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CONTENT

THE EFFECT OF SOY PHYTOESTROGEN DAIDZEIN AND FSH ON PORCINE OVARIAN CELL PROLIFERATION AND STEROIDOGENESIS

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Abstract

The soy isoflavone daidzein is a phytoestrogen with potent estrogenic activity. Nevertheless, the effect of soy and its component daidzein on ovarian cell functions has been studied insufficiently. The aim of our study was to examine the effect of daidzein, FSH and their combination on porcine ovarian granulosa cell proliferation and steroidogenesis. For this purpose we studied the effect of daidzein (at doses 0, 1, 10 and 100 µM), FSH (at doses 0, 0.01, 0.1, 1 IU/ml) and combination of FSH (at doses 0, 0.01, 0.1, 1 IU/ml) + daidzein (50µM) on proliferation and steroid hormones release by cultured porcine ovarian granulosa cells. Expression of proliferation related peptide (PCNA) was detected by Western blotting. Release of progesterone (P4) and testosterone (T) was analysed by EIA. It was observed that addition of daidzein significantly reduced P4 (at 1, 10 and 100 µM) and promoted T (at 100 µM) release by porcine ovarian granulosa cells. Granulosa cells proliferation was stimulated by daidzein (at 50 µM). Administration of FSH resulted in stimulated granulosa cells proliferation (at 0.1 and 1 IU/ml) and increased release of both, P4 (at 0.01 and 0.1 IU/ml) and T (at all doses added). Daidzein addition suppressed FSH-stimulated secretion of P4 (at 0.01 and 0.1 IU/ml) and T (at 0.1 and 1 IU/ml). On the other hand, proliferation stimulating effect of FSH (at 0.1 and 1 IU/ml) was partially increased by daidzein treatment (at 0.01 IU/ml). Our observations demonstrate that daidzein directly affects ovarian granulosa cells proliferation and steroidogenesis. The involvement of daidzein in stimulation of both basal and FSH-induced proliferation and T secretion suggest, that daidzein may promote ovarian follicle development. On the other hand, the ability of daidzein to decrease both basal and FSH-stimulated P4 release suggest, that it can prevent ovarian cell luteinisation associated with increase in P4 output. These observation might indicate the potential influence of soy-containing diet on female reproductive processes.

Keywords: phytoestrogens, daidzein, FSH, ovary, steroidogenesis, proliferation

Introduction

The consumption of soy products in the world is increasing now (Cederroth et al., 2012). Soy-containing foods contain estrogenic substances called phytoestrogens. The female reproductive system is dependent on hormones, whereas phytoestrogens can interfere with hormonal regulation of reproduction (Jefferson, 2010). The soy isoflavone daidzein is a phytoestrogen with potent estrogenic activity (Cederroth et al., 2012). The administration of daidzein resulted in reduction of both, basal and FSH-stimulated progesterone (P4) production, but did not affect estradiol release by porcine ovarian granulosa cells (Nynca et al., 2013). The effect of daidzein on testosterone (T) release by ovarian cells have not been studied yet. Daidzein action on proliferation of adult healthy ovarian cells has not been studied as well, although daidzein-induced promotion of human ovarian cancer cell (Schmitt et al., 2001) and chicken embryonic ovarian germ cells (Liu et al., 2006) has been reported. Therefore, the ability of daidzein to influence ovarian functions and to modify the action of their physiological stimulators - gonadotropins remain to be studied insufficiently. Follicle-stimulating hormone (FSH) is a gonadotropin which plays a key role in promotion of ovarian cells proliferation and steroidogenesis (Jiang et al., 2003; Yu et al., 2005) and thus of follicular growth, development (Sirotkin, 2014). Due to contemporarily wide consumption of soy products and their potential influence on reproductive processes, the influence of daidzein on basal and FSH-induced ovarian cell proliferation and steroidogenesis requires extensive studies.

The aim of our study was: (1) to confirm the action of daidzein in regulation of P4 secretion and the role of FSH in regulation of ovarian processes (2) to examine the effect of daidzein on porcine ovarian granulosa cells proliferation (PCNA expression) and T release, (3) to examine whether the effect of FSH can be modified by daidzein treatment.

Material and methods

Isolation and culture of granulosa cells

Ovaries of non-cycling pubertal gilts, about 180 days of age, were obtained after slaughter at a local abattoir. They were washed several times in 95% alcohol and thereafter in sterile 0.9% NaCl. Granulosa cells were aspirated by syringe and sterile needle from follicles 3–5 mm in diameter and separated from follicular fluid by centrifugation for 10 min at x 200g. Cells were then washed in sterile DMEM/F12 1:1 medium (BioWhittakerTM, Verviers, Belgium), resuspended in the same medium supplemented with 10% fetal calf serum (BioWhittakerTM)

and 1% antibiotic-antimycotic solution (Sigma, St. Louis, MO, USA) at a final concentration 10^6 cells/ml medium. Portions of this cell suspension were dispensed to 24-well culture plates (Nunc™, Roskilde, Denmark, 1 ml.well-1). The plate wells were incubated at 37.5 °C in 5 % CO_2 in humidified air until 60-75% confluent monolayer was formed (3-5 days), at which point the medium was renewed. Further culture was performed in 1 mL culture medium in 24-well plates (medium for EIA) as described previously. After medium replacement experimental cells were cultured in the presence of daidzein (Santa Cruz Biotechnology, USA) alone at concentrations of 1, 10 and 100 µM/ml or FSH (Sigma-aldrich spol s.r.o., St. Louis, MO, USA) at concentrations of 0.01, 0.1 and 1 IU/ml alone and in combination with daidzein (50 µM). Control cells were cultured without daidzein or FSH. After two days in culture, the medium from the 24-well plates was gently aspirated and frozen at -24°C to await EIA.

SDS–PAGE – western immunoblotting

SDS-PAGE-Western immunoblotting was performed as described previously (Sirotkin et al., 2006). Samples were transferred to nitrocellulose membrane parablot NCP (Macherey-Nagel, Düren, Germany) after electrophoresis by using a mini trans-blot system (Bio-Rad Labs, Richmond, CA, USA). Non-specific binding of antibodies was prevented by incubation of membranes in 5% bovine serum albumin BSA (Amersham International plc, Little Chalfont, Bucks, UK). The membranes were incubated with 3% H_2O_2 in order to quench an endogenous peroxidase. Primary mouse monoclonal antibodies against PCNA (the dilution in TTBS 1:500, from Santa Cruz Biotechnology, Inc., Santa Cruz, USA) and secondary polyclonal rabbit antibodies raised against mouse IgGs, labelled with horseradish peroxidase (Sevac, Prague, Czech Republic; dilution 1:1000) were used. Thereafter, the immunoreactive bands were visualized using the detection reagents Upstate Visualizer Western Blot (Temecula, CA, USA) and exposed on ECL Hyper-film (Amersham International). The molecular weights of the detected fractions were evaluated by their comparison with 100 kB standard (Fermentas, Vilnius, Lithuania). Expression of signals were quantified by den-sitometry using 1-D Manager software (TDI, Ltd., Madrid, Spain).

Immunoassay

Concentrations of P4 and T were determined in 25-100 µL samples of incubation medium by EIA, previously validated for use in culture medium, using antisera against steroids produced in the Institute of Animal Science, Neustadt, Germany. P4 concentrations were measured by using EIA as described previously (Prakash et al., 1987). Rabbit antiserum against P4 was obtained from Research Institute for Animal Production, Schoonoord, Netherlands. It cross-reacted b0.1% with 17 β-estradiol, dihydrotestosterone, testosterone and 17 β-hydroxyprogesterone. Sensitivity was 12.5 pg/mL. Inter- and intraassay coefficients of variation did not exceed 3.3% and 3.0%, respectively. T was assayed by using EIA according to Münster (1989). Sensitivity was 10 pg/mL. The antiserum cross-reacted b96% with dihydrotestosterone, b3% with androstenedione, b0.01% with progesterone and estradiol, b0.02% with cortisol and b0.001% with corticosterone. Inter- and intra-assay coefficients of variation were 12.3% and 6.8% respectively.

Statistical analysis

Each experimental group was represented by four culture wells. The samples intended for EIA were processed separately, while samples intended for SDSPAGE–Western immunobloting were pooled before processing. Differences between groups were evaluated using t-test using statistical software Sigma Plot 11.0 (Systat Software, GmbH, Erkhart, Germany). Values represent the mean ± SEM. Differences were compared for statistical significance at the P - level less than 0.05 ($P<0.05$).

Results

Our results demonstrated stimulatory effect of FSH on P4 (at 0.01 and 0.1 IU/ml) and T (at 0.01, 0.1 and 1 IU/ml) release. Administration of daidzein alone inhibited P4 release at all doses (1, 10 and 100 µM), meanwhile T secretion was increased (at 100 µM). Moreover, daidzein addition (50 µM) suppresed FSH-stimulated secretion of P4 (at 0.01 and 0.1 IU/ml) and T (at 0.1 and 1 IU/ml) (Tab. 1,2).

Increased accumulation of PCNA was observed after treatment with FSH (at 0.1 and 1 IU/ml) and daidzein (50 µM) when given alone. Moreover, daidzein addition (50 µM) increased FSH-stimulated (0.01 IU/ml) PCNA accumulation.

Table 1. Effect of daidzein on porcine ovarian cells steroidogenesis (EIA)

Supplement	P4 secretion ng/10^6 cells/day	T secretion pg/10^6 cells/day
Daidzein 0 µM	10,53 ± 0,22	114,29 ± 10,99
Daidzein 1 µM	5,45 ± 0,61 **A**	197,31 ± 42,53
Daidzein 10 µM	6,36 ± 0,30 **A**	123,18 ± 22,98
Daidzein 100 µM	5,61 ± 0,32 **A**	200,26 ± 40,12 **A**

Table 2. The secretion of steroid hormones in porcine ovarian granulosa cells treated with FSH and combination of FSH and daidzein (EIA)

Supplement	P4 secretion ng/10^6 cells/day	T secretion pg/10^6 cells/day
FSH 0 IU/ml	11,33± 0,40	114,29 ± 10,99
FSH 0,01 IU/ml	19,62 ± 2,52 **A**	173,27 ± 13,86 **A**
FSH 0,1 IU/ml	13,02 ± 0,45 **A**	254,17 ± 44,48 **A**
FSH 1 IU/ml	9,20 ± 2,34	349,78 ± 90,66 **A**
FSH 0 IU/ml + daidzein 50 µM	5,10 ± 0,25 **B**	191,91 ± 38,82 **B**
FSH 0,01 IU/ml + daidzein 50 µM	7,21 ± 1,08 **B**	450,34 ± 146,24
FSH 0,1 IU/ml + daidzein 50 µM	4,42 ± 0,17 **B**	42,30 ± 20,04 **AB**
FSH 1 IU/ml + daidzein 50 µM	8,47 ± 1,56	85,38 ± 20,16 **B**

Legend: All the values represent progesterone (P4) or testosterone (T) release, means ± SEM, A- indicates significant ($p<0,05$) effect of daidzein or FSH alone: differences between cells cultured with daidzein (1, 10 and 100 µM/ml) or FSH (0.01, 0.1 and 1 IU/ml) and control (without additions) cells. B – indicates significant ($p<0,05$) modulatory effect of daidzein (50 µM/ml) on FSH (0; 0,01; 0,1 and 1 IU/ml) action: differences between corresponding groups of cells cultured with and without daidzein.

Conclusion

Our observations: (1) confirm the previous data on stimulatory effect of FSH on ovarian cells proliferation and steroidogenesis and inhibitory effect of daidzein on both, basal and FSH-stimulated P4 secretion by porcine ovarian granulosa cells, (2) demonstrate for the first time the involvement of daidzein in up-regulation of porcine ovarian granulosa cells proliferation and T release, (3) show the first time that daidzein can prevent FSH-stimulated release of T and promote the stimulatory action of FSH on cell proliferation. The involvement of daidzein in stimulation of both basal and FSH-induced proliferation and T secretion suggest, that

daidzein may promote ovarian follicle development. On the other hand, the ability of daidzein to decrease both basal and FSH-stimulated P4 release suggest, that it can prevent ovarian cell luteinisation associated with increase in P4 output. These observations might indicate the potential influence of soy-containing diet on female reproductive processes.

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THE EFFECT OF AGE AT FIRST CALVING ON MILK PRODUCTION OF CZECH FLECKVIEH COWS

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Abstract

In this thesis we dealt with effect of age at first calving on milk production of Czech Fleckvieh at first and second lactation. We analyzed set of about 5 000 pieces of animals from our prominent breeding of Czech Fleckvieh. We evaluate dairy cows, which calve from 26th to 36th month of their life. Next criterion of choice was the length of both first and second lactation, which should be at least 280 days. These criteria fulfil overall 3 155 dairy cows. Their milk yield per lactation was converted to milk yield per day. Regarding the 1st lactation the highest milk yield per day was set in group of dairy cows, which calved in 36th month of age (23.57 kg of milk per day), and the lowest dairy milk yield in group calved in 32nd month of age (21.51 kg of milk per day). If we focus on the milk yield in the 2nd lactation, we will find out, that the highest milk yield reached dairy cows, which calved in 27th month (25.87 kg of milk per day) and the lowest milk yield reached dairy cows, which calved in 35th month (22.64 kg of milk per day). We can thus state, that milk yield in 1st lactation of dairy cows calved sooner is average, but in the 2nd lactation the yield was higher.

Key words: Czech Fleckvieh, milk yield, age at first calving

Introduction

The breeding of heifers from birth till calving constitute the 2nd highest article of costs in dairy farm and all this without profit until the lactation (Heinrichs, 1993). Length of rearing depends mostly on reached increases in weight and on concenption of heifer in optimal weight and age. The opinions on optimal age at first calving are not united and unchangeable by far (Mourits et al., 2000). This is why so many researches are focused on way, how to minimize costs connected with increase or decrease of unproductive period of animal´s life. The curtailment of unproductive period can be reached by decreasing of age of 1st calving under present recommendation. This can be reached by increasing of daily growth in this period (Hoffman, 1997), which could lead in breeding to lower average age of 1st calving. Although this strategy can lead to acceleration of the productive life, it was proved, that

increasing of daily growth influences negatively the development of the mammary gland (Radcliff et al., 1997; Sejrsen et al., 1982) and milk yield in 1st lactation (Lammers et al, 1999; Radcliff et al., 2000; Van Amburgh et al., 1998).

In summary of current literature dealing with relation between intensity of heifers´ growth and their milk yield in 1st lactation, was found out, that maximal production and amount of proteins was in daily growth about 800g/day of Holstein heifers (Zanton a Heinrichs, 2005). In our thesis we will follow up, how the age of 1st calving will affect yield in 1st and 2nd lactation of Czech Fleckvieh.

Material and methods

The set of dairy cows was analyzed to determine the effect of 1st calving age. The set consisted of about 5 000 pieces from breeding of Czech Fleckvieh with higher than average milk yield. From this set was chosen those dairy cows, which had both 1st and 2nd lactation longer than 280 days. Overall we analyzed 3 155 pieces of cows. These dairy cows were divided into groups according to their age of calving from 26 up to 36 months. Milk yield per lactation was observed and subsequently convert to lactation per day due to different length of lactation. The resulting data were processed according to statistically-mathematical methods in program Statistica 10.

Results and discussion

In table 1 we can see the effect of 1st calving age on milk yield in 1st lactation. There is only little difference between groups with the highest daily milk yield 23.57 kg (calving age 36 months) and the lowest daily milk yield 21.51 kg (calving age 32 months). This difference was 2.06 kg daily, which is 628.3 kg per whole lactation. But regarding to maximal daily yields, those were reached in group of cows calved in age of 36 months (23.57 kg) and 33 months (23.35). This means that they were mating in age of 27 and 24 months.

Table 1. The effect of 1st calving age on milk yield in 1st lactation

age at first calving	average daily milk yield	min.	max.	Sx	P
26 months	22.03	11.28	36.68	3.86	NS
27 months	21.96	11.30	33.31	3.58	NS
28 months	22.33	11.16	34.68	3.70	NS
29 months	22.11	12.82	32.15	3.56	NS
30 months	22.24	9.74	30.64	3.58	NS
31 months	22.35	14.16	33.98	3.98	NS
32 months	21.51	13.11	29.18	3.26	NS
33 months	23.35	14.95	34.88	4.23	NS
34 months	22.14	11.84	34.19	4.40	NS
35 months	21.62	15.34	27.53	3.36	NS
36 months	23.57	17.50	38.17	4.26	NS

According to results we can state, that slightly higher yield had those dairy cows, which were calved in later age. But this difference was not so striking. We can therefore conclude, that from economical point of view it is better sooner calving of dairy cows, which is also confirmed by Chládek et al. (2013), who states, that in 1st lactation there was not any essential difference in yield of cows calved in 24th month nor with cows calved in 32nd month of age. Sooner calving without negative effect on yield in 1st lactation confirm even studies of Mourits et al. (2000) who found out, that with increment 0.9 kg/day during prepubertal heifers period and with maximal reached increment 1.1 kg/day during postpubertal period it is practically possible to reach first calving in 20.5th month with body weight 563 kg, which is equal to consecutive incomes $107 per heifer per year. Authors (Britt et al., 1998, Mourits et al. 1999) demonstrate in their studies of Holstein cattle, that no disadvantages were found for early calving till 22 months of age, as long as the growth zones in breeding of heifers are abided.

In table 2 we can see the effect of 1st calving age on milk yield in 2nd lactation. There can be seen some tendency to higher yield of dairy cows mating in sooner period. The highest daily milk yield was reached in group calved in 27 month of age (25.87 kg of milk) and the lowest daily milk yield was reached in group calved in 35 month of age (22.64 kg of milk). The difference in milk yield was 985.15 kg of milk per whole lactation. Dairy cows calved from 26 to 30 months and in 33rd month produced over 25 kg of milk per day.

Table 2. The effect of 1st calving age on milk yield in 2nd lactation

age at first calving	average daily milk yield	min.	max.	Sx	P
26 months	25.59	11.71	39.25	4.49	NS
27 months	25.87	13.67	40.49	4.58	NS
28 months	25.81	9.91	39.71	4.35	NS
29 months	25.26	8.05	38.10	4.59	NS
30 months	25.05	14.96	36.21	4.32	NS
31 months	24.84	11.52	34.95	4.47	NS
32 months	23.83	13.46	35.79	4.61	NS
33 months	25.54	15.49	39.24	4.81	NS
34 months	23.86	16.82	30.62	3.57	NS
35 months	22.64	16.51	31.34	3.75	NS
36 months	23.80	15.66	37.27	4.36	NS

Dairy cows in 2nd lactation produced more milk than dairy cows in 1st lactation and the highest difference was in group of cows calved in period from 26 to 30 months of age, where it was average 3 kg of milk higher than in 1st lactation. This confirms also study of Ettema and Santos (2004), who say, that early calved heifers have lower yield in 1st lactation, but higher lifelong yield. In study of Wathes et al. (2008) the optimal fertility with preservation of maximal yield was reached in age of 24 to 25 months with Holstein dairy cows. But dairy cows calved in age of 22 to 23 months were best in overall yield and in life length (longevity) over 5 years, partially because heifers with good fertility have brilliant reproductive ability even later.

Conclusions

Finally we can state, that the age of dairy cows in calving in range of 26 to 36 months has not very large effect on average daily yield in 1st and 2nd lactation. We can however observe some tendency to increased yield in 2nd lactation of those dairy cows, which were calved earlier. On the contrary dairy cows calved in age of 34 months had almost 2 litres of milk less than dairy cows calved earlier. But it is important to realize, that with extension of non-productive age the costs will rise about 1200 CZK per month and cow.

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IMPACT OF ACRYLAMIDE ON OXIDATIVE STRESS PARMATERES IN HEART MUSCLE AND LUNGS

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Abstract

Acrylamide (ACR) is carcinogenic, mutagenic and toxic. ACR is delivered to the body by three ways. One of the frequently way is delivery to the organism with a food processed in high temperatures and rich in carbohydrates. It can also be delivered to the body by inhalation or by dermal way. The experiment was conducted on mice Swiss, 8 weeks old which were injected intraperitoneally with acrylamide at dose 20 mg/kg b. w. In the studied organs (heart and lungs) was determined levels of antioxidants (glutathione (GSH), glutathione peroxidase (GPx), superoxide dismutase (SOD).

Introduction

Acrylamide (ACR) is generated in food subjected to the thermal processing in the low humidity like baking, frying and grilling as a result of the Maillard reaction between free amino acid asparagine and carbonyl groups of reducing sugars (Stadler at al. 2002, Taeymans at. al 2004). Foods rich in both of these precursors are derived from plants such as potatoes and cereals. Acrylamide could not be generated from animals products. For example the content of acrylamide in potatoes is 150 up to 4000 µg/kg. More than 30% of the food in Europe and the United States contain acrylamide. Research on acrylamide showed the consumption of the medium dietary intake is 0.5 mg / kg body weight per day for adults and for children of 0.6 mg / kg body weight per day (Claus at. al 2008, Mucci at. al 2008, Pelucchi at. al 2011). According to a recent study conducted in South Poland value for whole group was 0.85 ± 0.82 µ/g acrylamide/kg/bw, per day (Zając at. all 2013). Acrylamide has been classified by International Agency for Research on Cancer (IARC) as a substance with potential carcinogenic. The European Union has classified acrylamide as a substance carcinogenic, mutagenic and toxic for reproduction. In the body, with the participation of liver monooxygenases Cytochrome P450 (mainly isoenzyme CYP2E1), acrylamide is transformed to more reactive acrylamide epoxy mold – glicydamide (GA) (Tareke at. al 2008) One of the mechanisms of action of acrylamide in the body is inducing the reactive oxygen species generation and disturbing redox balance by depleting the cellular pool of glutathione (Rodriguez-Ramiro at al., 2011). Oxidative stress may affect cell function, trigger cell death

and contribute to development and pathology of human diseases including cancer and neurodegenerative disorders (Halliwell 2011).

Materials and methods

The experiment was conducted on mice Swiss, 8 weeks old, fed standard diet and grown in 12:12 LD photoperiod, n=5. Animals from experimental groups were injected intraperitoneally with acrylamide at dose 20 mg/kg body weight and segregated into 4 groups: 2 controls injected with physiological saline and 2 experimental groups which were analysed after 24 and 48 h respectively. Level of antioxidants (glutathione (GSH), glutathione peroxidase (GPx), superoxide dismutase (SOD)) was measured by using a spectrophotometric method. The results were compared using MANOVA followed by Tukey's test. The significance level was established at $p<0.05$.

Results

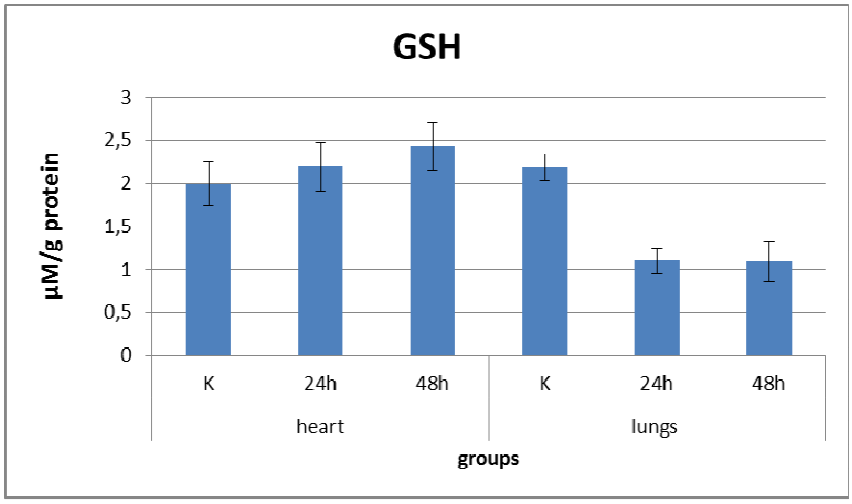

Figure 1. GSH (gltutathione) activity µM/g protein after ACR (acrylamide) in doses 20 mg / kg bw, after 24h and 48h.

Levels of glutathione in heart increased after the administration of ACR. In the contrast, the effective in the lungs was opposite. Glutathione was observed with a gradual increase over time, and these differences were statistically significant (F=5,0334, p=,03937). Effect of ACR in heart was statistically significant at the significance level F=131,50, p=,00000. In lungs time of exposition of ACR was no significantly effect, but impact of ACR was statistically significant (F=113,83, p=,00000) (Fig. 1).

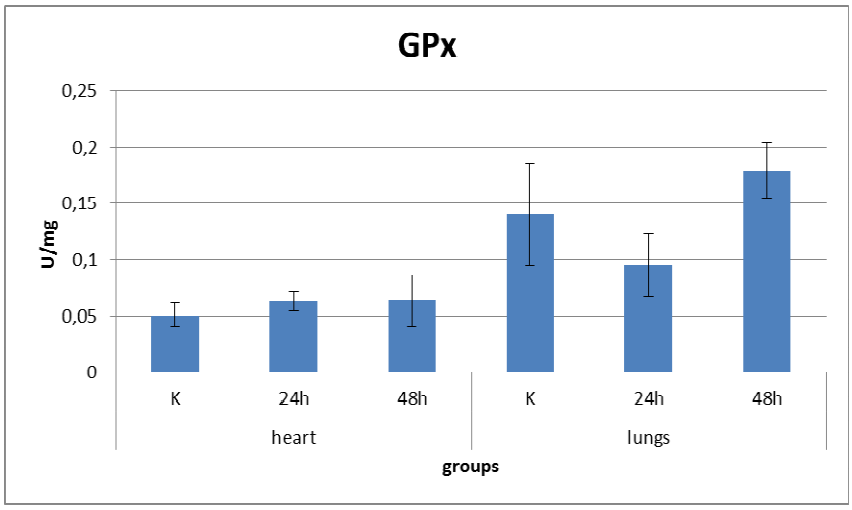

Figure 2. GPx (glutathione peroxidase) activity U/mg after ACR (acrylamide) in doses 20 mg / kg bw, after 24h and 48h.

ACR dose had no effect on the concentration GPx in the heart. Significant differences were observed between actions of ACR in lungs (F=6,4060, p=,02224). In lungs impact of ACR was dependent on the time, and caused an increase and a decrease of enzyme (Fig. 2).

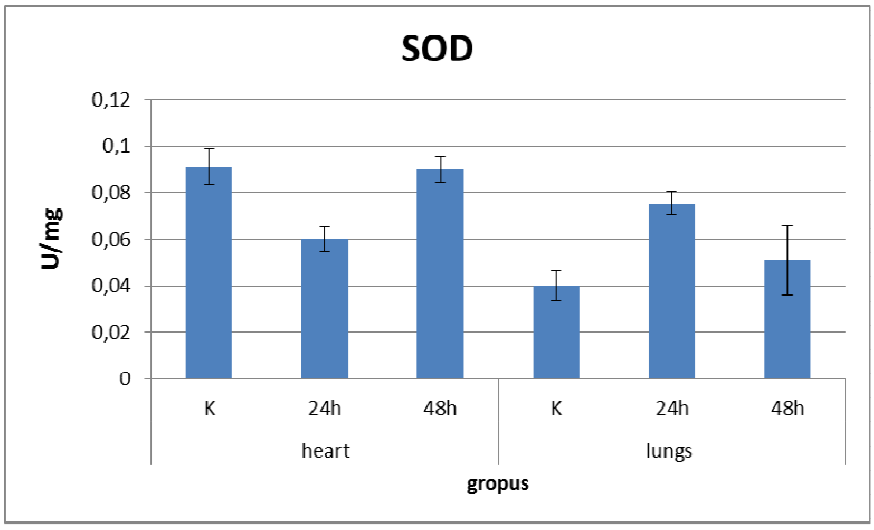

Figure 3. SOD (superoxide dismutase) activity U/mg after ACR (acrylamide) in doses 20 mg / kg bw, after 24h and 48h.

After 24 ACR dose resulted in a decrease in SOD activity in heart and after 48h differences have been observed compared to the control groups. Effect of ACR of enzyme activity in heart was statistically significant (F=32,395, p=,00003). In lungs was not observed statistically significant, but enzyme activity increased after 24h and 48h. In 24h level of SOD was highest than after 48h.

This results showed that acrylamide has impact on oxidative stress, and this same redox balance in heart muscle and lungs. Our research suggest that influence of acrylamide depends on the duration of action.

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DIFFERENCES IN IRON CONCENTRATIONS BETWEEN LUNG TUMOR AND ADJACENT TISSUES

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Abstract

Lung cancer is found to be the main type of cancer and permanently growing problem in the whole world. Research have shown that many trace elements are involved in activation or inhibition of numerous biological processes and consequently, can directly or indirectly mediate in carcinogenesis. Human samples consisted of healthy, as well as, tumorous lung tissue from 77 patients diagnosed with cancer. The iron concentrations were measured with flame AAS in 10 mL solution prepared on the basis of mineralized samples.

Introduction

Lung cancer is found to be the main type of cancer and permanently growing problem in the whole world (Erbaycu et al., 2007). It is estimated that lung cancer is annually accountable for 1.5 million of deaths (Cobanoglu et al., 2010). That disease is more frequent in men than in women (Alwahaibi et al., 2011; Kuo et al., 2006; Roggli et al., 2000 and Takemoto et al., 1991). In spite of fact that the accurate cause of lung cancer is unknown (Alwahaibi et al., 2011), there are a growing number of evidences that cigarette smoking, oxidative stress and dietary habits such as consumption of micronutrient and vitamin C may play crucial role in its formation (Lee and Jacobs, 2005). Additionally, research have shown that many trace elements are involved in activation or inhibition of numerous biological processes and consequently, can directly or indirectly mediate in carcinogenesis (Drake II et al., 1989). Especially, the lack of Cu, Fe and Zn, which are recognized as enzyme cofactors, is supposed to be involve in carcinogenic stress generation (Mulware et al., 2013).

Fe composes inseparable element of plant and animal life (Cobanoglu et al., 2010) because of its crucial role in an appropriate cell functioning (Zhou et al., 2005). Notwithstanding iron is a compound which is involved in redox reactions therefore can participate in free radicals generation (Cobanoglu et al., 2010). It is also known that alveolar macrophages which are presented in airway epithelial cells and have an active iron metabolism are involved in cancer

pathogenesis (Pascolo et al., 2013). It must be point out that iron acts as a potential carcinogenic compound only in free form (Lee and Jacobs, 2005). Studies have shown higher risk of lung cancer in relation to high dietary iron intake (Zhou et al., 2005), nevertheless Milman et al. (1991) research did not found any significant sex differences in iron levels among patients with lung cancer.

Iron status generally differs between gender and alters during the lifetime (NNR, 2012). However, iron concentrations in cancerous tissues, as well as, the influence of above mentioned factors on Fe levels is hardly studied in the literature. In this study, the aim was to investigate the differences in iron levels between healthy and tumorous lung tissue from men and women classified in two age groups.

Material and methods

This study was approved by Bioethical Commission of the Medical University in Katowice, Poland. Human samples group consisted of healthy, as well as, tumorous lung tissues from - 77 patients (31 men and 46 women) diagnosed with cancer and undergoing pulmonary resection in the Pulmonary and Thoracic Surgery Center in Bystra, Poland. After the excision, samples were placed in plastic vials and stored at -20° C until the further analysis.

Metal analysis was done in the laboratory of the Institute of Biology in the Pedagogical University of Cracow, Poland. To choose accurate analysis path, samples were divided according to the donors sex and age group (first – 44-60 years old, second - 61 years old and more). All samples were dried for 14 days at 60°C to obtain constant dry weight and mineralized in the hot nitric acid (65%, Baker Analyzed) using open mineralizer (Velp Scientifica DK-20). Iron concentrations were measured with flame atomic absorption spectrometer (PerkinElmer AAnalyst 200) in 10 mL solution prepared on the basis of mineralized samples. The detection and quantification limits for Fe were 0.019 mg/L and 0.032 mg/L, respectively.

The homogeneity of variance was checked with the Levene test. According to the obtained results, we carried out nonparametric tests.and analyzed each factor separately with U Mann Whitney test with changed significance level to 0.017 according to Bonferoni's correction. Excel 2010 PL (Microsoft) and Statistica 10 En (StatSoft) were used in all the analyses.

Results

In our studies we noted the significant higher iron concentration in non-affected lung tissues than in tumor tissues (Figure 1). What is more, we observed no statistically significant

differences in iron concentrations in tumor tissues neither between men and women nor age groups (Figure 2, Figure 3). Nevertheless, slightly higher median iron levels were in tumor tissues from women (222.12 ppm vs. 212.19 ppm).

In contrast to our data most researchers stated that higher iron levels are higher in cancerous tissues than normal tissues (Ionescu et al., 2006; Siddiqui et al., 2006). However, Mulware (2013) claimed that some organs represents lower iron levels in cancerous tissues. In case of lungs, affected tissues contain more Fe than normal ones (Farah et al., 2010). Nevertheless, in those studies they compared lung tumor tissues with those collected from healthy people, whereas we used tumor and adjacent tissues.

It is known that the iron concentration alters with human age (Zacharski et al., 2000) and, also, Fe can be accumulated in tumor tissues (Mulware, 2013). Whereas we noted no significance differences in iron tumor lung concentrations between age groups. Sex differences in iron concentrations have been reported in many research. However, Adachi et al. (1991) confirmed our findings that there is no significance differences in Fe levels between men and women.

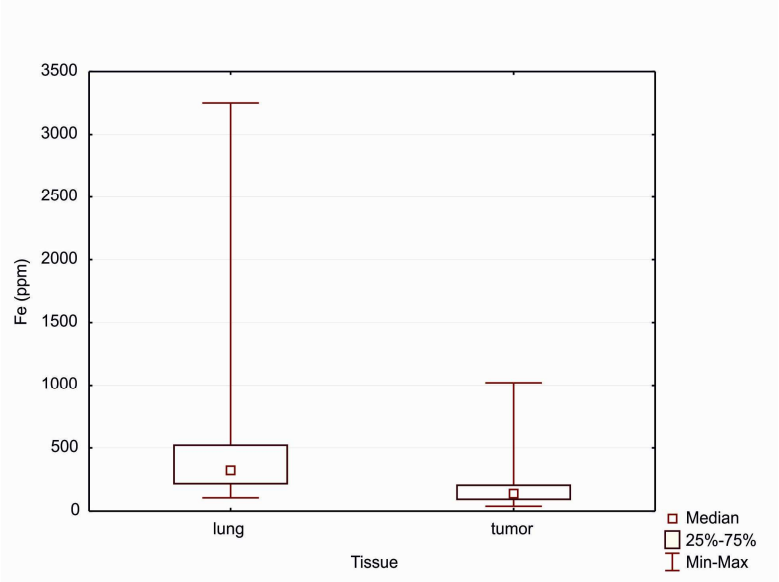

Figure 1 Median iron concentrations in lung tumor and adjacent tissues

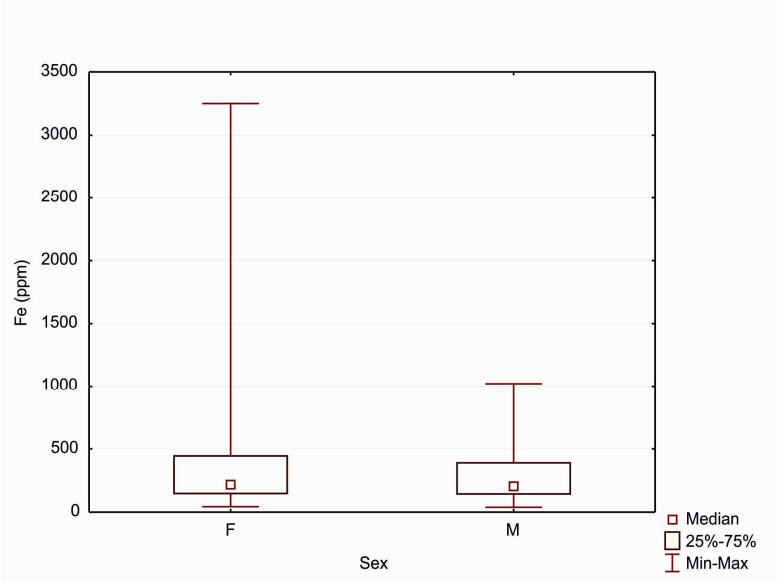

Figure 2 Median iron concentrations in lung tumor tissues from women (F) and men (M).

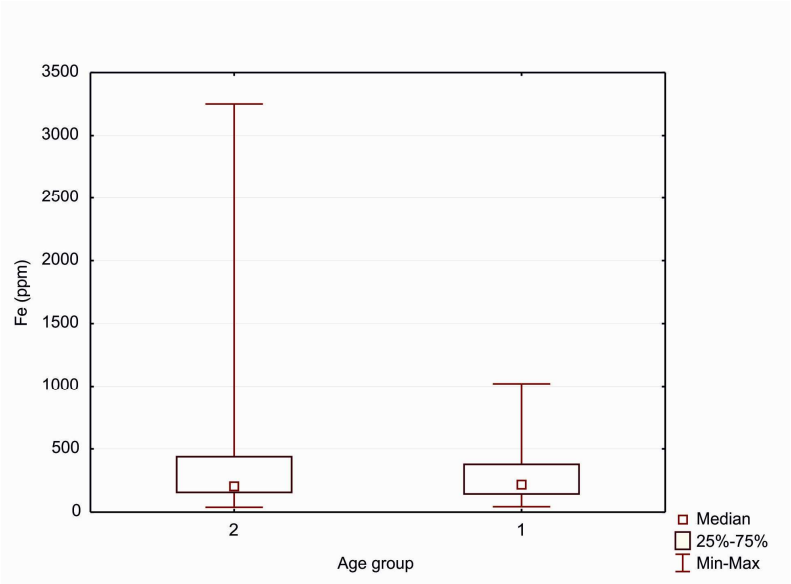

Figure 3 Differences in iron concentrations in lung tumor tissues from younger (1) and older (2) patients.

Conclusion

We found that there is a significant difference in iron concentrations between lung tumor and adjacent tissues. What is more, sex and age have no significant influence on Fe level in lung tumor tissues. However it is necessary to carry out further studies which will compare iron concentrations in tissues from patients under the age of 40.

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SEX-, SEASONAL- AND AGE-DEPENDENT CHANGES IN PLASMA ADRENOCORTICOTROPIC HORMONE (ACTH) CONCENTRATIONS IN THE EUROPEAN BEAVER (*Castor fiber L.*)

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Abstract

The European beaver (*Castor fiber L.*) is the largest free-living rodent of Euroasia and belongs to the group of long day breeders. The phenomenon of seasonal breeding may be related to seasonal changes in the secretion of ACTH - the main hormone that controls the secretion of glucocorticoids. The aim of our experiment was to examine the sex-, seasonal- and age-dependent changes in plasma ACTH levels. The results of our study showed that the level of stress-induced ACTH in the beavers remained constant independently on the season and gender. However, we noticed the strong upward trend in the concentration of ACTH in females (prebreeding) in November.

Introduction

The European beaver (*Castor fiber L.*) is the largest free-living rodent of Euroasia. The beavers have a breeding season (long day breeders) that is initiated at a time when the environment will allow for the best survival of the young. The mating takes place in late Winter and juveniles are born in May and June. The peak activity of a beavers family is in the Spring, during raising of offspring and in the Autumn, when individuals collect food for the Winter, build and repair burrows and lodges. During the Winter, beavers do not hibernate (Czech 2010). In many species of free-living vertebrates there are observed annual changes in the secretion of glucocorticoids which are a major component of the classic endocrine stress response (Romero 2002). Glucocorticoids help animals to respond to environmental dangers and protects an organism through inducing a variety of behavior and physiological changes (Reeder and Kramer 2005). The phenomenon of seasonal functioning of the adrenal glands may be related to seasonal changes in the secretion of ACTH - the main hormone that controls the secretion of glucocorticoids. A few publications, analyzing changes in the level of ACTH in domestic animals (horses, sheep, geese), indicate the existence of seasonality, although the results of these studies are not clear. Therefore, the aim of our experiments, conducted on a population of free-living male and female beavers, was to find gender-, seasonal- and age-dependent changes in plasma ACTH levels.

Material and Methods

Blood samples were collected under general anesthesia in April (8 males and 5 pregnant females), July (4 males and 6 females) and November (6 males and 5 females). In July (2 males and 3 females) and in November (3 males and 2 females), the blood samples were also collected from young beavers (the first year of life). The blood for the determination of the plasma hormone concentrations was collected from the carotid artery. The level of ACTH was measured by enzyme-linked immunosorbent assay (ELISA). Analyses were performed using Statistica software (StatSoft Inc., USA). Data (ng/mL; mean±SEM) were analysed by two-way ANOVA followed by the Duncan post hoc test. The level of significance was set at $p<0.05$ for all analyses.

Results

Sex and seasonal changes in plasma ACTH concentration were not observed. However, we noticed the strong tendency (main effect, $p=0.0521$) in seasonal-dependent changes in females. The hormone concentration was higher ($p<0.05$) in females in November than in April and in July. Additionally, we observed higher plasma ACTH level in the old than the young males in November ($p<0.05$).

Conclusion

In the present study, we report for the first time, the concentration of ACTH, as well as sex-, seasonal- and age-related changes in stress-induced plasma ACTH levels in the European beavers during the breeding season in April (males and pregnant females), in July, out of season (postbreeding) and in November, when the beavers begin preparing for Winter (prebreeding). Our data suggests that the levels of ACTH might depend on the season but not on the sex.

Acknowledgments

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CALVES FROM 1. TO 35. DAY OF AGE - SELECTED BIOCHEMICAL PARAMETERS

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Abstract

45 calves of the Black Pied breed, reared in the Agrokomplex Nitra, were selected at random in the trial. The blood of calves was taken by puncture from v*ena jugularis* regularly three hours after morning feeding at following ages: 1, 7, 14, 21, 28, 35 days. The copper, iron, urea and total protein contents were determined photometrically by help of the Bio-La-tests. The results were sorted by sex and live weight at birth. Fe levels were age downward trend throughout follow-up. Significant differences ($p < 0.001$) was recorded between (1:7, 1:14, 1:21, 1:28 and 1:35) days of age calves. Other differences were statistically insignificant. Cu levels were age upward trend throughout follow-up. Significant differences ($p < 0.001$) was recorded between (1:7, 1:14, 1:21, 1:28 and 1:35) days of age calves. Other differences were statistically insignificant. Urea had an upward trend with age. Significant differences ($p < 0.001$) was recorded between (1:7, 1:14, 1:21, 1:28 and 1:35) days of age calves. Other differences were statistically insignificant. We recorded total protein levels relatively balanced. In total protein, we did not detect any significant differences. We recorded no statistical differences between gender or birth weight of the monitored indicators. We confirmed a significant effect of age on changes in the levels of iron, copper and urea. The influence of gender and body weight at birth, we not confirmed.

***Keywords** : calves, blood serum, iron, copper, urea, total protein, age, gender, body weight at birth*

Introduction

Lack of nutrients is manifested the most in young animals, in particular in first days of life and later at transition from dairy nutrition to plant one. In these „critical periods“ different metabolic and consequently also health disorders of calves may be apparent.
Microelements, being components of various ferments, hormones, vitamins and other substances. take part within the metabolic processes, in growth, reproduction, immunologic activity, efficiency and particularly, not last in the general health condition of an organism.

Detecting the level of total protein may be important, inter alia, to the prediction of disease and mortality. Bami et al. (2008) studied the effects of parenteral administration of iron and copper on hematological parameters, weight gain, and health of neonatal dairy calves at 7, 14, 21 and 28 days of age. The effect of sodium butyrate (SB) supplementation in milk replacer (MR) or in starter mixture (SM) on concentration of blood parameters in calves from age 0 – 21 days studied Górka el al., (2011). Hammon et al., (2002) studied feed intake, growth performance, and metabolic and endocrine traits were studied in male calves fed unlimited (GrAL; n = 7) amounts of colostrum for 3 d after birth and mature milk up to d 28 and were compared with calves fed commonly recommended amounts of colostrum and milk (GrRS; n = 7). Blood samples were taken on d 1, 2, 3, 7, 14, 21, and 28 to measure several metabolites and hormones. Hesari et al., (2012) studied the effects of the copper injection and injection timing in pregnant dairy cows at dry period on the hematology, some serum metabolites, health and growth of their calves. Jugular blood was taken from all calves 24–48 h after birth and at 7, 14, 21 and 28 days of age for measuring hematological and biochemical parameters.
Khan et al., (2007) studied on Holstein calves fed milk either a conventional method or a step-down (STEP) method on biochemical parameters at age from 7 to 63 days.
Lee et al., (2009) studied the performance of female Holstein calves fed either whole milk (WM) or milk replacer (MR) having similar gross composition. Calves on both treatments were fed (1.8 L/feeding) for 4 times daily for the first 25 day of age. Feed intake, growth, and health variables were monitored until calves were 70 day of age.
Mohri et al., (2007) investigated the blood composition of growing calves in order to evaluate the need for defining reference values for different age groups. Thirty two Holstein calves were taken blood sampled. Blood samples were taken within 24–48 h following birth and at 14, 28, 42, 56, 70 and 84 days of age.
Naseri et al. (2011) added from the age of 14 days, 10 mg/kg, respectively 20 mg/kg of copper to milk. Blood samples were taken by puncture of the jugular vein at 14, 30, 60 and 80 days. Indicate that age has significant effects on the values of most measured parameters ($p<0.05$) except WBC, lymphocyte, total protein, and fibrinogen.

Material and Methods

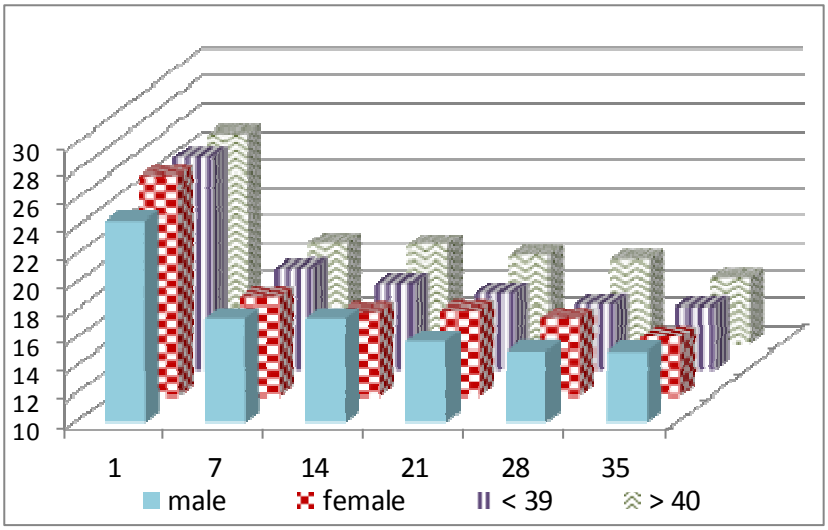
45 calves of the Black Pied breed, reared in the Agrokomplex Nitra, were selected at random in the trial. The blood of calves was taken by puncture from v*ena jugularis* regularly three hours after morning feeding at following ages: 1, 7, 14, 21, 28, 35 days. Blood serum was obtained by centrifuging the blood at 3000 revolutions per minute

during 30 minutes. The copper, iron, urea and total protein contents were determined photometrically by help of the Bio-La-tests.

The results were sorted by sex and live weight at birth (<39 kg respectively> 40 kg) and were calculated the basic variational-statistical characteristics and ANOVA, differences in the content of individual parameters are tested by using t-test and Scheffe's test.

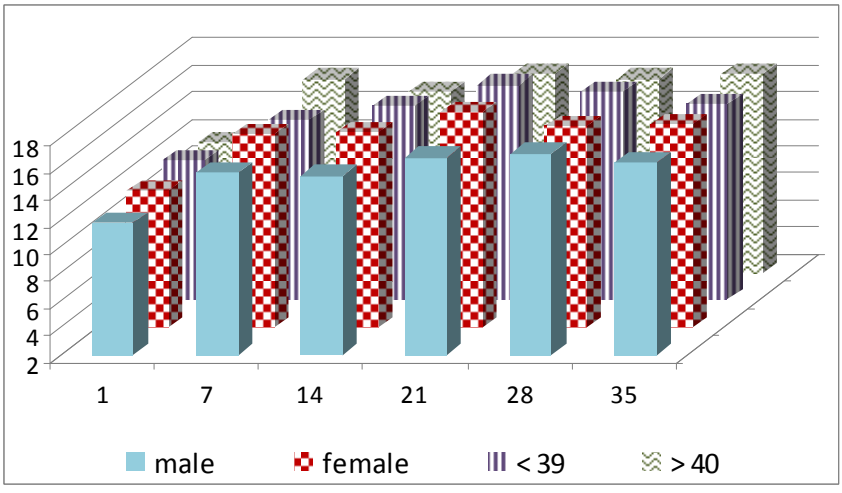
Results and discussion

Figure 1. Changes in the levels of iron during the experiment.



The average iron levels (Fig.1) ranged in bullocks from 25.30 μmol.l^{-1} aged 1 day to 14.25 μmol.l^{-1} at age 35 days, in heifers from 25.83 μmol.l^{-1} to 14.25 μmol.l^{-1} in calves with birth weight <39 kg from 25.30 μmol.l^{-1} to 14.54 μmol.l^{-1} , weighing > 40 kg from 25.09 μmol.l^{-1} to 14.77 μmol.l^{-1} . Fe levels were age downward trend throughout follow-up. Significant differences ($p < 0.001$) was recorded between (1:7, 1:14, 1:21, 1:28 and 1:35) days of age calves. Other differences were statistically insignificant.

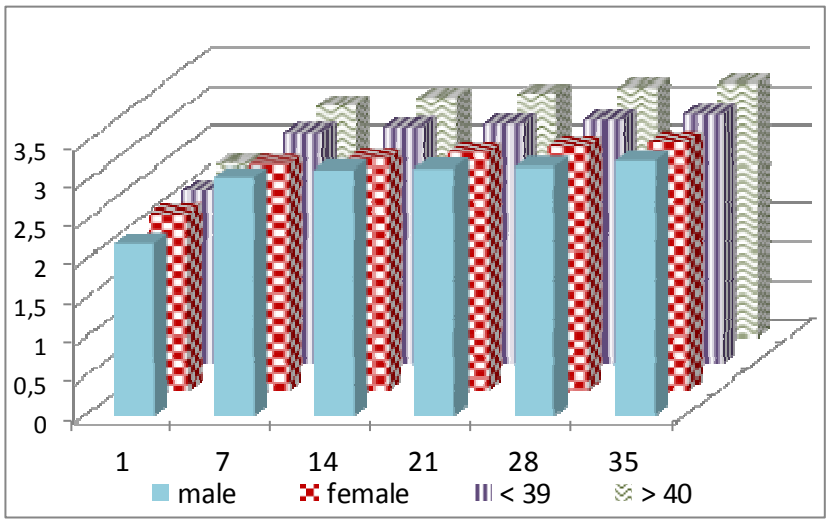
Figure 2. Changes in the levels of copper during the experiment.



For bullocks, the average copper levels (Fig.2) ranged from 11.76 μmol.l^{-1} aged 1 day to 16.26 μmol.l^{-1} at age 35 days, in heifers from 12.16 μmol.l^{-1} to 17.06 μmol.l^{-1} in calves with birth weight < 39 kg from 12.36 μmol.l^{-1} to 16.55 μmol.l^{-1} , weighing > 40 kg from 11.67 μmol.l^{-1} to 16.78 μmol.l^{-1}. Cu levels were age upward trend throughout follow-up. Significant differences ($p < 0.001$) was recorded between (1:7,

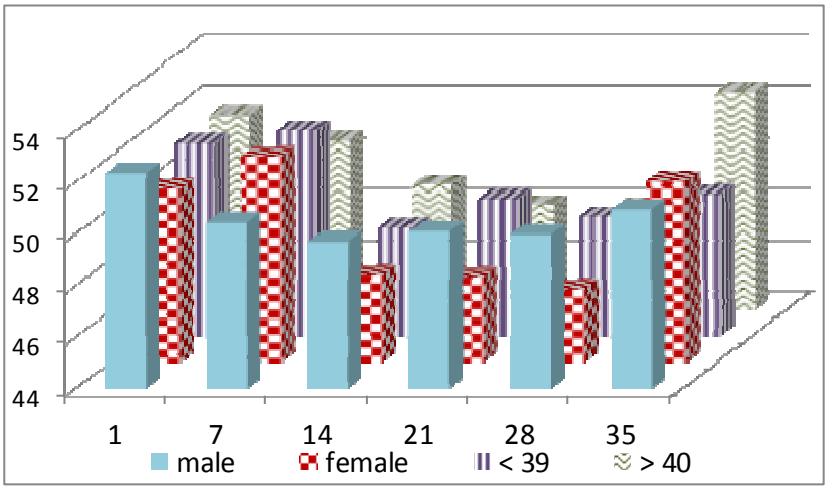
1:14, 1:21, 1:28 and 1:35) days of age calves. Other differences were statistically insignificant.

Figure 3. Changes in the levels of urea during the experiment.



Levels of urea (Fig. 3) in bullocks ranged from 2.21 mmol.l^{-1} to 3.27 mmol.l^{-1}, heifers from 2.26 mmol.l^{-1} to 3.19 mmol.l^{-1} , in calves with birth weight <39 kg from 2.23 mmol.l^{-1} to 3.20 mmol.l^{-1} in calves weighing> 40 kg from 2.24 mmol.l^{-1} to 3.26 mmol.l^{-1}. Urea had an upward trend with age. Significant differences ($p < 0.001$) was recorded between (1:7, 1:14, 1:21, 1:28 and 1:35) days of age calves. Other differences were statistically insignificant.

Figure 4. Changes in the levels of total protein during the experiment.



We recorded total protein levels relatively balanced moving in bullocks from 49.71 g.l^{-1} (day 14) to 42.37 g.l^{-1}, heifers from 46.85 g.$^{l-1}$ (day 28), in calves with weight <39 kg from 48.19 g.l^{-1} to 51.98 g.l^{-1} and calves weighing > 40 kg from 47.96 g.l^{-1} to 52.42 g.l^{-1}. In total protein, we did not detect any significant differences.

We recorded no statistical differences between gender or birth weight of the monitored indicators.

Bami et al. (2008) investigated the levels of total protein, Fe and Cu aged 7, 14, 21 and 28 days. The Fe content of our findings are consistent, whereas the total protein found above and below the Cu levels.

Levels of Fe, Cu and total proteins, stating Hesari et al. (2012) are consistent with our findings. Insignificant differences in the age dynamics of Fe and Cu are in conflict with our findings. Likely to have been caused by injecting Cu dry cows standing.

Unlike Mohri et al. (2007) were recorded at a decreasing level of Fe, for urea slight increase in values. In total protein had consistently balanced level with us.

In accordance with our findings Naseri et al., (2011) indicate that age has a significant effect on the levels of iron, copper and other indicators except total protein, WBC, lymphocytes, and fibrinogen.

In accordance with our findings Gorka el al., (2011) reported more or less balanced levels of total protein from birth until the age of 21 days.

Hammon, et al. (2002) provides for urea with age increasing tendency of its levels in total proteins more or less balanced by the value, which is consistent with our findings.

Our findings in rising levels of urea are consistent with data reported by Khan el al., (2007), Lee et al., (2009) as well as more or less balanced level of TP.

Conclusion

- We confirmed a significant effect of age on changes in the levels of iron, copper and urea.
- The influence of gender and body weight at birth, we not confirmed.
- The largest changes in the dynamics of studied parameters take place, as a role, during the first 7 days of calf life. From this viewpoint the period from birth up to 7 days of age could be regarded as „the critical period".

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PATULIN - INDUCED CHANGES IN HAEMATOLOGICAL PARAMETERS OF MALE AND FEMALE RABBITS AFTER 2 WEEKS EXPOSURE

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Abstract

The subject of the present study was to determinate the effect of single dose of patulin on haematological parameters of rabbits: white blood cell count (WBC), lymphocytes count (LYM), medium size cell count (MID), granulocytes count (GRA), lymphocyte percentage (LYM%), medium size cell percentage (MI%), granulocytes percentage (GRA%), red blood cell count (RBC), haemoglobin (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red cell distribution width (RDWc), platelet count (PLT), platelet percentage (PCT), mean platelet volume (MPV) and platelet distribution width (PDWc). Fourteen rabbits of Californian broiler line were used in this experiment. Animals were divided into four groups, two control groups C, C1 and two experimental groups E, E1. Rabbits were fed with a granular feed mixture (FM). Experimental groups received patulin in injectable form at 10 $\mu g.kg^{-1}$ for 14 days 2 times a week. The investigation was performed with haematology analyzer Abacus junior VET (Diatron®, Vienna, Austria). Significant changes were observed in females groups in lymphocytes (LYM), lymphocyte percentage (LYM%), red blood cell count (RBC), haemoglobin (HGB). In case of male, significant decrease ($P < 0.05$) was detected only of MID in E1 group in comparison with C1 group. Our results revealed that females were more sensitive to patulin exposure in comparison to males.

Introduction

Mycotoxins are secondary metabolites of fungal origin (Šimůnek, 2004; Serra et al., 2005; Frisvad et al., 2006). Patulin is a frequently found food contaminant mainly produced by the fungi *Aspergillus* and *Penicillium* (Glaser et al., 2012; Malíř et al., 2003; Frisvad and Thrane, 2006; González et al., 2007). Patulin has bacteriostatic, bactericidal and fungicidal effects. It is toxic to plants and animals cells (Toman et al., 2003; Sabater-Vilar, 2004), exhibits carcinogenic (Herzig, 2002; Sabater-Vilar, 2004), mutagenic and teratogenic activity (Sugiyanto et al., 1993; Schumacher et al., 2005), activate gastrointestinal disorders, anaemia,

swelling and haemorrhage of various organs (Jesenská, 1987; Rimárová, 2002; Sabater-Vilar, 2004; Mahfoud et al., 2002). The genotoxic (Alves et al., 2000; de Melo et al., 2011; Liu et al., 2003; Pfeiffer et al., 1998) and cytotoxic (Schumacher et al., 2005) properties are believed to be due to the high reactivity of patulin to cellular nucleophiles. It reacts fast with sulfhydryl groups and more slowly with amino functions (Lee and Roschenthaler, 1986) of proteins and glutathione (Pfeiffer et al., 1998; Schumacher et al., 2006).

The aim of this study was to determine the effect of patulin treatment on haematological parameters of female and male rabbits.

Material and Methods

Animals and diet

Fourteen adult rabbits of Californian broiler line were used in experiment. Rabbits were obtained from an experimental farm of the Animal Production Research Centre in Nitra, Slovak Republic. Rabbits (in the age of 4 months, weighing 3.5 – 4.0 kg) were housed in individual flat-deck wire cages (area 0.34 m^2). The animals were healthy and their condition was judged as good at the commencement of the experiment. Conditions of their care, manipulations and use corresponded to the instruction of EC no. 178/2002 and related EC documents, and they were approved by local ethics commission. Animals were kept in cages, at standard conditions (temperature 20 – 22°C, 14 h light period). Drinking water and feeding mixture for all animals was provided on an *ad libitum* basis. Animals were divided into four groups, two control groups C, C1 and two experimental groups E, E1 (Table 2). Rabbits were fed with a granular feed mixture (Table 1). Experimental groups received patulin in injectable form at 10 µg.kg^{-1} for 14 days 2 times a week (Table 2).

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

Component	
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}

Table 2. Concentration of patulin in experimental groups

	Concentration of patulin (μg.kg^{-1})	Gender
C (n=4)	0	♀
C1 (n=3)	0	♂
E (n=4)	10	♀
E1 (n=3)	10	♂

Blood sampling and analyses

Blood samples from *vena auricularis* were taken from all animals. In whole blood, selected haematological parameters as total white blood cell count (WBC), lymphocytes count (LYM), medium size cell count (MID), granulocytes count (GRA), lymphocyte percentage (LYM%), medium size cell percentage (MI%), granulocytes percentage (GRA%), red blood cell count (RBC), haemoglobin (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red cell distribution width (RDWc), platelet count (PLT), platelet percentage (PCT), mean platelet volume (MPV) and platelet distribution width (PDWc) were measured using haematology analyzer Abacus junior VET (Diatron®, Vienna, Austria).

Statistical analyses

The obtained results were statistically interpreted using statistical software SIGMA PLOT 11.0 (Jandel, Corte Madera, CA, USA), on the basis of the T-test of independent samples for difference estimation at confidence level $p < 0.05$ was used.

Results

The results of blood haematological parameters are summarized in Table 3 and Table 4.

Table 3. Haematological parameters of female rabbits

parameter	C		SD	E		SD
WBC	9.8089	±	1.7774	9.7434	±	2.5197
LYM	**5.7049[a]**	**±**	**1.2762**	**2.5177[b]**	**±**	**2.2523**
MID	**0.2452[a]**	**±**	**0.1013**	**0.6393[b]**	**±**	**0.2849**
GRA	3.8589	±	0.9096	6.5864	±	3.7962
LY%	**58.1750[a]**	**±**	**7.4848**	**28.3500[b]**	**±**	**23.1179**
MI%	**2.4500[a]**	**±**	**0.6137**	**6.3250[b]**	**±**	**1.3376**
GR%	39.4000	±	7.6503	65.3250	±	22.5212
RBC	**5.9983[a]**	**±**	**0.1315**	**5.4352[b]**	**±**	**0.1171**
HGB	**142.5507[a]**	**±**	**4.7370**	**128.6232[b]**	**±**	**8.2466**
HCT	32.3680	±	2.6960	30.5846	±	1.9723
MCV	54.0484	±	5.6491	56.2797	±	3.6115
MCH	23.7500	±	1.3178	23.6500	±	1.4480
MCHC	441.7554	±	22.2355	420.5613	±	2.7927
RDWc	19.7000	±	1.0583	19.3750	±	0.6551
PLT	201.5155	±	65.7249	235.2967	±	82.5502
PCT	0.1104	±	0.0405	0.1377	±	0.0537
MPV	5.4250	±	0.2754	5.8000	±	0.3559
PDWc	26.6500	±	1.3988	29.1250	±	1.6958

[a,b] – means in the same line with the different letters are different at the level $P < 0.05$

WBC - total white blood cell count (10^9/l); LYM - lymphocytes count (10^9/l); MID - medium-size cell count; GRA - granulocytes count (10^9/l); LYM% - lymphocyte percentage; MID% - medium-size cell percentage; GRA% - granulocytes percentage; RBC - red blood cell count (10^{12}/l); HGB - haemoglobin (g/l); HCT - haematocrit (%); MCV - mean corpuscular volume (fl); MCH - mean corpuscular haemoglobin (pg); MCHC - mean corpuscular haemoglobin concentration (g/l); RDWc - red cell distribution width (%); PLT - platelet count (10^9/l); PCT - platelet percentage; MPV - mean platelet volume (fl); PDWc - platelet distribution width (%), C – control group, E1 (0,5 %), E2 (1 %), E3 (1,5 %) – experimental groups. The values shown are the mean ± SD (standard deviation).

Table 4. Haematological parameters of male rabbits

parameter	C1		SD	E1		SD
WBC	11.6869	±	1.2517	10.4560	±	1.7655
LYM	3.4350	±	1.9408	6.1348	±	1.5424
MID	**0.7814[a]**	**±**	**0.1133**	**0.3664[b]**	**±**	**0.2203**
GRA	7.4705	±	3.0061	3.9548	±	2.5847
LY%	30.5667	±	18.3745	60.3667	±	19.2048
MI%	6.7000	±	0.6000	3.5333	±	2.0207
GR%	62.7667	±	18.6065	36.1000	±	18.2261
RBC	6.2087	±	0.4975	6.4500	±	0.3398
HGB	140.2372	±	7.9002	148.3311	±	2.2047
HCT	31.8424	±	1.7321	33.7055	±	0.9361
MCV	51.3611	±	1.6374	52.3039	±	1.4158
MCH	22.6000	±	0.5196	23.0333	±	0.8622
MCHC	440.4380	±	8.6856	440.1859	±	5.7677
RDWc	18.7000	±	1.0536	19.2667	±	0.3215
PLT	283.6453	±	21.0003	240.7194	±	62.4640
PCT	0.1595	±	0.0129	0.1436	±	0.0407
MPV	5.6333	±	0.0577	5.9333	±	0.2082
PDWc	29.7667	±	1.7010	29.9000	±	0.5196

[a,b] – means in the same line with the different letters are different at the level $P < 0.05$

WBC - total white blood cell count (10^9/l); LYM - lymphocytes count (10^9/l); MID - medium-size cell count; GRA - granulocytes count (10^9/l); LYM% - lymphocyte percentage; MID% - medium-size cell percentage; GRA% - granulocytes percentage; RBC - red blood cell count (10^{12}/l); HGB - haemoglobin (g/l); HCT - haematocrit (%); MCV - mean corpuscular volume (fl); MCH - mean corpuscular haemoglobin (pg); MCHC - mean corpuscular haemoglobin concentration (g/l); RDWc - red cell distribution width (%); PLT - platelet count (10^9/l); PCT - platelet percentage; MPV - mean platelet volume (fl); PDWc - platelet distribution width (%), C – control group, E1 (0,5 %), E2 (1 %), E3 (1,5 %) – experimental groups. The values shown are the mean ± SD (standard deviation).

The sex of a rabbits variously affected haematology and serum biochemistry variables (Çetin, et al., 2009). In our experiment female rabbits were more sensitive to patulin treatment when compared to males. In case of female, significant decreases were observed in lymphocytes

(LYM), lymphocyte percentage (LYM%), red blood cell count (RBC), haemoglobin (HGB). The values of medium size cell count (MID), medium size cell percentage (MI%), were statistically higher ($P > 0.05$) in the experimental group in comparison with the control group. In case of male, significant decrease ($P < 0.05$) was detected only of MID in E1 group in comparison with C1 group.

Minimal changes in haematological parameters in males may be caused naturally by higher level of testosterone. Selmanoglu et al. (2004) found, that patulin caused an increase (66.6%) of testosterone levels in males.

Testosterone and other androgenic steroids are known as stimulators of erythropoiesis (Shahidi and Diamond, 1961). Testosterone stimulates red blood cell formation, thus it seems that it is the reason, why we did not found any significant changes in males in RBC. On the contrary, females showed significant reduction in RBC in the experiemtnal group against the control.

Significant change of HGB ($P < 0.05$) in females was observed in experimental group (128,6232 g/l) in comparison with the control group (142,5507 g/l) in our study.

Similarly, Gbore and Akele (2010) found that concentration of haemoglobin of female rabbits significantly decreased after fumonisin administration.

Significant increase ($P < 0.05$) of MID and MI% in females was found in the experimental group in comparison with the control group.

In our previous results (Emrichova et al., 2013b), the values of MID and MI% in males rabbits showed significant increase after short term (2 weeks) application of patulin in combination with strawberry leaves. This haematological parameter was not influenced after long term (4 weeks) application of patulin in combination with strawberry leaves in our another study (Emrichova et al., 2013a). Parabathina et al. (2011) found similar results in their study with rutin and quercetin on rabbits. Authors observed increase in eosinophils after 28 days of treatment. Slight increase of MI% was found in experiment with pesticide bendiocarbamate applied to rabbits (Capcarova et al., 2010).

We observed significantly lower level of LYM and LY% ($P < 0.05$) in experimental group of males as in the control group. Patulin caused a decrease in the cell viability (Liu et al., 2003) in human embryonic kidney cell line. Patulin in the 5.0 and 7.5μM concentrations were found in human lymphocytes to cause cytostasis effect after 48 h treatment with patulin (Donmez-Altuntas et al., 2013). In another *in vivo* study, Keblys et al. (2003) found significant changes in lymphocyte proliferation in pigs. A similar reaction of patulin *in vitro* is described in the

experiments of the Escoula et al. (1988), using the lymphocytes isolated from mice and rabbits.

Conclusion

Our results demonstrated that short term exposure of patulin influenced mostly haematological parameters of female rabbits. Minimal changes in haematological parameters in males may be caused by naturally higher protective level of testosterone. Further experimental studies are needed to define the specific mechanisms of action.

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THE ROLE OF AMYGDALIN IN REGULATION OF BITTER TASTE PERCEPTION AND OTHER PHYSIOLOGICAL RESPONSES IN RABBIT

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Abstract

Amygdalin is a glycoside present in some seeds and fruits, such as apricot (*Prunus armeniaca*) seeds. It is an ingredient found in foods and a by-product of fruit processing that can be used for other purposes, namely oil extraction and animal feeding. It has also been studied over the years for its potential on pharmaceutical use, namely for the prevention of chronic diseases such as psoriasis, cardiovascular diseases, neurological disorders, certain inflammatory processes, and some types of cancers, although raising controversy, due to its benefits/toxicity effects. Furthermore, amygdalin is a bitter substance and thus likely to interfere with the taste cells via intracellular receptors.

Introduction

Characteristic and effects

Amygdalin (D-mandelonitrile-β-D-gentiobioside), $C_{20}H_{27}NO_{11}$, is composed of two molecules of glucose, one of benzaldehyde (Chang et al., 2006). Apricot (*Prunus armeniaca*) seeds are a natural source of amygdalin, a glycoside also present in bitter almonds (*Prunus dulcis*) and black cherry (*Prunus serotina*) seeds, conferring bitter taste to fruits. It is an ingredient found in foods and a by-product of fruit processing that can be used for other purposes, namely oil extraction and animal feeding. This compound has been used in traditional Eastern medicine for its health benefits, although raising controversy, due to its benefits/toxicity effects. Nevertheless, amygdalin has been studied over the years for its potential on pharmaceutical use, namely for the prevention of chronic diseases such as psoriasis, cardiovascular diseases, neurological disorders or certain inflammatory processes, including Alzheimer's, atherosclerosis and several cancers (Jiagang et al., 2011; Chen et al., 2013; Guo et al., 2013; Perez, 2013). Its relation with chronic diseases and inflammation occurs through a role in apoptosis (Chen et al., 2013), proliferation (Guo et al., 2013), angiogenesis (Mirmiranpour et al., 2012) and inflammation signaling pathways such as TNF-alpha and interleukins (Lin and Lin 2011). These interferences result also in changes in lipid

metabolism (Fon Tacer, 2007). Furthermore, amygdalin is a bitter substance and thus likely to interfere with the taste cells via intracellular receptors. Bitter taste research in farm animals, namely by studying the genes related to bitter taste perception in sheep and water buffalo is described in previous study (Ferreira et al., 2013a,b). It is interested in enlarging the focus of research to other production animals and also extends the studies to the effects of natural bitter tastants such as amygdalin on these animals. The rabbit represents a promise animal model in biomedical research and its production has been increasing especially in Europe, due to the growing rabbit meat consumption (Dalle and Szendro, 2011).

Metabolism

Beta-glucosidase, one of the enzymes that catalyses 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 vivo the enzyme complex emulsion containing the enzymes β-D-glucosidase, benzocyanase, and others, degrades the amygdalin into four components: hydrocyanic acid, benzaldehyde, prunasin, and mandelonitrile, which are absorbed into the lymph and portal circulations (Chang and Zhang, 2012). The enzymatic breakdown of amygdalin occurs most rapidly in alkaline conditions. The β-glucosidase may be deactivated in the acid environment of the stomach but can then be partially reactivated in the alkaline environment of the gut (JECFA, 1993). Cyanogenic glycosides can also be hydrolysed by gut flora. Amygdalin is metabolized by the body to produce cyanide, a very rapid poison which impairs cellular respiration leading to a cascade of events culminating in death (Ballantyne and Marrs, 1987).

Mechanism of the effect

Recent data indicated that amygdalin reduced proliferation potential, decreased mitochondrial activity of cervical cancer cells, accumulated cells in G1 phase and lead to their death (Jarocha and Majka, 2011). Amygdalin induces apoptotic cell death by caspase-3 activation through the down-regulation of anti-apoptotic Bcl-2 protein and the up-regulation of pro-apoptotic Bax protein in DU145 and LNCaP prostate cancer cells (Chang et al., 2006).

Conclusion

The contribution suggests a broader knowledge underlying the different metabolic pathways activated by exposition to amygdalin. This could support the development of new therapeutics application of this substance. Also it can point to a dosage threshold between beneficial

therapeutics and toxicity. In addition we will also infer whether apricot seeds supplementation can represent a sustainable production interest, similarly to other by-products from the food industry, such as tomato (Peiretti et al., 2013), and hence infer its economic potential.

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EFFECT OF LEPTIN GENE ON SERUM LEPTIN CONCENTRATION AND BEEF QUALITY IN CZECH FLECKVIEH BULLS

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Abstract

The aim of this work was to evaluate the effect of leptin genotype on concentration on leptin serum and beef quality parameters of Czech Fleckvieh bulls. Significant ($p < 0.05$) differences was found between leptin genotype groups CC and TT on content of intramuscular fat (1.84 – 1.77 %) and muscle pigments (3.84 – 3.45 $mg.g^{-1}$), on energy value (5840 – 5616 $kJ.kg^{-1}$) and water holding capacity (81.97 – 79.01 %). We found non-significant effect of leptin genotype on serum leptin concentration but we proved the correlation between serum leptin and muscle pigments and area of MLT.

Introduction

Leptin, a 167-amino acid hormone, is primarily secreted by white adipose tissue (Williams et al., 2002). The leptin gene located on bovine chromosome 4, encodes leptin, a peptide hormone, which is synthesized and secreted by adipose tissue (Zhang et al., 1994). Leptin is a hormone affecting the regulation of body composition, energy balance and meat quality in mammals (Tian et al., 2013). The concentration of leptin could be seen as an indicator of marbling, back fat depth and yield and quality grade in feedlot cattle (Geary et al., 2003). Markers at promoter regions of the bovine leptin gene have been shown to be associated with carcass and meat quality traits (Nkrumah et al., 2005). Sochor et al. (2005) compared the beef quality of the Czech Fleckvieh bulls with the Charolais, Meat Simmental and Blond d´Aquitane breeds. The authors did not discover any significant differences in nutritional parameters among the individual breeds. However, the differences among the breeds in the water-holding capacity and size of the MLT were significant ($p < 0.01$). Serrano et al. (2005) stated that the average percentage of proteins in beef was 20.5%. Moloney et al. (2011) fed bulls a concentrated feed ration and found that the percentage of proteins in the meat was significantly ($p < 0.05$) higher (23%) than in animals fed grass silage (19%). Zapletal et al. (2009) evaluated the amount of intramuscular fat in the meat of the Czech Fleckvieh and Monbéliarde cattle. The authors did not prove that the breed had a significant ($p < 0.05$) effect on the content of intramuscular fat. Bartoň et al. (2006) presented similar conclusions in terms of the content of intramuscular fat in meat of bulls of the Charolais, Hereford and Meat

Simmental breeds. The size of the MLT area according to Moon et al. (2006) is 73.59 ± 2.50 cm^2. Sami et al. (2004) proved a relation ($p < 0.05$) between the grade class for meatiness based on the seurop system and size of the MLT area.

The aim of this study was to evaluate associations of leptin gene on serum leptin concentration and beef quality traits in Czech Fleckvieh cattle.

Methods

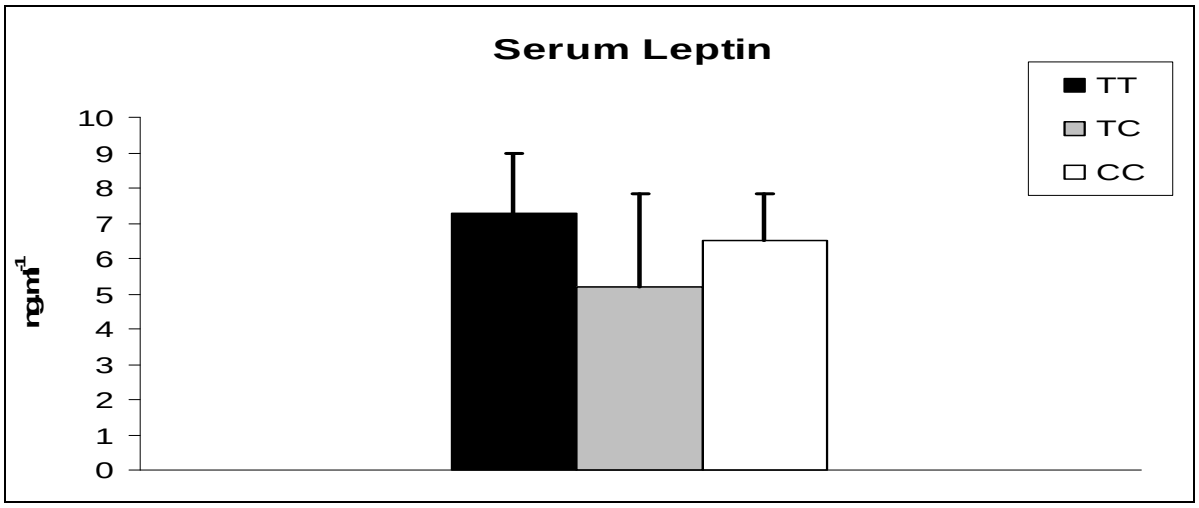
In this work, the results of 210 Czech Fleckvieh bulls were evaluation. Three experimental groups of these animals were created depending on different leptin genotypes (CC, n=83; TC, n=66 and TT, n=61). They were fed an identical feed ratio based on an ad libitum intake of maize silage and a limited amount (3 kg per bull per day) of a concentrate which consisted of 50% crushed barley and 50% of soya meal with a mineral and vitamin additive. Bulls were slaughtered at the 620 days of age. The average weight gain during fattening was 822 ± 108 g per day. The carcasses (weight ranged from 395 to 463 kg) were classified to the class of meatiness "U" and "R" and the class of fattiness "2". Blood samples of all bulls in experimental groups were collected from *vena jugularis externa* at age of bulls' average 240 days old, between 8.00 and 9.30 am. Blood was sampled into the test tube with silicone gel separator and coagulation accelerator (Dispolab, Czech Republic). Blood samples were centrifuged (10 min, 4 ºC, 2000 g) and the separated serum was stored at -20^{o}C until analysed. Blood sampling was performed randomly in bulls kept in particular groups. Leptin concentration was analyzed by ELISA using Bovine Leptin (LEP) E90084Bo kit (USCN Life Science, China). The beef samples of *musculus longissimus et thoracis (MLT)* were excised from carcass at the half cutting level between 9th and 10th rib. Sample of MLT was taken and used to assess the nutritional and technological parameters of meat quality. The laboratory analyses were based on the Czech norm CSN 570185 (1963).

Experimental data were assessed using STATISTICA software, version 10.0 (StatSoft, Inc., Tulsa, Oklahoma, USA), where the genotypic effects of leptin gene on concentration of leptin in blood and meat quality parameters was carried out using the GLM procedure. Correlations between leptin and beef parameters quality were evaluated by means of the correlation coefficient.

Results

The effect of leptin genotype on other serum parameters was non-significant found (Fig. 1). The highest concentration of serum leptin was recorded in TT group (7.27 ng.ml^{-1}), lowest in group of TC leptin genotype (5.21 ng.ml^{-1}). The mean value of plasma leptin from the present research (ranged between 5.21 and 7,27 ng.ml^{-1}) was greater than leptin concentration reported by Daix et al. (2008) in a different beef cattle breed, whereas it was comparable with values reported by Yamada et al. (2003).

Figure 1. Serum leptin concentration of bulls divided into 3 groups depending leptin genotype



The chemical analysis of the MLT (Table 1) revealed several significant differences between leptin genotypes. The TT samples had the lowest content of dry matter (26.14 ± 0.11%). A significantly ($p < 0.05$) higher level of dry matter (27.03 ± 0.27%) was found in CT genotype. Content of the total protein was from 20.87% (TT genotype) to 21.25% (CC genotype). No differences in protein content were observed by Velik et al. (2008) between Fleckvieh and Fleckvieh x Charolais. Bartoň et al. (2010) cite the same content of protein in the Czech Fleckvieh bulls (21.18 ± 1.3 %). Significant ($p < 0.05$) differences were approved between genotype CT (2.51%) and CC (1.84%), respectively CT (2.51%) and TT (1.77%) in intramuscular fat (IMF) content. The TT samples had the lowest ($p < 0.05$) content of water holding capacity (79.01 ± 0.51%) than meat from group of bulls with genotype CC (81.97 ± 1.14%). Statistically significant effect of leptin genotypes on the colour of beef was found. The TT samples had the lowest ($p < 0.05$) content of myoglobin pigments (3.45 ± 0.11 mg.g^{-1}) than meat from group of bulls with genotype CC (3.84 ± 0.14 mg.g^{-1}). A comparable result evaluation of beef colour was reported by Huuskonen et al. (2010). Li et al. (2013) indicated non-significant ($p > 0.05$) effect of leptin gene on colour of beef. The area of MLT showed

non-significant ($p > 0.05$) growing trend among categories of leptin genotypes CC < CT < TT, respectively 84.20 < 86.06 < 86.65 cm^2.

Table 1. B*eef quality in Czech Fleckvieh bulls*

Trait	Genotypes					
	CC		CT		TT	
	LSM	± SE	LSM	± SE	LSM	± SE
Dry matter (%)	26.43[a]	0.15	27.03[b]	0.27	26.14[a]	0.11
Intramuscular fat (%)	1.84[a]	0.16	2.51[b]	0.33	1.77[a]	0.13
Total protein (%)	21.25	0.14	21.20	0.15	20.87	0.15
Ash (%)	1.07	0.01	1.05	0.01	1.08	0.01
Energy value ($kJ.kg^{-1}$)	5840[a]	618	5798	592	5616[b]	507
Water holding capacity (%)	81.97[a]	1.14	81.39	0.83	79.01[b]	0.51
Pigments ($mg.g^{-1}$)	3.84[a]	0.14	3.63	0.11	3.45[b]	0.11
Area of MLT (cm^2)	84.20	15.62	86.06	14.08	86,65	15,93

Table 2. *Correlation between serum leptin concentration and beef quality*.

	IMF*	Protein	Ash	Energy value	Pigments	WHC**	Area of MLT
Leptin	0,1705	-0,1852	0,5434	0,0917	0,9017	0,0893	0,9439
	p=,715	p=,691	p=,207	p=,845	p=,006	p=,849	p=,001

* IMF = intramuscular fat; ** WHC = water holding capacity

The correlations between leptin concentration in blood plasma and quality parameters are shown in Table 2. Plasma leptin was not significantly correlated with content of intramuscular fat, protein, ash, energy value and water holding capacity. A positive ($p < 0.01$) relationship was found between leptin concentration and content of pigments ($r = 0,9017$) and area of MLT ($r = 0,9439$).

Conclusion

In this study, the effects of single nucleotide polymorphism of leptin gene were investigated. We found non-significant effect of leptin SNP on serum leptin concentration. The effect of leptin gene on beef quality was proved in Czech Fleckvieh cattle population. Bulls with TT genotype had the lowest content of intramuscular fat. Beef had lowest water holding capacity and was lighter than beef from groups of leptin gene CC. The content of the total protein, ash

and area of MLT was not affected by leptin gene. The correlation was found among plasma leptin and pigments and area of MLT.

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ACCUMULATION OF MERCURY IN BLADDER OF WOMEN AND MEN

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Abstract

Bladder cancer characteristically causes blood (redness) in the urine. Tobacco smoking is the main known contributor to urinary bladder cancer; in most populations, smoking is associated with over half of bladder cancer cases in men and one-third of cases among women. There is a linear relationship between smoking and risk, and quitting smoking reduces the risk. Passive smoking has not been proven to be involved. Healthy, unchanged histopathologically, tissues coming from the bladder (n=21 samples) and were taken during the autopsy. Cancerous tissues coming also from the bladder (n=11 samples) and were taken during surgery. The content of mercury was measured using the cold vapor atomic absorption spectrometry (CVAAS). Each sample was analyzed three times for mercury content. For men (50-90 years old) mean content of mercury in control tissues was 0,011±0,009 ppm; in tissues away from the tumor mean content of mercury was 0,095±0,066 ppm; in tissues surrounding the tumor mean content of mercury was 0,063±0,054 ppm; in tumor tissues mean content of mercury was 0,081±0,07 ppm. For women (50-90 years old) mean content of mercury in control tissues was 0,006±0,003 ppm; in tissues away from the tumor mean content of mercury was 0,223±0,015 ppm; in tissues surrounding the tumor mean content of mercury was 0,431±0,055 ppm; in tumor tissues mean content of mercury was 1,403±0,532 ppm. Studies clearly showed that the lowest mercury content have control tissues of bladder, both in men and women. Higher mercury content in cancerous tissues may indicate to accumulation of this metal in the human bladder during carcinogenesis.

Introduction

Human exposure to metals is common due to their ubiquity, wide use in industry, and environmental persistence. Historically, the heaviest metal exposures occurred in the workplace or in environmental settings in close proximity to industrial sources. Among the general population, exposure to a number of metals is widespread but generally the level of exposure is substantially lower. For this reason, epidemiologic evidence for the carcinogenicity of metals derives mainly from highly exposed occupational groups, with some studies of populations with unusual exposures. (Goyer, 1986; Ennever, 1994; Hayes, 1997).

Some experimental studies suggest mercury may be carcinogenic, but the risks have not been adequately evaluated in human populations (Ennever, 1994; IARC, 1987; IARC 1993).

Assessing the download of mercury in 1993-2002 found that it contains in the range of 2,2 to 9,2 µg/day in women, and from 2,5 to 15,0 µg/day in men (Marzec, 2003). Committee FAO/WHO defined the weekly tolerable intake of mercury by man, which should not exceed 5 µg/kg, but in Poland it amounts to about 9-33 µg (Kabata-Pendias and Pendias, 1999). Among the products that regularly consume meat of pigs has an average of 0,002 $\mu g \cdot g^{-1}$ś.m. Hg, cattle 0,003 $\mu g \cdot g^{-1}$ś.m. Hg, game animals 0,006 $\mu g \cdot g^{-1}$ś.m. Hg, chickens 0,001 $\mu g \cdot g^{-1}$ś.m. Hg, and the milk less than 0,001 $\mu g \cdot g^{-1}$ś.m. Hg (Szkoda and Żmudzki, 2003). Large animals excrete mercury slowly - for human t ½ is approximately 70 days, and for fish about 400-1000 days (Marzec, 2006). In Spain states the highest levels of mercury probably due to the high consumption of fish (almost 40 % of people eat fish more than twice a week) (Diez et al., 2009).

Material and Methods

Tissues were taken during surgery and post-mortem from Military Hospital, PROSMED Health Center and Oncology Hospital in Cracow. Permission for research was given by Local Bioethical Commission. Fragments of tissues healthy, cancerous, adjacent normal and tissues away from the tumor were taken from bladder and then frozen. Average mass of each sample hesitated from 0,5-1g. Samples were taken from 32 (n=32) patients, among them 11 were cancerous tissues (n=11 tumor tissues, n=11 tissues away from the tumor and n=11 tissues surrounding the tumor) and 21 control tissues. Mercury contents were detected using CVAAS methods (Fot. 1).

Fot. 1. Mercury Analyzer.

All results were expressed in micrograms per gram of dry mass of the tissue. Hypothesis falsification were made by U Mann-Whitney test, and Kruskal-Wallis ANOVA.

Results

In our study we determined presence of mercury content in control and cancerous tissues of human bladder. Results are shown in figure 1 and 2.

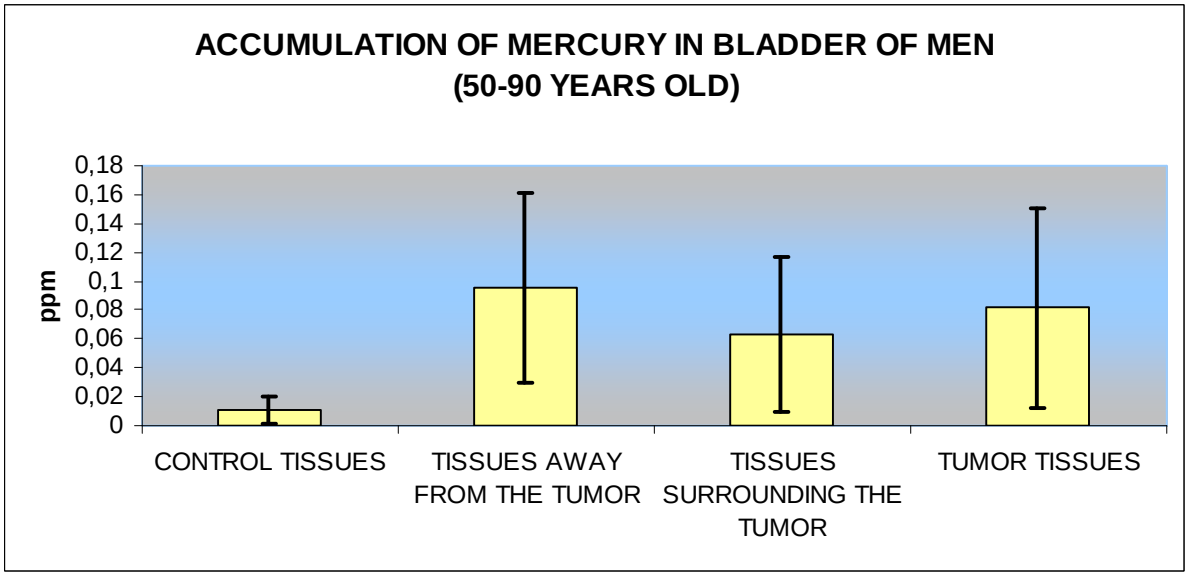

Figure 1. Accumulation of mercury in bladder of men (50-90 years old).

For men (50-90 years old) mean content of mercury in control tissues was 0,011±0,009 ppm; in tissues away from the tumor mean content of mercury was 0,095±0,066 ppm; in tissues surrounding the tumor mean content of mercury was 0,063±0,054 ppm; in tumor tissues mean content of mercury was 0,081±0,07 ppm.

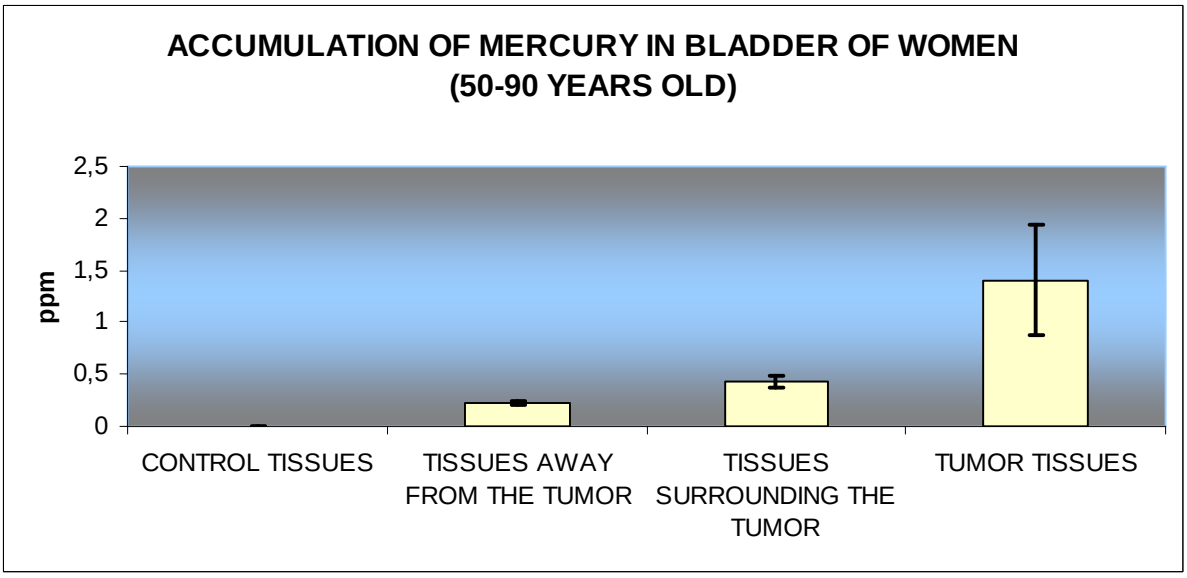

Figure 2. Accumulation of mercury in bladder of women (50-90 years old).

For women (50-90 years old) mean content of mercury in control tissues was 0,006±0,003 ppm; in tissues away from the tumor mean content of mercury was 0,223±0,015 ppm; in tissues surrounding the tumor mean content of mercury was 0,431±0,055 ppm; in tumor tissues mean content of mercury was 1,403±0,532 ppm.

Conclusion

Regarding the bladder tissues, they accumulate mercury to relatively small extent. In previous studies suggest that tumor tissues of men and women contain more mercury than control tissues (women young healthy 0,01 $\mu g \bullet g^{-1}$ś.m. Hg, women young sick 0,02 $\mu g \bullet g^{-1}$ś.m. Hg, women older healthy 0,01 $\mu g \bullet g^{-1}$ś.m. Hg, women older sick 0,02 $\mu g \bullet g^{-1}$ś.m. Hg, men young healthy 0,01 $\mu g \bullet g^{-1}$ś.m. Hg, men young sick 0,01 $\mu g \bullet g^{-1}$ś.m. Hg, men older healthy 0,01 $\mu g \bullet g^{-1}$ś.m. Hg, men older sick 0,03 $\mu g \bullet g^{-1}$ś.m. Hg) (Głogowska et al., 2012). Studies clearly showed that the lowest mercury content have control tissues of bladder, both in men and women. Higher mercury content in cancerous tissues may indicate to accumulation of this metal in the human bladder during carcinogenesis.

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THE IMPACT OF AMYGDALIN ON THE SECRETORY ACTIVITY OF PORCINE OVARIAN GRANULOSA CELLS IN THE PRESENCE OR ABSENCE OF DEOXYNIVALENOL

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Abstract

Amygdalin is a controversial anti-tumor natural product found in plants of the *Rosaceae* family that has been used as an alternative cancer drug for many years. One of the most widely distributed trichothecene contaminating food and animal feed is deoxynivalenol (DON) produced by *Fusarium* species. The aim of the present *in vitro* study was to compare effects of different natural compounds - amygdalin and deoxynivalenol and their combination at selected doses on secretory activity of porcine ovarian granulosa cells in vitro. The progesterone release by ovarian granulosa cells was significantly ($P \leq 0.05$) increased only in experimental group with the highest dose of amygdalin combined with DON, but amygdalin itself did not cause statistical differences in the release of progesterone compared to control group without addition of substances. On the other hand, the release of 17-β-estradiol by granulosa cells was influenced by amygdalin addition itself at the highest dose as well as amygdalin in combination with mycotoxin deoxynivalenol. Our findings suggest possible involvement of presented natural compounds in the regulation process of steroidogenesis and contribute to knowledge about interaction between two different natural substances.

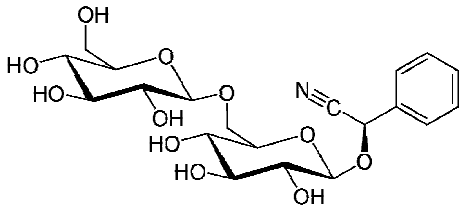
Keywords: amygdalin, deoxynivalenol, steroid hormones, ovarian granulosa cells.

Introduction

Amygdalin, commonly referred to as Vitamin B_{17} or Laetrile, is a cyanogenic glycoside which can be found in different plant species mainly in the seeds of apricots and bitter almonds. Amygdalin (D-mandelonitrile-β-D-gentiobioside, Fig. 1), $C_{20}H_{27}NO_{11}$, is composed of two molecules of glucose, one of benzaldehyde, which induces an analgesic action, and one of hydrocyanic acid, which is an anti-neoplastic compound (Ames et al., 1981; Chang et al., 2006). It has been used as a traditional drug because of its wide range of medicinal benefits, including curing or preventing cancer, relieving fever, suppressing cough, and quenching thirst. 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. From these many reasons it has been proposed to be effective in both the prevention and the treatment of cancer in humans (Ames et al., 1981; Zhou et al., 2012).However, the Food and Drug Administration (FDA) has not approved amygdalin as a cancer treatment owing to insufficient clinical evidence of its efficacy and potential toxicity. Despite the failure of clinical tests to demonstrate the anticancer effects of amygdalin in the U.S.A. and in Europe, amygdalin continues to be manufactured and administered as an anticancer therapy in northern Europe and Mexico (Chang et al.,2006; Kwon et al., 2010).

Figure 1. Chemical structure of amygdalin.



Deoxynivalenol (DON) is one of the most important and occurring *Fussarium* mycotoxin (Łazicka and Orzechowski, 2010; Klem et al., 2007). Occurrence of this mycotoxins is mainly in grains such as wheat, barley and maize (Creppy, 2002). DON could be rapidly absorbed after oral administration passively throughout the gastrointestinal tract and actively in the kidneys, liver, muscle, adipose tissue and reproductive tissues. Thus, mycotoxin exposure that alters granulosa cells steroid hormone production may also alter oocyte development, ovulation, reproductive tract function and pregnancy outcome (Medveďová et al., 2011).

The aim of the present *in vitro* study was to compare effects of different natural compounds - amygdalin and deoxynivalenol and their combination at selected doses on secretory activity of porcine ovarian granulosa cells in vitro.

Material and methods

Preparation, culture and processing of granulosa cells from ovaries

Ovaries from non-cyclic gilts were obtained from healthy Slovakian White gilts without obvious reproductive abnormalities. Isolated ovaries were transported to the laboratory in containers at 4ºC and washed in sterile physiological solution. 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^{TM}, Verviers, Belgium) and resuspended in the same medium supplemented with 10% fetal calf serum (BioWhittaker^{TM}, Verviers, Belgium) and 1% antibiotic-antimycotic solution (Sigma, St.

Louis, Mo, USA) at the final concentration of 10^6 cells/mL (as detected by haemocytometer). Portions of the cell suspension were dispensed to 24-welled culture plates (Nunc™, Roskilde, Denmark, 1ml/well; for Enzyme Linked ImmunoSorbent 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 similar 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, Sigma-Aldrich, St. Louis, Mo, USA) combined with deoxynivalenol (1000 ng/ml) (Romer Labs Division Holding GmbH, Tulln, Austria) 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 17-β-estradiol were assayed using ELISA (Dialab, Wiener Neudorf, Austria) according to the manufacturer´s instructions.

Statistical Analysis

Each experimental group was represented by four culture wells of granulosa cells. Assay of hormone level in the incubation media was performed in duplicate. Significance of differences between the control and experimental groups were evaluated by one-way ANOVA and t-test using statistical software Sigma Plot 11.0 (Jandel, Corte Madera, USA). The data are expressed as means ± SEM. Differences were compared for statistical significance at the P – level less than 0.05 ($P \leq 0.05$).

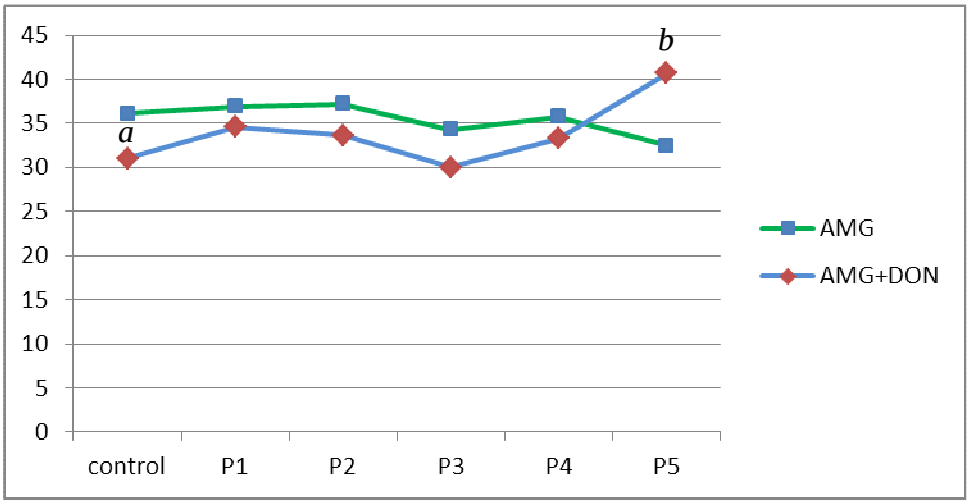
Results

Release of progesterone by granulosa cells from non-cyclic porcine ovaries.

The secretion of steroid hormone progesterone by porcine ovarian granulosa cells after addition of amygdalin alone (1, 10, 100, 1000, 10 000 µg/ml) and its combination with mycotoxin deoxynivalenol (1000 ng/ml) was determined (Fig. 2). The release of progesterone by granulosa cells was not significantly ($P \geq 0.05$) affected after amygdalin administration in any experimental group compared to control without addition of natural substance. However, amygdalin at the highest dose (10 000 µg/ml) combined with deoxynivalenol (1000 ng/ml) caused significant ($P \leq 0.05$) stimulation of the progesterone secretion by porcine ovarian granulosa cells. No significant differences in the release of the steroid hormone progesterone by ovarian granulosa cells were observed between control group without addition of both

natural compounds and experimental groups with 1, 10, 100, 1000 µg/ml of amygdalin treatment combined with 1000 ng/ml of DON.

Figure 2. The effect of amygdalin and its combination with deoxynivalenol on progesterone release by porcine ovarian granulosa cells.

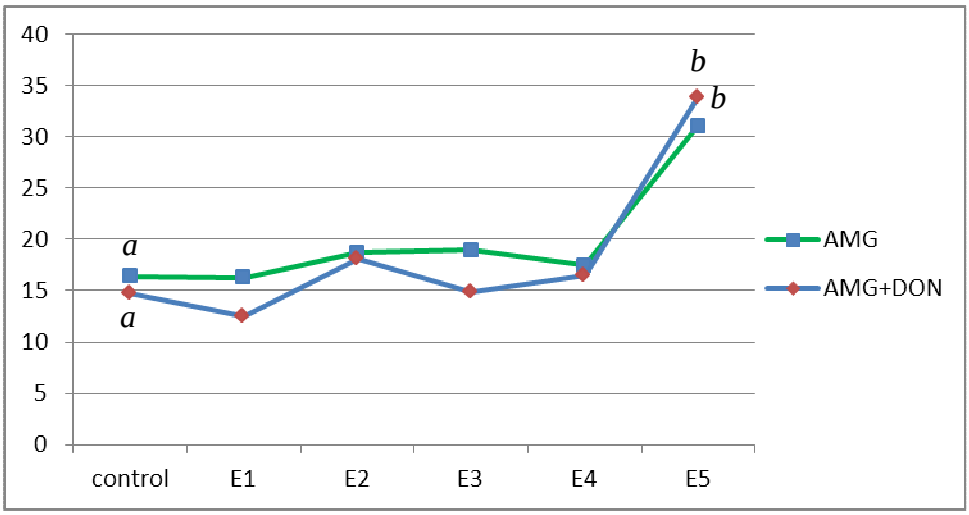


Control represents culture medium without amygdalin and deoxynivalenol addition. Experimental group P1 represents culture medium with amygdalin (1 µg/ml) plus deoxynivalenol (1000 ng/ml), other groups represent culture medium with amygdalin P2 (10 µg/ml) P3 (100 µg/ml), P4 (1000 µg/ml) and P5 (10 000 µg/ml) plusin each group deoxynivalenol (1000 ng/ml). Signs a,b denote values significantly different from control group (P≤0.05) evaluated by one-way ANOVA and t-test. ELISA. (ng/ml).

Release of 17-β-estradiol by granulosa cells from non-cyclic porcine ovaries.

The release of steroid hormone 17-β-estradiol by granulosa cells from non-cyclic porcine ovaries after addition of natural compounds amygdalin (1, 10, 100, 1000, 10 000 µg/ml) alone and in combination with deoxynivalenol (1000 ng/ml) was detected (Fig. 3). Significant (P≤0.05) increase of 17-β-estradiol releasing by porcine ovarian granulosa cells after amygdalin treatment was found in experimental group E5 with the highest dose (10 000 µg/ml) of amygdalin compared to control. We have observed similar result in case of amygdalin combined with doexynivalenol, where was found out significant (P≤0.05) stimulation of the release of 17-β-estradiol by granulosa cells at the concentration of amygdalin 10 000 µg/ml combined with DON (1000 ng/ml) compared to the control group without addition of substances.

Figure 3. The effect of amygdalin and its combination with deoxynivalenol on 17-β-estradiol release by porcine ovarian granulosa cells.



Control represents culture medium without amygdalin and deoxynivalenol addition. Experimental group E1 represents culture medium with amygdalin (1 µg/ml) plus deoxynivalenol (1000 ng/ml), other groups represent culture medium with amygdalin E2 (10 µg/ml), E3 (100 µg/ml), E4 (1000 µg/ml) and E5 (10 000 µg/ml) plus in each group deoxynivalenol (1000 ng/ml). Signs a,b denote values significantly different from control group (P≤0.05) evaluated by one-way ANOVA and t-test. ELISA. (pg/ml).

Discusion

In the present study, possible response of porcine ovarian granulosa cells to amygdalin addition and its combination with mycotoxin deoxynivalenol (DON) was examined. Granulosa cells isolated from ovaries were able to survive, growth in culture medium and release steroid hormones after experimental addition of natural compound amygdalin and mycotoxin deoxynivalenol. The results from our observation demonstrate that the release of both steroid products from granulosa cells was influenced by natural compound amygdalin combined with trichothecene deoxynivalenol.

Natural plant origin products like amygdalin are still a major part of traditional medicine (Nabavizadeh et al., 2011). 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 (Zhou et al., 2012). Previous studies examined the effects of natural compounds on different parts of animal reproductive system (Kolesárová et al., 2012; Tanyildizi and Bozkurt, 2004; Yasui et al., 2003; Randel et al., 1992). Our recent *in vitro* investigation showed that the release of steroid hormone progesterone by granulosa cells from cyclic and non-cyclic porcine ovaries was not affected by the amygdalin addition (1, 10, 100, 1000, 10 000 µg/mL) (Halenár et al., 2013) The results from this *in vitro* study showed that amygdalin combined with deoxynivalenol caused significant (P≤0.05) dose-dependent stimulation of steroid hormone secretion by granulosa cells from non-cyclic porcine ovaries.

Many studies have described the dose-dependent effects of different mycotoxins on the secretion activity of porcine (Medveďová et al., 2011, Maruniaková et al., 2013, Ranzenigo et al., 2008) and rats ovarian cells (Kolesárová et al., 2011). Steroid secretion by porcine ovarian granulosa cells after deoxynivalenol addition was examined by Medveďová et al. (2011). The release of progesterone by porcine ovarian granulosa cells was stimulated by DON addition at the doses 1000 ng/mL but not at 10 and 100 ng/mL.

There are several evidence suggesting that addition of some natural compounds can affected the secretion of steroid hormones by ovarian granulosa cells *in vitro*.

Conclusion

This examination was focused on the potential effects of reportedly anti-tumor natural substance amygdalin combined with mycotoxin deoxynivalenol on secretion of steroid hormones by granulosa cells from porcine non-cyclic ovaries. Amygdalin treatment combined with DON caused increase of steroid hormones release by ovarian granulosa cells. Our findings suggest possible involvement of presented natural compounds in the regulation process of steroidogenesis and contribute to knowledge about interaction between two different natural substances.

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THE EFFECTS OF BOVINE SERUM ALBUMIN ON MOTILITY PARAMETERS OF BULL SPERMATOZOA

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Abstract

The aim of this study was to investigate the effects of Synthetic Bovine Serum Albumins (BSA) on spermatozoa. We used bovine semen from 5 adult breeding bulls (Slovak Biological Services, Nitra, Slovak Republic). The semen samples were evaluated using Computer-Assisted Sperm Analysis (CASA). Spermatozoa motility differences between the control groups and BSA was significant ($P<0.01$) only at 0 h at the room temperature. However increased parameters of both motility and progressive motility were observed at hour 24 with significant differences ($P<0.001$) at thermostat temperature.

Introduction

Artificial insemination (AI) is one of the most widespread biotechnological methods of animal reproduction through which rapid genetic improvement in livestock have been achieved. One of the most important achievements in dairy farming after the introduction of artificial insemination is the cryopreservation of bull semen, which has enabled the worldwide distribution and use of desired genetic lines at a reasonable cost (Polge *et al*. 1949; Smith *et al*. 1950; Manjunath *et al*. 2002).

Production-tested sires with high fertilizing capacity are essential in order to assure optimal reproductive efficiency for the livestock and artificial insemination industry. To assess semen quality evaluation of sperm concentration and motility has been used, however provides limited information about the potential fertility of sires (Elliot, 1978; Correa *et al*., 1997; Rodriguez-Martinez and Larsson, 1998; Zhang *et al*., 1998; Brahmkshtri *et al*., 1999). Another criteria for estimation of semen quality is computerized analysis of motility and acrosome integrity which have been related to non-return rates of bulls, but correlations are not high or even consistent (Kjaestad *et al*., 1993; Farrell *et al*., 1998; Januskauskas *et al*., 2000a,b; Arlindo, 2005).

This study is focusing on substances that are used to protect and improve the quality of Insemination. The main scientific objective of this work was the determination of the parameters of sperm motility of prepared semen samples by using the BSA (Synthetic Bovine Serum Albumins).

Materials and Methods

Bovine semen samples were obtained from 5 adult breeding bulls (Slovak Biological Services, Nitra, Slovak Republic). The samples had to accomplish the basic criteria given for the corresponding breed. The semen was obtained on a regular collection schedule using an artificial vagina. After collecting the samples were stored in the laboratory at room temperature (22-25°C). Each sample was diluted in physiological saline solution (PS) (sodium chloride 0.9% w/v, Bieffe Medital, Italia), using a dilution ratio of 1:40, depending on the original spermatozoa concentration.

Obtained data were statistically analyzed using PC program GraphPad Prism 3.02 (GraphPad Software Incorporated, San Diego, California, USA). Descriptive statistical characteristics (mean, minimum, maximum, standard deviation and coefficient of variation) were evaluated. T-test and Wilcoxon matched pairs test were used for statistical evaluations. The level of significance was set at *** (P<0.001); ** (P<0.01) and * (P<0.05).

Table 1. Composition of diluents

BSA (synthetic Bovine Serum Albumins)
4.2 mL Triladyl
3 mL BSA (20%)
2 mL Fructose
3 mL Trehalose
20 mL Redestilated water

Computer-Assisted Sperm Analysis (CASA)

The motility analysis was carried out using a CASA system – SpermVision^{TM} program (MiniTűb, Tiefenbach, Germany) with the Olympus BX 51 microscope (Olympus, Japan) at cultivation times 0 h, 2 h, 4 h, 6 h and 24 h at room temperature (RT), thermostat (T) according to the methods described by Massanyi et al., 2009 and Lukac et al., 2011. Each sample was placed into the Makler Counting Chamber (deph 10 µm, Sefi-Medical Instruments, Izrael) and the following parameters evaluated: the percentage of motile spermatozoa (motility > 5 µm/s; MOT), the percentage of progressively motile spermatozoa (motility > 20 µm/s; PROG). This study was performed in five replicates at each concentration (n = 5). At least 1000 spermatozoa were analyzed in each sample. All results were compared with control samples among experiments samples for statistical significance.

Results and Discussions

We mainly discuss the evaluation of percentage of motile and progressively motile spermatozoa with BSA (composition stated in Table 1) at the room and thermostat temperatures (Table 2). Spermatozoa motility was significant ($P<0.01$) at 0 h at room temperature and was the highest (76.91%) in compare to control value (71.22%) at hour 2. The progressive motility difference was significantly higher ($P<0.01$) at the 2nd hour as well. The motility and progressive motility were significant ($P<0.01$and $P<0.05$, respectively) at hour 4 and the highest significant (55.01%, 46.74%; $P<0.001$, $P<0.001$ respectively) at hour 24, where as the control value shows motility (21.29%) and progressive motility (7.974%) at the same hour. Eventually the highest stimulating effects of BSA on the spermatozoa motility and progressive motility were observed after 24 hours at thermostat temperature.

In the aerobic incubation reactive oxygen species causes the sperm plasma membrane, which is rich in polyunsaturated fatty acids and is susceptible to peroxidative damage, loss membrane integrity, decreased sperm motility, and eventually loss in fertility (Alvarez *et al.*, 1987). Ashrafi et al. suggested that the protection effects of BSA on spermatozoa are associated with an increment in antioxidant enzymes activity, total antioxidant capacity, total thiols (Ashrafi et al., 2013).

The present study shows the effectiveness of BSA in maintaining and improving the motility and progressive motility of spermatozoa from hour 0 to hour 24 of incubation. Thus, we may suggest that BSA is effective in preventing the rapid loss of motility that normally occurs during incubation of spermatozoa. Similar observations had been made on stallion (Kreider *et al.;* 1985, Klem *et al.;* 1986), rabbit (Sariozkan *et al.;* 2013) and Mahabadi goat (Naijian *et al.;* 2013).

It can be concluded that the 3 ml BSA along with other components mentioned in Table 1 protects and improving the percentage of motile and progressively spermatozoa at the thermostat temperature and seems to maintain them at room temperature according to control value.

Table 2. The percentage of motile and progressively motile spermatozoa

Parameter	MOT RT		PROG RT		MOT T		PROG T	
	Ctrl	BSA	Ctrl	BSA	Ctrl	BSA	Ctrl	BSA
0 h								
x	78.28	70.83**	70.82	68.87	79.06	79.48	73.73	77.49
S.D.	14.00	7.273	15.67	7.225	13.27	12.02	14.71	12.20
2 h								
x	71.22	76.91	64.42	71.49**	74.00	75.85	72.31	72.84
S.D.	11.72	7.252	13.41	9.097	16.11	11.57	15.19	11.47
4 h								
x	73.78	71.97	67.53	66.66	76.18	71.29**	70.12	66.71*
S.D.	10.48	10.65	11.76	11.02	14.26	11.19	14.34	10.99
6 h								
x	75.94	72.10	68.29	67.16	82.24	78.55**	74.73	73.87
S.D.	7.435	9.588	7.749	9.520	4.810	7.236	6.970	7.501
24 h								
x	65.32	64.35	54.21	54.05	21.29	55.01***	7.974	46.74***
S.D.	11.84	14.61	14.55	16.04	10.48	16.14	9.510	17.09

*** (P<0.001); ** (P<0.01) and * (P<0.05).

BSA: Synthetic Bovine Serum Albumins

Ctrl: Control samples

MOT: Motility (motility > 5 µm/s) -%

PROG: Progressive motility (motility > 20 µm/s) -%

RT: Room temperature

S.D.: Standard Deviation

T: Thermostat temperature

x: Mean

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RESISTANCE OF *ESCHERICHIA COLI* ISOLATED FROM PHEASANTS (*PHASIANUS COLCHICUS*) IN THE REGION OF EASTERN MORAVIA AND SLOVAKIA

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Abstract

This article investigates antibiotic resistance of 180 strains of *Escherichia coli* isolated from wild pheasants in the region of Eastern Moravia and Slovakia. Research evaluating 180 strains of *E. coli* showed 130 strains resistant to ampicillin (72.22%), 160 strains resistant to cephalothin (88.89%), and 10 strains resistant to chloramphenicol as well as sulfamethoxazole-trimethoprim (5.56%). Forty strains were found to be resistant to Tetracycline (22.22%). In case of antibiotics such as cefoperazone-sulbactam, ciprofloxacin, colistin, gentamicin and piperacillin-tazobactam, all strains as sensitive.

Laboratory tests clearly illustrate the increasing resistance of *Escherichia coli* bacterial strains to ampicillin, cephalothin and tetracycline, while many Czech and foreign microbiological studies have been calling attention to this problem as well.

Introduction

Wild birds may serve as a significant reservoir of pathogenic bacteria. In the intestinal tract, these microorganisms multiply and spread to humans by various means. Synanthropic birds are mainly important in this respect because they occupy environment modified by humans, however, hunted game birds are important as well (Walsh, 2003). The most common bacterial human pathogens of particular importance settling in the digestive tract of synanthropic birds are *Salmonella*, *Escherichia*, *Campylobacter*, *Listeria* and *Chlamydia*. (Craven et al., 2000) Synanthropic birds can also represent a source of parasites and viral diseases such as avian influenza (Alexander, 2000).

One of the main reasons for the resistance of microorganisms is unreasonable use of antibiotics in human and veterinary medicine.

The most important mechanism of bacterial resistance leading to inhibition of bactericidal activity of beta-lactam antibiotics (penicillins, cephalosporins, monobactams and carbapenems) currently includes the ß-lactamases. Resistance to β-lactam antibiotics in the *Enterobacteriaceae* family is becoming an increasingly important issue. The most common

mechanism of its formation is the production of the aforementioned β-lactamases. In addition to the original β-lactamases, which occurred in Gram-negative microbes in the early '60s, an increasing number of enzymes with broad spectrum of activity (ESBL) emerge (Jacoby and Medeiros, 1991).

In the last decade, the clinical veterinary medicine experienced an introduction of quinolones, which market penetration was marked by introduction of enrofloxacin, the first original veterinary fluorinated quinolone for the Small Animal Practice used in 1992 in the Czech Republic. Although *Escherichia coli* is sensitive to fluoroquinolones, there are strains with reduced sensitivity as well as resistant strains (Ozeki, 1997).

Monitoring the prevalence of resistance in indicator bacteria such as faecal Escherichia coli in different populations, animals, patients and healthy humans, makes it feasible to compare the prevalence of resistance and to detect transfer of resistant bacteria or resistance genes from animals to humans and vice versa (van den Bogaard and Stobberingh, 2000).

The aim of our research was to determine antibiotic resistance of 180 examined strains of *Escherichia coli* isolated from rectal swabs of wild pheasants in Eastern Moravia and Western Slovakia between 2010 and 2013. Study results were compared with the current state of antibiotic resistance in the Czech Republic and in the world according to available data in the literature.

Materials and methods

Investigated strains of *Escherichia coli* (180 strains) were collected from wild pheasants occupying the region of Eastern Moravia (100 strains) and Western Slovakia (80 strains).

The strains were cultured from rectal transport swabs on MacConkey agar and the identification was confirmed using a Triple sugar agar (Imuna, S. Michalany, Slovakia) and EnteroTest 24 (Pliva-Lachema, Brno, Czech Republic).

In vitro susceptibility of the isolates against antimicrobial agents was determined by the standard disk diffusion procedure (EUCAST). The following antibiotic discs (Oxoid Ltd., Besingstoke, Hampshire, GB) were used: amoxicilin – clavulanic acid 2:1, 30 µg, ampicilin, 10 µg, cephalothin, 30 µg, ciprofloxacin, 5 µg, colistin sulphate, 10 µg, gentamicin, 10 µg, chloramphenicol, 30 µg, neomycin, 30 µg, piperacillin – tazobactam 10:1, 110 µg, streptomycin 10 µg, sulbactam – cefoperazon, 105 µg, sulphamethoxazole – trimethoprim, 25 µg, tetracycline, 10 µg.

Results and discussion

The study yielded the following results (Table 1): From the 180 examined strains of *E. coli*, 130 strains were ampicillin-resistant (72.22%) and the remaining 50 strains were ampicillin-intermediate. Out of all examined strains, 160 strains (88.89%) showed resistance to cephalothin, the other 20 strains were ampicillin-intermediate. Ten *E. coli* strains were resistant to chloramphenicol and trimethoprim-sulfamethoxazole (5.556%) and the rest of strains were sensitive to these antibiotics. Intermediate sensitivity to amoxicillin-clavulanic acid was determined in 10 strains; all other strains were found to be sensitive. In case of the antibiotics neomycin and streptomycin, 40 strains were identified as intermediately sensitive; all other strains were identified as sensitive. For cefoperazone-sulbactam, ciprofloxacin, colistin, gentamicin and piperacillin-tazobactam, all strains were sensitive. Forty strains (22.22% of the strains) were determined to be resistant, while 50 strains were intermediately sensitive and 90 strains were sensitive.

The research confirmed the seriousness of increasing resistance of *Escherichia coli* bacterial strains to ampicillin (72.22% resistant strains) and cephalothin (88.89% resistant strains). The study has also demonstrated considerable resistance to tetracycline (22.22% of resistant strains). There were 10 bacterial strains of *E. coli* which were found to be resistant to chloramphenicol and sulfamethoxazole-trimethoprim (5.556%) at the same time, while other strains were sensitive to these antibiotics. No intermediate or resistant strains of *E. coli* were found for cefoperazone-sulbactam, ciprofloxacin, colistin, gentamicin and piperacillin-tazobactam antibiotics.

This study showed that resistant strains of *Escherichia coli* can also be found in wild animals (such as pheasants). It can be assumed that the presence of these strains is given by the increasing resistance of bacteria to antimicrobial drugs worldwide. The resistant bacteria get into the gastrointestinal tract of animals via food and proliferate there. Faeces can consequently contaminate surface water or infect humans. It is likely that similar reason accounts for the presence of resistant *E. coli* strains that can be observed in the investigated pheasants.

In the Czech Republic, the situation regarding antibiotic resistance is quite serious. For example, between the years 2001-2005, there was a twofold rise of antibiotic resistance of *E. coli* to fluoroquinolones, ciprofloxacin resistance increased from 8% to more than 20% and the upward trend continues (Nyc et al., 2011). In case of ciprofloxacin antibiotics, as well as cefoperazone, colistin, gentamicin and piperacillin-tazobactam, our research showed no intermediate or resistant strains.

Our research results of *Escherichia coli* investigation concur to some extent with the research of *E. coli* fluoroquinolone-resistant strains which was carried out in 2003 - 2005 on five poultry farms and two calf farms in Central Moravia. Total of 239 strains of *E. coli* were isolated on poultry farms and the frequency of fluoroquinolone-resistant strains (resistant to ciprofloxacin and ofloxacin) amounted to 3% and the frequency of resistant strains to tetracycline and ampicillin was 48% and 29% respectively (Kolar et al., 2005). If we compare the results with our laboratory investigation, a comparative agreement in case of ciprofloxacin (no resistant strains) and tetracycline (22.22% resistant strains) can be stated. However, in case of ampicillin, much higher resistance (72.22% of the strains) has been observed.

The results of our research with ampicillin also partially correspond with other prior study of Kolar et al. (2002) analysing the incidence of bacterial strains resistant to antibiotics in poultry in the Czech Republic between 1999 and 2000. There were 51% of *Escherichia coli* strains resistant to ampicillin, 31% were piperacillin-resistant and even 97% were tetracycline-resistant. In 10% of examined strains, an increasing resistance to ciprofloxacin and ofloxacin has been observed as well.

Interesting results for comparison were provided by a study carried out in Slovakia, which examined the dynamics of microbial resistance to quinolone antibiotics (nalidixic acid, ciprofloxacin and enrofloxacin) of commensal *Escherichia coli* isolated from healthy broiler chickens from several different farms. Isolates exhibited high resistance to nalidixic acid and ciprofloxacin, enrofloxacin. The study showed that resistance to quinolone antibiotics (ciprofloxacin and enrofloxacin) is Slovakia in animal *E. coli* isolated from broilers is very high. The resistance levels were approximately 80% for ciprofloxacin and 50% for enrofloxacin (Kmet et al., 2007). Our laboratory investigation results mostly correspond to the study results from 2008 of strains resistant to ciprofloxacin (with a high degree of quinolone resistance), which were also resistant to other antibiotics, such as ampicillin (64%), tetracycline (39%), streptomycin (60%), neomycin (14%), gentamicin (14%) and chloramphenicol (7%). However, these strains were also sensitive to ceftazidime-clavulanic acid or ampicillin-sulbactam (Kmet and Kmetova, 2010). This largely corresponds to values obtained in the laboratory examination of *E. coli* strains, in which the ampicillin resistance was 72.22%, tetracycline resistance was 22.22% and chloramphenicol resistance was 5.556%, however, there were no strains found which were resistant to streptomycin, neomycin and gentamycin. Similarly, in terms of amoxicillin-clavulanic acid and cefoperazone-sulbactam, no resistant strains were found.

Conclusion

Results of our research show that *Escherichia coli* from wild birds also follows well the trend of increasing microbial resistance to antibiotics. Of the 180 examined *E. coli* strains, most were resistant to cephalothin and ampicillin (88.89% and 72.22% respectively), other strains were intermediately sensitive and no strains was sensitive to these antibiotics. Moreover, the resistance of 40 strains to tetracycline (22.22% of strains) is not negligible. The obtained results are certainly not accidental given the serious situation of antibiotic resistance in the Czech Republic and Slovakia.

Table 1. *Antibiotic resistance of Escherichia coli – numbers of strains*

Antibiotic (μg / disc)	R	S	I
Amoxycilin – clavul. 2:1 (30)	0	170	10
Ampicilin (10)	130	0	50
Cephalothin (30)	160	0	20
Cefoperazon – sulbact. (10.5)	0	180	0
Ciprofloxacin (5)	0	180	0
Colistin (10)	0	180	0
Gentamicin (10)	0	180	0
Chloramphenicol (30)	10	170	0
Neomycin (30)	0	150	30
Piperacillin – tazobactam 10:1 (110)	0	180	0
Streptomycin (10)	0	160	20
Sulfamethoxazole trimethoprim (25)	10	170	0
Tetracycline (10)	40	90	50

(R – resistant, S – sensitive, I – intermediate)

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THE EFFECT OF COMMENCEMENT OF FEED RESTRICTION ON BIOCHEMICAL BLOOD PARAMETERS IN BROILER RABBITS

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Abstract

The objective of this study was to examine the effect of starter period of the one week intensive feed restriction on biochemical blood parameters in growing rabbits Hyplus. Rabbits after weaning (at 35 days of age) were divided into three groups: ADL - *ad libitum fed*, R50 – rabbits restricted on 50 g of feed per rabbit per day and R65 – rabbits restricted on 65 g of feed per rabbit per day. Feed restriction was applied from 42 to 49 days of age in the first experiment and from 35 to 42 days of age in the second experiment. Before and following feed restriction, rabbits were fed *ad libitum*. The significant interaction between group and age ($P \leq 0.007$) was detected in cholesterol in experiment 1. The highest cholesterol concentration had R50 group at 56 days of age, while the lowest ADL at 63 days of age. In experiment 2 interactions was observed in glucose ($P \leq 0.002$); the highest at 42 days of age (9,65 mmol/l), the lowest at 70 days of age (6,53 mmol/l), both in ADL, in NEFA ($P \leq 0.022$) with the highest values in R65 at 42 days of age (0,78 g/l), and the lowest in R50 at 49 days of age (0,45 g/l) and in TAG ($P \leq 0.046$) with the highest values in ADL at 70 days of age (1,37 mmol/l) and the lowest in R50 at 42 days of age (0,55 mmol/l). The effect of feeding regime was determined in NEFA, cholesterol and TAG in rabbits with restriction which started a week after weaning.

Keywords: rabbit, feed restriction, biochemical parameters, blood

Introduction

Feed restriction is applied in rabbit females to avoid excessive fattening, reproduction troubles and supports reproductive results (Rommers et al., 2004). Limited feed intake in growing rabbits enhances compensatory growth and improves feed efficiency (Tůmová et al., 2003; Di Meo et al., 2007; Gidenne et al., 2012), reduces fat in carcass (Gondret et al., 2000; Tůmová et al., 2003; 2007) and it can be used as a prevention of rabbit digestive disorders (Boisot et al., 2003; Di Meo et al., 2007). Biochemical blood parameters are particularly important as characteristics of the metabolism. The basic rabbit blood biochemical characteristics include total proteins, glucose, non-esterified fatty acids (NEFA), cholesterol, urea and

triacylglycerides (TAG). The main function of the proteins is to maintain the osmotic pressure, transportation of substances throughout the body, immune response and regulation of enzymes (Kaneko et al., 1997). Glucose is the energy source (Kaneko et al., 1997). The amount of NEFA released into blood can be used as an indicator of body reserves mobilization (Fortun-Lamothe, 2006). Cholesterol is an important structural component of the cell plasma dynamics and it is required for normal cell function (Króliczewska et al., 2011). Urea is a chemical denaturant which is widely exploited for investigating the conformational stability of proteins (Khan et al. 2013). High urea concentration in rabbit blood is associated with kidney diseases (Archetti et al., 2008). TAGs are the most abundant lipids in the body. They are stored in adipocytes which are used as a reserve of chemical energy for tissues.

A variety of factors can affect the biochemical parameters in animals, including the breed, sex, age and seasonal variations (Wells et al., 1999; Çetin et al., 2009). There is lack of information about the effect of feed restriction on biochemical characteristics in rabbits.

Rommers et al. (2004) who examined the effect of feed restriction in rabbit does observed similar NEFA and plasma urea nitrogen levels in *ad libitum* and restricted rabbits during the time of feed restriction. In following period, they found higher concentration of NEFA and TAG in *ad libitum fed* rabbits. Tůmová et al. (2011) in our previous experiment detected significant differences in rabbits with various feeding technique. Restricted groups had lower concentration of NEFA, urea and TAG, and higher serum cholesterol.

The aim of this study was to evaluate the effect of different starter period of one week intensive feed restriction on blood biochemistry in the growing rabbits.

Material and methods

Two experiments with different commencement time of feed restriction were performed. A total of 192 rabbits in the first experiment and 81 rabbits in the second experiment were used. Rabbits from both experiments were weaned at 35 days of age and divided into three groups: ADL (*ad libitum* fed rabbits), R50 (rabbits with feed restriction on 50 g per rabbit per day) and R65 (rabbits with feed restriction on 65 g per rabbit per day). In the first experiment, feed restriction was applied from 42nd to 49th days of age. In the second experiment, restricted feed intake was realized from 35th to 42nd days of age. Rabbits were fed *ad libitum* before and following restriction period. The commercial pelleted diet with 90.1% dry matter, 17.1% crude protein, 20.7% crude fibre and 2.8% fat was used. Water was available *ad libitum* throughout the experiments. Animals were kept in microclimate suitable for growing rabbits.

Blood samples were collected at the age of 49, 56, 63 and 70 days for experiment 1 and at 42, 49 and 70 days of age in experiment 2. Samples were taken from eight rabbits per each group and experiment, i.e. a total of 96 samples from the experiment 1 and 72 samples from the experiment 2. Blood serum was separated by centrifugation (2700 g/10 minutes), thereafter it was frozen at –70°C until assay. Biochemical parameters (total protein, glucose, non-esterified fatty acids, cholesterol, urea and triacylglycerides) were determined using commercial sets Randox (Randox Laboratories Ltd., UK) and spectrophotometer Libra S22 (Biochrom Ltd., UK). The values of blood biochemical parameters were determined by counting from the absorbances.

Data were processed by two-way analysis of variance ANOVA (interaction between group and age) using GLM procedure of SAS (SAS Institute Inc, 2003). The significance of differences between groups was tested by the Duncan test. P-value $P \leq 0.05$ was considered significant for all measurements.

Results and discussion

Results of the effect of feed restriction applied one week after weaning (from 42 to 49 days of age) are shown in Table 1. The significant interaction between group and age ($P \leq 0.007$) was detected in cholesterol content; the highest concentration of cholesterol was measured in the R50 group at 56 days of age (4.41 mmol/l), while the lowest in the ADL at 63 days of age (1.30 mmol/l).

Age of rabbits significantly affected ($P \leq 0.001$) concentration of total proteins with the lowest values at 63 days and the highest at 70 days of age. However, Archetti et al. (2008) obtained higher values of total protein in older rabbits than in the younger ones (48 vs. 42 g/l).

Concentration of glucose was not influenced by any of monitored factors which is in agreement with Yamada et al. (2004) who showed that concentration of glucose in rabbits did not differ with increasing age. On the other hand, Rommers et al. (2004) observed lower concentration of glucose in restricted rabbits than in the *ad libitum* fed ones.

Concentration of NEFA was significantly affected by feeding regime ($P \leq 0.003$) with lower values in restricted groups, and by age ($P \leq 0.001$). NEFA concentration in restricted rabbits decreased with age, which corresponds with the results of Rommers et al. (2004) and Van Harten and Cardoso (2010).

Triacylglyceride (TAG) concentration was significantly affected by feeding regime ($P \leq 0.048$) with higher values in ADL. The results correspond with Rommers et al. (2004) and Van Harten and Cardoso (2010) who obtained the effect of feed restriction on concentration of

TAG in rabbits. There was also the significant effect of age (P≤0.001) on TAG concentration. Concentration of TAG increased with rising age.

Table 1. The effect of feed restriction applied from 42 to 49 days of age on biochemical blood parameters

Group	Age	Total protein (g/l)	Glucose (mmol/l)	NEFA (g/l)	Cholesterol (mmol/l)	Urea (mmol/l)	TAG (mmol/l)
ADL	49	50.95	7.95	0.61	2.37^{c}	7.88	0.91
	56	55.25	7.93	0.44	3.69^{ab}	7.59	0.60
	63	47.51	7.29	0.55	1.30^{d}	6.03	1.58
	70	54.71	8.71	0.45	1.43^{d}	7.79	1.34
R50	49	44.46	7.63	0.54	3.31^{b}	6.77	0.46
	56	50.23	7.79	0.39	4.41^{a}	7.55	0.48
	63	44.88	8.03	0.35	1.55^{d}	6.59	1.23
	70	59.42	7.09	0.39	1.95^{cd}	7.04	1.27
R65	49	49.09	6.71	0.45	4.24^{a}	8.09	0.56
	56	48.74	7.24	0.48	4.22^{a}	7.43	0.64
	63	46.58	8.35	0.37	1.56^{d}	6.90	1.46
	70	50.70	8.55	0.28	1.31^{d}	7.46	1.07
RMSE		7.05	1.36	0.14	0.73	1.19	0.41
Significance							
Group		0.158	0.582	0.003	<0.001	0.252	0.048
Age		<0.001	0.337	0.001	<0.001	0.007	<0.001
Group×age		0.127	0.053	0.129	0.007	0.352	0.591

RMSE: root mean square error
^{a,b,c,d}P≤0.05
NEFA – non-esterified fatty acids, TAG - triacylglycerides

Results of the limited feed intake applied immediately after weaning (from 35 to 42 days of age) are shown in Table 2. Contrary to the results of the first experiment, significant interactions between group and age were observed in concentration of glucose, NEFA and TAG. The highest (P≤0.002) glucose concentration was detected in *ad libitum* fed rabbits at 42 days of age (9.65 mmol/l), while the lowest in the same group at 70 days of age (6.53 mmol/l). The significantly highest (P≤0.022) concentration of NEFA was measured in rabbits restricted on 65 g per rabbit per day immediately after the end of feed restriction (0.78 g/l), while the lowest content was found in R50 group at 49 days of age (0.45 g/l). The significantly (P≤0.046) highest serum TAG were measured in ADL rabbits at 70 days of age (1.37 mmol/l), whereas the lowest concentration of TAG was detected in R50 group immediately after period of feed restriction (0.55 mmol/l).

The significant effect of feeding technique was not observed in any biochemical characteristics. Rommers et al. (2004) and Van Harten and Cardoso (2010) detected lower NEFA and TAG in restricted rabbits.

Table 2. The effect of feed restriction applied from 35 to 42 days of age on biochemical blood parameters

Group	Age	Total protein (g/l)	Glucose (mmol/l)	NEFA (g/l)	Cholesterol (mmol/l)	Urea (mmol/l)	TAG (mmol/l)

ADL	42	45.89	9.65^{a}	0.46^{b}	2.17	5.69	1.22^{ab}
	49	47.41	8.92^{ab}	0.55^{b}	2.21	6.11	0.93^{bcd}
	70	55.04	6.53^{d}	0.49^{b}	1.48	8.68	1.37^{a}
R50	42	45.64	8.08^{bc}	0.60^{ab}	1.97	5.32	0.55^{d}
	49	47.04	8.79^{ab}	0.45^{b}	2.28	6.03	1.15^{ab}
	70	50.82	7.93^{bc}	0.54^{b}	1.71	9.75	1.22^{ab}
R65	42	45.06	8.35^{bc}	0.78^{a}	2.05	6.26	0.68^{cd}
	49	47.65	7.31^{cd}	0.50^{b}	2.09	6.31	0.96^{bc}
	70	51.47	7.38^{cd}	0.46^{b}	1.42	9.30	1.06^{abc}
RMSE		2.75	1.13	0.18	0.44	1.39	0.40
Significance							
Group		0.098	0.084	0.340	0.547	0.518	0.054
Age		<0.001	<0.001	0.037	<0.001	<0.001	0.004
Group×age		0.168	0.002	0.022	0.676	0.544	0.046

RMSE: root mean square error
$^{a,b,c,d}P \leq 0.05$
NEFA – non-esterified fatty acids, TAG - triacylglycerides

Serum cholesterol and urea were significantly affected ($P \leq 0.001$) only by age. However, in chickens Rajman et al. (2006) observed that limited feed intake reduced concentration of cholesterol.

Conclusion

Results of the experiments show that the commencement of feed restriction in the growing rabbits affects biochemical measurements. The significant interactions between group and age were detected in the cholesterol concentration in rabbits with limited feed intake which started a week after weaning whereas in rabbits restricted immediately after weaning, interactions were observed in glucose, NEFA and TAG. In the first case, feeding technique had significant effect on fat metabolism (i.e. concentration of cholesterol and TAG), which indicated a decrease in fat accumulation in restricted groups in compare to *ad libitum* fed rabbits. On the other hand, feed restriction applied from 35 days of age not only affected fat metabolism but also energetic metabolism.

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EFFECT OF CADMIUM ON THE PROGESTERONE PRODUCTION OF HUMAN ADRENOCORTICAL CARCINOMA (nci-h295R) CELL LINE

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Abstract

In this study the NCI-H295R human adrenocortical carcinoma cell line was used as an *in vitro* biological model to study the effect of cadmium ($CdCl_2$) on the progesterone production. The cells were cultured for 48 h with addition of 1.90, 3.90, 7.80, 15.60, 31.20 or 62.50 μM of $CdCl_2$ and compared to control group (medium without $CdCl_2$). The cell viability was measured by the MTT (metabolic activity) assay. Quantification of progesterone directly from aliquots of the medium was performed by enzyme linked immunosorbent assay (ELISA). Cadmium decreased progesterone release in the whole applied range even at the lowest concentration (1.90 μM) of $CdCl_2$, while the cell viability remained relatively high (> 75 %) up to 7.80 μM of $CdCl_2$ and significantly (P 0.01) decreased from 15.60 μM or higher concentration of $CdCl_2$. Findings of the present study confirm the endocrine disruptive effect of cadmium and its toxicity can affect other points of the steroidogenesis pathway.

Introduction

Cadmium (Cd) is an industrial and environmental contaminant unique among metals because of its non biodegradable nature, long environmental persistence, extremely protracted biological half-life, low rate of excretion from the body and predominant storage in soft tissue (primarily liver and kidney) (IARC, 1993; National Toxicology Program, 2000; Joseph, 2009). This heavy metal has a diversity of toxic effects, including nephrotoxicity, teratogenicity, carcinogenity and endocrine toxicities. Cadmium has a strong preferential affinity for the liver and the kidney over a wide range of exposure levels. In general, about 50 % of the total body burden is found in these two organs (Hammond and Beliles, 1980). It causes tissue damage in humans and animals and many toxicological studies have found the functional and structural changes in the kidneys, liver, lungs, bones, ovaries and fetal effects (Friberg et al., 1986; Kukner et al., 2007; Massányi et al., 2007). Reproductive organs, such as the testis and placenta, are sensitive to the toxic effects of Cd (Takiguchi and Yoshihara, 2006). Its action may be either direct, affecting the gonads and accessory organs, or indirect via interference with the hypothalamus-pituitary-gonadal axis (Paksy et al., 1992). As a well-

known endocrine disrupting chemical, Cd is not only a regulator of hypothalamus and pituitary hormone secretion (Lafuente et al., 2003; 2004), but also disrupts steroidogenesis including the syntheses of androgen, progesterone and oestrogen, leading to suppression of reproductive functions (Takiguchi and Yoshihara, 2006; Ji et al., 2010).

In the present study the human adrenocortical carcinoma cell line NCI-H295R was used as a model system for detection of toxic effect of $CdCl_2$ on the production of progesterone. These cells represent unique *in vitro* model system having the ability to produce all steroid hormones found in the adult adrenal cortex and the gonads, allowing testing the effects of corticosteroid synthesis and the production of sex steroid hormones (Gazdar et al., 1990). The objective of our study was to determine the effects of cadmium chloride ($CdCl_2$) on the progesterone production of human adrenocortical carcinoma cell line (NCI-H295R).

Material and Methods

The NCI-H295R human adrenocarcinoma cell line was obtained from the American Type Culture Collection (ATCC, Manassas, USA). The cells were cultured in a certified laboratory (National Institute of Chemical Safety, OGYI/31762-9/2010, Budapest, Hungary) according to previously established and validated protocols (Hecker et al., 2006; OECD, 2011). The adrenocortical carcinoma cells were grown (37 ºC, with a 5 % CO_2 atmosphere) in a 1:1 mixture of Dulbecco´s Modified Eagle´s Medium and Ham´s F-12 Nutrient mixture (DMEM/F12) (Sigma-Aldrich, St. Louis, USA) (1:1) supplemented with 1.20 g/l $NaHCO_3$ (Sigma-Aldrich, St. Louis, USA), 5.00 ml/l of ITS+Premix (BD Biosciences, San Jose, USA) and 12.50 ml/l of BD Nu-Serum (BD Biosciences, San Jose, USA). The medium was changed 2-3 times per week and cells were detached from flasks for sub-culturing using sterile 0.25 % trypsin-EDTA (Sigma-Aldrich, St. Louis, USA). Cell density was determined using a hemocytometer and adjusted with culture medium to a final concentration of 300.000 cells/ml. The cell suspensions were plated (with final volume of 1.00 ml/well) into sterile plastic 24-well plates (TPP, Grainer, Germany) for steroid measurements. For cytotoxicity evaluation the cells (100 µl/well) were seeded into 96-well plates (MTP, Grainer, Germany) (Knazicka et al., 2013). After a 24 h attachment period the cell culture medium was removed from the plates and replaced with a new medium supplemented with 1.90; 3.90; 7.80; 15.60; 31.20 or 62.50 µM cadmium chloride ($CdCl_2$; Sigma-Aldrich, St. Louis, USA) and cell cultures were maintained for 48 h.

The viability of the cells exposed to $CdCl_2$ was evaluated by the metabolic activity (MTT) assay (Mosmann, 1983). This colorimetric assay measures the conversion of a yellow

tetrazolium salt (3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), to blue formazan particles by mitochondrial succinate dehydrogenase of intact mitochondria of living cells. Formazan can then be measured spectrophotometerically. Following the termination of $CdCl_2$ exposure, the cells were stained with MTT (Sigma-Aldrich, St. Louis, USA) at concentration 0.20 mg/ml. After 2 h incubation (37 ºC, with a 5 % CO_2 atmosphere), the cells and the formazan crystals were dissolved in 150 µl of acidified (0.08 M HCl) isopropanol (CentralChem, Bratislava, Slovak Republic). The optical density was determined at a measuring wavelength of 570 nm against 620 nm as reference by a microplate reader (Anthos MultiRead 400, Austria). The data were expressed in percentage of the control group (i.e. optical density of formazan from cells not exposed to $CdCl_2$). Enzyme linked immunosorbent assay (ELISA) was used for the quantification of progesterone directly from the aliquots of the medium. The ELISA kits were purchased from Dialab GmbH (Wiener Neudorf, Austria). According to the manufacturer's data the sensitivity of progesterone assay was 0.05 ng/ml, and the intra- and inter-assay coefficients of variation were ≤ 4.00 and ≤ 9.30 %, respectively. The absorbance was determined at a wavelength 450 nm using an Anthos MultiRead 400 (Anthos MultiRead 400, Austria) microplate reader.

Obtained data were statistically analyzed by the PC program GraphPad Prism 6.00 (GraphPad Software Incorporated, San Diego, California, USA). Descriptive statistical characteristics (arithmetic mean, minimum, maximum, standard deviation and coefficient of variation) were evaluated. 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); * (P<0.05).

Results and Discussion

According to several research studies, Cd can affect multiple points of the steroidogenesis pathway, inhibiting enzymes important for hormone synthesis (Paksy et al., 1997b; Takiguchi and Yoshihara, 2006). Recently, the effects of Cd on steroidogenesis have been described, but results vary depending on the experimental model, time-duration of exposure and the dose used. Therefore, the general objective of this study was to provide other information of its impact on the steroidogenesis. Specifically, we examined progesterone production of adrenocortical carcinoma cells in relation to $CdCl_2$ concentrations.

The recent studies conducted using cultured human placental trophoblastic cells suggest that Cd reduces progesterone synthesis by inhibiting the gene expression of the low-density lipoprotein (LDL) receptor, which controls the internalization of cholesterol into

steroidogenic cells (Jolibois et al., 1999), cytochrome $P450_{SCC}$, which coverts pregnenolone to progesterone (Kawai et al., 2002). On the other hand, some reports have indicated that Cd administered to female rats during estrus and diestrus resulted in increased serum progesterone level (Piasek and Laskey, 1994; Paksy et al., 1997a, b) and stimulated progesterone synthesis in both cultured porcine granulosa cells (Varga et al., 1993) and JAR choriocarcinoma cells, a malignant trophoblast cell line (Powlin et al., 1997).

The results of our present study indicate dose-dependent decreases in progesterone production of NCI-H295R cell culture following a 48 h *in vitro* $CdCl_2$ exposure detected at low concentration. The lowest amount of progesterone was significantly detected in groups with the highest doses (≥ 31.20 μM) of $CdCl_2$ (Table 1), which these concentrations have a cytotoxic effect. The control mean progesterone production (100 %) was 26.72±9.68 $ng.ml^{-1}$. These results clearly confirm previous experimental studies by Forgacs et al. (2011) and Ocztos et al. (2011), who observed the effect of Ni^{2+}, Hg^{2+} and Cd^{2+} on the progesterone and testosterone production of H295R cells.

Table 1. Effect of 48 h $CdCl_2$ exposure on progesterone production ($ng.ml^{-1}$) of NCI-H295R cell culture.

Groups	**Control Ctrl**	**1.90 F**	**3.90 E**	**7.80 D**	**15.60 C**	**31.20 B**	**62.50 A**
				$CdCl_2$ (μM)			
Progesterone ($ng.ml^{-1}$)							
x	26.72	23.99	20.97	22.93	9.85	5.34*	2.49**
minimum	15.24	11.77	10.89	8.15	4.88	2.44	1.25
maximum	42.88	37.51	35.07	32.53	12.25	6.88	3.88
S.D.	9.68	10.33	9.17	10.60	2.70	1.58	0.90
CV (%)	36.24	43.05	43.71	46.25	27.37	29.55	36.15
%	100.00	71.20	78.47	85.80	36.86	19.99	9.31

Legend: x – arithmetic mean, S.D. – standard deviation, CV (%) – coefficient of variation. * $P \square 0.05$, ** $P \square 0.01$.

Similar results were also found in our previous study with mercury (Knazicka et al., 2013). The authors Paksy et al. (1992, 1997b) found a decrease of progesterone production by human granulosa cells after Cd treatment *in vitro*. Cadmium did not cause a significant alteration in progesterone accumulation during 4 h incubation periods. Following 24 h Cd decreased progesterone production (Paksy et al. 1992). Massanyi et al. (2000) examined the effects of $CdCl_2$ on ultrastructure and steroidogenesis in cultured porcine ovarian granulosa cells *in vitro*. In the evaluation of steroidogenesis they found that $CdCl_2$ induced an increase in progesterone production and a decrease in 17β-oestradiol production, which are required for further stages of reproduction. Subsequently structural and functional alterations in the ovarian granulose cells after $CdCl_2$ administration were confirmed.

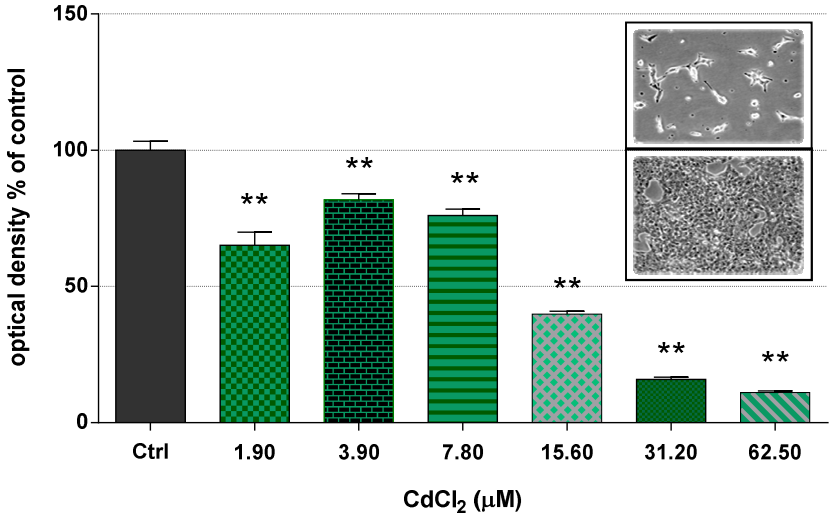

Figure 1. The viability of NCI-H295R cell culture after 48 h $CdCl_2$ exposure.
Legend: Cytotoxicity was assessed using the MTT assay following $CdCl_2$ exposure. Bar values represent the aritmetic mean (±SD) optical density in % of (untreated) controls. The number of replicate wells was 21-32 at each point. A decline in optical density reflects a decline in cell viability. Statistical difference between the values of control and treated cells in indicated by asterisks ** P□0.01.

Our experiment showed that the cytotoxic effect was significantly (P□0.01) detected at all concentrations of $CdCl_2$ (Figure 1). However, the cell viability remained relatively high (> 75 %) up to 7.80 μM of $CdCl_2$ and significantly (P□0.01) decreased from 15.60 μM or higher concentration of $CdCl_2$. These results are in agreement with previous study Tchounwou et al. (2001) indicating the high degree of $CdCl_2$ toxicity to human liver carcinoma cells (HepG2).

Conclusion

Data obtained from this *in vitro* study indicate that the release steroid hormone - progesterone by adrenocortical carcinoma cells is associated with the dose of cadmium administration. All chosen concentrations of $CdCl_2$ decreased the progesterone production. The results suggest that low concentrations of progesterone can cause affect their metabolites, whose production is conditioned by steroid enzymes. Therefore, further studies are needed to clarify the precise mechanism of action of cadmium on the sexual steroid production.

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THE USE OF BULK MILK SAMPLE AS AN ORIENTATION INDICATOR OF HEALTH CONDITION OF CZECH FLECKVIEH DAIRY COWS

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Abstract

This paper investigates the use of bulk milk sample as an orientation indicator of health condition of Czech Fleckvieh dairy cows (farm GenAGRO Říčany, a.s.). Bulk milk samples were analysed during the period of 1.12.2013 to 25.2.2014 (n=87). The analysis included following parameters relationships: lactose content, chloride content, calcium content, somatic cell count and chloride-lactose ratio. Based on the results were found statistically significant relationships (P□0.05) between lactose content and somatic cell count and between (P□0.05) somatic cell count and chloride-lactose ratio (and also between chloride-lactose ratio and chloride content - P□0.001). Other relationships between measured parameters were statistically non-significant (P>0.05).

Introduction

Changes in milk composition (content of lactose, minerals, enzymes, or somatic cells count) can be attributed to disease onset (Hamann, Krömker, 1997). When number of somatic cells (SCC) in milk increases (often due to inflammation of the mammary gland or due to stress), there are always (greater or lesser) changes in its composition (Zadražil, 2002; Hovorková, 2007). For SCC in raw milk is given as standard in 1 ml ≤ 400 000 SC (Doležal et al., 2000). There is a negative correlation between SCC and lactose (L) content in milk - among the most sensitive indicators of udder diseases belongs lactose content (Gajdůšek, 1996; Lukášová et al., 1999). Average milk contains 4.6 $g.100g^{-1}$ of L. When is its level reduced then concentrations of chloride (Cl) and sodium ions in milk rise. The average Cl content in milk is in range 0.8-1.4 $g.l^{-1}$ (Harding, 1996). Koestler (1920) used the ratio of Cl ions and L to indicate normal or mastitis milk (McSweeney, Fox, 2009). In normal bulk milk samples is chloride-lactose (Cl/L) ratio 1.7–2.2. Values greater than the upper limit mentioned points secretory disorders in the mammary gland (Šustová, 2005). Some authors present upper limit to 3 (McSweeney, Fox 2009). Among the minerals what level declines during mastitis is included calcium (Ca) – its content in cow's milk is in average 1,2–1,37 $g.l^{-1}$ (Gajdůšek, 2003; Genčurová et al. 1997). From a technological point of view the content of Ca in milk is one

of the critical factors affecting cheese production (Lukášová, Smrčková, 2003). In healthy cows' milk is content of minerals quite stable (Gajdůšek, 2003).

Material and Methods

During the period of 1.12.2013 to 25.2.2014 were analysed bulk milk samples (sampled daily) obtained in herd of Czech Fleckvieh cows (n=87) from farm GenAGRO Říčany, a.s. Cows were fed total mixed ration *ad libitum* and were in various stage of lactation.

Analysis of samples was performed in the laboratory of Department of Animal Breeding at Mendel University in Brno. **Lactose (L)** content was measured on instrument Julie C5 Automatic (Scope Electric) working on the principle of thermo analysis. **Chloride (Cl)** content in milk was determined after the addition of nitric acid by titration argentometric. Chlorides were precipitated by excess silver nitrate solution and for reverse titration was used a solution of ammonium thiocyanate. For the determination of **calcium (Ca)** content was used complexometric titration with EDTA, 2Na. **Somatic cell count (SCC)** was determined in Laboratory for the analysis of milk – Brno using a fluoro-opto-electronic method. **Chloride-lactose ratio (Cl/L)** (x) number was determined according to the formula:

$$x = \frac{a.100}{b.10}$$

a…chloride ions content ($g.l^{-1}$)

b…lactose content in milk ($g.100g^{-1}$)

Results and discussion

Tab. 1 provides average values of monitored parameters; **Tab. 2, Fig. 1** indicates correlation relationship between monitored parameters. Between L content (average value was found 4.73 $g.100g^{-1}$) and SCC (average value was found 110 $ths.ml^{-1}$) was found statistically significant difference (P□0.05; r = -0,25). With this result, that with the increasing SCC decreased L content agree Auldist et al. (1995); Ogola et al. (2007). A similar relationship (P□0.05 r = -0.29) also found Javorová et al. (2013). Between SCC and Cl/L (average value was found 1.79) was also found statistically significant difference (P□0.05; r = 0.21) which means with increasing SCC increased Cl/L too. McSweeney, Fox (2009) agree with this statement, Rogers et al. (1989) in their research also indicated a positive correlation relationship (P<0.01). Statistically significant relationship (P□0.001; r = -0.97) was (logically) detected between Cl/l and Cl content (average content was found 0.85 $g.l^{-1}$).

Other measured parameters relations among themselves were statistically non-significant (P>0.05). Some authors evidence statistically relationship between SCC and Ca (Ogola et al., 2007; Rogers et al., 1989) and also between Ca and Cl (Gajdůšek, 2003). Hanuš et al. (1992); Gaucheron (2005) also point that content of L and minerals is auxilliary indicator for detection of the mammary gland secretion disorders.

Table 1. *Average values of monitored parameters*

Parameter	Unit	Average	Min.	Max.	S_x
L	$g.100g^{-1}$	4.73	2.90	5.00	0.31
SCC	$ths.ml^{-1}$	110	7	306	69.7
Ca	$g.l^{-1}$	1.17	0.93	1.43	0.10
Cl	$g.l^{-1}$	0.85	0.64	0.96	0.30
Cl/L	-	1.79	1.34	2.57	0.65

Table 2. *Correlation relationship between monitored parameters*

Parameter	L	SCC	Ca	Cl	Cl/L
L	-	-0.25*	0.01	0.03	-0.20
SCC	-0.25*	-	0.13	0.16	0.21*
Ca	0.01	0.13	-	-0.10	-0.10
Cl	0.03	0.16	-0.10	-	0.97***
Cl/L	-0.20	0.21*	-0.10	0.97***	-

*= P<0.05, ***= P<0.001

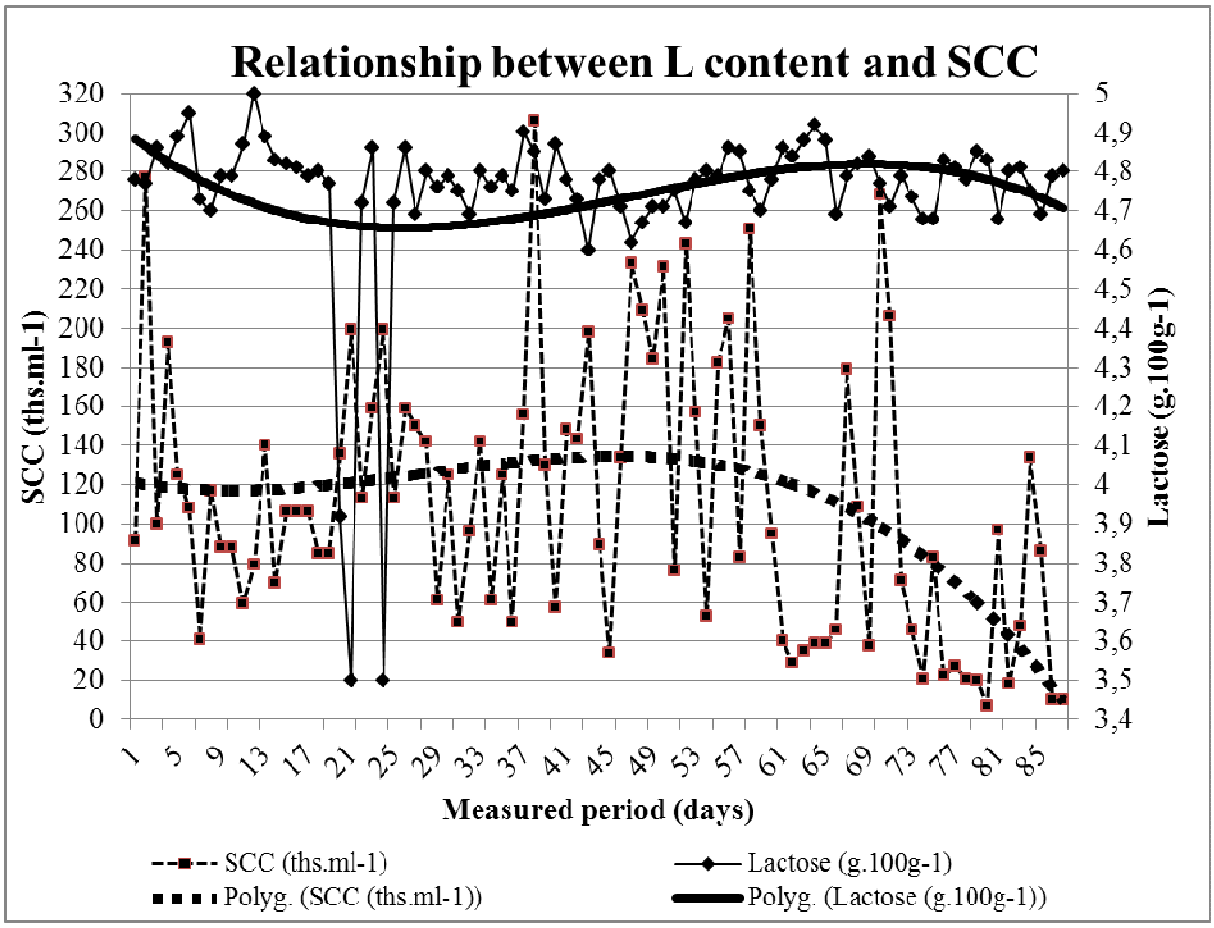

Figure 1. The relationship between SCC and L during the measured period

Conclusions

The aim of this study was to evaluate bulk milk sample (measured parameters were content of lactose, calcium, chloride, somatic cell count and chloride-lactose ratio) as an orientation indicator of the health condition of Czech Fleckvieh dairy cows. During the monitored period was found statistically significant relationships between lactose content and somatic cell count and between somatic cell count and chloride-lactose ratio (and also between chloride-lactose ratio and chloride content). Other relationships between measured parameters were statistically non-significant.

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GREEN TEA POLYPHENOLS EFFECT ON PORCINE OVARIAN CELLS

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Abstract

The aim of our study was to examine the the potential effect of green tea polyphenols (GTPP) on cultured porcine ovarian granulosa cell functions – proliferation, apoptosis and steroidogenesis. It was found, that doses of GTPP diminished number of cells containing marker of proliferation (PCNA) and raised the percentage of apoptotic (bax-positive) cells, enhanced progesterone release and affected testosterone release in granulosa cells. This results suggest a direct effect of epigallocatechin gallate on basic ovarian functions.

Introduction

Plant polyphenols are natural antioxidants and most of their pharmacological properties are considered to be due to their antioxidant action (Ames et al., 1995). Green tea polyphenols (GTPP) are known for their preventive, antibacterial and therapeutic effects, anticancer and apoptosis inducing-properties (Blanko et al., 2003). This molecules prevents neural cell death (Reznichenko et al., 2005) and induces chromosomal damage in lymphoblastoid cell lines (Sugisawa and Umegaki, 2002). Polyphenols inhibited the volume of growth hormone, leptin, insulin-like growth factor I and prolactin on male rats (Kao et al, 2000).

This green tea extract may induced reductions in the levels of sex steroids hormones as testosterone and estradiol and possible negative effects on reproductive performance (Kao et al., 2000) on granulosa cell functions (Basini et al., 2005) and *in vitro* fertilization in swine Spinaci et al., 2006).

Cell proliferation is the amount of cells in culture or in the body can be divided. The extent of DNA synthesis is marker for proliferation (Wyllie et al., 1998). Involving the protein to cell proliferation include PCNA (Tomanek and Chronowska, 2006). This protein is localized in the cell nucleus (Makarevich et al., 2000) and located in granulosa cells of gilts (Sanislo et al. 2001). Addition of polyphenols supresses proliferation of porcine granulosa cells (Basini et al., 2005), viability, and anchorage-independent growth (Kang et al., 2010).

Apoptosis is programmed death of cells. This process eliminates unnecessary and useless cells from the body (Wyllie et al., 1998). Apoptosis is supported by group of caspases, which include BAX (Zwain and Amato, 2001). This protein is localized especially in mitochondria and located in granulosa cells of porcine (Sanislo et al. 2001).

Progesterone (P4) is an ovarian steroid produced by ovarian granulosa cells (Kolesárová et al., 2010) of pigs and contributes to regulation of ovarian follicular development and remodelling (Mahajan, 2008). This progestin is essential for normal ovarian cycle of females (Hagan et al., 2009). Another hormone produced in ovary is testosterone (T) (Delort et al., 2009). T is steroid hormone as well as P4 are necessary as a precursor for the synthesis of estrogen (Sirotkin, 2014). Androgens, primarily testosterone, are promoting proliferation of follicular cells, recruitment and development of ovarian follicles up to preovulatory stage, either stimulate or suppress development of Graafian follicles and their ovulation, increase apoptosis and follicular atresia at different stages of follicullogenesis and either promotes or suppress oocyte nuclear maturation. Testosterone treatments altered release of progesterone, estradiol, by cultured ovarian cells (Sirotkin et al., 2003; Sirotkin, 2014).

The results concerning effect of GTPP on reproductive processes and regulators are poor, and the available data are limited mainly on studies of non-ovarian or ovarian cancer cells. Effect of apoptosis and T release due to GTPP has not been studied in porcine granulosa cells.

The aim of our study was to examine the effect of GTPP treatment at doses 1, 10 and 100 μg/ml on the basic ovarain functions – proliferation (accumulation of markers of proliferation PCNA), apoptosis (BAX) and secretory activity (steroid hormones of P4 and T) of porcine granulosa cells (GCs) *in vitro.*

Material and method

Preparation, culture and processing of granulosa cells from ovaries

Granulosa cells were collected from the ovaries of prepubertal Slovakian White gilts, after slaughter at a local abattoir. After aspiration and isolation of granulosa cells, these cells were then washed in sterile DMEM/F12 1:1 medium (BioWhittakerTM, Verviers, Belgium), resuspended in the same medium supplemented with 10% fetal calf serum (BioWhittakerTM) and 1% antibiotic-antimycotic solution (Sigma, St. Louis, MO, USA) at a final concentration 10^6 cells/ml medium. Portions of the cell suspension were dispensed to 24-well culture plates (NuncTM, Roskilde, Denmark, 1 ml suspension/well; for EIA) or 16-well chamber slides (Nunc Inc., International, Naperville, USA, 200 μl/well, for immunocytochemistry). Both, the plate wells and chamber slides were incubated at 37 ° C and 5% CO2 in humidified air until

60-75% confluent monolayer was formed (3-5 days), at which point the medium was renewed. Further culture was performed in 2 ml culture medium in 24-well plates (medium for RIA) or 200 µl/medium in 16-well chamber slides, (cells for immunocytochemistry) as described previously. After medium replacement experimental cells were cultured in the presence of green tea polyphenols (GTPP) (Changsha Sunfull Bio-tech. Co, Hunan China) at concentrations of 0, 1, 10 and 100 µg/ml. GTPP was dissolved in culture medium immediately before their addition to the cells. After two days in culture, the medium from the 24-well plates was gently aspirated and frozen at -24°C to await EIA. After removing the medium from chamber slides, cell were washed in ice-cold PBS (pH 7.5), fixed in paraformaldehyde (4% in PBS, pH 7.2-7.4; 60 min) and held at 4°C to await immunocytochemistry.

Immunocytochemical analysis

Following washing and fixation, the cells were incubated in the blocking solution (1% of goat serum in phosphate-buffered saline – PBS) at room temperature for 1 h to block nonspecific binding of antiserum. Afterwards, the cells were incubated in the presence of monoclonal antibodies against either PCNA (marker of proliferation) and BAX (marker of apoptosis) (all from Santa Cruz Biotechnology, Inc., Santa Cruz, USA; dilution 1:500 in PBS) for 2 h at room temperature at overnight at 4°C. For the detection of binding sites of primary antibody, the cells were incubated in secondary swine antibody against mouse IgG labelled with horse-radish peroxidase (Servac, Prague, Czech Republic, dilution 1:1000) for 1 h. Positive signals were visualized by stanning with DAB-substrate (Roche Diagnostics GmbH, Manheim, Germany). Following DAB-staining, the cells on chamber-slides were washed in PBS, covered with a drop of Glycergel mounting medium (DAKO, Glostrup, Denmark); then coverslip was attached to a microslide. Cellular presence and localization of PCNA and BAX positivity in cells was proved on the basis of DAB-peroxidase brown staining. A ratio of DAB-HRP- stained cells to the total cell number was calculated.

Immunoassay

Concentrations of P4 and T were determined in 20 µl samples of incubation medium by EIA. The concentrations of P4 and T were assayed using Emzymeimmunoassay (EIA) according to Prakash et al. (1987) for P_4 and Münster (1989) for T. All EIA were validated for use in samples of culture medium.

Statistical Analysis

Significant differences between the experiments were evaluated using one-way ANOVA followed by paired Wilcoxon-Mann Whitney test, Sigma Plot 11.0 software (Systat Software,

GmbH, Erkhart, Germany). Differences from control at P< 0.05 were considered as significant.

Results

- *proliferation and apoptosis* (Immunocytochemistry)(Tab.1).

In our study 10 and 100 µg/ml doses of GTPP significantly (P<0,05) inhibited the percentage of cells containing PCNA.

A number of porcine granulosa cells containing BAX was stimulated by GTPP treatment at all used doses.

Table.1.The percentage of cells containing markers of proliferation (PCNA) and apoptosis (BAX) after GTPP treatment (Imunocytochemistry).

Supplement	% of cells contained	
	PCNA	bax
Control (no additon)	49,86±1,3 (3311)	49,8±1,24 (3939)
GTPP 1 µg/ml	45,13±2,17 (903)	61,75±1,71* (886)
GTPP 10 µg/ml	34,6±3,98* (597)	64,88±3,0* (890)
GTPP 100 µg/ml	41,0±3,53* (658)	68,71±1,77* (809)

All the values represent % of cells containing particular antigen, means ± SEM, *- significant (P<0.05) differences with control (cells not treated with GTPP). In the brackets is a number of counted cells.

- *steroidogenesis* (EIA)(Tab.2).

In our study 10 µg/ml dose of GTPP increased the P4 secretion by porcine ovarian granulosa cells.

In the case of secretion of T by porcine ovarian GCs stimulatory effect of GTPP (at the dose 1 µg/ml) and inhibitory effect (at the dose 10 and 100 µg/ml) was found.

Table 2. The secretion of steroid hormones by porcine ovarian granulosa cells after GTPP treatment (EIA).

Supplement	P4 secretion	T secretion
	ng/10^6cells/day	pg/10^6 cells/day
Control (no additon)	81,20±6,28	344,46±79,20
GTPP 1 µg/ml	172,00±28,40	965,00±29,90*
GTPP 10 µg/ml	250,00±5,00*	154,00±20,50*
GTPP 100 µg/ml	146,70±47,30	156,00±15,20*

All the values represent P4 or T release, means ± SEM, *- significant (P<0.05) differences with control (cells not treated with GTPP).

Conclusion

The present study suggest a possible stimulatory effect of GTPP on the release of progesterone and change in the testosterone level, inhibitory impact on proliferation (accumulation of PCNA) and stimulatory influence on apoptosis (accumulation of bax) on granulosa cells of porcine ovary. Our results suggest a direct effect of GTPP on steroidogenesis, proliferation and apoptosis in porcine ovaries. Our study is the first evidence between GTPP treatment and its increased effect on testosterone release. Taken together, these data suggest that GTPP can suppress porcine reproductive (ovarian) function – suppress ovarian cell proliferation, promote their apoptosis and alter release of steroid hormones.

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NUTRITIONAL VALUES OF FEMALE RABBITS MEAT AFTER ADMINISTRATION OF QUERCETIN

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Abstract

The aim of the present study was to determine the effect of intramuscular application of quercetin in various doses on selected parameters characterizing the content of nutrients (content of total water, content of proteins, fat and energy value) of rabbit's meat. Animals were divided into four groups (n=5 in each group), one control group (C) and three experimental groups (E1, E2 and E3) in both sexes. Experimental groups received quercetin in dose 10 $\mu g.kg^{-1}$ (of body weight) in E1 group, 100 $\mu g.kg^{-1}$ in E2 group, and 1000 $\mu g.kg^{-1}$ in E3 group for 90 days 3 times a week. Control group received injection water. After 90 days animals were slaughter. Meat quality was analysed from a sample of *Musculus longissimus dorsi* (100 g) for parameters characterizing the content of nutrients. The analysis was detected using Infratec 1265 (Tecator, Sweden) 48 hours post mortem. Energy value (EV) was detected by measured fat content, protein and corresponding coefficients EV=16,75 x content of protein + 37,68 x content of fat. Energy value was determined from the measured fat content, proteins and compent coefficients. Application of quercetin had slight or no effect on rabbit meat and differences among the groups were insignificant (P>0.05). Further investigations regarding the extent to which they could withstand oxidative changes under stress conditions are needed, especially without support from an external antioxidant supply.

Keywords: flavonoids, quercetin, rabbit, meat quality

Introduction

Meat and meat products have always been important for healthy growth and development because they are good sources of proteins, essential amino acids, minerals, vitamins as well as fats (Biesalski, 2005). Rabbit meat offers excellent nutritive and dietetic properties (Dalle Zotte, 2002; Dalle Zotte, 2004; Combes, 2004; Combes and Dalle Zotte, 2005; Hernàndez and Gondret, 2006). Although synthetic antioxidants were once widely used in the meat industry,

consumer concerns with safety and toxicity drove the industry to find natural sources (Coronado et al., 2002). Some herbs and spices (rosemary, sage, green tea, clove, cinnamon, nutmeg, rose petals) could be efficient food ingredients in improving the shelf life of (mainly processed) meats vulnerable to oxidative changes because they contain many phytochemicals that are potential sources of natural antioxidants, including flavonoids, tannins, phenolic acids, and phenolic diterpenes, while also promoting anti-inflammatory, antimicrobial and anticancer activities (Zhang et al., 2010). Quercetin is a flavonoid, which is found in vegetables, fruits, and other dietary sources (Pawlikowska-Pawlęga et al., 2003). It is marketed as a diet supplement with anti-inflammatory, antiviral, immunomodulatory, and antioxidant properties (Egert, et al., 2008; Liu et al., 2012). To reduce the negative impacts of reactive oxygen species (ROS), several mechanisms have to be integrated. Endogenous and exogenous antioxidants work synergistically to neutralize the action of ROS. The endogenous antioxidant network includes enzymatic and non-enzymatic mechanisms. Antioxidant enzymes convert superoxide anion radicals, through H_2O_2 to H_2O, thereby minimizing the production of hydroxyl radicals which are the most potent ROS in biological systems (Halliwell et al., 1995).

The aim of this study was to investigate the effect of quercetin applied intramuscularly on specific parameters of meat quality of rabbis.

Material and methods

Animals and diet

Adult female rabbits (n=20) of meat line M91, maternal albinotic line (crossbreed New-Zealand White, Buskat Rabbit, French Silver) and paternal acromalictic line (Crossbreed Nitra's Rabbit, Californian Rabbit, Big Light Silver) were used in the experiment. Rabbits were obtained from an experimental farm of the Animal Production Research Centre Nitra, Slovak Republic. Rabbits (age 4 months, weighing 4.0–4.5 kg) were housed in individual flat-deck wire cages (area 0.34 m^2) under a constant photoperiod of 14 h of day-light. The temperature (18–20°C) and humidity (65 %) of the building were recorded continually by means of a thermograph positioned at the same level as the cages. Rabbits were healthy and their condition was judged as good at the commencement of the experiment. Adult rabbits were fed diet of a 12.35 $MJ.kg^{-1}$ of metabolizable diet composed of a pelleted concentrate (Table1).

Animals were divided into four groups (n=5 in each group), one control group (C) and three experimental groups (E1, E2 and E3) in both sexes. Experimental groups received quercetin (Sigma Aldrich, Saint Louis, USA) in injectable form (intramuscularly) at 10 $\mu g.kg^{-1}$ in E1 group, 100 $\mu g.kg^{-1}$ in E2 group, and 1000 $\mu g.kg^{-1}$ in E3 group for 90 days 3 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 the State Veterinary and Food Institute of Slovak Republic, no. 3398/11-221/3.

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

Component	
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 $MJ.kg^{-1}$

Procedures

After 90 days animals were slaughtered, and samples of *Musculus longissimus dorsi* were collected. The meat samples were collected one hour after slaughter, wrapped in aluminium foil and stored at 4^{o}C for 24 hours. Meat quality was analysed from a sample of *Musculus longissimus dorsi* (100 g) for parameters characterizing the content of nutrients (content of total water, content of proteins, fat and energy value). The analysis was detected using Infratec 1265 (Tecator, Sweden) 48 hours post mortem. Energy value (EV) was detected by measured fat content, protein and corresponding coefficients

EV=16.75xcontent of protein + 37.68 x content of fat

Statistical analyses

The data used for statistical analyses represent means of values obtained in three blood collections performed on separate days. 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 11.0 (Jandel, Corte Madera, CA, USA) was used. Differences were compared for statistical significance at the level $P<0.05$.

Results and Discussion

Results of total water, proteins, fat and energy values are presented in Table 2. Analysis of the chemical composition of rabbit meat indicated that received quercetin in injectable form had no effect on the nutritional composition (of total water, protein, lipids and energy value) of the *Musculus longissimus dorsi* female rabbits. The observations of Kremer et al. (1999) lead to conclude that slowing the rate of pH decline but not altering ultimate pH can improve the water holding capacity of fresh pork muscle. Dietary quercetin addition did not alter Hunter L* (paleness) but decreased Hunter a* (redness) scores (5.6, 5.0, and 5.0) of the muscles. Different natural ways to improve the oxidative stability of rabbit meat have also been studied, leading to increased stability against the oxidative changes of rabbit meat (López-Bote et al., 1998). Paci et al. (2001) tested the antioxidant capacity of the polyphenol oleuropeine from olive oil leaves on rabbit meat but failed to observe any reduction in the meat's susceptibility to oxidation. There are a variety of supplemental antioxidants employed in practical rabbit nutrition of which tocopherol (TOH) and vitamin C are the most widely used. TOH protects cellular membranes against oxidative damage. It reacts or functions as a chain-breaking antioxidant, thereby neutralizing free radicals and preventing oxidation of lipids within membranes (Morrissey et al., 1994 and McDowell, 2000).

Table 2. Effect of quercetin on selected parameters of nutrient content in samples of *musculus longissimus dorsi* of rabbits.

parameter	**C**	**E1**	**E2**	**E3**
Proteins $g.100g^{-1}$	23.06±0.65	22.63±0.64	23.00±0.95	23.33±1.02
Fat $g.100g^{-1}$	2.93±1.19	4.26±1.39	3.13±2.59	2.10±1.24
Total water $g.100g^{-1}$	73.00±0.62	72.10±0.80	72.86±1.20	73.56±0.64
Energy value $kJ.100g^{-1}$	496.22±33.77	539.87±41.47	503.14±82.27	469.96±33.49

C - control group; E1 10 $\mu g.kg^{-1}$, E2 100 $\mu g.kg^{-1}$, E3 1000 $\mu g.kg^{-1}$ – experimental groups; mean ± SD (standard deviation)

Conclusions

In conclusion, administration of quercetin through intramuscular injection to broiler rabbits had no effect on the selected parameters of meat quality (*M.. longissimus dorsi*). Detailed physiological mechanism of effect of quercetin should be further studied.

Acknowledgments

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EFFECT OF GENETIC SELECTION FOR HIGH AND LOW EGG TESTOSTERONE ON IMMUNE RESPONSE UNDER LIMITED FOOD CONDITIONS

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Abstract

Maternal testosterone modulates the whole range of phenotypic characteristics, including body growth and proactive behaviour. Therefore it has a potential to modify allocation of resources within organism and consequently act at the expense of immune system. In our experiment we subjected Japanese quail, selected for high (HET) and low (LET) egg testosterone, to quantitative (food dilution with 30% of sawdust) and qualitative (decrease of crude protein content) food restriction and evaluated responsiveness of cell-mediated immunity and acute phase response under various rates of food deprivation. Both kinds of food restrictions decreased body weight of birds. Quantitative restriction had no or even positive effects on immune system, while qualitative restriction clearly exceeded physiological limits and caused immunosuppression. Although we repeatedly observed higher body weight of quail from the HET line, they showed a comparable immune responsiveness to their counterparts from the LET line even under severe restriction. Therefore we presume that the effect of selection for high egg testosterone on immune system does not represent a limitation during offspring development.

Introduction

Shortage of food resources is a common phenomenon in nature. Organisms must respond to this unpredictable challenge, for example by re-allocation of accessible internal resources among various organ systems. Competition between the immune system and body growth was reported (Brzęk and Konarzewski, 2007), as well as a negative impact of food restriction on cell-mediated immunity (Lochmiller et al., 2003) and humoral response in birds (Bunchasak et al., 2005). The data confirm a potential of limited food intake to influence immunocompetence of organisms.

Maternal testosterone stimulates early postnatal growth (Müller et al., 2008), proactive behaviour (Müller et al., 2012) and development of secondary sexual characteristics (Eising et al., 2006). All these effects are supposed to enable transfer of information about relevant

environmental changes to the next generation (Groothuis and Schwabl, 2008). Except of these organisational effects, maternal testosterone is often discussed in relation to its possible immunosuppressive influence. Testosterone negatively influences immune-responsiveness in adult birds (Peters, 2000). However, studies exploring effect of maternal testosterone on the immune system of offspring provide ambiguous results. This diversity of results can point out to the possibility that effects of maternal testosterone depend on environmental conditions experienced by offspring during their early postnatal development.

Food restriction itself might represent an important modulating factor in interactions of maternal testosterone and immune system. The need for re-allocation of limited food resources to processes stimulated by higher levels of maternal testosterone could cause inadequate supplementation of immune system. Re-allocation hypothesis is based on these presumptions (Lochmiller and Deerenberg, 2000) and it provides one possible explanation for immunosuppressive effects of maternal testosterone as well as the variability of obtained results.

In our research we analysed effect of quantitative (food dilution with 30% of sawdust) and qualitative (decrease of crude protein content) food restriction on parameters of immune response in Japanese quail selected for high and low maternal testosterone content.

Material and methods

The study was conducted on F5 and F6 generation of Japanese quail (*Coturnix japonica*) selected for high and low testosterone content in egg yolk (Okuliarova et al., 2011) bred at the Institute of Animal Biochemistry and Genetics, Slovak Academy of Sciences, Ivanka pri Dunaji. After hatching, chicks were weighted, individually labelled and separated into boxes according to the genetic line and diet. Chicks were kept under constant light and temperature provided by an infrared lamp. In all experiments quail had *ad libitum* access to food and water during the first three days of experiment. In the first and the second experiment, the half of experimental animals was subjected to 30% quantitative restriction (70% of feeding mash HYD13 + 30% of sawdust from hard wood) from the fourth day of age. In case of the third experiment, qualitative restriction was used (control: 260 g/kg crude protein content, 11.5 MJ/kg metabolizable energy; restricted group: 150 g/kg crude protein content, 11.0 MJ/kg metabolizable energy). Animals were regularly weighted.

We used 40 animals from each line to test immunological parameters. In the first experiment, cell-mediated immunity induced by phytohemagglutinine (PHA) administration was tested. The second experiment was focused on evaluation of acute phase response to

lipopolysaccharide (LPS) administration and in the third experiment complex immune responsiveness to both stimulants was evaluated.

Phytohemagglutinine was subcutaneously injected into the right wing web (100 μg PHA, Sigma, USA in 20 μl of phosphate buffer saline – PBS). Left wing was used as control, administered only with the same amount of PBS. Thickness of wing web was measured before and 24 hours after PHA injection with a micrometre (Vogel, Germany), with precision of 0.001 mm. The results were evaluated as PHA index.

$$PHAindex = \frac{PostPHA - postPBS}{(prePBS + prePHA)/2}$$

[I.] PostPHA – thickness of the wing post PHA application, postPBS – thickness of the wing post PBS application, prePBS - thickness of the wing before PHA application, prePHA - thickness of the wing before PHA application.

Acute phase response was evaluated after intaperitoneal injection of LPS. The half of experimental animals was administered with 1.5 mg of LPS (Sigma, USA) per kilogram of body weight in 100 ěl of PBS. Control group received only 100 ěl of PBS. Animals were killed by quick decapitation three hours after the LPS/PBS administration and blood was collected into heparinised tubes. Part of blood was used for preparation of blood smears in order to establish the ratio of heterophils to lymphocytes. Blood smears were dyed and fixed by the Pappenheim panoptic method, two smears per animal. The rest of plasma was centrifuged and stored under -20°C.

Corticosterone concentration in plasma was measured by radioimmunoanalysis (commercial kit DRG, Germany). Use of this kit was validated in our laboratory for Japanese quail (Okuliarová et al., 2010). Unspecific plasma antibody concentration was evaluated by enzyme-linked immunosorbent assay (ELISA) according to Grindstaff et al. (2006).

Results were checked for normal distribution and evaluated by hierarchical multifactorial analysis of variance. Group differences were analysed by LSD post hoc tests.

Results

Both food restrictions influenced the physiological state ofquail, as demonstrated by decreasing of their body weight (qualitative restriction 1: $F_{(1,354)} = 8.32$; $p < 0.01$; qualitative restriction 2: $F_{(1,216)} = 5.24$; $p < 0.05$; quantitative restriction: $F_{(1,360)} = 352.70$; $p < 0.001$) (Tab. 1). Repeated analysis of variance proved line differences in body weight after quantitative restriction 1 from 14 days of age ($p < 0.001$) and in qualitative restriction from day 11 till the end of experiment (day 11: $p < 0.05$; day 14: $p < 0.01$; day 19: $p < 0.001$). We found no sex-specific differences.

In the experiment with quantitative restriction, statistical analysis revealed significant differences between food groups ($F_{(1,73)}$ = 13.483; $p < 0.001$) and lines ($F_{(1,73)}$ = 10.118; $p < 0.01$) in cell-mediated immunity. Quail from the restricted group reached higher PHA index in comparison with the control group, while we measured the thicker wing web swelling in quail from the HET line regardless the food group (Fig. 1). Protein restriction decreased the immune response after PHA injection ($F_{(1,34)}$ = 16,68; $p < 0,001$). In this experiment we noticed a tendency to interaction between the line and food group. Quail from the LET line under control food conditions tended to have a higher cell-mediated immunity in comparison with their HET counterparts ($F_{(1,34)}$ = 3,79; p = 0,058). There were no intersexual differences.

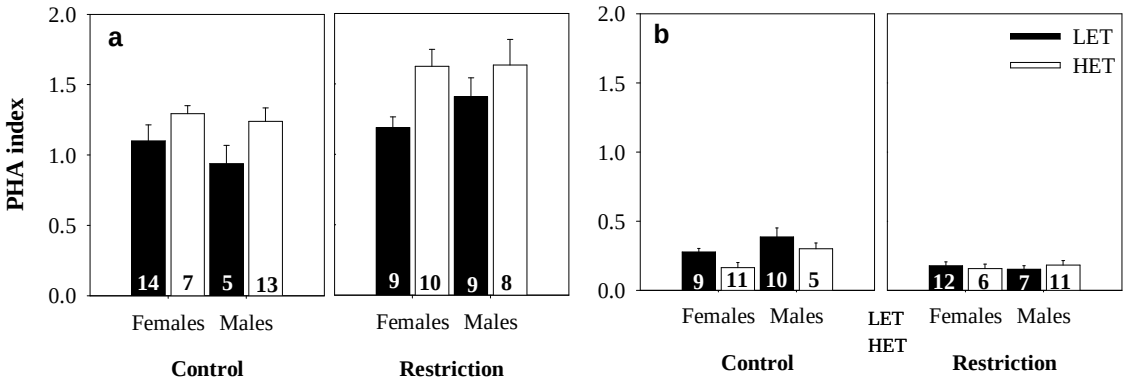

Figure 1. Phytohemagglutinine (PHA) index of Japanese quail selected for high (HET) and low (LET) egg yolk testosterone content. a – quantitative restriction, b – qualitative restriction. Values are expressed as mean ± SEM.

Quantitative restriction had no significant effects on parameters of acute phase response three hours after LPS injection and we detected no differences between genetic lines in this experiment (Tab. 2). Treatment with LPS increased corticosterone concentrations in both control and restricted groups with no line differences.

Qualitative restriction increased the heterophil/lymphocyte ratio and synergically with LPS injection tended to raise plasma corticosterone concentrations. Administration LPS modulated all parameters of the acute phase response since it increased plasma corticosterone and He/Ly ratio and decreased unspecific immunoglobulin levels. Effects of genetic line manifested separately from effects of food restriction. We observed the lower He/Ly ration in HET line, whereas plasma immunoglobulins were higher in this line in comparison with quail from LET line (Tab. 2). Statistical analysis revealed no interactions among the observed factors.

Table 1. Body weight of Japanese quail selected for high (HET) and low (LET) testosterone content. Values are expressed as mean ± SEM.

	Group	n	1. day	SEM	4. day	SEM	7./8. day	SEM	11. day	SEM	14. day	SEM	19. day	SEM
Qualitative restriction 1	HET	65	6.03	0.08	13.29	0.22	22.40	0.34			47.41	0.58		
	LET	61	5.72	0.06	13.23	0.19	22.85	0.34			47.23	0.61		
	Control	67	5.87	0.07	13.24	0.19	23.54	0.28			48.90	0.41		
	Restriction	59	5.89	0.08	13.28	0.22	21.57	0.36			45.53	0.70		
Qualitative restriction 2	LET	40	6.15	0.06	12.01	0.20	23.50	0.36			47.73	0.59		
	HET	40	6.09	0.10	11.85	0.18	24.27	0.36			49.78	0.61		
	Control	40	6.10	0.07	11.95	0.17	24.74	0.25			49.64	0.51		
	Restriction	40	6.14	0.10	11.90	0.21	23.03	0.41			47.86	0.70		
Quantitative restriction	HET	40	6.51	0.08	11.04	0.25	16.59	0.55	28.16	1.40	36.59	2.04	53.09	2.98
	LET	40	6.23	0.09	11.25	0.24	15.92	0.48	26.40	1.35	34.08	1.98	48.88	3.07
	Control	40	6.36	0.09	11.32	0.24	18.68	0.36	35.00	0.64	47.04	0.76	68.84	0.94
	Restriction	40	6.39	0.08	10.96	0.24	13.82	0.34	19.55	0.60	23.63	0.75	33.12	1.15

Table 2**.** Acute phase response of Japanese quail selected for high (HET) and low (LET) testosterone content measured 3 hours after lipopolysaccharide treatment.

Cort – plasma corticosterone concentration, He/Ly ratio – heterophil/lymphocyte ratio, IgY – plasma immunoglobuline Y. Values are expressed as mean ± SEM.

	Quantitative restriction						**Qualitative restriction**					
	Cort		**He/Ly ratio**		**IgY**		**Cort**		**He/Ly ratio**		**IgY**	
	$F_{1,42}$	p	$F_{1,42}$	p	$F_{1,42}$	p	$F_{1,40}$	p	$F_{1,40}$	p	$F_{1,40}$	p
Line	0.740	0.395	0.197	0.659	0.037	0.848	0.085	0.772	6.358	**<0.050**	9.605	**<0.010**
Restriction	0.528	0.472	0.827	0.368	0.635	0.430	3.233	0.081	15.890	**<0.001**	0.786	0.382
Treatment	1.606	0.212	26.765	**<0.001**	0.198	0.659	10.498	**<0.010**	33.508	**<0.001**	5.026	**<0.050**
Line x Restriction	0.010	0.923	1.846	0.181	0.017	0.897	2.268	0.141	0.089	0.767	0.074	0.787
Line x Treatment	0.298	0.588	0.074	0.787	0.066	0.798	0.012	0.915	0.138	0.712	0.017	0.897
Restriction x Treatment	0.890	0.351	0.011	0.916	0.027	0.871	3.679	0.062	0.005	0.944	0.743	0.393
Line x Restriction x Treatment	0.008	0.931	1.263	0.267	0.159	0.692	2.782	0.103	0.000	0.984	1.161	0.287

Both kinds of food restriction influenced body weight and physiological state of quail. Mild quantitative restriction had no or even positive effects on the immune system, while qualitative restriction clearly exceeded physiological limits and caused immunosuppression. However, we noticed no serious impairment of immune system of quail selected for high egg testosterone kept under severe restriction, even though we repeatedly observed higher growth in his line. Throughout our experiments we observed various effects of genetically raised egg testosterone on immune parameters, ranging from positive up to negative. Therefore we suspect that genetic selection alone does not have a consistent impact on their immune system and effects of selection can be modulated by environmental conditions and possibly by other maternal substances, like maternal antibodies.

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DIFFERENCES IN SERUM AND EGGSHELL MINERALS IN LAYING HENS AND BROILER BREEDERS

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Abstract

This study was carried out to investigate the differences in eggshell quality parameters, eggshell minerals content and blood serum minerals concentration in two production types (laying hens and broiler breeders). In the experiment 96 birds were used, 48 of laying hens Lohmann Brown and 48 of broiler breeders Cobb 500. Significant differences were determined in egg weight ($P \leq 0.001$) with higher values in broiler breeders. On the other hand, eggshell percentage ($P \leq 0.001$), thickness ($P \leq 0.006$), specific gravity ($P \leq 0.003$) were higher in laying hens. Significantly higher eggshell P ($P \leq 0.002$) and Mg ($P \leq 0.001$) content were determined in broiler breeders. Significantly higher serum Mg concentration ($P \leq 0.001$) and Zn concentration ($P \leq 0.001$) were detected in laying hens.

Keywords: Eggshell quality, shell mineral content, Serum mineral laying hens, broiler breeders.

Introduction

The shell quality remains one of the most important factors affecting poultry industry. In laying hens, egg weight and shell quality are important for the producers because these traits are related to the economics of production, moreover a high quality of both external and internal characteristics need to be maintained in broiler breeder eggs because these influence embryo development (Tůmová and Gous 2012).The egg shell quality characteristics varied between production types, with the weight of shell as a proportion of egg weight (shell percentage) being significantly higher ($P \leq 0.002$) in laying hens compared to broiler breeders (Tůmová and Gous (2012). Eggshell can be influenced by many factors in which minerals content is very important. The importance of minerals is reflected in changes of arrangement pattern of shell membrane fibers in relation to the structural composition of the eggshell. Mineralized eggshell is about (96%) calcium carbonate; the remaining components include the organic matrix (2%) as well as magnesium, phosphorus and a variety of trace elements (Nys et al. 2004).

Calcium is the major structural element in the eggshell and large amounts of Ca are required to synthesize the shell, the calcification process is always associated with a decline in serum concentration of both total and ionized Ca. Pavlík et al. (2009) reported that increasing serum Ca level was associated with decreasing eggshell strength and thickness. Identically, Řezáč et al. (2000) determined the highest serum Ca levels in laying hens producing eggs with damaged shells. In contrast Hester et al. (1980) reported that a decrease of blood plasma Ca levels had no significant effect on eggshell quality. Phosphorus is the second main mineral in the eggshell; it does not only occur in vesicles in the shell cuticle but is incorporated at very low concentrations into the outer regions of the eggshell. Phosphorus concentration then increases until eggshell termination, supporting the concept of the role of phosphorus in termination of eggshell formation (Cusack et al. 2003). In the case of phosphorus plasma level, no significant difference was found between egg-type hens and meat-type hens (Suchý et al. 2004). Although the eggshell is composed of calcium carbonate, metabolism of both Ca and P is closely related such that a deficiency in one can interfere with proper utilization of the other. The shells of the hens with magnesium deficient diets were thinner than those receiving sufficient magnesium. Magnesium incorporated into the developing shell of the domestic hen only in regions of high organic content (Cusack et al. 2003). Zink as well have a great effect on eggshell. In broiler breeder hens the use of amino acid complexes of Zn increased specific gravity and reduced amount of cracked eggs (Hudson et al. 2004).
The main aim of the study was to compare eggshell quality, eggshell mineral composition and serum mineral concentration in laying hens and broiler breeders.

Material and methods

In total, 96 birds were used in the experiment which lasted seven weeks. Birds were placed into four environmental chambers in which temperature and humidity were controlled. In each chamber 24 birds were housed in individual cages, 12 of Lohmann Brown laying hens, the remaining 12 birds in each chamber were Cobb 500 broiler breeder hens. The lighting regime used consisted of 14 h light and 10 h darkness, with lights being turned on at 5:00 am and off at 7:00 pm. Laying hens were given a commercial laying feed *ad libitum* (174 g/kg crud proteins, 11.65 MJ ME, 32.6 g/kg Ca, 4.8 g/kg P, 2.3 g/kg Mg and 168 ppm Zn). Broiler breeders received 160 g of a high protein broiler breeder feed daily (174 g/kg crud protein, 11.31 MJ ME, 32.8 g/kg Ca, 5 g/kg P, 2.3 g/kg Mg and 180 ppm Zn).

Blood and egg sampling

Blood samples were collected separately at 7:30 morning. Over a period of three weeks, blood samples were taken from 12 birds per strain, being 288 samples in total. Selected birds were bled from a wing vein with a frequency of no less than once a week. Eggs for shell quality measurements were collected on the day of blood collection from the birds sampled.

Blood and egg analyses

Biochemical parameters analyzed in serum were calcium, phosphorus, magnesium and zinc. Minerals were determined photometrically in a Libra S 22 spectrophotometer (Biochrom Ltd., UK) using standard commercial kits (Randox Laboratories Ltd., Crumlin, UK). Eggs were weighed individually at the day of collection. The method used to measure specific gravity was method of Richards and Swanson (1965). The shell weight was determined by a method described by Skřivan et al. (2013). Shell thickness was evaluated by taking the mean of 3 measurements in the equatorial region using a micrometer (Mitutoyo UK Ltd.). After completenig the physical measurements, shells were analyzed for Ca, P, Mg and Zn content. Ca and P were determined by a method described by Englmaierová et al. (2013). AOAC International (2005) procedures were used to determine the crud protein, Mg and Zn in feed mixtures and eggshells. Mineral elements were analysed on Varian ICP spectrophotometer (Varian, Inc.).

Statistical analysis

Data collected during the experiment were analyzed by SAS program (SAS 2003) using the general analysis of variance Anova method. Differences between strains were analyzed by t-test. A P-value of $P<0.05$ was considered significant for all measurements.

Results and discussion

Eggshell quality characteristics were varied between production types (Table 1). Egg weight was significantly higher in broiler breeders eggs than laying hens

Table 1. Mean eggshell parameters in laying hens and broiler breeders.

	Laying hens	Broiler breeders	RMSE	P
Egg weight (g)	60.13^{b}	68.18^{a}	7.22	0.001
Eggshell percentage (%)	11.80^{a}	10.88^{b}	1.74	0.001
Eggshell thickness (mm)	0.314^{a}	0.298^{b}	0.037	0.006
Specific gravity	1.08^{a}	1.07^{b}	0.020	0.003
Shell index (g / 100cm^2)	9.87	9.50	1.45	0.113

^{a,b}statistically significant differences ($P \leq 0.05$) within columns are indicated by different superscripts

On the other hand eggshell percentage ($P \leq 0.001$), eggshell thickness ($P \leq 0.006$), and specific gravity ($P \leq 0.003$) were higher in laying hens than broiler breeders, these results correspond with Tůmová and Gous (2012) who found higher eggshell parameters in laying hens. Moreover, we found that shell index did not differ in both production types. Minerals content play an important role in eggshell quality. In this study we evaluated the minerals content in both production types (Table 2).

Table 2. Mean eggshell minerals content in laying hens and broiler breeders.

	Laying hens	Broiler breeders	RMSE	P
Ca (g/kg)	346.8	348.8	1.97	0.537
P(g/kg)	1.16[b]	1.38[a]	0.042	0.002
Mg (g/kg)	3.35[b]	4.00[a]	0.052	0.001
Zn (mg/kg)	48.3	42.1	2.11	0.070

[a,b]statistically significant differences ($P \leq 0.05$) within columns are indicated by different superscripts

We found out that no significant differences in Ca and Zn content between laying hens and broiler breeders, however, P and Mg content were significantly higher ($P \leq 0.002$, $P \leq 0.001$ sequentially) in broiler breeders.

Blood serum minerals concentration was also evaluated in this study in both production types (Table3). No significant differences in serum Ca concentration between laying hens and broiler breeders were detected. On the other hand, Suchý et al. (2004) reported that the average plasma levels of calcium in meat-type hens were highly significantly ($P \leq 0.01$) lower in most cases compared with those of egg-type hens.

Table 3. Mean serum minerals content in laying hens and broiler breeders.

	Laying hens	Broiler breeders	RMSE	P
Ca (mmol/L)	5.01	4.55	2.68	0.053
P (mmol/L)	1.87	1.84	0.659	0.563
Mg (mmol/L)	1.48[a]	1.29[b]	0.356	0.001
Zn (mmol/L)	82.11[a]	61.80[b]	17.63	0.001

[a,b]statistically significant differences ($P \leq 0.05$) within columns are indicated by different superscripts

Pavlík et al. (2009), Řezáč et al. (2000) reported that increasing serum Ca levels in laying hens were associated with decreasing eggshell strength and thickness. On the other hand, Hester et al. (1980) reported that a decrease in blood plasma Ca levels had no significant effect on eggshell quality. No significant differences have been found in serum phosphorus

between laying hens and broiler breeders, similar results were reported by Suchý et al. (2004). Moreover Pavlík et al. (2009); Boorman and Gunaratne (2001) observed that there is in fact no relationship between plasma phosphorus levels and eggshell weight. Furthermore serum concentration of Mg and Zn were significantly higher ($P \leq 0.001$) in laying hens. Kaya et al. (2001) found a positive correlation between plasma zinc concentrations and egg production.

Conclusion

In this study we observed significant differences between laying hens and broiler breeder in content of P and Mg in the eggshell; however, there was no effect of production types on Ca and P concentration of blood serum. Significant differences in Mg and Zn concentration in blood serum were detected. These results could help to produce eggs with better eggshell quality because of the differences between strains in eggshell percentage, eggshell thickness and eggshell strength.

Acknowledgement

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INSECT ANTI-STRESS HORMONES AND THEIR ROLE IN KEEPING OF METABOLIC HOMEOSTASIS

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Insects possess a well-organised and relatively well-explored endocrine system producing three basic groups of insect hormones. (1) Juvenile hormones of sesquiterpene origin produced by the corpora allata a gland situated near the brain, and controlling insect metamorphosis and reproduction. (2) Steroid hormones called ecdysteroids (or earlier moulting hormones) produced mainly in prothoracic gland localized in the first thoracic article and head. Ecdysteroids control larval moulting and metamorphosis, and reproduction in adults. And, (3) a huge group of peptide neurohormones (dozens or a few hundreds representatives) produced by neurosecretory cells of CNS mainly of brain. These hormones are involved in a control of practically all aspects of insect life.

Adipokinetic hormones represent an important group of insect neurohormones. These hormones belong to the AKH/RPCH (adipokinetic hormone/red pigment concentrating hormone) arthropod peptide family. In insects, the AKHs are synthesised, stored and released by neurosecretory cells from the corpora cardiaca, a neuroendocrine gland connected with the brain. AKHs comprise 8 to 10 amino acids (Gäde et al., 1997) and their signal transduction at the cellular level including specific membrane receptor (Park et al., 2002; Staubli et al., 2002) is well documented in the fat body (Gäde et Auerswald, 2003). The AKH peptides behave as typical stress hormones by stimulating catabolic reactions (mobilise lipids, carbohydrates and certain amino acids), making energy more available (Gäde et al., 1997), and by suppressing processes that are momentarily less important and could, if allowed to continue, even draw on the mobilized energy. It is evident that the kind of AKH-mobilized energy substrate is species specific depending on general metabolism of the particular insect species. For example in the fruit fly *Drosophila melanogaster*, ablation of AKH secreting cells results in a profound decrease in circulating carbohydrate levels (Isabel et al., 2005), while in the firebug *Pyrrhocoris apterus* injection of AKH significantly increases a level of haemolymph lipids (Kodrík et al., 2000). However, AKH peptides are pleiotropic, with a number of actions that boost their main roles in stress energy metabolism, and that are responsible for keeping of metabolic homeostasis (Kodrík, 2008). Among other functions the AKHs stimulate heart beat (Scarborough et al., 1984) and general locomotion (Kodrík et al., 2000), enhance immune responses (Goldsworthy et al., 2002), regulate starvation-induced foraging behaviour of

Drosophila (Lee et Park, 2004), participate in the activation of the anti-oxidant mechanisms (Kodrík et al., 2007; Velki et al., 2011; Krishnan et Kodrík, 2012), and as found recently, enhance also a food intake and digestive process in insect gut (Kodrík et al., 2012), and activity of salivary glands (Vinokurov et al., 2014). On the other hand the AKHs inhibit synthesis of RNA (Kodrík et Goldsworthy, 1995), proteins (Carlisle et Loughton, 1979) and lipids (Gokuldas et al., 1988). On the physiological level the AKHs inhibit the egg maturation (Lorenz, 2003).

It is supposed the AKH stress response is complex involving metabolic, biochemical, physiological and behavioural levels. Its probable course is schematically shown in Fig. 1.

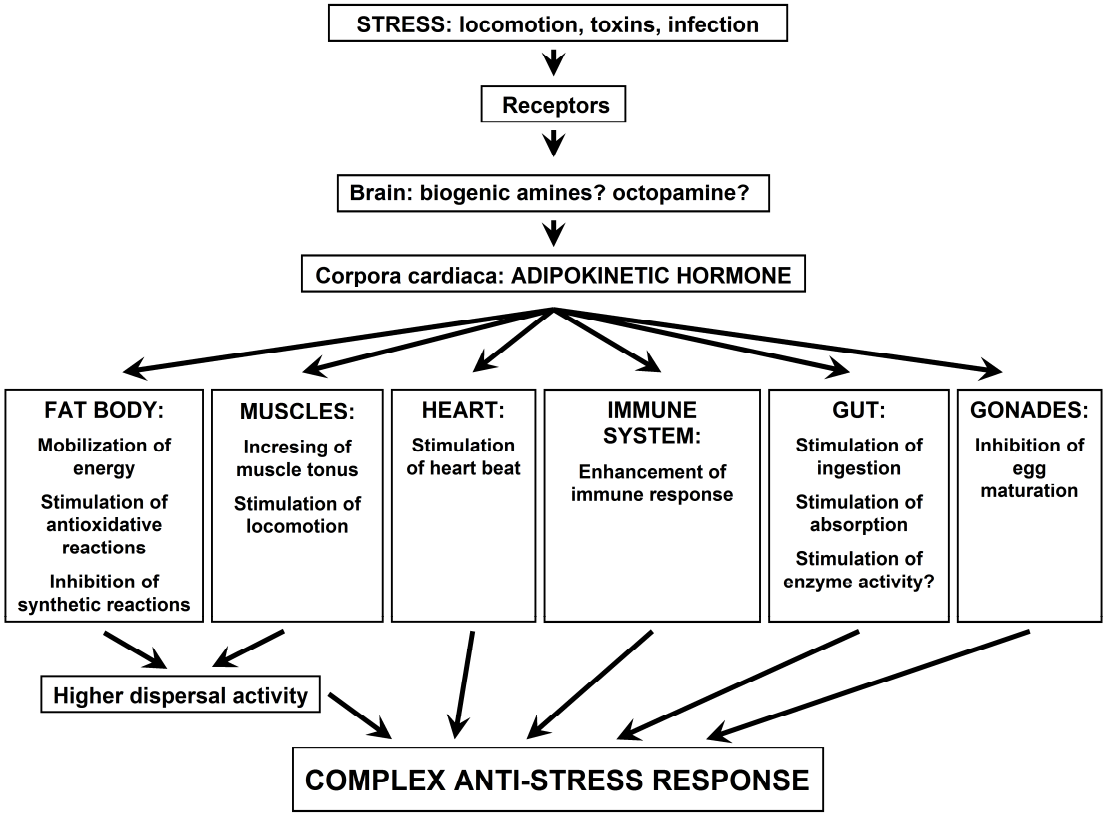

Figure 1. Hypothetic model of anti-stress response in insects controlled by adipokinetic hormones.

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ASSESSMENT OF APOPTOTIC OVARIAN CELLULAR MECHANISM INDUCED BY MYCOTOXIN T-2 TOXIN *IN VITRO*

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Abstract

Occurrence of mycotoxins in food and feed is a worldwide problem and therefore study of these toxins could be beneficial for better understanding of mechanism of their toxic effects in organism. The aim of this study was to examine the effect of various doses of T-2 toxin on process of apoptosis and apoptotic marker Bax in porcine ovarian granulosa cells *in vitro*. In our experiments the percentage of apoptotic cells and cells containing apoptosis related peptide Bax were not significantly ($P>0.05$) affected by treatment of T-2 toxin at the doses 10, 100 and 1000 ng/ml during 24 h period. The results showed that trichothecene as T-2 toxin did not modulate apoptotic marker Bax and process of apoptosis in porcine ovarian granulosa cells *in vitro* but it was a pilot study in this field and it is necessary to realize more *in vitro* and *in vivo* experiments. Therefore, study of *Fusarium* toxin T-2 toxin is very required issue, because they could also play role in failures of reproductive functions as well as endocrine system of domestic animals.

Introduction

Mycotoxins which are contaminants of animal feed can impair growth and/or reproductive efficiency. This is especially prominent in prepubertal gilts (Dänicke, 2002). On the other hand application of chemical additives containing organic acids, organic salts and inorganic salt was sufficient to inhibit mycotoxins formation (Biro et al., 2009). T-2 toxin is considered to be the most common trichothecene mycotoxin belonging to type A and is produced predominantly by *Fusarium sporotrichioides* and *F. langsethiae* (EFSA, 2011; Kokkonen et al., 2010). Oral, parenteral and cutaneous exposures to T-2 toxin induce lesions in various

tissues as hematopoietic, lymphoid and gastrointestinal tissues and suppress reproductive organ functions (Williams, 1989; IARC, 1993; Sharma, 1993). This toxin is rapidly adsorbed and rapidly excreted without any accumulation in any tissue (Schlatter, 2009). T-2 toxin has potent direct dose-dependent effects on porcine ovarian functions (Caloni et al., 2009). Apoptosis plays an essential role in survival of the organisms and is considered to be an imperative component of various processes including normal cell turnover, proper development and functioning of the immune system, multiplication of mutated chromosomes, hormone-dependent atrophy, normal embryonic development, elimination of indisposed cells and maintenance of cell homeostasis (Reed and Tomaselli, 2000; Elmore, 2007). The aim of this study was to examine the effect of various doses of T-2 toxin on process of apoptosis and presence of apoptotic marker Bax in porcine ovarian granulosa cells *in vitro*.

Material and Methods

Ovarian granulosa cells

Ovarian granulosa cells were isolated by centrifugation for 10 min at 200xg followed by washing in sterile DMEM/F12 1:1 medium and resuspended in the same medium supplemented with 10 % fetal calf serum and 1 % antibiotic–antimycotic solution at a final concentration of 10^6cells/ml (determined by haemocytometer). Portions of the cell suspension were dispensed to Lab–Tek 16–welled chamber slides (Nunc Inc., International, Naperville, USA, 200 µl per well) for analysis (Kolesarova et al., 2010). The plate wells were incubated at 37.5 ºC and 5% CO_2 in humidified air until a 75% confluent monolayer was formed (5 days). At this point, the medium was renewed and ovarian granulosa cells were incubated with 10 % fetal calf serum (BioWhittaker™) with T-2 (Romer Labs Division Holding GmbH, Tulln, Austria) at the various doses 0, 1, 100, 100 ng/ml. After 24h the culture media from chamber slides were aspirated and prepared for imunofluorescene (Kolesarova et al., 2010).

Imunofluorescence

Visualization of the primary antibody binding sites was achieved with fluorescein isothiocyanate (FITC) – green fluorescent antibody stain (Sevac, Prague, Czech Republic; dilution 1:1000). Chamber slides stained with peroxidase/diaminobenzidine (DAB) (Roche Diagnostics Corporation, IN, USA, 10%) reagent were mounted with Glycergel (DAKO, Carpinteria, CA, USA). The general cell morphology, presence and localization of specific immunoreactivity in cells, as well as the counting of the percentage of cells containing specific immunoreactivity was determined by fluorescente microscope (Kolesarova et al., 2010).

Significance of differences between the control and experimental groups were evaluated by one-way ANOVA and t-test using the statistical software Sigma Plot 11.0 (Jandel, Corte Madera, 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 and Discussion

In our experiments the percentage of apoptotic cells and cells containing apoptosis related peptide Bax were not significantly ($P>0.05$) affected by treatment of T-2 toxin at the doses 10, 100 and 1000 ng/ml but tendency of decreasing of these parameters was detected (Figs. 1, 2).

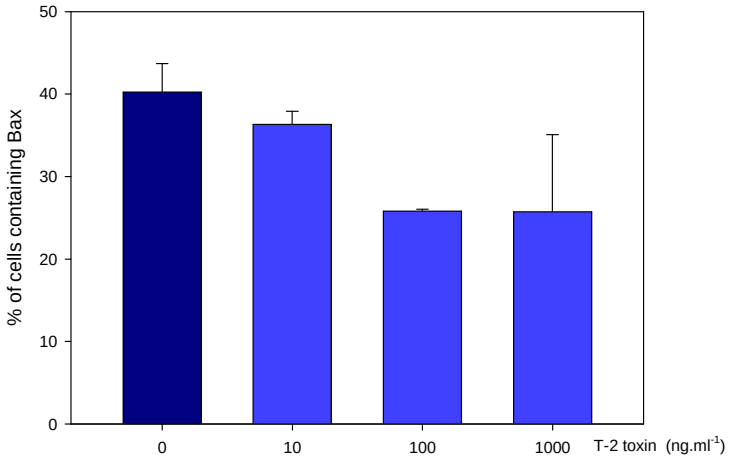

Figure 1. Percentage of cells containing Bax after 24 hours long treatment of T-2 toxin. The data are expressed as means ± SEM. Imunofluorescence method.

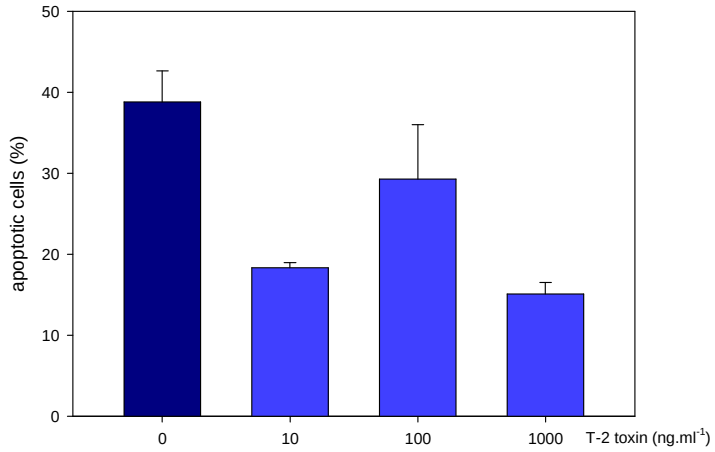

Figure 2. Percentage of apoptotic cells after 24 hours long treatment of T-2 toxin. Imunofluorescence method.

Membrane-active properties of various mycotoxins determine their toxicity and incorporation of mycotoxins into membrane structures causes various detrimental changes, resulting in alterations in second messenger systems through damaging membrane receptors (Surai et al.,

2008). The amphipathic nature of trichothecenes allows these toxins to cross the cell membrane and interact with different organelles such as the mitochondria (Pace, 1983; Schappert and Khachatourians, 1986) and endoplasmic reticulum (ER) (Yang et al., 2000). Mechanistically, a balance between cell survival and apoptosis may be mediated by oxidative stress. Oxidative stress refers to a pathological state when pro-oxidants (reactive oxygen or nitrous species) are not neutralized adequately by antioxidant defenses (Townson et al 2012). Oxidative stress is certainly involved in the toxicities of trichothecene mycotoxins including T-2 toxin (El Golli et al., 2006). Our results did not show significant ($P>0.05$) effect of T-2 toxin at the doses 10, 100 and 1000 ng/ml on the percentage of apoptotic cells and cells containing apoptosis related peptide Bax. On the other hand results of previous *in vivo* study clearly show that the number of apoptotic cells in rabbits was slightly increased by T-2 toxin (Lesniak et al., 2013). Oxidative stress is the underlying mechanism by which T-2 toxin causes DNA damage and apoptosis (Chaudhary et al., 2009). Fang et al. (2012) demonstrate that T-2 toxin induces oxidative stress and apoptosis in differentiated murine embryonic stem cells (ES) cells, and ROS-mediated mitochondrial pathway plays an important role in T-2 toxin induced apoptosis. Wu et al. (2011) demonstrated that T-2 toxin-induced cells apoptosis was accompanied by the upregulation of p53 mRNA and protein level in a dose-dependent manner in granulosa cells. Increases in Fas, p53 and the pro-apoptotic factor Bax protein and mRNA expressions and a decrease of the anti-apoptotic factor Bcl-xL were observed in a dose-dependent manner after exposures to 1~20 $ng.ml^{-1}$ T-2 toxin, while the expression of the anti-apoptotic factor Bcl-2 was unchanged (Chen et al., 2008). Moreover T-2 toxin increases the expression of p53, a pivotal apoptotic protein, and other proteins such as Bax, Bcl-2, cytochrome-c involved in mitochondrial apoptotic pathway (Chaudhari et al., 2009a).

Currently, occurrence of mycotoxins in food and feed is a worldwide problem and therefore study of these toxins could be beneficial for better understanding of mechanism of their toxic effects in organism. Results in this study showed that trichothecene as T-2 toxin did not modulate apoptotic marker Bax and process apoptosis in porcine ovarian granulosa cells *in vitro* but it was a pilot study in this field and it is necessary to realize more *in vitro* and *in vivo* experiments. Therefore, study of *Fusarium* toxin T-2 toxin is very required issue, because they could also play role in failures of reproductive functions as well as endocrine system of domestic animals.

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NATURAL PROTECTIVE AND TOXIC SUBSTANCES MODIFY OVARIAN FUNCTIONS

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Abstract

Acute ingestion of high levels of mycotoxins can be very harmful to the animal, long term consumption of low concentrations of mycotoxins can also be damaging. There are several ways of reducing mycotoxin concentrations both pre and post-harvest, including the addition of feed additives such as, but not limited to, natural clays, yeasts, and enzymes. Natural protective substances may inhibit ovarian toxicity induced by toxic substances – mycotoxins. Tissue and cell cultures are of increasing interest in the evaluation of toxicological risks of contaminated compounds and their possible elimination. It is for this reason the solution of the problem in the field of examining effects of the potential protective natural substances in the animal organism and their mechanism of the effect is highly relevant.

Introduction

Protective substance - resveratrol

Natural protective substances may inhibit reproductive toxicity induced by mycotoxins (Kolesarova et al., 2012a). Resveratrol (3,5,4´-trihydroxystilbene) attracted little interest until 1992, when it was postulated to explain some of the cardio-protective effects of red wine (Das et al., 2010; D'Mello et al., 1999). It is a natural polyphenol widely present in plants and in particular in the skin of red grapes and in wine, resveratrol antioxidant properties have been well demonstrated, with a wide range of biological effects (Karuppagounder et al., 2009). It is beneficial against diverse cardiac diseases including ischemic heart disease, hypertrophy, heart failure, atherosclerosis, hypertension, diabetes and obesity (Bertelli and Das, 2009; Mukherjee et al., 2009). Resveratrol also has protective role in endothelial cells by modulating mitochondrial oxidative stress (Ungvari et al., 2009). It promotes apoptosis to reduce rat ovarian theca-interstitial cells growth in vitro (Wong et al., 2010). Zhou et al. (2011) explored the molecular mechanisms of resveratrol-induced apoptosis in pancreatic

cancer cells. These data indicate resveratrol a potent medicine to treat human pancreatic cancer. The most prominent mechanism of action is probably through its ability to perform intracellular signaling and altering gene expression. Resveratrol can alter a variety of genes thereby changing the ‘‘death signal’’ into a ‘‘survival signal’’ (Das and Maulik, 2004). Svechnikov et al. (2009) present investigation has demonstrated that resveratrol and its analogues structure-dependently attenuated steroidogenesis in Leydig cells through suppression of the expression of StAR and cytochrome P450c17. Furthermore, systemic administration of resveratrol showed slightly toxic effect (Baur and Sinclair, 2006).

Toxic substance – mycotoxin

Mycotoxins are natural and very stable toxins, with relatively low-molecular weight secondary metabolites of fungal origin, which can contaminate a large variety of feed mixtures (Labuda et al., 2009), grains and foodstuffs worldwide (Ranzenigo et al., 2008). They are found in a variety of foods and beverages, including both plant-based products and animal products. Among the first ones, its presence in cereal grains (corn, wheat, barley, oats, rye, rice, etc.) and beans (coffee, cocoa, soy, etc.) are harmful to animals and humans (Abouzied et al., 1991). Addition of biological and biochemical additives to ensiled matter of corn (Biro et al., 2009) and alfalfa (Galik et al., 2008) may reduce the concentration of mycotoxins in fermented silages. However, not all fungi produce mycotoxins and among the toxigenic species, some only produce one type of mycotoxin, while others are able to produce several. Cereals can be mostly contaminated by *Fusarium* species, a group of toxin-producing molds (Larsen et al., 2004; Giraud et al., 2010).

Secretory activity of ovarian granulosa cells

In the study of Kolesarova et al. (2012) stimulatory effect of RSV on progesterone release by GCs was recorded after resveratrol treatment at the dose of 50 μg/ml, while doses of 30 and 10 μg/mL did not affect the release of the steroid hormone. On the other hand, inhibitory actions of resveratrol analogs on steroidogenesis in Leydig cells indicate novel mechanisms of action of these compounds, which may be of potential therapeutic interest, where suppression of androgen action is needed (Svechnikov et al., 2009). On the other hand, progesterone release was stimulated by toxic doses of DON (2000, 3000 and 5000 ng/ml) used in the experiments (Kolesarova et al., 2012a). The effect of DON at the various doses (10, 100 and 1000 ng/ml) in relation to progesterone release by porcine GCs have been examined in previous study (Medvedova et al., 2011). Findings of Kolesarova et al. (2012a)

are in accordance with previous report of Medvedova et al. (2011). DON at the highest dose (1000 ng/ml) significantly stimulated ($P<0.05$) progesterone release by GCs but not at the lower doses (10 and 100 ng/ml) (Medvedova et al., 2011). The effect of the DON was found to be concentration dependent (Medvedova et al., 2011; Ranzenigo et al., 2008). In the study of Kolesarova et al. (2012a) RSV in combination with DON at the highest doses (50 µg/mlof RSV and 5000 ng/ml of DON) stimulated progesterone release by GCs. Stimulatory effect of alone DON on the progesterone release by GCs was lower in comparison with RSV in combination with DON. In previous study curcumin (polyphenolic antioxidant purified from turmeric) and resveratrol (polyphenol obtained from grapes) were evaluated for possible protection against liver injury induced by aflatoxin B(1) in rats (El-Agamy, 2010).

Conclusion

The results of Kolesarova et al. (2012) indicate, (1) the dose-depended stimulatory effects of RSV, DON and combination of RSV with DON on release of steroid hormone progesterone and (2) reduction of the stimulatory effect of DON by RSV. Acute ingestion of high levels of mycotoxins can be very harmful to the animal, long term consumption of low concentrations of mycotoxins can also be damaging. There are several ways of reducing mycotoxin concentrations both pre and post-harvest, including the addition of feed additives such as, but not limited to, natural clays, yeasts, and enzymes. Natural protective substances may inhibit ovarian toxicity induced by toxic substances – mycotoxins. Tissue and cell cultures are of increasing interest in the evaluation of toxicological risks of contaminated compounds and their possible elimination. It is for this reason the solution of the problem in the field of examining effects of the potential protective natural substances in the animal organism and their mechanism of the effect is highly relevant.

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ANALYSIS OF GSH CONCENTRATION IN SELECTED BRAIN STRUCTURES OF MICE AFTER INTRAPERITONEAL INJECTION OF ACRYLAMIDE

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Glutathione (GSH) is very important antioxidant. Composted of three amino acids - cysteine, glycine, and glutamate - glutathione can be found in virtually every cell of the human body. The highest concentration of glutathione is in the liver, making it critical in the body's detoxification process. GSH is a substance which is responsible for defence system. A lot of factors e.g. heavy metals, radiation, certain medications, and the normal process of aging can cause free-radical damage to healthy cells and deplete glutathione. Glutathione depletion has been correlated with lower immune function and increased vulnerability to infection due to the liver's reduced ability to detoxify. As the generation of free radicals exceeds the body's ability to neutralize and eliminate them, oxidative stress occurs. A primary function of glutathione is to alleviate this oxidative stress (Pompella, 2003).

International Agency for research on Cancer classified acrylamide as probable human carcinogen (Chico et al., 2006; Mustafa et al., 2008). Acrylamide is a good example of such toxic ingredients of human diet. It is present in numerous popular food products. Very high acrylamide contents were found in fried potatoes (170-2287 µg/kg) and potato chips (50-3500 µg/kg). High acrylamide contents occurred also in breakfast cereals (30-1400 µg/kg) or coffee (170-351 µg/kg). Toxic influence of acrylamide was documented in several studies (Szczerbina et al., 2007). Numerous reports indicate the involvement of free radicals in acrylamide cytotoxicity. Acrylamide as popular neurotoxine interferes balance of our organism and in serious imbalance in prooxidant/antioxodant state of cells (Szczerbina et al., 2007).

The aim of our work was to estimate concentration of GSH in selected brain structures after acrylamide: the right hemisphere, the left hemisphere, cerebellum and brainstem.

The experiment was carried out on 24 male mice of Swiss strain, average body weight 25 to 26 g, fed standard diet with unlimited access to water. The measurements were performed after 48, 72 and 192 hours after acrylamide injection in two doses – 40 mg/kg and 80 mg/kg. The animals were segregated into three experimental and one control group. The brain structures were homogenized in 1 ml of cooled phosphate buffer pH=7,4 with EDTA. Next the homogenates were centrifuged at speed 15.000 for 15 min at 4°C. The supernatant which was obtained from brain homogenates has been purified from protein. Then, mixed and refrigerated for 10 minutes and prepare to board composition 180 ml H2O + 15 ml + 20 ml EDTA Tris + 20 ml + 10 ml of the supernatant DTNB. After 10 minutes incubation at 4°C the extinctions were measured at a wavelength of λ = 412 nm using microplate reader SUNRISE TECAN.

One-way analysis of variance (ANOVA) and the Dunnett´s multiple comparison test were used for statistical evaluations. We noticed influence of acrylamide on acetylcholinesterase activity in selected brain structures. There were significant changes in the activity of Glutathione concentration in each structure after 48, 72 and 192 hours. This changes were after both doses of acrylamide (40 mg/kg and 80 mg/kg). Both doses of acrylamide caused decreased GSH content. In most of the structures the significance level was at $p<0,001$.

It seems that the decrease of GSH content activity was associated with toxic properties of acrylamide.

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SYNERGISTIC EFFECTS OF □-GLUCAN AND RESVERATROL ON PLATELET AGGREGATION

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Abstract

In the last years β-glucan and resveratrol are investigated in both veterinary and human medicine. Many findings about their immunomodulatory effects and effects on blood parameters can be found in literature. Recent studies show that biological effect of β-glucan and resveratrol is often similar and can be mediated via identical mechanisms. Our work follows previous information about significant effect of resveratrol on platelet activity in laboratory rats. The aim of this study was to verify these data in growing piglets and newly investigate the role of resveratrol during simultaneous β-glucan supplementation.

Materials and Methods

6-week-old Large White breed piglets were divided into one control group (C) and two experimental groups: R (resveratrol) and RG (resveratrol + β-glucan). Resveratrol isolated from *Polygonum cuspidatum* root and β-glucan gained from Saccharomyces cerevisiae were soluted in 15% alcohol and administered to the piglets by gastric probe in a daily dose 3 $mg/kg^{0.75}$ of body weight. Control piglets obtained only alcohol solution. Blood samples were collected by puncture of v. jugularis and tested for number of platelets, platelet aggregation and speed of platelet aggregation after stimulation by ADP and cationic propylgallate.

Results

In comparison to control piglets number of platelets dropped markedly in both experimental groups. After one week of supplementation (R: $P<0.05$; RG: $P<0.01$) and after 2 weeks (both R and RG: $P<0.01$). Aggregation induced by ADP was significantly reduced after 2 weeks in both experimental groups (R: $P<0.01$; RG: $P<0.05$) while aggregation induced by cationic propylgallate was significantly reduced only in RG after 2 weeks ($P<0.05$). Speed of aggregation (slope) induced by ADP significantly decreased after 2 weeks (both R and RG: $P<0.01$). Speed of aggregation (slope) induced by cationic propylgallate significantly decreased in both experimental groups (R: $P<0.05$; RG: $P<0.01$).

Conclusion

Results suggest that neither suppressive effect of resveratrol on platelet numbers nor its "aspirin-like" effect is inhibited by simultaneous administration of β-glucan. On the contrary we demonstrated synergistic effect of both agents in experimental piglets.

EFFECT OF TREHALOSE ON THE MORPHOLOGICAL AND ACROSOMAL CHANGES OF DOGS SPERMATOZOA

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Abstract

Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) is a non-reducing disaccharide consisting of two glucose moieties joined together by an alpha-1, 1 glucosidal bond. In our work, we focused on microscopic examination of large breed dogs ejaculates. The effect of trehalose in relation to morphological changes and changes in the acrosome was verified. In the study semen from 5 large dog breeds was used. The collected samples were exposed to various concentrations of trehalose (TAT: 20 $mg.ml^{-1}$, TBT: 10 $mg.ml^{-1}$, TCT: 5 $mg.ml^{-1}$). The occurrence of morphologically abnormal spermatozoa was quantified microscopically at 1000x magnification. Comparing among groups showed significant increase in the acrosomal changes (AC) in the group of TAT compared to the control group TKT ($p<0.001$), and to the TCT group ($p<0.05$). It was found, that incidence of dilated acrosome significantly increased with increasing the concentration of trehalose (20 $mg.ml^{-1}$), when compared to the control group ($p<0.001$). A similar trend was observed by a comparison TAT group to TRT resp. TCT group ($p<0.01$). Our study indicated, that analyzed additive have effect on the analyzed parameters of spermatozoa morphology. Detected acrosomal changes are related to additive concentration.

Key words: *trehalose, acrosome, morphological changes, dog*

Introduction

Enlargement of holding dogs of various breeds brings new tasks imposed on Andrology, whose aim is the development of advanced spermatological methods to detect functional changes that cause dog fertility reduction. The current andrologic methods for evaluating quality of semen are based on macroscopic examination considering microscopic evaluation of spermatozoa morphology. However, these methods cannot determine the fertilization capacity of the spermatozoa and to predict fertility in dogs sufficiently. Therefore, new methods, based on examining the functional purpose of assessing sperm fertilizing ability, are still being developed.

Several studies have been conducted of different concentration of trehalose on semen parameters in ram (Aisen et al., 2002; Jafaroghli et al., 2011), goat (Khalili et al., 2009), boar (Hu et al., 2009) and in dog semen (Yildiz et al., 2000).

Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) is a non-reducing disaccharide consisting of two glucose moieties joined together by an alpha-1, 1 glucosidal bond (Patist and Zoerb, 2005) and is used as a non-penetrating cryoprotectant of bovine spermatozoa (Hu et al., 2010). It does not pass through cell membranes, but osmotically reduces the intracellular concentration of water and also cryoprotectants, which results in reduction of their toxicity (Chrenek et al., 2009; Ahmad and Aksoy, 2010). For these reasons, it is successfully used as an additive in the basic protocols of freezing of dogs spermatozoa to improve their motility and fertilizing ability (Asano et al., 2010).

In the nature is widespread, it exists naturally in insects, plants and fungi as well as some bacteria (Aisen et al., 2000).

In comparison with other sacharides, it is chemically stable and resistant to acids. In nature, it protects cells from damage by ice crystals creating during freezing.

In our work, we focused on microscopic examination of large breed dogs ejaculates. The effect of trehalose in relation to morphological changes and changes in the acrosome was verified.

Material and Methods Experiments were realized in *in vitro* conditions. In the study semen from 5 large dog breeds was used. They were typical representatives of large breeds (Boxer – 6 years old, Border Collie – 2 years old, Leonberger – 2 years old, Greyhound – 6 years old, Siberian Husky – 4 years old) comming from healthy rearing, regularly vaccinated and integrated to the further breeding. Semen collections were realized at the owners of animals and in the veterinary clinic. Samples were transported to the laboratory in 15 minutes at 37°C in thermos. Subsequently the experiments were realized at the Department of Animal Physiology of the SUA in Nitra.

The collected samples were exposed to various concentrations of trehalose (TAT: 20 $mg.ml^{-1}$, TBT: 10 $mg.ml^{-1}$, TCT: 5 $mg.ml^{-1}$). One sample served as a control TKT (dilution with saline) and one sample was diluted with commercial diluent for canine ejaculate (CANIPRO^{TM} chill10, Minitub Slovakia TRT). The samples were kept at 37 ° C throughout the analysis.

The occurrence of morphologically abnormal spermatozoa was quantified microscopically at 1000x magnification, and the following abnormal morphological changes were evaluated:

SDH – separated heads, AC – acrosomal changes, KTF – knob-twisted flagellum, FT – flagellum torso, FB – flagellum ball, LH – large head, SH – small head, PPS – % of physiological spermatozoa and PMC – % of morphological changed spermatozoa.

The changes that we observed the acrosome were detached acrosome (DTA), dilated acrosome (DLA), acrosome with undulate membrane (UMA).

Results and Discussion

The basic standard for working with ejaculate in the laboratory is to provide optimal conditions for the in vitro cultivation (Balaban et al., 1999; Jankovičová et al., 2006), which is a complex process in view of its further processing.

By morphological dog sperm analysis, as well as by other animal species, abnormalities are classified as primary and secondary (Ball et al., 1983; Johnson, 1991; Veznik et al., 2003).

Of the many morphological and functional studies ejaculates was determined limit 20% incidence of abnormal sperm in the insemination dose, and this also applies to ejaculate dog (Oettle, 1993; Veznik et al., 2003). It's a limit which can be considered satisfactory in terms of the fertilization effect.

On the basis of the morphological analysis of sperm in the group without addition of trehalose (TKT), we found that the total number of sperm were analyzed 87.75 ± 2.41% of physiological spermatozoa (PPS) and 12.25 ± 2.41% of morphological changed spermatozoa (PMC). Other groups have non-significant lower number of physiologically normal sperm against control group.

Comparing among groups showed significant increase in the acrosomal changes (AC) in the group of TAT compared to the control group TKT ($p<0.001$), and to the TCT group ($p<0.05$). Monitoring of other parameters has not identified significant differences between groups but we recorded increased values in all parameters, compared to the control group except LH parameter (Table 1).

Table 1. Incidence of morphologically changed forms of spermatozoa (%) after the addition of trehalose

Parameter	**TAT (20 mg.ml^{-1})**	**TBT (10 mg.ml^{-1})**	**TCT (5 mg.ml^{-1})**	**TKT (control)**	**TRT (Canipro^{TM})**
SDH	0.91 ± 0.74	0.99 ± 0.47	0.74 ± 0.42	0.58 ± 0.32	1.08 ± 0.69
AC	**5.41 ± 0.74^{AC}**	4.00 ± 0.72	**3.25 ± 0.74^{A}**	**2.16 ± 0.43^{C}**	3.17 ± 0.33
KTF	4.25 ± 0.96	3.41 ± 1.91	4.91 ± 2.38	2.16 ± 1.23	3.25 ± 1.90
FT	2.50 ± 1.94	2.25 ± 1.10	2.83 ± 0.88	2.08 ± 1.20	2.08 ± 1.26
FB	1.00 ± 0.72	1.08 ± 0.63	1.33 ± 0.94	1.25 ± 0.74	1.33 ± 0.55
SH	0.49 ± 0.19	0.42 ± 0.42	0.75 ± 0.42	0.41 ± 0.17	0.66 ± 0.66
LH	0.42 ± 0.42	0.49 ± 0.19	0.50 ± 0.33	0.74 ± 0.42	0.58 ± 0.57
PPS	81.66 ± 3.45	84.25 ± 3.42	83.25 ± 2.23	87.75 ± 2.41	86.08 ± 1.23
PMC	18.33 ± 3.46	15.75 ± 3.42	16.75 ± 2.24	12.25 ± 2.41	13.91 ± 1.23

SDH – separated heads, AC – acrosomal changes, KTF – knob-twisted flagellum, FT – flagellum torso, FB – flagellum ball, LH – large head, SH – small head, PPS – % of physiological spermatozoa and PMC – % of morphological changed spermatozoa
A $p<0.05$; B $p<0.01$; C $p<0.001$

It was found, that incidence of dilated acrosome significantly increased with increasing the concentration of trehalose (20 mg.ml^{-1}), when compared to the control group ($p<0.001$). A similar trend was observed by a comparison TAT group to TRT resp. TCT group ($p<0.01$). In addition, significantly higher occurrence of acrosome with undulate membrane in TAT group was found in comparison to control group ($p<0.05$). Presence of such acrosome changes was decreased in groups with lower concentration of trehalose (Table 2).

Table 2. Acrosomal changes (%) in observed groups after the addition of trehalose

Parameter	**TAT (20 mg.ml^{-1})**	**TBT (10 mg.ml^{-1})**	**TCT (5 mg.ml^{-1})**	**TKT (control)**	**TRT (Canipro^{TM})**
DTA	2.00 ± 0.82	3.00 ± 1.63	1.50 ± 0.58	1.00 ± 0.82	2.00 ± 0.82
DLA	**8.50 ± 1.00^{BC}**	5.75 ± 0.96	**4.00 ± 0.82^{B}**	**3.25 ± 0.96^{C}**	**4.50 ± 1.91^{B}**
UMA	**5.75 ± 1.50^{A}**	3.25 ± 0.50	4.25 ± 2.06	**2.25 ± 1.50^{A}**	3.25 ± 0.96

DTA - detached acrosome, DLA - dilated acrosome, UMA - acrosome with undulate membrane
A $p<0.05$; B $p<0.01$; C $p<0.001$

Yildiz et al. (2000) investigated the effect of saccharides (fructose, galactose, glucose, xylose, lactose, trehalose, maltose, saccharose, raffinose), Tris diluent with citric acid and glycerol on motility, sperm viability and acrosome integrity of dog spermatozoa. They found that monosaccharides increase total motility ($p<0.05$) together with viability and don´t affect the integrity of the acrosome. Trehalose, xylose and fructose significantly increased the number of active spermatozoa in comparison to other saccharides ($p<0.01$). Disaccharides, except lactose, decreased motility and there has been a demonstrable increase of acrosomal changes ($p<0.01$), which correlates with our results.

It was found, that trehalose affects the membrane integrity of spermatozoa, but also protects the sperm before freeze-shock during freezing. Molinia et al. (1994) and Matsuoka et al.

(2006) dealt with this subject. They found, that after defrosting rams spermatozoa, motility of spermatozoa which has been added trehalose is higher. The results were better than in the groups in which glucose was added.

Yamashiro et al. (2007) investigated the effect of trehalose-yolk diluent and Tris diluent on spermatozoa motility and acrosome integrity of frozen semen. In the experiment, the dogs Poodle breed were used. They found that after defrosting motility in semen with the additive trehalose with egg yolk has significantly improved.

Hu et al. (2009) dealt with effect of trehalose on the parameters of sperm motility in boars and found that after addition of 100 mM of trehalose higher mobility, acrosomal stability and membrane integrity were observed. Oxidative stress after freezing and re-defrosting was also reduced.

Conclusion

Our study indicated, that analyzed additive have effect on the analyzed parameters of spermatozoa morphology. Detected acrosomal changes are related to additive concentration. Further studies with different concentrations of aditive, temperatures, breeds will be needed to minimize morphological changes in spermatozoa.

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BLOOD SERUM ENZYMATIC ACTIVITY OF CZECH PIED BULLS DEPENDING ON SNP OF LEPTIN GENE

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Abstract

The present study was designed to evaluate the effect of leptin gene single nucleotide polymorphism on catalytic ALT, AST, ALP, GGT concentration in blood serum of Czech Pied bulls. Single nucleotide polymorphism of leptin gene in 58 Czech Pied bulls, were investigated. Resulting genotypes in the exon 2 were CC (48.3%), CT (36.2%), and TT (15.5%). There were no differences in serum AST, GGT and ALP catalytic activity among the genotypes. However, activity of ALT in serum of bulls with TT genotype was significantly higher than those of TC and CC genotypes.

Introduction

Leptin is an adipose-derived hormone that regulates a wide variety of physiological processes. As a signal from energy stores to the hypothalamus, it is involved in food-intake regulation and energy homeostasis. Reciprocally, there is long-term regulation of leptinemia by nutritional status and short-term regulation by food intake (Chilliard et al., 2001). Leptin impairs several metabolic actions of insulin, namely stimulation of glucose transport, glycogen synthesis and lipogenesis. Moreover, leptin diminishes insulin secretion of pancreatic beta cells and induces insulin resistance (Remesar et al., 1997).

Single nucleotide polymorphisms (cytosine/thymine (C/T) substitution detected at position 528 in the bovine leptin promoter region) in the leptin gene have been associated, among others, with serum leptin concentration (Liefers et al., 2002). It is known, that leptin has the potential to stimulate liver cells (Briscoe et al., 2001), and other results indicate that leptin may have preventive effect against progression of hepatic injury (Canbakan et al., 2008).

The present study was designed to evaluate the effect of leptin gene single nucleotide polymorphism on catalytic ALT, AST, ALP, GGT concentrations in blood serum of Czech Pied bulls.

Material and Methods

Animals and blood sampling

The experiment were performed in 58 Czech Pied bulls at 240 ± 9 days of age, Which were divided in three experimental groups depending on different leptin genotypes (CC, n=28; TC, n=21; TT, n=9). Blood for hormone and metabolite analyses was collected randomly from *vena jugularis externa* of bulls in three groups between 8.00 and 9.30 a.m., and sampled into the test tube with silicon gel separator and coagulation accelerator (Dispolab, Czech Republic). Serum was separated by centrifugation with 2,000 x g for 10 min at 4°C, and was stored at -20 ^{o}C until analyzed.

Leptin genotypes analysis

Blood samples (2 ml) were collected into tubes with EDTA stored at -20 °C. Genomic DNA was isolated from the samples using the QIAamp DNA Blood Mini Kit (Qiagen Inc., Valencia, CA, USA). The quality of DNA was verified by 1% agarose gel electrophoresis and sequential visualization with ethidium bromide. Genotypes were determined based on molecular genetic analysis of single-nucleotide polymorphism (SNP) in the exon 2 of the bovine leptin gene (transition C → T) (Buchanan et al., 2002). For testing, we used our own methodology. PCR primers were designed based on the nucleotide sequence of bovine leptin gene (GenBank U50365) (FW:5'TCGTTGTTATCCGCATCTGA3', REV: 5'TACCGTGTGTGAGATGTCATTG 3'). PCR was performed in 12.5 µl volumes containing 25 ng of bovine genomic DNA, 1x HotStarTaq Master Mix (Qiagen) and 0.2 µM of each forward and reverse primer. A PCR thermal profile consisted of pre-denaturation at 95 °C for 2 min; followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, elongation at 72°C for 30 s; and final extension at 72°C for 7 min. The PCR products of 278 bp in size were separated on 3% agarose gel and sequenced using the ABI PRISM 3100-Avant Genetic Analyzer. The polymorphic locus (C/T) is located at position 204 base of the fragment.

Enzymes analysis

Aspartate aminotransferase AST, alanin aminotransferase ALT, alkaline phosphatase ALP and Gamma-glutamyl transferase (GGT) were analysed on Konelab T20xt automatic analyser (Thermo Fisher Scientific, Finland) using currently available commercial kits (Biovendor-Laboratorni medicina, Czech Republic).

Statistical evaluation

Changes in serum leptin and trace elements were analyzed by one-way ANOVA for factors leptin genotype. ANOVA was followed by post-hoc Fischer LSD test. All statistical analyses

were performed by Statistica 8.0 statistical software (StatSoft Inc., Tulsa, USA). Data presented mean ± SE. The overall level of statistical significance was defined as $P < 0.05$.

Results and Discussion

Based on the results of one-way ANOVA, significant effect of leptin genotype on ALT blood serum catalytic concentration [F(2,55) = 7.517, P = 0.023] was determined (Fig. 1). Using Fisher's LSD post-hoc test, significantly lower ($P < 0.05$) ALT activity were recorded in bulls of TT group (0.551 ± 0.013 µkat.l^{-1}) compared to CC (0,381 ± 0,016 µkat.l^{-1}) and CT group (0.389 ± 0,018 µkat.l^{-1}). No significant effects of leptin genotype on AST, ALP and GGT were found (Fig. 2, 3 and 4).

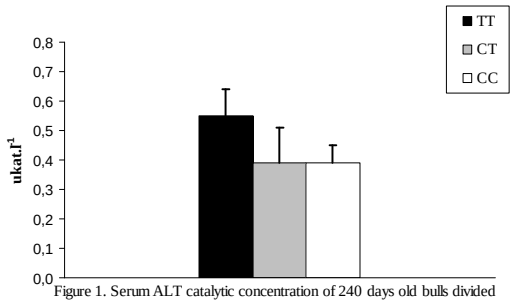

Figure 1. Serum ALT catalytic concentration of 240 days old bulls divided into 3 groups depending on leptin single nucleotide polymorphism (TT, CT, CC)

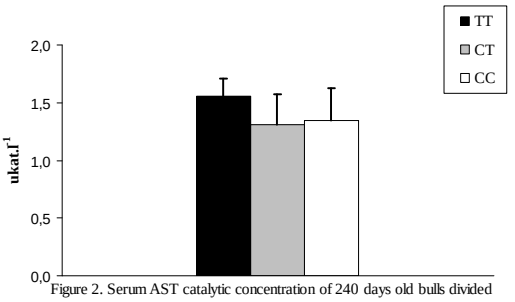

Figure 2. Serum AST catalytic concentration of 240 days old bulls divided into 3 groups depending on leptin single nucleotide polymorphism (TT, CT, CC).

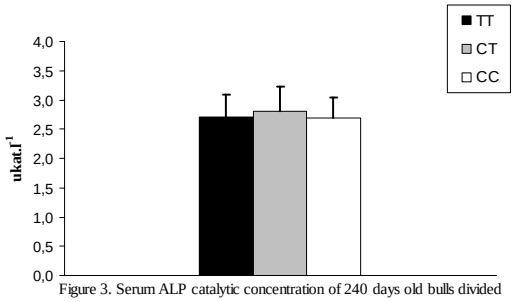

Figure 3. Serum ALP catalytic concentration of 240 days old bulls divided into 3 groups depending on leptin single nucleotide polymorphism (TT, CT, CC)

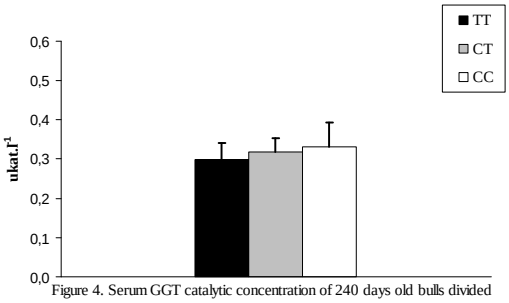

Figure 4. Serum GGT catalytic concentration of 240 days old bulls divided into 3 groups depending on leptin single nucleotide polymorphism (TT, CT, CC)

As mentioned above, leptin may have a preventive effect against progression of hepatic injury. Rising of catalytic activity of enzymes (ALT, AST, ALP and GGT) in blood serum could show on injury or damage of the liver tissues due to hepatobiliary diseases. In several studies (Buchanan et al., 2002, Liefers et al., 2002) the T allele was associated with higher level of leptin in the blood. Based on this information it would be expected, that serum activity of enzymes would be lower in TT bulls compared to other experimental groups. By contraries, significantly higher catalytic concentrations of ALT were found in the TT group. Ikejima et al. (2001) observed higher ALT level after application of leptin in mice. Similarly, Leclercq et al. (2002) found, that the circulating leptin levels are increasing in chronic liver

injury. There are no consistent results in the research field, but Wang et al. (2000) note, that serum total leptin level may not correlate closely with the biological action of leptin in the liver.

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ACRYLAMIDE IMPACT ON THE ALBUMIN LEVEL IN TESTES OF MICE

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Abstract

In 1994, the IARC Committee acknowledged acrylamide as a substance with potentially carcinogenic effect on humans and classified it to a group 2A (IARC, 1994). The concentration of acrylamide depends on the content of asparagine, reducing sugars (glucose, fructose) and temperature. Albumin is the main protein of blood plasma. Albumin has an important function – they belong to major antioxidants. Synthesis of albumin occurs mainly in the liver. Albumin represent 50% of all plasma proteins and is responsible for water distribution between plasma and the rest of extracellular fluid of the body. The research was conducted on mice SWISS, 8 weeks old, weight 26 g. The aim of this study was to estimate the level of albumin in testes of mice after acrylamide injection. The animals were segregated into five experimental groups. As a result of experiment we observed a linear increase of albumin level in testes of mice after acrylamide injection. The highest concentration was recorded after eight weeks from the first injection of acrylamide.

Introduction

Acrylamide (ACR) is an organic compound H2C=CH-CON H2. The research showed that the highest concentration of acrylamide was in french fries and potato chips i.e. respectively 170-2287µg/kg and 50-3500 µg/kg. Highest concentration of acrylamide was found in cereal products such as breakfast cereals i.e. 30-1400 µg/kg. Acrylamide was found in baby food, powdered milk,baby biscuits (Erkekoglu and Baydar, 2010). On the other hand another source of acrylamide is smoking (Schettgen et al, 2003). Daily intake from foodstuff was estimated in the range of 0.3-0.8 mg/kg bw per day (Seal et al., 2008). Acrylamide may be formed as a product of Maillard reaction between reducing sugars and amino acids, mainly asparagines in foodstuffs with high temperature (Taeymans et al., 2004; Stadler et al., 2002). Maillard reaction causes browning of food and produces taste and flavor. Intensive studies revealed that this compound may cause neurotoxic, cytotoxic and genotoxic effects (Seal et al., 2008). Carcinogenic properties are shown by acrylamide monomer exclusively. Acrylamide in the form of a polymer is not toxic. Metabolism of acrylamide is complicate. Its biotransformation occurs in the liver, where acrylamide is conversed to glycidamide (more

reactive derivative of epoxide) via cytochrome P450 (Żyżelewicz et al., 2010). Only 10% of the compound is removed in the urine, while 90% is metabolized. Animal model studies indicated significant correlation between the concentration of acrylamide in food and number of acrylamide adducts with hemoglobin (Szczerbina et al., 2008). The half-life of acrylamide in the body is 2-7 hours, which indicates a relatively slow excretion of the substance (Sörgel,2002).

Albumins are proteins widely distributed in the plasma. Albumin assists in many important body functions i.e. antioxidant action and transport of valid metabolites. Albumin is an ideal carrier of small molecule metabolites. The penetration of albumin into the interstitial fluid enables the contact with the majority of the body's cells. Important property of albumin is related to the capture of reactive oxygen species. The presence of the sulfhydryl groups makes albumins important component of plasma antioxidant barrier and antioxidant system. Acrylamide molecule has a double bond which easily react with molecules containing an unpaired electron such as albumin sulfhydryl.

Results

The main aim of our work was to estimate the level of albumin in testes of mice exposed to acrylamide. The research was conducted on mice SWISS, 8 weeks old, weight 26 g. The animals were fed with standard diet and grown in 12/12 light/dark photoperiod. The animals from experimental groups were injected intraperitoneally with acrylamide at dose of 80 mg/kg body weight once a week. Animals were segregated into five experimental groups. The animals from control group were injected intraperitoneally with physiological saline and decapitated after 24h. The first experimental group was decapitated after 24h, the second experimental group was decapitated after two weeks, the third experimental group was decapitated after four weeks, the fourth experimental group was decapitated after eight weeks. Total number of animals was n=25. The level of albumin was measured using a spectrophotometer UV-VIS Merck "Genesys 10UV", by absorbance at 578nm with the Bio-La Test (Albu L 500).

Normality of decomposition was checked with the Shapiro-Wilk test. To determine the differences between the groups ANOVA test was used. The homogeneity of variance was calculated using Levene's test at a significance level $p<0.05$.

We observed a linear increase in the level of albumin in each experimental group compared to the control $F_{(4, 21)}=344,80$, $p=0$. The studies showed an increase in the level of albumin in all

experimental groups. The largest increase was observed in the fourth experimental group decapitated after eight weeks of the experiment. Albumin has sulfhydryl groups and they can perform the functions of antioxidants (Miller and Jedrzejczak, 2001). They can be engaged during oxidative stress. On the other hand albumins play a role of the amino-acids reserve (Nicholson et al., 2000). Significant correlations show the figure 1.

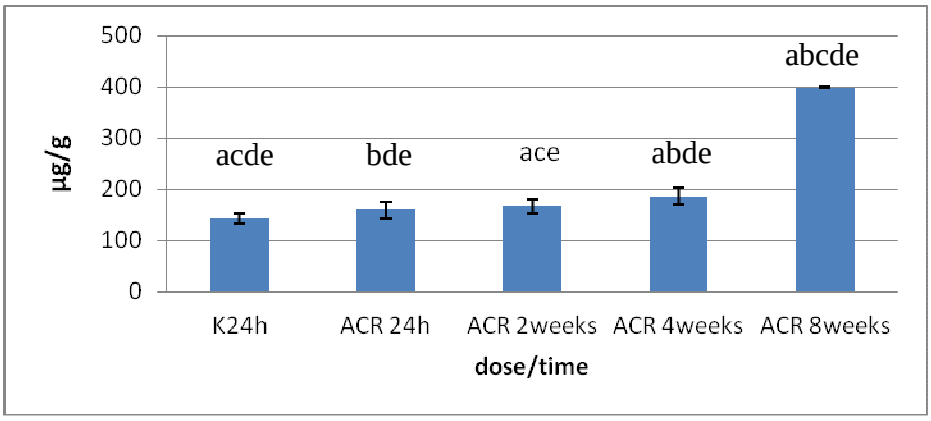

Figure 1.The level of albumin [µg/g] in testes of mice after acrylamide injection

a,b,c,d,e – The significant level $p<0,05$

Thus the increase in the concentration of albumin in the testes of mice may indicate elevated demand for protein substrates and may express the adaptation/repair processes.

Oxidative stress induced by free radicals and xenobiotics such as acrylamide functional proteins may be damaged thereby triggering the protein reserves, which are albumin.

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ASTROCYTES RESPONSES TO IMPAIRED GLUCOSE CONDITIONS MODULATED BY mTOR INHIBITION

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Abstract

The purpose of the study was to explore the influence of mTOR inhibitor on astrocytes activity in various glucose conditions *in vitro*. Rat primary cortical astrocytes were incubated for 24 hours in medium with different concentrations of glucose and mTOR inhibitor - rapamycin. Concentration of IL-6 and BDNF in the culture medium were estimated by ELISA test. Levels of both parameters were significantly increased in the medium with higher concentration of glucose than in control groups while addition rapamycin significantly reduced concentration of IL-6 and BDNF. The changes of astrocytes activities showed a modulating effect of mTOR inhibitor depending also on the glucose conditions. Astrocytes are cells that are able to react in response to peripheral glucose abnormalities and their biochemical features are related to mTOR pathway.

Introduction

Astrocytes are the main homeostatic cells of the central nervous system and play a wide role in physiology and pathology of the CNS (Verkratsky et al., 2013). Astrocytes exert a number of important functions during development and as a part of tripartite synapse regulate synaptic transmission through releasing active molecules, including glutamate, ATP, GABA, D-serine (Perea et al., 2009; Sofroniev & Vinters, 2010). Strong evidence, particularly from studies on cell culture, suggests also astrocytes role in regulating BBB properties (Perea et al., 2009). Neurosupportive role of astrocytes may be changed by contribution to an inflammatory cascade (Ong et al., 2013). Reactive astrocytes are capable of producing a variety pro-inflammatory mediators, including interleukin-6 (IL-6), interleukin-1β (IL-1 β), tumor necrosis factor-α (TNF- α) and neurotrophic factor like brain-derived neurotrophic factor (BDNF) (Lisi et al., 2011). Astrocytes activation has been implicated in the pathogenesis of several neurological conditions, such as neurodegenerative diseases e.g. Alzheimer's disease (AD) (Verkratsky et al., 2013), infection, trauma or ischemia (Lisi et al., 2011). The latest results confirm also a deleterious role of activated astrocytes in conditions related to impaired glucose and insulin states (Liao et al., 2011). Strong reaction of astrocytes was observed in streptozotocin-induced type 1 diabetic rat (Coleman et al., 2004).

One of several kinases modulating astrocytes activity is mTOR (mammalian target of rapamycin) belonging to a large family of serine/threonine kinases. It is involved in maintaining cell homeostasis by controlling the transcription, translation, autophagy, proliferation and coordinating the catabolic and anabolic processes (Kapahi et al., 2010; Sengupta et al., 2010). Kinase functions as a catalytic subunit in two different complexes: mTORC1 and mTORC2 (Kapahi et al., 2010). The most widely used inhibitor of mTOR pathway both *in vivo* and *in vitro* is rapamycin - a drug belonging to macrolide group and revealing anticancer and immunosuppressive activity (Minor et al., 2010).

Due to the broad spectrum of action mTOR kinase has become an important target of the research in the last decade. Impaired activity of mTOR is mentioned as one of the pathological factor in the development of neurodegenerative diseases (Perycz et al., 2007), associated with metabolic disorders (de la Monte & Wands, 2008; Carvalho et al., 2013). Although numerous studies have been done about influence of the mTOR inhibitor on functions of neurons (Cao & Obrietan, 2010), the effect of this drug on the most frequently occurring cells in the central nervous system (CNS) - astrocytes has not been so far fully understood.

In our experiment we studied the changes of the astrocytes activity based on concentration of IL-6 and BDNF after exposure to the mTOR kinase inhibitor and different concentration of glucose in *in vitro* conditions.

Material and Methods

The study was performed on primary cortical actrocytes from fetal Sprague-Dawley rats (Life Technologies, Invitrogen, Poland). The first step of the experiment was the multiplicative growth of astrocytes for 14 days. Cell were seeded at 2 x 10^4cell/cm^2. Cultures were successfully grown in 25cm^2 flasks to 100% confluence in 85% Dulbecco's Modified Eagle Medium containing 4.5g/L glucose and 15% fetal bovine serum (Life Technologies, Invitrogen, Poland). Standard physical growth conditions for rat primary cortical astrocytes (37ºC in a humidified atmosphere of 5% CO_2 in air) were used. Medium was changed every 4 days. The next stage of the experiment was 24 hours incubation of the cells in four different mediums: CONTROL (normoglycemic: 22.5mM of glucose), H1 (45mM of glucose) and H2 (67.5mM of glucose) with supplementation of 1nM (R1) and 10nM (R2) of rapamycin. Glucose and rapamycin used in cell culture were from Sigma-Aldrich (Poland).

Culture medium was collected and IL-6 and BDNF concentrations were estimated by commercial enzyme-linked immunosorbent assay according to the manufacturer's instruction (Sigma-Aldrich, Poland; BDNF Emax ImmunoAssay System, Promega, USA).

The results were expressed as means ± standard deviations (SD). Comparisons of the group means were made using ANOVA followed by Tukey's post-hoc test (PQStat 1.4.6 Program, Poland). $P<0.05$ and $P<0.01$ were considered statistically significant.

Results

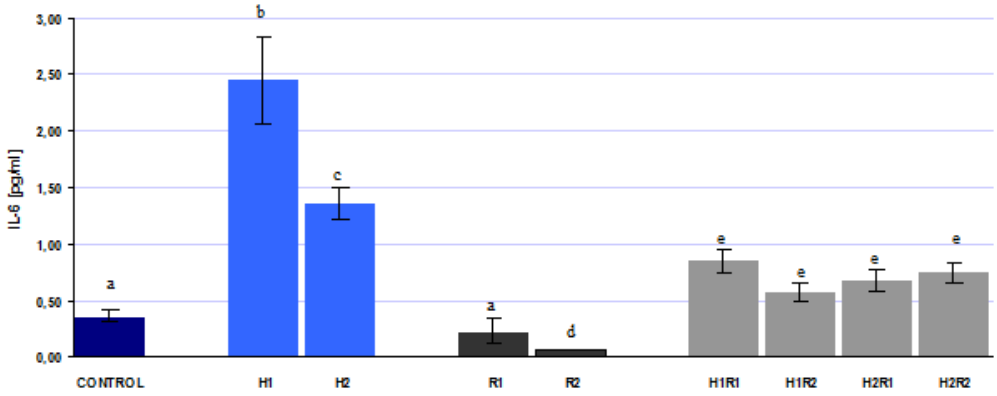

Figure 1. Concentration of IL-6 after 24 hours incubation in control (22.5 mM), H1 (45 mM) and H2 (67.5 mM) glucose conditions and with supplementation of 1nM (R1) and 10nM (R2) of rapamycin, and supplementation of rapamycin to H1 and H2 medium (X±SD, values with different superscript letters differ significantly, $P<0.05$).

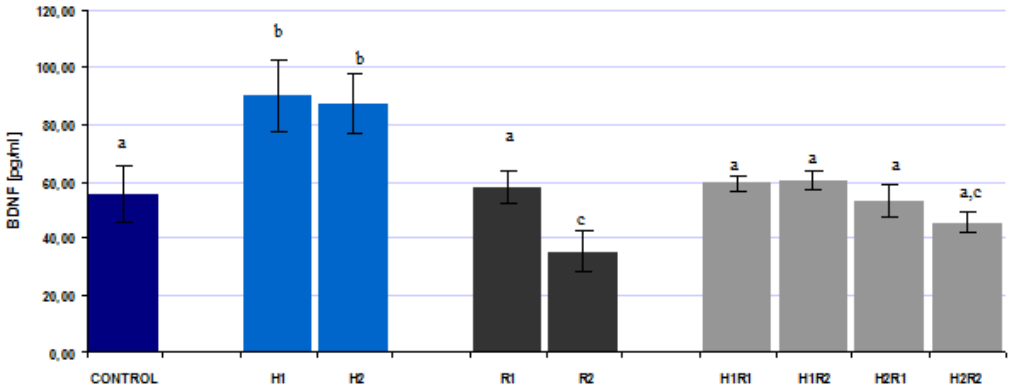

Figure 2. Concentration of BDNF after 24 hours incubation in control (22.5 mM), H1 (45 mM) and H2 (67.5 mM) glucose conditions and with supplementation of 1nM (R1) and 10nM (R2) of rapamycin, and supplementation of rapamycin to H1 and H2 medium (X±SD, values with different superscript letters differ significantly, $P<0.01$).

Both concentration of glucose in medium significantly increased the level of IL-6 compare to control (H1:2.45±0.39 and H2:1.35±0.14 vs. 0.37±0.05 pg/ml, $P<0.05$). Higher dose of rapamycin decreased the medium level of IL-6 from 0.37±0.05 pg/ml to 0.06±0.01 pg/ml ($P<0.05$). Addition of rapamycin to H1 and H2 medium attenuated effect of glucose and the level of IL-6 was almost reduced to the value observed in control group.

Addition of glucose, independently of dose, significantly increased the secretion of BDNF (H1:89.99±12.63 and H2:87.28±10.49 vs. 55.32±9.65 pg/ml, $P<0.01$). BDNF concentration was significantly lower only in R2 group (35.42±7.05 pg/ml vs. 55.32±9.65 pg/ml) and addition rapamycin to H1 and H2 mediums reduced BDNF concentration to the control value.

Discussion and conclusions

The results of our pilot investigations emphasize the role of astrocytes as cells responsible for maintenance the energy homeostasis in the central nervous system and suggested that their neuroprotective effects are dependent on the activity of mTOR kinase.

mTOR kinase is one of the most important factor that participates in the regulation of the processes contributing to the nervous system physiology and pathology e.g. by controlling protein translation, autophagy and microtubule dynamics (Verkratsky et al., 2013). Changed mTOR activity has been reported in many CNS diseases (Perycz et al., 2007).

Ramapycin is a perfect agent that can decrease level of IL-6 which only emphasizes its immunosuppressive features and mTOR role in immune system (Soliman, 2013) what we also noted in ours investigations (Fig. 1.). IL-6 is thought to be a major mediator of chronic inflammation associated with aging and aging-related diseases including Alzheimer disease (Bhat et al., 2012). Level of this cytokine is increased in the CNS of AD patients and *in vitro* studies have confirmed that exposure of astrocytes to Aβ peptides triggers senescence and that senescent astrocytes produce high quantities of IL-6 (Glass et al., 2010).

Our results suggested that although in the many pathological CNS states higher concentration of IL-6 is the main factor of neurodegeneration, in our case increased level of this cytokine might be a neuroprotective factor in hyperglycemia. We also suggested that in short period of time IL-6 can positively stimulate production and secretion of BDNF similarly to other pro-inflammatory cytokines. Prevoius results indicate that some pro-inflammatory molecules like TNF-α, associated to various pathological conditions are capable to stimulate rat primary astrocytes by inducing the expression of BDNF (Saha et al. 2006).

The brain-derived neurotrophic factor (BDNF) belongs to neurotrophin family and is the most extensively widespread trophic factor in the brain (McAllister et al. 1999). BDNF is an important modulator of neuronal function and survival. An enhanced expression of BDNF in the CNS after various injures suggests its neuroprotective role (Saha et al. 2006).

Concentration of astrocytes BDNF can be up-regulated in response to several neuroprotective agents (Cardile et al. 2003).

We observed that hyperglycemic conditions in the short period of time can stimulate neurosupportive mechanism by increasing secretion of BDNF (Fig. 2.). Our findings seem to be consistent with previous results, in which the efficacy of BDNF in regulating glucose and energy metabolism were confirmed. Intraperivetricular and peripheral administration of BDNF revealed hypoglycemic feature of this neurotrophin and reduced both food intake and blood glucose, improved hyperglycemia and insulin resistance in db/db mice (Nakagawa et al. 2002). We suggest that elevated level of glucose causes an increase in the synthesis and secretion of BDNF which is one of the first mechanism that protects cells against high glucose level. It was confirmed that the increased risk of dementia in diabetes mellitus type II and obesity could be linked to chronic hyperglycemia, peripheral insulin resistance, oxidative stress and increased production of pro-inflammatory cytokines (de la Monte & Wands 2008).

Our study revealed that mTOR kinase is a potent factor which can modulate the activity of astrocytes particularly in glycemic abnormalities. Decreased activity of this kinase has positive influence on CNS functions. Rapamycin, as a mTOR ihibitor, kept concentration of IL-6 at the control level during glycemic abnormalities (Fig. 2.) what may help to maintain homeostatic conditions. A similar response of BDNF after addition rapamycin was observed in impaired glucose conditions, although inhibition of mTOR in normoglycemia caused decrease in BDNF concentration. It is known that in neurons BDNF induced activation of mTOR what is essential for the upregulation of local protein synthesis in neuronal dendrites (Takei et al. 2004). We suggested that mTOR kinase pathway may influence activity of astroglia through modulating BDNF synthesis and secretion.

The data showed a significant modulating effect the mTOR inhibition on the activity of astrocytes. Glial cells may be a good therapeutic target in preventing the development of neurodegeneration by using an appropriate concentration of mTOR inhibitor.

Acknowledgments

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IMMUNOLOCALIZATION OF MMP-7, MMP-9 AND TIMP-3 IN THE RABBIT OVARY

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Abstract

The aim of the study was to investigate the localization of MMP-7, MMP-9, and TIMP-3 in the rabbit ovary. Adult female rabbits of meat line M91 (n=5) were used in the experiment. Immunohistochemical analysis revealed the presence of MMP-7 and TIMP-3 in the zona pellucida, basement membrane, theca interna and ovarian stroma. Immunostaining for MMP-9 was found predominantly in the zona pellucida and stroma. The intensity of immunopositive reaction for examined members of the MMP system depended on the stage of follicle development and regression. The results suggest that the MMP system by reorganizing the extracellular matrix, particularly the basement membrane, may regulate follicular development and atresia in the rabbit ovary.

Introduction

Numerous studies have established that the matrix metalloproteinases (MMPs) are key enzymes involved in the control of extracellular matrix (ECM) turnover during tissue growth, morphogenesis and regression (Curry et Osteen, 2003; Vu et Werb, 2000; Kessenbrock et al., 2010). The architectural features and substrate preference categorize the MMP family members into classes, i.e. the collagenases (MMP-1, MMP-8, MMP-13), gelatinases (MMP-2, MMP-9), stromelysins (MMP-3, MMP-10, MMP-11), matrilysins (MMP-7, MMP-26), membrane type MMPs (MT-MMP-1,-2,-3,-4,-5,-6), and other less characterized members (Page-McCaw et al., 2007). The gelatinases, stromelysins and matrilysins are capable of degrading major constituents of basement membranes, including type IV collagen, laminin and fibronectin (Stamenkovic, 2003).

The site and the extent of ECM remodeling depend on the local balance between the MMPs and their associated endogenous inhibitors such as liver-derived α2-macrroglobulin and the tissue inhibitors of metalloproteinases (TIMPs) (Murphy, 2008). TIMP-1, -2, -3, and -4 act selectively on different MMPs. Thus, the activity of MMPs can be regulated at multiple

levels: gene expression, conversion from zymogen to active enzyme, and, eventually, the presence of specific inhibitors.

The MMP system has been revealed to play critical role in reproductive functions in humans and diverse animal species (Gabler et al., 2001; Bai et al., 2005, Leśniak et Hrabia, 2012). It has been postulated to regulate dynamical structural changes that occur in the ovary and uterus. In addition, the expression of MMPs in reproductive tissues has been reported to be cell-, tissue- and reproductive cycle-dependent (Curry et Osteen, 2003; Goffin et al., 2003). Although, the participation of MMP family members in reproductive functions was strongly suggested the information regarding localization of the MMPs and TIMPs in the ovary is scarce. In the rabbit ovary only localization of MMP1 was reported (Tadakuma et al., 1993). Therefore, the aim of our study was to determine the immunolocalization of MMP-7, MMP-9 and TIMP-3 in the ovary of mature rabbits.

Materials and methods

Adult female rabbits (n = 5) of meat line M91 (age: 240 days) were housed in individual flat-deck wire cages under a constant photoperiod of 12h of day-light, the temperature 20–24°C and humidity 55 ± 10 %. Institutional and national guidelines for the care and use of animals were followed, and all experimental procedures were approved by the State Veterinary and Food Institute of Slovak Republic, no. 3398/11-221/3 and ethics committee. Immediately after slaughter, the ovaries were fixed in freshly prepared 4% (v/v) buffered formalin, processed and embedded in paraffin wax. Tissue sections were examined for MMP-7, MMP-9, and TIMP-3 localization.

Ovarian sections (6 μm thick) were deparaffinized in xylene, rehydrated in ethanol and rinsed with water. Endogenous peroxidase activity was blocked with 0.5% H_2O_2 in methanol. After washing with water, slides were heated in citric buffer (pH 6.0, 75°C, 20 min). Nonspecific binding was blocked using 5% (v/v) normal goat serum in TBST buffer (Tris buffer saline + 0.1% Tween 20, RT, 10 min). Slides were incubated over night (4°C) with specific rabbit anti-MMP-7 (Santa Cruz Biotechnology), anti-MMP-9 (Abcam) or anti-TIMP-3 (Abcam) antibody at 1:100 dilution. Then ovarian sections were washed with TBS and incubated with biotinylated goat anti-rabbit antibody (40 min, dilution 1:200) followed by Vectastain ABC kit (30 min). Color reaction was developed with diaminobenzidine (DAB) and slides were analyzed under a light microscope (Jena Zeiss, Germany).

Results

Immunoreactivity for MMP-7

Rabbit ovaries demonstrated immunoreactivity for MMP-7 mainly in the zona pellucida, basement membrane and theca interna of developing follicles as well as in the connective tissue of stroma between follicles and interstitial cells (Fig. 1, A and B). In the primary and secondary follicles, MMP-7 protein was at background levels in the granulosa cells but substantially increased in tertiary follicles (Fig. 1, C). Atretic follicles, exhibited MMP-7 expression primarily in the basement membrane, theca interna, and the remnant of zona pellucida (Fig. 1, D and E).

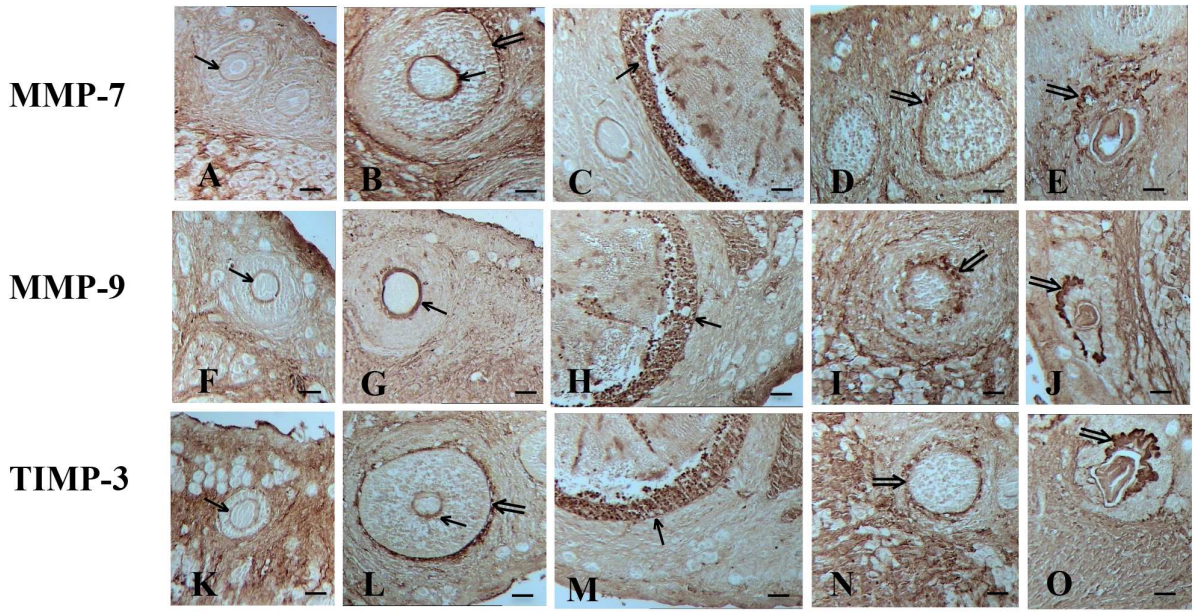

Figure 1. Immunolocalization of MMP-7, -9 and TIMP-3 in the rabbit ovary. Representative photomicrographs of growing follicles scattered within the stroma (A, B, C, F, G, H, K, L and M) and degenerating atretic follicles (D, E, I, J, N, and O). Thin arrows indicate zona pellucida (A, B, F, G, K, and L) or granulosa cells (C, H, and M). Thick arrows indicate basement membrane and theca interna (D, E, I, J, N, and O). Scale bar represents 50 μm.

Immunoreactivity for MMP-9

Immunoreactivity of MMP-9 protein was observed in the stroma and zona pellucida (Fig. 1, F and G). Expression of MMP-9 in the granulosa and theca cells of developing follicles was low and approximated background levels. However, in tertiary follicles, MMP-9 was detected in granulosa layer in considerable amount (Fig. 1, H). In atretic follicles, MMP-9 was

localized mainly to the basement membrane and theca layer, as well as the remnant of zona pellucida (Fig. 1, I and J*).*

Immunoreactivity for TIMP-3

TIMP-3 was detected in the zona pellucida, basement membrane, theca interna, and stroma (Fig. 1, K and L), with low to undetectable amount in granulosa layer, which increased as the follicle matured (Fig. 1, M). Immunopositivity for TIMP-3 was also found in the remnant of zona pellucida, basement membrane and theca interna of atretic follicles (Fig. 1, N and O).

Discussion

The current study is the first showing immunohistochemical localization of MMP-7, -9 and TIMP-3 in the rabbit ovary. The MMP-7 was localized predominately in the theca layer of follicles, an area that undergoes reconstruction during follicular growth. Negligible amount of matrilysin was present in the granulosa layer of growing follicles followed by substantial increase as the follicle matured. These results are in line with observations by Chaffin et al. (1999) that reported relatively low abundance of MMP-7 mRNA in periovulatory granulosa cells of the monkey, that increased after hCG administration, suggesting a regulatory role of progesterone in MMP-7 mRNA expression. In our study, immunolocalization of matrilysin was also found in the basement membrane of atretic follicles, and markedly increased at the latter stages of follicular atresia. Taking into account recent studies, that confirmed changes in the content of particular components of the basement membrane of atretic follicles, such as nidogen-2 (Hatzirodos et al. 2014), and laminins (van Wezel et al., 1998), MMP-7 may be one of the players in the eventual regression of the atretic follicles. Moreover, MMP-7 may be involved in the induction of cell apoptosis, by the cleavage of ligands or receptors that transduce proapoptotic signals, as apoptosis is believed to be the major process of cell death occurring in the atretic follicles (Yang et Rajamahendran, 2000; Rodgers et Irving-Rodgers, 2010), and interaction between MMP-7 and Fas ligand has been documented (Mitsiades et al., 2001).

Follicles rest in an extracellular environment of collagen, laminin, and fibronectin, major substrates for MMPs, and correlative data implicate the MMP system in the follicular growth. For example, Bagavandoss (1998) noted that MMP-9 immunostaining elevated at the latter stages of follicular development in the rat ovary. Immunoreactive MMP-9 was absent in the neonatal rat ovary during early follicular growth. After stimulation of follicular growth with equine chorionic gonadotropin (eCG), gelatinase B was found in the thecal and interstitial

tissue (Bagavandoss, 1998). Localization of MMP-9 transcripts after eCG administration revealed MMP-9 mRNA in the theca layer as well as in the stroma. Accordingly, present findings demonstrate strong immunoreactivity for gelatinase-B in the rabbit ovarian stroma, but on the other hand, very weak staining in the wall of developing follicles. Additionally, our research showed presence of MMP-9 protein in the zona pellucida, which separates the oocyte from the follicular cells. This prominent layer is rich in polysaccharides and is believed to be elaborated by both oocyte and follicular cells. It persists even after the degradation of the oocyte during atresia of the follicle. Of particular interest is the finding that MMP-9 was immunolocalized in atretic follicles. Similarly, Huet et al. (1998), reported elevated activity of MMP-9 in ovine atretic follicles following hypophysectomy. It is suggested that proteolytic enzymes such as MMP-9 may facilitate the basement membrane breakdown characteristic of the latter stages of atresia.

Localization of MMPs and TIMPs during follicular growth and atresia corresponds with sites that must be remodeled as the follicle grows and regresses. We found strong immunostaining for TIMP-3 in the stroma, basement membrane and theca interna of the secondary follicles, whereas in the granulosa cells TIMP-3 appeared in tertiary follicles. The results are in agreement with the observations by Curry et al. (2001) in the rat ovary. Authors revealed that TIMP-3 mRNA was present in the granulosa layer of certain follicles but it was absent in the granulosa cells of adjacent follicles. What is more, authors reported that with continued follicular development, TIMP-3 mRNA became highly expressed in the granulosa cells of healthy preovulatory follicles. The results obtained in the present investigation has also reveled the presence of TIMP-3 in the atretic follicles of the rabbit ovary, what may suggest the indirect involvement of TIMP-3 in the follicular atresia by mediating MMP action or direct action including the induction of apoptosis.

In conclusion, present results indicate that MMP system by reorganizing the extracellular matrix, particularly the basement membrane, may regulate follicular development and atresia in the rabbit ovary.

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IN VITRO EFFECT OF BISPHENOL A (BPA) ON BOVINE SPERMATOZOA

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Abstract

Our objective was to evaluate the effect of bisphenol A (BPA) at doses - 1, 10, 100 and 200 µg/mL dissolved in 1% ethanol on the spermatozoa motility and viability. The spermatozoa motility was determined by CASA (Computer Assisted Semen Analyzer) system using the Sperm Vision™ program. The viability of the cells exposed to BPA was evaluated by the metabolic activity (MTT) assay after 6 h of *in vitro* cultivation. The initial spermatozoa motility (0 h) showed decreased value in all doses of bisphenol A and the significant differences (P<0.001) were observed between the groups 100 and 200 µg/mL of BPA and the control group. The highest concentrations of bisphenol A also decreased spermatozoa motility significantly (P<0.001) after 2 h of *in vitro* cultivation. After 4 h of *in vitro* cultivation was determined significant decreased (P<0.001) in all experimental groups containing BPA. The viability of bovine spermatozoa detected by the MTT cytotoxicity assay was slightly increased at the lowest dose of bisphenol A (1 µg/mL). The lowest survival of bovine spermatozoa was determined and significant difference (P<0.001) was found out only after the addition of 200 µg/mL of BPA. In conclusion, the results from our experiments suggested the toxic effects of bispehnol A in the highest doses on motility and viability of bovine spermatozoa.

Introduction

The toxic effect of various environmental contaminants on male reproductive system such as heavy metals – copper (Xu et al., 1985; Skandhan et al., 1992; Eidi et al., 2010; Kňažická et al., 20013), lead and cadmium (Patra et al., 1999; Hernández-Ochoa, 2005; Arabi and Mohammadpour, 2006; Tvrdá et al., 20013) or industrial chemicals such as alkylphenols (Nimrod and Benson, 1996; Nagao, et al. 2001; Gong and Han, 2006; Uguz et al., 2009; Lukáčová et al., 2012; Lukac et al., 2013) were found.

Bisphenol A, one of the environmental contaminants used in the manufacture of plastics and other products, is released largely into the environment (Olea et al., 1996). The majority of studies on BPA have focused on their endocrine disrupting and potential adverse effects on

the developing reproductive system. Accumulation of bisphenol A in male reproductive organs has some clinical implications since exposure to low doses of bisphenol A during fetal live has been shown to decrease the efficiency of spermatozoa production in the offspring of male mice. Maternal exposure to bisphenol A at 2-20 µg/kg body weight in CF-1 mice reduced the efficiency of sperm production, decreased epididymal weight and increased the weights of preputial and prostate glands (vom Saal et al., 1998; Sakagami et al., 1999; Takahashi and Oishi, 2001).

The objective of this study was to determine the effect of various concentrations of BPA during time periods (0 h, 2 h and 4 h) on the motility and viability of bovine spermatozoa.

Material and methods

Eight bovine semen samples were obtained from adult breeding bulls (Slovak Biological Services, Nitra, Slovakia). Spermatozoa were incubated with various concentrations of bisphenol A (BPA; Sigma-Aldrich, Bratislava, Slovakia) dissolved in 1% ethanol (Sigma-Aldrich, Bratislava, Slovakia) (group 1; 10; 100; 200 µg/mL of BPA). The control (Ctrl) group cultured with physiological saline solution (medium without BPA) was compared to the experimental groups (exposed to different concentrations of BPA). Positive control group (P Ctrl) was cultured with 1% ethanol. Spermatozoa were cultivated in the laboratory at room temperature (22-25°C).

The motility analysis was carried out using a CASA (Computer Assisted Semen Analyzer) system – SpermVision^{TM} program (MiniTűb, Tiefenbach, Germany) with the Olympus BX 51 microscope (Olympus, Tokyo, Japan) at cultivation times 0 h, 2 h and 4 h.Each sample was placed into the Makler Counting Chamber (deph 10 µm, Sefi-Medical Instruments, Haifa, Izrael) and percentage of motile spermatozoa (motility > 5 µm/s; MOT) was evaluated. This study was performed in eight replicates at each concentration (n = 8). At least 1000 spermatozoa were analyzed in each sample.

The viability of the cells exposed to BPA *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 (MTT) to purple formazan particles by mitochondrial succinate dehydrogenase of intact mitochondria of living cells. The amount of formazan was measured spectrophotometrically. In brief, the cultured $3.0x10^6$ cells/mL in 96-well plates (NUNC, Denmark) were stained with MTT tetrazolium salt (Sigma, St. Louis, USA). MTT was dissolved in PBS (Dulbecco´s Phosphate Buffer Saline, Sigma, St. Louis, USA) at 5 mg/mL

and added to the cells (in 20 µL per well). After 1.5 h of incubation (37ºC), the cells and the formazan crystals were dissolved in 80 µL of isopropanol (2-propanol, p.a. CentralChem, Bratislava, Slovakia). Optical density was determined at a measuring wavelength of 570 nm against 620 nm as reference by a microplate ELISA reader (Multiscan FC, ThermoFisher Scientific, Finland). The data were expressed in percentage of control (i.e., optical density of formazan from cells not exposed to BPA).

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

Evaluation of bovine spermatozoa motility

The initial spermatozoa motility (0 h) showed decreased value in all doses of bisphenol A (BPA) in comparison to the control group without BPA. The significant differences (P<0.001) were observed between the groups containing the highest doses of BPA (100 and 200 µg/mL) and the control group without BPA (70.53% and 42.00% versus 92.30%). No significant difference was found out between positive control group and control group. The results are presented in the Table 1. The highest concentrations of BPA decreased spermatozoa motility significantly (P<0.001) also after 2 h of *in vitro* cultivation (48.16% and 5.82% in comparison to 87.49%). In this time, it was found out slightly increased of motility at the dose 1 µg/mL compared to the control group (87.77% versus 87.49%). After 4 h of *in vitro* cultivation was determined significant decreased (P<0.001) in all experimental groups containing BPA (1; 10; 100 and 200 µg/mL) (73.41%; 75.39%; 25.20% and 1.27% versus 84.65%).

Table 1. Bovine spermatozoa motility exposed to bisphenol A during several time periods.

Groups	**Ctrl**	**P Ctrl**	**1**	**10**	**100**	**200**
			µg/mL of bisphenol A			
Time 0						
x	92.30	91.71	91.32	88.11	70.53[A]	42.00[A]
minimum	82.55	81.81	82.92	79.48	43.33	2.77
maximum	98.73	98.30	98.50	96.96	83.33	70.39
S.D.	4.01	3.46	3.29	4.29	10.82	21.63
CV (%)	4.34	3.77	3.61	4.87	15.34	51.50
Time 2						
x	87.49	87.77	86.25	83.45	48.16[A]	5.82[A]
minimum	69.69	66.66	72.72	61.66	12.82	0.0
maximum	97.26	96.77	97.22	92.85	78.72	18.18
S.D.	6.55	6.12	5.57	6.31	22.72	5.79
CV (%)	7.49	6.97	6.45	7.56	47.17	99.57
Time 4						
x	84.65	81.75	73.41[A]	75.39[A]	25.20[A]	1.27[A]
minimum	72.50	67.85	42.42	41.17	0.0	0.0
maximum	96.29	94.11	90.00	89.65	59.09	10.52
S.D.	4.21	5.68	10.45	8.75	19.91	2.63
CV (%)	4.97	6.95	14.23	11.61	79.02	206.22

Legend: x – mean, SD – standard deviation, CV (%) – coefficient of variation

[A]$P<0.001$; [B]$P<0.01$; [C]$P<0.05$

Evaluation of bovine spermatozoa viability

The viability of bovine spermatozoa detected by the MTT cytotoxicity assay was slightly increased at the lowest dose of bisphenol A (1 µg/mL), while the doses 10, 100 and 200 µg/mL had the negative effect on the cell viability after 6 h of *in vitro* cultivation. The lowest survival of bovine spermatozoa was determined and significant difference ($P<0.001$) was found out only after the addition of 200 µg/mL of BPA.

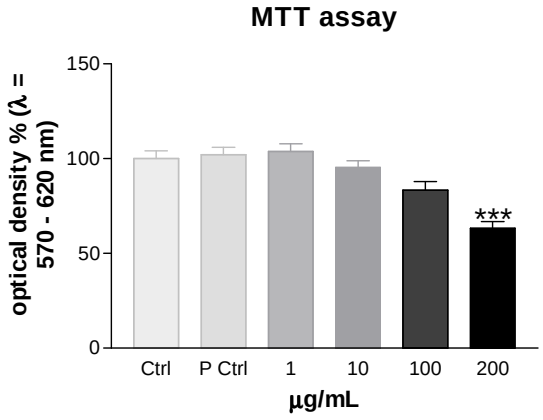

Figure 4. Effect of various concentrations of bisphenol A dissolved in 1% ethanol on the viability of bovine spermatozoa after 6 h. Legend: Each bar represents the mean (± SD) optical density (λ = 570 - 620 nm) as percent of controls, which represented 100%. The level of significance was set at $P<0.001$.

The majority of *in vivo* and *in vitro* experiments on BPA have focused on their endocrine disrupting and potential adverse effects on the developing reproductive system. It was found out that environmental relevant doses of BPA increased the prostate–weight in mice (Nagel et al., 1998) and decreased in daily spermatozoa production in mice (vom Saal et al., 1998; Nakahashi et al., 2001). Chitra et al. (2003) administrated orally BPA to male rats at the dose levels of 0.2, 2 and 20 µg/kg body weight per a day for 45 days and found out that BPA significantly decreased weights of the testis and epididymis. Administration of bisphenol A at various doses decreased the epididymal spermatozoa motility and spermatozoa count in dose-dependent manner.

There are few studies examining the effects of BPA on spermatozoa in *in vitro* conditions, therefore we can compare the impact of BPA and other endocrine disruptors where the experiments were carried out *in vitro*. For example study of Uguz et al. (2009), that examined the effect of nonylphenol (NP) (1, 10, 100, 250 and 500 µg/mL) and found out that the highest doses of NP were highly detrimental for motility of rat epididymal spermatozoa. Our experiment confirms these findings for the highest doses of BPA. Another study Lukacova et al. (2013) presents similar results for the doses of octylphenol (OP) – 100 and 200 µg/mL) added in the same concentrations to bovine spermatozoa.

Conclusion

Reproductive system influences many environmental toxicants that can enter humans and animals through food, drinking water, air and skin contact and present the serious risk the health and reproduction. One of these chemicals is bisphenol A that can cause the abnormalities in male reproduction. The results obtained from our *in vitro* study confirm that the doses 100 and 200 µg/mL of bisphenol A have the detrimental effect on spermatozoa motility during short-term cultivation. Bisphenol A in these doses is also able to decreased viability of spermatozoa after 6 h of *in vitro* cultivation.

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IMPACT OF WEIGHT ON ADULT TURKEY SPERMATOZOA MOTILITY DURING *IN VITRO* CULTIVATION

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Abstract

The aim of this study was to analyze the impact of body weight of turkey males on spermatozoa motility parameters during *in vitro* cultivation at 5°C. Turkey toms were selected for weight at 18 weeks of age and divided into two groups. The first heavier group was marked as white (W) and the second lighter group was marked as blue (B). Individual motility parameters were recorded at two time periods: 0 and 30 minutes. Each sample was evaluated using the Computer Assisted Semen Analyzer (CASA) system. In all spermatozoa motility parameters (MOT, PRO, VCL, DAP) similar or higher values were founded in lighter group B compared to heavier group W in both times (0 and 30 minutes). Body weight has no significant effect on spermatozoa motility.

Keywords: turkey, weight, spermatozoa, motility, CASA

Introduction

At a global scale, modern turkey breeding industry is manifesting prosperity. The turkey is becoming more and more preferred source for poultry meat production (Oblakova, 2007). In heavy broiler breeder males, the decline in reproduction is considered a multifactorial problem. It is influenced by numerous factors including hormonal levels, the development of testicular tissue, behaviour, locomotion (mobility) and physical body composition (Fragoso et al., 2013). The body weight of the roosters affects the fertility values (Hocking and Duff, 1989). This one effect was confirmed by a study Haunshi et al. (2012). In their research two chicken breeds were evaluated. Aseel breed with significantly ($P \leq 0.001$) higher body weight, absolute and relative testes weights had significantly higher semen volume ($P \leq 0.05$) and spermatozoa motility ($P \leq 0.01$) as compared to Kadaknath. Also Mohan et al. (2011) confirmed a fact, that in heavier chicken breeds, higher semen volume and spermatozoa concentration were detected. Animals with a low body weight often have underdeveloped testes and are considered subfertile from the point of a certain threshold (Ross, 2004). The relationship between body weight and testicular mass was observed by Pizzari et al. (2004).

Body weight is one of the important economic traits (Haunshi et al., 2012). The aim of this study was to analyze the influence of body weight of turkey males on spermatozoa motility parameters during *in vitro* cultivation at 5°C.

Material and methods

Biological material

In this study semen was obtained by penal massaging of the turkeys of the line Big 6 (BUT – British United Turkeys Ltd., Chester, United Kingdom) and divided into two groups. Turkey males were selected for weight at 18 weeks of age. The first group of the average weight 19.7 kg was marked as white (W). In this group the best growth potential was supposed. The second group from individuals about 2.5 kg lighter than group W was composed. It was marked as blue (B). Semen samples were a mixture of several groups of identical individual turkeys (n = 5).

Sample preparation

Semen was diluted in a ratio of 1 part of semen and 100 parts of physiological solution (Sodium chloride 0.9% Braun, B. Braun Melsungen AG, Melsungen, Germany). The letter corresponding of turkey weight group: W – White and B – Blue. Samples were cultured at 5°C and recorded at two time periods: 0 and 30 minutes. The experiment was realized in 8 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 our study the following parameters were evaluated – total motile spermatozoa (MOT) [%], progressively motile spermatozoa (PRO) [%], distance average path (DAP) [μm] and curvilinear velocity (VCL) [$\mu m.s^{-1}$] in two time periods.

Statistical analysis

Obtained data were statistically analyzed using PC program Excel and a statistics package GraphPad 5 using t–test. Statistical significance was indicated by p values of less than 0.05; 0.01 and 0.001.

Results

The resulting values of spermatozoa motility and progressive motility are presented in Figure 1. There were no significant differences in motility and progressive motility between heavier group (W) and lighter group (B) at time 0 minute. After 30 minutes of cultivation spermatozoa motility values was statistically significant ($p<0.01$) lower in group W. Similar result in progressive motility was observed. Analysis of velocity curved line (VCL) between samples W and B revealed no significant differences at time 30 minutes (Figure 2). Significantly lower values ($p<0.01$) showed group W compared to lighter group B at time 0 minutes. Analyzing the distance average path (DAP) as well as VCL very equal values were founded (Figure 3). At time 0 minutes significantly lower values ($p<0.01$) in heavier group W than in group B were detected. After 30 minutes of cultivation in both samples similar values were reported.

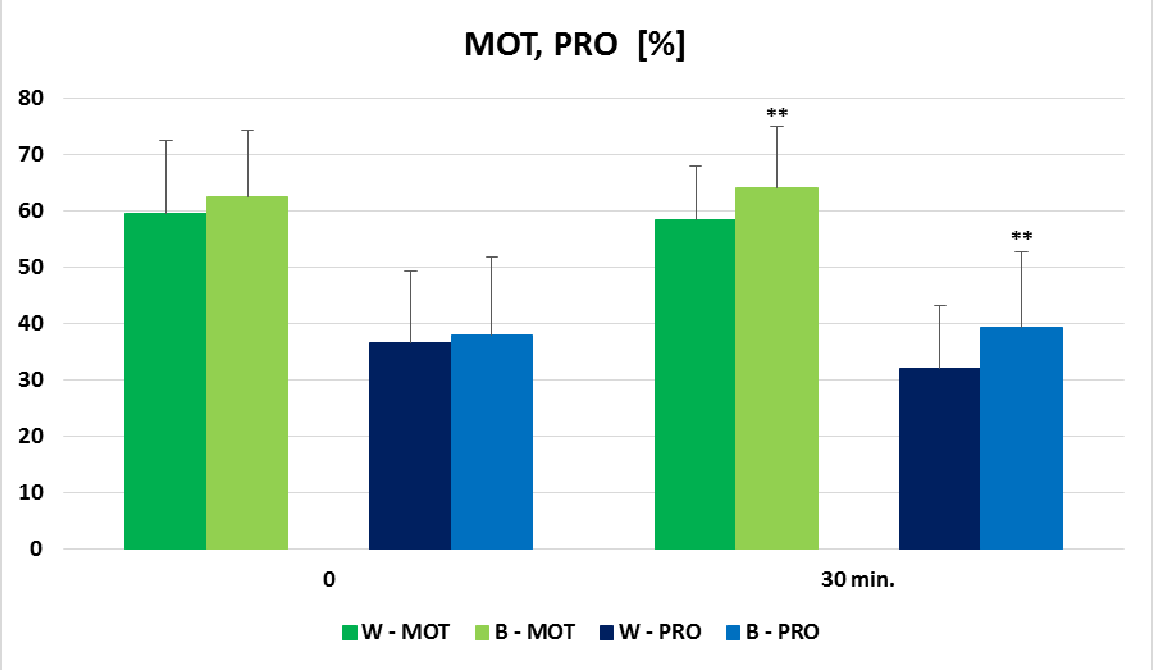

Figure 1. Spermatozoa motility (MOT) and progressive motility (PRO) in turkey (n=40) in different weight groups (Group W ≥ 19.70 kg; group B ≤ 17.20 kg) at different times (0 and 30 minutes). Significant differences *p<0.05; **p<0.01; ***p<0.001.

Relationships between fertility and some parameters in male broiler breeders (body and testicular weight, histology and immunohistochemistry of testes, spermatogenesis and hormonal levels) was the aim of Fragoso et al. (2013) study. There was a significant difference in spermatozoa production between the subgroups of roosters at age 55 weeks. Males in the overweight category had an average production of 7600 x 10^4, while the males in

the underweight category produced an average of 2300 x 10^4 spermatozoa per gram of testicular tissue.

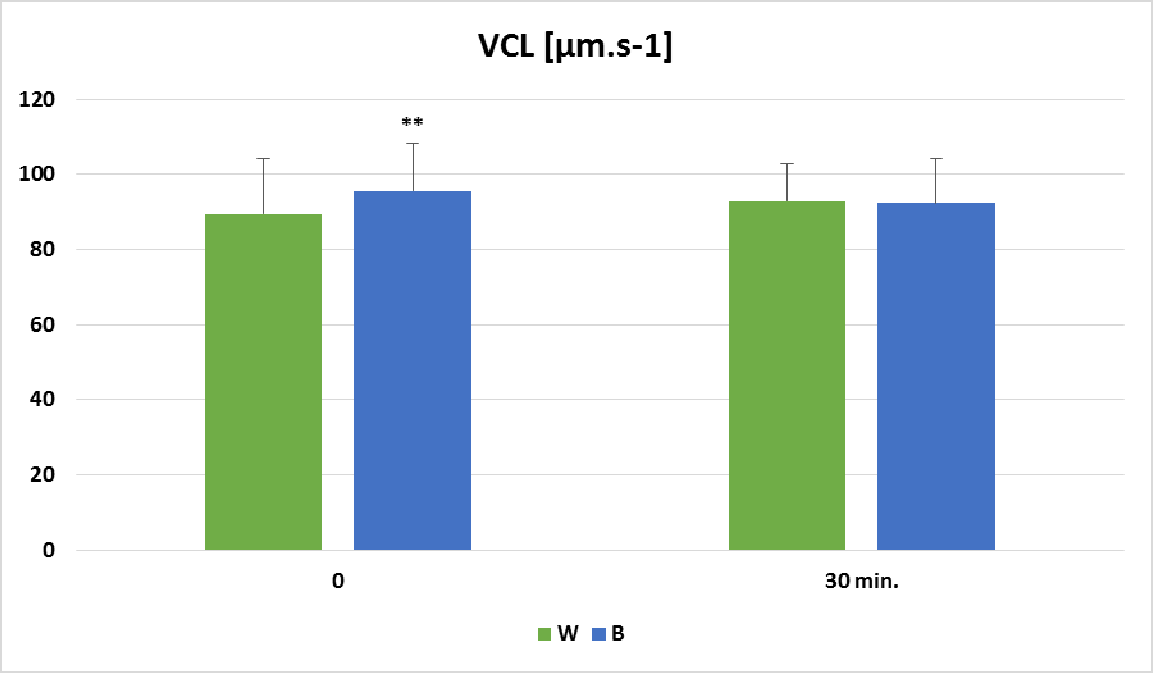

Figure 2. Velocity curved line (in µm.s^{-1}) in turkey (n=40) in different weight groups (Group B ≤ 17.20 kg; group W ≥ 19.70 kg) at different times (0 and 30 minutes). Significant differences *p<0.05; **p<0.01; ***p<0.001.

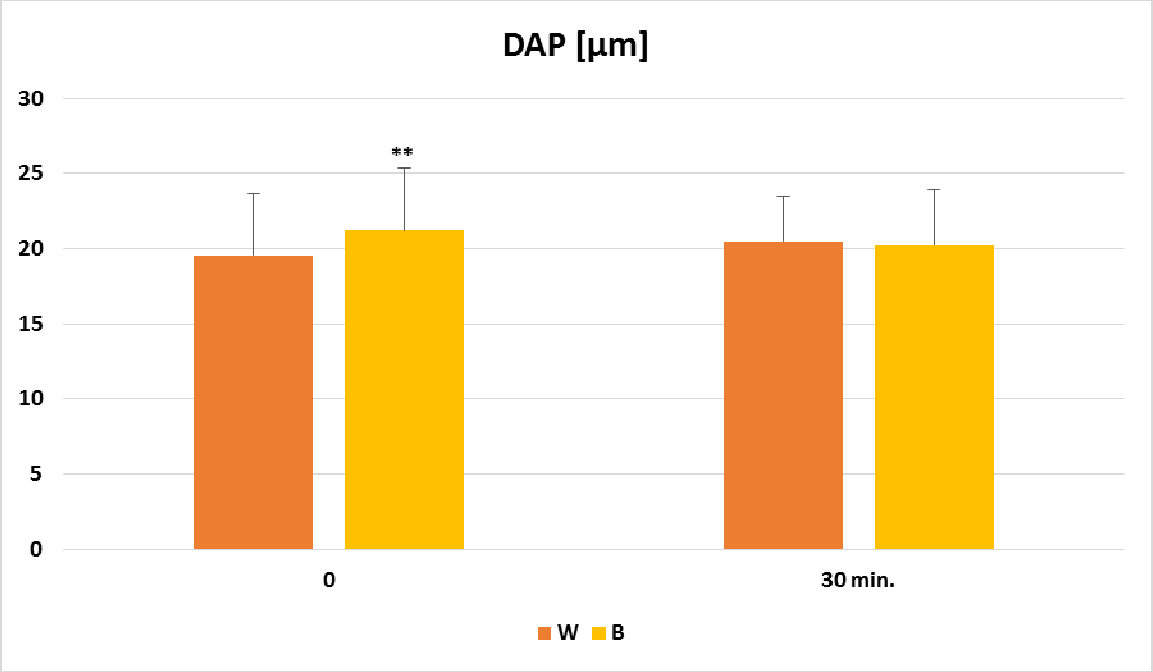

Figure 3. Distance average path (in µm) in turkey (n=40) in different weight groups (Group B ≤ 17.20 kg; group W ≥ 19.70 kg) at different times (0 and 30 minutes). Significant differences *p<0.05; **p<0.01; ***p<0.001.

Froman and Feltmann (1998) were compared spermatozoa morphology, fertilizing ability, and ATP content between phenotypes. Fertility and spermatozoa ATP content differed ($p < 0.001$) between phenotypes, whereas sperm morphology did not ($p > 0.05$). Neither body weight nor the combined weight of the testes was correlated with sperm mobility ($r = -0.02$ and 0.01, respectively).

Conclusion

The body weight and its influence on selected spermatozoa parameters were assessed in this study. In all parameters (motility, progressive motility, velocity curved line and distance average path) were founded similar or higher values in lighter group B compared to heavier group W in both times of cultivation (0 and 30 minutes). As a result, this leads us to the conclusion, that heavier body weight of monitored male turkeys no significant effect on spermatozoa motility was detected.

Acknowledgments

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EFFECT OF THE SEASON ON THE OCCURRENCE OF FOOTPAD DERMATITIS IN BROILER CHICKEN

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Abstract

The aim of the study was to evaluate the effect of the season on incidence of footpad dermatitis in broiler chicken. Ross 308 and Cobb 500 hybrids were used in this experiment. This monitoring was performed in two houses on the same farms. Broilers on the both farms were it the same age and were fattened in the same time. The incidence of footpad dermatitis was evaluated in 8 batches. The evaluation was conducted throughout the year. Temperature and humidity of litter were observed in the fourth week of age. Samples of litter were collected from 10 locations in each hall. Scoring of the paws was done in slaughterhouse according to six-point scale (0-5) Ask (2010). To facilitate the evaluation of the paws damage the numbers in scoring groups were summarized as follows: negligible damage (0+1), intermediate damage (2+3) and severe damage (4+5).

There was found statistically significant difference ($P<0.05$) was between the season and litter moisture and temperature. Statistically significant difference ($P>0.05$) was found between the season and classification of paws by six-point scoring system by Ask (2010).

Key words: broiler, footpad dermatitis, paws, season

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Introduction

Footpad dermatitis (FPD) first became an issue for the poultry industry in the 1980s, but it surely existed long before that time. Even though FPD was firstly described in the 1980s, this period was the beginning of the development of the broiler paw market and greater attention was being given to paw quality. Recently chicken paw prices have escalated due to an insatiable demand for high-quality paws in export markets (Shepherd and Fairchild, 2010). FPD is a condition that is characterized by inflammation and necrotic lesions, ranging from superficial to deep on the plantar surface of the footpads and toes. Deep ulcers may lead to abscesses and thickening of underlying tissues and structures (Greene et al., 1985). The ulcers can cause swelling, redness, and heat under the skin and cause the surface area to thicken

(Meluzzi et al., 2008). It is likely that FPD causes pain and therefore has a negative effect on bird welfare (Jong, 2012). Animal welfare audits in Europe often use foot, hock, and breast burn-lesions as an indicator of housing conditions and the general welfare of the birds (Haslam et al., 2007). Concerns about the welfare of broilers have led to a new European Broiler Welfare Directive to be implemented by June 2010 (Ask, 2010).

Birds spend most of their lives in direct contact with litter material. High-moisture litter (i.e., >30%), type or quality of bedding material in broiler production systems has been clearly associated with an increasing incidence and severity of FPD (Cenzig et al., 2011). The weather influences litter quality. High relative humidity, both outdoors and inside the house is associated with poor litter quality (Berg, 2004).

The time of year flocks are raised has been suggested as a contributing factor associated with the incidence of FPD. Dermatitis has been found more frequently during winter months than in summer and footpad condition has a high correlation with relative humidity inside and outside the broiler house. These seasonal effects are most likely caused by an increase in broiler house relative humidity, which is due to decreases in ventilation rates typically observed in cold weather as operations try to avoid reducing house temperature and save of heating costs (Shepherd and Fairchild, 2010).

The aim of the study was to evaluate the effect of the season on the occurrence of footpad dermatitis in broiler chicken.

Material and methods

Incidence of footpad dermatitis was performed in two houses on the same farm in Starý Petřín. Ross 308 and Cobb 500 hybrids were used in this experiment. Broilers in both houses were always it the same age (+/- 2 days). The stocking density was max 39 kg/m2 at the end of fattening. Chopped wheat straw was used as litter.

Temperature and humidity of litter were observed in the fourth week of age. Samples of litter were collected from 10 locations in each houses - 2 x under the ventilators, 2 x between ventilators, 2 x in the middle of the hall, 4 x in every corner of the hall under drinker system. Temperature was also measured in these places by contact thermoneter. Samples of the litter were dried at 65 ° C and litter moisture was calculated.

The incidence of footpad dermatitis was evaluated in 8 batches (February, March, April, June, July, October, September and December). These months were divided into seasons - spring, summer, autumn and winter.

Broiler chickens were slaughtered in the slaughterhouse Modřice Vodňanská Drůbež, a.s. company. Scoring of the paws was done in slaughterhouse according to six-point scale (0-5) Ask (2010). To facilitate the evaluation of the paws damage the numbers in scoring groups were summarized as follows: negligible damage (0+1), intermediate damage (2+3) and severe damage (4+5).

Data obtained from this experiment were analyzed using the single factor analysis of variation. Data were followed by LSD test using the software package Unistat 5.1 (UNISTAT Ltd, ENGLAND).

Results and discussion

The incidence of FPD was evaluated in two houses throughout the year in each season. In the **Table 1** there is expressed the influence of season on temperature and moisture of litter. The average temperature of the litter in the 4th weeks of broilers age was 31.1°C in spring, 31.7°Cin summer, 30.8°C in autumn and 29.6°C in winter. The highest temperature of the litter was in summer and significantly the lowest temperature of the litter was in winter season ($P<0.05$). Statistically significant difference in litter temperature ($P<0.05$) was found between autumn and summer, winter and spring, winter and summer and winter and autumn.

The average moisture of the litter in the 4th weeks of age broilers was 44.6% in spring, 42.9% in summer, 42.8% in autumn and 47.9% in winter. The highest moisture of litter was in winter and the lowest moisture of litter was in autumn season. Statistically significant difference in litter moisture ($P<0.05$) was found between winter and summer and winter and autumn. Season had influence on litter moisture.

Table 1. Effect of season on temperature and moisture of litter

	Season*			
Parameters	**Spring**	**Summer**	**Autumn**	**Winter**
Litter temperature (°C)	**31.1^{b,c}**	**31.7^{c}**	**30.8^{b}**	**29.6^{a}**
Litter moisture (%)	**44.6^{a,b}**	**42.9^{a}**	**42.8^{a}**	**47.9^{b}**

*Different letters show statistically significant differences ($P<0.05$).

Abd El-Wahab (2012) assumed that the “critical moisture content” for the development of FPD lesions is about 35 % litter moisture content. Furthermore, doubling exposure time (4 - 8h) led to only slightly increased severity of FPD for the low litter moisture contents (35 and

50 % moisture) and a higher rise for the wettest litter treatment (65 % moisture) at the end of the trial.

Table 2 shows the percentages of different degrees of damage of paws in the halls according to the season. Paws without damage or with slight damage were included in group 0+1. Percentage of paws in group 0+1 was in spring 20.6%, in summer 16.3%, in autumn 52.8% and in winter 42.9%. The highest number of healthy paws and paws with slight damage were in autumn. The lowest litter humidity was measured in the same season. On the other hand paws classified in group 4+5 are considered as paws with severe damage. Ulcer occurs over almost the entire plantar surface (25-80%) (Ask, 2010). Moreover, lesions on the paws may be a gateway for bacteria which might affect carcass quality (Mayne et al., 2007). Percentage of paws with severe damage (group 4+5) was in spring 38.1%, in summer 56.9%, in autumn 16.2% and in winter 23.3%. The highest numbers of paws with severe damage were observed in summer and spring. In these seasons litter moisture and litter temperature were high.

Table 2. Percentage of paws according to season

Percantage of paws in scoring groups	**Season***			
	Spring	**Summer**	**Autumn**	**Winter**
0+1	**20.6^{a,b}**	**16.3^{a}**	**52.8^{c}**	**42.9^{b,c}**
2+3	**41.3^{b}**	**26.8^{a}**	**31.0^{a,b}**	**33.9^{a,b}**
4+5	**38.1^{a,b}**	**56.9^{b}**	**16.2^{a}**	**23.3^{a}**

*Different letters show statistically significant differences ($P<0.05$).

Thus, the higher litter moisture and a higher incidence of severe damage of paws were in summer and spring. In general, a high incidence of FPD can be produced in broilers by increasing the moisture level of the litter, as suggested earlier by Harms et al. (1977). According to research conducted with broilers and turkeys, litter conditions (i.e., type, particle size, and moisture level) are significant factors in the development of FPD (Cengiz et al., 2011).

Martland (1985) also reported that FPD lesion scores increased rapidly following wetting litter after 1 wk. More recently, Mayne et al. (2007) showed a similar effect in turkey poults and concluded that water alone was sufficient to cause FPD in a very short time. However, the FPD lesions appeared to regress (i.e., improve) in birds with time, especially with improvements in litter conditions.

Severity of FPD is reduced in warm, dry seasons and elevated in cooler months. This may result from elevated litter humidity, for example, due to reduced ventilation when it gets

colder (Shepherd and Fairchild, 2010). Greene et al. (1985) and Haslam et al. (2007) reported studies in which incidence of paws lesions was greater in cold weather.

Not all research has found the incidence of FPD elevated in winter months. Wang et al. (1998) observed no cases of FPD in White Leghorn chickens when outside temperatures were between 9°C and 15°C, but more birds with FPD were found when the temperature was warmer, between 20°C and 26°C. Musilova et al. (2013) reported the most severe damage of the paws in the spring.

As the conditions under which broilers are raised vary between different parts of the world, it is extremely difficult to give efficient general advice on how to prevent contact dermatitis (Berg, 2004). One thing that is common among most previous research is that litter moisture is a significant factor in the onset of FPD.

Conclusion

There was found statistically significant effect ($P<0.05$) of season on both on litter moisture and temperature. The study showed the highest litter temperature in summer and spring and also the highest damage of paws in these two seasons. Lower incidence of paws damage was obtained in autumn and winter. Statistically significantly lower damage of paws was found in winter and autumn in comparison with summer ($P>0.05$) using classification of paws by six-point scoring system by Ask (2010)

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UP-REGULATED IMMUNOREACTIVITY IN RATS PRENATALLY EXPOSED TO INCREASED ANGIOTENSIN 2 LEVELS IN MATERNAL CIRCULATION

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Suboptimal conditions experienced *in utero* can permanently change the developmental program of an individual and be manifested by an increased risk of neurodegenerative, metabolic and cardiovascular diseases in adulthood. Common link among these disorders is frequently accompanied by a disrupted balance between pro- and anti- inflammatory immune mechanisms resulting in a subclinical inflammation. To investigate how an exposure to adverse prenatal environment can affect the immune status in adulthood, we analysed the progeny of female rats with experimentally increased levels of plasma angiotensin 2 (ANG) during the pregnancy. We assigned 12 female Wistar rats into two equal groups, one was sham operated and one was implanted with osmotic minipumps (Alzet) continuously releasing ANG 2 (2 µg/kg/h) 14 days from day 6 of pregnancy. Progeny of these females was assigned to control (C) and ANG rats, respectively. For immunological measurements, venous blood of 15 week-old rats was analysed by flow cytometry. Reduced numbers of CD8+ T cells and a tendency to increased CD4+/CD8+ ratio was found in the circulation of ANG rats as compared to controls. Moreover, oxidative burst of granulocytes induced by phorbol myristate acetate was higher in ANG than C rats. No differences between both groups were found in plasma levels of pro-inflammatory cytokines (TNF-α, IL-6). Our results demonstrate changed peripheral distribution and functional activity of immune cells in rats prenatally exposed to increased ANG 2 in the maternal circulation. These data may indicate development of subclinical inflammation in prenatally-treated rats increasing their vulnerability to pathological processes in adulthood. Supported by the grants VEGA 1/0686/12, APVV-0291-12.

POSITIVE EFFECT OF TAURINE ON NEW ZEALAND WHITE RABBIT SPERMATOZOA MOTILITY IN VITRO

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Abstract

This study was constructed to investigate the effect of taurine on rabbit spermatozoa motility *in vitro*. Semen was collected by the means of artificial vagina. Obtained semen was pooled and subsequently exposed to 1.5 mM, 3 mM, 6.25 mM and 12.5 mM taurine. Sample analysis was performed with the help of CASA technology to assess overall spermatozoa motility, progressive motility, curvilinear velocity, beat cross frequency and amplitude lateral head displacement. Obtained data express a positive impact of taurine addition on all analyzed motility characteristics. For rabbit spermatozoa motility maintenance the most beneficial showed to be the addition of 1.5 mM and 3 mM taurine.

Key words: rabbit, spermatozoa, taurine, motility

Introduction

Artificial insemination (AI) is thought to be one of the most advanced approaches in animal breeding. This approach requires semen of acceptable characteristics and sufficient motility (Brun et al., 2002; Ducci et al., 2002). Methods such as computer-assisted semen analysis (CASA) have been, and are still, widely used for determining a broad range of motility parameters (Mortimer, 2000), some of which were proven to be directly implicated in fertilization rate (Fréour et al., 2010). Many times in the AI, it comes to situations where it is not possible to use the ejaculate immediately after collection. Instead, semen is stored in cold for several hours *in vitro*. In such cases, however, accumulation of free oxygen radicals occurs in semen followed by a gradual drop of spermatozoa motility as a consequence of membrane lipid peroxidation (Perumal et al., 2013). Studies of many research groups are therefore focused on finding novel compounds such as antioxidants capable of eliminating unfavorable effects of oxidative stress and thus maintaining spermatozoa motility *in vitro* (Yun et al., 2013). Among them, a very good candidate seems to be taurine, (2-aminoethane sulfonic acid), one of the most abundant amino acids in organs throughout the whole body (Ripps et al., 2012). Taurine is known for exerting beneficial effects on cells through its

antioxidant properties (Chen et al., 2012, Marcinkiewicz et al., 2014), as well as the ability to improve mitochondrial function by stabilizing the electron transport chain and inhibiting the generation of reactive oxygen species (Schaffer et al., 2009). The addition of taurine to the semen of farm animals like boar (Jang et al., 2006) dog (Michael et al., 2007), ram (Bucak et al., 2007), stallion (Ijaz et al., 1995) or bull (Perumal et al., 2013) emerged as useful for maintenance of spermatozoa motility and overall cell integrity. This study was conducted to assess the ability of taurine to maintain selected motility parameters in New Zealand White rabbit spermatozoa after its *in vitro* storage in cold over a short time period.

Material and Methods

Semen of 5 sexually mature and healthy New Zealand White rabbits was collected three times by the means artificial vagina. After each collection ejaculates with overall spermatozoa motility ≥ 80% were pooled and used for further sample preparation. Taurine work solutions were created by dissolving of appropriate amounts of taurine (Sigma-Aldrich, Japan) in sodium chloride physiological solution (Braun Melsungen, Germany) to a final concentration of 1.5 mM, 3 mM, 6.25 mM and 12 mM. Then, semen aliquots were diluted with corresponding taurine work solutions to get experimental groups. Control group was prepared by semen dilution in physiological solution. All samples were diluted in a ratio of 1:5.

Finally, samples were stored at 5 °C and only aliquots of them were incubated at 37 °C in the time of 15 min. and subsequently used for further assessment. Sample analysis was performed by CASA technology equipped with the SpermVision 3.5 software and the Makler Counting Chamber (Sefi-Medical Instruments, Germany). Motility measurements were carried out at a point in time 0, 2, 4 and 24 hours after semen dilution. Overall motility (MOT), progressive motility (PRO), beat cross frequency (BCF), curvilinear velocity (VCL) and amplitude of lateral head displacement (ALH) were evaluated. All experiments were repeated three times.

Statistics

Analysis of variance was used for statistical comparisons to identify overall taurine effects. Significant changes were determined by post hoc comparisons of means using Student-Newman-Keuls test. Statistical significance was set at a confidence level of 95%. Obtained data are present as mean ± standard deviation. All the computations were done in SigmaPlot 12.0.

Results

Obtained results of CASA analysis are concluded in Fig. 1. Motility assessment performed immediately after taurine addition revealed no impact of taurine on analyzed parameters in

experimental groups. Protective effect of taurine was revealed for the first time after 2 hours of semen exposure to taurine. Here, taurine protected spermatozoa against decrease of MOT and PRO at 1.5 mM, while ALH, VCL and BCF were significantly higher compared to the control group at all tested taurine concentrations. However, taurine best protected rabbit spermatozoa against decrease of motility mostly at 1.5 mM. 4 hours after taurine application the highest significant rise in MOT, PRO as well as ALH values was recorded in semen containing 6.25 mM taurine. With regards to the VCL and BCF parameters, these were significantly increased compared to the control, reaching their peak at 1.5 mM and 3 mM, respectively. Beneficial effect of taurine in maintaining spermatozoa motility in experimental groups was yielded also 24 hours after its inclusion into the semen. While MOT, PRO and ALH were best maintained at 6.25 mM, for BCF and VCL the most suitable showed to be taurine concentration of 1.5 mM. These findings clearly demonstrate the ability of taurine to stably sustain rabbit semen motility upon one day after its application.

Figure 1. Motility of rabbit spermatozoa exposed to different taurine concentrations

Parameter (mean ± SD)	Time (hours)	0 (control)	1.5	3	6.25	12.5
		Taurine concentration (mM)				
MOT	0	73.63 ± 4.08	74.7 ± 8.89	74.99 ± 5.01	78.44 ± 4.05	79.17 ± 5.38
PRO		54.49 ± 10.1	55.65 ± 9.41	59.08 ± 7.86	60.07 ± 6.4	60.69 ± 5.1
VCL		109.73 ± 9.95	106.59 ± 9.72	121.27 ± 10.94	113.73 ± 7.18	112.35 ± 14.88
ALH		3.16 ± 0.3	3.22 ± 0.28	3.2 ± 0.41	3.12 ± 0.28	3.06 ± 0.39
BCF		34.64 ± 2.61	34.41 ± 1.64	36.7 ± 2.04	37.58 ± 2.09	35.17 ± 1.17
MOT	2	63.3 ± 6.36	71.87 ± 7.28*	66.57 ± 6.95	67.18 ± 5.21	65.86 ± 7.16
PRO		51.08 ± 8.32	62.48 ± 6.41*	52.74 ± 7.11	52.71 ± 6.57	54.7 ± 6.77
VCL		93.28 ± 9.27	128.76 ± 9.56*	107.99 ± 9.3*	118.02 ± 17.77*	118.05 ± 11.63*
ALH		3.26 ± 0.25	3.92 ± 0.24*	3.6 ± 0.32*	3.57 ± 0.41*	3.89 ± 0.32*
BCF		31.33 ± 2.85	34.73 ± 2.03*	33.58 ± 1.81*	34.99 ± 2.35*	34.91 ± 2.3*
MOT	4	59.32 ± 7.18	63.76 ± 9.88	63.93 ± 7.18	79.41 ± 4.92*	63.19 ± 6.32
PRO		42.03 ± 8.72	50.66 ± 9.32*	51.83 ± 7.95*	69.73 ± 6.31*	48.25 ± 7.78*
VCL		83.85 ± 11.7	120.1 ± 13.22*	118.86 ± 12.65*	119.67 ± 11.76*	110.62 ± 11.94*
ALH		2.9 ± 0.43	3.92 ± 0.47*	3.64 ± 0.45*	4.09 ± 0.43*	3.51 ± 0.39*
BCF		29.84 ± 3.26	33.92 ± 2.28*	34.39 ± 2.01*	34.13 ± 1.64*	32.8 ± 2.47*
MOT	24	34.35 ± 6.96	58.16 ± 8.27*	56.59 ± 7.17*	74.09 ± 4.68*	57.39 ± 5.25*
PRO		20.23 ± 6.06	47.24 ± 8.75*	43.09 ± 7.58*	62.23 ± 5.13*	43.7 ± 6.13*
VCL		69.02 ± 17.05	118.98 ± 10.74*	110.93 ± 10.17*	115.44 ± 7.76*	99.65 ± 12.49*
ALH		2.12 ± 0.52	3.01 ± 0.5*	3.52 ± 0.34*	3.63 ± 0.25*	3.22 ± 0.39*
BCF		30.61 ± 4.29	38.76 ± 2.91*	35.14 ± 2.53*	36.12 ± 2.21*	34.46 ± 1.88*

Legend: SD – standard deviation, * $p \leq 0.05$

The presence of generally lower motility values in the control groups could be explained by the fact, that during semen storage *in vitro*, it comes to the generation of free oxygen radicals (Aitken et al., 2012). This further leads to the formation of oxidative stress linked to

mitochondrial dysfunction (Das and Sil, 2012, Crompton and Andreeva, 1993, DiMonte et al., 1992, Menzie et al., 2012, Perfeito et al., 2012). The beneficial effects of taurine, as seen in experimental groups, could be a result of its antioxidant properties (Chen et al., 2012, Marcinkiewicz and Kontny, 2012, Aruoma et al., 1988), as well as its ability to improve mitochondrial function by stabilizing the electron transport chain and inhibiting the generation of reactive oxygen species (Schaffer et al., 2009, Jong et al., 2012). Furthermore, taurine restores respiratory chain activity and stimulates the synthesis of ATP (Schaffer et al., 2009, Jong et al., 2012), consequence of which is an increase of total sperm motility (Piomboni et al., 2012).

Conclusion

Based upon our results it can be concluded that 1.5 mM, 3 mM, 6.25 mM and 12.5 mM taurine supplementation of rabbit semen presents a perspective way for maintaining analyzed spermatozoa motility characteristics over a short time period *in vitro*. For overall spermatozoa motility maintenance the most beneficial emerged the addition of 1.5 mM and 6.25 mM taurine.

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THE EFFECT OF PUNICALAGIN ON STEROID SECRETION OF RABBIT FRAGMENTS.

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Abstract

Pomegranate is a well-known edible fruit in different part of world. The mainly protective compound of pomegranate is able to be punicalagin. Punicalagin is the predominant ellagitannin of *Punica granatum* and present in two anomers; punicalagin A and B. Punicalagin is metabolised to ellagic acid (antioxidant) and microorganism of human 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 observed effect of natural antioxidants from pomegranate to secretion function of rabbit ovaries. Our research was focused on examination of the effect of punicalagin to steroid secretion. Our biological material was non-cycling ovarian fragments from rabbits. The fragments were cultivated during 24h with various doses (1, 10 and 100 $\mu g.ml^{-1}$) of protective compound – punicalagin. Steroid hormones of female reproductive system - 17β-estradiol, progesterone and testosterone were evaluated by ELISA. 17β-estradiol had significant (P >0.05) differences between control and experimental group with addition of 10 $\mu g.ml^{-1}$ of punicalagin. Progesterone and testosterone were evaluated like non-significant (P >0.05) changes. The effects of phytochemicals of pomegranate on animal cells were not found but it was only the pilot study.

Introduction

Pomegranate belongs to the Punicaceae family and is one of the oldest edible fruits (Melgyjero and Salazar, 2003). Pomegranate is a well-known source of many valuable substances, such as ellagitannins – punicalagins and punicalins (Gil et al., 2000). Punicalagin (2,3-hexahydroxy-diphenoyl-4,6-gallagylglucose) is a high molecular weight (MW 1084) water-soluble compound isolated from pomegranate husk. Punicalagin is the predominant ellagitannin of *Punica granatum* and present in two anomers; punicalagin A and B (Seeram et al., 2005). Hydrolysis of ellagitannins (punicalagins and punicalins) releases ellagic acid, some of which is absorbed, while a portion is further metabolized to urolithins A and B by

human intestinal microflora, which are subsequently excreted in both urine and feces (Seeram et al., 2006; Larrosa et al., 2006). Punicalagins and ellagic acid are responsible for antioxidant activity and healthy benefits of pomegranates (Tyagi, 2012).

In the study we have examine possible dose-dependent effect of punicalagin on secretion of steroid hormones by ovarian fragments of rabbits.

Material and Metods

Material

Adult female rabbits (n = 5) of meat line M91, maternal albinotic line (crossbreed Newzealand white, Buskat rabbit, French silver) and paternal acromalictic line (crossbreed Nitra's rabbit, Californian rabbit, Big light silver) from an experimental farm of the Animal Production Research Centre Nitra, Slovak Republic were used. Rabbits (age: 150 days, weighing 4.00 ± 0.5 kg) were housed in individual flat-deck wire cages under a constant photoperiod of 12h of day-light, the temperature 20–24°C and humidity 55 ± 10 %.

Cultivation of fragments

Ovaries were collected and transported to the laboratory at the ambient temperature in a glass container within 30 minutes of slaughter. There after ovaries were washed in washed in sterile physiological solution and dissected using a blade knife to 8 approximately equal parts (weight 4.8-5.6 mg). These ovarian fragments were washed again 2 times in sterile physiological solution and these ovarian fragments were cultured in medium DMEM/F12 1:1 (BioWhittaker TM, Verviers, Belgium) supplemented with 10 % fetal calf serum (BioWhittaker TM) and 1 % antibiotic–antimycotic solution (Sigma, St. Louis, MO, USA) and without (control group) or with punicalagin treatment at various concentrations 1, 10 and 100 $\mu g.ml^{-1}$ during 24 h.

Test of steroid hormone and Statistical methods

Progesterone, 17β-estradiol and testosterone were evaluated by ELISA (DIALAB, Microwell Method). The absorbance was determined at a wavelength 450 nm on the microplate ELISA reader (Thermo Scientific Multiskan FC, Vantaa, Finland). The results were evaluated by One Way ANOVA test by statistical program Sigma Plot 12.0 (Jandel, Corte Madera, USA). The values are presented average ± SEM.

Results and discussion

We are describing influence of punicalagin on steroid secretion in female reproductive organs of rabbits. Some studies are focused on cell lines and carcinoma cell lines, where are significantly results in secretion of estrogen. Secretion of 17β-estradiol is shown on **Fig. 1**, there is shown dose-dependent influence of punicalagin to ovarian fragments. 17β-estradiol levels were decreased in each concentration of punicalagin. The secretion of ovarian fragments from rabbits was significantly (P >0.05) changed by punicalagin at the dose of 10 $\mu g.ml^{-1}$. According to Larrosa et al. (2006) urolithins (possible metabolite of punicalagin) could display estrogenic and/or antiestrogenic activity. Wang et al. (1994) described pomegranate or several flavonoids found in pomegranate have been shown to competitive inhibit aromatase in a human preadipocyte cell culture system. Punicalagin or its metabolite can have effect to secretion of 17β-estradiol. Phenols from pomegranate was inhibited on 55% estrogen activity by normal human breast epithelial cell – the autors Kim et al. (2002) were using lyophilized fresh pomegranate juice.

But in other side punicalagin is very important antioxidant. Its antioxidant effect is described in more studies. Aqil et al. (2012) has studied punicalagin from pomegranate husk and its protection against oxidative DNA damage.

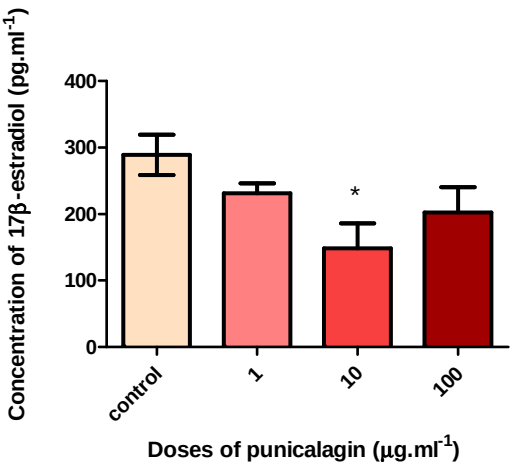

Figure 1. Doses-dependent effect of punicalagin on 17β–estradiol ($pg.ml^{-1}$) secretion by ovarian fragments from rabbit non-cycling ovaries. Significant (P>0,05) different among control and experimental groups. The results were evaluated by One Way ANOVA test.

Secretion of progesterone was changed and it is shown on the **Fig. 2**. The progress of punicalagin is non-significant (P>0,05) among control and experimental groups. In the groups with additional punicalagin 1 $\mu g.ml^{-1}$ and 10 $\mu g.ml^{-1}$ is increase, but the results is non-significant (P>0,05). It does not exist more studies, which are focused on secretion of

progesterone. Punicalagin belongs to group of polyphenol like quercetin, resveratrol and other natural substances studied in our previous studies (Kolesarova et al., 2012). There is a one study focused on natural polyphenols: resveratrol, epicatechin and quercetin – the study of polyphenol did not observe bind to steroid receptors (Damianaki et al., 2000).

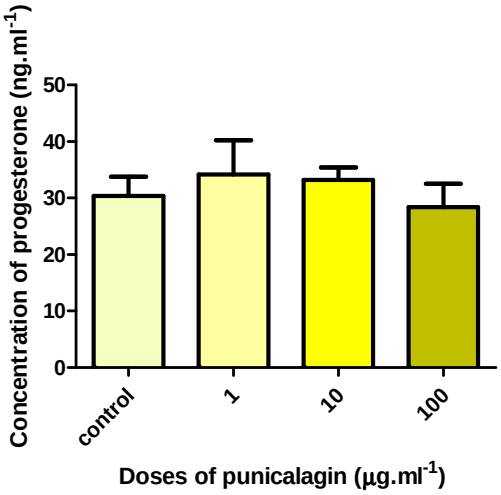

Figure 2. Doses-dependent effect of punicalagin on progesterone (ng.ml^{-1}) secretion by ovarian fragments from rabbit non-cycling ovaries. Non-Significant (P>0,05) different among control and experimental groups. The results were evaluated by One Way ANOVA test

On the **Fig. 3** is presented dose-dependent effect of punicalagin to testosterone secretion. The measurements were non- significant (P>0,05) among control and experimental groups. The additional of punicalagin had been had decreased effect to secretion of testosterone.

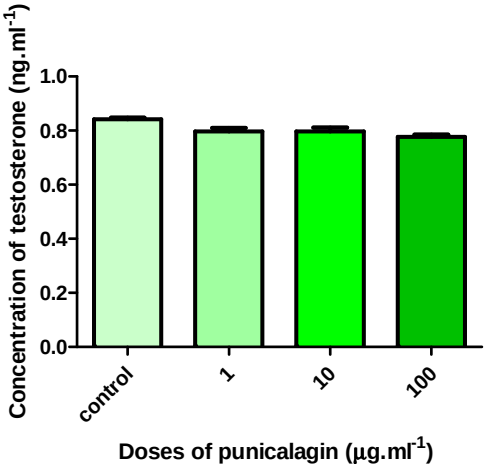

Figure 3. Doses-dependent effect of punicalagin on testosterone (ng.ml^{-1}) secretion by ovarian fragments from rabbit non-cycling ovaries. Non-Significant (P>0,05) different among control and experimental groups. The results were evaluated by One Way ANOVA test

The synthesis of testosterone is able to be inhibited by used doses of punicalagin, but the results non-significant. Previous studies are focused on gene expression – gens responsible to

synthesis of more enzymes, which are important in process of steroidogenesis. According to Hong et al. (2008) pomegranate polyphenols of gene expression were involved in androgen-synthesizing enzymes and may be of particular importance in androgen-independent prostate cancer cells. Testosterone could be converted to estrogen and some polyphenols are inducted conversion testosterone to estrogen. By Wang et al. (2005) the administration of flutamide, an anti-androgen, could not significantly suppress the MCF-7aro cell growth induced by testosterone; however, the pure estrogen receptor antagonist could revert the testosterone-induced cell growth to the baseline. This indicated that the conversion of testosterone to estrogen was responsible for the induction of the estrogen receptor positive cell proliferation.

Conclusion

The research is focused on possible effects of phytochemicals from pomegranate on animal cells. Punicalagin and its metabolite ellagic acid or urolihins as bioactive compound of pomegranate have shown their influence on physiological functions of animals. More phytochemical – antioxidants have been shown influence to secretory activity of reproductive cells. Previous studies have shown a possible anti-proliferation, pro-apoptotic and anti-inflammatory effect on different animal cells. There is a pilot study which suggests possible effect of punicalagin on steroid secretion by rabbit fragments. It is necessary to realize more research in the areas of phytochemicals.

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EFFECT OF BODY FRAME ON QUANTITATIVE AND QUALITATIVE PARAMETERS OF CANINE FRESH SEMEN

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Abstract

The effect of body frame on the quality parameters of dog ejaculate has been evaluated in this study. Total 90 semen samples were being evaluated. The dogs were divided into three groups according to the withers height (small: < 34.99 cm, medium: 35.00 – 42.99 cm and large: > 43.00 cm). Semen samples were collected by manual stimulation into the glass beaker. Immediately after collection, macroscopic and microscopic analysis has been conducted for all samples, which included finding out: volume of ejaculate, sperm activity, concentration of sperm and morphology of sperm. Volume of ejaculate was being measured using the graduated cylinder. Sperm activity was evaluated by subjective method, according to the percentage of motile sperm in the fresh ejaculate and concentration of sperm by haematocytometry method using Bürker counting chamber. Morphological evaluation of sperm was conducted on preparations Farelly stained method. Monitored factors have been expressed in weighted average and standard error. Based on the results we can state that the body frame of dogs had the verifiable effect on quality parameters of their ejaculate. Highly statistically significant differences ($P < 0.01$) have been demonstrated, in case of volume of ejaculate, concentration of sperm, total sperm count, total number of morphologically normal sperm, degenerated sperm and number of sperm with a damage of the flagellum. Then, statistically significant differences ($P < 0.05$) have been proved in case of volume only. And finally, statistically significant differences have not been proved ($P > 0.05$) in case of sperm activity, number of immature sperms and sperms with a damage of the head.

Key words: canine fresh semen, volume of ejaculate, sperm activity, concentration of sperm, total sperm count, body frame.

Introduction

There are approximately 2 million dogs of different breeds bred in the Czech Republic. So, a quality control of their reproduction function (Kutzler, 2005) is becoming the most important precondition for successful breeding work. The present situation, when there is no selection on quality of dog semen, is a significant risk for the future of dog breeding as such.

Fortunatelly, thanks to modern methods used in the evaluation of ejaculate, we are able to determine semen quality, and thus, to some extent affect the chance of successful fertilization (Root Kustritz, 2007). The most attention should be given to the sperm activity, sperm cell viability, concentration of sperm and total sperm count in the fresh ejaculate. The results of this research could be used as a feedback for objective assessment of reproductive level of breeding dogs. There is also a possibility to use the ejaculate in the future, both for a season of infertility of aliving stud male dogs, thus even after his death (Rijsselaere et al., 2002).

Material and methods

Totaled 90 samples of the fresh ejaculate from 30 male dogs were evaluated. The dogs were divided into three groups according to the withers height (small: < 34.99 cm, medium: 35.00 – 42.99 cm and large: > 43.00 cm). Semen samples have been collected by manual stimulation. Immediately after collection, macroscopic and microscopic evaluation has been conducted for all samples, which included finding out volume of ejaculate, sperm activity, concentration of sperm and morphology of sperm. Volume of ejaculate was being measured using the graduated cylinder. Concentration of sperm was evaluated by haematocytometry method using Bürker counting chamber (Veznik et al. 2004) and sperm activity then by subjective method according to the percentage of motile sperm in the native ejaculate. The percentage of sperm with progressive direct movement after the head has been evaluated. Morphological evaluation of sperm was conducted on preparations Farelly stained method under the oil immersion (Root Kustritz, 2007). The total sperm count has been calculated by multiplying volume by concentration of sperm (Prinosilova et al., 2012). Monitored characteristics have been expressed in weighted average and standard error.

Results

The volume of the ejaculate is not itself an indicator of dog semen quality. However, its measuring is an essential part of the calculation of the total count of sperm contained (Root Kustritz, 2007) and this value is highly important for an objective semen evaluation, because from total sperm count, we can determine other possible manipulation with ejaculate (e.g. dilution, production of insemination doses, preservation by chilling or freezing, etc.). Svoboda et al. (2001) report that physiological amounts of canine ejaculate is should be located in range from 1 to 40 ml, with the average value about 7 ml. Dostal et al. (2001) were more specific. They stated that the average volume of ejaculate from them examined dogs

was ranged from 4.5 to 8.5 ml. Our measured values were very similar to theirs, because the volume of ejaculate was moved in variation range from 4.15 ± 0.54 to 12.26 ± 0.73 ml. Highly statistically significant difference ($P < 0.01$) was found between value of small dogs (A: 4.15 ± 0.54 ml) and values of groups of large dogs (C: 12.26 ± 0.73 ml). Statistically significant difference ($P < 0.05$) was proved between group of medium dogs (B: 8.65 ± 0.98 ml) and other groups. In the case of volume and total sperm count, the effect of body frame of the dogs was most pronounced, when the values of both parameters were increased with increasing body frame of dogs. With regard to the total sperm count, the highly statistically differences ($P < 0.01$) were observed between group of small dogs (A: 542.87 ± $0.65.10^6$ sperms) and medium dogs (B: 1149.19 ± $150.89.10^6$ sperms), as well as between group of small dogs and group of large dogs (C: 1967.12 ± $163.58.10^6$ sperms).

Progressive direct movement after the head is one of the most important indicators of sperm fertilization ability and is a functional indicator of biological full value (Louda et al., 2001). Root Kustritz (2007) says that the normal percentage of motile sperm in the fresh semen of healthy dog should be 70.00 % or more. This fact was confirmed by Veznik et al. (2003) who stated that the minimum activity of dog spermatozoa intended for further use may not fall below 70.00 %. This condition was fulfilled by all of our collected individuals, when the activity of their sperm was moving in variation range from 76.78 % to 77.76 %. The results of all monitoring groups were very so close (A: 77.00 ± 1.39 %, B: 77.76 ± 1.74 % and C: 76.78 ± 0.84 %). In this parameter of canine fresh semen quality, statistically significant differences were not observed.

Svoboda et al. (2001) reported that the concentration of sperm cell should be 300.10^3. mm^{-3} to $800.10^3.mm^{-3}$ in the native ejaculate of healthy dog, while the total ejaculate should contain $300.10^3.mm^{-3}$ to $1000.10^3.mm^{-3}$ sperm. The concentration values of small dogs (A: 153.25 ± 12.75 $.10^3.mm^{-3}$) and large dogs (180.19 ± 9.84 $.10^3.mm^{-3}$) were quite close and statistically significant difference was found between them. On the other hand, between the group of medium dogs (B: 316.83 ± 27.45 $.10^3.mm^{-3}$) and other groups, even highly statistically significant difference ($P < 0.01$) was proved.

Johnston et al. (2001) reported that dogs ejaculate should have a minimally 80.00 % of morphologically normal sperm. Rijsselaere et al. (2003) noted on the need of optimal value of this parameter, because their study showed that, if the number of morphologically normal sperm was around 75.80 % in the fresh semen, then the value decreased to 48.30 %, after the freezing. This condition was met by all monitored groups of dogs, when their value of

morphologically normal sperm was moving in variation range from 72.60 ± 1.87 % (group A) to 84.24 ± 0.84 % (group B). Between values of these groups, highly statistically significant difference ($P < 0.01$) was proved. Next highly statistically significant difference was found between group B (84.24 ± 0.84 %) and group C (80.43 ± 1.08 %). The most morphological defects were represented by defects of the flagellum. In this case, the worst result was found in group of the small dogs (A: 13.27 ± 0.84 %). Between this value and value of ~~a~~ medium dogs (B: 7.53 ± 0.55 %) and large dogs (C: 8.33 ± 0.41 %), the highly statistically significant differences ($P < 0.01$) were found. Next highly statistically differences were demonstrated in case of degenerated sperms among groups of small dogs (A: 1.23 ± 0.26 %), medium dogs (B: 0.47 ± 0.09 %) and large dogs (C: 0.54 ± 0.07 %). In the case of immature sperms, as well as the number of sperms with damage of the head, the statistically differences have not been proved ($P > 0.05$).

Table 1. The effect of body frame on quality of the canine fresh semen.

MONITORING FACTORS		*Small (group A)* $\bar{x} \pm s_x$	*Medium (group B)* $\bar{x} \pm s_x$	*Large (group C)* $\bar{x} \pm s_x$
Volume of ejaculate	*ml*	$4.15^{b,C} \pm 0.54$	$8.65^{a,c} \pm 0.98$	$12.26^{A,c} \pm 0.73$
Sperm activity	*%*	77.00 ± 1.39	77.76 ± 1.74	76.78 ± 0.84
Concentration	$10^3.mm^{-3}$	$153.25^{B} \pm 12.75$	$316.83^{A,C} \pm 27.45$	$80.19^{B} \pm 9.84$
Total sperm count	10^6	$542.87^{B,C} \pm 65.58$	$1149.19^{A} \pm 150.89$	$1967.12^{A} \pm 163.58$
Normal sperm	*%*	$72.60^{B,C} \pm 1.87$	$84.24^{A} \pm 0.84$	$80.43^{A} \pm 1.08$
Immature sperm	*%*	4.67 ± 0.73	3.10 ± 0.50	3.41 ± 0.32
Degenerated sperm	*%*	$1.23^{B,C} \pm 0.26$	$0.47^{A} \pm 0.09$	$0.54^{A} \pm 0.07$
Damaged flagellum	*%*	$13.27^{B,C} \pm 1.72$	$7.53^{A} \pm 0.55$	$8.33^{A} \pm 0.41$
Damaged head	*%*	4.43 ± 0.84	1.26 ± 0.18	3.62 ± 0.75

A, B, C – between values with different letters in a column in each section were proved statistical highly significant differences ($P < 0.01$).
a, b, c – between values with different letters in a column in each section were proved statistical evidential differences ($P < 0.05$).

Conclusion

Based on the above results, we can conclude~~,~~ that the body frame has a significant effect on the qualitative and quantitative parameters of canine fresh semen, because the most of observed differences were highly statistically significant. Highly statistically significant differences ($P < 0.01$) have been demonstrated, in the case of volume of ejaculate, concentration of sperm, total sperm count, total number of morphologically normal sperm, degenerated sperm and number of sperm with damaged of the flagellum. Then, statistically significant differences ($P < 0.05$) have been proved in the case of semen volume only. And

finally, statistically significant differences have not been proved ($P > 0.05$) in the case of sperm activity, number of immature sperms and sperms with damaged of the head.

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REVIEW: EFFECT OF SELECTED FLAVONOIDS AND REACTIVE OXYGEN SPECIES ON SPERM

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Abstract

Quercetin, resveratrol and curcumin are flavonoids with diverse properties, including antioxidant potential. Antioxidant properties have been attributed to the ability of scavenging reactive oxygen species; a relation between the chemical nature of flavonoids and their antioxidant activity has been established, in particular the number and the position of hydroxyl groups influence their antioxidant power. Every ejaculate is contaminated with potential secretors of ROS. In the male reproductive system, ROS can derive from leukocytes or from the spermatozoa themselves.

Introduction

Flavonoids are a large group of polyphenolic compounds found in many plant-based foods, such as fruits, vegetables, wine and tea, consumed regularly by humans and animals. These compounds have been shown to prevent a number of chronic and degenerative diseases, including cancer and cardiovascular diseases, and to exert antioxidant activity (Kessler et al., 2003; Yao et al., 2004; Wang et al., 2005). Antioxidant properties have been attributed to the ability of scavenging reactive oxygen species (ROS); a relation between the chemical nature of flavonoids and their antioxidant activity has been established, in particular the number and the position of hydroxyl groups influence their antioxidant power (Celik et Arinc, 2010).

It is well known that the production of low level of ROS supports some main functions of sperm, such as capacitation, acrosome reaction, zona pellucida binding and oocyte fusion (Lamirande et al., 1997). Nevertheless, uncontrolled ROS production and defects in the balance between ROS concentration and the antioxidant scavenging system cause “oxidative stress”.

Reactive Oxygen Species

Generation of ROS in living organisms may be caused by several mechanisms including ionizing radiation (Sadani and Nadkarni 1997, Zhang, Zheng et al. 1998), bioactivation of xenobiotics (Akiyama 1999), inflammatory cells (Villegas, Kehr et al. 2003), increased cellular metabolism (Hollan 1996), decompartmentalization of transition metal ions (Huang,

Tseng et al. 2001), activation of oxidases and oxygenases (Davydov 2001), and loss of antioxidant capacity (Hsu, Liu et al. 1998, Aitken and Sawyer 2003).

Every ejaculate is contaminated with potential secretors of ROS. In the male reproductive system, ROS can derive from leukocytes or from the spermatozoa themselves. Most semen specimens contain variable numbers of leukocytes, with neutrophils noted as the predominant type (Aitken 1995, Saleh and Agarwal 2002). Activated neutrophils generate and release ROS in high concentrations to form cytotoxic reactions against nearby cells and pathogens (Ochsendorf 1999). Leukocytospermia has been associated with decreased sperm concentration, motility and morphology, as well as decreased hyperactivation and defective fertilization (Moskovtsev, Willis et al. 2007). Spermatozoa have also been noted to generate ROS independently of leukocytes (Baker, Krutskikh et al. 2003) and the ability of sperm to generate ROS depends on the maturation level of the spermatozoa.

Sperm membranes are rich in polyunsaturated fatty acid, a feature rendering them susceptible to oxygen-induced damage mediated by lipid peroxidation (LPO) (Sikka, 2001). Lipid peroxidation is an important pathophysiological process occurring in numerous diseases and stress conditions and results in a series of degenerative processes affecting the organization and function of various cellular components (Aitken, Clarkson et al. 1989). Oxidative stress is an important factor in the etiology of poor sperm function, inducing morphological alterations (Aziz et al., 2004) and oxidative damage to DNA, membranes and proteins (Stadtman et Levine, 2003; Moustafa et al., 2004; Aitken et De Luliis, 2007). The LPO caused by ROS such as H_2O_2 is the limiting factor of the lifespan of mammalian spermatozoa. In addition, an excess of ROS and free radical generation have frequently been detected in the seminal plasma and sperm of infertile men (Baker et Aitken, 2005; Pasqualotto et al., 2008). Seminal plasma and sperm contain a panel of protective antioxidants, such as the glutathione peroxidase/reductase system, superoxide dismutase, catalase and low-molecular weight antioxidants, vitamin E, vitamin C, urate and albumin, which scavenge ROS and circumvent possible cellular damage (Tremellen, 2008). Many studies have investigated the possible effect of this process on the loss of sperm functional parameters (Geva, Bartoov et al. 1996, Zabludovsky, Eltes et al. 1999, Rajesh Kumar, Doreswamy et al. 2002).

Flavonoids

Flavonoids are characterized by a phenyl benzo(γ)pyrone-derived structure consisting of two benzene rings (A and B) linked by a heterocyclic pyran or pyrone ring (C) (Kühnau, 1976 and Morand et al., 1998). Flavonoids are plant phenolic compounds with strong

antioxidant properties found in many dietary sources such as tea, onion, broccoli, apple and green beans (Anjaneyulu and Chopra, 2004). Flavonoids can prevent oxidative damage as a result of their ability to scavenge reactive oxygen species such as hydroxyl radical and superoxide anion (Galati et al., 2002) and metal chelating (Pedrielli and Skibsted, 2002). In plants, the flavonol aglycone is most commonly present conjugated at the 3-position of the unsaturated C-ring with a sugar moiety, forming O-β-glycosides such as quercitrin or rutin (Merck, 2001). Quercetin can be obtained from plants via extraction of the quercetin glycosides followed by hydrolysis to release the aglycone and subsequent purification. Flavonols exhibit numerous biological and pharmacological effects, including anti-oxidant, chelation, anti-carcinogenic, cardioprotective, bacteriostatic, and secretory properties (Gross et al., 1996, Middleton et al., 2000 and PDRNS, 2001). In plants, these compounds are involved in energy production (Theoharides et al., 2001) and exhibit strong anti-oxidant properties, possibly protecting plants against harmful ultraviolet rays (Wiczkowski et al., 2003).

Quercetin

Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is the most abundant dietary flavonoid in fruit and vegetables **(**Russo et al., 2012). According to the literature, the daily intake of quercetin varies widely. In the western world, the average daily intake of quercetin has been estimated to be between 20 mg and 40 mg, although it can increase up to 500 mg/day in individuals who ingest large quantities of apples, onions and tomatoes (Manach et al., 2005, Manach et al., 2004 and Russo et al., 2012). Furthermore, quercetin is available as a dietary supplement, and ingestion of 1 g/day or even more has been reported (Manach et al., 2005 and Russo et al., 2012). Quercetin is a member of a family of flavonoid polyphenols with more intense antioxidant activity than vitamins C and E, as well as lesser toxicity. Quercetin has also a pro-oxidant effect and this depending on its concentration, which is indicative of its hermetic behaviour with lower doses displaying mostly antioxidant properties (Watjen et al., 2005). Phenolic compounds act as free radical scavengers and, at times, as metal chelators, acting both in the initiation step, as well as in the propagation of oxidative process (Stojanovic, Sprinz et al. 2001). Quercetin improved stallion sperm motility and acrosome integrity, and markedly enhanced the zona binding ability of spermatozoa while also reducing DNA fragmentation in cryopreserved spermatozoa (Gibb, Butler et al. 2013). Quercetin was reported to ameliorate the cadmium induced oxidative toxicity of germ cells in male mice (Bu et al., 2001) also cytotoxicity in Sertoli-germ cell cultures due to atrazine was countered by

quercetin intervention (Abarikwu et al., 2012). Similarly, oxidative damage induced by diethylstilbestrol to hamster spermatogenic cells was prevented by simultaneous treatment with quercetin (Li et al., 2010). However, a recent contradictory report indicates that quercetin administration to male mice increased the generation of reactive oxygen species and lipid peroxidation in the testis with concomitant decrease in sperm count and motility in a dose dependant manner (Ranawat et al., 2013).

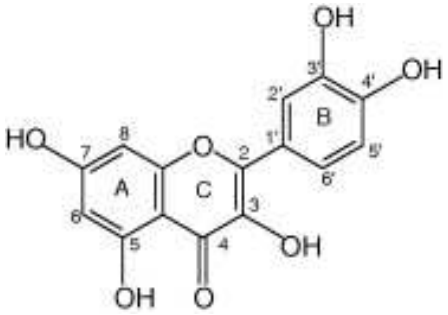
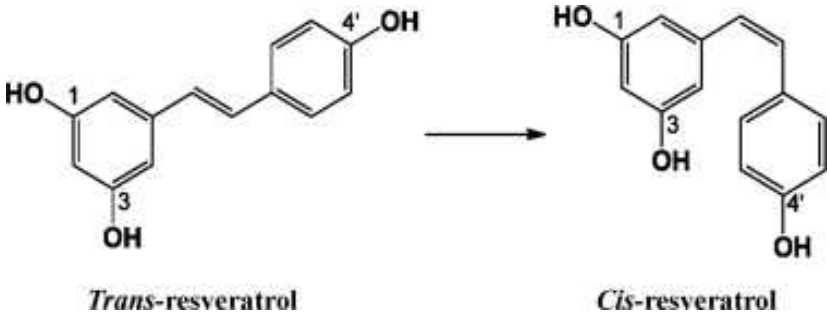


Figure 2 Chemical structure of quercetin

Resveratrol

Other flavonoid substances with antioxidant action have been described. With regard to semen supplementation, resveratrol (3,5,4′-trihydroxystilbene) is a natural polyphenol found mainly in red grapes and red wine that plays an important role as a lipid soluble antioxidant, acting as an effective scavenger of superoxide ($O2^{-}$), hydroxyl (OH) and metal-induced radicals (Joe, Liu et al. 2002, Sarlos, Molnar et al. 2002, Collodel, Federico et al. 2011, Schmatz, Perreira et al. 2012). Resveratrol also exhibits a protective effect against ethanol induced lipid peroxidation in testes (Kasdallah-Grissa, Mornagui et al. 2006) and lipid peroxidation induced by tert-butyl hydroperoxide in spermatozoa, protecting sperm chromatin and membranes (Collodel, Federico et al. 2011), and preventing DNA damage caused by ROS (Revel, Raanani et al. 2001). Resveratrol inhibited lipid peroxidation of ram spermatozoa most effectively when applied at low concentrations (15 μg/109 spermatozoa), as demonstrated by the TBARS test (Sarlos, Molnar et al. 2002). Resveratrol inhibited the BαP-diol-epoxide (BPDE) adduct formation in sperm DNA, and apoptotic and necrotic cell death (Revel, Raanani et al. 2001).



Trans-resveratrol *Cis*-resveratrol

Figure 3 Chemical structure of *trans*- and *cis*-resveratrol.

Curcumin

Curcumin is a bright yellow compound found in turmeric, which is derived from the rhizomes of the plant *Curcuma longa* Linn, a perennial herb of the *Zingerberaceae* family (Ammon and Wahl 1991). Curcumin is a lipophilic polyphenol that is insoluble in water (Ruby, Kuttan et al. 1995). Curcumin has been shown to scavenge free radicals (Sharma 1976). Under *in vitro* conditions, curcumin significantly inhibited the generation of ROS such as superoxide anions and H_2O_2, and nitrite radical generation by activated macrophages, which plays an important role in inflammation (Mathuria and Verma 2008). Curcumin lowered the production of ROS *in vivo* (Joe and Lokesh 1994). The antioxidant mechanism of curcumin is attributed to its unique conjugated structure, which includes two methoxylated phenols and an enol form of diketone; the structure of curcumin shows typical radical-trapping ability as a chain-breaking antioxidant (Masuda, Maekawa et al. 2001). Supplementation of fresh bull semen with curcumin significantly increased the post-thaw sperm content of GSH (Bucak, Baspinar et al. 2012). Administration of curcumin to male rodents challenged by reproductive toxicant appears to have a protective effect toward testicular function and fertility (Mathuria and Verma 2008, Sahoo, Roy et al. 2008).

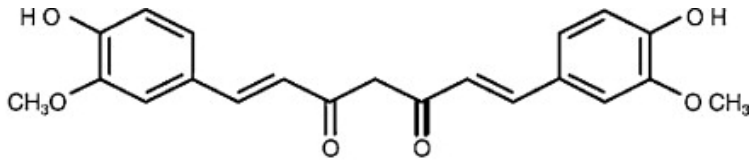


Figure 4 Chemical structure of curcumin

Conclusion

Antioxidants can improve semen quality. One of the many antioxidants could be flavonoids like quercetin, resveratrol, curcumin and etc. Based on other authors' results, flavonoids might be used as an alternative drug for the treatment of male infertility problems. This ROS scavenging antioxidants could be provided in feed, added directly to semen extender or added to insemination catheters. However, the exact mechanism of action and dose analysis need to be further investigated.

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THE EFFECTS OF TCDD AND/OR GENISTEIN ON THE VIABILITY AND INCIDENCE OF APOPTOSIS IN GRANULOSA CELLS ISOLATED FROM LARGE PORCINE FOLLICLES

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Introduction

Endocrine disrupting chemicals are present in the environment and influence reproductive processes by interfering with steroid hormone biosynthesis/secretion and/or receptor signaling and gene regulation. The current study was designed to examine whether the changes in steroid hormone production caused by low doses of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and/or genistein (G) were related to the induction of apoptosis in porcine granulosa cells obtained from large (≥8 mm) porcine follicles.

Materials and Methods

For estimation of P_4 and E_2 secretion, granulosa cells were first precultured for 40 h and then cultured (phenol-free Eagle's medium, 5% CS, 0.05 mg/ml gentamycin and 60 U/ml nystatin) for additional 48 hours with TCDD (100 pM) and/or genistein (50 or 500 nM). Medium concentration of steroid hormones was measured by radioimmunoassay. The effects of TCDD and/or G on the incidence of apoptosis were measured in granulosa cells, first precultured (40 h) and then cultured (phenol-free Eagle's medium, 5% CS, 0.05 mg/ml gentamycin and 60 U/ml nystatin) with TCDD (100 pM) and/or G (500 nM) for three, six or 24 hours. After culture, the cells were stained with the use of FITC Annexin V Apoptosis Detection Kit - FITC labeled Annexin V (5 µl/ 1×10^6 cells) and propidium iodide (PI; 5 µl/ 1×10^6 cells) which was followed by flow cytometry. Annexin V^-/PI^+ cells were recognized as necrotic, Annexin V^+/PI^+ as late apoptotic, while Annexin V^+/PI^- cells were recognized as early apoptotic cells. Statistical analysis was performed using one-way ANOVA for repeated measurements followed by the LSD test.

Results

Higher dose of G (500 nM) and TCDD combined with G (50 or 500 nM), in contrast to TCDD or lower dose of G (50 nM) alone, decreased P_4 secretion by granulosa cells from large follicles. TCDD and/or G did not affect E_2 secretion. TCDD (100 pM) increased the number of live granulosa cells after three hours of culture, whereas genistein (500 nM)

abolished this effect of TCDD. At the same time point, TCDD decreased the number of necrotic cells. TCDD and genistein used alone and in combination did not affect the percentage of live, necrotic, early and late apoptotic granulosa cells in the remaining time points.

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Conclusions

In contrast to TCDD (100 pM) and G (50 nM) applied alone, the use of the combined compounds decreased P4 production by porcine granulosa cells from large follicles after 48 hours of culture. TCDD increased the number of apoptotic cells and decrease the number of necrotic cells after 3 hours of culture, but not after 6 or 24 hours. Therefore, TCDD and genistein induced changes in granulosal steroidogenesis did not correspond to the changes in the incidence of apoptosis.

THE EFFECTS OF TCDD AND/OR GENISTEIN ON THE VIABILITY AND INCIDENCE OF APOPTOSIS IN GRANULOSA CELLS ISOLATED FROM MEDIUM PORCINE FOLLICLES

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Introduction

Environmental estrogens such as dioxins (e.g., 2,3,7,8-tetrachlorodibenzo-p-dioxin; TCDD) and phytoestrogens (e.g., genistein; G) affect reproductive processes including granulose cell functions in humans and animals. The current study was designed to examine whether the changes in steroid hormone production caused by low doses of TCDD and/or G were related to the induction of apoptosis in porcine granulosa cells obtained from medium (3-6 mm) porcine follicles.

Materials and Methods

For estimation of P_4 and E_2 secretion, granulosa cells were first precultured for 40 h and then cultured (phenol-free Eagle's medium, 5% CS, 0.05 mg/ml gentamycin and 60 U/ml nystatin) for additional 48 hours with TCDD (100 pM) and/or genistein (50 or 500 nM). Medium concentration of steroid hormones was measured by radioimmunoassay. The effects of TCDD and/or G on the incidence of apoptosis were measured in granulosa cells, first precultured (40 h) and then cultured (phenol-free Eagle's medium, 5% CS, 0.05 mg/ml gentamycin and 60 U/ml nystatin) with TCDD (100 pM) and/or G (500 nM) for three, six or 24 hours. After culture, the cells were stained with the use of FITC Annexin V Apoptosis Detection Kit - FITC labeled Annexin V (5 µl/ 1×10^6 cells) and propidium iodide (PI; 5 µl/ 1×10^6 cells) which was followed by flow cytometry. Annexin V^-/PI^+ cells were recognized as necrotic, Annexin V^+/PI^+ as late apoptotic, while Annexin V^+/PI^- cells were recognized as early apoptotic cells. Statistical analysis was performed using one-way ANOVA for repeated measurements followed by the LSD test.

Results

The higher concentration of genistein (500 nM) alone and in combination with TCDD (100 pM) decreased P_4 secretion by granulosa cells isolated from medium follicles. Higher dose of genistein (500 nM) alone as well as TCDD combined with G (50, 500 nM) significantly

decreased E_2 secretion by the cells. TCDD and/or genistein (500 nM) did not affect the number of live, early apoptotic, late apoptotic or necrotic cells after 3, 6 and 24 hours.

Acknowledgment

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Conclusions

TCDD (100 pM) amplified the inhibitory effect of 500 nM of genistein on P_4 secretion by granulose cells isolated from medium follicles. Although when applied separately, TCDD (100 pM) and lower concentration of genistein (50 nM) did not affect E_2 secretion, but in combination they decreased E_2 secretion. No treatment affected the number of apoptotic cells. Therefore, TCDD and genistein induced changes in granulosal steroidogenesis did not correspond to the changes in the incidence of apoptosis.

SUPPLEMENTAL LIGHTING ON DAIRY PERFORMANCE IN THE TROPICS

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Abstract

Milk yield and milk composition were compared in 4 cows during a 58-day period in mid lactation in central Thailand. Controls received natural lighting which averaged 11 hours of daylight and 13 hours of darkness (11L:13D), while treated cows received supplemental fluorescent lighting to a photoperiod of 16L:8D. All cows were housed indoors in open-sided barns and were fed a mixture of grass hay, meal concentrate and molasses. Machine milking took place twice daily. Treated cows on the 16L:8D photoperiod produced 4.2% more fat-corrected milk (9.9±2.1 kg/d) than controls (9.8±1.9 kg/d) after weeks 5-8 only but non-significant, and ate 4.7% more grass hay (8.7 vs. 8.2 kgDM/d). Milk fat averaged 4.3±0.11% in both groups and milk protein 3.0±0.14%. IGF-1 levels were non-significantly higher in the treated group (49.25±10.3 vs. 45.25±9.9 ng-100ml). More cows per groups, a longer trial and individual monitoring of hay intake, and milk composition will be needed to confirm the current results which nevertheless suggest valuable responses in tropical dairy cows to supplemental lighting.

Introduction

At high latitudes, short days in winter have been identified as a limitation to milk production, and responses of 8-10% in milk yield and DM intake have been reported to long-supplemental photoperiods (typically, 16 h light/day: Miller *et al*., 1999). Dahl *et al*. (1997) have proposed increased circulating levels of Insulin-like-growth factor-1 (IGF-1), a galactopoietics hormone in ruminants, as the principal regulatory mechanism in this photoperiodic response.

In general, dairy cows in Thailand are commonly kept permanently indoors under shade and shelter for safety and fed by the cut-and-carried pasture (Tudsri and Sawasdipanit, 1993). It would thus be a simple matter to supplement their photoperiod with artificial light, but no information is available on the efficacy of this technique under tropical conditions, or with tropical genotypes, which in other respects (eg. hair shedding) are less photo-reactive than temperate breeds (Yates, 1957). The current experiment was thus undertaken in central

Thailand with cows of the dominant local dairy type (75% crossbred Friesian) fed and housed under typical local conditions.

Materials and Methods

Experimental site, and climate

The experiment was conducted at Muaklek, Saraburi Thailand (14°50´N, 101°10´E) at an altitude 220 m and where natural daylength varied only from 11.2 to 11.5 h/d during the experimental period. Annual rainfall in the area is 1192 mm. Daily recordings in a Stephenson screen adjacent to the cow barns reveals mean values of 29.1 and 17.0°C (maximum and minimum dry bulb temperatures), 66% relative humidity, 1.3 m/s wind velocity and a total rainfall of 23.5 mm.

Experimental design, treatment and management

Two groups of 4 Friesian-cross lactating cows, weighing 384-389 kg, yielding 10.5 kg milk/day in the 4th month of lactation were balanced for weight and milk yield and allocated to Treatment 1 (Control-natural lighting for the area: 11L:13D photoperiod) or Treatment 2 (Natural lighting + supplemental fluorescent lighting each evening until 22.00 h: a 16L:8D photoperiod).

Light intensity at eye level averaged 350 lux (Dahl *et al.,* 1997). Each group was housed, unrestrained, in one of the 2 identical open-sided barns (12 m x 7 m wide; 3.5 m high), and was grouped fed a ration of protein supplement (90.5%DM, 16.4%CP, 73% in vitro dry matter digestibility (IVDMD): at the rate of 1 kg/1.5kg milk produced), 1 kg of molasses (78.4%DM, 3.6%CP, 15.6 MJ/kgDM) and Guinea grass hay (91%DM, 5.7%CP, 33%IVDMD) *ad libitum* ;Treatment 1 was 30 m distant from Treatment 2 and was shielded from any stray artificial lighting coming from the latter barn.

Mechanical milking took place at 05.00 and 15.30 h daily and prior to milking all cows were thoroughly showered and fed their concentrate and molasses. The experiment ran for 58 days from December 13, 1999 to February 9, 2000. Milk yield was recorded daily for each cow, and from each milking a sample was taken, bulked and analysed weekly for fat and protein content using the Milkoscan Tester. All milk yields were compared on a 4% fat-corrected basis (FCM). The intake of grass hay was calculated as the difference between hay offered and the uneaten residual.

Blood urea nitrogen (BUN) was used to assess the nitrogen status (Blowey *et al.,* 1973). Ten ml jugular blood samples were taken from all cows on 19 January 2000 at 07.30 h, and again at 13.30 h. The heparinised samples were centrifuged and the plasma was stored at -20°C for

the late BUN (Tiffany *et al.*,1972) and glucose analysis (Slein, 1963), both using the Cobas Mira, Roche. To accommodate the BUN and glucose technique, morning supplementation was delayed until after milking on that day only. The following day, 20 January 2000, separate 10ml jugular blood samples were drawn at 13.30 h. and the plasma stored at -20°C for plasma IFT-1 determination (IGF-1 OCTEIA ELISA 96 Wells (Immunodiagnostic Systems, UK).

Statistical analyses on milk production, blood metabolites, liveweight and plasma IFG-1 changes were carried using at t-test under a Pair-comparison design with the animals as the experimental unit (SAS 1985).

Results and Discussion

Feed intake and blood metabolites

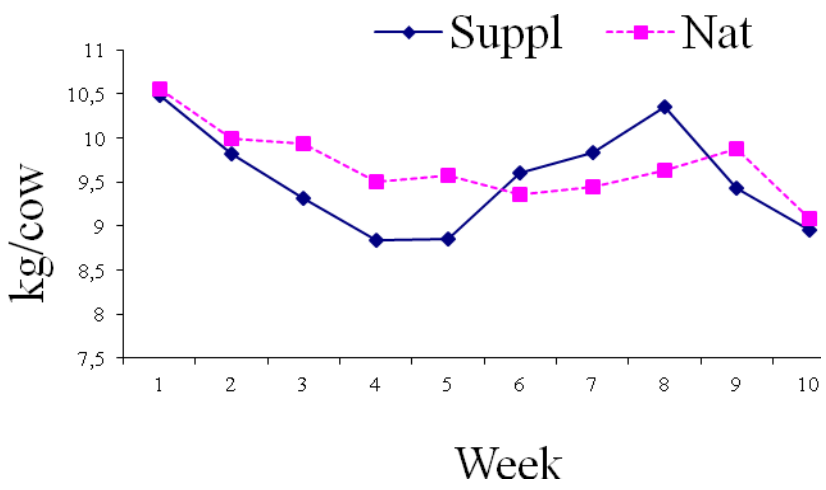
The low CP and digestibility of the grass hay fed in this study would, alone, have presumably limited milk production, but the supplementation with protein meal and molasses lifted the intake of experimental cows above that required (NRC, 1988) for maintenance and production. The BUN and glucose levels recorded support that conclusion. Neither parameter was affected by treatments, and at the morning sampling BUN values averaged 14.8±6.3 mg%. Afternoon values were slightly higher, and averaged 18.6±6.6 mg%; both values being above the average range of 12.3 to 17.8 mg% reported by Rowlands *et al.* (1977). Glucose values at the corresponding times averaged 52.4±2.0 and 55.2±3.8 mg%, respectively; both values well above the averages quoted by Payne *et al.*(1974) of 42-45 mg%. From these data, we can conclude that the experimental cows were well fed, relative to their level of production, and that they should thus have been in position to respond to any galactopoietics stimulus provided by the artificial long photoperiod (16L:8D) in Treatment 2. The intake if grass hay DM was higher in Treatment 2 than Treatment 1 (estimated as 8.7 and 8.2 kgDM/cow/d) on a group-fed basis), a result that is consistent with early work in higher latitudes (Tanida *et al*., 1984) and in Thailand (Tudsri and Sawasdipanit, 1993) which showed that 24h/d lighting for security reasons led to an increase in feed intake. Cows in Treatments 1 and 2 weighed 389±6.3 and 384±6.3 kg at the start of the experiment, and gained 28.3 and 15.4 kg during experimentation, respectively. The lesser (non-significant) gain in weight in Treatment 2 is consistent with those cows producing more milk, and partitioning more nutrients to milk production than cows of Treatment 1. Under normal conditions (for example, Treatment 1), supplementary feeding of cows in mid-lactation would be expected to

lead to more energy being partitioned to body energy reserves, as was apparently the case here.

Milk yield and composition, and circulating IGF-1

Average daily FCM yields averaged 9.9±2.1 and 9.8±1.9 kg/cow in Treatments 1 and 2, respectively (non-significant), but when the week by week data is examined (Figure 1), it can be seen that FCM yield in Treatment 2 was relatively low at the first 4 week, but then recovered to an overall 4.2% gain in week 5 to 8.

Figure 1. Average daily FCM yields of cows in both treatments.



This indicates that the lower FCM yield at the first 4 week would possibly be a direct physiological mechanism which acts by reversing a natural trend for cows to visual stress and adaptation resulting in low eating activity (Phillips and Schofield, 1989). However, total DM intake of light supplemented cows was apparently higher. More cows per treatment will be necessary in future work to confirm the existence of such a trend, but the pattern in Treatment 2 is consistent with the known fact that photoperiodic responses in ruminants are delayed, and slow-acting (Yates, 1956) Milk fat and protein levels (4.3±0.11 vs. 4.3±0.12% and 3.0±0.16 vs 3.0±0.11%, respectively in Treatments 1 and 2) were not significantly affected by treatment under the current conditions. Similarly, IGF-1 levels were not significantly affected by treatment, but values in Treatment 2 (49.25±10.3 ng/100ml) were higher than those in

Treatment 1 (45.25±9.9 ng/100ml). The latter result is consistent with the work of Dahl et al. (1977), which showed that long photoperiods led to increased circulating levels of IGF-1.

There are number of reports in the temperate zone and higher latitudes (Peters *et al.,* 1981; Mercek and Swanson, 1984; Bilodeau *et al.,* 1989; Dahl *et al.,* 1997), that supplemental lighting can increase milk yield. The current result from a 9-week study (duration limited by outside circumstances) indicate a similar situation in tropical dairy cows of largely Friesian genotype. The facts that a 4.2% increase in yield was recorded in Treatment 2 and that the trend apparent in DM intake and IGF-1 levels are consistently in the expected direction, support the need for more extensive studies involving a much larger sample size, cows in early lactation and cows that are individually recorded for all parameters. Artificially supplemental photoperiod appear to have real potential to improve feed intake and milk yield in tropical countries where cows are normally housed for thermoregulatory and security reasons.

Conclusion

It can be expressed that this experiment has possibly provided a support for the use of photoperiod as an effective, non-invasive method to enhance milk production. Details on stage of lactation, number of cows and hormonal responses from PRL and IGF-1 are needed to an increase in milk production under tropical conditions.

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HEMATOLOGIC PROFILE FROM MOUFLONS (*OVIS MUSIMON*) DEPENDING ON SEASON

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Abstract

The aim of our work was to investigate haematological parameters of mouflons. The experimental study included a total of 10 mouflons, they were only 5 years age females. Most animals proved to be clinically healthy. The middle level of hemoglobin was observed and detected in interval 12,18 – 13,06 $g.dl^{-1}$ with the lowest middle level in spring and with the maximum middle level in autumn. The middle level of hematocrit (*Hk*) was observed in seasons which were detected in interval 0,371 – 0,384 $l.l^{-1}$ with the lowest middle level in spring and with the maximum middle level in summer season. The middle level of erythrocyte (*Er*) was observed in seasons and detected in interval 8,07 – 9,37 $T.l^{-1}$ and middle level of lymphocyte (*Ly*) observed in seasons which were detected in interval 62,92 – 66,18 % with the lowest middle level in winter and with maximum middle level in autumn.

Key words: erythrocytes, female, lymphocytes, mouflons

Introduction

Mouflons are primarily native from Mediterranean region. Since 1730 the first mouflons were carried in to Europe from Sardinia and Corsica islands. On the sustenance are these animals less demanding than other wild living animals. They are more demanding on the climatic conditions. They are modest, because feeds on only with herbs, campestral herbs, campestral fruits, leafs and in winter season to mouflons suffices only dry grass and hay. They are hardy towards frost but unfavourably suffer high snow (Hell et al., 2008).

Hematological parameters observation are using as an aid in clinical diagnostics. The haematological parameters are used for utilization on the metabolic status valuation in animals (Sakalová, et al., 2011). Causes of hematological variability that cause differences at selected individuals may be clear or very slightly and they are given genetics, environment, age and sex of individuals. The aim of this paper is to point to another parameter of

interaction on the animal body and even the impact of the season on the hematological status of mouflons.

Material and Methods

The experimental group consisted of 10 mouflon females in ages 5 year of the South-East area in Slovakia. Wild living animals were kept in menagerie during year. Water intake was unrestricted. The blood was taken from jugular vein using by vacuum tubes. In the blood, by normal laboratory procedure was setting the haematological profile of blood components (Er, Htk, MCV, Hb, Lc). The obtained samples were examined by the Centre of Excellence Laboratory of Animal Ecology and man at Prešov University in Prešov. The obtained values were analysed and subsequently evaluated. Based on the evaluation, data was determined by middle level of standard variation, minimal and maximal levels. Based on the determination, minimal and maximal middle variations were compared relevant samples, measured in reported seasons and set a correlation coefficient of significance.

Results

Middle level standard variation, minimum and maximum values of selected hematological parameters in blood of mouflons shows Table 1.

Table.1. Selected haematological indicators in mouflons blood.

Winter season (n=10)				
Parameter	**Hb**	**Hk**	**Er**	**Ly**
Benchmark	7,22 - 13,9	0,27 - 0,40	5,2 - 11,2	56,4 - 69,9
	$g.dl^{-1}$	$l.l^{-1}$	$T.l^{-1}$	%
□x	12,4	0,382	8,07	62,92
std	1,222	0,060	1,616	3,759
min	10,4	0,28	5,3	57,3
max	14,3	0,49	10,6	69,1
Spring season (n=10)				
□x	12,18	0,371	8,13	63,27
std	1,040	0,057	1,485	4,122
min	10,4	0,27	5,7	57,2
max	14,1	0,47	10,5	70,1
Summer season (n=10)				
□x	12,97	0,384	9,09	65,66
std	1,044	0,042	1,294	2,859
min	11,2	0,29	7,1	60,4
max	14,3	0,44	10,9	69,8
Autumn season (n=10)				
□x	13,06	0,375	9,37	66,18
std	0,838	0,032	1,323	2,424
min	11,5	0,29	7,2	62
max	14	0,41	11,1	69

n – number of individuals, Hb – hemoglobin, Hk – hematokrit, Er – erythrocyte, Ly – lymphocyte, □x –median, std – standard variation, min – minimum, max – maximum.

Hematological profile

Middle level of hemoglobin (*Hb*) was observed in seasons and detected between 12,18 – 13,06 $g.dl^{-1}$ with the lowest median level in spring and maximum middle level in autumn. Among the sample of these seasons were not found significant differences. Middle level of hematokrit (*Hk*) was observed in seasons and detected between 0,371 – 0,384 $l.l^{-1}$ with lowest median level in spring and maximum middle level in summer. Among sample of these seasons were found significant differences. Middle level of erythrocyte (*Er*) was observed in seasons and detected between 8,07– 9,37 $T.l^{-1}$ with the lowest median level in winter and with maximum middle level in autumn. Among observed sample of these seasons were found significant differences. Middle level of lymphocyte (*Ly*) was observed in seasons and detected between 62,92 – 66,18 % with the lowest median level in winter and with the maximum middle level in autumn. Among observed sample of these seasons were found significant differences.

Conclusion

Hematological results of the investigation presented in this work are primary study a broader research on the impact of seasonality on the haematological status of wild game. The results of this work show that it is necessary to point out the quantitative and qualitative haematological profile in wild mouflon females. Determined middle hematologic values ranged in hemoglobin 12,18 – 13,06 $g.dl^{-1}$, hematokrit was 0,371 – 0,384 $l.l^{-1}$, erythrocyte was 8,07 – 9,37 $T.l^{-1}$ and lymphocyte 62,92 – 66,18 %. The results in our experiment showed interaction of animal organism and the impact of the season on the hematological status of experimental female mouflon.

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EFFECT OF COPPER ON PORCINE OVARIAN GRANULOSA CELLS

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Abstract

The objective of the present study was to investigate the secretion of progesterone and insulin-like growth factor I of porcine ovarian granulosa cells after Cu addition *in vitro* and to outline a potential intracellular mediator (cyclin B1) of its effects. This study also examined the *in vitro* apoptotic potential of Cu on porcine ovarian granulosa cells. Ovarian granulosa cells were incubated with copper sulphate ($CuSO_4.5H_2O$) at the doses ranging from 0.33–2.0 $\mu L.mL^{-1}$ for 18 h and compared with control group without Cu addition. Results showed that P_4 release by granulosa cells was inhibited while the release of IGF-I and cyclin B1 was stimulated significantly ($P < 0.05$) by $CuSO_4.5H_2O$ addition at the dose 2.0 $\mu L.mL^{-1}$. Also, addition of $CuSO_4.5H_2O$ at the lowest dose used in the study (0.33 $\mu L.mL^{-1}$) significantly ($P < 0.05$) decreased apoptosis in granulosa cells. Obtained data suggest dose dependent effect of Cu on secretion of steroid hormone progesterone and growth factor IGF-I, and expression of cyclin B1 as marker of proliferation of porcine ovarian granulosa cells. Results also indicate dose dependent effect of Cu on apoptosis of porcine ovarian granulosa cells and, that the effect of Cu on ovarian cell proliferation could be mediated by IGF-I and cyclin B1.

Introduction

Copper (Cu) is an essential trace element and plays an important role in plant, animal and human nutrition (Roychoudhury et al., 2008). Copper is strongly bioaccumulated and is readily absorbed after ingestion, inhalation, and dermal exposure (Binkowski et al., 2013; Gerhardsson et al., 2002; Roychoudhury et al., 2009). One of the most frequent forms of Cu is copper sulphate ($CuSO_4$) - it a naturally occurring inorganic salt and is widely used in agriculture as fungicide, algaecide, herbicide and molluscicide (Roychoudhury and Massanyi, 2008; NPIC, 2013). Examinations of $CuSO_4$-poisoned animals showed signs of acute toxicity

in the spleen, liver and kidneys (Clayton and Clayton, 1981). High Cu concentrations in the animal organism have also been associated with harmful consequences on the reproductive system, although the role of Cu in reproductive cells remains largely unknown (Massanyi et al., 2012; Roychoudhury et al., 2009; Roychoudhury et al., 2010; Roychoudhury and Massanyi, 2008). Our previous reports of exposure to metals suggest changes in hormonal secretion of porcine and chicken ovarian granulosa cells (Capcarova et al., 2009; Kolesarova et al., 2010b).

Progesterone (P_4) is essential for normal ovarian cycles and contributes to regulation of ovarian follicular development and remodeling (Kolesarova et al., 2010a,b). It is produced by ovarian granulosa cells and the *corpus luteum* (Kolesarova et al., 2009, 2010a,b). Insulin-like growth factor I (IGF–I), is an important stimulator of mitosis and ovarian steroid production by ovarian granulosa and theca cells, which is required for normal oocyte development and hormonal feedback signalling to the hypothalamus and pituitary (Kolesarova et al., 2009, 2010b). IGF-I is produced by porcine ovarian granulosa cells, beside other organs (Kolesarova et al., 2010a,b, 2011b). The process of ovarian cell proliferation, growth and development also involves cell cycle peptides, especially proliferation related peptide cyclin B1 (Kolesarova et al., 2011a). The fate of the ovarian follicle not only depends on a delicate balance between the expression of factors promoting follicular cell proliferation, growth and differentiation, but also of those inducing apoptosis (Jiang et al., 2003). Our previous studies also reported metal-induced alterations in cyclin B1 expression in porcine ovarian granulosa cells (Kolesarova et al., 2010a,b, 2011a,b). The objective of the present study was to investigate the secretion of progesterone and insulin-like growth factor I of porcine ovarian granulosa cells after Cu addition *in vitro* and to outline a potential intracellular mediator (cyclin B1) of its effects. This study also examined the *in vitro* apoptotic potential of Cu on porcine ovarian granulosa cells.

Material and methods

Preparation, culture and processing of granulosa cells from ovaries

Porcine ovaries obtained from healthy Slovakian White gilts at the early and mid-follicular phase of the estrous cycle without visible reproductive abnormalities were collected, transported to the laboratory at 4°C and washed in sterile physiological solution. Follicular fluid was aspirated from 3 to 5 mm follicles and granulosa cells were isolated by centrifugation for 10 min at 200 × g followed by washing in sterile DMEM/F12 1:1 medium

and resuspended in the same medium supplemented with 10% fetal calf serum and 1% antibiotic-antimycotic solution at a final concentration of 10^6 cells.mL^{-1}. Portions of the cell suspension were dispensed to well plates and chamber slides and were incubated at 37.5 ◦C and 5% CO_2 in humidified air until a 75% confluent monolayer was formed (5–7 days). At this point, the medium (1 mL per well plates or 200 µL medium in 16-welled chamber slide cells) was renewed and ovarian granulosa cells in culture media were incubated 18 h with the same supplements (10% fetal calf serum, 1% antibiotic-antimycotic solution) and with or without chemical substance copper sulphate - $CuSO_4.5H_2O$ (purity ≥98.0%; molecular weight 249.69; Sigma-Aldrich, St. Louis, USA) at the doses 0.33, 0.40, 0.50, 1.0 and 2.0 µL.mL^{-1} (Table 1). After 18 h the culture media from wells plates were collected for RIA, 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 −4 ◦C to await immunocytochemical analysis.

Immunoassay

Concentrations of P_4 and IGF-I were determined in 25–100 µL incubation medium by RIA. These substances were assayed using RIA kits. All RIAs were validated for use in samples of culture medium. RIA assay sensitivity for P_4 was 0.05 ng.mL^{-1} while inter- and intra-assay coefficients of variation did not exceed 9.0% and 5.8%, respectively. RIA assay sensitivity for IGF-I was 2 ng.mL^{-1} while inter- and intra-assay coefficients of variation did not exceed 6.8%, and 6.3%, respectively.

Immunocytochemistry

Signalling substances within granulosa cells plated on chamber slides were detected using immunocytochemistry. The ImmunoCruz Staining System and primary mouse monoclonal antibodies against cyclin B1 were used as directed by the manufacturer at a dilution of 1:500. Visualisation of the primary antibody binding sites was achieved with a secondary rabbit polyclonal antibody against mouse IGs, labelled with horseradish peroxidase (dilution 1:1000) and diaminobenzidine (DAB) reagent (10%). Chamber slides stained with peroxidase/DAB reagent were mounted with Glycergel mounting medium. The general cell morphology, presence and localization of specific immunoreactivity in cells, as well as the counting of the percentage of cells containing specific immunoreactivity were determined by light microscopy.

TUNEL assay

MEBSTAIN Apoptosis kit Direct was used to detect terminal deoxynucleotidyl transferase (TdT)–mediated dideoxy uridinetriphosphate (dUTP) nick end labelling (TUNEL) reactions. At

Table 1**.** Copper concentrations used in the study

Group	**$CuSO_4.5H_2O$ (µL)**	**Medium (mL)**	**Dilution Rate**	**Concentrations of $CuSO_4.5H_2O$ ($\mu L.mL^{-1}$)**
Control	0	1	0:1	0
A	2	1	1: 500	2
B	1	1	1:1000	1
C	0.5	1	1:2000	0.5
D	0.4	1	1:2500	0.4
E	0.33	1	1:3000	0.33

the end of the culture, the chamber slides were subjected to the TUNEL assay using an *in situ* cell death detection kit. The general cell morphology and percentage of TUNEL–positive cells in each culture was determined by visual evaluation of cultures and counting of TUNEL–positive and TUNEL–negative cells using light microscope and Leica fluorescent microscope.

Statistical analysis

Significant differences between the control and experimental groups were evaluated by using two-way ANOVA, paired t-test or Chi-square test (χ^2) using statistical software Sigma Plot 11.0. The data are expressed as means ± SEM. Differences from control at $P < 0.05$ were considered as significant.

Results

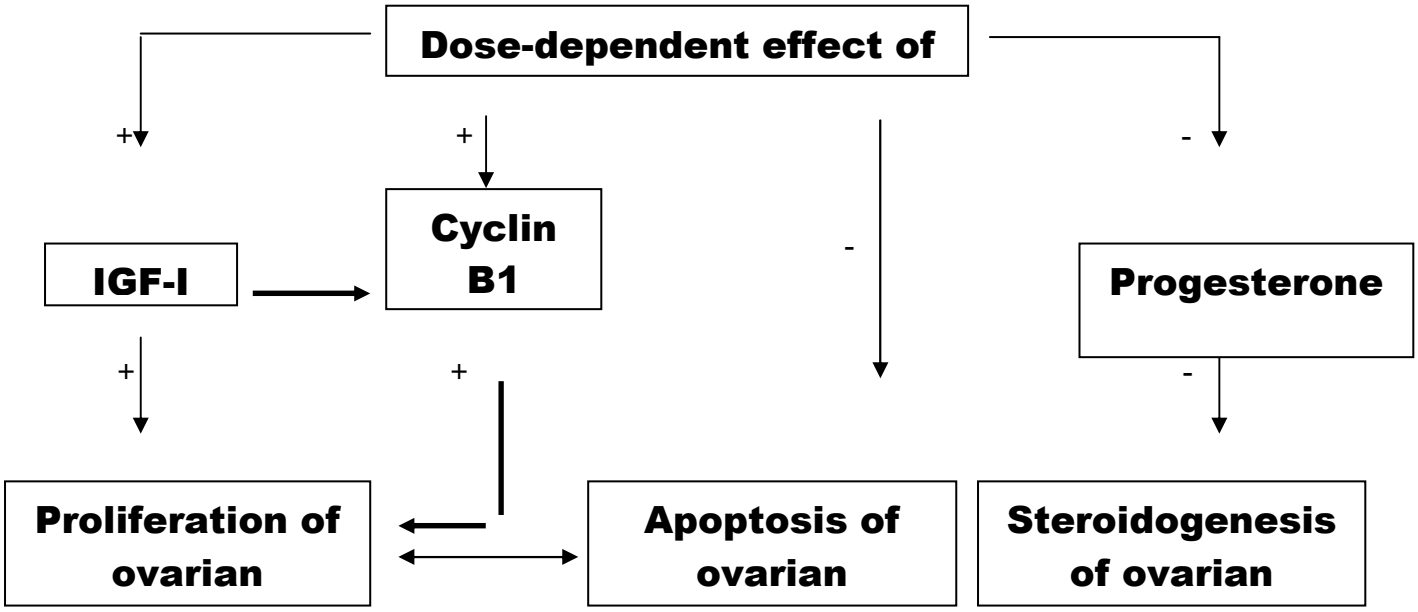
Secretion of P_4 by granulosa cells was significantly ($P<0.05$) inhibited in the experimental group A after Cu addition at the dose 2.0 $\mu L.mL^{-1}$ (0.64 ± 0.08 $ng.mL^{-1}$) in comparison to control group (3.31 ± 0.09 $ng.mL^{-1}$). P_4 release by granulosa cells was similar to that of control group in other experimental groups B (2.62 ± 0.05 $ng.mL^{-1}$), C (3.36 ± 0.32 $ng.mL^{-1}$), D (4.097 ± 0.486 $ng.mL^{-1}$) and E (2.59 ± 0.23 $ng.mL^{-1}$) after Cu addition. The P_4 inhibition by Cu addition was in the group with the highest dose of Cu. IGF–I release by granulosa cells was

significantly ($P<0.05$) stimulated in the experimental group A after Cu addition at the dose 2.0 µL.mL^{-1} (86.10±2.27 ng.mL^{-1}) in comparison to control group (34.42±7.34 ng.mL^{-1}). P_4 release by granulosa cells was similar to that of control group in other experimental groups B (26.86±1.11 ng.mL^{-1}), C (21.38±1.65 ng.mL^{-1}), D (22.06±3.23 ng.mL^{-1}) and E (27.06±2.99 ng.mL^{-1}) after Cu addition. The IGF-I stimulation by Cu addition was in the group with the highest dose of Cu. Examination by light microscopy revealed presence of proliferation associated substances in the cells after Cu addition. Cyclin B1 expression was increased significantly ($P<0.05$) by Cu addition at the highest experimental dose of 2.0 µL/mL used in the study in group A (37.25±5.09%) in comparison to control group (17.62±6.02%) without Cu addition. Expression of cyclin B1 in granulosa cells was similar to that of control group in case of other experimental groups B (22.00±5.50%), C (25.00±4.54%), D (24.12±4.16%) and E (24.25±3.15%) after Cu addition. Presence of apoptotic ovarian granulosa cells was decreased significantly ($P<0.05$) by Cu addition at the lowest experimental dose of 0.33 µL.mL^{-1} used in the study in group E (23.05±12.41%) in comparison to control group (69.54±13.74%) without Cu addition. Presence of apoptotic granulosa cells was similar to that of control group in other experimental groups A (39.98±7.49), B (39.38±17.14%), C (35.69±9.12%) and D (35.70±8.00%) after addition of Cu.

Conclusion

Present study indicate possible involvement of Cu in the regulation of secretion activity, proliferation and apoptosis in porcine ovarian granulosa cells. In conclusion, obtained data suggest dose dependent effect of Cu on secretion of steroid hormone progesterone and growth factor IGF-I, and expression of cyclin B1 as marker of proliferation of porcine ovarian granulosa cells. Results of the study also indicate dose dependent effect of Cu on apoptosis of porcine ovarian granulosa cells and, that the effect of Cu on ovarian cell proliferation could be mediated by IGF-I and cyclin B1. Figure 1 oulines the interference of Cu in the pathways of proliferation of porcine ovarian granulosa cells through hormonal and intracellular peptide cyclin B1.

Figure 1. Possible signalling pathways of ovarian functions through hormonal and intracellular substance cyclin B1 affected by copper compound



Acknowledgements

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THE CONCENTRATIONS OF GSH IN THE MOUSE EYE LENS TREATED WITH UV RADIATION AND ETHYL ALCOHOL.

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Recently observed dangerous decrease of the ozonosphere and high technological development caused that living organisms are increasingly exposed to the harmful effects of UV radiation. Eye, because of its function, is particularly vulnerable to destruction. This specialized sensory analyzer is sensitive to electromagnetic waves of length of about 400-800 nm. The spectrum of UV radiation absorbed by the light-sensitive structures of the eye, damage them at the cellular, physiological and molecular level. Disorder in lens metabolism manifests itself as lens opacities and eventually cataract. Exposure to UV radiation, aging body and also food ingredients such as alcohol are causes of the opacities of lens epithelium. Another well knows factor inducing oxidative stress in eye structures is ethanol. This factor is little known, although its interactions with UV in eye lenses have not been studied so far. In eye lens glutathione is the most important antioxidant, therefore its reactions with free radicals may be a source of interactions between both tested factors.

The aim of my research was to analyze GSH content in lenses of male mice (SWISS) treated with UV radiation and ethyl alcohol. The protein content was estimated with Bradford's method. Both parameters were estimated using the microplate reader.

My results shows that alcohol ($F = 4{,}32$, $p = 0{,}006$) and UV radiation ($F = 48{,}08$, $p = 0$) induces a strong antioxidant response in mouse eye lens. In lens mice exposed ethyl alcohol (5%, 15%), concentration of GSH was higher than in control group. UV radiation caused a decrease of GSH concentration in control and experimental groups. My research indicates no interaction between alcohol and UV radiation.

THE EFFECT OF ORAL ADMINISTRATION OF STRAWBERRY LEAVES ON SELECTED PARAMETERS OF SPERM MOTILITY OF RABBITS

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Abstract

The aim of present study was to analyse the motility and progressive motility of rabbit's sperm after strawberry leaves inclusion to the feed mixture in various doses. Animals were divided into four groups, one control group C (n=3) and three experimental groups E1, E2 and E3 (n=4 in each group). Rabbits were fed with a granular feed mixture (FM) with strawberry leaves in various doses: C 0%; E1 0.5%; E2 1.0%; E3 1.5%. Semen collection was performed using an artificial vagina. All samples were analysed using CASA (Computer Assisted Semen Analysis) system – SpermVision (Minitüb, Tiefenbach, Germany) combined with Olympus BX 51 microscope (Olympus, Japan) and following parameters were evaluated: percentage of motile spermatozoa (motility>5 μm.s^{-1}) and percentage of progressive motile spermatozoa (motility>20μm.s). Motility was significantly higher in the control group (82.17%; p□0.05) as compared with the E3 group (72.39%). Progressive motility was significantly lower in the experimental group E3 39.61%) (p□0.05) as compared with the control group C (73.12%), E1 (77.53%) and E2 (59.46%). Samples treated with 1.0% (E2 59.46%) of strawberry leaves were significantly different (p□0.05) from E1 group (77.53%).

Introduction

Various beneficial biological effects of strawberry fruits consumption have been documented, such as an increase of the serum antioxidant capacity in humans (Cao et al., 1998), anti-carcinogenic activity (Carlton et al., 2001; Wedge et al., 2001), anti-thrombotic effects (Naemura et al., 2005), etc. Oxidative stress manifests when reactive oxygen species (ROS), that include hydroxyl radicals, superoxide anions and hydrogen peroxide (Combelles et al., 2009; Sharma and Agarwal, 1996; Tremellen, 2008) overwhelm the antioxidant defence system in cells. Spermatozoa were the first cell type showing a potential susceptibility to oxidative damage (MacLeod, 1943). They are particularly vulnerable to oxidative stress caused by the imbalance between ROS and antioxidant scavenging systems of the male

reproductive tract. Spermatozoa are particularly vulnerable to oxidative stress as they are characterized by: 1) high polyunsaturated fatty acid content; 2) intrinsic deficiencies in intracellular antioxidant enzymes; and 3) limited capacity of DNA repair, (De Lamirande and Gagnon, 1995; Padron et al., 1997). Only few data are available on their effects in vitro (Lewin and Lavon, 1997; Gavella and Lipovac, 1998). Therefore, it is not clear whether the beneficial effects of the therapy on sperm physiology were due to an improvement of spermatogenesis or also to a direct effect on spermatozoa. The aim of present study was to analyse the motility and progressive motility of rabbit's sperm after strawberry leaves inclusion to the feed mixture in various doses.

Material a methods

Animals

Adult male rabbits (n=30), 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 the 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 energy composed of a pelleted concentrate. Animals were divided into four groups, one control group C (n =3) and three experimental groups E1, E2 and E3 (n=4 in each group). Rabbits were fed with a granular feed mixture (FM) with strawberry leaves in various doses: C 0%; E1 0.5%; E2 1.0%; E3 1.5%. 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.

Semen collection and analysis

Semen collection was performed using an artificial vagina. All samples were analysed using CASA (Computer Assisted Semen Analysis) system – SpermVision (Minitüb, Tiefenbach, Germany) combined with Olympus BX 51 microscope (Olympus, Japan) and following parameters were evaluated: percentage of motile spermatozoa (motility>5$\mu m.s^{-1}$) and percentage of progressive motile spermatozoa (motilit >20μm.s).

Statistical analysis

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

The results of effect of strawberry leaves on rabbit sperm motility and progressive motility are summarized in Table 1. Motility was significantly higher in the control group (82.17%; p□0.05) as compared with the E3 group (72.39%). Progressive motility was significantly lower in the experimental group E3 39.61% (p□0.05) as compared with the control group C (73.12%), E1 (77.53%) and E2 (59.46%). Samples treated with 1.0 % (E2 59.46%) of strawberry leaves were significantly different (p□0.05) from E1 group (77.53%). The results of Kaedei et al. (2012) indicated that motility and penetrability of boar spermatozoa are improved by co-incubation with 50 μm epigallocatechin gallate (EGCG), but the effects vary with individual boars. Supplementation of 0.5 mM of vitamin C plus polyphenol yielded the highest percentages of sperm motility and viability; however, there was no beneficial effect on the plasma membrane and acrosomal integrity of the spermatozoa (Wittayarat et al., 2012).

Table 1. The percentage of total motility and progressive motility of rabbit´s spermatozoa after strawberry leaves administration

Parameter	C	E1	E2	E3
Total motility %	82.17±5.85^{b}	78.89±9.85	73.68±8.87	72.39±6.29^{a}
Progressive motility %	73.12±6.52^{b}	77.53±4.70^{bA}	59.46±11.86^{bB}	39.61±8.00^{a}

C - control group; E1,E2,E3,E4 – experimental groups; mean ± SD (standard deviation), a-b, A-B - the different letters mean significant differences in the line ($p<0.05$)

Conclusion

Sperm qualitative parameters were negatively affected by strawberry leaves inclusion. The progressive motility was affected mostly. The addition of antioxidant agents did not improve sperm quality. To our knowledge there is just little data in the literature regarding addition of strawberry leaves to the feed mixture for rabbits and their effect on sperm quality. To complete the research intention different doses should be used in future experiments.

Acknowledgments

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HIPPOCAMPAL NEUROGENESIS AND BEHAVIOUR IN PRENATALY IRRADIATED RATS

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Abstract

Clinical experience shows high frequency of cognitive impairment and behavioral disorders in children after brain tumor radiotherapy. One of possible causes of this damage may be the reduction of the intensity of postnatal neurogenesis.

The aim of this work was to study the effects of very low doses of ionizing radiation applied during the most sensitive phase of embryonic development of the CNS. Pregnant female rats were irradiated with a dose of 1 Gy of gamma-rays on the 17-th day of gravidity. The behavior of the male progeny was tested on spatial memory in Morris´s water maze (MWM) and on anxiosity in elevated plus maze (EPM) at the age of 2 and 3 months along with the offspring of sham-irradiated mothers of the same age. We found, that the irradiation influenced negatively the long-term memory in MWM and significantly increased the level of anxiety measured in EPM in 2 months, but not in 3 months aged rats. The level of mitotic activity in hippocampus was assessed as the number of cells labeled with 5-bromo-2-deoxyuridine in the subgranular zone (SGZ) and the hilus of *gyrus dentatus (GD)*. The number of these cells was reduced under the influence of irradiation in aged rats 3 weeks. To determine the number of mature neurons we used NeuN antibody staining. We observed the highest reduction of NeuN-positive cells in CA1 region and in hilus of the hippocampus in irradiated animals aged 2 months, which corresponds with behavioral changes observed. To determine the temporal dynamics of neurogenesis from postnatal stages to adulthood we compared the numbers of cells among the age groups. With age, there was a significant reduction of BrdU-positive cells in irradiated and also in control animals, but the number of NeuN-positive cells significantly decreased with age only in irradiated animals, what may be associated with impaired neurogenesis. Our results indicate, that prenatal low-dose gamma irradiation can influence the mitotic activity in brain markedly in young animals, which may results in reduced number of mature neurons in older animals and thus affect their long term memory and emotional status. These findings could contribute to the understanding of incorporation of newly created neurons into the already existing neuronal networks.

Introduction

The the subventricular zone (SZ) and the subgranular zone (SGZ) of the *gyrus dentatus* of the hippocampus (GDH) are the two places in the mammalian brain, where the formation of new neurons from neuronal stem cells (neurogenesis) during the adult age has been proved unambiguously (Cameron et al.,1993). However, the mechanisms of the integration of new neurons into the existing neuronal network and their exact role in brain functions are to be clarified. Ionizing radiation is known to selectively damage or kill dividing cells and is often used as a methodical tool to stop cell proliferation (Hellström et al. 2009).

The aim of our experiments was 1. to assess, whether a whole-brain irradiation with a very low dose of gamma-rays during the sensitive period of the embryonic development of brain will suppress the level of hippocampal neurogenesis in adult rats and 2. to see, if in this case an impairment of hippocampal-dependent behaviour will occur.

Material and Methods

Six female Wistar rats were irradiated with a whole-body dose of 1 Gy of gamma-rays on the 17th day of pregnancy. Four pregnant rats were sham-irradiated on the same day of gravidity. The male progeny of irradiated (n = 32) and of sham-irradiated mothers (n = 17) was used in further experiments.

The level of neurogenesis in two different areas of the hippocampus was established in animals aged 3 weeks, 2 and 3 months. The total number of dividing cells was assessed by 5-brom-2´- deoxyuridine (BrdU) labelling, using 3 doses of BrdU (100 mg/kg) applied during 3 consecutive days. Two hours after the last injection the brains of rats were washed by saline solution and fixed. The treatment was performed under chloralhydrate anaesthesia. The brains were taken out, weighted and post-fixed. Slices of the brain 33 µm sick were cut on cryostat. Seven slices were randomly chosen for further analysis. The BrdU-labeled cells in SGZ and in the *hilus* of *DG* were visualised by immunohistochemical staining.

The numbers of mature neurons in the SGZ of CA1-region and of *hilus* were established by NeuN-staining according the method of Kim et al. (2008).

Both BrdU- and NeuN-labeled cells were counted microscopically using the computer software Image Tool (UTHSCSA, San Antonio, USA).

Behavioural analyses.

The groups of rats aged 2 months and 3 months were tested 1 week before the analysis of neurogenesis. The groups of irradiated and control rats were tested for learning and memory in Morris´s water basin (MWB) and for the level of anxiety and exploratory behavior in

elevated plus maze (EPM). On the 1st day of the experiment, the rats were tested 3-times in 3-hour intervals (testing of learning and short-time memory). A repeated test was performed on the 8th experimental day (long-term memory testing).The time spent in the open arms and the number of defecation boluses (anxiety), the number of rearing (exploratory behavior) and the number of crossing the center (motor activity) were evaluated.

Results.

Irradiation of developing rat embryos on the 17th day of embryogenesis caused a statistically highly significant decrease of neurogenesis in *gyrus dentatus* of the hippocampus in animals aged 3 weeks, in comparison with controls, but no differences were observed in animals aged 2 or 3 months (Fig.1).

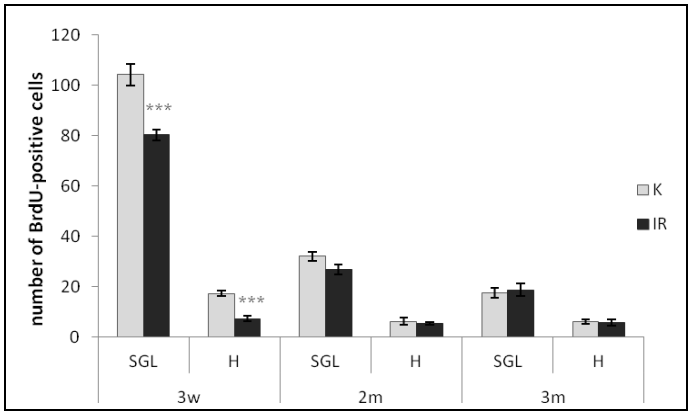


Figure 1. Number of BrdU-positive cells in the subgranular zone (SGZ) and hilus (H) of the hippocampus of irradiated (IR) and control (K) rats aged 3 weeks (3w), 2 months (2m) and 3 months (3m). *** - $P < 0.001$

The number of mature neurons in CA1-region and in hilus of the hippocampus was statistically significantly decreased in irradiated rats aged 2 months, but not at age of 3 weeks or 3 months, compared with controls of the same age (Fig.2). The exploratory behavior was significantly lower and the level of anxiety was higher in EPM-test in irradiated animals aged 2 moths, than in controls. These differences were not present in animals aged 3 weeks (Figs.3, 4 and 5).

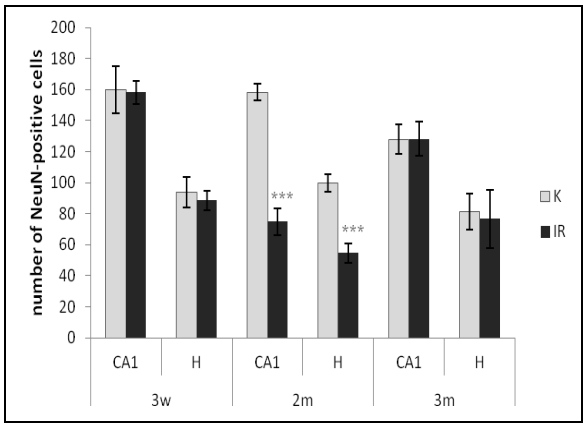


Figure 2. Number of NeuN-positive cells in the CA1-area and hilus (H) of the hippocampus of irradiated (IR) and control (K) rats aged 3 weeks (3w), 2 months (2m) and 3 months (3m). *** - P < 0.001

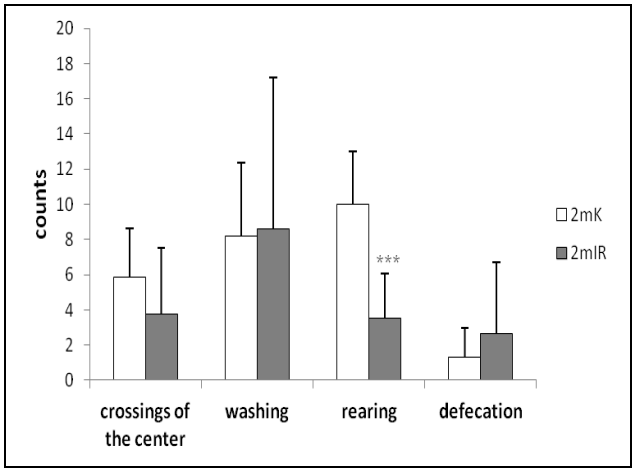
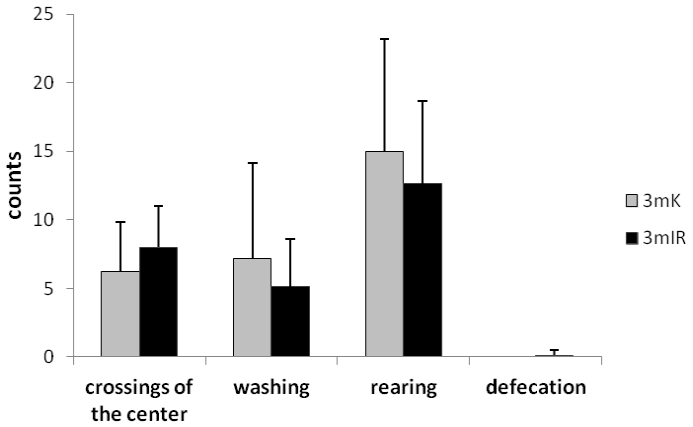


Figure 3. Behavioural parameters in groups of irradiated (2mIR) and control (2mK) rats aged 2 months in elevated plus maze measured during a 5-minute test. *** - P < 0.001

The ability of learning and the short-term memory in MWM were not altered by radiation. The long/term memory was significantly decreased in irradiated animals aged 2 months, but not in 3 weeks old rats (Fig.6). A significant correlation between the number of NeuN-stained neurons in CA1-region and the cognitive performance in the MWM was found (Fig.8 and 9).



Fiure 4. Behavioural parameters in groups of irradiated (3mIR) and control (3mK) rats aged 3 months in elevated plus maze measured during a 5-minute test.

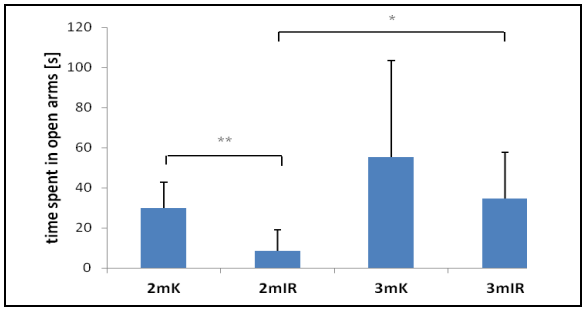


Figure 5. Time spent in the open arms of the elevated plus maze in irradiated (IR) and control (K) rats aged 2 (2m) or 3 months (3m) during a 5-minute test. Data given as arithmetic mean ± SD. ** - P< 0.01, * - P < 0.05

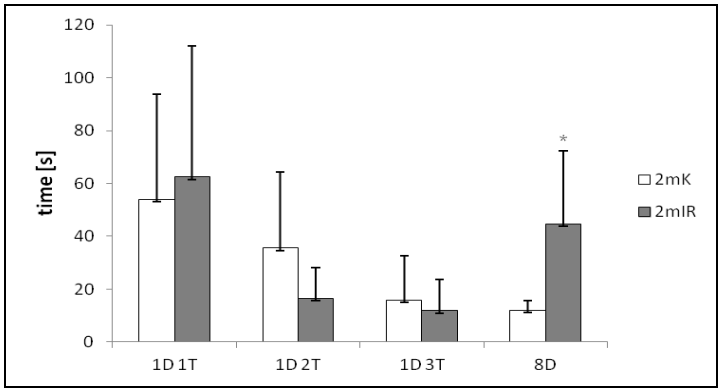


Figure 6. Time needed to find the hidden platform in Morris´s water basin in irradiated (2mIR) and control (2mK) rats aged 2 months tested on the 1st experimental day (1D) during 3 sessions (1T, 2T,3T) in 3-hour intervals and on the 8th experimental day. Data given as arithmetic means ± SD. * - P < 0.05.

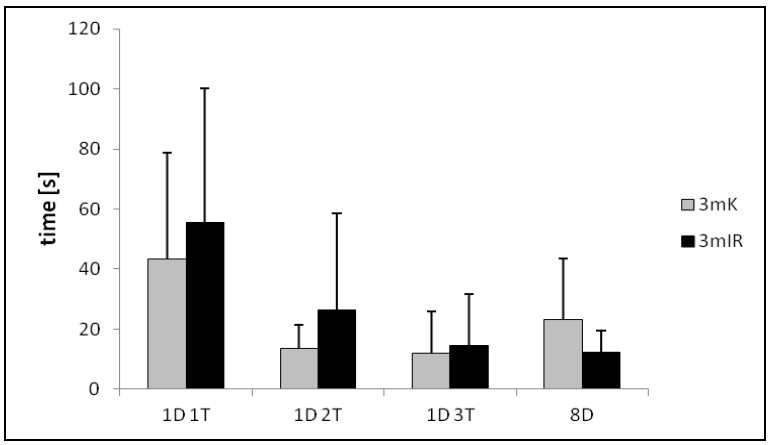


Figure 7. Time needed to find the hidden platform in Morris´s water basin in irradiated (3mIR) and control (3mK) rats aged 3 months tested on the 1st experimental day (1D) during 3 sessions (1T, 2T,3T) in 3-hour intervals and on the 8th experimental day. Data given as arithmetic means ± SD. * - P < 0.05.

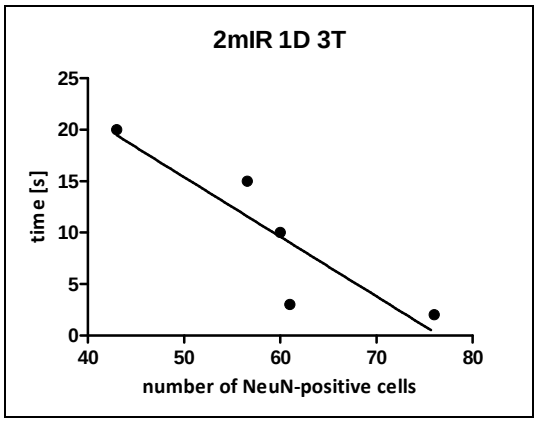


Figure 8. The relationship between the number of NeuN positive cells in the CA1 region of the hippocampus and the short-time memory performance in irradiated rats aged 2 months in 3rd session on the 1st experimental day given as the time needed to find the hidden platform in MWM. The linear regression coefficient b = - 0.5799, the correlation coefficient r = - 0.8572, $P < 0.05$.

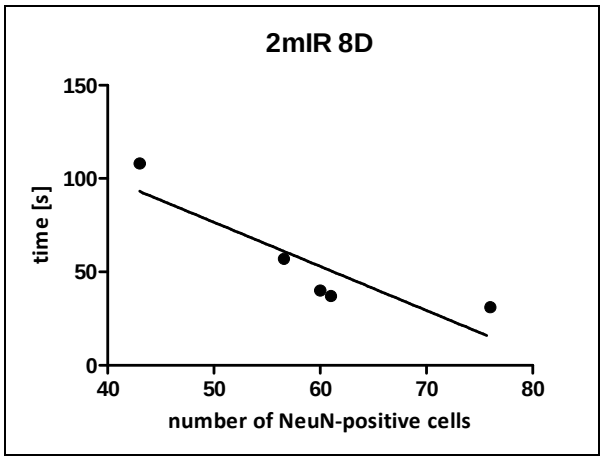


Figure9. The relationship between the number of NeuN-positive cells in the CA1 region of the hippocampus and the long-time memory performance in irradiated rats aged 2 months on the 8th experimental day, given as the time needed to find the hidden platform in MWM. The linear regression coefficient b = - 2,3614, the correlation coefficient r = -0.88679, $P < 0.01$.

Conclusions.

Our results show, that irradiation of the developing brain with a very low dose of IR can couse a markedly decrease of the level of neurogenesis in SGZ of GDH in early phases of life. Later, in young adult rats we found a significant decrease of the number of mature neurones in CA1-region and in hilus of the hippocampus which corresponds with lower level of explanatory behaviour and a higher level of anxiety and with decreased performance in spatial memory test in these animals. In parallel, both the neuronal changes and the changes in behavioral parameters dissapear with age. Our data support the earlier findings (reviewed by Abrous et al., 2005), that HN plays an important role in hippocampaly-related cognitive functions. Our results also show, that a radiation dose as low as 1 Gy received during the sensitive phase of brain development can couse an impairment of the postnatal development of mental functions.

Acknowledgement

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THE EFFECT OF AGE ON SERUM AND EGGSHELL MINERALS IN LAYING HENS AND BROILER BREEDERS

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Abstract

The aim of study was to evaluate the effect of age on concentration of calcium, phosphorus, zinc and magnesium in serum and in the eggshell of laying hens and broiler breeders at the beginning and at the end of laying cycle. In this experiment were included 24 Lohmann Brown laying hens, at 22 weeks of age, and the other 24x being 83 weeks old at the beginning of the experiment. Cobb 500 broiler breeder hens, 24 of which were 36 weeks old, and the other 24, 64 weeks of age at the beginning of the experiment. The serum levels of phosphorus were significantly ($P < 0.008$) affected by age. Concentration of zinc (82.48 µmol/l for young and 81.54 µmol/l for old hens) and magnesium (1.50 mmol/l for young and 1.47 mmol/l for old hens) in serum was significantly ($P < 0.001$) influenced by strain with higher values in Czech hen. A significant influence of strain on content of phosphorus ($P \leq 0.002$) and magnesium ($P < 0.001$) in eggshell was detected. There were no significantly differences in calcium and zinc content in eggshell.

Keywords: laying hens, broiler, minerals, serum, eggshell

Introduction

With respect to animal welfare and quality production over the whole egg-laying period it is necessary to maintain the metabolic profile of layers within physiological ranges characteristic of particular hybrid combination, age and production type.

Shell quality can be influenced by many factors including mineral nutrition. Calcium, magnesium and phosphorus are major inorganic constituents of the avian eggshells (Cusack et al., 2003). Simons (1976) found small amounts of potassium, copper and zinc in the palisade layer of the eggshell. The presence of sodium, potassium, magnesium, zinc and copper was confirmed also in the shell membranes (Wedral et al., 1974). Insufficient supplies of calcium and phosphorus also reduce growth rate and bone calcification, and excess Ca intake also induces similar, but moderate reductions in growth and bone ash (Shafey et al. 1990; Hurwitz et al. 1995). The calcium content in eggshell is 372 mg/kg (Kebreab et al., 2009), phosphorus 1.27 - 1.5 mg/kg and magnesium 3.17 - 3.63 mg/kg (Tůmová, 2011).

Important elements are also zinc and manganese. Plasma mineral concentrations during the laying period can be influenced by many factors; such as laying rate and energy requirements (Suchý et al., 2001), partial quantitative feed restriction (Sahin a nd Kucuk, 2001), mineral supplements (Eren et al., 2004), production type (Suchý et al., 2004) or age of hens (Cerolini et al., 1990; Gyenis et al., 2006). Minerals are transported by the blood throughout the body. The calcium concentration in serum was found in the range from 4.88 - 5.25 mmol/l, phosphorus 1.82 - 2.17 mmol/l and magnesium 1.44 - 1.52 mmol/l (Tůmová, 2011).

The aim of study was to evaluate the effect of age on minerals concentration in serum and in the eggshell in laying hens and broiler breeders.

Materials and methods

In total, 96 birds were used in the experiment which lasted 7 weeks. Birds were placed into four environmental chambers in which temperature was controlled. 24 Lohmann Brown laying hens, six being 22 weeks of age, and the other 24 birds being 83 weeks old at the beginning of the experiment. Cobb 500 broiler breeder hens, 24 of which were 36 weeks old, and the other 24, 64 weeks of age at the beginning of the experiment. The lighting regime consisted of 14 h light and 10 h darkness, with lights being turned on at 05:00 h and off at 19:00 h. Laying hens were given a commercial laying feed *ad libitum* (174 g/kg crude protein, 11.65 MJ ME, 32.6 g/kg Ca, 4.8 g/kg P, 2.3 g/kg Mg and 168 ppm Zn). Broiler breeders received 160 g per day of a high protein broiler breeder feed daily (174 g/kg crude protein, 11.31 MJ ME, 32.8 g/kg Ca, 5.0 g/kg P, 2.3 g/kg Mg and 180 ppm Zn).

Selected birds were bled from a wing vein with a frequency of no less than once a week. In total 288 samples of blood were collected.

Biochemical parameters analysed in serum were calcium, phosphorus, magnesium and zinc. Minerals were determined photometrically in a Libra S 22 spectrophotometer (Biochrom Ltd., UK) using standard commercial kits (Randox Laboratories Ltd., Crumlin, UK).

Shells were analysed for calcium, phosphorus, magnesium and zinc content. Calcium and phosphorus were determined by a method described Englmaierová et al. (2013). AOAC International (2005) procedures were used to determine the crude protein, magnesium and zinc in feed mixtures and egg shells. Mineral elements were analysed on Varian ICP spectrophotometer (Varian, Inc.).

Resultant values were statistically analysed by SAS (SAS Institute INC., 2003) program and an analysis of variance (ANOVA) was used for resultant values evaluation. There was studying the interaction of age and strain.

Results and discussion

There were no significant interactions of serum and eggshell in mineral concentration. Table 1 shows the result of concentration of minerals in serum. The significant effect of strain and age was not detected in concentration of calcium. This parameter was higher in egg – type hens compared to meat - type hens. Suchý et al. (2004) found the average plasma levels of calcium in broiler breeders 5.00 – 6.22 mmol/l. These concentration were highly significantly ($P \leq 0.01$) lower compared to those of laying hens (5.92 – 7.19 mmol/l). Pavlík et al. (2009) observed that plasma calcium significantly increased with age. Gyenis et al. (2006) stressed that blood mineral results levels may be different as a function of the methodology applied, by breed or genetic line. The serum levels of phosphorus were significantly (P 0.008) affected by age. The highest values were detected in older hens from both strains (laying hens 1.94 mmol/l, broiler breeders 1.92 mmol/l). On the other hand Pavlík et al. (2009) detected that mean values of plasma phosphorus in laying hens with age, which corresponds with the results of Straková et al. (1994) and Eren et al. (2004).

Table 1. Concentration of minerals in serum

Strain	**Age**	**Ca (mmol/l)**	**P (mmol/l)**	**Zn (µmol/l)**	**Mg (mmol/l)**
Egg - type hens	young	4.87	1.80	82.48	1.50
	old	5.12	1.94	81.54	1.47
Meat - type hens	young	4.53	1.75	63.93	1.24
	old	4.58	1.92	58.92	1.33
Significance					
Strain		0.054	0.561	0.001	0.001
Age		0.538	0.008	0.403	0.354
Strain x Age		0.687	0.728	0.522	0.077

The concentration of zinc and magnesium were significantly (P 0.001) influenced by strain. However Pavlík et al. (2009) noted that the age of laying hens or the phase of the laying cycle had an effect on changes in mineral levels. Considerably higher concentration of zinc was determined in laying hens (82.48 µmol/l for young and 81.54 µmol/l for old hens) compared to broiler breeders (63.93 µmol/l for young and 58.92 µmol/l for old hens). Similarly, serum magnesium was significantly (P 0.001) higher in laying hens.

Table 2. Concentration of minerals in eggshell

Strain	Age	Ca (g/kg)	P (g/kg)	Zn (mg/kg)	Mg (g/kg)
Egg - type hens	young	349.69	1.21	4.81	3.35
	old	343.39	1.12	4.85	3.36
Meat - type hens	young	346.14	1.40	3.86	3.85
	old	351.56	1.36	4.57	4.16
Significance					
Strain		0.535	0.002	0.070	□ 0.001
Age		0.732	0.331	0.324	0.078
Strain x Age		0.064	0.700	0.318	0.082

Content of minerals in the eggshell is described in Table 2. The effect of age was not discovered in any of monitored characteristics. A significant influence of strain on phosphorus content ($P \leq 0.002$) and magnesium (P □ 0.001) in eggshell was detected. The content of phosphorus was higher in broiler breeders hens as well as concentration of magnesium compared to egg - type hens. Content of calcium and zinc in the eggshell were not influenced by strain on age. Cusack et al. (2003) in their study describes that a greater increase in magnesium concentration the outer region of eggshells from older birds was found.

Conclusions

In our study any significant interaction between strain and age of hens in concentration of minerals in serum and eggshell was not found. The serum levels of phosphorus were significantly affected by age with higher values in older hens. Concentration of zinc and magnesium in serum was also significantly influenced by strain. The higher concentration of minerals was found in laying hens. The effect of age on content of minerals in the eggshell was not discovered in any of monitored elements. A significant influence of strain on content of phosphorus and magnesium in eggshell was detected, where higher values were measured in broiler breeders.

Acknowledgments

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THE PHYSIOLOGICAL RESPONSE OF TSIGAI AND IMPROVED VALACHIAN TO MACHINE MILKING.

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Abstract

The physiological response of ewes to machine milking stimulation causing occurrence of milk ejection reflex is important evidence of required reaction of ewes to udder stimuli. Therefore milk flow kinetic (one or two emission, steady state – plateau) especially in breeds with lower milk yield, and residual milk volume could be considered as sufficient trials for physiological evaluation of adaptability of ewes to milking. Tsigai and Improved Valachian showed relatively high occurrence one emission milk flows (depending on the stage of lactation it ranged from 20 to 70 %) and higher residual volume indicating less effective milk removal.

Introduction

Improved Valachian (IV) and Tsigai (T) represent main breeds of sheep reared in Slovakia for milk and lamb production. Both breeds are considered as animals with high endurance and adaptability to walking and natural conditions. They are often crossed with Lacaune (LC) or purebred selection through the genetic evaluation done with the purpose of improving their milk production and milkability (Oravcová et Peškovičová, 2008). Therefore the knowledge about physiological response of above mentioned breeds or cross-breeds to machine milking are crucial from the milk production point of view and management system before and during machine milking. The aim of presented work was to summarise our results from the adaptability and response of T, IV and their cross-breeds with LC to machine milking depending on the stage of lactation and possible reared factors.

Milk is produced in the secretory cells of the udder parenchyma continuously. Between milking the milk is divided into two anatomical parts of the udder. Part of milk is stored in secretory alveolus lumen and alveolar and intraalveolar ducts – this is called the alveolar compartment (Davis et al., 1998). Alveolar fraction is fixed by capillary forces and requires an active expulsion into the cistern (Bruckmaier et Wellnitz, 2008) through the tactile stimulation of the teat causing the release of oxytocin from the neurohypophysis. The second part of milk represents the cisternal fraction, which has already been transferred from the

alveoli to the milk cistern between milkings and is immediately obtainable for milk removal without occurrence of milk ejection by overcoming the teat canal sphincter by vacuum of milking system (Marnet et McKusick, 2001). Alveolar milk has a higher milk fat content as compared to cisternal milk in dairy ewes (Castillo et al., 2008) and dairy cows (Tančin et al., 2007) and thus the milk ejection is crucial for removing the milk from the alveolar compartments.

From the milk distribution between alveolar and cisternal part of udder point of view the small ruminants could be considered as animals with relatively larger volume of cistern as compared with cow. Most acceptable is volume of cisternal milk ranged from 38 % to 47 % of the total milk volume or even more, when milking interval is 12 h (McKusick et al., 2002) but in some animals the volume of citernal milk could be higher. Larger volume of milk in cistern would imply that milk ejection at the time of milking is less important compared to dairy cows (Bruckmaier et al., 1994). However, for the complete and fast milk removal, the ejection reflex is important for ewes too (Mačuhová et al., 2012; Antonič et al., 2013).

Physiology of milk ejection

The main condition for fast and complete milking and achieving maximal production is the induction of milk ejection. Complete milk ejection during each suckling or milking is a prerequisite to maintain milk synthesis and secretion on a high level throughout lactation (Tančin et Bruckmaier, 2001). During milking, the alveolar milk, which is the richest in fat, can be excluded only after the contraction of the myoepithelial cells surrounding the alveoli mediated by oxytocin (Casu et al., 2008).

Milk ejection is an innate reflex that is not under conscious control of the animal (Tančin et Bruckmaier, 2001) and oxytocin is secreted from neuro-pituitary by neuro-endocrine mechanisms into the blood (Lollivier et al., 2006) as a response to stimulation of mammary gland by suckling, hand or machine milking (Bruckmaier et Wellnitz, 2008). Even the stimulus of having teat cup liner attached without pulsation is usually sufficient to evoke oxytocin release (Tančin et Bruckmaier, 2001). The blood oxytocin concentration is not related to milk yield. However, determination of blood oxytocin concentrations in ewes may be useful in determining the efficiency of milking machine providing optimal stimulation of mammary glands (Olechnowicz, 2012).

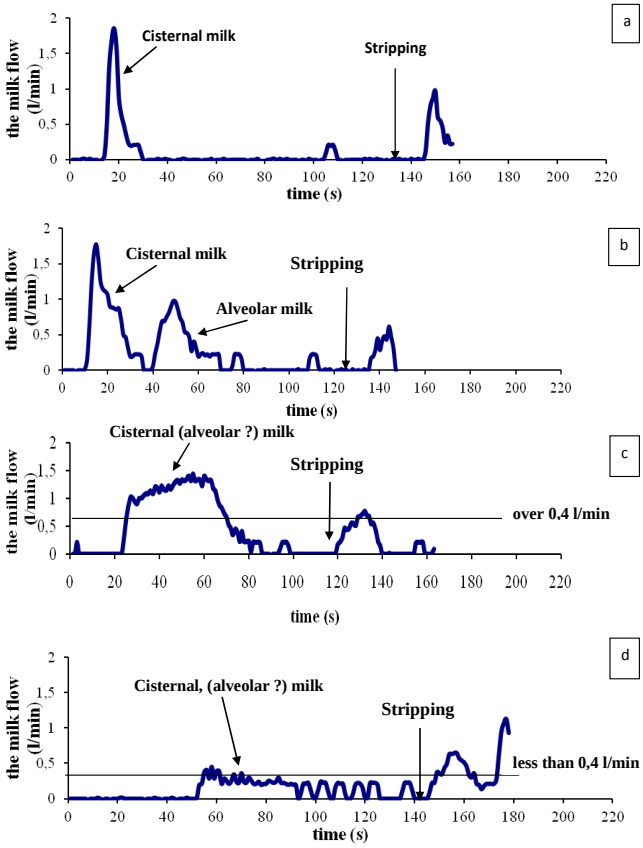

Figure 1. Milk flow curves:
a) non-bimodal (one emission) (1P)
b) bimodal (two emissions) (2P)
c) plateau (PL)
d) low plateau (PLN)

Types of milk flow curves

Milk emission is one of the most interesting criteria for studying milkability in the machine milking of dairy sheep and its main traits are considered to be relevant for the design of milking machines and to adopt the optimal milking routine in each breed (Mačuhová et al., 2008). Milk flow patterns depend on physiological response of animal to machine stimulation, milk production and anatomical and functional characteristics of the teat such as the canal length and diameter or sphincter tightness and elasticity (Tančin et al., 2007; Casu et al., 2008). A good milk flow curve should mean quick and complete milking, with a high milk flow rate and an effective ejection of alveolar milk under the action of the oxytocin. Bruckmaier et al. (1997) and Mačuhová et al. (2008) classifieds four types of milk flow curves (Figure 2). Animals exhibiting no significant oxytocin release always had one, and generally short, milk surge (Marnet et al., 1998, Figure 2a). Oxytocin release results in alveolar emission of milk causing bimodal milk flow patterns – first emission of milk represent cisternal and second one alveolar milk volume – when cisternal volume is limited and/or milk flow is high (Figure 2b). With increasing milk production, increasing volume of cisternal compartment or with low milk flow due to limitation of teat canal, the bimodal milk flow patterns are no longer visible and replaced by steady state (plateau) milk flow patterns (Figure 2c). Such ewes could have a milk ejection but second emission is masked probably due to the filling of cistern by alveolar milk before milk from cistern is removed (Marnet et al., 1998). Few ewes with plateau milk flow could response without milk ejection and distinction between the ewes becomes difficult especially when the ewes with plateau showed lower peak flow (Tančin et al., 2011, Figure 2d).

The influence of breed on frequency of individual milk flows

The breed of ewes significantly influences the physiological response of ewes to machine milking stimulation. The above mentioned response is mainly evaluated by milk flow kinetics described in Figure 1. In the literature there are available data from different breeds raised in the world. The main focus is concentrated on LC as an important dairy breed of ewes. Marnet et al., (1998) according to the classification of milk flows, observed two separate milk emissions in 56 % of ewes and a single emission in 33 % of ewes and plateau milk flow in 11 % LC ewes. Also Such et al., (1999) observed, the 83 % of the LC ewes presented the ejection reflex during the emission of the machine milk, but only 38 % of the Manchega ewes showed a second peak. Also, Rovai et al (2002) found a higher percentage of 1P in Manchega, a Spanish breed less adapted to machine milking compared to LC breed.

The physiological response through the milk flow kinetic evaluation was also intensively studied in our breeds (Tančin et al., 2011, Table 1). Obtained results showed that our breeds of ewes are mainly one peak ewes. The highest percentage of one peak milk flow as noted in T and IV (depending on the stage of lactation it ranged from 20 to 70 %, Figure 3) indicate a possibly lower sensitivity to udder stimulation by machine milking (Tančin et al., 2011, Mačuhová et al., 2012).

Concerning milking ability between breeds and crosses from the more detail study of milk flow kinetic in our breeds and crossbreds (Tančin et al., 2011), the highest occurrence of 1P milk flow was found in TS and IV (1P, 2P, PL: 58 %, 36 %, 6 % and 35 %, 50 %, 15 %, respectively) (Table 2). On the other hand, LC and its crosses (TS x LC and IV x LC) showed the lowest occurrence of 1P (18 %, 24 %, 60 %; 13 %, 42 %, 45 %; and 15 %, 43 %, 42 %, respectively; Table 2). The highest percentage of 1P milk flow as noted in TS and IV indicate a possibly lower sensitivity to udder stimulation by machine or both breeds are less adapted to machine milking. From our another study (Mačuhová et al., 2012) we supported above mentioned results. We found out higher resudual milk volume in T and IV (16,47±1,44 %, 15,99±1,78 %, resp.) than in Lacaune (9,86±1,16 %) indicating less effective milk removal of T and IV.

Table 1. LSM for milk emission traits per morning milking by genotype (Tančin et al., 2011).

Trait	Genotype				
	TS N=48 $\mu_i \pm s_{\mu_i}$	IV N=48 $\mu_i \pm s_{\mu_i}$	LC N=47 $\mu_i \pm s_{\mu_i}$	TS50xLC50 N=52 $\mu_i \pm s_{\mu_i}$	IV50xLC50 N=48 $\mu_i \pm s_{\mu_i}$
Total milk yield (l)	0.35±0.02^{a}	0.32±0.02^{a}	0.52±0.02^{bc}	0.55±0.02^{b}	0.47±0.02^{c}
Machine milk yield (l)	0.27±0.02^{a}	0.26±0.02^{a}	0.36±0.02^{b}	0.40±0.02^{b}	0.37±0.02^{b}
Stripped milk yield (l)	0.08±0.02	0.06±0.02^{A}	0.16±0.02^{B}	0.15±0.02^{B}	0.10±0.02
Machine milking time (s)	45.9±3.06^{a}	46.6±3.02^{a}	56.2±3.00^{b}	60.3±2.86^{b}	52.7±2.94^{ab}
Latency time (s)	11.3±0.98	12.9±0.97	14.0±0.96	14.9±0.92	13.2±0.95
Maximum flow (l/min)	1.07±0.10	0.88±0.10	0.99±0.10	0.98±0.10	1.06±0.10
Time to maximum milk flow (s)	17.5±2.37	21.4±2.34	22.7±2.33	26.2±2.22	22.6±2.28
Machine milk to 30s (l)	0.20±0.02	0.16±0.02	0.22±0.02	0.21±0.02	0.22±0.02
Stripped/total milk (%)	23.3±3.09	19.9±3.06^{A}	33.3±3.04^{B}	27.8±2.89	24.3±2.98

a,b –differences among means with different superscripts significant at P<0.05
A, B - differences among means with different superscripts significant at P<0.07

Table 2. Distribution of ewes according to milk flow profile, breeds and stages of lactation. For kinetics see Figure 1. (Tančin et al., 2011)

		Distribution (%) of animals over months			
	Kinetics	May	June	July	August
Tsigai	2P	50	42	25	25
(TS)	1P	42	42	75	75
	PL	8	16	-	-
	2P	23	62	54	31
TS x LC	1P	-	-	15	38
	PL	77	38	31	31
Lacaune	2P	8	50	9	25
(L)	1P	-	8	36	33
	PL	92	42	55	42
Improved	2P	59	68	50	25
Valachian	1P	16	16	42	67
(IV)	PL	25	16	8	8
	2P	42	42	42	50
IV x LC	1P	-	16	16	25
	PL	58	42	42	25

On the basis of our results we could conclude that for better evaluation of Tsigai and Improved Valachian ewes for machine milking it will be necessary to perform more detail study of milkability and physiological responses to milking conditions. Though we could find out relatively good potencial of mentioned breed for milking, for better using in dairy practice it is important to increase the milkability parameters.

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COMPARISON OF SELECTED BLOOD BIOCHEMICAL PARAMETERS OF DAIRY COWS AT THE BEGINNING AND IN THE MIDDLE OF LACTATION

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Abstract

The aim of this work was to monitor the relations between selected blood biochemical parameters of dairy cows at the beginning and in the middle of lactation and to observe the correlations between parameters within groups. In total, 31 Holstein cows were chosen, which were divided into 2 groups: First group - beginning of lactation (BL) (n=15), Second group - middle of lactation (ML) (n=16). The average values of indicators in blood profile of the internal milieu of monitored cows were in the physiological normal range, except a elevated level of total proteins (in both groups), sodium (in both groups) and cholesterol (in ML) when compared to biochemical reference ranges. The D-BHB level in BL group was also slightly increased (1,015 mmol.l^{-1}). Obtained levels of triglycerides in both groups were a little bit lower in both groups (0,16 mmol.l^{-1}). Concentration of alanine aminotransferase (ALT) was significantly higher in ML group (0,5038 µkat.l^{-1}) when compared to BL group (0,3233 µkat.l^{-1}). Significant difference of cholesterol concentration was observed among the groups BL (3,869 mmol.l^{-1}) and ML (7,283 mmol.l^{-1}). Correlation showed high positive dependence between bilirubin and triglycerides in BL group (0,887; $p<0.001$), urea and ALT in ML group (0,742; $p<0.001$) and between chlorides and sodium also in ML group (0,977; $p<0.001$).

Introduction

During the dairy cow production cycle, the transition period is critical due to the several endocrine and metabolic changes that accompany parturition and the initiation of lactation (Çağdaş, 2013; Grummer et al., 2004; Smith et Risco, 2005).

Lactation has a great impact on the intensity of metabolism and on metabolic parameters in the blood (Filipejová et Kováčik, 2009; Milinković-Tur et al., 2005). Economically most important diseases of high-producing dairy cattle include metabolic disorders - ketosis, acidosis, and others. E.g. metabolic alkalosis causes a reduction in cattle tissue oxygen consumption, energy deficit, acid-base and water-electrolyte balance disturbances (Nicpoń et Hejłasz, 2005).

The diagnosis of metabolic disorders is very difficult. Several methods have been developed to detect them. One of the most important method is metabolic profile test (MPT) (Stengärde, 2008). Using MPT is possible in preclinical stages, even before the appearance of clinical signs of disease (Andrews, 2000). Their early diagnosis and preventive measures could prevent or at least alleviate clinical symptoms of disorders and hence their impact on the economy of farming (Kantíková et Balážik, 2003).

The gist of MPT is the determination of the diagnostically relevant metabolites of body fluids (Slanina, 1993) of a representative group of individuals at different stages of the reproductive cycle. Metabolic test consists of various indicators, eg. hematocrit, albumin, globulin, glucose, total cholesterol, aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), calcium, inorganic phosphorus or magnesium, etc. (Kida , 2002).

The aim of this work was to monitor the relations between selected blood biochemical parameters of dairy cows at the beginning and in the middle of lactation and to observe the correlations between parameters within groups.

Material and methods

In total, 31 Holstein cows were chosen, which were divided into 2 groups:

First group - beginning of lactation (BL) (n=15),

Second group - middle of lactation (ML) (n=16).

Blood samples collection for biochemical analysis was performed 2 hours after the morning feeding by *vena jugularis* puncture. Blood was captured directly into centrifuge tubes and centrifuged. Energetic (glucose - GLU, d-beta-hydroxybutyrate - D-BHB, triglycerides - TG), nitrogenous (total proteins - TP, UREA), hepatic (aspartate aminotransferase - AST, alanine aminotransferase - ALT, bilirubin - BILI, cholesterol - CHOL) and mineral (sodium - Na, potassium - K, chlorides - Cl^-) profiles were determined in the blood serum. 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 the devices Microlab 300 (villa Scientific, Dieren, The Netherlands) and EasyLite Plus (The Hague, The Netherlands).

The results were evaluated by GraphPad Prism 5. Unpaired t-test and correlation were used.

Results and discussion

The average values of indicators in blood profile of the internal milieu of monitored cows (table 1) were in the physiological normal range, except a elevated level of total proteins (in both groups), sodium (in both groups) and cholesterol (in ML) when compared to Serum Biochemical Reference Ranges (Merck Sharp & Dohme Corp, 2012).

Parameter	Units	BL		ML		Reference Ranges
		Mean	S.D.	Mean	S.D.	
GLU	$mmol.l^{-1}$	**4,013**	0,5617	**4,081**	0,3229	2,2-5,6
TG	$mmol.l^{-1}$	**0,16**	0,0713	**0,1625**	0,0526	0,17-0,51
D-BHB	$mmol.l^{-1}$	**1,015**	0,7052	**0,6868**	0,2999	0-0,96
AST	$\mu kat.l^{-1}$	**1,992**	1,655	**1,727**	0,4904	1,0-2,083
ALT	$\mu kat.l^{-1}$	**0,3233^{A}**	0,1071	**0,5038^{B}**	0,0934	0,115-0,583
BILI	$\mu mol.l^{-1}$	**4,249**	3,803	**2,474**	2,218	0-27
CHOL	$mmol.l^{-1}$	**3,869^{A}**	1,423	**7,283^{B}**	1,611	1,6-5,0
TP	$g.l^{-1}$	**84,2**	9,144	**89,53**	8,251	67-75
UREA	$mmol.l^{-1}$	**4,387**	1,228	**4,875**	0,9441	3,6-8,9
Na	$mmol.l^{-1}$	**144,6**	7,533	**145,6**	10,49	136-144
K	$mmol.l^{-1}$	**3,801**	0,2575	**3,856**	0,3692	3,6-4,9
Cl^-	$mmol.l^{-1}$	**103,3**	1,983	**105,3**	8,509	99-107

Table 1. Comparison of blood biochemical parameters of dairy cows at the beginning (BL) and in the middle of lactation (ML). A,B - different letters within the row mean significant differences among the groups at level $p<0.001$

The D-BHB level in BL group was also slightly increased (1,015 $mmol.l^{-1}$). It could point to the starting subacute ketosis, but the level of glucose was not decreased yet (hypoglyceamia is typical for ketosis) (Kováč et al., 2001). This value of D-BHB is very close to the cut-point of 1,2 $mmol.l^{-1}$ that LeBlanc et al. (2005) found associated with a risk of LDA (left displaced abomasus) in post-parturient cows. Values of D-BHB very similar to our results were observed by Quiroz-Rocha et al. (2009).

Obtained levels of triglycerides in both groups are a little bit lower in both groups (0,16 $mmol.l^{-1}$) than those published by Slanina et al. (1992) and those used as reference range. Low blood levels of lipoproteins indicate lipomobilisation, fat production and release of lipoproteins as transport forms of fat in the liver. This situation occurs for example during steatosis as well as prolonged lack of energy in food (Balabámová et al., 2009).

Concentration of alanine aminotransferase (ALT) was significantly higher in ML group (0,5038 $\mu kat.l^{-1}$) when compared to BL group (0,3233 $\mu kat.l^{-1}$) (figure 1).

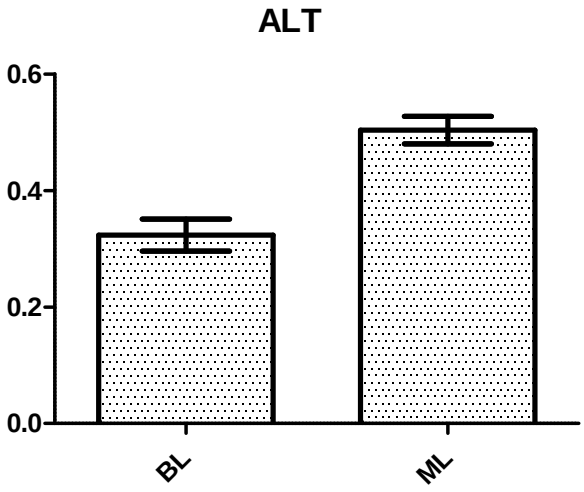

Figure 1. Concentration of ALT in groups BL and ML (in mmol.l^{-1}). ALT - alanine aminotransferase; BL - beginning of lactation; ML - middle of lactation

Significant difference of cholesterol concentration was observed among the groups BL (3,869 mmol.l^{-1}) and ML (7,283 mmol.l^{-1}) (figure 2). Similar results were described by Filipejová et al. (2011). Low serum cholesterol in BL group is related to the synthesis of lipoproteins. In the postpartum period there is an increase in circulating high density lipoproteins (HDL) and a markable decrease in low density lipoproteins (LDL), as well as reductions in very low density lipoproteins (VLDL). Low density lipoproteins contain the largest proportion of cholesterol among the lipoproteins (Bruss, 1997).

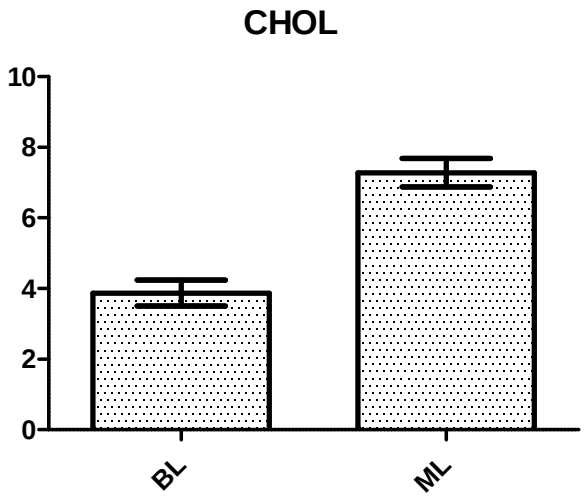

Figure 2. Concentration of cholesterol in groups BL and ML (in mmol.l^{-1}). CHOL - cholesterol; BL - beginning of lactation; ML - middle of lactation

Correlation showed strong positive dependence between bilirubin and triglycerides in BL group (0,887; $p<0.001$), urea and ALT in ML group (0,742; $p<0.001$) and between chlorides and sodium also in ML group (0,977; $p<0.001$). Similar experiment was provided by Kalaitzakis (2010), where correlation between bilirubin and triglycerides was not significant.

Conclusion

In this work, the relations between selected blood biochemical parameters of dairy cows at the beginning and in the middle of lactation were monitored and the correlations between parameters within groups were observed. We detected some significant differences in cholesterol and ALT concentration between BL and ML group and strong positive dependence between bilirubin and triglycerides in BL group, urea and ALT in ML group and between chlorides and sodium in ML group. For more objective results, it is necessary to do further research on more subjects.

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THE EFFECT OF CRUDE PROTEIN SOURCE AND FEEDING TECHNIQUE ON PERFORMANCE, HEALTH STATUS AND CARCASS TRAITS OF GROWING RABBITS

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Abstract

Two experimental isonitrogenous weaning diets (SBM diet and WLS diet) were formulated. The SBM diet contained soybean meal (70 g/kg) as the main CP source, whereas the WLS diet was based on white lupin seeds (105 g/kg). A total of 160 Hyplus rabbits (33 days of age) were divided into 4 groups according to diet and feeding technique (*ad libitum* SBM, restriction SBM, *ad libitum* WLS and restriction WLS group of rabbits). The feeding programme was applied as follows: both the AL SBM rabbits and AL WLS rabbits were fed *ad libitum* during the whole fattening period, whereas the R SBM and R WLS rabbits had a limited feed intake between 33rd and 47th day after weaning (75% of *ad libitum* intake). Afterwards, all restricted rabbits were fed *ad libitum* until the end of fattening period (75 days of age). No significant interaction between dietary CP source and feeding technique was observed in terms of the growth performance of rabbits, whereas the highest hot carcass weight was observed in AL WLS rabbits. Results confirmed that the weaning diet based on WLS fed *ad libitum* for the whole fattening period is safer than the weaning diet containing SBM in terms of the digestive health of weaned rabbits. Feed restriction had no favourable effect on rabbit health in the entire trial, regardless of the CP source.

Introduction

It is well known that growing rabbits are very susceptible to digestive disorders. Because the use of a feed supplemented with antibiotics was banned by EU, there is a need to find alternative solutions to control the disease in order to fulfil current EU legislation. Among them, looking for new feeding strategies, that will increase the resistance of rabbits to digestive pathologies, is getting to the centre of interest (De Blas et al., 2012).

In this regard, a suitable dietary crude protein source (CP) for intensively reared broiler rabbits seems to be an important factor. Recently Volek et al. (2014) have reported that a suitable dietary CP source for growing-fattening rabbits, as a replacement of SBM, seem to be white lupin seeds (WLS) (*Lupinus albus* cv. Amiga).

Another way how to limit digestive disorders of weaned rabbits may be the application of post-weaning feed intake limitation strategy, as clearly proved by Gidenne et al. (2012). However, there are some contradictory results in the literature regarding the favourable effect of feed restriction on digestive health of the young rabbit in terms of the entire fattening period (Birolo et al, 2013). Furthermore, some disadvantages of feed restriction such as the lower slaughter weight and dressing-out percentage are observed in restricted rabbits (Gidenne et al., 2012). Thus, the post-weaning feed intake limitation strategy should be farther studied in relation to growth performance or carcass traits, as well as digestive health of rabbits in terms of the entire fattening period. To our knowledge, for example, no information regarding of the interaction of dietary CP source and feed restriction on growth and digestive health of growing rabbits is available.

The aim of this study was to evaluate the effect of CP source in combination with feeding technique on performance, health status and carcass traits of growing-fattening rabbits.

Material and Methods

Two weaning diets containing SBM (SBM diet) or WLS (WLS diet) as the main CP source were formulated (Table 1). The levels of CP, starch, neutral detergent fibre (NDF), acid detergent fibre (ADF) and lignin/cellulose ratio were similar for both diets. No medication was included in the feed or in the drinking water. Diets were offered as pellets.

A total of 160 Hyplus rabbits, 33 days old at the beginning of the trial, were randomly allocated to four groups (AL SBM group, R SBM group, AL WLS group and R WLS group; 40 rabbits per group) and fed one of the two experimental diets for 42 days, at which time the rabbits were 75 days old. Rabbits were housed in wire net cages (80 x 60 x 45 cm), four per cage. The feeding programme was applied as follows: both the AL SBM rabbits and AL WLS rabbits were fed *ad libitum* during the whole fattening period, whereas the R SBM and R WLS rabbits had a limited feed intake between 33rd and 47th day after weaning. Afterwards, all restricted rabbits were fed *ad libitum* until the end of the fattening period. Restricted rabbits were fed at a level of 75 % of the *ad libitum* groups. Briefly, feed intake was recorded daily during the whole fattening period, thus the restrictive feed ration was calculated every day during the restriction period, based on previous average daily feed intake of *ad libitum* fed rabbits. In addition to the growth performance, health status of rabbits was checked daily and the Health Risk Index (HRI, sum of morbid and dead rabbits) was calculated afterwards according to the methodology of Fernández-Carmona et al. (2005).

Table 1. Ingredient and chemical composition (g/kg as-fed basis) of weaning diets based on soybean meal (SBM diet) or white lupin seeds (WLS diet)

	SBM diet	WLS diet
Ingredient (g/kg)		
Alfalfa meal	300	300
Soybean meal, CP (480 g/kg)	70	0
White lupin seeds, CP (350 g/kg)	0	105
Wheat bran	330	330
Sugar beet pulp	70	50
Oats	150	125
Barley	50	60
Vitamin supplement	10	10
Dicalcium phosphate	5	5
Limestone	10	10
Salt	5	5
Chemical composition (g/kg)		
Dry matter	889	894
Crude protein	163	161
NDF	368	376
ADF	183	187
Lignin (ADL)	44	49
Lignin / cellulose ratio	0.32	0.33
Ether extract	37	45
Starch	159	157

At the end of the experiment, 15 rabbits per group were used for the evaluation of carcass traits according to the methodology recommended by Blasco and Ouhayoun (1996).

All analyses were performed in duplicate. Diets were analysed by AOAC (1984) methods. Data of the performance and carcass traits were examined by analysis of variance using the GLM procedure of the SAS (2012). Health status was assessed using the chi-square test. All differences were considered significant at $P < 0.05$.

Results and discussion

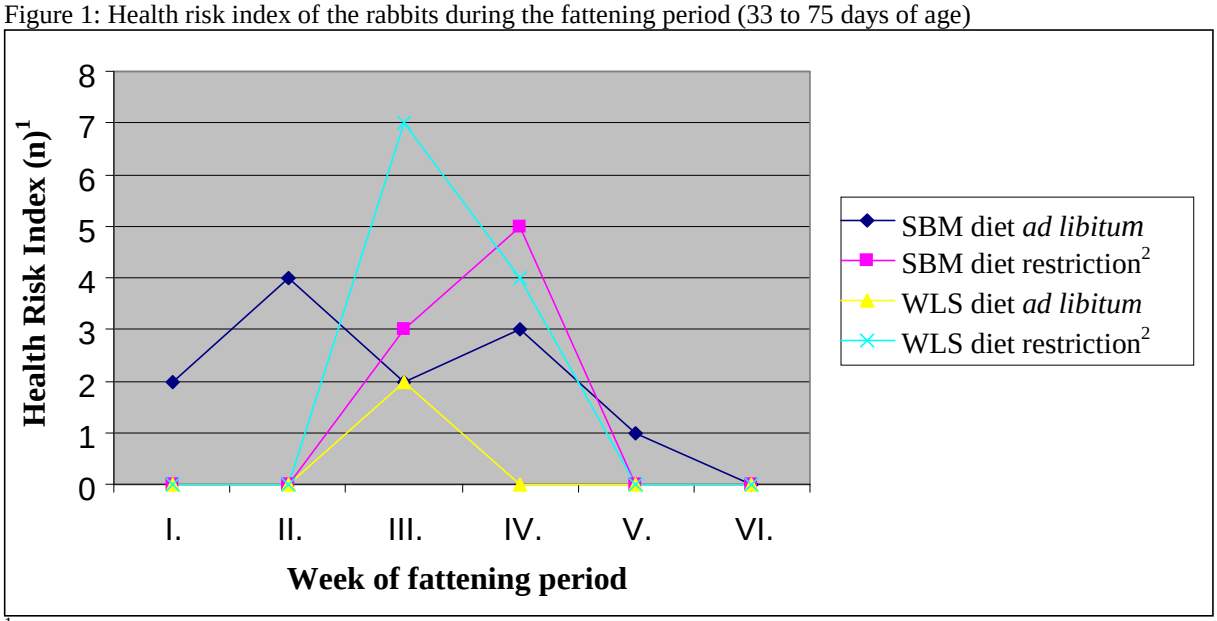
Data on the effect of feed restriction (AL SBM + AL WLS rabbits *versus* R SBM + R WLS rabbits) on the growth performance are not presented in table. Over the whole fattening period, as rather expected, feed restriction reduced significantly both the feed intake (147.8 *vs.* 154.2 g/d for the restricted and *ad libitum* fed rabbits, respectively) and the slaughter weight (2843 *vs.* 2927 g for the restricted and *ad libitum* fed rabbits, respectively), but feed conversion was not affected by the feeding technique. This is consistent with the findings of other authors (Gidenne et al., 2012). On the other hand, no significant interaction between dietary CP source and feeding technique was observed in terms of the growth performance of rabbits (Table 2).

Table 2. Growth, intake and feed conversion of the rabbits according to the dietary CP source and the feeding technique (33 to 75 days of age)

	Crude protein x feeding technique					
	SBM diet		WLS diet			
	AL	R	AL	R	RMSE	*P*-value
Live weight (g)						
33 d of age	769	775	788	764	55	0.432
47 d of age	1461	1327	1527	1339	85	0.356
75 d of age	2903	2827	2952	2863	112	0.865
Feed intake (g/day)						
33 to 47 d of age	103.0	77.7	108.1	78.9	7.6	0.409
47 to 75 d of age	178.3	182.8	180.6	185.5	8.6	0.938
33 to 75 d of age	152.6	146.9	155.9	149.0	6.1	0.771
Weight gain (g/day)						
33 to 47 d of age	49.4	39.4	52.8	41.1	5.0	0.620
47 to 75 d of age	39.0	36.9	39.3	39.3	4.8	0.526
33 to 75 d of age	50.8	47.3	51.4	51.2	4.8	0.316
Feed conversion						
33 to 47 d of age	2.18	2.12	2.13	2.01	0.30	0.765
47 to 75 d of age	4.65	5.02	4.64	5.03	0.76	0.956
33 to 75 d of age	3.04	3.15	3.06	3.07	0.38	0.698

AL – *ad libitum*, R – restriction (between 33 and 47 day of age), RMSE – root mean square error (9, 9,10 and 8 cages for the AL SBM, R SBM, AL WLS and R WLS group of rabbits, respectively)

Figure 1: Health risk index of the rabbits during the fattening period (33 to 75 days of age)



[1]Healt Risk Index = sum of ill and dead rabbits. Mortality and morbidity were associated with diarrhoea; [2]restriction between 33 and 47 days of age.

Figure 1 presents Health Risk Index of rabbits during the fattening period. We clearly found a favourable impact of the limited feed intake on the digestive health of the rabbits during the

restriction period. However, this effect was not persistent after returning to an *ad libitum* intake, which concurs with the results of the other authors (Gidenne et al., 2012). In fact, after re-alimentation we observed a sharp impairment of the digestive health of the previously restricted rabbits, regardless of the CP source used. Thus, the feed restriction did not affect rabbit health in the entire trial, which is in agreement with previous report in rabbits (Birolo et al., 2013). On the other hand, the significantly lower Health Risk Index was observed in the rabbits fed the *ad libitum* weaning diet based on WLS for the entire fattening period than in AL SBM rabbits, which is consistent with our previous report (Volek et al., 2014). However, it is noteworthy that when the post-weaning feed intake limitation strategy is used, the favourable effect of WLS on the digestive health of weaned rabbits, after returning to an *ad libitum* intake, is not observed. Further studies should be carried out to elaborate on this point. Data on the effect of feed restriction (AL SBM + AL WLS rabbits *versus* R SBML + R WLS rabbits) on the carcass traits of rabbits are not presented in table. There were a significantly lower reference carcass weight and dressing-out percentage and a significantly higher drip loss percentage in restricted rabbits, which is consistent with the other authors (Gidenne et al., 2012).

Table 3. Carcass traits of the rabbits according to the dietary CP source and the feeding technique (75 days of age)

	Crude protein x feeding technic					
	SBM diet		WLS diet			
	AL	R	AL	R	RMSE	*P*
Cold carcass weight(g)	1719	1711	1889	1768	111	0.070
Reference carcass weight (g)	1386^{b}	1379^{b}	1532^{a}	1414^{b}	95	0.041
Dressing out (%)	58.1	57.5	58.9	57.8	1.3	0.448
Perirenal fat (%)	1.7	1.8	1.9	2.1	0.5	0.634
Drip loss (%)	2.5	2.9	2.3	2.9	0.5	0.563

AL – *ad libitum*, R – restriction (between 33 and 47 d of age), RMSE – root mean square error (n=15 rabbits per group). a,b $P < 0.05$

There was an interaction between CP source and feeding technique in terms of both the hot carcass weight and reference carcass weight (Table 3). Thus, the highest hot carcass weight and reference carcass weight was observed in AL WLS rabbits, probably associated with the lowest Health Risk Index, and thereby the higher growth rate in these rabbits.

Conclusion

No significant interaction between dietary CP source and feeding technique was observed in terms of the growth performance of rabbits. Results confirmed that the weaning diet based on

WLS fed *ad libitum* for the whole fattening period is safer than the weaning diet containing SBM in terms of the digestive health of weaned rabbits. Feed restriction had no favourable effect on rabbit health in the entire trial, regardless of the CP source. Therefore it is necessary to carry out further studies that would describe the conditions under which the feed restriction is effective in terms of increasing the resistance of rabbits against digestive disorders during the whole fattening period.

Acknowledgement

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THE RESPONSE OF EWES WITH BIMODAL MILK FLOW TO STRESS LOAD

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Abstract

Eleven cross breed ewes of Tsigai and Improved Valachian breeds with Lacaune with only bimodal milk flow type were selected on the basis of their milk flow kinetic from two pre-experimental usual milking. The last usual milking was considered as control. Before experimental milking, each ewe was exposed to social isolation (free movement in parlour alone for at least 4 min). Afterwards the ewe was fixed into a milking stall and disturbed by an unknown person standing in front of its head and gently touching it for a period of extra 2 min before cluster attachment on udder. Milking under stress decreased the total milk yield ($P < 0.001$), machine milk yield ($P < 0.02$), machine stripping ($P < 0.02$) and milking time ($P < 0.01$). Inappropriate handling, such as milking under stress can significantly reduce milk production.

Introduction

Handling with animals should be careful, because it can be very stressful for them. Animals can be stressed by either psychological stress (restraint, handling, novelty) or physical stress (hunger, thirst, fatigue, injury, thermal extremes) (Grandin, 1997). Stress is generally caused by fear. Once established, the fearful reaction is relatively permanent (LeDoux, 1994). Animal reaction may interact by previous handling experiences. Reducing stress during handling will provide advantages of increasing productivity (Grandin, 1998). The presence of an aversive handler during a cows milking reduced milk yield, increased residual milk and lead to elevated heart rates (Rushen et al., 1999). Reaction of animals may vary depending on the environment. The movement patterns and the behavior of isolated ewes were different from those observed when they were located within a social group (González et al., 2013). Routine handling can affect the milk ejection efficiency. The disorders of milk ejection reflex as a consequence of some stress exposure can lead to incompletely milk removal from the udder (Tančin et Bruckmaier, 2001; Negrao et Marnet, 2003). When animals are disturbed or stressed during milking, adrenaline and other related chemicals began to inhibit the milk flow from the udder at central and peripheral level (Tančin et Bruckmaier, 2001; Marnet et McKusick, 2001). In ewes, different studies demonstrated the worsening of production performance in response to stressful management procedure (Sevi et al., 2001; Caroprese et

al., 2010). The variability of milk ejection is influenced significantly by breeds and cross-breeds (Mačuhová et al., 2012; Tančin et al., 2011), but presence of different milk flow kinetic may be considered as a relatively stable response of individual ewes to usual milking stimuli (Tančin et al., 2011) that could be used for experimental purposes.

Material and Methods

The reaction of ewes to specific psychological stress (social isolation before and disturbance during milking) was tested in July. We examined how the animal perceives this manipulation and how it could affect milking efficiency. Eleven cross breed ewes of Tsigai and Improved Valachian breeds with Lacaune (genetic portion of Lacaune was between 25 – 50 %) were selected from the flock on the basis of their milk flow kinetic from the two pre-experimental usual milking. Data of the last usual milking were considered as control milking. For this experiment only ewes with bimodal (2 emissions) milk flow type were selected. Milking under stress condition (experimental milking) of individual ewes was realized during the evening machine milking. Before experimental milking, each ewe was exposed to social isolation (free movement in parlour alone) in dairy parlour for at least 4 min. Afterwards the ewe was fixed into milking stall and disturbed by an unknown person standing in front of its head and gently touching it for a period of extra 2 min before cluster attachment on udder. Before and during experimental milking, the ewes did not have access to the concentrates as usual. Ewes were milked at parallel milking parlour with 12 standard units and with following milking parameters: pulsation rate 160 cycles per min., milking vacuum 39 kPa and pulsation ratio 50:50. During usual milking ewes received 0.1 kg per head concentrate in parlour. Four electronic jars (1.5 L each) collecting total milk produced at the milking were used for individual recording of milk flow kinetic. There was a 2-wire compact magnetostrictive level transmitter within each jar (NIVOTRACK) (NIVELCO Ipari Elektronika Rt, Budapest, Hungary) connected to the computer (Tančin et al., 2011). Milk level in the jar was continuously measured by a transmitter recording signal on the computer every second. Measured changes of height level of milk were transformed into values, from which were detected parameters of milkability (TMY – total milk yield, MMY – machine milk yield, MS – machine stripping, MT – milking time, LT – latency time, MMF – maximum milk flow rate) and types of milk flow (B - bimodal, two emissions, N – non-bimodal, one emision, PL - plateau, steady flow, PLL – plateau with low peak flow) were evaluated according to Bruckmaier et al. (1997) and Mačuhová et al. (2008).

Results

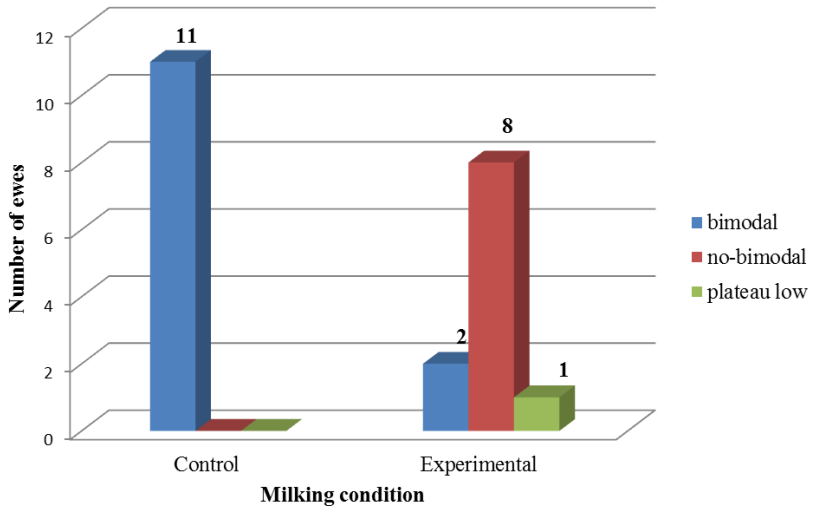
Statistically significant decrease of TMY, MMY, MS and MT (Tab. 1) was recorded under stressful conditions compared to control milking. Similar effect of unknown person on these parameters of milking efficiency during machine milking was recorded also by Kulinová *et al*. (2012). The LT was not influenced by stress load; probably as a consequence of sufficient amount of milk in the cistern directly available for milking and very small effect of catecholamine on contraction of teat sphincter to postpone the beginning of milk flow (Bruckmaier et al., 1991). Large variability in observed parameters among ewes during milking under stressful conditions could be related to different response of animals to stress (Caroprese et al., 2010). A changing of milk flow type is a response on milking under stressful conditions. Lower parameters of milking efficiency during stress response could relate to decrease of B and increase of N and undesirable PLL milk flow types (Fig. 1). The increased occurrence of N milk flow type could indicate inhibition of oxytocin release as a consequence of stress effect. The higher occurrence of N milk flow type was found out during stress load in ewes also by other authors (Marnet and McKusick, 2001; Kulinová et al., 2012).

Table 1. The milking parameters during control milking and milking under stressful conditions (experimental).

	Milking condition (n=11)		
Factor	**Control**	**Experimental**	**P**
TMY, (L)	0.289 ± 0.72	0.185 ± 0.97	0.001
MMY, (L)	0.158 ± 0.74	0.104 ± 0.67	0.016
MS, (L)	0.130 ± 0.06	0.081 ± 0.43	0.017
MT, (s)	61 ± 18	38 ± 13	0.006
LT, (s)	17 ± 8	19 ± 11	0.701
MMF, (L)	0.651 ± 0.37	0.683 ± 0.34	0.736

TMY – total milk yield, MMY – machine milk yield, MS – machine stripping,
MT – milking time, LT – latency time, MMF – maximal milk flow rate

Figure 1. Frequency of distribution of milk flow types during different milking conditions.



Conclusion

Inappropriate handling such as stress caused by social isolation of ewes shortly before milking and disturbing of animals during milking has a significantly negative effect on milking efficiency of ewes with bimodal milk flow response to machine milking.

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COMPARISON OF TWO COMMERCIAL KITS FOR THE ELIMINATION OF RABBIT SPERMATOZOA WITH DAMAGED MEMBRANE VIA MACS TECHNIQUE

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Abstract

The objective of this preliminary study was to compare the effectiveness of two available commercial kits: Annexin V MicroBead Kit (AnV) and Dead Cell Removal Kit (DCR) for the elimination of spermatozoa with damaged membrane. Sexually mature and clinically health rabbit bucks (n = 2) and does (n = 36) of broiler New Zealand White were used for the experiments. The control (unseparated) and magnetically separated spermatozoa were used for artificial insemination of hormonally treated rabbit does. MACS separation of spermatozoa yields two fractions: negative (AnV^- and DCR^-) and positive (AnV^+ and DCR^+). No differences in spermatozoa motility and viability between two different negative fractions (AnV^- and DCR^-) were found. Similarly, no differences in kindling rate and number of liveborn kits were observed between negative fractions (AnV^- and DCR^-). In conclusion, the cheaper Dead Cell Removal Kit could be used for the future experiments with the same efficiency as Annexin V MicroBead Kit for the elimination of spermatozoa with damaged membrane from the rabbit ejaculate.

Introduction

In most normal, viable eukaryotic cells, the negatively charged phospholipid phosphatidylserine (PS) is located in the cytosolic leaflet of the plasma membrane lipid bilayer (Koopman et al., 1994). PS redistribution from the inner to the outer leaflet is an early and widespread event during apoptosis (Koopman et al., 1994; Martin et al., 1995). However, in necrosis, PS becomes accessible due to the disruption of membrane integrity (Martin et al., 1995). PS exposure also serves as a trigger for the recognition and removal of cells by macrophages (Fadok et al., 1992; 2000). Annexin V is a 35kD phospholipid-binding protein and a major cell membrane component of macrophages and other phagocytic cell types. Annexin V has a high affinity to PS in the presence of physiological concentrations of calcium (Ca^{2+}) (Moss et al., 1991) and has already been used to isolate cells with exposed PS using MACS MicroBeads (Kuypers et al., 1996; Rickers et al., 1998). The MACS Annexin V

MicroBead Kit (Miltenyi Biotec) have been also used in studies aimed on the elimination of spermatozoa with exposed PS from human (Said et al., 2006) or rabbit ejaculates (Vasicek et al., 2014). Nowadays, there is similar product on the market called MACS Dead Cell Removal Kit (Miltenyi Biotec). Dead Cell Removal MicroBeads recognize an antigen in the plasma membrane of apoptotic as well as dead cells. Thus, using MACS Dead Cell Removal Kit, even early apoptotic cells with an intact cellular membrane are removed. Moreover, recently the similar method for magnetic sorting of mammalian spermatozoa with damaged membranes using carboxyl-group functionalized magnetic particles conjugated to propidium iodide was patented by Fox et al. (2012). Therefore the aim of this preliminary study was to compare the effectiveness of two available commercial kits for the elimination of spermatozoa with damaged membrane.

Materials and Methods

Sexually mature (6 – 36 month old) and clinically health rabbit bucks (n = 2) and does (n = 36) of broiler New Zealand White (NZW) line reared in a partially air-conditioned hall of a local rabbit farm at APRC Nitra (Animal Production Research Centre, Lužianky, Slovak Republic) were used in the experiments. The treatment of the animals was approved by the Ministry of Agriculture and Rural Development of the Slovak Republic, no. SK P 28004 and Ro 1488/06-221/3a.

Semen samples from 14 NZW bucks were collected using an artificial vagina. Each sample of fresh ejaculate was evaluated for the concentration and motility using Sperm Vision™ (Minitübe, Tiefenbach, Germany), a computer assisted sperm motion analyser (CASA). For the magnetic separation and artificial insemination (AI), the worst 2 bucks were chosen basing on the motility parameters.

Before magnetic sperm separation, the sperm cells were diluted at the ratio of 1:8 and carefully filtered through a 30 µm filter (Celltricks) in order to remove cell clumps. Filtered semen samples from both bucks were then divided into 3 groups as follows: control (unseparated) group, then magnetically separated spermatozoa using Annexin V MicroBead Kit (AnV; Miltenyi Biotec, Germany) and magnetically separated spermatozoa using Dead Cell Removal Kit (DCR; Miltenyi Biotec, Germany) according to the producer manual. The MiniMACS Magnetic Cell Sorting system (Miltenyi Biotec, Germany) was used for MACS assay of rabbit spermatozoa. MACS separation of spermatozoa yields two fractions: negative (AnV^- and DCR^-) and positive (AnV^+ and DCR^+).

The semen samples from the control group, negative (AnV^-, DCR^-) and positive (AnV^+, DCR^+) fractions were placed into Standard Count Analysis Chamber Leja 20 micron (MiniTüb, Tiefenbach, Germany) and evaluated using the CASA system (Sperm Vision™) under a Zeiss Axio Scope A1 microscope. In each sample the percentage of motile spermatozoa (motility >5 µm/s) were evaluated.

The semen samples from the control group, negative (AnV^-, DCR^-) and positive (AnV^+, DCR^+) fractions were also analysed for the viability using flow cytometer FACSCalibur (BD Biosciences, USA). Propidium iodide (Molecular Probes, USA) was used to distinguish cells with the intact membrane (live cells) from dead cells (with damaged membrane).

The unseparated (control group) and MACS separated (AnV^-, DCR^-) rabbit semen from both bucks (Buck I and II) were then used for the artificial insemination (AI). Briefly, females of NZW rabbits (n = 36) were inseminated either with fresh doses of filtered semen (control; n = 3; 0.5 ml I.D. per female) or with magnetically separated semen (n = 3 for each negative fraction; 0.5 ml I.D. per female). The insemination doses with concentration at least $0.04x10^9$/ml were used for the insemination. PMSG at 25 I.U. (Sergon, Bioveta, Czech Republic) was administered to each doe 48 hours before AI. Immediately following AI 2.5 µg of synthetic GnRH (Supergestran, Ferring-Pharmaceuticals, Czech Republic) was intramuscularly injected into each doe. The kindling rate (the ratio of kindled does to the number of inseminated does) and number of liveborn kits were determined at parturition.

Statistics

The experiments were replicated 2 times for each buck (January to March). Obtained data were evaluated statistically using a one-way ANOVA (Duncan's multiple range test) in SigmaPlot software (Systat Software Inc., Germany) and expressed as means ± SE. *P*-values at $P<0.05$ were considered as statistically significant.

Results and Discussion

The spermatozoa motility of Buck I decreased ($P<0.05$) after magnetic separation in both negative fractions (AnV^- and DCR^-) in comparison to the control group (Table 1). On the other hand, spermatozoa motility of Buck II decreased ($P<0.05$) only in the DCR^- fraction in comparison to the control group or AnV^- fraction (Table 2). However, in our previous experiments the motility of spermatozoa of individual bucks was not statistically different between control and the MACS separated group (Vasicek et al. 2011). This discrepancy between these two studies could be due the quality of rabbit ejaculates used for the experiments since in the previous study ejaculates with the best quality in terms of the

spermatozoa motility were used. Moreover, we assumed that the lower spermatozoa motility values in both negative fractions (Table 1 and 2) could be not affected by MACS separation itself, but by the semen handling procedure (e.g. storage time, temperature, centrifugation etc.) before the separation. Anyway, there were no differences in spermatozoa motility between two different negative fractions (AnV^- and DCR^-) in both bucks (I and II). Furthermore, we observed significantly lower spermatozoa motility ($P<0.05$) in positive fractions (AnV^+ and DCR^+) in comparison to the control groups or negative fractions (Table 1 and 2).

Table 1. *In vitro* parameters of unseparated (control) or magnetically separated rabbit spermatozoa (Buck I) using two commercial kits.

PARAMETER **BUCK I**	**MOTILITY %**	**LIVE CELLS %**	**DEAD CELLS %**
Before MACS (Control)	44.56 ± 7.04^{a}	59.95 ± 6.07^{a}	$32.16 \pm 8{,}36^{a}$
AnV^-	$22.49 \pm 0.27^{b, c}$	58.28 ± 2.04^{c}	28.46 ± 2.91^{c}
AnV^+	$2.38 \pm 2.38^{b, d}$	$18.32 \pm 7.25^{b, d}$	$71.36 \pm 11.94^{b, d}$
DCR^-	$24.11 \pm 0.64^{b, e}$	56.06 ± 0.61^{e}	31.89 ± 2.03^{e}
DCR^+	$9.81 \pm 0.72^{b, f}$	$14.17 \pm 1.92^{b, f}$	$76.88 \pm 7.47^{b, f}$

AnV^- - Annexin V-negative fraction, AnV^+ - Annexin V-positive fraction, DCR^- - Dead cell removal kit-negative fraction, DCR^+ - Dead cell removal kit-positive fraction; Results are expressed as means ± SE; ^{a} vs. ^{b}, ^{c} vs. ^{d} and ^{e} vs. ^{f} were statistically significant at $P<0.05$.

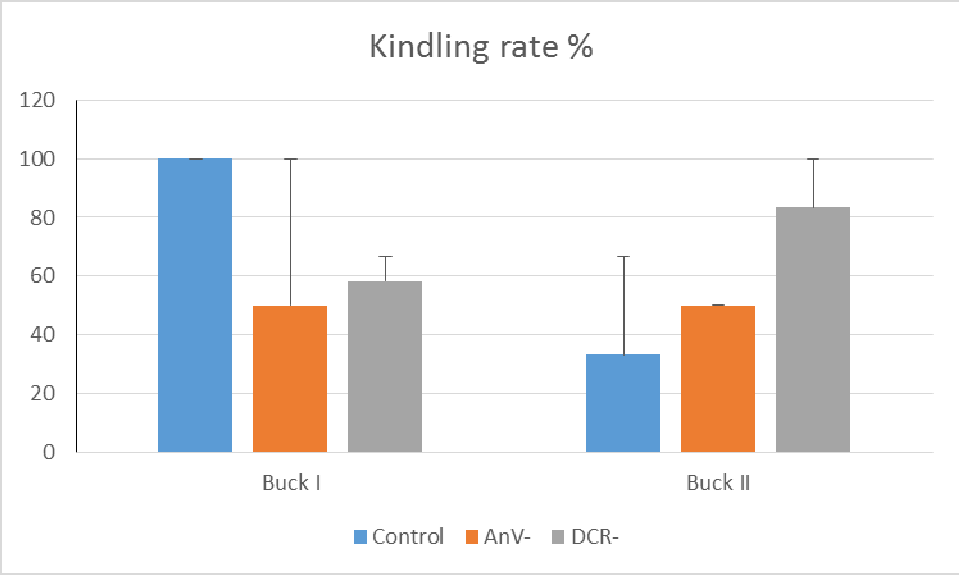
Similar results were obtained by the evaluation of spermatozoa viability before and after MACS separation. The percentage of live cells (both bucks I and II) in the negative fractions (AnV^- and DCR^-) were similar to control group, but significantly higher ($P<0.05$) in comparison to the positive fractions (AnV^+ and DCR^+; Table 1 and 2). Moreover, the negative fractions (AnV^- and DCR^-) had significantly lower ($P<0.05$) percentage of dead cells (both bucks I and II) in comparison to the positive fractions (AnV^+ and DCR^+) with no differences in comparison to the control group (Table 1 and 2). Again, there were no differences in the percentage of live or dead cells between AnV^- and DCR^- fraction in both bucks (I and II). Thus, the MACS technique evidently divided rabbit spermatozoa into two subpopulations: one (AnV^- and DCR^- sperm) with the better semen quality and the other (AnV^+ and DCR^+ sperm) with significantly worse semen quality in terms of sperm motility and viability (Table 1 and 2).

Table 2. *In vitro* parameters of unseparated (control) or magnetically separated rabbit spermatozoa (Buck II) using two commercial kits.

PARAMETER BUCK II	MOTILITY %	LIVE CELLS %	DEAD CELLS %
Before MACS (Control)	77.89 ± 6.10^{a}	81.43 ± 3.55^{a}	14.78 ± 1.78^{a}
AnV^{-}	62.22 ± 3.49^{c}	82.72 ± 3.30^{c}	10.99 ± 1.34^{c}
AnV^{+}	15.28 ± 10.52^{b}	$17.77 \pm 0.53^{b, d}$	$71.30 \pm 3.40^{b, d}$
DCR^{-}	$38.85 \pm 18.29^{b, d}$	89.13 ± 2.84^{e}	6.19 ± 2.81^{e}
DCR^{+}	3.38 ± 1.38^{b}	$5.98 \pm 3.13^{b, f}$	$91.57 \pm 3.71^{b, f}$

AnV^{-} - Annexin V-negative fraction, AnV^{+} - Annexin V-positive fraction, DCR^{-} - Dead cell removal kit-negative fraction, DCR^{+} - Dead cell removal kit-positive fraction; Results are expressed as means ± SE; ^{a} vs. ^{b}, ^{c} vs. ^{d} and ^{e} vs. ^{f} were statistically significant at $P<0.05$.

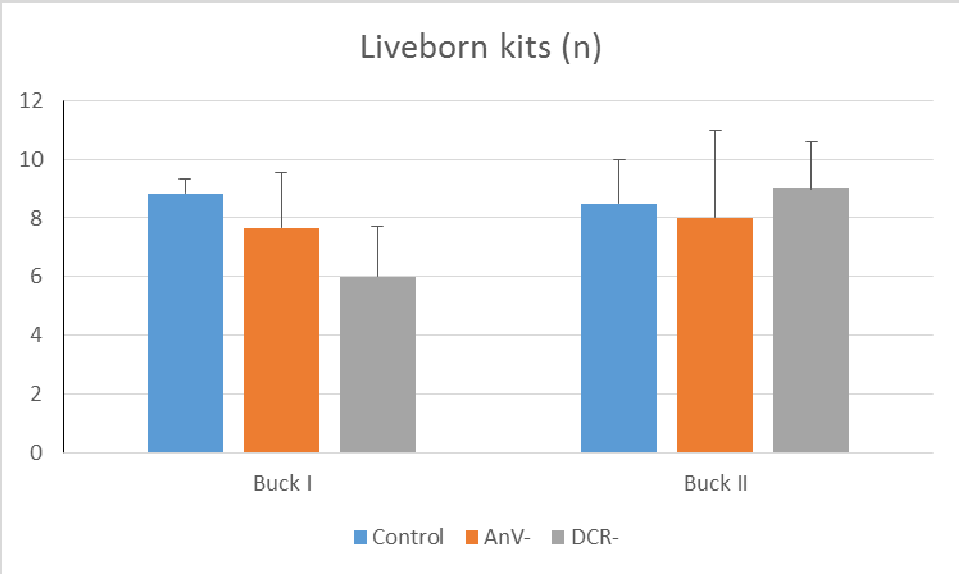
Figure 1. Percentage of kindled does after insemination with unseparated (control) or magnetically separated rabbit spermatozoa (Buck I) using two commercial kits.



AnV^{-} - Annexin V-negative fraction, DCR^{-} - Dead cell removal kit-negative fraction

The MACS technique in both bucks (I and II) did not affect the reproductive parameters of rabbit does (Fig. 1 and 2). Although we found some differences in kindling rate between the control group and negative fractions (AnV^{-} and DCR^{-}), they were not statistically significant (Fig. 1). Moreover, data in Fig. 2 clearly showed that independently of the semen used for insemination (unseparated or MACS separated semen) each doe born about 8 kits. Similar results were observed in our previous study (Vasicek et al., 2014).

Figure 2. Average number of liveborn kits after insemination with unseparated (control) or magnetically separated rabbit spermatozoa (Buck I) using two commercial kits.



AnV$^-$ - Annexin V-negative fraction, DCR$^-$ - Dead cell removal kit-negative fraction

Conclusion

These preliminary results indicate that the MACS Dead Cell Removal Kit could be used for the future experiments with same efficiency as the other commercial MACS Annexin V MicroBead Kit. Moreover, the Dead Cell Removal Kit is cheaper than the other kit. However, due to the high variability in some observed results, further experiments are required in order to prove the real effectiveness of DCR kit for the elimination of spermatozoa with damaged membrane from the rabbit ejaculate.

Acknowledgement

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THE EFFECT OF THE HEIGHT IN THE CROSS OF CZECH FLECKVIEH COWS ON THE PREFERENCE OF CUBICLE ROW IN SELECTED BREEDING

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Abstract

The aim of study, the effect of the height in the cross of czech fleckvieh cows on the preference of cubicle row was evaluated. The frequency of standing cows and cows lying on the right and left side (laterality) was determined. Experiment was conducted in farm GenAgro Ricany a. s.. The objective of monitoring was one section - one quarter of stable (n = 98 cows), where 103 cubicles are arranged in three rows (32 cubicles in row A – close to feed alley; 33 cubicles in row B – in the middle; and 38 cubicles in row C – located peripherally, close to the side wall). Cows were divided by actual of milk yield into 3 groups (< 138 cm; 139-142 cm; 143 < cm). Height in the cross was detected using the linear traits.

It was confirmed, that the height in the cross has influence on the preference of cubicle row and on resting behavior of dairy cows. It was found evidential preference of cubicle row located close to the feed alley higher dairy cows. Smaller dairy cows then have preferred a cubicle row was located peripherally, close to the side wall. The cows showed significant tendency towards left-side laterality.

Material and methods

The aim of study, the effect of the height in the cross of czech fleckvieh cows on the preference of cubicle row was evaluated. The frequency of standing cows and cows lying on the right and left side (laterality) was determined. Experiment was conducted in farm GenAgro Ricany a. s. (49°12´30.370´´N, 16°23´43.092´´E). Observation was carried out at weekly intervals from 8. 6. 2011 to 30. 5. 2012. The objective of monitoring was one section - one quarter of stable (n = 98 cows), where 103 cubicles are arranged in three rows (32 cubicles in row A – close to feed alley; 33 cubicles in row B – in the middle; and 38 cubicles in row C – located peripherally, close to the side wall). A total of 1 876 observations were analysed. Cows were divided by actual of milk yield into 3 groups (< 138 cm; 139-142 cm; 143 < cm). Height in the cross was detected using the linear traits. To the resulting program was used MS Office Excel 2003 and Statistica 10.0.

Introduction

Although the process of domestication brought about a number of important, or even essential, changes in farm animal performance or exterior, their environmental requirements remained relatively invariable throughout their phylogenesis. The impact of environmental factors on domesticated animals is extremely complex and difficult to define. The more altered the original environmental conditions, the greater responsibility of the breeder to provide adequately for animals' needs (Chládek, 2004).

The main objective a dairy cattle is milk production, which plays an important and irreplaceable role in human nutrition for its high nutrtitional value (Frelich et al., 2001). Cows that lie are healthier and more productive (Thorne, 2008). The time cows spend lying down then can be used to evaluate the quality of barns and it should be emphasised in this context that the access to a comfortable lying area is one of the most important design criteria in the field of dairy cow housing (Ito et al., 2009).

Results

All dairy cows (n = 1 876) were divided into 3 groups. Most of individual monitoring was in the group with the height in the cross between 139 – 142 cm (n = 820), least in the group with the height in the cross 143 < cm (n = 347). All the observed parameters significantly differed between rows A, B and C. From **Tab. 1** is evidently, that dairy cows with the height in the cross 143 < cm preferred mostly of cubicle row A and least preferred cubicle row C. This difference was high statistically significant ($p < 0.01$). Dairy cows with the height in the cross < 138 cm conversely preferred mostly cubicle row C and least preferred cubicle rows A and B. This difference was statistically significant ($p < 0.05$). Dairy cows with an average height in the cross (139 – 142 cm) occupied all three rows evenly. This difference was not statistically significant ($p > 0.05$).

All cows in row A and C identically have preferred when lying left side more than the right. This difference was high statistically significant ($p < 0.01$). In contrast, in a row B gave dairy cows preference lying at the side of right. This difference was high statistically significant ($p < 0.01$).

Wagner – Storch ET AL. (2003) who says that the preference of peripheral rows of stalls may be due to a better ventilation of air near sidewalls. On the contrary, Natzke et al. (1982) observed that the inner rows of stall are preferred to the outer ones. In the study of Doležal (2003) the cows preferred the rows situated close to the feed table rather than the outer rows

further from it. These results were confirmed by GAWORSKI et al. (2003). Večeřa et al. (2011) also observed the tendency of cows to occupy the first (closest) or second (middle) row from the feed table, given the choice, when coming from the milking parlour. Večeřa et al. (2012) further specified that the rows closest to the feed table and middle rows were preferably occupied by cows in late lactation.

A non-significant tendency towards a left-side preference was also found by Hrouz et al. (2007) where 53–70 % of their experimental animals preferred the left side to rest on. Tucker et al. (2009) observed a left side laterality in free-housed dry cows; however, the authors admited that cows in pens or on pasture may exhibit no laterality. Although the cows show no overall laterality as a group, they still may have a strong preference as individuals (Gibbons et al., 2012). Zejdová et al. (2011) found out that older cows (lactation 4 and older) preferred left side more often than younger cows (lactation 2 and 3). In our experiment, the cows preferring the left side had a higher milk production. We speculated that this was due to the anatomical differences in the left and right lung. A greater respiration capacity of the right lung allowed better lung ventilation in cows lying on their left side.

Table 1. The effect of Height in the cross on the preference of cubicle row

Height in the cross (cm)	**Position**		**Cubicle row**			**significant**	∑
			A (n = 32)	**B (n = 33)**	**C (n = 38)**		
< 138	**number**		**233**	**234**	**242**	*	**709**
	lying	left	123^{A}	72^{A}	155^{A}	**	350
		right	82^{B}	123^{B}	56^{B}	**	261
	standing		28	39	31	**NS**	98
139 – 142	**number**		**299**	**271**	**250**	**NS**	**820**
	lying	left	162^{A}	98^{A}	148^{A}	**	408
		right	109^{B}	138^{B}	55^{B}	**	302
	standing		28	35	47	**NS**	110
143 <	**number**		**150**	**109**	**88**	**	**347**
	lying	left	78	39^{a}	56^{A}	**	173
		right	58	61^{b}	23^{B}	**	142
	standing		14	9	9	**NS**	32
Overall			**682**	**614**	**580**	*	**1876**

*Values within the row differ if marked with * ($p < 0.05$) and ** ($p < 0.01$) or difference is not significant (NS)*
Values within the column differ if marked with different letters a, b ($p < 0.05$); A, B ($p < 0.01$) or difference is not significant (NS)

Conclusion

It was confirmed, that the height in the cross has influence on the preference of cubicle row and on resting behavior of dairy cows. It was found evidential preference of cubicle row located close to the feed alley higher dairy cows. Smaller dairy cows then have preferred a cubicle row was located peripherally, close to the side wall. The cows showed significant tendency towards left-side laterality.

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EFFECT OF MILKING FREQUENCY ON MILK PRODUCTION OF HOLSTEIN COWS DURING WINTER AND SUMMER PERIOD

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Abstract

The objective of this study was to determinate the effect of milking frequency on milk production of Holstein cows during winter and summer. Total number of cows was divided into two groups by milk yield. Almost 40 % of cows milked more than 25 kg of milk per day per cow so cows are milked twice during the morning. The other more than 60 % of dairy cows milked per day less than 25 kg of milk per day, and these cows are milked once during the morning. In summer and winter period were monitoring the average daily temperature in the barn (BAT) and the stable relative humidity (RH). The study lasted 177 days (data analyzed daily) in the summer (from 1 June 2013 to 31 August 2013) and in winter period (from 1 December 2013 to 23 February 2014). Data comes from University farm. The farm is situated in Žabčice (GPS49°0'51.786"N, 16°36'14.809"E). Based on the correlation of milk production data of Holstein breed was found with increasing BAT reduces average morning milk yield per twice milked cow on the second milking at 8.00 a.m. ($r = -0.32$, $P < 0.01$), total average morning milk yield per once milked cow ($r = -0.23$; $P < 0.05$) and total morning milk yield ($r = -0.41$, $P < 0.001$). In milk production data was observed that with decreasing RH in summer reduces average morning milk yield per twice milked cow on the second milking at 8.00 a.m. ($r = 0.23$; $P < 0.05$), average morning milk yield per once milked cow ($r = 0.31$, $P < 0.01$, total morning milk yield per twice milked cow on the second milking at 8.00 a.m. ($r = 0.37$, $P < 0.001$) and total morning milk yield per twice milked cow ($r = 0.26$; $P < 0.05$). Other analyzed parameters were not affected by BAT or RH ($P > 0.05$).

Key words: Holstein cows, milking frequency, milk production, barn airspace temperature, relative humidity

Introduction

Milk production is a complicated physiological property, which primarily related to the anatomical formation of the udder (with development organs, the activities of organs, blood and circulatory system, with breathing and transformation of nutrients) (Vaněk, Štolc, 2002). The amounts of milk affect the internal and external factors. Between the external factors

include the level of nutrition, human factors, technology of stabling, frequency of milking and seasonality (Senka, 2011).

It is generally known that it is unnatural to highly productive dairy cows were milked twice a day. More frequent milking (three times a day or more) increases the production of milk, lactation curve is higher and very balanced course, the result is the growth of performance of dairy cows (Fleischmannová, 2005). Simply put, more frequent milking reduces the pressure in the mammary gland and milk production accelerates, from the physiological viewpoint (Anonym 1, 2003). Rabold, Achesen a Haschek et al. (2002) said that with increasing frequency milking increases quality of the milk and total milk yield, compared with twice daily milking about 12 to 15 % (Doležal et al., 2000). Pařilová (2006) even report an increase milk yield about 6 to 25 % per lactation.

The climate in the barn is one of the key factors potentially affecting animal performance. The climate in the barn decides on milk production, purity and health of the cows (Brestenský and Mihina, 2006). According Kadlečík and Kasarda (2007) are especially important parameters temperature, relative humidity and light.

The aim of the study was evaluate the effect of milking frequency on milk production of Holstein cows in winter and summer period.

Material and methods

Measured dates came from University farm in Žabčice (GPS49°0’51.786”N, 16°36’14.809”E), which reared with Holstein breed. During the 177 days were collected data after the morning milking in the summer (from 1 June 2013 to 31 August 2013) and in winter period (from 1 December 2013 to 23 February 2014).

The total number of dairy cow was divided into two groups according to the average daily milk yield. The first group of cows (average of 152 cows, i.e. almost 30 %), with milk yields of 25 l of milk per day were milked twice during the morning (at 4.00 a.m. and 8.00 a.m.). The second group of cows (average 304 cows), with a maximum milk yield 25 kg per day was milked once (at 5.00 pm). All cows were milked again in the afternoon. This study does not deal with afternoon milk yield. The cows were stabling in free boxing with bedding of straw and dairy cows were fed a TMR ("total mix ratio"). Barn airspace temperature (BAT) represents the average of temperatures in control days. It was measured every 15 minutes by 3 sensors with HOBO data logger (Onset Computer). RH in barn was recorded the same sensors and in the same intervals like BAT. Milk production (used as a average morning milk yield per cow and total morning milk yield) was obtained from the computer database of

university farm in Žabčice. MS Office Excel 2003 and Unistat version 1.5 were used to evaluate the results of the data.

Results and discussion

Values of mean, minimum, maximum and standard deviation of BAT, RH, average morning milk yield one cow and total morning milk yield of Holstein cows with twice and once morning milking are presented in Table 1.

Table 1. Barn airspace temperature, relative humidity, milk production of twice and once morning milking of Holstein cows in summer and winter period

Parameter		Hour	unit	n	$\bar{x}$	min	max	SD
Bioclimate	***BAT***	-	*°C*	177	13,98	-2.60	29.65	8.62
	RH	-	*%*	177	68.39	35.82	95.11	16.91
Number of cows	***Twice MM***	-	-	177	152	145	163	3.92
	Once MM	-	-	177	304	232	337	31
Average morning milk yield per cow	***Twice MM***	*4.00 a.m.*	*kg/cow*	177	16.28	13.53	17.92	0.67
		8.00 a.m.		177	6.28	5.10	7.21	0.39
		∑		177	22.56	19.26	24.52	0.91
	Once MM	*5.00 a.m.*		177	14.30	12.58	16.63	0.60
Total morning milk yield	***Twice MM***	*4.00 a.m.*	*kg*	177	2462	1976	2741	136
		8.00 a.m.		177	952	770	1109	67
		∑		177	3399	2168	3801	231
	Once MM	*5.00 a.m.*		177	3656	3124	4214	291

Note: BAT – barn airspace temperature, RH – relative humidity, MM – morning milking

It was selected for 177 days with a range of BAT from -2.6 ° C to 29.65 ° C, with an average BAT 13.98 ± 8.62 °C. Neutral range of BAT is considered in cattle 4 - 16 ° C (Dolejš et al., 2002). This means that cows were exposed to a heat stress in some summer periods. Temperature 20 °C is considered a risk for the creation of heat stress (Zejdová et al., 2013). RH was measured from 35.82 % to 95.11 %, with an average of 68.39 ± 16.91%, in these days. RH in the barn should be in the range of 40 – 80 %. The relative values should not exceed 85% in the barn (Zejdová et al., 2013). This means that dairy cows were exposed higher than its optimum value of RH in some periods.

Values of coefficients of correlation of milk production of twice and once morning milking of Holstein cows and BAT in summer and winter period are presented in Table 2.

Table 2. Values of coefficients of correlation of milk production of Holstein cows milked twice and once during the morning and barn airspace in the barn in summer and winter period

Parameter		Hour	unit	BAT (°C) in winter	BAT (°C) in summer
Average morning milk yield per cow	Twice MM	4.00	kg/cow	-0.12 N.S.	0.03 N.S.
		8.00		0.09 N.S.	-0.32**
		∑		-0.6 N.S.	-0.12 N.S.
	Once MM	5.00		0.02 N.S.	-0.23*
Total morning milk yield	Twice MM	4.00	kg	-0.15 N.S.	-0.13 N.S.
		8.00		-0.001 N.S.	-0.41***
		∑		-0.12 N.S.	-0.20 N.S.
	Once MM	5.00		0.05 N.S.	-0.05 N.S.

*Note: Signification: N.S. - P > 0.05; * - P < 0.05; ** - P < 0.01; *** - P < 0.001*
BAT – barn airspace temperature, RH – relative humidity, MM – morning milking

The table shows the effect of BAT on milk production. Unusual warm winter (without cold stress) had a positive (statistically insignificant) effect on dairy cows and milk production. Statistically significant effect on milk production BAT was demonstrated in the summer because high temperatures causing heat stress. Based on the correlation of milk production data of Holstein breed was found with increasing BAT reduces average morning milk yield per twice milked cow on the second milking at 8.00 a.m. ($r = -0.32$, $P < 0.01$), total average morning milk yield per once milked cow ($r = -0.23$; $P < 0.05$) and total morning milk yield ($r = -0.41$, $P < 0.001$). Milk production of cows is influenced by environmental factors, especially high temperature during summer (Brouček et al., 2009). Many times was stated that high yielding cows that are at the top of lactation are particularly sensitive to heat stress (Doležal et al., 2000). Metabolic heat production increases as the productive capacity of dairy cows improves (Purwanto et al., 1990). Bernabucci et al. (2002) found a 10 % lower milk yield in summer than in spring. Cows with high production were probably less sensitive to the effects of high ambient temperatures (Brouček et al., 2009).

Values of coefficients of correlation of milk production of cow milked twice and once during the morning and RH in summer and winter period are presented in Table 3. In milk production data was observed that with decreasing RH in summer reduces average morning milk yield per twice milked cow on the second milking at 8.00 a.m. ($r = 0.23$; $P < 0.05$), average morning milk yield per once milked cow ($r = 0.31$, $P < 0.01$, total morning milk yield per twice milked cow on the second milking at 8.00 a.m. ($r = 0.37$, $P < 0.001$) and total morning milk yield per twice milked cow ($r = 0.26$; $P < 0.05$).

Table 3. Values of coefficients of correlation of milk production of Holstein cows milked twice and once during the morning and relative humidity in summer and winter period

Parameter		Hour	unit	RH (°C) in winter	RH (°C) in summer
Average morning milk yield per cow	Twice MM	4.00	kg/cow	-0.17 N.S.	-0.06 N.S.
		8.00		-0.15 N.S.	0.23*
		∑		-0.20 N.S.	0.06 N.S.
	Once MM	5.00		-0.09 N.S.	0.31**
Total morning milk yield	Twice MM	4.00	kg	-0.12 N.S.	0.16 N.S.
		8.00		-0.05 N.S.	0.37***
		∑		-0.11 N.S.	0.26*
	Once MM	5.00		-0.08 N.S.	-0.10 N.S.

*Note: Signification: N.S. - P > 0.05; * - P < 0.05; ** - P < 0.01; *** - P < 0.001*
BAT – barn airspace temperature, RH – relative humidity, MM – morning milking

High temperatures and summer's depression of milk yield is a serious problem in conjunction with higher relative humidity can reduce milk production by 10 to 35% of the annual average (Vokřálová and Novák, 2005).

Conclusion

The aim of the study was evaluate the effect of milking frequency on milk production of Holstein cows in winter and summer period. Based on the correlation of bulk milk samples of Holstein breed was found that with increasing barn airspace temperature reduces average morning milk yield per cow, total morning milk yield. In milk production data was observed that with decreasing RH in summer reduces average morning milk yield per cow and total morning milk yield. We conclude that to heat stresses (to high BAT, low RH) are much more sensitive cows with high milk yield than cows with low yield.

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BIOTRANSFORMATION OF MELAMINE INTO CYANURIC ACID IN LAYING HENS

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Abstract

The objective of this work was to investigate whether melamine is biotransformed into cyanuric acid in laying hens. Addition of 100 mg of melamine per kg of feed used for experimental layers over the period of 5 weeks led to the presence of the melamine residue in their eggs, liver, kidney, and breast and thigh muscles. Although cyanuric acid wasn't directly added into feed used for experimental layers, its presence also was detected in eggs, liver, and kidney of these layers. On the basis of our results, it can be concluded that melamine was biotransformed into cyanuric acid in the hens' body.

Introduction

Melamine (2, 4, 6-triamino-1, 3, 5-triazine) is widely used to make plastics, laminates, and coating. Besides that, melamine is present as a trace contaminant in nitrogen supplements used in animal feeds (e.g., urea) and can also be found as a metabolite and degradation product of the pesticide and veterinary drugs (EFSA, 2010). Cyanuric acid (2, 4, 6-trihydroxy-1, 3, 5-triazine), a structural analogue of melamine, is also a by-product of the production of plastics and is also used as water disinfectant in pools (WHO, 2008; Wu and Chen, 2009).

The illegal adulteration of feed and milk used for infant formula (often to mask insufficient protein content) with either melamine alone or melamine containing cyanuric acid resulted in the illness and death of children and companion animals due to the nephrotoxicity associated with the accumulation of melamine-uric acid or melamine-cyanuric acid crystals in the kidneys (US-FDA, 2007; WHO, 2009).

Risk assessment of melamine and cyanuric acid in poultry was already conducted on laying hens, broiler chickens, ducks, and Japanese quails (Bai et al., 2010; Gao et al., 2010; Wang et al., 2012; Zhang et al., 2012). Previous poultry research has shown that melamine is absorbed from the diet and subsequently can be found in poultry tissues, blood, and eggs.

According to Dorne et al. (2013), cyanuric acid may be produced by microbial degradation of melamine in the gastrointestinal tract. This process may occur mainly in ruminants (Dorne et

al., 2013) and has not yet been proved in poultry (Dong et al., 2010; Zhang et al., 2012). Recently, however, the biotransformation of melamine into cyanuric acid has been confirmed in experimental rats. Specifically, the presence of *Klebsiella terrigena* (a bacterial species) in intestinal tract was determined as a main "culprit" responsible for this biotransformation (Zheng et al., 2013).

The objective of our study was to investigate whether melamine is biotransformed into cyanuric acid in laying hens.

Material and Methods

Birds and experimental design

Twenty ISA Brown laying hens were used in the experiment. Hens were divided into the EXP group (n=10) and the control group (n=10). The experimental period lasts 6 weeks (from the 36th week of age). Before the experiment, all hens were fed the N1 commercial diet without melamine. From week 1 to 5, EXP hens were fed the N1 diet with the addition of 100 mg of melamine (Sigma-Aldrich, France) per kg of feed. Thereafter, the EXP hens were fed the N1 diet without melamine. Hens in the control group were fed the N1 diet without melamine during entire period of the experiment. Layers were housed individually in cages in the accredited experimental stable of the Department of Animal Nutrition, University of Veterinary and Pharmaceutical Sciences Brno. The experimental procedures were approved by the Animal Welfare Committee.

Sample collection and analyses

The eggs of all layers were collected daily. The samples of egg white and egg yolk were prepared from the total week egg production, using a homogenizer. The samples were freeze-dried, homogenized by grinding and stored in a fridge at 5 °C for subsequent analysis for melamine and cyanuric acid. Analysis was performed with 0.5 g of homogenized sample that was extracted using 20 ml of the DEA extraction medium (water/acetonitrile) and agitated vigorously for 1 min. The mixture was centrifuged for 10 min and then filtered through the nylon filter with a pore size of 0.45 mm. A sample of 200 ml was taken from the solution and evaporated at 70 °C to dryness using liquid nitrogen. The residue was dissolved in 200 ml of pyridine and mixed with 200 ml of a silanizing reagent (SYLON BFT) and 100 ml of the benzoguanamine internal standard. The entire mixture was stirred and incubated for 45 min at 70 °C.

At the end of the experiment (Week 6), all hens were stunned and exsanguinated by cutting their jugular veins. Melamine and cyanuric acid were determined in samples of the liver, kidney, breast muscle, and thigh muscle. Tissue samples were homogenized, dried in an oven and ground. Samples were stored in a freezer until the samples were prepared for analysis.
The resultant sample was analysed by gas chromatography - triple quadrupole mass spectrometry (GC-MS/MS). The detection limit of the assay was 0.2 mg of melamine and/or cyanuric acid per kg of dry matter basis.

Results and Discussion

Experimental layers fed the melamine contaminated diet laid eggs with the presence of both melamine and cyanuric acid residues (Table 1). Within EXP layers, the melamine and cyanuric acid residues were detected during the entire period of the experiment. Their residues in laid eggs were found very fast after melamine contamination of feed. Concerning the melamine residue in eggs, our results are in accordance with findings published by Bai et al. (2010) and Chen et al. (2010). Moreover, also in week 6, one week after cessation of experimental contamination of feed, detected levels of melamine and cyanuric acid residues (more than 0.2 mg/kg of dry matter basis) were found. In eggs of control layers no detectable residues of melamine and cyanuric acid were found.

Table 1. Presence of melamine and cyanuric acid residues in eggs during the experiment.

Layers	Residue	Week of experiment					
		1	2	3	4	5	6*
Experimental	Melamine	+	+	+	+	+	+
	Cyanuric acid	+	+	+	+	+	+
Control	Melamine	ND					
	Cyanuric acid						

* = one week after cessation of the feed contamination in experimental layers.
+ = detected, detection limit of the assay was 0.2 mg per kg of dry matter basis; **ND** = not detected.

Feeding the melamine contaminated diet over 5 weeks to EXP layers led to the presence of melamine residue in all evaluated body tissues of layers (Table 2). Moreover similar to eggs, the cyanuric acid residue was detected in layer tissues, namely in the liver and kidney.

These findings demonstrate that melamine was biotransformed into cyanuric acid in hen bodies, what is not in accordance with results published by Dong et al. (2010) and Zhang et al. (2012) in poultry. Concerning control layers, no melamine and cyanuric acid residues were detected in their body tissues.

Table 2. Presence of melamine and cyanuric acid residues in hen tissues at the end of experiment.

Layers	Residue	Tissue			
		Liver	Kidney	Breast muscle	Thigh muscle
Experimental	Melamine	+	+	+	+
	Cyanuric acid	+	+	ND	ND
Control	Melamine	ND			
	Cyanuric acid				

+ = detected, detection limit of the assay was 0.2 mg per kg of dry matter basis; **ND** = not detected.

Conclusion

Melamine was detected in eggs of experimental layers during whole assessment period, even one week after cessation of melamine contamination in feed. At the end of experiment, all analyzed body tissues of experimental layers contained the detectable level of melamine residue.

Although cyanuric acid wasn't directly added into feed used for experimental layers, the presence of the cyanuric acid residue was detected in their eggs, liver, and kidney.

On the basis of our results, it can be concluded that melamine was biotransformed into cyanuric acid in the body of laying hens.

Acknowledgments

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MOLECULAR IDENTIFICATION OF PARAMECIUM BURSARIA SYMBIONTS USING RPS11-RPL2 GENE CLUSTER FRAGMENT AS A MARKER

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Keywords: *Paramecium bursaria*, endosymbiosis, *rps11-rpl2* gene cluster, *Chlorella vulgaris*, *Chlorella variabilis*, *Micractinum reisseri*

Abstract

Symbiosis of green algae with protozoa has been studied for over 100 years. Endosymbionts are widely distributed in ciliates such as *Paramecium* ,*Stentor*, *Euplotes*. *Paramecium bursaria*, a freshwater ciliate, typically harbors hundreds of symbionts inside the cell within perialgal vacuoles. The relationship between host and algae is stable and mutually beneficial. Endosymbiotic green algae have been identified as named or unnamed species of *Chlorella* Beij., *Zoochlorella* K. Brandt or referred to as *Chlorella*-like species or *Zoochlorellae*. *Paramecium bursaria* contains symbionts belonging either the so-called American or European group. The aim of this work was to identify symbionts of *Paramecium bursaria* strains originating from different places all over the world, confirm the genetic autonomy of the American and European groups and to investigate the occurrence of correlation between *Paramecium bursaria* strain's belonging to any of five syngen and the species of endosymbiont, the strain possess. We studied 10 strains of *Paramecium bursaria* containing symbionts. The fragment of *rps11-rpl2* cluster gene was amplified by polymerase chain reaction using primers set: UCP2F and UCP2R. In the present study, we confirmed the genetic autonomy of the American and European groups and we did not reveal the occurrence of correlation between *Paramecium bursaria* strain's belonging to any of five syngen and the species of endosymbiont, the strain possess. Furthermore, we described the symbionts as *Chlorella vulgaris*, *Chlorella variabilis* and *Micractinum reisseri*.

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HOW CAN PRENATAL ENVIRONMENT INFLUENCE BLOOD PRESSURE CONTROL IN ADULTS

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Blood pressure is controlled by a complex of feed-back loops and set points that are formed during ontogeny. Prenatal environmental conditions may change settings of these control systems with a subsequent hypertension development later in postnatal life. In this way the prenatal environment in interaction with well known risk factors (increased salt intake, smoking, obesity, diabetes, life style) may explain the increasing incidence of this cardiovascular syndrome in developed countries.

To understand better possible mechanisms we have analyzed in our studies consequences of exposure of pregnant female rats to hypoxia (10 %) during 1) the second embryonic week or 2) days 19 and 20 of a pregnancy on changes of blood pressure and heart rate in offspring of these females. In another set of experiments we exposed pregnant female rats to exogenous angiotensin 2 infused continuously via osmotic minipumps (Alzet) continuously releasing angiotensin 2 (2 µg/kg/h) for 14 days, from day 6 till the end of pregnancy. Blood pressure and heart were measured by radiotelemetry (DataSciences, USA) in conscious mature male offspring. Pressure transmitters (TA11PA-C40, DSI, USA) were implanted into the aorta under isofluran anesthesia and data were collected continuously for one week. For immunological measurements, venous blood of 15 week-old rats was analysed by flow cytometry. At the end of experiment we determined hormonal parameters such as plasma renin activity, aldosterone, triiodothyronine and thyroxine levels by radioimmunoassay in plasma. Kidneys were used for morphological and immunohistochemical analyses.

All measured parameters (blood pressure, heart rate, locomotor activity) exhibited distinct circadian rhythms wit significantly higher values during the dark than light-time. Exposure to hypoxia during the final stages of embryonic development (H), but not during the second embryonic week, resulted in significant higher systolic blood pressure in comparison to control (C) group during the light (C: 107 ± 2 mm Hg, H: 116 ± 3 mm Hg, $p < 0.05$) and the dark (C: 114 ± 3 mm Hg, H: 125 ± 3 mm Hg, $p < 0.05$ phase). Maternal treatment with angiotensin 2 increased blood pressure in progeny in a similar extent as the perinatal hypoxia.

Moreover, we observed differences in the activity of the renin-angiotensin-aldosterone system (RAAS) in progeny treated during embryonic development in comparison with controls.
Our result suggest that changed functional morphology of kidney, changes in the RAAS activity and infiltration of target organs with lymphocytes and monocytes with a subsequent sub-clinical inflammation may explain increased blood pressure in progeny. We hypothesize that prenatal hypoxia at the end of pregnancy and the changed activity of RAAS system can significantly increase blood pressure in progeny probably via an increased sympathetic drive and modulations of central mechanisms of blood pressure control can be involved.

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THE MODIFICATION OF MICROCIRCULATION IN SPONTANEOUSLY HYPERTENSIVE RATS

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Abstract

Changes in microcirculation are connected with hypertension. However, it is still unclear, if thesechanges area cause or a consequence of hypertension. To explore more this problem weanalyzed density of capillaries in spontaneously hypertensive rats (SHR) kept under constant light and after administration of melatonin (2.5 mg/kg for 4 weeks and 10 mg/kg in last week). The density of capillaries was visualized by immunofluorescence method with labeled lectin (*Fluorescein-lycopersicon esculentum lectin*). We observed a higher density of capillaries in SHR than in Wistar rats. After exposure to constant light we found a higher density of capillaries in Wistar rats as compared to control conditions (12D:12L). Administration of exogenous melatonin to SHR rats did not induce significant changes in the density of capillaries. In next studies we will focus on microcirculation in the kidney and brain since these organs are the key regulator of blood pressure.

Introduction

Microcirculation is one of the most important parts of the cardiovascular system and it consist of three structures: capillaries, arterioles and venules. Capillaries are composed only from one layer of endothelial cells that create the membrane responsible for the selective permeability of capillaries. This membrane controls the transport of oxygen and nutrition from vessels to tissues and organs. Besides perfusion, microcirculation is involved in the control of blood pressure (Tuma et al., 2008). Measurement of intraluminal pressure shows thatdevelopment of hypertension isaccompanied only by a small increase of blood pressure in large vessels and therefore this part of circulation cannot significantly contribute to an increased resistance. On the other hand, small arteries and arterioles show a significant increase in blood pressure indicating the importance of this part in an increased vessel resistance (DeLano et al., 1991). Therefore, constriction of small arterioles and arteries can be one of primary causes of hypertension development and it is associated with rarefaction and structural changes in this part of cardiovascular system in late stages of hypertension development (Hutchins et al., 1996).

Rarefaction,the lower density of capillaries and arterioles in hypertension, is often found in hypertensive individuals as well as normotensive people witha genetic predisposition to hypertension. It is unclear if this phenomenon is a structural (disappearance of vessels) or functional decrease of the vessels (there are vessels, but they are not perfused) (Steeghs et al., 2008). The lower density of vessels is consideredas a mechanism contributing to the negative impact of hypertension, such as stroke or infarct of myocardium (Paglieri et al., 2004).

Melatonin is a hormone produced bythe pineal gland and its production exhibits circadian rhythms in circulation.Rhythmic melatonin production depends on the amount and the quality of light, since melatonin is produced only during the night (Reiter et al., 2007). Melatonin has a pleiotropic effect on many physiological systems. Melatonin can have some beneficial effect on endothelium and structure of vessels since it can increase NO production. On the other side, there arelimited data on the effects of melatonin on endothelial cells. (Paulis and Simko, 2007). Melatonin can act through two types of receptors that are coupled with G-proteins, MT1 and MT2 receptors (Benova and Zeman, 2007), but also with unspecific mechanism coupled with the strong antioxidant capacity of melatonin, which contribute to the proper function of endothelium (Pechanova et al., 2007).

Material and methods

In experiment 1, 20 Wistar rats and 20 spontaneously hypertensive rats (SHR, Dobra Voda, SR) were used. At the beginning of experiment rats were 5 weeks old. Rats were divided into 4 groups each containing 10 animals: SHR control, SHR constant light, Wistar control, Wistar constant light. Each group consisted of 5 male and 5 female rats.Control animals were kept under 12L:12D light/dark regimen and experimental animals were kept underconstant light (LL). Experiment lasted for 6 weeks.

In the experiment 2, SHR rats (Dobra Voda, SR) were used. Ten mature male rats were divided into 2 groups: control SHR (SHR) and melatonin SHR (SHRM). SHR rats were administered with melatonin in doses (2.5 mg/kg for 4 weeks and 10 mg/kg in last week). Experiment lasted for 6 weeks.

The density of capillaries was measured on the slices of skeletal muscle *m. soleus*. After the preparation, the muscle was inserted into a medium TissueTek (Histolab AB, Sweden) and stored at -80°C. Slice thickness was 10µm and the slice was kept perpendicular to the longitudinal axis of the muscle. In the next step, slices were incubated with a fluorescent labeled lectin (*Fluorescein-lycopersicon esculentum lectin*, Vector Laboratories, Burlingame, USA). The concentration of antibodies was optimized at 1:300. A cell nucleus was visualized

by 6-diamino-2-phenylindol (DAPI; Sigma-Aldrich, USA) in concentration 1:10000. Density of capillaries was observed with fluorescent microscope Zeiss Axio Scope.A1 (Zeiss, Germany) (magnification 200x) and photographs were taken with a camera AxioCam ICm1 (Zeiss, Germany). For observation was used color filter with wavelength 495nm for lectin and 365nm for DAPI. Five slices from each muscle was documented, wherein from every slice was made 10 images for DAPI and 10 images for lectin. For the evaluation of capillary density we used program ImageJ (National Institute of Mental Health, Bethesda, Maryland, USA). On each photography 3 randomly selected squares with size 100x100µm were located on the image, wherethe density of capillaries was measured.

Data was analyzed in statistic software STATISTICA 7 (Statsoft Inc., USA). The analysis of variance (ANOVA) was used for the comparison of the density of capillaries and differences between groups were evaluated by LSD post-hoc tests.

Results

Significant differences in capillary density (Figure 1) were recorded in skeletal muscle *m. soleus* between SHR (11.52 ± 0.42) and control Wistar rats (7.00 ± 0.26). These results contrast with published data reporting occurrence of rarefaction in hypertension (Steeghs et al., 2008). However, Hudllet et al. (2005) observed the higher density of capillaries in young prehypertensive rats compared to controls. In the same way, our results can be explained by young age of rats. Sex-differences were not recorded. The significant increase in the density of capillaries ($p < 0.05$) was found in normotensive rats after exposure to constant light (7.56 ± 0.23) as compared to control 12L:12D conditions (6.44 ± 0.39).

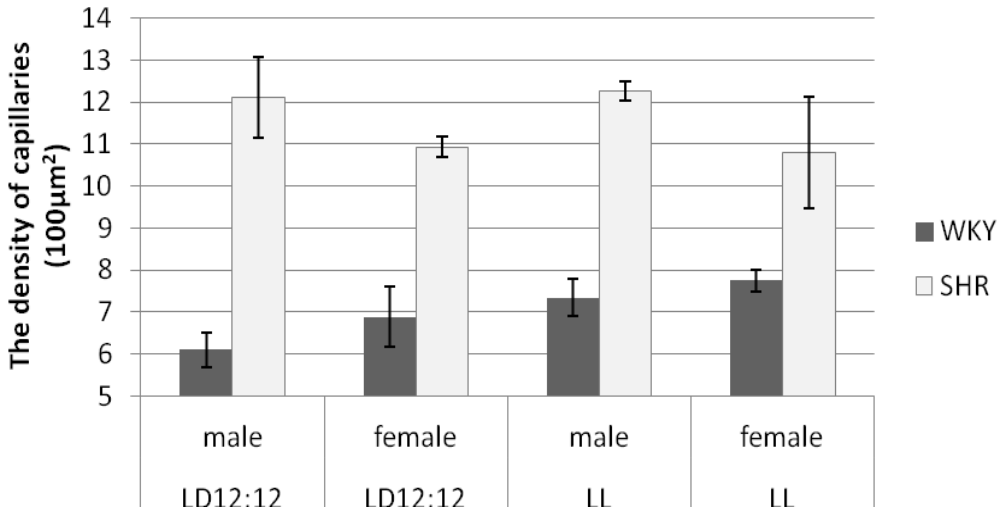

Figure 1. Differences in the density of capillaries in skeletal muscle *m. soleus* between sponataneously hypertensive rats (SHR) and normotensive rats (WKY) under standard light regimen (12D:12L) and constant light (LL).). Data are shown as mean ± SEM.

We did not find significant changes (p = 0.736) in capillary density in skeletal muscle *m.soleus* between control group (7.40 ± 0,50) and group after the melatonin administration (7,23 ± 0,17). However, it is not known how important are the changes in capillary density in skeletal muscle for blood pressure control, because an adequate blood flow is regulated mainly by the metabolism of this tissue. We expect taht for the system blood pressure control the microcirculation in kidneys is much more important that will be studied closer in future studies.

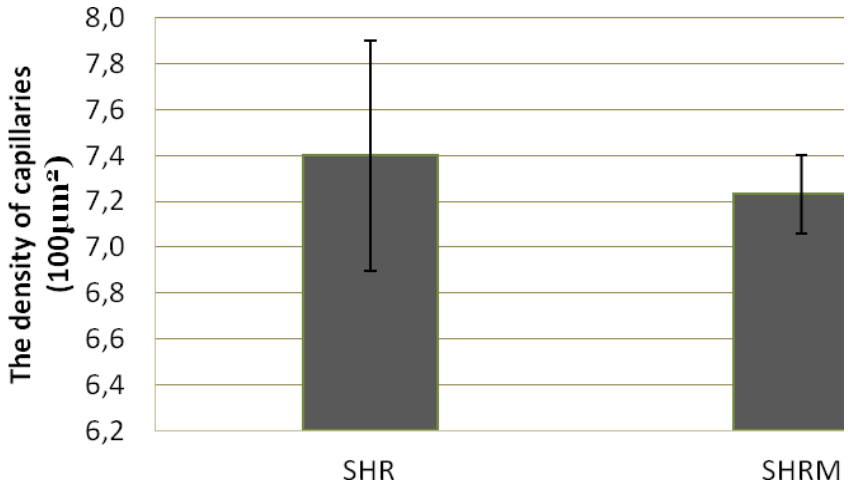

Figure 2. Differences in capillary density of skeletal muscle *m. soleus* between spontaneously hypertensive rats (SHR) and spontaneously hypertensive rats with melatonin administration (SHRM). Data are shown as mean ± SEM.

Conclusion

In these experiments we found changes in the capillary density between SHR and normotensive rats. We observed significant changes in the density of capillaries in skeletal muscle *m. soleus* after exposition to constant light, while after the direct administration of melatonin we did not found significant changes.

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VYTVOŘENÍ PARTNERSKÉ SÍTĚ VZDĚLÁVÁNÍ A VÝZKUMU V OBLASTI MASTITID

CZ.1.07/2.4.00/17.0026

Dotační titul: OP Vzdělávání pro konkurenceschopnost
Prioritní osa: 7.2 Terciární vzdělávání, výzkum a vývoj
Oblast podpory: 7.2.4 Partnerství a sítě
Doba řešení: 1.1.2012 – 31.12.2014
Přidělené finanční prostředky: 6 918 878 Kč
Koordinátor projektu: Ing. Petr Sláma, Ph.D.
Partneři projetu: Jihočeská univerzita v Českých Budějovicích
Společnost mladých agrárníků České republiky
Bentley Czech s.r.o.

Anotace projektu:

Hlavním cílem projektu je vytvoření partnerské sítě mezi institucemi terciárního vzdělávání, výzkumnými organizacemi a subjekty soukromého sektoru, které se zabývají vzděláváním a výzkumem v oblasti mastitid. Dalším cílem je prohloubit vzájemnou spolupráci mezi jednotlivými subjekty a přispět tak k lepšímu přenosu poznatků. Do projektu budou zapojeny také zahraniční univerzity a výzkumné instituce, které jsou předpokladem zkvalitnění vzdělávání cílové skupiny. Tyto cíle budou naplňovány prostřednictvím následujících **klíčových aktivit:**

1. Komunikační a interaktivní platformy
2. Vzdělávací a tréninkové moduly
3. Stáže a odborné praxe
4. Odborná stáž na Kasetsart University, Thajsko
5. Odborná stáž na National Institute of Animal Health, Japonsko
6. Odborná stáž na Slovenskej poľnohospodárskej univerzite v Nitre, Slovensko
7. Odborná stáž v Centru výskumu živočíšnej výroby Nitra, Slovensko

Tento projekt je spolufinancován z Evropského sociálního fondu a státního rozpočtu České republiky

Inovace studijních programů AF MENDELU směrem k internacionalizaci studia

CZ.1.07/2.2.00/28.0020

Doba řešení: 1.6.2012 – 31.5.2015

Koordinátor projektu: Jiří Skládanka

Žadatel projektu: Mendelova univerzita v Brně

Partner projektu: AgroKonzulta – poradenství, s.r.o.

Obsah projektu: Projekt přispívá k tvorbě studijních materiálů oboru Všeobecné zemědělství, který je na AF MENDELU akreditován v češtině a angličtině. Pro anglickou výuky nejsou k dispozici didaktické pomůcky nutné pro dosažení požadovaných znalostí a dovedností absolventů. Učební texty v angličtině jsou také nutné pro další akreditace. Projekt je dále zaměřen na inovaci oborů fytotechnických a zootechnických, jejichž předměty se prolínají v mezioborovém Všeobecném zemědělství. Vytvořené studijní texty budou reagovat na aktuální měnící se ekonomické podmínky a na environmentální problematiku, zejména změny klimatu. Pro vyšší uplatnění absolventů na trhu práce budou realizovány stáže u budoucích zaměstnavatelů. Projekt je určen pro učitele VŠ a studenty AF MENDELU. Cílů projektu bude dosaženo tvorbou e-learningových prezentací, skript, posterů, DVD nahrávek, atlasů a dalším jazykovým vzděláváním učitelů. Plánované aktivity bude realizovat tým VŠ učitelů z AF MENDELU a odborníků z firmy AgroKonzulta – poradenství, s.r.o.

Klíčové aktivity:
KA1 Inovace fytotechnických programů směrem k modernizaci didaktických metod
KA2 Inovace zootechnických programů směrem k modernizaci didaktických metod
KA3 Inovace studijních programů s možností mezioborových studií směrem k výuce v AJ
KA4 Zvyšování jazykových kompetencí akademických pracovníků
KA5 Zapojení odborníků ze zahraničí do přípravy studijních programů v anglickém jazyce
KA6 Podpora stáží studentů u budoucích zaměstnavatelů
KA7 Inovace předmětů v cizích jazycích napříč obory AF

Rozpočet projektu: celkové náklady 20 092 755,20 Kč

Tento projekt je spolufinancován z Evropského sociálního fondu a státního rozpočtu České

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