EFFECT OF AMYGDALIN ON ANEUPLOIDY INCIDENCE IN RABBIT

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ABSTRACT

The aim of this study was to examine possible effect of amygdalin substance applied to rabbit by different ways on the occurrence of aneuploidy. Totally 30 adult females of the New Zealand White line were used in the experiment. Animals were divided into four experimental groups and one control group, each consisted of five individuals. In groups P1 and P2, isolated amygdalin substance was administered intramuscularly (P1: 0.6 mg.kg⁻¹; P2: 3 mg.kg⁻¹ of body weight), whilst in P3 and P4 the animals were fed by a mixture of commercial diet and apricot seeds (P3: 60 mg.kg⁻¹; P4: 300 mg.kg⁻¹ of body weight). Experimental treatment lasted one month of continuous amygdalin application (one experimental dose applied per day in each group). Aneuploidy assay was performed on peripheral blood lymphocytes arrested at the metaphase stage by colcemid solution (0.6μg.ml⁻¹). The average occurrence of diploidy versus aneuploidy (presented by hypodiploid nuclei) was detected as follows: P1 - 76 % vs 21.6 %; P2 - 72.8 % vs 27.2 %; P3 - 82.4 % vs 17.6 %; P4 - 70.4 % vs 29.6 %; Control - 70.4 % vs 26.8 %. Low incidence of polyploid cells was found in P1 (2.4 %) and control (2.8 %) groups. In conclusion, no significant effect of amygdalin on aneuploidy occurrence in rabbit blood cells was found.

Key words: amygdalin; aneuploidy; lymphocyte; chromosome; rabbit

INTRODUCTION

Amygdalin, the substance hidden under various nicknames, including: vitamin B17, nitriloside, mandelonitrile, etc. (Fukuda et al., 2003) is widely distributed in plants, especially in the Rosaceous plant seed, such as: apricot and peach (Santos Pimenta et al., 2014). It can hydrolyze and generate prunasin and mandelonitrile under the glucosidase action, such as amygdalase and prunase, and ultimately decomposed into benzaldehyde and hydrocyanic acid. Amygdalin itself is non-toxic, but its production of hydrocyanic acid decomposed by some enzymes is poisonous substance (Suchard et al., 1998). There are number of studies describing the effect of amygdalin and derived substances on the recipient body. For example: anti-tussive and anti-asthmatic effects by the amygdalin decomposition to hydrocyanic acid which could inhibit the respiratory center to a certain level (Chang et al., 2005; Do et al., 2006); effects on the digestive system by inhibition of the pepsin activity (Song and Xu, 2014); analgesic effect by inhibiting prostaglandins E2 and nitric oxide synthesis (Yang et al., 2007; Paoletti et al., 2013); promoting apoptosis of human renal fibroblast by enhancing the activity of type I collagenase (Guo et al., 2013); improving the immune function of organism by the significant increase of polyhydroxyalkanoates inducing human peripheral blood T lymphocyte proliferation (Baroni et al., 2005); the anti-tumor effect of amygdalin presented by the hydrocyanic acid, which is an anti-tumor compound formed from the amygdalin decomposition (Kwon et al., 2003). In other studies, amygdalin significantly inhibited sperm hyaluronidase activity and spermatozoon motility of bull sperm in vitro (Tanyildizy and Bozkurt, 2004). Recent data indicated that amygdalin reduced proliferation potential, decreased mitochondrial activity of cervical cancer cells, accumulated cells in the G1-phase and led to their death.

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Received: June 22, 2016
Accepted: August 17, 2016

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Various ways of amygdalin application play a different role in recipient organism, what was confirmed by Moertel et al. (1981), who demonstrated in human, that intravenous infusion of amygdalin produced neither cyanidemia nor signs of toxicity, but oral administration resulted in significant blood cyanide levels.

Cytogenetic analysis plays an important role in livestock breeding. The chromosomal abnormalities, caused by various factors, are generally divided into structural and/or numerical changes. Commonly observed aneuploidies are hypo (loss) and hyper (gain) haploidy, as a result of meiotic and mitotic non-disjunction events (Goepfert et al., 2000). Normal organisms are characterised by the presence of aneuploid cells at very low frequency. In rabbits, the rate of aneuploidy varies depending on the tissue of origin, from 5 % in in vitro fertilized oocytes (Asakawa et al., 1988), 16-18 % in blood cells (Zartman and Fechheimer, 1967; Parkanyi, 1981), 35-51 % in bone marrow (Parkanyi, 1981; Curlej et al., 2007), up to 56-83 % in embryos (Austin, 1967; Shi et al., 2004; Curlej et al., 2010). Diseases, assisted reproductive technologies, or genetic manipulations might cause an increase in the incidence of aneuploidy (Hegedus et al., 2008).

The present study was focused to reveal the effect of various amygdalin concentrations, administrated by different ways, to evoke the aneuploidy on the rabbit model. To achieve the paper goals chromosomes were recovered from lymphocyte’s nuclei isolated from the peripheral blood.

**MATERIAL AND METHODS**

**Animals**

A total of 30 adult rabbit females (20 individuals assigned to the experimental groups P1-P4 and 10 rabbits selected to control group) of the New Zealand White line were used in the experiment. The animals were bred at the farm of the Research Institute for Animal Production, NAFC Nitra. Prior to start of the experiment, animals were selected according to similar age and good health status which was continuously monitored from the start up to the end of the experimental treatment. Environmental conditions were maintained using a control system for the light (16:8 light/dark photoperiod), air ventilation and the temperature (18-25 °C). Animals were fed ad libitum with treated/ non-treated commercial rabbit diet. The experiment was lasted one month of continuous amygdalin application (one experimental dose applied per day in each group) as described in the text below.

**Experimental groups**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>Intramuscular application of isolated amygdalin substance at the final concentration of 0.6 mg.kg⁻¹ of body weight.</td>
</tr>
<tr>
<td>P2</td>
<td>Intramuscular application of isolated amygdalin substance at the final concentration of 3 mg.kg⁻¹ of body weight.</td>
</tr>
<tr>
<td>P3</td>
<td>Feeding by a mixture of commercial diet and apricot seeds at the final concentration of 60 mg.kg⁻¹ of body weight.</td>
</tr>
<tr>
<td>P4</td>
<td>Feeding by a mixture of commercial diet and apricot seeds at the final concentration of 300 mg.kg⁻¹ of body weight.</td>
</tr>
</tbody>
</table>

**Chromosome preparation**

Venous blood samples were collected aseptically from the intermediate auricular vein (vena auricularis caudalis) of the experimental (on the last day of experimental treatment) and control animals using sterile needles and heparinized plastic syringes. Three drops of blood were added to 4 ml of the complete blood cultivation medium PB Max Karyotyping (Gibco BRL). The blood cultures were incubated at 37 °C for 72 h. Colcemide at the final concentration of 0.6 μg.ml⁻¹ (Gibco BRL) was added 60 min prior to cell harvest. After the hypotonic treatment with 0.075 M potassium chloride solution (Gibco BRL) for 15 min and fixation in modified Carnoy’s solution (3 : 1, methanol : acetic acid), the resuspended cells were placed onto frozen glass microslides, air-dried and stained for 6 min with 2 % Giemsa solution (Gibco BRL). Stained microslides were observed under the Leica light microscope. The chromosomal analysis was carried out using 25 c-metaphase spreads per rabbit taken from chromosome microphotographs (Parkanyi et al., 2004).

The χ² test was used for statistical evaluation of the results.

**RESULTS AND DISCUSSION**

The study was focused on examining a possible effect of amygdalin substance applied to rabbit at different concentrations and by different ways on the occurrence of aneuploidy. Proportion of the cells with diploid chromosome sets in the cell nuclei was ranged from 70.4 % in the P4 group to 82.4 % in the P1 group of rabbits (Table 1). The same occurrence of diploid cells (70.4 %) was found in the control group. Aneuploidy was represented exclusively by hypodiploid cells for experimental groups as well as for control group. The lowest percentage of aneuploidy was found in P3 rabbits (17.6 %), whilst the P4 group exhibited the highest
presence of aneuploid nuclei (29.6 %) in comparison to control animals (26.8 %). According to the results of χ²-test, no significant differences were found. Polyploid cells were found at low percentage in P1 (2.4 %) and control (2.8 %) groups.

Previous studies, performed specifically to reveal the rate of chromosome number variability, show that almost all tissues exhibit aneuploid cells (Iourov et al., 2008). The incidence of chromosomal aneuploidies may depend on many factors, such as the source of cells, animal species, animal age, cell culture conditions and genetic manipulations (Czepulkowski, 2001). Aneuploidy in the present study was represented by hypodiploid cells (2n < 44). Average value of hypodiploid cells regardless to experimental groups was represented by 24 % (21.6, 27.2, 17.6 and 29.6, respectively). Control group exhibited 26.8 %. These relatively small differences among the groups may be explained by a random selection of evaluated nuclei. Higher proportion of hypodiploid cells (at average 41.47 % and 54.8 %, resp.) for non-treated rabbits have been recorded in the study of bone marrow cells, published by Curlej et al. (2007). Parkanyi et al. (2004) detected 38 % and 26 % presence of hypodiploid cells (average value 32 %) in the blood lymphocytes of control rabbits. Occurrence of cells with normal chromosome sets (diploid cells) in the present study is represented by average value of 75.4 % for experimental groups (measured values 76 %, 72.8 %, 82.4 %, and 70.4 %, resp.) in comparision to 70.4 % for control group.

Numbers of studies have been focused to reveal potential action of amygdalin substance to cell culture, especially those from cancer tissues. But still there is a lack of scientific records derived from the experiments with the model organisms about effect of amygdalin-derived substances on the structure or number of chromosomes in the cell nuclei. Studies by other authors suggest that the amygdalin in “safety” concentrations and admission way affect the viability of human cervical cancer HeLa cells (Chen et al., 2013). Such information brings a therapeutic option to use of this substance. Nevertheless, the anti-tumor mechanism of amygdalin is not completely clear (Song and Xu, 2014). Clinical trials and large retrospective studies showed some adverse reactions after large dose application, such as gastrointestinal tract reaction and headache (Barwina et al., 2013; Yang et al., 2013; Karabulutlu, 2014). The ways of amygdalin application to rabbits in our study were chosen basing on the knowledge, that the toxicity of oral administration route is far greater than the intravenous route. The mean lethal dose (LD50) of amygdalin in rats was reported to be 880 mg.kg⁻¹ body weight by oral administration (Adewusi and Oke, 1985; Park et al., 2013). For the rabbits, mice and dogs the maximum tolerance dose for oral amygdalin intake has been published as 75 mg.kg⁻¹ (Zhang et al., 1986).

Table 1: Evaluation of chromosomal number from c-metaphase spreads

<table>
<thead>
<tr>
<th>Rabbit groups</th>
<th>Diploidy</th>
<th>Hypodiploidy</th>
<th>Polyploidy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1</td>
<td>76.0 % (n = 95)</td>
<td>21.6 % (n = 27)</td>
<td>2.4 % (n = 3)</td>
</tr>
<tr>
<td>P2</td>
<td>72.8 % (n = 91)</td>
<td>27.2 % (n = 34)</td>
<td>-</td>
</tr>
<tr>
<td>P3</td>
<td>82.4 % (n = 103)</td>
<td>17.6 % (n = 22)</td>
<td>-</td>
</tr>
<tr>
<td>P4</td>
<td>70.4 % (n = 88)</td>
<td>29.6 % (n = 37)</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td>70.4 % (n = 176)</td>
<td>26.8 % (n = 67)</td>
<td>2.8 % (n = 7)</td>
</tr>
</tbody>
</table>

* n – the number of evaluated metaphase plates


In conclusion, according to our results, amygdalin applied at chosen concentrations orally or intramuscularly, showed no significant adverse effect represented by extra-creation of aneuploid cells in rabbits.
ACKNOWLEDGEMENT

This work was supported by the Ministry of Education, Science, Research and Sports of the Slovak Republic no. 1/0022/13 and Slovak Research and Development Agency of the Slovak Republic no. APVV-0304-12.

REFERENCES


GENETIC CHARACTERIZATION OF NITRA RABBITS AND ZOBOR RABBITS

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ABSTRACT

In an attempt to characterize the two rabbit breeds concerning the shift on the genetic level we studied microsatellite and genetic markers associated with coat color. These two local rabbit breeds, Nitra rabbit (Ni) and Zobor rabbit (Zo) with different phenotypes have a common origin. Based on the highest polymorphic six microsatellites we chose more than 300 rabbits microsatellites. In two analysed experimental groups Ni (n = 8) and Zo (n = 8) the number of detected alleles for six chosen microsatellites was ranged from two to four alleles (4 alleles per INRACCDDV0106, 3 alleles per INRACCDDV0183, 4 alleles per INRACCDDV0259, 4 alleles per Sol08, 2 alleles per SOL28 and 3 alleles per Sol33). Genetic markers were chosen based on the unique characteristics of the two analyzed breeds: mutations c.585delG in the MLPH gene (dilution of coat color), c.5_6insA in the ASIP gene (characters of wild type rabbit) and c.1118C → A in the tyrosinase gene (albinism). The mutation c.585delG in Ni rabbits is represented only by homozygous genotype del/del. Ni is a rabbit breed with a blue-grey coloring, therefore, in principle, other genotype for c.585delG is not expected in this breed. For Zo breed, only G/G homozygous genotype should be represented, but even in that small group of Zo rabbits (n = 8) one heterozygote del/G has occurred, with the recessive allele hidden under the black phenotype. Concerning ASIP gene, from 8 Ni rabbits 7 animals were homozygous ins/ins, which were blue-coated, and only one was Wt/ins, which was Ni wild-blue, whilst Wt/Wt homozygote was not represented. In Ni and Zo breeds the mutation c.1118C → A in the tyrosinase gene was represented only by CC homozygotes, whereas albinos Hyla hybrid  was represented only by AA homozygotes. Here we propose steps to make more efficient the process of cross-breeding of the analyzed Ni and Zo rabbit breeds in order to stabilize and then standardize these two unique rabbit breeds, which could could be preserved in the gene bank for possible future use.

Key words: coat color; cross-breeding; genetic marker; microsatellite; rabbit

INTRODUCTION

Rabbit breeds with different names may sometimes have recent common origin, while in other cases their uniqueness has been eroded by cross-breeding. Breeds of rabbits as well as all animals are characterized by the standard of breed, by a set of precisely defined characteristics or parameters established on the basis of phenotypic assessment. The aim of this study is to shift this characterization on the genetic level through a microsatellite and genetic markers associated with coat coloring in two local rabbit breeds with common origin but so uniquely different phenotype, Nitra rabbit (Ni) and Zobor rabbit (Zo).

Microsatellite markers are useful tool recommended by ISAG / FAO, used for the analysis of genetic diversity in many programs of the livestock preservation (FAO, 2011). Research program ResGen-CT95-60 in 1995-2000 was similarly focused on rabbits in which the European Union was working to inventory, evaluate, conserve and utilize the genetic resources of European rabbits (Bolet et al., 2000), but Slovakia did not participate in this program. For this study, polymorphic rabbit microsatellites

Received: February 19, 2016
Accepted: August 17, 2016

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Sol08, SOL28, Sol33 were chosen from articles written by Rico et al. (1994) and Surridge et al. (1997) and microsatellites INRACCDDV0106, INRACCDDV0183, INRACCDDV0259 were published by Chantry-Darmon et al. (2006).

Genetic markers were chosen based on the unique characteristics of the two analyzed breeds. As a genetic marker for similar study of differentation analysis, in particular in the case of the definition of genetic resources, traits such as reproduction must not be used such as reproduction characteristics, for which is the effort to achieve in all aspects of their a cost-effectived and balanced level, no go to extremes (Bolet et al., 2000).

In this case it was not possible to evaluate carcasses characteristics, because two similar meat breeds of rabbit were examined. Suitable markers associated with coat color had to be found. Today several genetic mutations associated with coloring are known, from which those were chosen for analysis, which define some of the unique characteristics of these two specific analyzed rabbit breeds. The purpose for breeding of these two breeds, Ni and Zo, was to create a white meat rabbit. First, Ni was bred from the cross-breeding of the French silver rabbit with Russian rabbit breed and subsequently with Californian rabbit. Zo breed is the result of cross-breeding of Ni with the New Zealand White rabbit. The uniqueness of Ni and Zo is in specific distribution of pigment areas (color pattern) and their different color. Ni breed is unique by Himalayan color pattern in blue color (nose, ears, feet and tail are blue with clear division between these areas and the white body color), originally in conjunction with characters of wild type rabbit. Ni has clear bright blood red eyes. The Himalayan drawing is typical for Russian rabbit and Californian rabbit, but in black color, while Ni has this color pattern in blue, as previously mentioned. Zo breed has a unique Zobor color pattern (head white, tail black and hindlegs cuffs black with clear division from the white body color). Zo has clear bright blood red eyes, too. Typical Zobor color pattern is a result of combination of Dutch color pattern and partial albinism.

The purpose of this study was the characterization of the unique features of Ni and Zo at the genetic level, which corresponds with genetic markers associated with coat color: mutations c.585delG in the MLPH gene (melanophiline gene) (dilution of coat color, Lehner et al., 2013), c.5 6insA in ASIP gene (characters of wild type rabbit, Fontanesi et al., 2010) and c.1118C → A in the tyrosinase gene (albinism, Aigner et al., 2000). Epistatic deletion c.585delG in the MLPH gene inhibits the full expression of pigment formation with manifestation of weaker coloring, called dilution of coat color. Dominantly inherited mutation c.5_6insA in the ASIP gene Wt is associated with the characters of wild type rabbit (white inside ears, white underside of the tail, as well as the whole ventral part of the body). Recessive inherited, but also epistatic to all mutation associated with color changes, mutation c.1118C → A in the tyrosinase gene, which in a homozygous recessive line up repressed all pigment production, including the eyes, so-called complete albinism.

We propose steps to make more efficient the process of cross-breeding of analyzed breeds Ni and Zo rabbit to be able to stabilize and then breed-standardize the genetic material of these two unique rabbit breeds, which could be preserved in the gene bank for possible future use.

Ni and Zo are the result of combination of genes of four high quality foreign breeds (Silver French, Russian, California and New Zealand White). In the breed Ni and Zo not only the combination of high quality genetic material is encoded, but at the same time adaptability of this gene combination to our environmental conditions, its efficient use of local natural food sources of forage and its resistance to the pathogenic pressure in our animal production. But choosing the breeds to be evaluated, we did not take into account the risk of extinction of the breed, but their historical status and their potential zootechnical interest (Bolet et al., 2000).

MATERIAL AND METHODS

Animals

The total set of analyzed animals was represented by total of 77 animals from 31 rabbit breeds (Table 4). In this study, three analyzed systems of three rabbit breeds Ni (n = 8), Zo (n = 8) and albinos of hybrid Hyla (n = 3) are considered the key results. From small populations of Ni and Zo rabbits the selection has been made so that the animals conform to the characteristics of the breed standard.

Isolation of DNA

Genomic DNA was extracted from blood using DNA Purification kit Maxwell16® (Promega).

Multiplex PCR

The sequence of six polymorphic microsatellite loci was chosen from published articles (Sol08, SOL28 and Sol33 by Rico et al., 1994 and Surridge et al., 1997 and INRACCDDV0106, INRACCDDV0183 and INRACCDDV0259 by Chantry-Darmon et al., (2006), Table 1). Sequences of microsatellites were obtained from GenBank (NCBI). Primers for multiplex PCR were chosen using software QDD2 (Meglécz et al., 2010).
Multiplex PCR conditions and fragment analysis

Multiplex PCR reactions were performed at a total volume of 16 µl using QIAGEN Multiplex PCR Kit (Qiagen), 10-50 ng genomic DNA as template and 0.2 μmol.l⁻¹ of each primer. The PCR profile included an initial denaturation step at 95 °C for 15 min, 28 cycles: 94 °C (60 s), 60 °C (90 s), 72 °C (60 s) and a final extension step of 30 min at 60 °C in C1000 thermal cycler (Bio-Rad). The PCR products were analysed on ABI PRISM 310 and the fragment analysis was performed using the Gene Mapper software 4.0.

PCR primers sequence for ASIP was taken from Fontanesi et al. (2010) and for MPLH from Lehner et al. (2013) (Table 2).

Table 1: Primer sequences for analysed microsatellite

<table>
<thead>
<tr>
<th>Microsatellite marker</th>
<th>GenBank. Acc. No.</th>
<th>Fluorescence labeling and sequence of forward and reverse primers in 5’-3’</th>
<th>No. of publ. alleles</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>INRACCDDV0106</td>
<td>AJ874448</td>
<td>6-FAM: TGGGGAGTGAACCAGCAGAT AATCCGACCCACCATTTTGAT</td>
<td>6</td>
<td>Chantry-Darmon et al., 2006</td>
</tr>
<tr>
<td>INRACCDDV0183</td>
<td>AJ874521</td>
<td>6-FAM: TCACCTTCTCAGCTGGCAT CGCCTCACTAAGCTGCGTG</td>
<td>7</td>
<td>Chantry-Darmon et al., 2006</td>
</tr>
<tr>
<td>INRACCDDV0259</td>
<td>AJ874589</td>
<td>VIC: CTTTGCAAGGCAATGGAAGCT ACTGAGACAGAGGAGAG</td>
<td>7</td>
<td>Chantry-Darmon et al., 2006</td>
</tr>
<tr>
<td>Sol08</td>
<td>X79217</td>
<td>NED: ATGGGCGCTTTTGGCTACCACTGC</td>
<td>1-10</td>
<td>Rico et al., 1994; Surridge et al., 1997</td>
</tr>
<tr>
<td>Sol28</td>
<td>X79216</td>
<td>PET: AACCAGGACGGAAGTGGATT ATTGAATGGGAGAGA</td>
<td>1-7</td>
<td>Rico et al., 1994; Surridge et al., 1997</td>
</tr>
<tr>
<td>Sol33</td>
<td>X94683</td>
<td>6-FAM: CCCATGTTGTGACTCAGATTTC GATGTCAATCTGGCCTCCC</td>
<td>1-15</td>
<td>Rico et al., 1994; Surridge et al., 1997</td>
</tr>
</tbody>
</table>

Table 2: Primer sequences for analysed chosen genetic markers for rabbit coat color

<table>
<thead>
<tr>
<th>Genetic marker</th>
<th>GenBank Acc. No.</th>
<th>Sequence of primers forward and reverse in 5’-3’</th>
<th>PCR product</th>
<th>Temp. of annealing</th>
<th>Restriction endonuclease</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASIP c.5_6insA</td>
<td>FN547134</td>
<td>CAGGAAGGCACATCCTACTT</td>
<td>95 bp</td>
<td>57 °C</td>
<td>EcoR I</td>
</tr>
<tr>
<td>MPLH c.585delG</td>
<td>KC791692</td>
<td>GTGAGCGAGGAGACGAGGAG</td>
<td>111 bp</td>
<td>60 °C</td>
<td>HpyF31 (Dde I)</td>
</tr>
<tr>
<td>TYR c.1118C→A</td>
<td>AF210660</td>
<td>EXT1 TGCTAATTTACTTGTGGATAG</td>
<td>131 bp</td>
<td>60 °C</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EXT 2 CATGTTGAGGGAGAAAATGGGATC</td>
<td>79</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

STAS-PCR

STAS-PCR for tyrosinase c.1118C-A (T373K) polymorphism. Briefly, two allele-specific internal primers (INT1, INT2) recognized with their 3’ nucleotide the allelic variation (Table 2). In order to increase the allele specific annealing an additional mismatch position 3 from the 3’ end of each primer was introduced (lower case). The external primers are located at different distances from point mutation, so that different fragment lengths were generated for each allele. In case of nt1118C and nt118A homozygotes only one fragment of 79 bp or 99 bp respectively was amplified, whereas in heterozygotes C/A both fragments were detected. Independently of the genotype an additional product (131 bp) of the common external primers (EXT1, EXT2) were amplified.
The PCR conditions

The PCR reactions were performed at a total volume of 25 μl using 1X Colorless GoTaq Flexi buffer, 2.0 mmol.l⁻¹ MgCl₂, 0.2 mmol.l⁻¹ of each dNTP, 1 U GoTaq G2 Hot Start DNA polymerase (Promega, USA), 50-100 ng genomic DNA as template and 0.4 mmol.l⁻¹ of each primer. The PCR profile included an initial denaturation step at 94 °C for 2 min, 35 cycles of 94 °C (20 s), 57 °C/60 °C (30 s), 72 °C (30 s) and a final extension step of 10 min at 72 °C in C1000 thermal cycler (Bio-rad).

RFLP

The appropriate restriction endonuclease (ThermoScientific, Germany): EcoRI for ASIP and HpyF31 (Dde I) for MLPH polymorphism was used to digest PCR products (Table 2).

Microchip electrophoresis of DNA fragments

PCR products were analysed by the automated microchip electrophoresis system MCE®-202 MultiNA (Shimadzu, Japan) with a DNA-500 kit according to the manufacturer’s protocol. A SYBR Gold fluorescent dye for DNA staining (Invitrogen, USA) and a 25 bp DNA Ladder (Invitrogen, USA) were used to determine the size of the restriction fragments.

RESULTS AND DISCUSSION

The overall population size of Ni and Zo rabbit breeds is very low. Stability of these two breeds is constantly eroded by cross-breeding efforts. Frequent is the occurrence of weak coloring intensity and the lack of compact coloring markings with the appearance of pigment spots on various parts of the body. The selection of the necessary amount of individuals to conform to the breed standard characteristics among them was difficult. However, it 8 specimen of Ni rabbits and 8 specimen of Zo rabbits were selected for the experiment. In order to find genetic variability and to define two breeds on the genetic level, from more than 300 rabbit microsatellites we chose the six highest microsatellite alleles ranges from 2 to 15 (Surridge et al., 1997). For chosen microsatellites 6 alleles per Sol33 (Surridge et al., 2006), 7 alleles per INRACCDDV0183 (Chantry-Darmon et al., 2006), 7 alleles per INRACCDDV0259 (Chantry-Darmon et al., 2006), 10 alleles per Sol08 (Surridge et al., 1997), 7 alleles per Sol28 (Rico et al., 1994) and 15 alleles per Sol33 (Surridge et al., 1997) are described.

In our two analysed rabbit experimental groups Ni (n = 8) and Zo (n = 8) the number of detected alleles for six chosen microsatellites ranges from 2 to 4 alleles (4 alleles per INRACCDDV0106, 3 alleles per INRACCDDV0183, 4 alleles per INRACCDDV0259, 4 alleles per Sol08, 2 alleles per SOL28 and 3 alleles per Sol33) (Table 3).

Here we avoided traditional statistical processing, determinations of heterozygocity and genetic distances which would lead to erroneous conclusions rather than to achieve reasonable results, both precisely because of the small numbers of analyzed groups of rabbits, and also because two populations that are genetically distant need not phenotypically differ (Edding and Laval, 1999), and vice versa.

Microsatellite is a mean particularly to distinguish individuals under paternity testing. For many species (cattle, buffalo, sheep, goats, horses, donkeys, camels, pigs and chickens) the sets of microsatellites to identify on the genetic level (FAO 2011) are recommended. For rabbits such microsatellite sets are not recommended yet, although the number of the rabbit microsatellites is described. Since in rabbits very intensive cross-breeding has been done, the origins of the rabbit breeds significantly overlap and this complicates the ability to find appropriate specific microsatellites. Nevertheless, we tried to look for characteristics of these two breeds through microsatellites too. No reallocation alleles in the study of rabbit breeds Ni and Zo were observed as is shown in Table 3. The results of genotyped rabbits for the three genetic mutations associated with change of the coat color c.585delG in MLPH gene, c.5_6insA in ASIP gene and c.1118C→A in tyrosinase gene are listed in the Table 5.

Table 4 shows the results of analysis of mutation c.585delG MLPH in the MLPH gene for 31 rabbit breeds with different coat coloring for a total of 77 animals. This mutation was described by Lehner et al. (2013) and Fontanesi et al. (2014). Dilution of coat color effect of this mutation was confirmed in breeds Ni, Czech Spot blue, Havana blue, Moravian blue, Gray-blue Rex, Dwarf grey-blue Rex, French Lop blue and Vienna blue (Table 5).

We also thought that coloring in New Zealand red is caused by interaction of the mutation c.585delG in the MLPH gene, too. It is possible however, that in this case the dilution or mixing of pigments is caused by another interactions. One known mutation is now associated with the dilution coat color c.111-5C→A gene MLPH (Lehner et al., 2013) but it was not the subject of the study.

Mutation c.585delG in Ni rabbits is represented only by homozygous genotype del/del. Ni is rabbit breed with blue-grey coloring, therefore, in principle, another genotype for c.585delG is not possible in this breed. On the contrary, it should be for Zo breed, which should be represented only by homozygous genotype G/G for c.585delG, therefore, any likelihood of excision
Table 3: Number of detected alleles for chosen microsatellites in Ni and Zo rabbits

<table>
<thead>
<tr>
<th>Microsatellite name</th>
<th>Rabbit of Nitra (n = 8)</th>
<th>Zobor rabbit (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Allele number</td>
<td>Allele size (bp)</td>
</tr>
<tr>
<td>INRACCDDV0106</td>
<td>4</td>
<td>184_188_190_193</td>
</tr>
<tr>
<td>INRACCDDV 0183</td>
<td>3</td>
<td>152_159_161</td>
</tr>
<tr>
<td>INRACCDDV 0259</td>
<td>4</td>
<td>105_116_118_120</td>
</tr>
<tr>
<td>Sol08</td>
<td>4</td>
<td>99_101_104_106</td>
</tr>
<tr>
<td>Sol28</td>
<td>2</td>
<td>100_102</td>
</tr>
<tr>
<td>Sol33</td>
<td>3</td>
<td>76_79_84</td>
</tr>
</tbody>
</table>

Table 4: Results of rabbit genotyping for the c.585 in the MLPH gene

<table>
<thead>
<tr>
<th>c.585delG in melanophilene gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>del/del</td>
</tr>
<tr>
<td>Rabbit of Nitra (8) del/del</td>
</tr>
<tr>
<td>Zobor rabbit (8) del/G, G/G</td>
</tr>
<tr>
<td>Californian (8) G/G</td>
</tr>
<tr>
<td>Dwarf Russian (Dwarf Himalayan) (1) del/G</td>
</tr>
<tr>
<td>Czech Spot (5) G/G</td>
</tr>
<tr>
<td>Czech Spot blue (1) del/del</td>
</tr>
<tr>
<td>Czech Spot red (1) G/G</td>
</tr>
<tr>
<td>German Giant Spot (1) del/G</td>
</tr>
<tr>
<td>Spot of Mecklenburg (1) G/G</td>
</tr>
<tr>
<td>Belgian Giant black (2) G/G</td>
</tr>
<tr>
<td>Dutch black (4) G/G</td>
</tr>
<tr>
<td>Havana blue (1) del/del</td>
</tr>
<tr>
<td>Moravian blue (2) del/del</td>
</tr>
<tr>
<td>Grey-blue Rex (2) del/del</td>
</tr>
<tr>
<td>Dwarf grey-blue Rex (1) del/del</td>
</tr>
<tr>
<td>French Lop blue (1) del/del</td>
</tr>
<tr>
<td>French Lop wild type (1) G/G</td>
</tr>
<tr>
<td>Blue of Vienna (2) del/del</td>
</tr>
<tr>
<td>Vienna rabbit (wild coloring) (4) G/G</td>
</tr>
<tr>
<td>European rabbit (3) G/G</td>
</tr>
<tr>
<td>Dwarf european rabbit (1) del/G</td>
</tr>
<tr>
<td>Big Light Silver (3) G/G</td>
</tr>
<tr>
<td>Big Chinchilla (3) G/G</td>
</tr>
<tr>
<td>Rabbit of Rhön (2) G/G</td>
</tr>
<tr>
<td>Dwarf Black (2) G/G</td>
</tr>
<tr>
<td>Dwarf Casto Rex (2) del/G</td>
</tr>
<tr>
<td>Thuringer (1) del/G</td>
</tr>
<tr>
<td>Dwarf Lynx (1) del/del</td>
</tr>
<tr>
<td>New Zealand Red (1) G/G</td>
</tr>
<tr>
<td>Fox-Rabbit white (1) del/G</td>
</tr>
<tr>
<td>Albinos-hybrid HYLA (3) G/G</td>
</tr>
</tbody>
</table>
offspring with dilution coat color would be eliminated. But even in that small group of Zo rabbits (n = 8), one heterozygote del/G is occurred with the recessive allele hidden under the black phenotype. Here is an example, that even if the individual is recognized as a breeding animal, it does not mean that it is “cleans” (homozygous) for a given reference characters, as many farmers/breeders believe. In case of two heterozygotes del/G for c.585delG in mating plans, their use brings 25 % likelihood of progeny with inadmissible coat color due to dilution. In this way, recognized heterozygotes for c.585delG need not be negatively selected as they will be properly combined with a truly „pure“ homozygote G/G, and all the offspring will have a 100 % full black pigment.

Concerning the ASIP gene, within our small experimental group of 8 pieces Ni 7 rabbits were homozygous ins/ins, these were Ni blue, only one was Wt/ins, and this was Ni wild-blue, whilst homozygote Wt/Wt was not represented. In this case it is necessary to point out one fact when assessing. Ni rabbit breed is bred in two color surges, blue and wild-blue. Blue Ni was bred from wild-blue, Ni blue has intense darker blue-grey color and is without characteristics of wild type rabbit. Currently there is a gradual reduction of wild-blue Ni rabbits, which is the original Ni rabbit with a unique combination of the Himalayan pattern in blue color with wild characters. Modern cross-breeding steps should be reviewed in order to prevent the elimination of unique color lines like that of the wild-blue Ni breed. In relation to the ASIP gene, another major problem was found in Zo breed too. In our small experimental group (n = 8) three Zo rabbits had clear signs of wildness (homozygotes ins/Wt). Characters of wild type rabbit are inadmissible in Zo rabbit breed, because white hairs move to the pigment black areas. When the allele Wt is present, black characters with white hairs create the effect of less intense coloring. C.5_6insA mutation in the ASIP gene is dominantly inherited and effect of allele Wt is clearly identifiable. According to this it is necessary to put more emphasis on checking wildness in the evaluation of breeding animals of Zo rabbit breed. A clear indicator of the presence of the Wt allele and therefore wildness is the white underside of tail.

Mutation c.1118C→A in the tyrosinase gene, a key enzyme for melanin production, is associated with the expression of complete albinism. In the breeds Ni and Zo mutation c.1118C→A in the tyrosinase gene is represented only by homozygotes CC, whereas albinos Hyla hybrid is represented only by AA homozygote. The results, therefore, confirm the complete suppression of pigment in albino AA assembly, and only homozygous genotypes CC for c.1118C→A in the tyrosinase gene in breeds Ni and Zo. In conjunction with suppression the production of pigment is not specifically defined, concepts/phenomena such as acromelanizmus, partial albinism and leucizmus are misunderstood and often confused, especially in these two breeds. These are therefore excellent experimental material for study and subsequent understanding of these concepts, but this is subject to further studies of the Ni and Zo breeds. Basing on the results of analysis of Ni and Zo rabbit

<p>| Table 5: Results of rabbit genotyping for three analyzed genetic mutations |
|---------------------------------|----------------|----------------|----------------|
|                                  | del/del | del/G | G/G |
| Rabbit of Nitra                 | 8       | 8    | -   |
| Zobor rabbit                    | 8       | -    | 1   |</p>
<table>
<thead>
<tr>
<th>Albinos</th>
<th>3</th>
<th>-</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ins/ins</td>
<td>ins/Wt</td>
<td>Wt/Wt</td>
</tr>
<tr>
<td>Rabbit of Nitra</td>
<td>8</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>Zobor rabbit</td>
<td>8</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Albinos</td>
<td>3</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>----------------</td>
<td>----------------</td>
<td>----------------</td>
</tr>
<tr>
<td></td>
<td>A/A</td>
<td>A/C</td>
<td>C/C</td>
</tr>
<tr>
<td>Rabbit of Nitra</td>
<td>8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Zobor rabbit</td>
<td>8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Albinos</td>
<td>3</td>
<td>3</td>
<td>-</td>
</tr>
</tbody>
</table>
breeds of three systems we propose following genetic assembly shown in the Table 6. For both color surges (blue and wild-blue) of Ni rabbits for c.585delG it should be homozygous assembly del/del, which determines the dilution coat color. For c.585delG of Zo rabbit breed it should be homozygous assembly G/G, ensuring full pigment production on the site-specific staining characters. For c.5_6insA of wild-blue Ni, which has characters of wild-type rabbits as a part of its breeding standard, it should be homozygous assembly Wt/Wt, whilst the ins/ins homozygous assembly is necessary for Blue Ni and Zo, which must be without characters of wild-type rabbit. This system is very effectively monitored thanks to the underside of the tail as effective indicator of wildness (only genotype ins/ins has coloring underside of tail in coloring rabbit breeds, but presence of only one Wt allele is associated with characters of wild-type rabbit). Regarding the last analyzed genetic system TYR_1118, both breeds are important to maintain in the clean homozygous assembly C/C, without the presence of allele A, which needs to be eliminated if clean lines are disrupted by cross-breeding to avoid useless of the negative selection of born albinos. Simply on the basis of the presence of albinos in the litter, without doing genetic analysis, it is possible to confirm whether the parental pair, both male and female, has heterozygous assembly A/C for c.1118C→A in the tyrosinase gene.

**CONCLUSION**

The implementation of genetic analyses to rabbit breeding is important in order to use all available information and knowledge of similar genetic studies for more efficient breeding techniques to avoid unnecessary false steps, such as the inclusion Marder breed in cross-breeding programs Ni in mistaken belief that the phenomenon acromelanism improves the coloring characteristics of the partial albinism.

**ACKNOWLEDGEMENT**

This work was supported by Slovak Research and Development Agency under contracts APVV-0556-11 and APVV-0044-12.

**REFERENCES**


EFFECTS OF DIETARY SUPPLEMENTATION WITH ISOFLAVONES ON EXTERIOR DEVELOPMENT AND TIBIA BONE QUALITY OF LAYING HENS

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1Ss. Cyril and Methodius University, Institute of Animal Science, Skopje, Macedonia
2Ss. Cyril and Methodius University, Faculty of Agricultural Sciences and Food, Skopje, Macedonia

ABSTRACT

The experiment was conducted to evaluate the effect of dietary supplementation with isoflavones on exterior development and tibia bone quality of laying hens. Eighty laying hens, 20 weeks old, were divided into 5 groups, 20 in each and fed with different amount of 300, 600, 1200 and 1800 mg.kg\(^{-1}\) supplemented isoflavones (SI) in the feed. Dietary isoflavones supplemented in different concentrations to the ISA Brown laying hens diet caused increase of the body weight (P < 0.05). The heaviest body weight was noticed in the group fed with supplementation of 1800 mg.kg\(^{-1}\) SI (2142.5 ± 190.5 g). In addition, shank length as an important measurement of skeletal development was significantly longest in the group fed with supplementation of 1800 mg.kg\(^{-1}\) SI (29.50 ± 1.73 cm). There were no differences in other exterior parameters among the experimental groups (P > 0.05). High SI treatment (1800 mg.kg\(^{-1}\) feed) had a beneficial effect (P < 0.05) on bone quality of aged laying hens (weight, volume, ash/tibia and calcium), but do not affect on the tibial phosphorus (P > 0.05). Results of the present study indicates that isoflavones are effective supplements to improve the body weight and calcium bone content during the late laying period, even though their supplementation was high.

Key words: isoflavones; genistein; daidzein; bone quality; laying hens

INTRODUCTION

Commercial laying hens have a problem with the loss of bone strength during their egg production cycle. When the hens reached sexual maturity, the osteoporosis initiated serious welfare problems (Webster, 2004; Whitehead and Fleming, 2000). In their ordinary diet the soybean and its products are commonly used as a protein source for layers. Soybean is a great source of isoflavones, such as genistein and daidzein, which are believed to be biologically active in several animals, including humans and poultry. Supplementation of daidzein improves the laying performance during post peak laying in Shaoxing duck (Zhao et al., 2005) and the amount of cracked eggs decreases and eggshell thickness and egg production increases (Ni et al., 2007). Sahin N. et al. (2006) reported that soybean isoflavones have a significant effect on the egg quality and bone mineralization in quails.

The experiment will thus provide knowledge on the effect of dietary supplementation with isoflavones on the tibia bone quality in aging laying hens and may provide as a means to increase the welfare of laying hens.

MATERIAL AND METHODS

Animal Treatments and Doses

The experiment was performed on ISA Brown laying hens in a commercial poultry farm. Eighty laying hens, 20 weeks old, were divided into 5 groups,
20 in each (4 cages for a group), set to a 16L:8D cycle and fed with different amount of supplemented isoflavones (SI) in the feed. The control group was blank and fed with no SI in the feed and the other 4 experimental groups was fed with SI in the feed in amount of 300, 600, 1200 and 1800 mg.kg$^{-1}$. Water was offered for *ad libitum* consumption throughout the experiment, which was conducted for 40 weeks. The experimental feed was supplemented with a concentrated product, 40 % isoflavones, produced by the North China Pharmaceutical Corporation. The isoflavone composition of the product is presented in Table 1.

The composition and nutritive value of the basal diet is presented in Table 2.

The laying hens received additional isoflavones from 20 to 60 weeks of age.

**Exterior Parameters**

The body weight of the laying hens was measured with a special digital balance at the beginning and at the end of the experiment. The body length, the breast circumference and the length of the legs were measured using the clot tape.

<table>
<thead>
<tr>
<th>Ingredient, g.kg$^{-1}$</th>
<th>Basal feed (BF) Phase I of egg production</th>
<th>Basal feed (BF) Phase II of egg production</th>
<th>Basal feed (BF) Phase III of egg production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yellow corn</td>
<td>40.01</td>
<td>40.24</td>
<td>43.13</td>
</tr>
<tr>
<td>Soybean meal, 44 % protein</td>
<td>18.96</td>
<td>15.60</td>
<td>14.43</td>
</tr>
<tr>
<td>Sunflower meal, 33 % protein</td>
<td>15.00</td>
<td>15.00</td>
<td>15.30</td>
</tr>
<tr>
<td>Wheat middlings</td>
<td>9.40</td>
<td>12.39</td>
<td>10.70</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>5.10</td>
<td>4.94</td>
<td>4.32</td>
</tr>
<tr>
<td>Methionine, 99 %</td>
<td>0.12</td>
<td>0.07</td>
<td>0.04</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>9.12</td>
<td>9.61</td>
<td>9.94</td>
</tr>
<tr>
<td>Monocalcium phosphate</td>
<td>1.10</td>
<td>0.90</td>
<td>0.76</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>0.15</td>
<td>0.20</td>
<td>0.30</td>
</tr>
<tr>
<td>Potassium carbonate</td>
<td>-</td>
<td>0.04</td>
<td>0.09</td>
</tr>
<tr>
<td>Zeolites</td>
<td>0.30</td>
<td>0.30</td>
<td>0.30</td>
</tr>
<tr>
<td>Salt</td>
<td>0.24</td>
<td>0.22</td>
<td>0.19</td>
</tr>
<tr>
<td>Vitamin and mineral mixture</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>Total</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
</tr>
</tbody>
</table>

**Table 1: Composition of the 40 % isoflavone product**

<table>
<thead>
<tr>
<th>Isoflavone</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Genistin</td>
<td>7.30</td>
</tr>
<tr>
<td>2. Genistein</td>
<td>1.26</td>
</tr>
<tr>
<td>3. Daidzin</td>
<td>22.12</td>
</tr>
<tr>
<td>4. Daidzein</td>
<td>1.74</td>
</tr>
<tr>
<td>5. Glycitin</td>
<td>8.01</td>
</tr>
<tr>
<td>6. Glycitein</td>
<td>0.45</td>
</tr>
<tr>
<td>Total</td>
<td>40.88</td>
</tr>
</tbody>
</table>

**Table 2: Composition and nutritive value of the basal feed (BF)**

**Chemical composition, calculated**

| Dry matter, g.kg$^{-1}$ | 904.30 | 904.00 | 903.10 |
| Metabolic energy, kcal.kg$^{-1}$ | 2800.00 | 2750.00 | 2750.00 |
| Crude proteins, g.kg$^{-1}$ | 179.90 | 169.40 | 165.00 |
| Crude fat, g.kg$^{-1}$ | 71.50 | 70.90 | 65.20 |
| Crude fiber, g.kg$^{-1}$ | 69.80 | 72.00 | 70.00 |
| Total ash, g.kg$^{-1}$ | 125.30 | 130.00 | 130.00 |
| Calcium, g.kg$^{-1}$ | 37.50 | 39.00 | 40.00 |
| Phosphorus (available), g.kg$^{-1}$ | 3.80 | 3.40 | 3.00 |
| Lysine, g.kg$^{-1}$ | 8.50 | 7.70 | 7.40 |
| DL Methionine, g.kg$^{-1}$ | 4.60 | 3.90 | 3.60 |
| Methionine + cystine, g.kg$^{-1}$ | 7.30 | 6.50 | 6.10 |
**Bone Sample Collection**

At the end of the experiment 5 laying hens from each experimental group were sacrificed. The laying hens were anaesthetized by inhalation with diethyl ether and killed. At necroscopy, the right tibia was excised from the body and defleshed without boiling. The tibia was then sealed in a plastic bag to minimize moisture loss and stored at -20 °C until analysis. The bone volume was taken by the weight change in water method. Tibias were measured on the air and in the water. The weight change equalled the weight of water replaced by the bone as the specific gravity of water is 1.0 g.cm\(^{-3}\). The weight was measured with a digital balance of 0.01 g accuracy. Bone length was measured with a calliper with 0.01 cm accuracy. The tibia was defatted, dried at 105 °C for 24 h and placed in a dessicator. The bone ash weight was obtained after ashing at 600 °C for 24 h. Bone ash concentration was calculated as bone ash weight per unit of volume. The content of calcium and phosphorus were determined by potassium per manganate titration and vanadate-molybdate colorimetric methods, respectively.

**Statistical analysis**

Statistical analysis was performed by Statgraph 3 software package. One-way analysis of variance (ANOVA) was used for the differences between groups. When the F values were significant, the Duncan’s Multiple Range Test was performed.

**RESULTS AND DISCUSSION**

The obtained results of the body weight and the exterior parameters are presented in Table 3.

There are some information (Payne et al., 2001; Jiang et al., 2007) which indicate that phytohormones added to a large amount to the feed of chicken cause changes in the body weight and appearance that have influence on some other exterior characteristics and the whole body conformation. In our experiment dietary isoflavones supplemented in different concentrations to the ISA Brown laying hens’ diet caused increase in the body weight (P < 0.05). The heaviest body weight was noticed in the group fed with supplementation of 1800 mg.kg\(^{-1}\) SI (2142.5 ± 190.5 g).

In addition, shank length is an important measurement of skeletal development (Yilmaz Dickmen and Ipek, 2006). It is related with the productive traits of the laying hens (Petek et al., 2000). The longest shank length was noticed in the group fed with supplementation Table 3: Body weight and exterior parameters of the ISA brown laying hens

<table>
<thead>
<tr>
<th></th>
<th>Basal feed (BF)</th>
<th>Basal feed (BF)</th>
<th>Basal feed (BF)</th>
<th>Basal feed (BF)</th>
<th>Basal feed (BF)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(BF)</td>
<td>(BF)</td>
<td>(BF)</td>
<td>(BF)</td>
<td>(BF)</td>
</tr>
<tr>
<td></td>
<td>+ 300 mg.kg(^{-1}) SI</td>
<td>+ 600 mg.kg(^{-1}) SI</td>
<td>+ 1200 mg.kg(^{-1}) SI</td>
<td>+ 1800 mg.kg(^{-1}) SI</td>
<td></td>
</tr>
<tr>
<td>Body weight, g</td>
<td>1987.5 ± 125.8(^{a, b})</td>
<td>1967.5 ± 100.1(^{a, c})</td>
<td>2045.0 ± 92.9(^{a})</td>
<td>1955.0 ± 92.6(^{a})</td>
<td>2142.5 ± 190.5(^{a})</td>
</tr>
<tr>
<td>Body length, cm</td>
<td>28.88 ± 1.55(^{a, b})</td>
<td>27.13 ± 0.85(^{a})</td>
<td>28.50 ± 1.29(^{a})</td>
<td>27.50 ± 1.29(^{a})</td>
<td>29.50 ± 1.73(^{a})</td>
</tr>
<tr>
<td>Breast circumference, cm</td>
<td>21.50 ± 1.73</td>
<td>20.63 ± 1.25</td>
<td>20.75 ± 0.65</td>
<td>20.75 ± 1.26</td>
<td>22.50 ± 1.29</td>
</tr>
<tr>
<td>Length of femur, cm</td>
<td>11.25 ± 0.96</td>
<td>11.00 ± 0.05</td>
<td>11.00 ± 0.82</td>
<td>10.75 ± 0.50</td>
<td>11.00 ± 0.71</td>
</tr>
<tr>
<td>Length of tibia, cm</td>
<td>20.50 ± 1.29</td>
<td>20.38 ± 0.95</td>
<td>22.50 ± 0.58</td>
<td>21.50 ± 0.58</td>
<td>21.38 ± 1.80</td>
</tr>
<tr>
<td>Length of metatarsus, cm</td>
<td>29.50 ± 1.29</td>
<td>29.00 ± 1.41</td>
<td>29.75 ± 0.50</td>
<td>29.50 ± 0.58</td>
<td>29.75 ± 1.50</td>
</tr>
</tbody>
</table>

SI – supplemented isoflavones; a,b,c – values in the same row with no common superscript differ significantly (P < 0.05).

of 1800 mg.kg\(^{-1}\) SI (29.50 ± 1.73 cm). This result is in agreement with the results of our previous study conducted with ISA Brown pullets (Gjorgovska et al., 2014).

There were no differences in other exterior parameters among the experimental groups (P>0.05).

There are few studies investigating the effects of the soy phytoestrogens on bone strength in animals, but this is one of the newest studies to investigate the effect of genistein and daidzein on the bone quality of laying hens during late egg production.

These results suggest that the high supplemented isoflavones treatment (1800 mg.kg\(^{-1}\) feed) has a beneficial effect (P < 0.05) on bone quality of aged laying hens (weight, volume, ash/tibia and calcium), but do not affect on the tibial phosphorus (P > 0.05). Sahin et al. (2007) reported that supplementation with soy isoflavones significantly improve bone mineral density in Japanese
Table 4: Bone quality and mineralization of the tibia (calculated on dry matter)

<table>
<thead>
<tr>
<th></th>
<th>Basal feed (BF)</th>
<th>Basal feed (BF)</th>
<th>Basal feed (BF)</th>
<th>Basal feed (BF)</th>
<th>Basal feed (BF)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(BF) + 300 mg.kg⁻¹ SI</td>
<td>(BF) + 600 mg.kg⁻¹ SI</td>
<td>(BF) + 1200 mg.kg⁻¹ SI</td>
<td>(BF) + 1800 mg.kg⁻¹ SI</td>
<td></td>
</tr>
<tr>
<td>1. Weight of the tibia, g</td>
<td>7.08 ± 0.56ᵃᵇ</td>
<td>6.71 ± 1.24ᵃᵇ</td>
<td>7.45 ± 0.77ᵃᵇ</td>
<td>7.06 ± 0.77ᵃᵇ</td>
<td>8.38 ± 1.01ᵃᵇ</td>
</tr>
<tr>
<td>2. Volume of the tibia, cm³</td>
<td>9.75 ± 0.50ᵃᵇ</td>
<td>8.38 ± 0.75ᵃᵇ</td>
<td>9.63 ± 0.75ᵃᵇ</td>
<td>9.83 ± 0.29ᵃᵇ</td>
<td>10.50 ± 0.58ᵃᵇ</td>
</tr>
<tr>
<td>3. Ash/tibia, g</td>
<td>4.10 ± 0.37ᵃᵇ</td>
<td>4.12 ± 0.62ᵃᵇ</td>
<td>4.18 ± 0.53ᵃᵇ</td>
<td>3.89 ± 0.56ᵇ</td>
<td>4.88 ± 0.74ᵃᵇ</td>
</tr>
<tr>
<td>4. Calcium, g</td>
<td>2.68 ± 0.17ᵃᵇ</td>
<td>2.53 ± 0.47ᵃᵇ</td>
<td>2.97 ± 0.27ᵃᵇ</td>
<td>2.61 ± 0.21ᵇ</td>
<td>3.31 ± 0.50ᵇ</td>
</tr>
<tr>
<td>5. Phosphorus, g</td>
<td>1.03 ± 0.32</td>
<td>1.16 ± 0.20</td>
<td>1.21 ± 0.42</td>
<td>1.22 ± 0.17</td>
<td>1.39 ± 0.15</td>
</tr>
</tbody>
</table>

SI – supplemented isoflavones; Ash concentration¹ (g.cm⁻³) (Ash concentration = ash weight/bone volume); a, b – values in the same row with no common superscript differ significantly (P < 0.05).

Recent studies also suggested that higher isoflavone intake is associated with increased mineral content (Saiafzadeh and Jahanian, 2013).

CONCLUSIONS

The results of the present study show that supplementation with 600 and 1800 mg isoflavones/kg diet increased the body weight significantly (P < 0.05). The longest shank length was noticed in the group fed with supplementation of 1800 mg.kg⁻¹ SI (P < 0.05). These findings also suggest that isoflavones have a stimulatory effect on calcium content in bone in groups supplemented with 1800 mg isoflavones.kg⁻¹ diet (P < 0.05). These results indicate that isoflavones are effective supplements to improve the body weight, body length and calcium bone content during the late laying period, even though their supplementation is high.

REFERENCES


SURFACE TEMPERATURE OF WARM-WATER PADS FOR HEATING PIGLETS IN FARROWING PENS

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²Slovak University of Agriculture in Nitra, Slovak Republic

ABSTRACT

The objective of this study was to evaluate the supplementary piglet heating on the basis of surface temperature of warm-water pads (WWP) in strawless farrowing house. Two WWPs (0.48 m²) were installed in each farrowing pen for piglet heating. Surface temperature of all double-size pads was measured by infrared thermometer (GIM 3590) in 120 farrowing pens during winter season. Average surface temperature of the pads was assessed in 8 groups in section rows (in the order of 1st till the 8th pen) including frequency distribution of temperatures in the range 34 – 43 °C (with scale of 1 °C), in the range 37 – 43°C and 39 – 41°C, and in the 3 specific temperature limits (< 37.8 °C, 37.8 – 38.9 °C, and > 38.9 °C). The data were analyzed using the Descriptive Statistics procedure and a General Linear Model ANOVA by the statistical package STATISTIX 10.0. Significance of differences between WWP in pens was determined by LSD All-Pairwise Comparisons Test (at α ≤ 0.01). Outdoor average air temperature was -0.6 °C and average temperature in the farrowing sections was 18.7 °C during the measuring period. Warm-water pads in the group of second pens had the highest average surface temperature 40.59 ± 0.96 °C. The pads in the eighth group of pens had the lowest temperature 37.91 ± 1.83 °C (P < 0.001), because these were situated at the external enclosure wall of stable. In these pens, internal wall was not thermally insulated sidelong the heating pads, as it was in the first group of pens. As far as the average surface temperature is concerned, almost 94.17 % of double-size pads had advantageous temperature (37 – 43 °C) and 67.50 % had the optimum temperature (39 – 41 °C). It is possible to get improvement of temperature conditions by additional insulation of the external enclosure wall in the area of pens as well as by optimal heat regulation of the pads basec on the position of lying piglets.

Key words: warm-water pads; temperature; farrowing pen; piglets

INTRODUCTION

The newborn pig is extremely susceptible to cold, damp conditions. It has little fat and hair, a thin skin and a small mass in relation to its body surface area (Roese and Taylor, 2006). Pigs have a normal temperature range of 38 – 40 °C. Piglets do not have the ability of thermoregulation at birth, because they need heat immediately to survive and grow (Blecha, 2001). Immediately after birth, a piglet’s temperature can drop by 1 °C or more within the first half-hour, depending on environmental conditions. Under favourable conditions, this decrease in temperature is regained in about 24 – 48 hours (Roese and Taylor, 2006).

This is particularly stressful for a newborn piglet which after birth experiences a sudden 15 – 20 °C decrease in ambient temperature (Herpin et al., 2002). Piglet after its birth leaves a warm draft-free environment of 38.9 °C which is the sow’s womb temperature and so for the newborn piglet even the temperature of 35 °C is cold during the first hours its life. At temperatures below 37.8 °C the piglet uses the sow milk also for warming its body (AHSI, 2010). For new born pigs in its resting (creep) area the temperature should be range from 32 °C (Lean, 1994; Curtis, 1995) to 35 °C (Xin and Zhang, 2000), which gradually decreases till 22 – 24 °C weaning (Herpin et al., 2002; Ilsters et al., 2009). When the temperature drops below the piglets’
thermo-neutral zone (34 – 36 °C), piglets try to increase their heat production by means of energetically demanding muscular shivering thermogenesis (Berthon et al., 1994), and they try to reduce their heat loss by social and individual thermoregulation (Vasdal et al., 2009).

Recommended optimum range of the air temperature in farrowing house for lactating sows is 16 – 22 °C only (Odehnalova et al., 2008; Pig Welfare Standards, 2012). The temperature of 22 °C is upper critical thermal limit for the lactating sow (Quiniou and Noblet, 1999). Because room temperature in the farrowing unit is usually kept within the sows’ thermal comfort zone (around 20 °C), it is necessary to provide a suitable temperature (30 – 34 °C) in the creep area by external heat sources and some sort of insulated flooring to avoid hypothermia in piglets. Several studies have found that newborn piglets prefer to huddle near the sow and littermates despite unfavourable thermal conditions in the sow area, instead of staying in the heated creep area during the first days after birth (Andersen et al., 2007; Vasdal et al., 2009). Hypothermia might often predispose piglets to starvation and crushing by the sow (Edwards, 2002), especially in the first three days of life when crushing risk is at its greatest (Berg et al., 2006).

Newborn piglets have remarkably higher requirements for warmth. Because the neonatal piglet requires quite a different environment from the sow, heated creep area (away from the sow) is essential in the farrowing pen (Roese and Taylor, 2006). It is necessary to use local heating of their area, the best by heated pads. To keep minimum ambient temperature of 20°C till piglets’ age of 1 month it is necessary to use local heating of their area.

Electric or warm-water pads for bottom local heating are used. Warmth transmission to piglets occurs by their body contact with the surface of heated pad. The surface temperature of pad (mat) is usually preset to certain levels (commonly 30 – 40 °C) according to the age of piglets, room temperature, and management style (Zhang and Xin, 2000a). Ilsters et al. (2009) suggested that the temperature of the resting place for piglets comprising of concrete floor panels should range from 35 °C to 40 °C. Zhang and Xin (2000b) proposed an acceptable mat surface temperature of 34 – 43°C for creep heating and for surface heating of piglets. de Baey-Ernsten et al. (1995) suggested a tolerable surface temperature range of 37 – 43 °C. If the goal is to increase piglets’ use of the creep area during the first critical days after farrowing, it seems important to increase the attractiveness of the area itself by using temperature gradients (Lynch, 1983; Barber and Bourne, 1987). Vasdal et al. (2010) showed that piglets had a significant preference for 42 °C compared to 34 °C (P < 0.05) when different types of infrared temperatures in the creep area at 24 hours of age were tested.

Recommended surface temperature of WWP at the birth of piglets is about 38 – 41 °C (MIK International, 2014). The laying position of the litter determines the WWP temperature’s control variable. Since the temperature needs of the litter cannot be established according to age, the lying position of the litter must be observed and the temperature adjusted as needed. According to DLG Fokus Test warm-water pads are tested from the point of view of the surface share with advantageous temperatures in the range of 37 – 43 °C and with optimum temperatures ranging from 39 °C to 41 °C (MIK International, 2009). From the viewpoint of comfort zone for piglets (AHSI, 2010), correct regulation of temperature, either optimum or advantageous surface temperatures of heated pads are important factors. The control of the surface temperature of heating areas is very important to keep the optimal temperature for the sucking piglets.

Fig. 1: Layout of the 1st farrowing section (from the total number of 5) with 3 x 8 farrowing pens with crate and warm-water pads for floor heating of piglets
1 to 8 - farrowing pens (pen groups according to their order), A - manipulating alley, B - feeding alley, C - warm-water pads, D - marginal lengthwise passage in stable
MATERIAL AND METHODS

The objective of this study was to evaluate the supplementary piglet heating on the basis of surface temperature of warm-water pads (WWP) in strawless farrowing house. In the farrowing house there were 5 sections with 3 rows of pens, 2 feeding and 2 manipulating alleys (Figure 1). In each row there was 8 farrowing pens with crate and a plastic slotted floor. The pens were situated across the alley in sections. Vacuum ventilation was regulated in all the sections.

The system of warm-water pads was used for supplementary heating of piglets. Two WWP with dimensions 400 x 600 mm were installed in each farrowing pen at the left or right side. They were mounted to the floor pen next to each other so as to create a long strip of 1 200 mm with a total area of 0.48 m² (double-size pad). Piglets had tipping covers at their disposal with plastic sheets. Warm-water pads in first farrowing pens were situated at the enclosure wall adjacent to marginal lengthwise passage of the farrowing unit. WWPs in the last group of (eighth) pens were situated in section rows at the external enclosure wall of the stable.

Surface temperature of double-size warm-water pad was measured in all the 120 farrowing pens by infrared thermometer GIM 3590 with an internal data logger (Greisinger Electronic GmbH, Regenstauf, Germany; accuracy ± 0.75 °C or ± 0.75 %, whichever was greater) in winter season. The measurements were taken vertically from a distance of 500 mm above the WWP surface as a whole by continuous scanning line in 4 selected longitudinal lines for a period of time about 11 seconds. Response time of this device was 150 ms, which correspond to a data set with about 70 values. Measured values of data sets (Max, Min, Avg) were continuous stored in device memory as the measurement protocols. Maximum, minimum and average values were related to the duration of measurement.

For data evaluation of 120 double-size pads farrowing pens were divided according to their order in the lateral section rows into eight longitudinal groups with 15 pens in each group. The average surface temperature of double-size pads was assessed in first to the eighth group of pens in the farrowing house including frequency distribution of temperature. Frequency distribution of surface temperature of WWPs was evaluated in the range 34 – 43 °C with scale of 1 °C (8 temperature zones with difference of 9 °C), than in the range from 37 °C to 43 °C and from 39 °C to 41 °C (as advantageous and optimum temperatures; MIK International, 2009) and in the three specific temperature limits (< 37.8 °C, 37.8 – 38.9 °C, and > 38.9 °C).

Temperature and the relative humidity of air were registered in the farrowing sections and in the external environment during the measuring period by electronic data loggers HDL (HIVUS Ltd., Žilina, Slovakia).

The data were analyzed using the Descriptive Statistics procedure and a General Linear Model ANOVA by the statistical package STATISTIX 10.0 was used (Analytical Software, Tallahassee, Florida, USA). Significance of differences between the average surface temperatures of warm-water pads in pens was determined by LSD All-Pairwise Comparisons Test (at α ≤ 0.001).

RESULTS AND DISCUSSION

Average temperature of air in the farrowing house was 18.7 ± 0.62 °C during the measuring period and average air temperature in the external environment was -0.6 ± 3.72 °C (Table 1). Indoor air temperature ranged from 17.4 °C to 20.6 °C and the outdoor air temperature ranged from -7.9 °C to 4.8 °C. Average relative humidity of air in the farrowing house was 54.6 ± 3.72 % and in external environment it was 73.8 ± 9.88 %. The indoor relative humidity of air ranged from 39.8 % to 64.6 % and the outdoor air relative humidity ranged from 49.1 % to 91.9 %. All the measured internal temperatures of air were suitable for sows (Odehnalova et al., 2008; Pig Welfare Standards, 2012) but not for sucking piglets. Registered room temperatures were lower than 22 °C which is the bottom temperature limit for piglets in the time of their weaning (Herpin et al., 2002) and supplementary heating by WWPs was essential to maintain the optimal temperature for the sucking piglets (Zhang and Xin, 2000;
Warm-water pads in the group of second pens in the farrowing house had the highest average surface temperature of 40.59 ± 0.96 °C (Table 2). The lowest temperature of 37.91 ± 1.83 °C (P < 0.001) was recorded for the pads in the group of eighth pens (last group of pens in section rows), which were situated at external enclosure wall. The internal wall in the area of these pens was not thermally insulated (by plastic plate) as most of the first group of pens (80 %). Warm-water pads in the group of the first group of pens, which were situated closest to the regulation unit, had average surface temperature of 40.20 ± 1.29 °C, i.e. by 0.39 °C lower than WWPs in the group of second pens in farrowing unit. However, this difference was non-significant statistically. Most likely it could be affected by the fact that the pads in the group of first pens were situated at the enclosure wall adjacent to marginal lengthwise passage in farrowing house. In this passage the temperature of air was lower than in the individual sections. Each of farrowing sections had four doors which were not always closed when the attendant staff was entering or exiting. The side pen walls in all the outlaying first pens in section rows (in the area with heating pads) were made from the ceramic tiles, but three farrowing pens in the middle section in this area did not have insulated wall in the form of plastic plate (like all the pens in the eighth in section rows). The surface temperature of ceramic tiles was lower than the surface temperature of the plastic plate. All mentioned factors could also affect the surface temperature of WWPs in the group of first farrowing pens.

Frequency distribution of 120 average surface temperatures of double-side pads (Table 3) indicated that the largest share of pad temperatures were in the range of 40 – 41 °C (40.8 %) and in the range of 39 – 40 °C (26.7 %). Ten pads (8.3 %) had surface temperatures lower than 38 °C and 16 pads (13.4 %) had surface temperatures higher than 41 °C. All measured surface temperatures were higher than 35 °C and lower than 43 °C, which corresponds with acceptable mat surface temperature range (34 – 43 °C) for creep heating as proposed by Zhang and Xin (2000b). Surface temperatures in the range of 37 – 43 °C, i.e. advantageous temperature (MIK International, 2009) were recorded for 113 double-size pads (94.17 %). This corresponds with a tolerable surface temperature range for surface heating of piglets as suggested by de Baey-Ernsten et al. (1995). Surface temperatures of 81 double-size pads (67.50 %) were in the range of 39 – 41 °C, which was also deemed as optimum temperatures by us (MIK International, 2009).

Table 2: Surface temperatures of double-size warm-water pads in farrowing pens according to their order in section rows (pen groups)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Order of pens in a row in sections (pen groups in farrowing house)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Average*</td>
<td>40.20&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>SD</td>
<td>1.29</td>
</tr>
<tr>
<td>Minimum</td>
<td>36.8</td>
</tr>
<tr>
<td>Maximum</td>
<td>41.9</td>
</tr>
</tbody>
</table>

*Significance of differences at P < 0.001, Data with an equal superscript are not significantly different from one another (LSD test at α = 0.001), n = 15 data sets (i.e. data set includes about 70 measured values).

Table 3: Frequency distribution of average surface temperatures of double-size warm-water pads in the farrowing house

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Range of surface temperatures in °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>4</td>
</tr>
<tr>
<td>%</td>
<td>3.3</td>
</tr>
</tbody>
</table>

*The lower limit of range includes the value and the upper limit excludes the value.
below 37.8 °C do not provide the comfort zone for piglets and a certain amount of milk received can be used for the heating of their body and not to growth (AHSI, 2010). Surface temperature of 38.7 °C or more may provide comfort zone for piglets and the value of 38.9 °C is the temperature of environment in the sow’s womb. The largest share (97 %) of all 120 double-size pads had the surface temperature above 38.9 °C, which is favourable in terms of providing temperature comfortable for piglets during the first critical days after farrowing.

CONCLUSION

Among 8 groups of 15 double-size warm-water pads (total 120 pads with dimensions 400 x 1200 mm) the lowest average surface temperature (37.9 °C) was recorded in pads in the group of eighth (last) farrowing pens in section rows (by their order), which were situated at external enclosure wall without thermal insulation of side pen wall from ceramic tiles in the area with local floor heating. All the measured surface temperatures were higher than 35 °C and lower than 43 °C. As far as the average surface temperature is concerned, almost 94.2 % of double-size warm-water pads had advantageous (acceptable) temperature in the range of 37 – 43 °C and 67.5 % of double-size pads had the optimum temperature in the range of 39 – 41 °C.

It is possible to get improvement of temperature conditions by additional insulation of the external enclosure wall in the area of pens as well as by optimal heat regulation of water heated pads based on the position of lying piglets.

ACKNOWLEDGEMENT

This study was supported by the departmental task of research and development RPVV 2 of the Ministry of Agriculture and Rural Development of the Slovak Republic. This article was possible through projects APVV of the Slovak Research and Development Agency Bratislava (0632-10 and 15-0060), and the project CEGEZ 26220120073 supported by the Operational Programme Research and Development funded from the European Regional Development Fund.

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Review

HEAVY METALS – ENVIRONMENTAL CONTAMINANTS AND THEIR OCCURRENCE IN DIFFERENT TYPES OF MILK

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ABSTRACT

The aim of this review is to summarize the findings about heavy metals and their effects on animal organism and to describe a source of contamination and inputs of heavy metals into food chain. Furthermore, the comparison of the occurrence of selected heavy metals in different types of milk on the basis of previous studies has been made. Based on available information and according to level of contamination, we want to draw an attention to suitability of using milk for further processing in selected areas of the world.

Key words: heavy metals; toxic elements; contaminants; milk

INTRODUCTION

Industrial progress, industrialization, urbanization and agricultural production have become permanent resources of extraneous chemicals for living organisms. In recent years, food safety is regarded as a carrier theme for food industry. The increase in agricultural production efficiency causes using of large quantities of chemical products not even in animal feed production, but also on farms with milk production. Due to non-compliance with right technologies, these substances are becoming a part of agricultural products, including milk from cows, ewes or goats. These contaminants are not a natural part of milk, but they are secreted into the milk from animal body. Success in food production significantly depends on the abundance of quality materials and good manufacturing practice. Therefore, an attention should be paid to understanding the problems and ways of possible penetration of contaminants into the food chain.

Heavy metals and trace elements

Heavy metals are widely dispersed in the environment. The toxicities induced by excessive levels of some of these elements, such as chromium (Cr), cadmium (Cd), lead (Pb) and mercury (Hg) are well known (Llobet et al., 2003). Heavy metals like cadmium, lead, mercury and arsenic are the major toxic metals posing a threat to human health. Their concentrations in animal organisms and their milk concentrations may increase very fast, although their excretion through milk is very low (Miller, 1971; Houpert et al., 1997). Their ecosystem accumulation (water-soil-plant-animal) makes them very toxic and leads to undesirable consequences for live organisms (Bogut et al., 2000; Piskorova et al., 2003). Free-living animals are important indicators of the environmental pollution with heavy metals (Kottferová and Koréneková, 1998). Sheep and cattle reared freely on pasture are also indicators of the environmental pollution (Gallo et al., 1996). Increased concentrations of heavy metals in body...
of domestic animals result in low fitness of animals, reproduction problems, immunity decline and occurrence of cancerous and teratogenic diseases (Bires et al., 1995).

In the local studies, cadmium, lead, copper, zinc, iron, chromium and manganese are found at toxic levels in soils and vegetables (Ghafoor and Rasool, 1999; Quadir et al., 2000). These studies guide to hypothesize that fodders grown on such soils will also accumulate these heavy metals, and animals reared on contaminated fodder will contain heavy metal residues in edible tissues, such as milk. The general public eating such contaminated edible products may accumulate toxic levels of heavy metals (Licata et al., 2004).

Pollution of the environment with metals, such as lead, is a world-wide problem. Lead alkyl additives into petrol are combusted and emitted into the atmosphere and can be responsible for high concentration of lead in some vegetation, roadside, soil, air, water and plants (Burguera and Randon, 1987).

Lead has been added to petrol (gasoline) as an anti-knocking agent since the 1920s in order to improve fuel performance and reduce wear on vehicle engines. Since this time, leaded petrol has been reported to cause more lead exposures than any other source worldwide (Landrigan, 2002). During the 1970s, health impacts associated with lead emissions from vehicles became a widely discussed issue. Many studies have reported that environmental lead emissions have resulted in significant health effects to the central nervous system, haem-synthesis, reproductive system, as well as psychological and neurobehavioral functions, and may even increase the risk of cancer (Bellinger, 2005; Fewtrell et al., 2004; Tong et al., 2000). It has been observed throughout the world that the lead content of various environmental components has been decreased after the replacement of leaded petrol (Bridbord and Hanson, 2009; Landrigan, 2002).

The use of leaded petrol resulted in the emission of large quantities of lead that are still present in the ambient environment, which may continue to cause concerns for health (Mielke et al., 2011). Although the United States were responsible for 80% of all leaded petrol sold globally prior to 1970 (Kristensen, 2015), Australia was a substantial consumer of leaded petrol products. The use of leaded petrol in Australia over a 70 years period, from 1932 to 2002 (Cook and Gale, 2005) was a major contributor to atmospheric lead levels (Kristensen, 2015). With the introduction of unleaded petrol in Australia in 1985 and the subsequent phase out of leaded petrol by 2002, ambient lead levels have fallen in metropolitan urban centers to levels less than 10% of Australia’s current guideline for lead in ambient air (0.5 µg.m$^{-3}$) (Abeeb et al., 2003).

Manufacturing processes, incineration of refuse and combustion of coal, are also the other sources which contribute to lead occurrence in the atmosphere; hence it is not surprising that lead levels are highest in area of intense industrialization (Burguera et al., 1988; Shakour et al., 2006). Lead is toxic to the blood and the nervous, urinary, gastric and genital systems. Furthermore, it is also implicated in causing carcinogenesis, mutagenesis and teratogenesis in experimental animals (Pitot and Dragan, 1996; Baht and Moy, 1997). Accumulation of lead in the organism produces damaging effects in the hematopoetical, hematic, renal and gastrointestinal systems (Correia et al., 2000). On the other hand, cadmium is also easily volatilized at the operating temperatures of common industrial processes, much of the cadmium in the atmosphere results from incineration of ferrous scrap and metallurgy processes (Thomas et al., 1972). Cadmium is considered to be one of the most toxic metals. In addition, it is implicated in high blood pressure (Perry et al., 1979), prostate cancer, mutations and fetal (embryonic) death (Pitot and Dragan, 1996). Chromium, nickel and cobalt are also toxic metals which are released to the environment. They originated from dumping industrial wastes in the rivers, as well as the application of phosphatic fertilizers (Venugopal and Luckey, 1978).

Toxicity of metal is closely related to age, sex, route of exposure, level of intake, solubility metal oxidation state, duration of exposure, frequency of intake, absorption rate and mechanisms/efficiency of extraction (Venugopal, 1978; Mertz, 1986).

Milk and milk products are the most diversified of the natural food stuffs in terms of composition and contain more than twenty different trace elements. Most of them are essential and very important, such as copper, zinc, manganese and iron (Schroeder, 1973; Somer, 1974). These metals are cofactors in many enzymes and play an important role in many physiological functions of man and animals. Lack of these metals causes disturbance and pathological conditions (Koh, 1986; Schuhmacher, 1991). The amount of metals in non-contaminated milk is admittedly minute, but their content may be significantly altered through manufacturing and packaging process. Also, metals that can contaminate different cattle feed and environment, such as lead, cadmium, chromium, nickel and cobalt, could be excreted into milk at various levels and cause serious problems (Abou-Arab, 1994, 1997).
The effect of toxic elements

Cadmium and lead are heavy metals which have caused most concern in terms of adverse effects on human health. This is because they are readily transferred through the food chains and are not known to serve any essential biological function (Liu, 2003). Children have been shown to be more sensitive to cadmium and lead than adults and the effects are cumulative. As a result, the regular absorption of small amounts of certain elements, such as lead, may cause serious effects on the health of growing children, including retardation of mental development and deficiencies in concentration, adverse effects on kidney function, blood chemistry and the cardiovascular system, as well as hearing degradation (Ataro et al., 2008; Salma et al., 2000).

Cadmium is ubiquitous environmental contaminant arising primarily from electroplating, plastics manufacturing, mining, paint pigments, alloy preparation, and batteries. Food is the most important source of cadmium in the non-smoking, non-occupationally exposed population (Järup, 2003). Cadmium 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 (Kukner et al., 2007; Massányi et al., 2007).

Table 1: Classification of elements according to toxicity (Toman et al., 2003)

<table>
<thead>
<tr>
<th>Low toxicity</th>
<th>Very toxic, relatively accessible</th>
<th>Toxic, low solubility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na</td>
<td>C</td>
<td>Be</td>
</tr>
<tr>
<td>K</td>
<td>P</td>
<td>Li</td>
</tr>
<tr>
<td>Mg</td>
<td>Fe</td>
<td>Nb</td>
</tr>
<tr>
<td>Ca</td>
<td>S</td>
<td>Te</td>
</tr>
<tr>
<td>H</td>
<td>Cl</td>
<td>Cd</td>
</tr>
<tr>
<td>O</td>
<td>Br</td>
<td>Be</td>
</tr>
</tbody>
</table>

Cadmium can induce both carcinogenic and non-carcinogenic effects on various organs including the lung, liver, kidney, bone and vascular system (Waalkes, 2003). At the cellular level, cadmium induces oxidative stress, cell proliferation and apoptosis (Tremellen, 2008; Turner and Lysiak, 2008). Cadmium is a known endocrine disruptor and reproductive toxicant (Henson and Chedrese, 2004; Cheng et al., 2011) which affects male fertility through altered function of hypothalamic-pituitary-testicular axis (Lafente, 2013) and/or through direct gonadotoxic and spermiotoxic effects (Thompson and Bannigan, 2008). The disruption of functional structures within the blood-testis (Wong et al., 2004; Siu et al., 2009) and blood-epididymis barrier (Toman et al., 2002; Dubé and Cyr, 2013) results in impaired spermatogenesis and sperm maturation processes associated with infertility (Cheng and Mruk, 2012). Cadmium has been suggested to have some of its toxic effects by disturbing metabolism of essential metals, such as selenium. Zinc and selenium are believed to be the antagonists of cadmium toxic effects (Toman et al., 2009).

Selenium is known due to its antioxidant role in living systems and, therefore, it is considered to be an essential element for humans and animals. However, routine selenium supplementation is not recommended.

Table 2: Mean ± SE values (mg.L⁻¹) of Cd, Cr, Ni and Pb in the milk of cattle and goats collected from two areas of Pakistan during the period of November, 2006- April, 2007 (Ijaz et al., 2009)

<table>
<thead>
<tr>
<th>Metal</th>
<th>Cattle milk</th>
<th>Goat milk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd</td>
<td>0.076 ± 0.014*</td>
<td>0.084 ± 0.003</td>
</tr>
<tr>
<td>Cr</td>
<td>1.066 ± 0.074*</td>
<td>1.152 ± 0.045</td>
</tr>
<tr>
<td>Ni</td>
<td>22.395 ± 0.988*</td>
<td>19.522 ± 0.011</td>
</tr>
<tr>
<td>Pb</td>
<td>18.870 ± 2.912*</td>
<td>42.687 ± 0.051</td>
</tr>
</tbody>
</table>

*Significantly (p < 0.05) different from the respective value.
NSNon-significantly (p > 0.05) different from the respective value.
if the Se intake is equal to 80 µg per day or greater (Burk, 2002). The best way of adequate selenium intake is via food and increase of its content in plants by soil or foliar applications is under investigation during the last decade (Ducsay and Ložek, 2006; Zhao and McGrath, 2009). The treatment with Se during Cd exposure has been demonstrated to have beneficial effects on Cd-induced toxicity (Kimáková et al., 2006; El-Sharaky et al., 2007). However, the co-effect of the trace elements on the toxicity caused by Cd is not yet well studied. Xiao et al. (2002) assessed the protective effect of Se and Zn on Cd-induced oxidative stress only in the kidney of the rat, besides, Cd was administrated using the intraperitoneal route.

Nickel is an essential trace element. Its industrial application has a broad spectrum and primarily it is used in alloys (Das et al., 2008). Nickel is also an essential trace metal that is vital for growth enhancement in very low doses for birds and mammals (Lukáč et al., 2009). High quantity of nickel is known to be injurious for animal and human health. Its effects on various aspects of reproduction have been previously described. Animal studies demonstrate that nickel has negative effects on the structure and function of the testis, seminal vesicles and prostate gland; there is similar report on adverse effect on spermatozoa (Pandey et al., 2000; Forgacs et al., 2001). Lukáč et al. (2014) reported about negative effect of nickel on spermatogenesis. The decrease in the relative volume of germinal epithelium indicates on alterations of the spermatozoa production.

Accelerated industrial and agricultural development considerably affects environmental emissions of selected toxic elements: cadmium, lead, mercury and arsenic (Lopez et al., 2002). Their concentrations in animal organisms and their milk concentrations may increase very fast, although their excretion through milk is very low (Houpert et al., 1997). Their ecosystem accumulation (water-soil-plant-animal) makes them very toxic and leads to undesirable consequences for live organisms (Bogut et al., 2000 Piskorova et al., 2003). Free-living animals are important indicators of the environmental pollution with heavy metals (Kottlerová and Koréneková, 1998). Sheep and cattle reared freely on pasture are also indicators of the environmental pollution (Galvo et al., 1996). It is also known that metal (Cd, Pb and Hg) excretion is significantly lower in the offspring (Oskarsson et al., 1995).

The content of heavy metals in different types of milk

Concentrations of heavy metals in milk were mainly described in cows (Cerkvenik et al., 2000; Pilsbacher and Grubhofer, 2002; Sikirič et al., 2003). Ijaz et al. (2009) compared the content of heavy metals (Cd, Cr, Ni, Pb) in cattle and goat milk in Pakistan. They found difference in concentrations of these metals, which are listed in Table 1. The goat milk was found strongly (p < 0.05) contaminated with Cd than cattle milk, 0.084 vs. 0.076 mg.L⁻¹, respectively.

Licata et al. (2004) and Triphathi et al. (1999) reported the levels of Cd in the milk of cattle 0.0228 and 0.00007 mg.L⁻¹ in area of Calabria-Italy and India. Baldini et al. (1990) (Rome-Italy), Cerutti (1999) (Milano-Italy) and Martino et al. (2001) (Spain) found Cd concentration in cattle milk in the range between 0.0002 and 0.03 mg.L⁻¹, which is quite lower than the measured values in the study of Ijaz et al. (2009). Ijaz et al. (2009) states in his study, that the higher values of Cd residues in the milk of goats (0.084 mg.L⁻¹), than in the cattle milk (0.076 mg.L⁻¹) are also in accordance with the values determined by Coni et al. (1996) in the milk of goat

Table 3: Metal content (mg.kg⁻¹) in buffalo's and cow's raw milk samples from area of Giza-Egypt (Enb et al., 2009)

<table>
<thead>
<tr>
<th>Metals</th>
<th>Buffalo’s milk</th>
<th>Cow’s milk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Range</td>
</tr>
<tr>
<td>Iron</td>
<td>0.980 ± 0.442</td>
<td>0.786-1.242</td>
</tr>
<tr>
<td>Copper</td>
<td>0.212 ± 0.102</td>
<td>0.188-0.542</td>
</tr>
<tr>
<td>Manganese</td>
<td>0.076 ± 0.044</td>
<td>0.051-0.916</td>
</tr>
<tr>
<td>Zinc</td>
<td>4.366 ± 0.814</td>
<td>3.966-6.814</td>
</tr>
<tr>
<td>Lead</td>
<td>0.084 ± 0.042</td>
<td>0.044-1.088</td>
</tr>
<tr>
<td>Cadmium</td>
<td>0.118 ± 0.086</td>
<td>0.094-0.142</td>
</tr>
<tr>
<td>Chromium</td>
<td>0.042 ± 0.022</td>
<td>0.036-0.058</td>
</tr>
<tr>
<td>Nickel</td>
<td>0.006 ± 0.010</td>
<td>0.003-0.009</td>
</tr>
<tr>
<td>Cobalt</td>
<td>0.008 ± 0.010</td>
<td>0.003-0.014</td>
</tr>
<tr>
<td>Tin</td>
<td>0.006 ± 0.010</td>
<td>0.004-0.010</td>
</tr>
</tbody>
</table>
(0.15 mg.L⁻¹) and by Licata et al. (2004) in cattle milk (0.0228 mg.L⁻¹). However, 0.05 mg.L⁻¹ of Cd in the milk of goat has been reported by Caggiano et al. (2005) in the region of southern Italy.

Ijaz et al. (2009) indicated in his work, that the higher milk concentration of heavy metals can be attributed to the use of sewage water for agricultural purposes. It has also been observed that the animals have direct access to this sewage water for drinking. The uptake of heavy metals in the soil, vegetables, fodder and other herbage produced in the investigated areas of Faisalabad city the main sewage drains may have a definite role in the contamination of the milk composition.

Enb et al. (2009) stated, that higher levels of heavy metals in buffalo’s and cow’s milk (Table 3) in his study may be attributed to the high contamination of animal feed and water by such pollutants. Abou-Arab (1994; 1997) indicated that these pollutants could be excreted into the milk at various levels and also could reach milk through handling procedures. In this respect, several studies have been carried out to assess metal contents in milk from different areas. Trickapi and Raghumath (1999) reported that metals (Zn, Cu, Pb and Cd) were detected in cow’s and buffalo’s milk samples at following levels: Zn 3.177-3.697 mg.L⁻¹, Cu 0.043-0.195 mg.L⁻¹, Pb 1.70-3.35 µg.L⁻¹ and Cd 0.07-0.10 µg.L⁻¹. However, Onianwa et al. (1999) determined levels of Zn 0.39-2.75, Cu 0.07-0.67, Fe 1.68-15.1, Cr 0.005-0.030, Pb 0.03-0.18, Cd 0.004-0.009, Co 0.03-0.12 and Ni 0.04-0.09 mg.L⁻¹ in cow’s milk in Nigeria. Regarding to the investigation of Lante et al. (2004), they reported that Zn (4.631 mg.L⁻¹), Cu(0.518 mg.L⁻¹), Fe(0.290 mg.L⁻¹), Mn(0.0291 mg.L⁻¹), Cr(0.004 mg.L⁻¹), Pb (5.23 µg.L⁻¹) and Cd (0.40 µg.L⁻¹) were detected at various levels in Italy. On the other hand, Caggiano et al. (2005) reported that metals in cow’s milk were detected in southern Italy at the following levels: Mn: 0.13; Cr: 0.20; Pb: 0.20 and Cd: 0.06 mg.kg⁻¹. The average lead concentration 7.9 ng.L⁻¹ in raw milk samples collected from milk tanker in Iran was recorded by Tajkarimi et al. (2008) and they ranged from 1 to 46 ng.L⁻¹.

In our study we found the levels of copper, mercury, nickel and lead in sheep and cow milk from selected area of Slovakia below the limit of quantification (LOQ) (Cu: < 0.50; Hg: < 0.002; Ni: < 0.10; Pb: < 0.010 mg.kg⁻¹) in spring and autumn season. We analyzed contents of some trace elements in sheep milk: Fe: 0.67 mg.kg⁻¹ in spring season; 0.69 mg.kg⁻¹ in autumn season, Mg 153 mg.kg⁻¹ in spring; 176 mg.kg⁻¹ in autumn and Zn 4.4 mg.kg⁻¹ in spring season; 3.8 mg.kg⁻¹ in autumn season. In cow milk we found content of Fe below the LOQ (< 0.50 mg.kg⁻¹) in both seasons, Mg 80 mg.kg⁻¹ in spring season; 103 mg.kg⁻¹ in autumn season and Zn 3.4 mg.kg⁻¹ in spring season; 4.1 mg.kg⁻¹ in autumn season (unpublished data).

According to Anutović et al. (2005), concentrations of selected toxic elements in ewe’s milk from the area of Slavonian region in Croatia can be different depending on lactation stage. In colostrum (2nd lactation day), Cd and Pb (0.011 and 0.035 mg.kg⁻¹, respectively) concentrations were significantly higher (P<0.01), whereas As (0.011 mg.kg⁻¹) concentrations were lower in comparison with milk on the 10th (Cd: 0.004; Pb: 0.022; As: 0.025 mg.kg⁻¹), 30th (Cd: 0.005 Pb: 0.024; As: 0.028 mg.kg⁻¹) and 60th (Cd: 0.006; Pb: 0.026; As: 0.025 mg.kg⁻¹) lactation day.

Rodriguez et al. (1999) determined concentrations of Pb and Cd in samples of human, cow (raw and pasteurized) and goat milk and powdered infant formula. The following mean Cd concentrations were recorded: in human milk - 2.70 µg.L⁻¹, in raw cow’s milk - 4.88 µg.L⁻¹, in pasteurized cow’s milk - 4.30 µg.L⁻¹, in goat’s milk - 7.81 µg.L⁻¹ and in powdered infant formula - 3.81 µg.L⁻¹.

Table 4: Toxic heavy metals and trace elements in milk from lactating cows reared in different environments of India (Patra et al., 2008)

<table>
<thead>
<tr>
<th>Toxic and trace elements in milk</th>
<th>Lead</th>
<th>Cadmium</th>
<th>Copper</th>
<th>Cobalt</th>
<th>Zinc</th>
<th>Iron</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unpolluted area (n = 52)</td>
<td>0.25 ± 0.03</td>
<td>0.033 ± 0.02</td>
<td>0.101 ± 0.006</td>
<td>0.18 ± 0.009</td>
<td>3.95 ± 0.40</td>
<td>5.10 ± 1.06</td>
</tr>
<tr>
<td>Closed lead zinc smelter (n = 14)</td>
<td>0.26 ± 0.047</td>
<td>0.052 ± 0.005</td>
<td>0.14 ± 0.007</td>
<td>0.23 ± 0.037</td>
<td>12.50 ± 0.73</td>
<td>8.68 ± 1.60</td>
</tr>
<tr>
<td>Phosphate fertilizer and mining areas (n = 21)</td>
<td>0.28 ± 0.039</td>
<td>0.037 ± 0.005</td>
<td>0.08 ± 0.006</td>
<td>0.25 ± 0.010</td>
<td>6.34 ± 0.63</td>
<td>4.11 ± 1.15</td>
</tr>
<tr>
<td>Coal mining areas (n = 46)</td>
<td>0.35 ± 0.024</td>
<td>0.057 ± 0.003</td>
<td>0.08 ± 0.004</td>
<td>0.21 ± 0.008</td>
<td>4.79 ± 0.31</td>
<td>2.19 ± 0.33</td>
</tr>
<tr>
<td>Steel manufacturing plant (n = 46)</td>
<td>0.50 ± 0.04</td>
<td>0.256 ± 0.02</td>
<td>0.09 ± 0.006</td>
<td>0.26 ± 0.020</td>
<td>3.68 ± 0.25</td>
<td>2.78 ± 0.42</td>
</tr>
<tr>
<td>Aluminium processing plant (n = 25)</td>
<td>0.65 ± 0.028</td>
<td>0.97 ± 0.003</td>
<td>0.05 ± 0.006</td>
<td>0.12 ± 0.014</td>
<td>3.04 ± 0.25</td>
<td>2.24 ± 0.13</td>
</tr>
<tr>
<td>Lead zinc smelter (n = 21)</td>
<td>0.85 ± 0.114</td>
<td>0.78 ± 0.014</td>
<td>0.08 ± 0.009</td>
<td>0.17 ± 0.013</td>
<td>6.18 ± 0.55</td>
<td>3.62 ± 0.95</td>
</tr>
</tbody>
</table>

Respective milk samples were collected from 201 lacting cows and allowed to graze around industrial units; P < 0.05.
The concentrations of Pb were following: in human milk - 8.34 µg.L\(^{-1}\), in raw cow’s milk - 14.82 µg.L\(^{-1}\), in pasteurized cow’s milk - 10.82 µg.L\(^{-1}\), in goat’s milk - 11.86 µg.L\(^{-1}\), and in powdered infant formula - 8.30 µg.L\(^{-1}\).

Data from this study were within the normal ranges for each kind of milk. The Cd and Pb concentrations in goat’s milk were significantly higher than the concentrations observed in the other kinds of milk, whereas human milk and powdered infant formula presented the lowest Cd and Pb concentrations. A considerable decrease in the concentration of Cd within the stage of lactation was observed. The concentrations of Pb and Cd in different kinds of milk also varied according to the time of year. The concentrations of Pb and Cd in different kinds of milk did not represent any risk to human health (infants or adults).

Tunegová et al. (2016) compared the content of selected toxic and trace elements in sheep and cow milk during two seasons (spring and autumn) in Slovakia. They reported that the content of Cd in cow milk was less than 0.0040 mg.kg\(^{-1}\) in spring season and less than 0.0040 mg.kg\(^{-1}\) in autumn season. The content of arsenic was below 0.030 mg.kg\(^{-1}\) in both seasons. In sheep milk they recorded content of Cd below 0.0040 mg.kg\(^{-1}\) and As below 0.030 mg.kg\(^{-1}\) in both seasons. The content of Cd was below 0.030 mg.kg\(^{-1}\) in sheep and cow milks. In cow milk, value of calcium was 915 mg.kg\(^{-1}\) in spring season and 1210 mg.kg\(^{-1}\) in autumn season. Content of calcium in sheep milk was 1770 mg.kg\(^{-1}\) in spring and 2170 mg.kg\(^{-1}\) in autumn.

Weglarzy (2010) reported that cows are susceptible to contamination of the environment with the lead, which deposits in meat and liver, especially at the beginning of the growing season. He found that there is more lead in the cow’s cheese than in milk with the difference being the highest in April and with decreasing during the growing season. In May, the amount of cadmium in milk was lower. Cadmium and lead were depositing mostly in liver, and their amount in this organ was higher in autumn than in spring. Furthermore, the content of cadmium in meat was much higher than the acceptable level.

**CONCLUSION**

The aim of this review was to document and compare the results of previous studies about prevalence of heavy metals in different types of milk and from different parts of the world. We pointed out effects of heavy metals on animal organism and their accumulation in human and animal organism. Majority of the literature sources shows that industry and agriculture are the most common sources of contaminants that are entering the food chain. Therefore, it is important to monitor the areas where milk is produced in order to avoid negative effects of these substances on animal and human organism and to improve the quality of milk, which is the raw material for further food processing.

**ACKNOWLEDGEMENT**

This article was financially supported by the VEGA grant, Ministry of Education, Science, Research and Sport of the Slovak republic No. 1/0292/14.
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Short communication

THE EFFECT OF CURCUMA LONGA PLANT EXTRACT ON THE RABBIT EMBRYO DEVELOPMENT IN VITRO

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ABSTRACT
The aim of this study was to evaluate the effect of Curcuma longa (CL) root extract on the rabbit embryo development in vitro. Totally 113 pronuclear stage zygotes were used in this experiment. Zygotes were divided into 4 groups: control (C; n = 28) and three experimental groups (E1, E2, E3; n = 31, 26 and 39, resp.) with addition of different concentrations of Curcuma longa extract to the culture medium (E1- 0.1 µg.ml⁻¹; E2- 0.01 µg.ml⁻¹; E3- 0.001 µg.ml⁻¹). Zygotes were cultured up to the blastocyst stage (120 h) in 5 % CO₂ at 37.5 °C. At the end of culture period the blastocysts were stained with DAPI fluorochrome for the total cell number determination. Evaluation of embryo developmental potential showed, that higher blastocyst rate was observed in the E2 (61.5 %) and E3 (60 %) groups compared to the control group (46.4 %). In the group with highest CL concentration in culture (E1- 0.1 µg.ml⁻¹) embryo development was stopped at the morula stage. In this group also the lowest (P < 0.001) number of cleaved embryos (19.4 %) compared to the control (60.7 %) and E3 group (82.1 %) was recorded. There were no differences in the blastocysts total cell number among the groups with lower CL concentrations (E2 77.81 ± 13.6; E3 89.25 ± 15.94) and control group (82.23 ± 21.75). On the basis of our results we suppose that Curcuma longa affects rabbit embryo development in a dose-dependent manner. Although lower concentrations showed positive effect, the highest concentration blocked embryo development at morula stage. It is necessary to determine since which concentration Curcuma longa may be toxic for normal embryonic development.

Key words: rabbit; embryos; Curcuma longa extract; in vitro development; DAPI staining

INTRODUCTION
Medical plants are widely used as a source of remedies for the treatment and prevention of many diseases as alternative therapeutic and medical tools (Kaur and Mondal, 2014). Natural products from some plants are used in pharmaceutical preparations either as pure compounds or as extracts (Araújo and Leon, 2001). One of them is Curcuma longa Linn. (Zingiberaceae family), well-known as turmeric, broadly grown in tropical areas of Asia and Central America (Ammon, 1991). This rhizome in powder form is widely used as a food additive for impart flavour and a yellow colour (Miquel et al., 2002). The major constituent, curcumin (diferuloylmethane) is the most important fraction of Curcuma longa (Araújo and Leon, 2001). It has been already demonstrated, that curcuminoids have anti-atherosclerotic (Olszanecki et al., 2005), anti-diabetic (Nabavi et al., 2015), anti-mutagenic, anti-cancer (Goel et al., 2008), antioxidant (Huang et al., 1994; Nishiyama et al., 2005; Wei et al., 2006; Kumar et al. 2007), anti-bacterial (Park et al., 2005), anti-inflammatory

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Received: June 20, 2016
Accepted: August 17, 2016
and anti-fertility (Mishra and Singh, 2009; Ammon and Wahl, 1991; Lantz et al., 2005) activities. Garg et al. (1974; 1978) found out, that aqueous extracts of turmeric rhizome show complete inhibition of embryo implantation in rats when fed orally. Curcumin has the potential for the use in development of novel intravaginal contraceptive (Rithaporn et al., 2003). Thakur et al. (2009) observed significant anti-fertilizing activity and decreasing of FSH and LH levels in blood plasma of albino rat females after oral administration of aqueous or ethanolic extracts from Curcuma longa. Many studies showed strong correlation between antioxidant activity and fertility (Ruder et al., 2009), as well as between free radical accumulation and reduction in fertility (Behrman et al., 2001). The aim of this study was to examine the effect of Curcuma longa Linn. on rabbit embryo development in vitro.

MATERIAL AND METHODS

Animals and superovulation

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. Sexually mature New Zealand White rabbit does from the Department of Small Farm Animals, APRC Nitra were used in this experiment. Superovulation of rabbit does was induced by intramuscular application of 50 IU PMSG (SERGON, Bioveta, a. s. Ivanovice na Hané, Czech Republic) and after 48 hours by 100 µl of HCG (Supergestran, Nordic Pharma s.r.o. Jesenice, Czech Republic) per doe. Before the HCG injection, all rabbit does were artificially inseminated by heterospermic dose of rabbit semen (0.5 ml/doe).

Egg recovery, culture and staining

At 19-20 h post-coitum, rabbit does were humanely slaughtered by the electrical stunning (Relco, Gewiss, Milano, Italy, alternating current 0.3 A/female, frequency 50 Hz, exposition 4s) and reproductive organs were expertly dissected. The pronuclear stage eggs were flushed from the oviducts with PBS (Gibco, Auckland, New Zealand) and subsequently morphologically evaluated. The selected eggs were placed into 4-well dishes (Nunc, Roskilde, Denmark) containing 500 µl of k-DMEM medium (Gibco) supplemented with three different concentrations of Curcuma longa (CL) extract (E1- 0.1 µg.ml⁻¹ CL; E2- 0.01 µg.ml⁻¹; E3- 0.001 µg.ml⁻¹) and cultured up to 120 hours post-coitum in 5 % CO₂ at 37.5 °C (the time point to reach the blastocyst stage). After the culture, embryos were washed in PBS with polyvinylpyrrolidone (PBS-PVP, 4 mg.ml⁻¹) for 3 x 5 min, stained with 4 µl of Vectashield mounting medium with DAPI (Vector Laboratories, Burlingames, CA, USA) and mounted between the microslide and coverslip. Total cell number was counted under a Zeiss fluorescence microscope equipped with a specific wave-length filter (Fig. 1).

Fig. 1: Representative image of the embryo cultured with the lowest concentration (0.001 µg.ml⁻¹) of Curcuma longa extract. A: light microscopy; B: fluorescence microscopy (cells stained by DAPI fluorochrome)
Statistical analysis

The data were analysed by Pearson’s Chi-square test.

RESULTS AND DISCUSSION

To examine the effect on embryo development we cultured rabbit pronuclear stage eggs in the medium enriched with three different concentrations of *Curcuma longa*.

The highest concentration of CL (E1 group) had negative effect on the embryo development, as was shown by decreasing (P < 0.001) cleavage rate (19.4 %) compared to the control and the other experimental groups (C: 60.7 %, E2: 69.2, E3: 82.1 %).

The lowest concentration of CL (E3 group) increased (P < 0.001) cleavage rate (82.1 %) when compared to control. Development to the blastocyst stage was completely stopped in the E1 group, whereas blastocyst rates in the E2 (61.5 %), E3 (60 %) and C (46.4 %) groups were not statistically different (Chi-square test).

Negative effect of *Curcuma longa* on reproduction was already reported on granulosa cells of porcine ovary, where it inhibited proliferation (accumulation of PCNA) and induced apoptosis (accumulation of bax) (Kádasi et al., 2012; Voznesenska et al., 2010; Bhaumik et al., 1999). In our study, the highest concentration (0.1 μg.ml⁻¹) of CL in the culture stopped embryo development at the morula stage. Possible explanation could be the stimulation of apoptotic process due to the toxicity of mentioned concentration in culture. At the blastocyst stage apoptosis is responsible for the elimination of undesirable cells during the normal embryonic development (Hardy et al., 2003). However, increased occurrence of apoptosis before or during the blastocyst stage probably removes important cell lineages, what might negatively affect embryonic development and lead to embryo degeneration (Long et al., 2000). Although, the objective of our study was not an evaluation of apoptosis incidence, the similar total cell number in each group indicates that there is no a developmental delay and increased apoptosis incidence. A similar conclusion was reported by Chen et al. (2010), who applied *Curcuma longa* to mouse embryo culture. On the basis of the blastocyst development evaluation by differential staining, the authors found that higher concentration (24 μM.ml⁻¹) of the CL extract induced apoptosis in the ICM but not in trophoblastic cells. Nevertheless, lower concentrations (6 and 12 μM.ml⁻¹) did not affect the apoptosis incidence or cell number. Likewise, in our study similar blastocyst cell numbers in the groups with lower CL concentration (E2: 77.81 ± 13.6; E3: 89.25 ± 15.94) and control group (82.23 ± 21.75) were found. Because none of the embryos were developed to blastocyst stage in the group with the highest concentration, counting of the total cell number in this group was not performed.

CONCLUSION

According to our results we can conclude that the highest concentration of *Curcuma longa* root extract added to culture medium negatively affects embryo cell number and terminates embryo development at the morula stage.

ACKNOWLEDGEMENT

This study was supported by the grants APVV-0854-11, KEGA-011UPJŠ-412014 and VEGA 1/0611/15.

Table 1: Effect of different Curcuma longa concentrations on rabbit embryo development

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of embryos (n)</th>
<th>Cleavage rate (%)</th>
<th>Morula rate (%)</th>
<th>Blastocyst rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>28</td>
<td>17/60.7b</td>
<td>4/14.3c</td>
<td>13/46.4f</td>
</tr>
<tr>
<td>E1</td>
<td>31</td>
<td>6/19.4a</td>
<td>6/19.4a</td>
<td>0/0c</td>
</tr>
<tr>
<td>E2</td>
<td>26</td>
<td>18/69.2b</td>
<td>2/7.7d</td>
<td>16/61.5f</td>
</tr>
<tr>
<td>E3</td>
<td>39</td>
<td>32/82.1b</td>
<td>7/17.9e</td>
<td>23/60.0f</td>
</tr>
</tbody>
</table>

Level of significance: P<0.001 a:b and e:f; P<0.01 c:d
C- control group, E1- 0.1 μg.ml⁻¹ of Curcuma longa in the culture, E2- 0.01 μg.ml⁻¹ of Curcuma longa in the culture, E3- 0.001 μg.ml⁻¹ of Curcuma longa in the culture
REFERENCES


THE EFFECT OF GREEN TEA ADDITION TO DIET ON WEIGHT GAINS OF RABBIT FEMALES

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ABSTRACT

The aim of present study was to evaluate the effect of different concentrations of green tea plant (Camellia sinensis) addition to the diet on the weight gains of rabbits. Rabbit females (n = 31) of New Zealand White breed were used in the experiment. Rabbit does in the control group (C; n = 12) were fed with a commercially available feed. In the experimental groups, 5 g (E1; n = 9) and 20 g (E2; n = 10) of green tea dried powder were added to 100 kg of commercially available feed. The lower weight gains per week (g) were observed in both experimental groups (E1; 229.7 ± 21.95 and E2; 223.09 ± 24.00 g, respectively) when compared to control (C; 242.58 ± 19.76 g). Total average weight gain was the highest in control (C; 2668.3 ± 97.61 g) when compared to the both experimental groups (E1, 2526.67 ± 79.64 g; E2, 2454.0 ± 118.09 g).

In conclusion, addition of green tea powder to the commercial diet for rabbit does had negative effect on the weight gains (g) per week and on the total average weight gains (g) during the fattening period.

Key words: rabbit females; green tea; weight gains

INTRODUCTION

Nowadays, biologically active substances and extracts are used worldwide particularly in terms of their stimulation and therapeutic effects (Park et al., 2014). More suitable composition of feed mixture or administration of natural additives at an appropriate concentration might be beneficial in livestock farming without negative effect on the environment and the animals as an individual (Abdel-Wareth et al., 2014). Plant polyphenols are natural antioxidants and most of their pharmacological properties are considered to be due to their antioxidant action (Ames et al., 1995). The most important polyphenolic compounds in green tea are catechins: epigallocatechin-3-gallate (EGCG), epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC), catechin and gallo catechin (GC) (Wang et al., 2013). Green tea contains also high levels of other bioactive phenols, such as caffeine. EGCG, the most abundant catechin in green tea, accounts for 65 % of the total catechin content. A cup of green tea may contain 100–200 mg of EGCG. Catechin and gallo catechin are present in trace amounts (Chu and Juneja, 1997).

Green tea polyphenols (GTPP) are known for their preventive, antibacterial and therapeutic effects, anticancer and apoptosis inducing-properties. These molecules prevent neural cell death and induce chromosomal damage in lymphoblastoid cell lines (Reznichenko et al., 2005; Sugisawa and Umegaki, 2002). Some epidemiological and clinical studies have shown the health benefits of EGCG on obesity...
and diabetes (Rains et al., 2011) and the underlying mechanisms involve modulations of energy balance, endocrine systems, food intake, lipid and carbohydrate metabolism (Chacko et al., 2010).

The studies have suggested that the extent of absorption of dietary polyphenols in the small intestine is relatively small (10–20 %). The majority of ingested polyphenols will reach the large intestine where they encounter the colonic microflora (Spencer, 2003). The colon contains microorganisms having an enormous catalytic and hydrolytic potential. This enzymatic degradation of flavonoids by the colonic microflora results in a huge array of new metabolites. These polyphenols do not interfere with the microflora of the colon (Zdunczyk et al., 2002).

Multiple in vitro studies on catechins report mechanisms consistent with protection against degenerative diseases (Crespy and Williamson, 2004). Green tea catechins (GTC) have been reported to possess multiple properties, such as cancer prevention, hypotensive effects, anti-viral and antioxidant properties, inhibition of plaque formation, anti-allergic potential and blood glucose-lowering effects (Katiyar and Mukhtar, 1996; Matsumoto et al., 1993).

Green tea has not been tested in rabbits yet, so the objective of this study was to evaluate the effect of different concentration of green tea powder addition to the diet on the total and average weight gains of the rabbit does.

MATERIAL AND METHODS

Animals

Two months old clinically healthy rabbit does of the New Zealand White line (NAFC Nitra, SR) were used in this experiment. The animals were housed in individual cages, under a constant photoperiod of 14 hours of light. Temperature and humidity in the building were recorded continuously by means of a thermograph positioned at the same level as the cages (average relative humidity and temperature during the year was maintained at 60 ± 5 % and 17 ± 3 °C). The rabbits were fed ad libitum and water was provided ad libitum with nipple drinkers.

Rabbit does (n = 31) were divided into three groups: control (C; n = 12) and two experimental groups (E1; n = 9 and E2; n = 10). The does in the control group were fed with a commercially available complete feed mixture. Green tea (right loose green tea, made in China and distributed by Oxalis, Czech Republic) was added at two different concentrations (E1: 5 g; E2: 20 g) to the 100 kg of the complete feed mixture in both experimental groups. The animals were fed for 77 days and weighted weekly.

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.

Statistical analysis

All values are expressed as means ± S.E.M. Differences between the control and experimental groups were evaluated by one-way ANOVA test using the SigmaPlot 11 software (Systat Software Inc., Erkharth, Germany).

RESULTS AND DISCUSSION

In our study the effect of different concentrations of green tea plant powder added to the diet on the total and average weight gains (g) of the rabbit does was evaluated.

The highest average weight gain of rabbit does per week (g) was recorded in the control group (C; 242.58 ± 19.76 g) when compared to the experimental (E1; 229.7 ± 21.95 and E2; 223.09 ± 24.00, respectively) groups (Figure 1). Similarly, total average weight gain (g) was the highest in the control group (C; 2668.3 ± 97.61 g) compared to the experimental (E1; 2526.67 ± 79.64 and E2; 2454.0 ± 118.09 g, respectively) groups, although these differences were not statistically significant (Figure 2).

Decreasing of weight in other animal species fed with green tea was also reported. In particular, in the study of Hamdaoui et al. (2003) the weight gains were evaluated in rats fed a commonly consumed Tunisian meal ‘bean seeds ragout’ (BSR), with or without beef and with black or green tea decoction. Both, black and green teas significantly reduced the weight gains, where the black tea decoction had the strongest effect.

![Fig. 1: Average weight gains (g) per week of rabbit does fed with green tea plant added into complete feed mixture](image)
Ito et al. (2008) found out that the administration of 0.5% catechins decreased the body fat accumulation and levels of serum cholesterol and bile acids. These results indicate that green tea catechins modulate lipid metabolism in obese, but also in the non-obese subjects.

Snoussi et al. (2014) found that chronic administration of green tea decoction (GTD) in rat fed high-fat diet reduced body weight gain, circulating triglycerides and cholesterol and improved glucose tolerance. On the other hand, administration of GTD did not affect food intake, suggesting that the reduced feed intake does not correspond to the reduction in weight gain.

Morita et al. (2009) administered green tea catechins to pregnant rats at two concentrations (2000 mg.kg\(^{-1}\) and 600 mg.kg\(^{-1}\) day) and observed the reduction in the weight gain (P < 0.05). However, heat-sterilized green tea catechin (GTC-H) administration did not affect mean gravid uterine weights or intrauterine growth and survival.

The results of Sayama et al. (2000) indicated that lipid metabolism in mice was suppressed by the administration of green tea powder and, thereby, the fatty accumulation and body weight increase was suppressed.

In mice fed a high-fat diet (60% energy as fat), supplementation with dietary EGCG treatment (3.2 g.kg\(^{-1}\) diet) for 16 weeks reduced body weight gain, body fat percentage and visceral fat weight (P < 0.05) compared to mice without EGCG treatment (Bose et al., 2008). Their results indicate that long-term EGCG treatment attenuated the development of obesity, symptoms associated with the metabolic syndrome and fatty liver. Short-term EGCG treatment appeared to reverse pre-existing high-fat-induced metabolic pathologies in obese mice. These effects may be mediated by decreased lipid absorption and decreased inflammation.

Similarly, Lu et al. (2012) reported beneficial effects of green tea polyphenols (GTP) on body weight via regulating obesity-related genes, anti-inflammation, anti-oxidant capacity and estrogen-related actions in high-fat-induced obese rats.

Weight gains observed in our experiment were slightly lower in the experimental groups compared to the control group, but the differences were not statistically significant. It can be explained by variability between females (very high S.E.M. values).

Similarly as in our study, Juśkiewicz et al. (2008) found out, that supplementation of a diet with green tea extract had no significant influence on elevated food intake and body weight loss.

**CONCLUSION**

In conclusion, addition of green tea powder to the commercial diet for rabbit does can potentially decrease the weight gains per week and the total average weight gains during the fattening period.

**ACKNOWLEDGEMENT**

This study was supported from the grant of the Slovak Research and Development Agency: APVV-0854-11, by the grants VEGA 1/0611/15 and KEGA-011UPJS-412014.

**REFERENCES**


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