection of amygdalin to adult male rabbits were assessed in this *in vivo* study (Figure 1). During 28 days treatment period, no significant ($P\geq0.05$) differences in serum levels of glucose were observed when compared to the untreated control group.

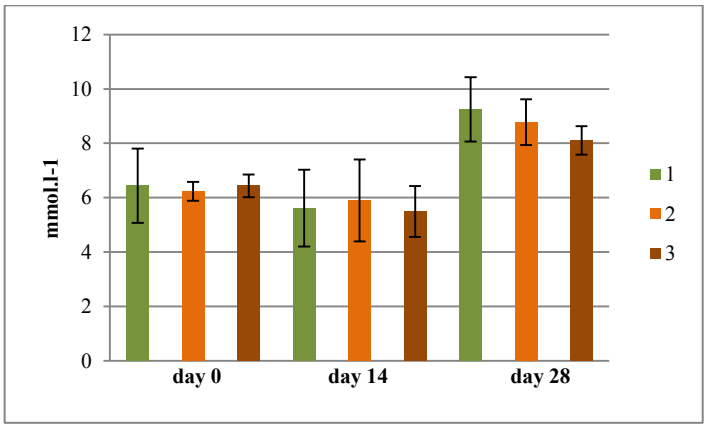

Figure 1 Serum levels of glucose during 28 days. 1 - control group (without amygdalin administration); 2 - group P1 (0.6 mg/kg); 3- group P2 (3.0 mg/kg)

All the measured values of serum glucose were compared to reference range 4.1 – 8.5 mmol.l^{-1} (**Merck Sharp & Dohme Corp., 2012**). Two groups did not comply this range – control group (9.25 mmol.l^{-1}) and P1 group (8.78 mmol.l^{-1}), both after 28 days of experiment. On the other hand, **Özkan *et al.* (2012)** indicate there are some differences in reference ranges between male (3.83-10.77 mmol.l^{-1}) and female (4.94-8.32 mmol.l^{-1}) rabbits. It has been reported that increased glucose levels in rabbits are generally due to various stress factors (**Melillo, 2007; Jenkins, 2008**).

The effect of short-term amygdalin administration on the plasma levels of triglycerides

Changes of triglycerides levels in response to amygdalin application were determined in this study as well. The effect of IM amygdalin aplication on rabbit serum levels of triglycerides is shown in Figure 2. The analysis has shown that the serum triglycerides levels of animals administered with 0.6 and 3.0 mg/b.w. amygdalin did not statistically ($P\geq0.05$) differ, when compared to the control group.

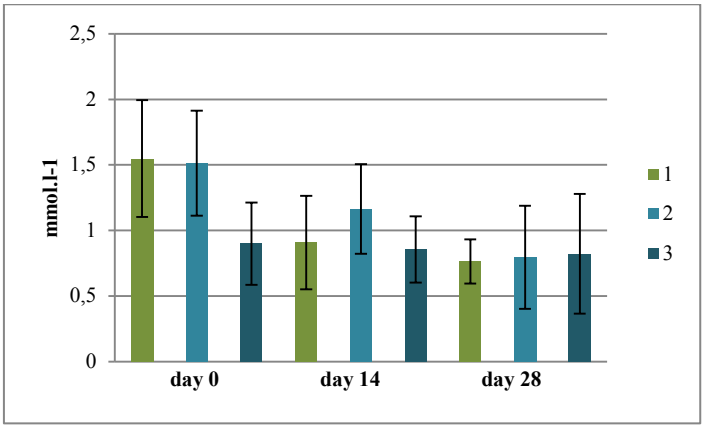

Figure 2 Serum levels of triglycerides during 28 days.
1 - control group (without amygdalin administration); 2 - group P1 (0.6 mg/kg); 3- group P2 (3.0 mg/kg)

However, a non-significant decrease of triglycerides levels in all groups during the experiment was observed. It could be connected to slightly increased concentration of glucose, since the secretion of triglycerides serves as an additional potential energy source in the form of circulating lipids (**Tuvdendorj *et al.*, 2015**).

The effect of short-term amygdalin administration on the plasma levels of cholesterol

Serum levels of cholesterol after short-term IM injection of amygdalin to adult male rabbits were assessed and are shown in Figure 3. During 28 days of experiment, no significant ($P\geq0.05$) differences in serum levels of cholesterol were observed when compared to the untreated control group.

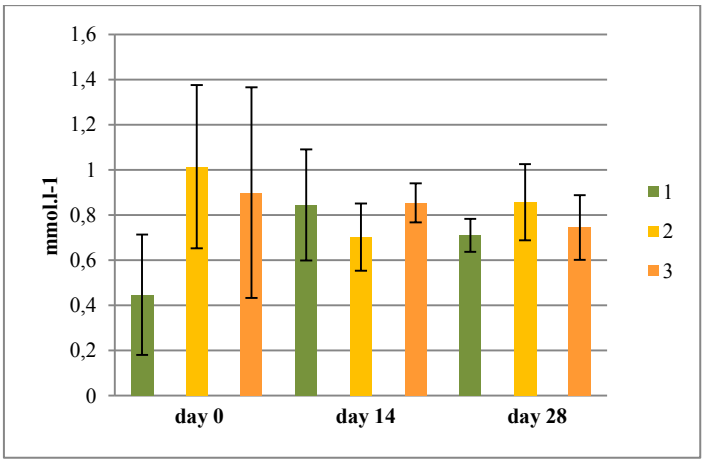

Figure 3 Serum levels of cholesterol during 28 days.1 - control group (without amygdalin administration); 2 - group P1 (0.6 mg/kg); 3- group P2 (3.0 mg/kg)

Cholesterol molecule plays an important role in organism, e. g. in incorporation to cell membranes, or as precursor of steroid hormones (**Vasudevan *et al.*, 2011**). Accorrding to veterinary manual of **Merck Sharp & Dohme Corp. (2012)** levels of serum cholesterol of all groups were not outside the reference range (0.3-2.1 mmol.l^{-1}) and no significant differences between the groups were noticed during our experiment. These results could partly explain possible modulatory impact of amygdalin on the steroid production in porcine ovaries *in vitro*, as **Halenár *et al.* (2015a, 2015b)** published in their study. Keishi-bukuryo-gan (a traditional Chinese herbal remedy containing seeds of *Prunus persica or P. persiba*) and its crude ingredients affected steroidogenesis in pre-ovulatory follicles (**Usuki, 1990, 1991**) in the rat ovary in vivo and in vitro.
There are just a few studies of blood chemistry changes after amygdalin administration *in vivo*. Similarly to our results, **Miller *et al.* (1981)** did not observe changes in blood chemistry of rats fed a diet containing 10% ground apricot kernels.
Our results showed some non-significant differences within the same groups between each blood collections. It is hypothesised that this difference may be due to the variations in some factors such as stress, blood collection methods and housing conditions (**Özkan *et al.*, 2012**).

CONCLUSION

It can be summarized, that amygdalin had no significant effect (negative or beneficial) on energetic profile of male rabbits in this *in vivo* study. Our results

showed some non-significant differences within the same groups between each blood collections, which may occur due to the variations in some factors such as stress, blood collection methods and housing conditions.

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IMPACT OF TILMICOSIN ON THE RABBIT SPERMATOZOA MOTILITY AND VIABILITY

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ABSTRACT

The aim of this study was to examine the effects of tilmicosin on rabbit spermatozoa viability and motility parameters during short-term *in vitro* incubation at 37°C. Semen samples were collected from adult New Zealand White rabbits and diluted with eight concentrations of tilmicosin: 0.300, 0.350, 0.400, 0.466, 0.500, 0.583, 0.600 and 0.700 mg.ml^{-1} (TIL 1 – 8) diluted in the physiological solution. The motility parameters were evaluated using the Computer Assisted Semen Analyzer system (Sperm Vision®) at five time periods: 0, 1, 2, 3 and 4 hours. Cell viability was measured using the metabolic activity MTT assay. Immediately at the beginning of incubation significantly higher values of motility (MOT) and progressive motility (PRO) were detected in samples TIL2, TIL5 and TIL7 compared to the control. Significantly lower values of tested motility parameters were observed after 1 and 3 hours of incubation. All concentrations of tilmicosin have no significantly negative effect on the parameters of the MOT, PRO and velocity curved line (VCL) after 4 hours of incubation *in vitro* at 37°C. Concentration of tilmicosin 0.350 mg.ml^{-1} has a positive impact on MOT (72.39 ± 15.62%, $p<0.001$) and PRO (54.28 ± 21.23%, $p<0.01$) of rabbit spermatozoa after 4 hours of incubation in vitro at 37°C. Supplementation of tilmicosin led to preservation of the cell over all time periods of the *in vitro* incubation. The results indicate that tilmicosin could be used to semen extenders without negative effects on rabbit spermatozoa motility and viability.

Keywords: Antibiotics, spermatozoa, motility, CASA

INTRODUCTION

Antimicrobial agents are of extraordinary importance for the control of bacterial growth in liquid-preserved ejaculates of farm animals. To counteract increasing bacterial resistance to conventional antibiotics, novel agents with different active mechanisms have to be developed **(Schulze, *et al.*, 2016)**. The bacterial presence in semen samples could be especially problematic in situations where the ejaculates are used for artificial insemination 24 h or more after collection **(Suarez and Pacey, 2006)**. Therefore, it is of great importance that the extended shipped ejaculates maintain the highest quality upon arrival. Bacterial contamination is routinely observed in raw, extended and stored semen produced for artificial insemination when semen is collected by the gloved-hand technique. A study reported that 62.5% of raw ejaculates and 79% of extended boar semen doses showed bacterial contamination **(Maroto Martín *et al.*, 2010)**. The incidence of bacterial presence in the germ line can impact semen quality, also affecting the DNA molecule **(Gonzalez-Marin, *et al.*, 2012)**. The inclusion of antibiotics in semen extenders is recommended for the control of several microorganisms that can be present in spermatozoa **(Bielansky, 2007; Thibier and Guerin, 2000)** and The World Organization for Animal Health recommends the inclusion of antibiotics in extenders used in cryopreservation of spermatozoa, in order to control bacterial contamination **(Madeira, *et al.*, 2014)**.
Tilmicosin, 20-deoxo-20-(3.5-dimethylpiperidin-1-yl) desmycosin, is a semisynthetic derivative of tylosin **(Giguere, *et al.*, 2013)**. Tilmicosin is a macrolide antibiotic developed for veterinary use. It is recommended for treatment and prevention of respiratory diseases in cattle, sheep, pigs, rabbits, chickens and turkeys and for the treatment of other diseases caused by tilmicosin-sensitive microorganisms **(WHO Technical Report Series, 2009)**. Tilmicosin is a narrow spectrum antibiotic and effective against Gram-positive pathogens **(Hogeveen, 2005)**, although some gram-negative bacteria are affected and the drug reportedly has some activity against mycoplasma. Preliminary studies have shown that 95% of studied isolates of *Pasturella haemolytica* are sensitive **(Plamb, 2008)**.
Antibiotics are necessary and mandatory additives in semen extenders for the liquid preservation of several animal species **(Bryła and Trzcińska, 2015)**. Although opportunistic contaminants of semen generally do not incur an important health risk for the inseminated females, they can affect spermatozoa quality **(Yániz *et al.*, 2010)**. Fertilizing capacity of spermatozoa can be directly affected by bacteria **(Morrell 2006)** that can impair the spermatoza motility, have the ability to adhere with spermatozoa, and can induce the acrosome reaction **(Qadeer, *et al.*, 2013)**.
The objective of our study was to examine the effects of tilmicosin, macrolide antibiotic, on rabbit spermatozoa viability and motility parameters *in vitro*.

MATERIAL AND METHODS

Biological material

In this study the ejaculates were collected from adult New Zealand White rabbits using artificial vagina **(Massányi *et al.*, 2008)**. The rabbit were kept in individual cages and fed *ad libitum* with a commercial diet.

Sample preparation

Semen was diluted in a ratio of 1 part of semen and 6 parts of physiological solution (Sodium chloride 0.9% Braun, B. Braun Melsungen AG, Melsungen, Germany) – control groups (TILC). At the same ratio semen was diluted with eight different concentrations of tilmicosin (purity 94%, Sigma-Aldrich, St. Louis, USA) solution: TIL1 – 0.300 mg.ml^{-1}; TIL2 – 0.350 mg.ml^{-1}; TIL3 – 0.400 mg.ml^{-1}; TIL4 – 0.466 mg.ml^{-1}; TIL5 – 0.500 mg.ml^{-1}; TIL6 – 0.583 mg.ml^{-1}.; TIL7 – 0.600 mg.ml^{-1}; TIL8 – 0.700 mg.ml^{-1} diluted in the physiological solution. All samples were cultured at 37°C and measured at five time periods: 0, 1, 2, 3 and 4 hours (Time 0 – 4). The experiment was realized in 6 replicates.

Analytical method

Each of thus prepared samples was evaluated using a Computer Assisted Semen Analyzer (CASA) system – Sperm Vision® (Minitub, Tiefenbach, Germany) equipped with a microscope (Olympus BX 51, Japan) to assess the spermatozoa motility. Each sample was placed into Makler Counting Chamber® (depth 10

µm, Sefi–Medical Instruments, Germany). In the present study the following parameters were evaluated – total motile spermatozoa (MOT) [%], progressively motile spermatozoa (PRO) [%] and velocity curved line (VCL) [µm.s^{-1}]. Within each of the measurement by the CASA system were evaluated motility parameters from minimum seven fields of Makler Counting Chamber.

Viability of rabbit spermatozoa exposed to tilmicosin *in vitro* was evaluated by the metabolic activity (MTT) assay (**Tvrdá *et al.*, 2015**). This colorimetric assay measures the conversion of 3-(4.5-dimetylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, USA) to purple formazan particles by mitochondrial succinate dehydrogenase of intact mitochondria of living cells. Formazan was measured spectrophotometrically by a microplate ELISA reader (Multiskan FC, ThermoFisher Scientific, Finland). The data are expressed in percentage of control (i.e. optical density of formazan from cells not exposed tilmicosin). Viability of the spermatozoa was tested in the samples TIL1, TIL2 and TIL8. Results from the analysis were collected during four repeated experiments for each concentration.

Statistical analysis

Obtained data were statistically analysed by PC program Excel and a statistics package GraphPad Prism 5 (GraphPad Software, Inc. La Jolla, California, USA) using one-way ANOVA with Dennett's posttest. Statistical significance was indicated by *p* values of less than 0.05; 0.01 and 0.001.

RESULTS AND DISCUSSION

The results of CASA analysis of selected motility parameters are presented in Figures 1-3. At the beginning of incubation significantly higher percentage of spermatozoa motility was detected in the samples TIL3 ($p<0.05$), TIL5 ($p<0.01$) and TIL7 ($p<0.001$) compared to control (TIL K). At the same time non-significant differences were observed in the others experimental samples at the Time 0. However after 1 hour of incubation negative effect of tilmicosin on the turkey spermatozoa motility was detected in the samples TIL3, TIL5, TIL6, TIL7 and TIL 8 in comparison to the control. With increasing time of incubation between tested samples and control similar values of motility were observed. Significantly ($p<0.001$) protective effect of tilmicosin on preservation of spermatozoa motility was found in the samples TIL2 (0.350 mg.ml^{-1}). After 4 hours of incubation percentage of motility was 72.69% in the sample TIL2 and 46.10% in the control sample (Figure 1).

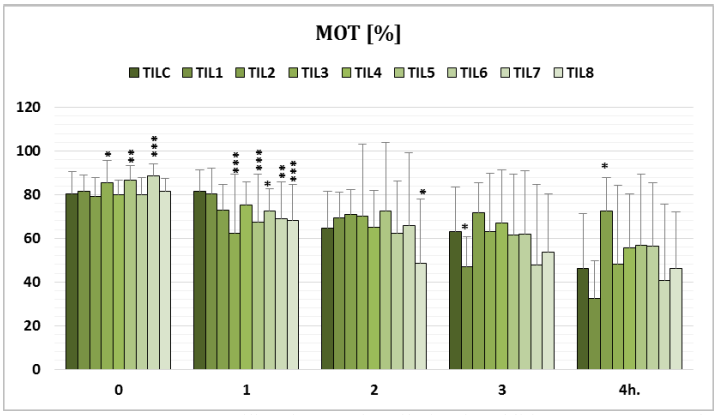

Figure 1 Spermatozoa motility (in %) after tilmicosin addition. TILC – control sample and TIL 1 – 8 (in this order): 0,300 mg.ml^{-1}; 0,350 mg.ml^{-1}; 0,400 mg.ml^{-1}; 0,466 mg.ml^{-1}; 0,500 mg.ml^{-1}; 0,583 mg.ml^{-1}.; 0,600 mg.ml^{-1}; 0,700 mg.ml^{-1}. Significant differences *$p<0.05$; **$p<0.01$; ***$p<0.001$.

Spermatozoa progressive motility (PRO) followed the tendency of spermatozoa motility. Measurement of progressive spermatozoa motility (PRO) showed statistically significant difference immediately at the beginning of incubation (Figure 2). The values of samples TIL3 (76.78%; $p<0.05$), TIL5 (78.43%; $p<0.01$) and TIL7 (81.19%; $p<0.001$) were significantly higher in comparison to the control sample TILK (66.12%). After 2 hours of *in vitro* incubation significant decrease of progressive motility was detected in all tested samples excepr sample TIL1 with the lowest concentration of tilmicosin (0.300 mg.ml^{-1}). With increasing time of incubation values of progressive motility between tested samples and control were balanced. Significantly higher ($p<0.001$) progressive motility was observed only in the sample TIL7 at the Time 4.

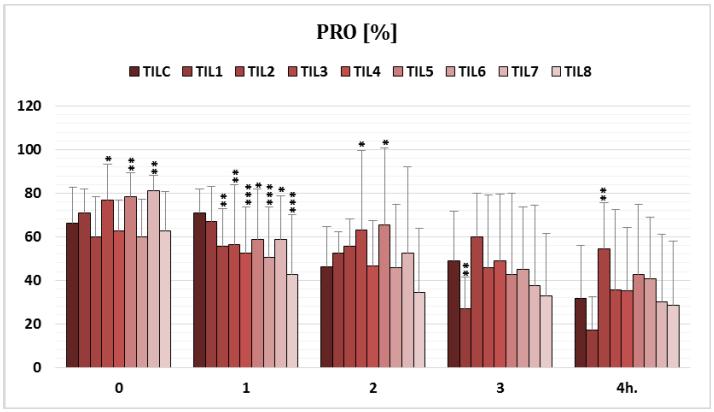

Figure 2 Spermatozoa progressive motility (in %) after tilmicosin addition. TILC – control sample and TIL 1 – 8 (in this order): 0,300 mg.ml^{-1}; 0,350 mg.ml^{-1}; 0,400 mg.ml^{-1}; 0,466 mg.ml^{-1}; 0,500 mg.ml^{-1}; 0,583 mg.ml^{-1}.; 0,600 mg.ml^{-1}; 0,700 mg.ml^{-1}. Significant differences *$p<0.05$; **$p<0.01$; ***$p<0.001$

The initial analysis of velocity curved line (VCL) proved significantly higher values in samples TIL2, TIL5 and TIL7 ($p<0.001$) compared to the control sample TILK. Significantly lower values of VCL were detected in samples TIL2, TIL4 – TIL8 in comparison to the control at the Time 1 and 3 (Figure 3.). At the other time of *in vitro* incubation balanced values of VCL were detected between tested samples and control group.

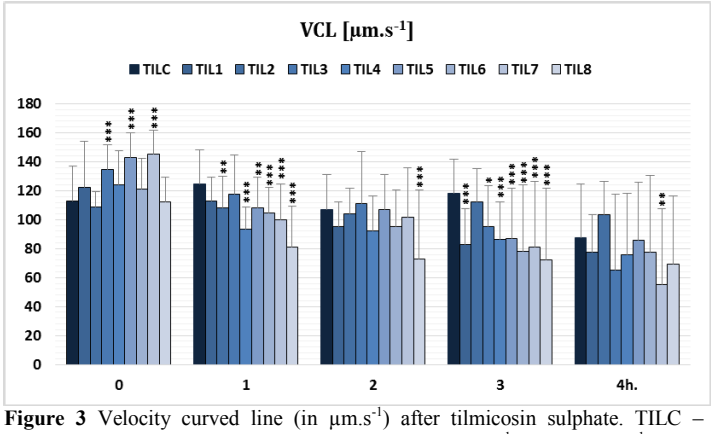

Figure 3 Velocity curved line (in µm.s^{-1}) after tilmicosin sulphate. TILC – control sample and TIL 1 – 8 (in this order): 0,300 mg.ml^{-1}; 0,350 mg.ml^{-1}; 0,400 mg.ml^{-1}; 0,466 mg.ml^{-1}; 0,500 mg.ml^{-1}; 0,583 mg.ml^{-1}.; 0,600 mg.ml^{-1}; 0,700 mg.ml^{-1}. Significant differences *$p<0.05$; **$p<0.01$; ***$p<0.001$

The impact of tilmicosin on viability of rabbit spermatozoa was measured in the samples TIL1 – 0.300 mg.ml^{-1}, TIL2 – 0.350 mg.ml^{-1} and TIL3 – 0 .700 mg.ml^{-1}. During the whole *in vitro* incubation the sample TIL 2 showed the most positive effect on the all monitored spermatozoa motility parameters. According to the MTT assay, significantly decrease of viability was not detected. Nevertheless at the Time 2 increasing of percentage of vital cells was observed (Figure 4). Similar to the CASA analysis, the MTT test revealed stimulation in the cell viability in the samples TIL1 and TIL2. The sample TIL1 (183.3%) showed significantly higher ($p<0.05$) values of viability in comparison to the control sample at the Time 2 and sample TIL2 at Time 4.

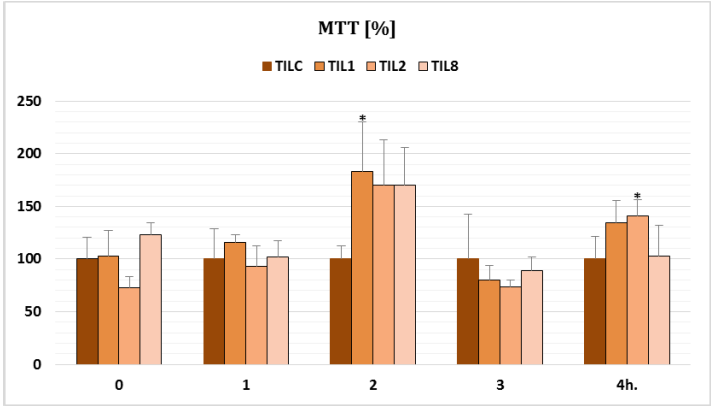

Figure 4 The effect of various doses of tilmicosin on the viability of rabbit spermatozoa. TILC – Control sample, TIL1 – 0.300 mg.ml^{-1}, TIL2 – 0.350 mg.ml^{-1} and TIL3 – 0 .700 mg.ml^{-1}.

The effect of bacteria on spermatozoa quality is still controversial. Semen is an ideal medium for the establishment and growth of several microorganisms including bacteria **(Bryła and Trzcińska, 2015)**. Microbial contamination reduces spermatozoa motility, shortens the storage time of viable semen, and decreases fertility rates, resulting in economic losses for AI facilities **(Bussalleu *et al.*, 2011)**. In contrast, some researchers have found that the presence of bacterial strains in semen samples does not usually impair semen quality **(Cottell *et al.*, 2000)** and that the bacterial contamination of the ejaculate has no significant effect on the farrowing rate or the total number of piglets born (**Reicks and Levis, 2008)**. Therefore it is very important to test not only the effect of bacterial strains on semen quality parameters of *in vitro* conditions but also testing the impact of the commercially used antibiotics.

Several studies have considered the effect of different antibiotics on the quality of stored semen. The data reported in study of **Gloria *et al.* (2014)** reveals that combinations of oflexacin (10-400 $mg.ml^{-1}$) and tylosin (10-250 $mg.ml^{-1}$), seems to have a negligible effect on spermatozoa motility ($p>0.05$). Furthermore, progressive motility was significantly higher for spermatozoa diluted with both antibiotic combinations compared with samples without antibiotics ($p<0.01$). Our results agree with **Gloria *et al.* (2014)** and confirmed that lower concentration of macrolide antibiotics, in our study tilmicosin, have no negative effect on spermatozoa motility. **Madeira *et al.* (2014)** showed that the combination of antibiotics (PES = 100,000 IU/ml penicillin and 0.100 $mg.ml^{-1}$ streptomycin; GTLS: 0.500 $mg.ml^{-1}$ gentamicin; 0.100 $mg.ml^{-1}$ tylosin; 0.300 $mg.ml^{-1}$ lincomycin and 0.600 $mg.ml^{-1}$ spectinomycin; CEF: 0.50 $mg.ml^{-1}$ ceftiofur sodium; ENR: 0.001 $mg.ml^{-1}$ enrofloxacin) in extenders at the tested concentrations did not influence the integrity of either spermatozoa membrane or acrosome, in both cooled and thawed ram spermatozoa. Nevertheless, spermatozoa motility was negatively affected with antibiotic addition. The reduction in spermatozoa motility may be attributed to the reduction of mitochondrial activity in spermatozoa, since quinolone antibiotics inhibit DNA gyrase **(Sárközy, 2001)**. This particular mechanism of action allows the elimination of strains resistant to antibiotics which act on the cell wall, the cytoplasmic membrane or on protein synthesis. **Qadeer *et al.* (2013)** suggested that neomycin, polymyxin, or colistin in combination with penicillin did not deteriorate semen quality and may be suggested to replace streptomycin in traditional antibiotic combination for cryopreservation of Nilli-Ravi buffalo bull spermatozoa. On the other side, combination of gentamycin, tylosin, lincomycin and spectinomycin have slightly significantly ($p<0.05$) negative effect on canine total spermatozoa motility **(Becher *et al.*, 2013)**.

The highest difference of toxicity between the cell lines and the spermatozoa were observed after application of the macrolide antibiotic tilmicosin. According to our measurements, the concentration of tilmicosin 0.350 $mg.ml^{-1}$ has a significantly positive effect on the motility and progressive motility and in the study of VERO cells survival of 25.79% was detected **(Fülöpová *et al.*, 2012)**. For the BHK-21 cells is the lethal dose a concentrations already 0.050 $mg.ml^{-1}$. At the concentration of 0.200 $mg.ml^{-1}$ only one third of cells was vital. The MTT assay showed that even the sample with the highest concentration of tilmicosin (0.700 $mg.ml^{-1}$) had not a negative effect on viability of rabbit spermatozoa. The samples TIL2 (0.350 $mg.ml^{-1}$) even showed significantly increase ($p<0.05$) of percentage of vital spermatozoa at the Time 4, which is in correlation with the results of motility analysis. During whole *in vitro* incubation no significant differences in this concentration of tilmicosin were detected.

CONCLUSION

According to our results it may be concluded that tilmicosin, a macrolide antibiotic, in concentrations from 0.300 to 0.700 $mg.ml^{-1}$ have not significantly negative effect on selected motility parameters after 4 hours of *in vitro* incubation at 37°C. Even concentration of tilmicosin 0.350 $mg.ml^{-1}$ showed significantly positive effect on motility and progressive motility of rabbit spermatozoa. Hereby neither the highest tested concentration of tilmicosin (0.700 $mg.ml^{-1}$) had negative impact on the mitochondrial metabolic activity. Therefore, we can recommend the use of tilmicosin for rabbit semen extenders.

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DO PUNICALAGINS HAVE POSSIBLE IMPACT ON SECRETION OF STEROID HORMONES BY PORCINE OVARIAN GRANULOSA CELLS?

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ABSTRACT

Punicalagin is a major component responsible for pomegranate's antioxidant properties. Punicalagin is the predominant ellagitannin of *Punica granatum* and present in two isomeric forms; punicalagin α and β. Punicalagins are metabolised to ellagic acid (antioxidant) and microorganisms present in colon can metabolize ellagic acid to urolithins – each other substances could be responsible for effect on cell intracellular mechanism. The aim of our study was to observe the effect of punicalagins on secretion of steroids hormones – progesterone and 17β-estradiol. Ovarian granulosa cells were cultivated during 24h without (control group) and with various doses (0.01, 0.1, 1, 10 and 100 $\mu g.ml^{-1}$) of pomegranate compounds – punicalagins. Steroid hormones progesterone and 17β-estradiol were evaluated by ELISA. Obtained results showed that the secretion of progesterone and 17β-estradiol by ovarian granulosa cells was not significantly ($P \geq 0.05$) influenced by punicalagins. In this pilot study no effects of punicalagins on porcine ovarian granulosa cells were found.

Keywords: Progesterone, 17β-estradiol, granulosa cells, punicalagins, steroidogenesis

INTRODUCTION

Punicalagins are ellagitannins in which gallagic and ellagic acid are linked to a glucose molecule **(Cerdá *et al.* 2003)**. Punicalagin has two isomeric forms in pomegranate: α and β. Chemical name of punicalagin is 2,3-(S)-hexahydroxydiphenoyl-4,6-(S,S)-gallagyl-D-glucose **(Tyagi *et al.* 2012)**. Punicalagins are phenolic compounds which are responsible for the main antioxidant activity of pomegranate (*Punica granatum*, Punicaceae) **(Syed *et al.* 2007)**. Punicalagin levels are widely variable in pomegranate juice and can range from as low as 0.014-1.5$g.l^{-1}$ depending on the fruit cultivar as well as processing and storage conditions **(Syed *et al.* 2007)**. Pomegranate peels and pulps have a higher total phenolic and antioxidant activity than juice **(Gözlekci *et al.* 2011; Elfalleh *et al.* 2011)**.
Punicalagin is a large molecule and knowledge about the fate of ellagitannins (include punicalagins) in human or animals is very sparse. In a recent study by **Cerdá *et al.* (2003)** the metabolism of punicalagins was described in rat. Punicalagins or metabolites of punicalagins were detected in faeces, urine and plasma. Native punicalagins were identified in plasma and urine, but at a very low concentration. The main metabolites present in biological fluids are those derived from punicalagins by hydrolysis and future conjugation. Rat microflora is able to metabolise ellagic acid derivatives to produce 6H-dibenzo[b,d]pyran-6-onemetabolites (urolithins) **(Cerdá *et al.* 2003)**. Punicalagins were metabolized and/or absorbed. This means that most of the ingested punicalagins have to be transformed to known (punicalin, ellagic acid, gallagic acid) or unknown metabolites or accumulated in tissue **(Cerdá *et al.* 2003)**. This *in vitro* study was focused on the secretion of steroid hormones, progesterone and 17β-estradiol, by porcine ovarian granulosa cells after punicalagin administration.

MATERIAL AND METHODS

Material

Ovaries (n=12 per experiment) of Slovakian White gilts were obtained from healthy animals without visible abnormalities. All experimental animals are kept under standard conditions at slaughterhouse in Myjava. Ovaries were transported to the laboratory at 4ºC and washed in sterile physiological solution.

Isolation of granulosa cells

Ovarian granulosa cells were used in this *in vitro* study. The suspension of the cells was centrifuged for 10 min at 200xg (to divide the follicular liquid from granulosa cells), washed in sterile DMEM/F12 1:1 medium with 10% fetal bovine serum and 1% antibiotic–antimycotic solution. The washing was repeated two or three times as needed. Viability of cells was determined using trypan blue. Ovarian cells were divided into and cultured in 24–well culture plates. The well plates were incubated at 37.5 ºC and 5% CO_2 in humidified air until a 75% confluent monolayer (5 days). At this point, the medium (1 ml per well) was removed and ovarian cells were incubated in a fresh medium with 10% fetal bovine serum, 1% antibiotic–antimycotic solution and without (control group) or with punicalagins (Sigma Aldrich, St. Louis, MO, USA) at 0.01, 0.1, 1, 10, 100 $\mu g.ml^{-1}$. The cultures were kept for 24 h. Following cultivation the media were aspirated from the well plates and analysed for steroids hormones.

Viability of granulosa cells

Viability of granulosa cells was assessed by trypan blue solution (0.4%). A reference sample (500 µl) was detracted from cell suspension. Trypan blue was added (100 µl) into the test tube with suspension in a ratio of 5:1. The suspension with trypan blues was gently mixed and incubated 5 min at room temperature. The total cells, vital and death cells were accounted using a haemocytometer from minimum 10 fields and the percentage of vital cells was assessed using a formula (vital cells/total cellsx100%).

ELISA (Enzyme-linked immunosorbent assay)

Quantification of hormones (progesterone and 17β-estradiol) was performed after exposure of punicalagins by enzyme linked immunosorbent assay (ELISA). The principle of this colorimetric method is a series of competitive reactions between antigens (hormones) in the sample with horseradish enzyme-labelled antigen for binding to the limited number of antibody sites within a 96-well microplate (Grainer, Germany). ELISA assays (Dialab, Wiener Neudorf, Austria) were performed according to the manufacturer's instructions. After 1 h incubation (37 ºC, 95 % air atmosphere, 5 % CO_2) the bound/free separation was performed by a simple solid-phase washing. The enzyme substrate (H_2O_2) and the TMB-Substrate were added. After the appropriate time was elapsed for maximum colour development, the enzyme reaction was stopped and the absorbancies were

determined. Hormone concentration in the sample was calculated based on a series of calibrators. The colour intensity was inversely proportional to the hormone concentration in the sample. The absorbance was determined at a wavelength 450 nm using a microplate ELISA reader (Thermo Scientific Multiskan FC, Vantaa, Finland). The results were evaluated by One Way ANOVA. Intra- and inter-assay coefficients for progesterone were ≤4 % and ≤9.3 %. For 17β-estradiol intra- and inter-assay coefficients were ≤9 % and ≤10 %. Sensitiveness for progesterone was 0.05 ng.ml^{-1} and 8.68 pg.ml^{-1} for 17β-estradiol..

RESULTS AND DISCUSSION

In our previous *in vitro* study punicalagins were described as a possible effector in the processes of ovarian steroidogenesis **(Packová *et al.*, 2015)**. **Nagata *et al.* (2007)** have shown that pomegranate juice is a potent inhibitor of CYP2C9 and CYP3A enzymes – these enzymes belong to cytochrome p450 group; and are responsible for cholesterol, steroid and lipid metabolism or synthesis. This study was focused on steroid hormones and the influence of punicalagins on the secretion of steroid hormones – progesterone and 17β-estradiol. Fig.1 describes the secretion of progesterone as the first important hormone of steroid synthesis. Punicalagin had no significant (P≥0.05) impact on secretion of progesterone. Similarly, **Ming *et al.* (2014)** described a significantly decreased progesterone in the samples (prostate cancer cell lines – 22RV1 and LNCaP) treated with pomegranate extracts. However in our previous study, punicalagin at 100 μg.ml^{-1} increased the progesterone secretion by rabbit ovarian cells **(Packová *et al.*, 2015)**.

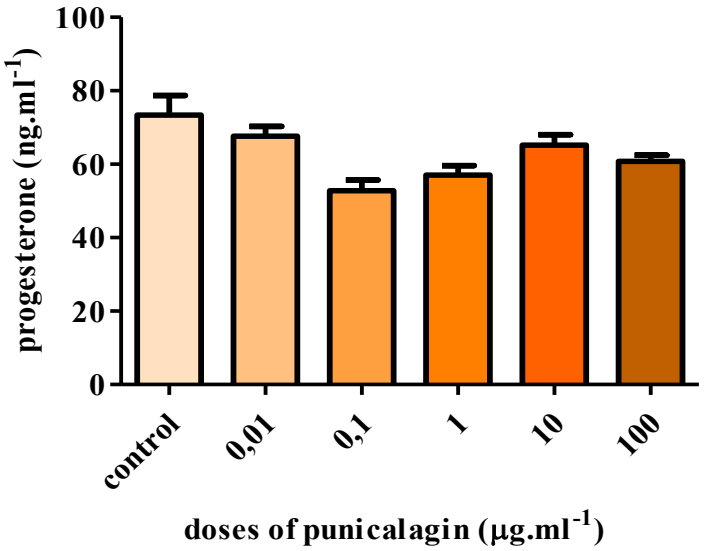

Figure 1 Effect of punicalagins (μg.ml^{-1}) on progesterone (ng.ml^{-1}) secretion by porcine ovarian granulosa cells. Non-significant (P≥0.05) differences among the control and experimental groups. The results were evaluated by One Way ANOVA.

The polyphenol from fermented pomegranate juice, pericarp and oil were shown to affect a blockade of endogenous active estrogen biosynthesis with subsequent inhibition of aromatase activity **(Toi *et al.* 2003)**. Fig. 2 shows secretion of 17β-estradiol by porcine ovarian granulosa cells after application of different doses of punicalagin, but these results were not significant (P≥0.05). Recently, **Ming *et al.* (2014)** used pomegranate extracts on prostate cancer cells to suggest that steroid biosynthesis might favour the backdoor pathway over the classical Δ4 and Δ5 pathways. They described the effect of pomegranate extract on androgen biosynthesis pathways specifically using two prostate cancer cell lines and prostate cancer mouse model. According to our previous study punicalagins - like mainly ellagitannins of pomegranate, had influence on secretion of 17β-estradiol. Punicalagins at 10 μg.ml^{-1} significantly (P<0.005) reduced the secretion of 17β-estradiol by rabbit ovarian fragments **(Packová *et al.*, 2015)**. Using lyophilized fresh pomegranate juice, **Kim *et al.* (2002)** reported that phenols from pomegranate inhibit 55% estrogen activity by normal human breast epithelial cells. Punicalagins or its derivatives (ellagic acid etc.) might have a possible effect on the process of ovarian steroidogenesis **(Packová *et al.* 2015)**. **Hong *et al.* (2008)** showed that pomegranate protectants (polyphenols) may be effective against prostate cancer by down regulation of genes involved in androgen synthesis. Crushed and dried seed produce oil with 80% punicic acid, the 18-carbon fatty acid, along with isoflavone genistein, the phytoestrogen coumestrol and the sex steroid estrone **(Syed *et al.* 2007)**.

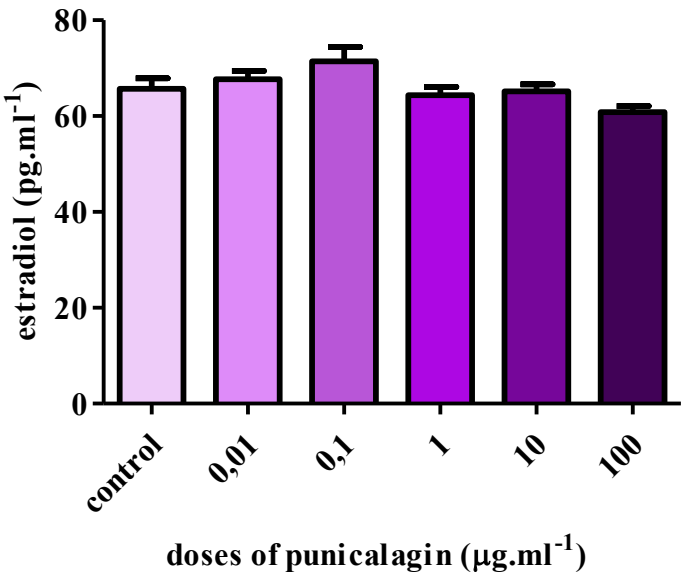

Figure 2 Effect of punicalagin (μg.ml^{-1}) on 17β-estradiol (pg.ml^{-1}) secretion by porcine ovarian granulosa cells. Non-significant (P≥0.05) differences among the control and experimental groups. The results were evaluated by One Way ANOVA test.

CONCLUSION

The research was focused on possible effects of punicalagins on porcine ovarian granulosa cells. Punicalagins or its derivatives (ellagic acid etc.), which were used in the present study might have no effect on steroidogenesis by porcine ovarian granulosa cells, but our previous studies with rabbit ovarian fragments have shown that punicalagin affects thesecretion of steroid hormones (progesterone and 17β-estradiol). Further research is necessary for a complex conclusion. There are more questions related to the effect of punicalagins or compounds from pomegranate on regulation of ovarian cells.

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