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QUALITY OF FRESH AND FROZEN-THAWED SEMEN FROM SLOVAK NATIVE RABBIT AND ITS STORAGE IN THE GENE BANK

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ABSTRACT

Aim of our work was to compare quality of fresh and frozen-thawed semen of four Slovak native rabbit breeds. In this study, semen from Nitra (n = 4), Zobor (n = 4), Holic Blue (n = 4) and Pastel Rex (n = 2) rabbit breeds was evaluated for possible inter-breed differences in fresh and frozen-thawed sperm quality traits. Individual male semen was diluted (v:v; 1:1) in a freezing medium composed of a commercial diluent, 16 % of DMSO, 4 % of Ficoll 70 and 2 % of sucrose and frozen in liquid nitrogen vapours before plunging into liquid nitrogen (LN_2). Different motility traits, viability and plasma membrane integrity of fresh and frozen-thawed semen were evaluated *in vitro* using CASA and flow-cytometry. Our results revealed several differences in motility parameters among the breeds of rabbit. Fresh sperm of Slovak Pastel Rex rabbit showed the lowest ($P \leq 0.05$) motility values when compared to the others. In terms of frozen-thawed semen, sperm total motility was similar among Nitra, Zobor and Pastel rabbit, while Holic showed higher ($P \leq 0.05$) total motility than Pastel. In addition, Pastel showed the lowest ($P \leq 0.05$) progressive movement among the breeds. Similarly to motility analysis, the highest ratio ($P \leq 0.05$) of dead sperm and plasma membrane damage among the fresh sperm samples was found in Pastel semen. On the other hand, both Nitra and Pastel rabbits showed lower ($P \leq 0.05$) viability of frozen-thawed semen than Holic and Zobor rabbit. In conclusion, this study confirmed variability in quality parameters measured *in vitro* among four Slovak native breeds of rabbit. Therefore, selection of good-quality insemination doses should be done in order to create a reserve of genetic variability of domestic rabbit in Slovakia.

Key words: rabbit; sperm; cryopreservation; quality trait

INTRODUCTION

Rabbit breeds vary extensively in weight, body conformation, fur type, coat colour, ear length, and this visible morphological variation dramatically exceeds the phenotypic diversity of their wild counterparts (Carneiro *et al.*, 2011). National breeds belong to the cultural heritage of the country in which they were bred. Slovak breeders gave rise to ten national rabbit breeds so far (Supuka *et al.*, 2012).

Due to the specificity of rabbit sperm membrane, fast cooling rates from room temperature to 5 °C are likely possible in this species. This fact might indicate that cold shock is not a major problem for rabbit sperm and that protocols for sperm cryopreservation in this species could be shortened (Moce *et al.*, 2003; Moce and Vicente, 2009). Nevertheless, differences between rabbit breeds in the resistance of sperm to cryopreservation process were reported (Moce *et al*., 2003). These differences among breeds and lines studied could be explained by genetic, age and environmental factors and also by the different evaluation criteria, sample size and semen processing methodologies applied (Safaa *et al*., 2008).

In our study, we aimed to evaluate quality of fresh and frozen-thawed semen of four Slovak native rabbit breeds (Nitra, Zobor, Holic Blue and Pastel Rex) in order to find possible differences among the breeds.

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MATERIAL AND METHODS

Chemicals

Unless stated otherwise, all chemicals were purchased from Sigma Aldrich (Germany).

Animals

Fourteen clinically healthy rabbit males of Nitra (n = 4), Zobor (n = 4), Holic blue (n = 4) and Slovak Pastel Rex (n = 2) breed were used in our study. Rabbit males were kept at the Institute of Small Farm Animals, Research Institute for Animal Production Nitra, NPPC (Nitra and Zobor rabbit) or at the Supuka Farm (Holic Blue and Slovak Pastel Rex). Animals were housed individually, fed with a commercial diet (KV; TEKRO Nitra s.r.o., Slovakia) and watered *ad libitum*. The photoperiod used was a ratio of 14L: 10D. The temperature in the halls was kept at 15 to 25 °C.

Semen collection and cryopreservation

Semen was collected from sexually mature rabbit males using a pre-heated artificial vagina once a week in a regular manner. The semen was transported to the laboratory in a water bath at 37 °C and processed individually. For each male, four semen samples were collected and used for cryopreservation and *in vitro* evaluation (fresh and frozen-thawed). Only ejaculates that exhibited white colour were used in the experiments. Samples containing urine and cell debris were discarded, whereas gel plugs were removed.

Semen from individual males was frozen using a rapid freezing method described previously (Kulíková *et al.*, 2014). Individual semen samples were cooled down to 5 °C for 90 min in a fridge to minimize cold-shock damage. After cooling, an aliquot of semen was diluted in a freezing medium (5 °C) up to the concentration of 500 x 10^6.mL^{-1} consisting of a commercial diluent (DMRS; Minitube, Germany) dissolved in a Milli-Q water (Milli Pore; Lambda Life a.s., Slovakia) and mixed with 16 % dimethyl sulfoxide (DMSO), 4 % of Ficoll 70 and 2 % sucrose in a ratio of 1:1 (v:v) to give the final concentration of 8 %, 2 % and 1 % of DMSO, Ficoll 70 and sucrose, respectively. Thereafter, the semen was loaded into 0.25 mL plastic straws and equilibrated at 5 °C for 45 min. The straws were suspended horizontally in liquid nitrogen vapours (LNV) 5 cm above the liquid nitrogen (LN) level for 10 min (-125 to -130 °C) before being plunged into the LN (-196 °C) for storage. For thawing, the straws were immersed into water bath at 50 °C for 10-13 s.

Motility assay

An aliquot taken from each fresh and frozen-thawed rabbit semen sample was used for motility analysis immediately after collection/thawing. Semen was diluted in a saline solution (0.9 % NaCl; Braun, Germany) in a ratio of 1:8 (v:v), immediately placed into Standard Count Analysis Chamber Leja (depth of 20 microns) (MiniTüb, Tiefenbach, Germany) and evaluated under a Zeiss Axio Scope A1 microscope using the CASA system (Sperm Vision™; MiniTübe, Tiefenbach, Germany). For each sample seven microscopic view fields were analysed for average concentration (CON; 1 x 10^6) and percentage of total motility (TM; motility > 5 $\mu m.s^{-1}$), progressively moving spermatozoa (PM; motility > 20 $\mu m.s^{-1}$), velocity curved line (VCL; $\mu m.s^{-1}$), velocity average path (VAP; $\mu m.s^{-1}$), velocity straight line (VSL; $\mu m.s^{-1}$), amplitude of lateral head displacement (ALH; μm), linearity (LIN; VSL/VCL), straightness (STR; VSL/VAP) and beat cross frequency (BCF; Hz).

Viability and plasma membrane integrity assay

To assess the viability and plasma membrane integrity of the frozen-thawed sperm each sample was fluorescently stained with SYBR-14 (viable sperm), propidium iodide (PI; necrotic sperm) and fluorescently-labelled lectin from peanut agglutinin (*Arachis hypogea*; PNA; plasma membrane damage). For SYBR/PI staining, approximately 1 × 10^6 sperm were added to 250 μL of phosphate-buffered saline (PBS) containing 100 nM SYBR-14 and incubated for 15 min in a dark. Afterwards, 4 μL of PI (50 $\mu g.mL^{-1}$) were added to reach the final concentration of 1 $\mu g.ml^{-1}$ PI. Thereafter, samples were immediately analysed using BD FACS Calibur flow cytometry analyser.

For PNA staining, approximately 1 ×10^6 sperm were washed in PBS and centrifuged at 300 g × for 6 min. The semen pellet was resuspended in 50 μL of PBS with PNA (0.05 $\mu g.mL^{-1}$) and incubated in a dark for 15 min. Thereafter, sperm were washed in PBS and centrifuged at 300 g for 6 min. The pellets were resuspended in PBS and 4 μL of PI (50 $\mu g.mL^{-1}$) was added to reach the final concentration of 1 $\mu g.ml^{-1}$ PI. Samples were immediately assessed using BD FACS Calibur flow cytometry analyser. At least, 10,000 events were analysed for each sample. The emitted green fluorescence of SYBR-14 or PNA positive cells and red fluorescence of PI positive cells were recorded in the FL-1 and FL-3 channels, respectively. The different labelling patterns in bivariate analysis (e.g. PNA/PI) identified four different sperm populations: viable sperm with intact plasma membrane (PNA^-/PI^-); viable sperm with damaged plasma membrane (PNA^+/PI^-); dead sperm with damaged plasma membrane (PNA^+/PI^+) and dead sperm with damaged plasma membrane and lost acrosome (PNA^-/PI^+).

Statistical analysis

Sperm quality among the four breeds was compared by a one-way ANOVA (Tukey test) using Sigma Plot

software (Systat Software Inc., Germany). Values at $P \leq 0.05$ were considered as statistically significant.

RESULTS AND DISCUSSION

Variations in the susceptibility of semen to cryogenic process among breeds (Auerbach *et al*., 2003; Mocé *et al.*, 2003; Long, 2006; Waterhouse *et al*., 2006) and males of the same breed (Thurston *et al*., 2002; Mocé *et al.*, 2005; Waterhouse *et al*., 2006; Lavara *et al*., 2013; Sellem *et al.*, 2015) have been reported for several species. Therefore, in order to facilitate long-term storage of good-quality insemination doses in a gene bank, regular assessment of fresh and frozen-thawed semen is necessary (Kulíková *et al*., 2017). Herein, we aimed to evaluate quality of fresh and frozen-thawed semen of four Slovak native rabbit breeds in order to evaluate potential differences among the breeds.

According to our results, fresh sperm of Slovak Pastel Rex (Pastel) showed the lowest ($P \leq 0.05$) TM, PM, VAP and BCF when compared to the others. Nitra and Zobor rabbit sperm showed higher ($P \leq 0.05$) VCL than Holic and Pastel rabbit. Sperm concentration, STR, LIN, VSL and ALH were similar among the breeds (Table 1).

Table 1: Concentration and motility parameters of fresh semen from four rabbit breeds

Fresh	CONC	TM	PM	VAP	VCL	VSL	STR	LIN	ALH	BCF
Nitra	0.747 ± 0.1	78.4 ± 1.1^{a}	65.8 ± 1.3^{a}	69.2 ± 2.0^{ab}	140.6 ± 4.6^{a}	51.3 ± 3.2	0.78 ± 0.02	0.41 ± 0.03	4.5 ± 0.1	30.7 ± 1.3^{a}
Zobor	0.88 ± 0.3	79.9 ± 0.8^{a}	66.6 ± 1.2^{a}	73.4 ± 3.6^{a}	142.5 ± 3.9^{a}	53.5 ± 6.1	0.72 ± 0.03	0.35 ± 0.03	4.7 ± 0.3	29.2 ± 1.8^{a}
Holic	0.82 ± 0.4	83.9 ± 2.1^{a}	72.4 ± 3.9^{a}	60.5 ± 2.4^{b}	111.9 ± 5.6^{b}	46.6 ± 2.6	0.76 ± 0.02	0.41 ± 0.02	4.5 ± 0.1	28.4 ± 1.1^{a}
Pastel	0.521 ± 0.1	66.3 ± 3.4^{b}	47.7 ± 0.3^{b}	53.1 ± 2.7^{c}	107.6 ± 6.8^{b}	39.7 ± 2.6	0.03 ± 0.01	0.36 ± 0.01	4.6 ± 0.2	22.7 ± 1.2^{b}

Different superscripts within column mean statistical difference ($P \leq 0.05$); ^{ab} vs ^{a,b} is not different.

Table 2: Motility parameters of frozen-thawed semen from four rabbit breeds

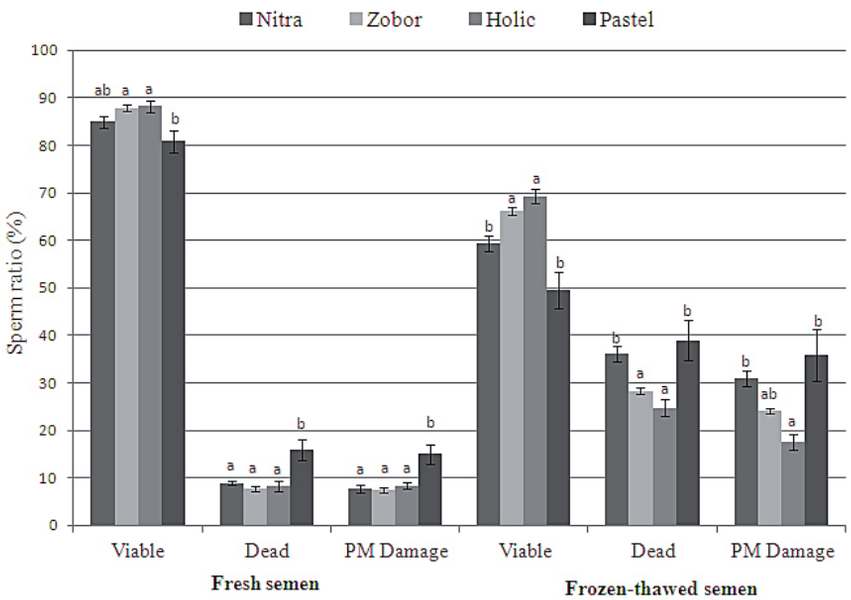
Frozen	TM	PM	VAP	VCL	VSL	STR	LIN	ALH	BCF
Nitra	38.4 ± 1.1^{ab}	25.7 ± 1.9^{a}	58.8 ± 2.1	121.7 ± 6.3	40.9 ± 3.1	0.69 ± 0.02	0.32 ± 0.02	5.1 ± 0.2	22.3 ± 0.8
Zobor	40.3 ± 2.2^{ab}	26.7 ± 2.1^{a}	54.1 ± 3.3	109.2 ± 5.4	35.1 ± 1.3	0.63 ± 0.02	0.32 ± 0.01	4.8 ± 0.2	19.7 ± 0.8
Holic	44.8 ± 2.6^{a}	32.9 ± 3.2^{a}	49.6 ± 2.5	103.5 ± 5.2	32.3 ± 1.8	0.71 ± 0.02	0.36 ± 0.01	4.5 ± 0.2	22.2 ± 0.3
Pastel	32.2 ± 3.5^{b}	16.3 ± 0.1^{b}	51.1 ± 0.7	104.3 ± 2.4	34.5 ± 0.1	0.67 ± 0.01	0.33 ± 0.01	5.6 ± 0.7	20.1 ± 0.3

Different superscripts within column mean statistical difference ($P \leq 0.05$); ^{ab} vs ^{a,b} is not different.

The total motility (TM) of frozen-thawed semen was similar among Nitra, Zobor and Pastel rabbit, while Holic showed higher ($P \leq 0.05$) TM than Pastel. In addition, Pastel showed the lowest ($P \leq 0.05$) progressive movement (PM) among the breeds. Other motility parameters were similar.

Similarly to motility analysis, the highest ratio ($P \leq 0.05$) of dead sperm and plasma membrane damage among the fresh sperm samples was found in Pastel semen. On the other hand, in terms of frozen-thawed semen, both Nitra and Pastel showed lower ($P \leq 0.05$) viability than Holic and Zobor rabbits (Figure 1). These results correspond with our previous study where significant effect of breed (Nitra vs Zobor) on proportion of frozen-thawed live/dead sperm was found (Kulíková *et al.*, 2017). On the other hand, ratio of plasma membrane damage was similar among frozen-thawed semen samples of Nitra, Zobor and Pastel rabbits. Only Holic showed lower ($P \leq 0.05$) damage when compared to Pastel and Nitra rabbits.

Altogether, fresh and frozen-thawed semen of Slovak Pastel Rex rabbit showed the lowest quality when compared to Nitra, Zobor and Holic rabbit. According to these results, insemination doses of Nitra, Zobor and Holic rabbit can be stored in a gene bank for later use after appropriate selection (post-thaw TM ≥ 35 %



PM – plasma membrane; Different superscripts mean statistical difference ($P \leq 0.05$); ^{ab} vs ^{a,b} is not different.

Fig. 1: Viability and plasma membrane integrity of fresh and frozen-thawed sperm from the four rabbit breeds

and PM ≥ 25 %). Pastel rabbit semen samples were not of desired quality in this study and therefore were discarded. Nevertheless, it should be taken into account that only two males of Pastel Rex rabbit were available in our experiment. Results of previous works indicate that relatively high part of the observed phenotypic variance is due to male-related sources of variation (Lavara *et al.*, 2013; Sellem *et al.*, 2015; Kulíková *et al.*, 2017). Therefore, semen quality assessment of higher number of males should be done to verify possibility of Pastel Rex semen to be stored in a gene bank of animal resources.

CONCLUSION

Our study confirmed variability in quality parameters measured *in vitro* among four Slovak native breeds of rabbit. Therefore, selection of good-quality insemination doses should be done in order to create a reserve of genetic variability of domestic rabbit in Slovakia.

ACKNOWLEDGEMENT

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FACTORS AFFECTING GROWTH IN NATIVE ORAVKA CHICKEN BREED

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ABSTRACT

The objective of this study was to analyse the effect of the most important factors affecting growth (body weights of 5-, 12- and 20-week old males and females) in Oravka chickens. In total, 359 individuals of conservation flock (operated by the National Agriculture and Food Centre – Research Institute of Animal Production Nitra) were studied. The mixed model included fixed effects of sex, age, rooster/breeding season, an interaction of sex and age nested within rooster/breeding season and random effect of bird due to repeated weights of each individual. All fixed effects highly significantly ($P < 0.01$) influenced body weight of birds; repeatability attributable to among-individual variation was estimated to be about 35 % proportion of variance. With respect to rooster/breeding season, body weights in 5-week old birds (between 384.62 ± 32.11 g and 572.04 ± 23.39 g for males vs. between 343.33 ± 35.73 g and 497.71 ± 26.10 g for females) were non-significant ($P > 0.05$); body weights in 12-week old birds (between 1299.51 ± 26.85 g and 1464.29 ± 23.39 g for males vs. between 1071.90 ± 25.27 g and 1134.83 ± 25.57 g for females) were either non-significant ($P > 0.05$) or significant ($P < 0.05$); body weights in 20-week old birds (between 2237.24 ± 31.17 g and 2633.76 ± 47.84 g for males vs. between 1615.77 ± 34.80 g and 2056.84 ± 37.56 g for females) were at least significant ($P < 0.05$).

Key words: genetic resources; chicken; body weight; growth factors

INTRODUCTION

Poultry breeding has undergone enormous changes during the last decades. The antagonistic relationship between meat and laying performance led the poultry industry to the separation of two lines of production: meat production and egg production (Siegman and Neumann, 2005).

Body weight, a high heritability trait, is an important economic factor mainly for broiler chickens reflecting the production level and economic benefits of a farm. The growth rate is related primarily to genetic factors, expression of which depends on the environmental factors (Gerken *et al*., 2003). A common practice in poultry production is to measure the increase in body mass of birds to control and modify the external conditions that affect their weight gain (Oliveira *et al.*, 2000; Agudelo Gómez *et al.*, 2008; Aggrey, 2009).

At present, with the growing demand for poultry products from extensive systems, an opportunity arises to increase the importance of native chicken breeds, which are particularly suitable for free range and organic farming because of their good adaptation to the local conditions. This is confirmed by the experience of many countries, in which native breeds of slow-growing chickens provide good-quality meat, which is in increasing demand (Fanatico *et al*., 2005 a, b; Youssao *et al.*, 2009; Smith *et al.*, 2012; Yin *et al*., 2013; Choo *et al.*, 2014; Walley *et al.*, 2015). Compared to fast-growing broilers, native chicken breeds and their hybrids show lower weight gain and smaller proportion of breast muscle in the carcass compared to fast-growing broilers, but their meat has many quality characteristics valued by modern consumers (Sokolowicz *et al.*, 2016).

Oravka chicken is a dual purpose breed of Slovakia provenience; originated with the intention

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of having been well adapted to less favourable environment. Population numbers of this breed, which has the status of the only native chicken in Slovakia, are available from the National/European Farm Animal Biodiversity System (http://efabis-sk.cvzv.sk) providing data on farm animal breeds from all around the world. Development of Oravka chicken started in 1950-ies under the guidance of the Research Institute for Poultry by combinatorial crossing of regional breeds with breeds of Rhode Island Red, New Hampshire and Wyandotte White (Chmelničná, 2004). The goal was to develop a breed suitable for harsh climatic conditions of northern Slovakia, which can be kept in free range. It is adapted for egg and meat production and was recognized as an independent breed in 1990. According to breed standard, it is of hard rectangular frame, body weight of males is between 2.8 and 3.3 kg, body weight of females is between 2.2 and 2.7 kg, egg laying ranges from 180 to 200 pcs per year; eggs are of a brownish shell, their average weight is about 55 g.

Some knowledge on growth ability in Slovak chicken breeds is available from earlier works (Malík and Malíková, 1993; Hrnčár *et al*., 2010). To take into account most recent data, this study is aimed at analysis of growth in Oravka birds in dependence on various effects that are assumed to have an influence on body weight. Also, Oravka's growth ability was compared with growth ability of some native and indigenous breeds kept worldwide.

MATERIAL AND METHODS

The birds of conservation flock are kept at the farm of the National Agriculture and Food Centre and operated by the Research Institute for Animal Production (RIAP) Nitra, considered as the *ex situ* flock, were included into the experiment. Breeding males, assumed to be of no genetic ties with breeding females, were used in mating each season (from 2011 to 2015). Thus, descendants of mating between breeding females produced at the farm of the RIAP Nitra and roosters produced out of it were weighed at age of 5, 12 and 20 weeks using the BAT 1 manual poultry scale (produced by VEIT Electronics, Czech Republic). Birds were kept in closed heating nurseries on deep litter (20 chickens per square meter) until 12 weeks of the age; afterwards, they were housed in unheated poultry house, also on deep litter (12 chickens per square meter). Birds were fed (*ad libitum*) the same feed for light chicken (according to age categories); water was available during the whole experiment. Because older birds had to cope with actual weather conditions (free range available) which might vary among seasons, season was considered as the effect that needs to be accounted for. Roosters as sires of next generation were replaced each season, therefore, variance of roosters was hardly possible to distinguish from variance of breeding seasons, and the overlapping effect of rooster/breeding season was considered. A total, 359 individuals that were weighed at least two times during the experiment (five-year duration from 2011 to 2015) were included in analysis. Over rooster/breeding season, individuals were distributed as follows: 90 (1), 47 (2), 80 (3), 54 (4) and 88 (5), respectively.

Statistical analysis was done using the SAS 9.2 statistical programme (2009); the mixed model methodology using MIXED procedure was applied to study the influence of effects causing variation of body weight in Oravka chicken. The model was as follows:

$$y_{ijkl} = \mu + S_i + A_j + R_k + S_i A_j (R_k) + u_l + e_{ijkl}$$

where:
y_{ijkl} – individual body weights
μ – intercept
S_i – fixed effect of sex class (male, female); $\sum_i S = 0$
A_j – fixed effect of age (5, 12, 20 weeks); $\sum_j A = 0$
R_k – fixed effect of rooster/breeding season (1, 2,... 5); $\sum_k R = 0$
$S_i A_j (R_k)$ – interaction of sex x age nested within rooster/breeding season; $\sum_{ijk} SAR = 0$
u_l – random effect of bird (1, 2,... 359); $u_l \sim N(0, I\sigma_u^2)$
e_{ijkl} – random error; $e_{ijkl} \sim N(0, I\sigma_e^2)$

Fixed effects included in the model were estimated using the Least Squares Means (LSM) method. Statistical significances of fixed effects were tested by Fischer's F-test; statistical significances of individual differences between estimated levels of fixed effects were tested by Scheffe's multiple range tests. Differences were considered significant when $P < 0.05$. Bird and residual error variances were estimated using the Restricted Maximum Likelihood (REML) method. Repeatability of body weight in Oravka chicken, estimated taking into account individual bird variances and residual variance: $r^2 = \frac{\sigma_u^2}{\sigma_u^{2+} \sigma_e^2}$ can be interpreted as the proportion of total variance attributable to among-individual variation.

RESULTS AND DISCUSION

Analysis of variance of fixed effects affecting body weight of chicken is given in Table 1. All fixed effects (sex, age of bird, rooster/breeding season as well as interaction between sex and age nested within rooster/breeding season) included in the model were of highly significant influence ($P < 0.01$). The difference in body weights between males (1433.24 ± 12.24 g) and females (1115.01 ± 10.24 g) was 318.22 ± 15.83 g

in favour to males (Table 2).

According to age, the differences in body weights of Oravka chicken found in this study were following: 829.21 ± 10.31 g (between 5- and 12-week old birds), 901.55 ± 15.89 g (between 12- and 20-week old birds) and 1730.76 ± 15.90 g (between 5- and 20-week old birds) in favour to birds of a higher age. Body weights of 5-, 12- and 20-week old birds were estimated as following: 420.80 ± 9.05 g, 1250.01 ± 9.05 g and 2151.56 ± 15.10 g (Table 2). Body weights of 12- and 20-week old birds were found similar to values reported by Hrnčár *et al.* (2010): 1128.53 ± 118.85 g and 1871.85 ± 146.86 g for Oravka breed in field test. Estimated body weights were higher than values reported by Galeano-Vasco *et al.* (2014) for Colombian Lohmann LSL chicken evaluated at the age of 36 days (301.23 ± 49.51 g), at the age of 85 days (902.33 ± 80.79 g) and at the age of 144 days (1561.72 ± 95.04 g). In addition, Zhao *et al.* (2015) reported both lower and higher body weights for indigenous China chicken breeds: 281.81 ± 69.32 g (Shaobo), 512.69 ± 79.96 g (Youxi) and 519.97 ± 88.63 g (Huaixiang) evaluated at the age of 5 weeks. With four varieties of native Assel chicken in Pakistan, Jatoi *et al.* (2014) reported almost two times lower body weights for 4-week old birds when comparing with 5-week old Oravka birds: 202.05 ± 4.29 g (Lakha), 219.79 ± 5.60 g (Mianwali), 229.60 ± 7.24 g (Mushki), 210.60 ± 5.90 g (Peshawari) indicating that body weights of 5-week old Pakistan chicken were unable to reach the same values as Oravka chicken. Also, body weights of 12-week old chicken were lower: 1062.50 ± 34.10 g, 1074.20 ± 25.42 g, 1088.30 ± 30.22 g, 997.30 ± 23.90 g. Similarly low body weights for 4- and 12-week old birds were reported by Adedeji *et al*. (2015) for purebred and crossbred chicken in Nigeria (kept at university operated poultry flock). Ekka *et al.* (2016), who analyzed body weights of native Hansli, Coloured synthetic male line chicken and their crosses from week 1 to week 8 under intensive rearing

Table 1: Analysis of variance of fixed effects on body weight

Source of variance	Body weight (g)		
	DF[2]	Mean Squares	*P*
Sex (S)	1	404.1	< 0.0001
Age (A)	2	7060.8	< 0.0001
Rooster/Breeding season (R)	4	24.2	< 0.0001
SxA (R)[1]	22	24.0	< 0.0001

[1]Interaction SxA nested within R, [2]Degrees of freedom

Table 2: Least squares means and standard errors ($\mu \pm s_{\mu}$) of body weights by sex, age and rooster/breeding season

Effect	Individual levels of investigated single effects				
Sex	Females (F)	Males (M)			
N	479	406			
Body weight (g)	1115.01 ± 10.04	1432.24 ± 12.24			
Scheffe's test	F:M^{++}				
Age	5 weeks	12 weeks	20 weeks		
N	357	356	172		
Body weight (g)	420.08 ± 9.05	1250.01 ± 9.05	2151.56 ± 15.10		
Scheffe's test	5:12^{++}, 20^{++}, 12:20^{++}				
Rooster/breeding s.	1	2	3	4	5
N	205	113	204	132	231
Body weight (g)	1393.24 ± 15.50	1239.23 ± 20.99	1188.87 ± 14.66	1286.87 ± 21.72	1262.42 ± 14.10
Scheffe's test	1:2^{++}, 3^{++}, 4^{++}, 5^{++}, 3:4^{++}, 5^{+}				

N - number of observations, $^{+}P < 0.05$, $^{++}P < 0.01$

system in India, reported body weights of 5-week old birds as follows: 970.02 ± 32.87 g, 317.77 ± 11.09 g and 589.30 ± 23.38 g. McCrea *et al.* (2014), who analyzed body weights of Delaware chicken from week 1 to week 15, reported values of about 500 g (5-week old) and 1600 g (12-week old). The body weight of 15-week old Delaware chicken was 2100 ± 40 g, that indicates that Oravka chicken, compared with this breed, were of a slower growth. In contrast, as these authors mentioned, broilers achieved body weight 2100 ± 40 g at the age of six weeks (despite Delaware and broiler chicken were raised under the same conditions).

According to rooster/breeding season (Table 2), body weights of birds in this study were following: 1393.24 ± 15.5 g (1), 1239.23 ± 29.99 g (2), 1188.87 ± 14.66 g (3), 1286.87 ± 21.72 g (4) and 1262.42 ± 14.1 g (5). The differences were highly significant ($P < 0.01$) or significant ($P < 0.05$) between rooster/breeding season (1) and the remaining ones (2, 3, 4, 5) and between (3) and (4, 5). The differences in body weights tended to decrease along with duration of the experiment, indicating an increasing conformity of body weights among birds. This needs, however, to be considered with caution due to limitations in available information.

Findings from analysis of interaction between sex and age nested within rooster/breeding season revealed that the differences were also higher in favour of males (Figure 1). The older birds, the higher differences between males and females were found. With 5-week old birds, body weights within individual rooster/breeding season (1, 2, 3, 4 and 5) were estimated as follows: between 384.62 ± 32.11 and 572.04 ± 23.39 g for males vs. between 343.33 ± 35.73 and 497.71 ± 26.10 g for females. The differences were non-significant. With 12-week old birds, body weights within individual rooster/breeding season were estimated as follows: between 1299.51 ± 26.85 and 1464.29 ± 23.39 g for males vs. between 1071.90 ± 25.26 and 1134.83 ± 25.57 g for females. The differences were either non-significant or significant. With 20-week old birds, body weights within individual rooster/breeding season were estimated as follows: between 2237.24 ± 31.17 and 2633.76 ± 47.84 g for males vs. between 1615.77 ± 34.80 and 2056.84 ± 37.56 g for females. The differences were significant. Similar values of body weights found in Oravka chicken were reported by Aggrey (2002) for unselected Athens-Canadian chicken population evaluated at the age of 36 days (417.41 ± 59.22 g for males vs. 355.13 ± 49.12 g for

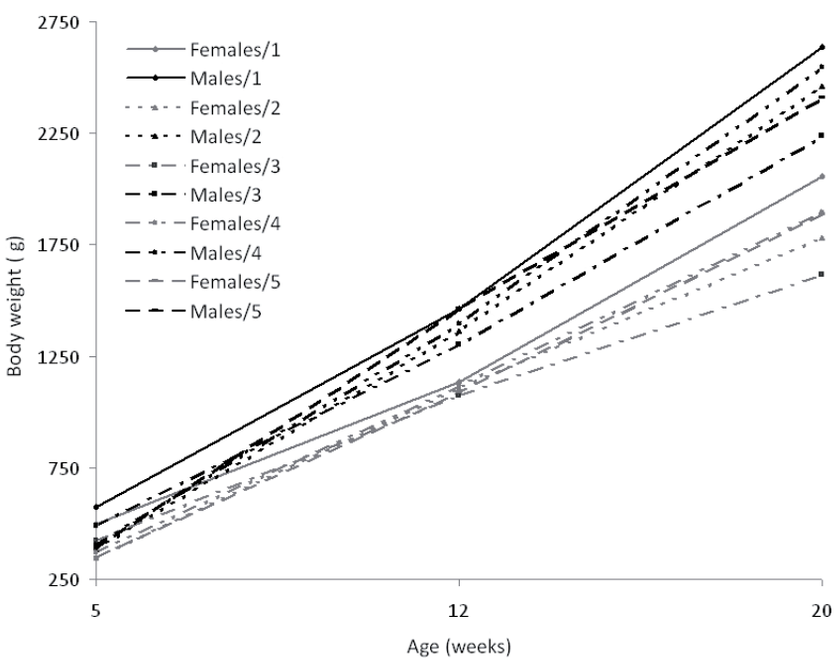

Fig. 1: Body weight according to sex (males, females), age (5-, 12- and 20-week old birds) and nested within rooster/breeding season (1, 2,… , 5)

females), at the age of 85 days (1326.49 ± 176.84 g for males vs. 1009.48 ± 130.78 g for females) and at the age of 141 days (2142.31 ± 243.44 g for males vs. 1619.34 ± 212.78 g for females). Malík and Malíková (1993) reported body weights for 12-and 20-week old birds of Oravka breed as follows: between 1090 g and 1280 g for males vs. between 1000 g and 1100 g for females, and between 1900 g and 2100 g for males vs. 1710 g and 1800 g for females, respectively. This comparison indicates that the ongoing breeders' preference of morphology traits did not influence Oravka chicken's growth in the negative way.

Repeatability treated as a random effect of bird was moderate, accounting for about 35 % of total variance of body weight.

CONCLUSION

Analyses showed significant effects of selected factors (sex, age, rooster and/or breeding season and interaction between sex and age nested within rooster/breeding season) on body weights in Oravka chicken breed. Along with increasing age of birds, body weights of males were higher than body weights of females. Further research taking into account body weights of birds weighed more often during growth phase of their life, and also including evaluation of body weights weighed at a higher age is needed to be done for describing growth curves in detail.

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EFFECTS OF DIETARY SUPPLEMENTATION OF COPPER SULPHATE AND COPPER OXIDE ON SOME EGG QUALITY PARAMETERS OF LAYING HENS

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ABSTRACT

A total of two hundred and twenty-four (224) Bovan Nera pullets of 20 weeks old were used for the 12-week experiment to determine the implications of two sources of inorganic copper supplementation in layers' diets. They were divided into 8 groups of twenty-eight (28) birds per treatment. Eight (8) isocaloric and isonitrogenous experimental diets of 17.63 % Crude Protein and 2592.80 kcal.kg^{-1} Metabolizable Energy were provided, each was supplemented with Copper Sulphate and Copper Oxide at four levels of inclusion (0, 100, 200 and 300 mg.kg^{-1}) and the pullets were randomly assigned to the diets in a 2 x 4 factorial experiment. Each treatment was replicated seven (7) times with 4 birds per replicate. All data collected were, therefore, subjected to 2 x 4 factorial analyses of variance. It was observed that the interaction of the different levels of inclusion of the two sources of copper had a significant ($p < 0.05$) effect on all the external egg qualities except the shell ratio. The different levels of inclusion with the two sources of copper also had a significant ($p < 0.05$) influence on the albumen height, albumen weight, yolk length, yolk index and egg mass. It can therefore be concluded that the hen could tolerate the two copper sources up to 300 mg.kg^{-1} without any deleterious effects on the egg qualities.

Key words: layer; egg; copper; quality; diet

INTRODUCTION

The economy of egg production could be affected highly by egg quality. Egg quality is a factor which contributes for better economy price of fertile and table eggs. Egg quality was regarded by Stadelman (1977) as characteristics important for consumers. Egg quality is presented by its weight, percentage of eggshell, thickness and strength of eggshell (Hanusova *et al*., 2015). Eggshell weight correlates to size of egg and thickness (Harms *et al*., 1990). According to Hanusova *et al*. (2015), egg internal quality is influenced by factors such as egg storage, bird strain and age, induced moult, nutrition, ingestion of contaminants and disease.

Layer nutrition plays a significant role in affecting both the internal and external egg qualities such as egg size, length, shell strength, yolk and albumen (Saldanha *et al.*, 2009). Copper (Cu) has been found to play an important role in eggshell membrane formation, which in turn influences eggshell structure, texture, and shape (Baumgartner *et al.*, 1978). Studies have shown that dietary supplementation of various Cu sources, such Cu Sulphate, Cu citrate, Cu chloride etc. in laying hens improved egg production, egg weight, reduced egg deformities, total cholesterol, triglycerides, low density-lipoprotein cholesterol and increased high density-lipoprotein cholesterol (Pesti and Bakalli, 1996; Miles *et al.*, 1998; Lim and Paik, 2003; Jegede *et al.*, 2011). Cu supplementation (125-250 ppm) in broiler diet was reported to confer improved feed consumption, body weight gain, feed conversion ratio and protein anabolism (Paik, 2001; Karimi *et al.*, 2011). Furthermore, Cu has a positive influence on the activities of some digestive enzymes such as trypsin, chemotrypsin, amylse and lipase (Tang *et al.*, 2013). However the maximum dietary tolerable level of copper for poultry

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was set at 300 mg.kg^{-1} (NRC, 1994) as excessive accumulation of copper in the body causes toxicity (Lapointe *et al*., 2011). The objective of the study was to evaluate the effects of two sources of dietary Cu and levels on some egg qualities of laying hens.

MATERIAL AND METHODS

Experimental Site

The study was carried out at the Poultry Unit, Teaching and Research Farm, The Federal University of Technology Akure, Nigeria. The geographical coordinates of the location is between 7° 17' North and 5° 9' East (Mapzoom, 2015). The climatic condition of Akure follows the pattern of southwest Nigeria where the climate is influenced mainly by the rain-bearing southwest monsoon winds from the ocean and the dry northwest winds from the Sahara desert. The rainy season lasts for about seven months (April to October). The rainfall is about 1524 mm per year. The atmospheric temperature ranges between 28 °C and 31 °C and mean annual relative humidity is about 80 % (Ajibefun, 2011). The experiment was conducted in accordance to the research ethics and guidelines of the Animal Production and Health Department of the institution.

Experimental Design and Diet

Eight (8) experimental diets were formulated in a 2 × 4 factorial arrangement such that they have varying inclusion levels of the two sources of copper, namely Copper Sulphate ($CuSO_4$) and Copper Oxide (CuO), sourced from BDH Chemical Ltd, Poole, United Kingdom. Each was supplemented at four levels of inclusion (0, 100, 200 and 300 mg.kg^{-1}) (Table 1). The formulated diets met the nutrient requirements of laying hens according to NRC recommendations (NRC, 1994). The proximate analyses of the diet samples were carried out according to AOAC (1995). The metabolizable energy (ME) of feed samples was calculated using the prediction equation by Pauzenga (1985) as follows: ME = (37 x CP + 81.8 x EE + 35.5 x NFE).

Experimental Animals

A total of two hundred and twenty-four (224) Bovan Nera pullets of 20 weeks old were divided into 8 groups of twenty-eight (28) birds per treatment and randomly assigned to the eight (8) treatment diets in a 2 x 4 factorial experiment. Each treatment was replicated seven (7) times with 4 birds per replicate. Feed was given according to body weight and age twice daily in line with the Bovan Nera management manual and drinking water was also provided *ad libitum*. All required managerial practices such as strict bio-security measures were ensured and also as and when due, appropriate vaccines and prophylactic treatments were administered. The birds were housed in an open-sided building in a thoroughly cleaned, washed and disinfected three tier cage system of 32 x 38 x 42 cm dimension. Four (4) birds were conveniently housed in a unit.

Data Collection

Egg collection was carried out thrice per day on days 21, 28, 35, 42, 49, 56 and 63 after the onset of lay from each treatment and taken for egg quality determination. A total of 35 eggs per treatment, 5 eggs per replicate were randomly selected on weekly basis within 24 hours of lay. Egg weight, yolk weight, shell weight and shell membrane were measured with sensitive scale calibrated in grams. The albumen weight was calculated by subtracting the sum of the weights of the shell and the yolk from the total egg weight. Shell thickness was measured with micrometer screw gauge and the shells air-dried for two days before weighting. Yolk index was determined as the ratio of the yolk height to the yolk width. Yolk height and width were measured with a ruler calibrated in centimeter with the aid of optical pins and mathematical compass. Yolk index and Haugh Unit (HU) was determined as described by Oluyemi and Robert (2000):

Yolk Index = (Yolk height/Yolk width) x 100

$HU = 100 \log (H + 7.57 - 1.7W^{0.37})$

Where H = observed albumen height in mm

W = observed weight of eggs in grams

The width and the length of the eggs were measured with the aid of vernier caliper calibrated in centimeters. Albumen heights in millimeters were taken with the aid of optical pin which was used for the calculation of Haugh Units.

The cholesterol levels of the eggs were evaluated using laboratory procedures for determining egg cholesterol. All the eggs from all the treatments on the last day of feeding trial were collected and weighed, hard cooked by immersion in boiling water for eight (8) minutes. The yolks were individually removed and individually weighed and oven-dried at 70 °C, pooled and blended.

Cholesterol determination was done using a commercial test kit for cholesterol analysis (Sigma diagnostic cholesterol reagent procedure No 352'Sigma Chemical Co., St Louis, MO, USA). All sample extracts were analyzed in triplicates. Cholesterol concentrations (mg) were determined from the absorbance read at 500 nm using a spectrophotometer (Idowu *et al*., 2006).

Table 1: Percentage composition of experimental diets

INGREDIENTS	Diets with CuO inclusion (%)			Diets with $CuSO_4$ inclusion (%)				
	T1	T2	T3	T4	T5	T6	T7	T8
Maize	40.00	40.00	40.00	40.00	40.00	40.00	40.00	40.00
Soya Meal	24.00	24.00	24.00	24.00	24.00	24.00	24.00	24.00
Corn Bran	14.00	14.00	14.00	14.00	14.00	14.00	14.00	14.00
Wheat Offal	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Limestone	8.52	8.52	8.52	8.52	8.52	8.52	8.52	8.52
Bone Meal	2.30	2.30	2.30	2.30	2.30	2.30	2.30	2.30
Lysine	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Methionine	0.35	0.35	0.35	0.34	0.35	0.35	0.35	0.34
Layer Premix*	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Salt	0.28	0.27	0.26	0.26	0.28	0.27	0.26	0.26
CuO	0.00	0.01	0.02	0.03	0.00	0.00	0.00	0.00
$CuSO_4$	0.00	0.00	0.00	0.00	0.00	0.01	0.02	0.03
Total	100	100	100	100	100	100	100	100
CALCULATED VALUES								
Crude Protein (%)	17.63	17.63	17.63	17.63	17.63	17.63	17.63	17.63
ME ($kcal.kg^{-1}$)	2592.80	2592.80	2592.80	2592.80	2592.8	2592.80	2592.80	2592.80
Ca (%)	3.90	3.90	3.90	3.90	3.90	3.90	3.90	3.90
Total Phosphorus (%)	0.51	0.51	0.51	0.51	0.51	0.51	0.51	0.51
Crude Fibre (%)	4.19	4.19	4.19	4.19	4.19	4.19	4.19	4.19
Crude Fat (%)	4.47	4.47	4.47	4.47	4.47	4.47	4.47	4.47
Lysine	1.21	1.21	1.21	1.21	1.21	1.21	1.21	1.21
Methionine	0.62	0.62	0.62	0.61	0.62	0.62	0.62	0.61
ANALYSED VALUES								
Crude Protein (%)	17.52	17.51	17.54	17.57	17.53	17.51	17.50	17.55
ME ($kcal.kg^{-1}$)	2602.77	2602.35	2604.93	2602.52	2603.85	2602.43	2603.25	2603.96
Crude Fibre (%)	7.15	6.98	6.99	7.05	6.99	7.35	7.42	7.51
Crude Fat (%)	2.16	2.25	2.16	2.59	2.39	2.13	2.01	2.20
Crude Ash (%)	11.43	11.57	11.28	11.98	11.60	11.87	11.43	11.78
Moisture (%)	11.66	11.82	11.91	11.78	11.92	10.99	11.18	10.97
NFE (%)	50.08	49.87	50.92	49.03	49.57	50.15	50.46	49.99

1 $mg.kg^{-1}$ (ppm) of $CuSO_4$/CuO = 0.0001 kg or %; NFE = Nitrogen Free Extract; ME = Metabolizable Energy

*Composition of premix: 2.5 kg of premix contains: Vit. A (10000000 iu), Vit. D3 (2500000 iu), Vit. E (12000 iu), Vit. B1 (2000 mg), Niacin (15000 mg), Vit. B6 (1500 mg), Vit. B12 (10 mg), Vit. K3 (2000 mg), Biotin (20 mg), Folic Acid (600 mg), Panthothenic Acid (7000 mg), Chlorine Chloride (150000 mg), Manganese (80000 mg), Iron (40000 mg), Copper (0 mg), Zinc (60000 mg), Selenium (150 mg), Iodine (1000 mg), Magnesium (100 mg), Ethoxyquine (500 g), BHT (700 g)

T1: Control diet without supplementary copper oxide; T2: Experimental diet supplemented with copper oxide at 100 ppm ; T3: Experimental diet supplemented with copper oxide at 200 ppm; T4: Experimental diet supplemented with copper oxide at 300 ppm; T5: Experimental diet without supplementary copper sulphate; T6: Experimental diet supplemented with copper sulphate at 100 ppm; T7: Experimental diet supplemented with copper sulphate at 200 ppm; T8: Experimental diet supplemented with copper sulphate at 300 ppm

Table 2: External egg qualities of laying hens fed diets supplemented with two sources of copper (mean ± SD)

TREATMENT	Level of Copper (mg)	Egg Weight (g)	Egg Length (mm)	Egg Width (mm)	Shell Weight (g)	Shell and Membrane (g)	Shell Membrane (g)	Shell Thickness (mm)	Shell Ratio	Egg Shape Index
CuO	0	51.51 ± 0.95	40.40 ± 0.61	28.63 ± 0.51	4.92 ± 0.11^{b}	6.00 ± 0.11	1.09 ± 0.03	0.30 ± 0.01	9.57 ± 0.15	0.71 ± 0.07
CuO	100	52.13 ± 0.70^{a}	41.20 ± 0.50	28.23 ± 0.52	4.76 ± 0.08	5.83 ± 0.10	1.07 ± 0.04	0.29 ± 0.01	9.14 ± 0.11^{b}	0.68 ± 0.07
CuO	200	50.90 ± 0.75	40.80 ± 0.52	27.81 ± 0.44^{b}	4.67 ± 0.09^{b}	5.68 ± 0.11^{b}	1.01 ± 0.03	0.28 ± 0.01^{b}	9.19 ± 0.16	0.68 ± 0.06^{b}
CuO	300	51.51 ± 0.64	40.10 ± 0.41^{b}	28.25 ± 0.41^{b}	4.90 ± 0.07	5.90 ± 0.08	1.00 ± 0.03^{b}	0.26 ± 0.01^{b}	9.53 ± 0.09	0.70 ± 0.05
$CuSO_4$	0	53.33 ± 0.70	41.10 ± 0.50	28.61 ± 0.51	5.18 ± 0.08^{a}	6.23 ± 0.09	1.05 ± 0.03	0.31 ± 0.01	9.73 ± 0.09	0.69 ± 0.09
$CuSO_4$	100	49.68 ± 0.59^{b}	40.50 ± 0.41	28.40 ± 0.43	4.73 ± 0.08	5.74 ± 0.09	1.01 ± 0.04	0.29 ± 0.01	9.52 ± 0.13^{a}	0.70 ± 0.06
$CuSO_4$	200	53.05 ± 0.92	42.30 ± 0.62	29.83 ± 0.50^{a}	5.07 ± 0.13^{a}	6.12 ± 0.14^{a}	1.06 ± 0.03	0.33 ± 0.00^{a}	9.64 ± 0.22	0.71 ± 0.07^{a}
$CuSO_4$	300	53.00 ± 0.75	43.00 ± 0.50^{a}	29.95 ± 0.51^{a}	5.01 ± 0.11	6.10 ± 0.11	1.10 ± 0.02^{a}	0.30 ± 0.01^{a}	9.44 ± 0.14	0.69 ± 0.07
MEAN SEPARATION										
Level of Copper										
0		52.49 ± 0.59^{a}	40.80 ± 0.40	28.62 ± 0.32	5.06 ± 0.07^{a}	6.13 ± 0.07^{a}	1.07 ± 0.02	0.30 ± 0.00^{a}	9.65 ± 0.09^{a}	0.70 ± 0.06
100		50.87 ± 0.47^{b}	40.90 ± 0.30	28.33 ± 0.31	4.74 ± 0.06^{b}	5.79 ± 0.06^{b}	1.04 ± 0.03	0.29 ± 0.00^{b}	9.34 ± 0.09^{b}	0.69 ± 0.05
200		52.03 ± 0.61^{ab}	41.60 ± 0.42	28.91 ± 0.41	4.88 ± 0.08^{ab}	5.91 ± 0.09^{b}	1.04 ± 0.02	0.30 ± 0.00^{a}	9.43 ± 0.14^{ab}	0.69 ± 0.05
300		52.18 ± 0.49^{ab}	41.40 ± 0.31	29.03 ± 0.32	4.95 ± 0.06^{a}	5.99 ± 0.07^{ab}	1.28 ± 0.05	0.28 ± 0.00^{b}	9.49 ± 0.08^{ab}	0.70 ± 0.04
Treatment										
CuO		51.54 ± 0.37	40.60 ± 0.22^{b}	28.24 ± 0.23^{b}	4.82 ± 0.04^{b}	5.86 ± 0.05^{b}	1.04 ± 0.02	0.28 ± 0.00^{b}	9.37 ± 0.06^{b}	0.69 ± 0.03
$CuSO_4$		52.20 ± 0.38	41.70 ± 0.32^{a}	29.23 ± 0.25^{a}	4.99 ± 0.05^{a}	6.04 ± 0.05^{a}	1.05 ± 0.02	0.31 ± 0.00^{a}	9.58 ± 0.07^{a}	0.69 ± 0.04
Statistical significance										
Treatment		0.20	0.002	0.004	0.01	0.01	0.59	< 0.0001	0.02	0.39
Level		0.13	0.27	0.45	0.01	0.01	0.78	< 0.0001	0.14	0.49
Treatment* Level		0.01	0.002	0.04	0.05	0.05	0.04	0.002	0.20	0.02

a,b: Means with different superscripts within column differ significantly ($p \leq 0.05$)

Data Analysis

All the data obtained were subjected to a 2 x 4 factorial analysis of variance (ANOVA) using the SAS Statistical Package (2008). The significant treatment means were compared using the Duncan Multiple Range test option of the same software. The procedure was reviewed and approved by the Federal University of Technology Akure Animal Care and Use Committee.

RESULTS

External egg quality

The external egg quality of laying hens fed diets supplemented with two sources of copper is shown in Table 2. The results revealed that the different levels of inclusion of the copper had a significant ($p \leq 0.05$) effect on the shell weight, shell + membrane, and the shell thickness. The highest significant values (5.06 ± 0.07 g, 6.13 ± 0.07 g and 0.30 ± 0.00 mm) for these qualities, were observed in the control. The other external egg qualities that were not significantly different ($p \geq 0.05$) had various highest numerical values at different levels of inclusion. The highest numerical values for egg weight (52.49 ± 0.59 g), shell ratio (9.65 ± 0.09) and egg shell index (70.18 ± 0.57) were observed in the control. The egg width (2.90 ± 0.03 cm) and shell membrane (1.28 ± 0.05 g) had numerical values ($p \geq 0.05$) observed at 300 mg of inclusion level while that of egg length (4.16 ± 0.04 cm) was noticed in 200 mg of inclusion level. There was significant difference ($p \leq 0.05$) in the qualities except for the egg weight, shell membrane and egg shell index due to the treatments with CuO and $CuSO_4$. Results showed significant and numerically higher values were observed in the hens treated with $CuSO_4$ for all the egg qualities examined.

The interaction of the different levels of inclusion of the two sources of copper had a significant ($p \leq 0.05$) effect in all the external egg qualities excluding the shell ratio. For the interaction between 100 mg CuO and 100 mg $CuSO_4$, significant difference ($p \leq 0.05$) was only observed in the egg weight with the significantly higher value (52.13 ± 0.70 g) observed in 100 mg CuO. In addition, the interaction of CuO and $CuSO_4$ at 200 mg also had a significant effect ($p \leq 0.05$) in the shell + membrane and egg shell index with the higher values (6.12 ± 0.14 g and 70.49 ± 0.71) in $CuSO_4$. Also, for the interaction of CuO and $CuSO_4$ at 300 mg, egg length and shell membrane had the higher values (4.30 ± 0.05 mm and 1.10 ± 0.02 g respectively) in $CuSO_4$ supplemented group. The egg width and shell thickness were significantly different in both 200 and 300 mg of CuO and $CuSO_4$ with the significantly higher values ($p \leq 0.05$) observed in $CuSO_4$.

Internal egg quality

The internal qualities of the eggs laid by the hens fed diets supplemented with two sources of copper are presented in Table 3. For the treatment with CuO and $CuSO_4$, the results revealed that the albumen height (7.5 ± 0.01 mm), yolk height (1.77 ± 0.01 mm) and yolk weight (12.87 ± 0.11 g) of eggs collected from hens fed $CuSO_4$ were significantly ($p \leq 0.05$) higher than those collected from hens fed CuO. The higher values for the albumen weight (14.17 ± 0.32 g), yolk length (2.95 ± 0.02 cm), egg mass (36.29 ± 0.34) and Haugh unit (99.98 ± 0.01) were observed in the eggs collected from the hens fed $CuSO_4$ and they were insignificantly different ($p \geq 0.05$) from CuO. The albumen length (6.75 ± 0.05 cm) and yolk index (0.60 ± 0.01) were observed to be higher ($p \geq 0.05$) for eggs obtained from hens treated with CuO.

All the yolk parameters (yolk height, weight, length and index) with albumen height were significantly ($p \leq 0.05$) influenced by the different levels of copper inclusion. Significantly highest values for albumen height (7.5 ± 0.01 mm) and yolk weight (13.00 ± 0.19 g) were observed in the control while yolk height (1.78 ± 0.02 mm) and yolk index (0.062 ± 0.01) were noticed to be highest ($p \leq 0.05$) at the 200 mg of inclusion. Significantly highest yolk length (2.99 ± 0.02 cm) was seen at the 300 mg. The other parameters were insignificantly ($p \geq 0.05$) different with their numerically higher values noticed at different levels: albumen length (6.84 ± 0.09 cm) at the control, Haugh unit (100.00 ± 0.01) at 100 mg, albumen weight (14.31 ± 0.49 g) at 200 mg, and egg mass (36.85 ± 0.45) at 300 mg.

The interaction of the different levels of inclusion with the two sources of copper had a significant ($p \leq 0.05$) influence on the albumen height, albumen weight, yolk length, yolk index and egg mass. This significant effect was observed in albumen height at the interaction of control, 100 and 200 mg of CuO and $CuSO_4$, with the highest significant mean (7.8 ± 0.01 mm) observed in 0 mg $CuSO_4$. For the albumen weight, yolk length, yolk index and egg mass, the significant ($p \leq 0.05$) effect of the interaction was noticed at the different levels of $CuSO_4$ but $CuSO_4$ control had the highest mean for albumen weight (14.85 ± 0.59 g) and egg mass (37.21 ± 0.75). Yolk index and yolk length have their significant highest means (0.062 ± 0.01 and 3.03 ± 0.03 cm) at 200 mg $CuSO_4$ and 300 mg $CuSO_4$, respectively.

Egg and excreta cholesterol level

Egg and excreta cholesterol level of the laying hens fed diets supplemented with the two sources of copper is shown in Table 4. For copper oxide, it was observed that the control had consistently the highest cholesterol values for the yolk (15.70 mg), whole egg (227.17 mg) and albumen (211.47 mg) except for the excreta where the lowest value for cholesterol was

Table 3: Internal egg qualities of laying hens fed diets supplemented with two sources of copper (mean ± SD)

TREATMENT	Level of Copper (mg)	Albumen Height (mm)	Albumen Weight (g)	Albumen Length (cm)	Yolk Height (mm)	Yolk Weight (g)	Yolk Length (cm)	Yolk Index	Egg Mass	Haugh Unit
CuO	0	7.30 ± 0.21^{b}	13.38 ± 0.59^{b}	6.90 ± 0.14	1.69 ± 0.03	12.96 ± 0.17^{b}	2.89 ± 0.06	0.059 ± 0.001^{b}	34.03 ± 0.98^{b}	99.97 ± 0.03
CuO	100	7.10 ± 0.20^{b}	14.46 ± 0.41	6.70 ± 0.08	1.75 ± 0.02	12.64 ± 0.17^{b}	2.94 ± 0.03	0.060 ± 0.001	36.57 ± 0.57^{b*}	99.95 ± 0.02
CuO	200	6.81 ± 0.22^{b}	13.81 ± 0.56	6.78 ± 0.09	1.77 ± 0.03	12.22 ± 0.19^{b}	2.90 ± 0.03	0.061 ± 0.001	35.40 ± 0.87	99.97 ± 0.02
CuO	300	7.10 ± 0.13	13.74 ± 0.43	6.66 ± 0.09	1.75 ± 0.02	12.77 ± 0.14^{b}	2.95 ± 0.03^{b}	0.060 ± 0.001	37.02 ± 0.58	99.98 ± 0.02
$CuSO_4$	0	7.82 ± 0.12^{a}	14.85 ± 0.59^{a}	6.78 ± 0.12	1.79 ± 0.02	13.03 ± 0.32^{a}	2.92 ± 0.03	0.061 ± 0.001^{a}	37.21 ± 0.75^{a}	99.97 ± 0.02
$CuSO_4$	100	7.40 ± 0.15^{a}	14.85 ± 0.59	6.78 ± 0.12	1.79 ± 0.02	13.03 ± 0.32^{b}	2.92 ± 0.03	0.061 ± 0.001	37.21 ± 0.75^{a*}	99.97 ± 0.02
$CuSO_4$	200	7.70 ± 0.25^{a}	14.76 ± 0.79	6.73 ± 0.18	1.79 ± 0.02	12.96 ± 0.21^{ab}	2.90 ± 0.03	0.062 ± 0.001	36.36 ± 0.73	99.97 ± 0.02
$CuSO_4$	300	7.41 ± 0.12	14.26 ± 0.58	6.80 ± 0.08	1.77 ± 0.01	13.03 ± 0.17^{ab}	3.03 ± 0.03^{a}	0.059 ± 0.001	36.65 ± 0.72	99.95 ± 0.02
MEAN SEPARATION										
Level of Copper										
0		7.52 ± 0.11^{a}	14.17 ± 0.42	6.84 ± 0.09	1.74 ± 0.02^{b}	13.00 ± 0.19^{a}	2.91 ± 0.03^{b}	0.060 ± 0.001^{ab}	35.74 ± 0.62	99.97 ± 0.01
100		7.21 ± 0.12^{b}	13.69 ± 0.36	6.66 ± 0.06	1.75 ± 0.02^{ab}	12.56 ± 0.11^{b}	2.95 ± 0.02^{ab}	0.060 ± 0.001^{b}	35.77 ± 0.36	100.00 ± 0.01
200		7.22 ± 0.20^{b}	14.31 ± 0.49	6.76 ± 0.10	1.78 ± 0.02^{a}	12.60 ± 0.14^{ab}	2.90 ± 0.02^{b}	0.062 ± 0.001^{a}	35.90 ± 0.56	99.96 ± 0.01
300		7.30 ± 0.12^{b}	13.97 ± 0.35	6.72 ± 0.06	1.76 ± 0.01^{a}	12.89 ± 0.11^{ab}	2.99 ± 0.02^{a}	0.059 ± 0.001^{b}	36.85 ± 0.45	99.96 ± 0.01
Treatment										
CuO		7.01 ± 0.13^{b}	13.86 ± 0.25	6.75 ± 0.05	1.74 ± 0.01^{b}	12.66 ± 0.08	2.92 ± 0.02	0.060 ± 0.001	35.89 ± 0.37	99.97 ± 0.01
$CuSO_4$		7.50 ± 0.10^{a}	14.17 ± 0.32	6.73 ± 0.06	1.77 ± 0.01^{a}	12.87 ± 0.11	2.95 ± 0.02	0.060 ± 0.001	36.29 ± 0.34	99.98 ± 0.01
Statistical significance										
Treatment		< .0001	0.43	0.85	0.03	0.03	0.20	0.58	0.41	0.25
Level		0.02	0.72	0.42	0.02	0.05	0.02	0.04	0.30	0.28
Treatment* Level		0.01	0.04	0.66	0.08	0.13	0.05	0.04	0.01	0.10

a,b: Means with different superscripts within column differ significantly ($p \leq 0.05$)

Table 4: Egg and excreta cholesterol levels of laying hens fed diets supplemented with two sources of copper (mean ± SD)

TREATMENT	Level of Copper (mg)	Yolk (mg)	Whole Egg (mg)	Albumen (mg)	Excreta (mg)
CuO	0	15.70 ± 0.16	227.17 ± 2.44	211.47 ± 2.30	134.16 ± 1.97
CuO	100	13.64 ± 0.18	192.20 ± 0.83	178.55 ± 0.72	148.86 ± 3.68
CuO	200	13.64 ± 0.08	187.55 ± 3.32	173.91 ± 3.25	183.90 ± 1.77
CuO	300	12.88 ± 0.11	177.58 ± 3.44	164.70 ± 3.52	202.28 ± 3.83
$CuSO_4$	0	15.27 ± 0.42	228.25 ± 1.94	212.98 ± 2.15	134.91 ± 1.21
$CuSO_4$	100	13.37 ± 0.29	189.11 ± 3.78	175.74 ± 3.99	156.53 ± 4.29
$CuSO_4$	200	12.35 ± 0.04	172.01 ± 4.15	159.66 ±4.12	185.33 ± 0.50
$CuSO_4$	300	12.26 ± 0.06	169.99 ± 4.67	157.74 ± 4.68	207.38 ± 0.59
MEAN SEPARATION					
Level of Copper					
0		15.49 ± 0.22[a]	227.71 ± 1.41[a]	212.23 ± 1.45[a]	134.28 ± 1.03[a]
100		13.51 ± 0.16[b]	190.65 ± 1.86[b]	177.15 ± 1.92[b]	152.70 ± 3.06[b]
200		12.99 ± 0.29[c]	179.78 ± 4.21[c]	166.79 ± 3.96[c]	184.62 ± 0.88[c]
300		12.57 ± 0.15[c]	173.79 ± 3.10[c]	161.22 ± 3.05[c]	204.83 ± 2.07[d]
Treatment					
CuO		13.96 ± 0.32[a]	196.12 ± 5.75[a]	182.16 ± 5.44[a]	167.30 ± 8.27
$CuSO_4$		13.31 ± 0.38[b]	189.84 ± 7.23[b]	176.53 ± 6.89[b]	170.91 ± 8.42
Statistical significance					
Treatment		0.0001	0.016	0.029	0.071
Level		< 0.0001	< 0.0001	< 0.029	< 0.0001
Treatment* Level		0.111	0.112	0.149	0.496

a,b: Means with different superscripts within column differ significantly ($p \leq 0.05$)

observed (134.16). Also it was observed that 300 mg copper oxide inclusion level consistently had the lowest cholesterol values for yolk (12.88 mg), whole egg (177.58 mg) and albumen (164.70 mg) except for excreta (202.28 mg) that had the highest cholesterol value. The same trend was also observed for copper sulphate where the control had the highest cholesterol values for yolk (15.27 mg), whole egg (228.25 mg) and albumen (212.98 mg) except for excreta (134.91 mg). The 300 mg inclusion level had consistently the lowest cholesterol values for yolk (12.26), whole egg (169.99) and albumen (157.74) but the highest cholesterol value was observed for the excreta (204.83). From the results, it was observed that an inverse relationship existed between values of egg cholesterol parameters and waste cholesterol values.

It was also observed that the cholesterol values for the parameters of the birds fed different levels of copper sulphate supplementary diet compared with the cholesterol levels in the parameters of the birds on copper oxide supplemented diet were lower. However, the cholesterol levels for the excreta for birds on copper sulphate diets had higher values than their counter parts on copper oxide supplementary diet (Table 4). This shows that copper sulphate was more effective in reducing yolk, whole egg and albumen cholesterol than copper oxide. In addition, it was observed from the results that dietary copper sulphate at the different levels of inclusion contributed to more loss of cholesterol in the excreta than copper oxide. Significant differences ($p \leq 0.05$) were observed for yolk, whole egg and albumen except for the excreta for the treatment. For the level of inclusion, the four parameters were significant ($p \leq 0.05$). However, for the interaction, significant difference was not observed for any of the parameters.

DISCUSSION

External egg quality

Copper (VI) sulphate ($CuSO_4$) had an increasing influence on the majority of the external qualities examined in this study as it was seen in the egg length,

egg width, shell weight, shell and membrane weight, shell thickness and shell ratio. Although these results were in contrast to Kaya and Macit (2012) who observed that the supplementation of copper in laying chicken diets did not have significant effect on egg quality traits, supplementation of $CuSO_4$ in the hens produced eggs with better external qualities. Egg quality traits such as weight, shell membrane and shell index were not affected by the main effects of Cu source. Shells from eggs laid by birds fed $CuSO_4$ were thicker than those of CuO. This was not in accordance with Holoubek *et al*. (2002) who reported an insignificant effect of copper on the values of eggshell weight and shell thickness.

The different levels of inclusion of the two copper sources in the diet of the hens showed alterations in few of the external egg qualities. These are the shell weight, shell and membrane weight as well as the shell thickness. Egg shell weight and shell thickness obtained for 200 $mg.kg^{-1}$ diets did not differ from the control group (0 $mg.kg^{-1}$ diet) but numerically lower shell weight was recorded in comparison with the control. Meanwhile, the linear improvements in majority of the external egg qualities, as a result of the three levels of copper sources, indicated that the hens can tolerate both CuO and $CuSO_4$ up to 300 $mg.kg^{-1}$ without adversely affecting the external qualities of the eggs. This was supported by the report of Thomas and Goatcher (1976) that dietary Cu concentrations as high as 480 $mg.kg^{-1}$ had no adverse effect on egg production and external qualities. The interaction of Cu source and level of supplementation indicated significant influence on all the external egg qualities evaluated excluding shell ratio. The better external egg qualities noticed in the hens fed $CuSO_4$ could be due to its ability to be absorbed and utilized more than CuO. Though the egg weight was not influenced statistically, it was observed to reduce with the different levels of the copper salts when compared with the control. This was in support of Pekel and Alp (2011) that postulated that supplementation with 250 $mg.kg^{-1}$ of $CuSO_4$ decreased egg weight. It means that the different levels of copper inclusion did not have any significant negative effect on the egg weight.

Internal egg quality

In this study, eggs laid by the hens fed $CuSO_4$ had longer albumen with no significant effect on the Haugh unit but a slight numerical improvement. This was against the findings of Idowu *et al.* (2006) who noted a significant effect in the Haugh unit of eggs collected from laying hens fed copper salts. Similarly, Jensen *et al.* (1978) found that adding Cu to the diet of laying domestic fowl affected egg quality significantly; this was reflected in an increase in the Haugh units of the eggs. This report was against the results of this study where the Haugh unit was not affected by the inclusion of copper.

Moreover, layers fed the diets supplemented with $CuSO_4$ presented improved yolk yield relative to the CuO treatment. This was in consonance with the observation reported by Jegede *et al.* (2011), that sulphate form of Cu resulted in higher yolk level. Meanwhile, in the experiment carried out by Kaya *et al.* (2013), no significant effect on egg quality traits, except for yolk color was observed following the dietary inclusion of copper into layers' diet. The different levels of copper supplementation had a great influence on the albumen height and all the yolk parameters examined. This inclusion of copper salts caused the albumen height and yolk weight to reduce relative to the control while the yolk height and length improved but Kaya *et al*. (2013) reported that it was only the yolk color, among the internal egg qualities, that was influenced when layers were fed diets supplemented with copper.

Egg and Excreta Cholesterol Level

Copper, according to Kim *et al.* (1992), Pekel and Alp (2011) and Jegede *et al.* (2015) regulates cholesterol biosynthesis by reducing hepatic glutathione concentration and thereby reducing cholesterol content of egg. The regulatory effect of copper was observed in the cholesterol level of egg; yolk, whole egg and albumen from birds fed copper diets as they were lower compared to those of eggs in birds on the control diet. These results support the findings of other authors (Al Ankari *et al*., 1998; Pesti and Bakalli, 1998; Balevi and Coskun, 2004; Idowu *et al.*, 2006; Lim *et al*., 2006; Jegede *et al.*, 2015). However, our results are not in support of Pekel and Alp (2011) who worked on laying hens and reported that dietary copper intake through the use of different copper compounds did not affect the cholesterol of egg yolk. It was observed from the results that egg yolk cholesterol reduced linearly as copper concentration increased which supports the report of Idowu *et al.* (2006).

The cholesterol content of egg has an inverse relationship with the cholesterol content in excreta in animals fed copper diet even due to the fact that excess cholesterol removed in the system of the animal is present in the excreta. The higher the concentration of copper in the diet of hens, the lower the cholesterol contents of their eggs and the higher the content in excreta. This explains the trend in excreta cholesterol in the results of the experiment. The results of excreta cholesterol was found to consistently increase as the copper dosage in diets increased linearly. These results support the findings of Leeson (2009). From the results, an inverse relationship was observed between the cholesterol values for the egg yolk and the cholesterol values for excreta for the different treatments and at different inclusion levels. This supports the findings of Idowu *et al.* (2006) in their work on laying hens who reported increase in the values of excreta cholesterol as the cholesterol values

in the egg parameters decreased. It was also observed that inclusion levels of copper in the diet of laying hens had significant effect on the cholesterol levels in their excreta in this study which supports the report of Idowu *et al.* (2006).

CONCLUSIONS

The two sources of copper did not influence the external egg qualities negatively but hens fed $CuSO_4$ had eggs with better external qualities especially shell thickness. Though the albumen height was influenced, the Haugh unit, being the unit for measuring the quality of an egg, was not affected. Yolks of eggs obtained from hens fed $CuSO_4$ were longer and heavier than those from hens fed CuO. Copper oxide was more effective in reducing the cholesterol level in eggs compared to copper sulphate, hence it promotes production of healthier eggs for consumption. Therefore, it can be concluded that the hen could tolerate the Cu sources up to 300 $mg.kg^{-1}$ which also supports the claim of NRC (1994).

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POSSIBILITIES OF CATTLE OVARIAN TISSUE CONSERVATION: A MINI-REVIEW

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ABSTRACT

Cryopreservation of ovaries, their surface tissues or ovarian follicles represents the main source of female gametes in the future. In case of serious damage to the animal (limb fractures and others), when it is necessary to slaughter the animal, the ovarian tissue, ovarian follicle or the entire ovary can be collected and frozen. After thawing, the biological material may be transplanted or cultured *in vitro* as a source of oocytes for *in vitro* fertilization and *in vitro* embryo production. Ovarian tissue can be cryopreserved in various forms, such as fragments, slices, hemi-ovaries or whole ovaries with a vascular pedicle for future vascular anastomosis. Whole ovaries tend to be more difficult to preserve due to their dense tissue structure, intricate vascular system and diversity of cell types, all of which results in poor heat transfer and uneven cooling rates. An alternative approach to harvesting and vitrifying oocytes would be to cryopreserve ovarian tissues instead of follicles. Dissection of the ovary into ovarian fragments followed by puncture of the follicles is considered an effective technique toobtain a high number of oocytes in excellent conditions. Therefore, attention must be given to preserving this yield of good oocytes. This method of ovarian tissue storage is very promising but insufficiently elaborated yet. Cryopreservation of ovarian surface tissues or ovarian follicles represents the future main source of female gametes. Last year, we have started investigations focusing on cryopreservation of ovarian fragments from Slovakian cattle breeds for the purposes of national gene bank of animal genetic resources.

Key words: cattle; ovary; oocyte; cryopreservation

INTRODUCTION

To preserve genetic diversity, notably for the conservation of endangered species and indigenous farm animal breeds, it is essential to create genome or genetic resource banks (GRBs) of male and female gametes and embryos, made up of a very large number of individual donors in good condition (Wildt, 2000).

Important role in solving the issue of the preservation of animal genetic resources belongs to a cryopreservation and subsequent long-term storage of genetic material from valuable breeds of livestock or genetically valuable individuals. Cryopreservation of ovaries, their surface tissues or ovarian follicles represents a main source of female gametes in future. In case of serious damage of the animal (limb fractures and others), when it is necessary to slaughter the animal, the ovarian tissue, ovarian follicle or the entire ovary can be collected and frozen. After thawing, the biological material may be transplanted or cultured *in vitro* as a source of oocytes for *in vitro* fertilization (IVF) and *in vitro* embryo production (IVP).

The population of females of Pinzgau cattle breeds in Slovakia according to FAO database is around 2000 units, what is considered as a critical state within the animal genetic resources. Moreover, in Slovakia there are no yet any frozen embryos of Pinzgau cattle, which represents history, landscaping and indispensable part of mountainous and mountain foothill areas of Slovakia. One solution to this problem is the cryopreservation of rare biological material, which will play an important role in addressing the issue of preservation of animal

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genetic resources. Cryopreservation of ovaries, their surface tissues or ovarian follicles represents the future main source of female gametes.

The ability to preserve oocytes and ovarian tissues in a healthy state for a desired period of time would have tremendous application in the field of biomedical research. Besides the application for the purpose of animal breeding, ovarian tissue banking in humans is also being considered in the hope of restoring fertility to patients who lose ovarian function due to chemo- or radiotherapy during cancer treatment (Newton, 1998; Paynter *et al.*, 1997).

SUCCESS OF CRYOPRESERVATION OF OVARIAN TISSUES AND CELLS

Methods developed for oocyte and ovarian tissue cryopreservation must protect structural and functional viability. To date, several strategies have been suggested to restore fertility using cryopreserved oocyte or ovarian tissue. These strategies describe cryopreservation of oocytes at either an immature (isolated from the ovary) or a mature (after ovulation) state. In regard to ovarian tissue, one strategy involves autologous orthotopic or xenogeneic transplantation of the frozen-thawed ovaries and monitoring the developmental potential and follicular dynamics *in vivo* (Aubard *et al.*, 1998; Gosden *et al.*, 1994a,b; Gunasena *et al*., 1997a,b; Harp *et al*., 1994; Oktay *et al*., 1998b; Weissman *et al*., 1999). The second strategy involves *in vitro* maturation, fertilization and culture of primordial follicles isolated from frozen-thawed ovaries (Carroll *et al*., 1990; Oktay *et al*., 1998a; Sztein *et al.*, 2000).

In most of the studies on human (Huang *et al.*, 2008; Wang *et al.*, 2008; Zhou *et al*., 2010) and cattle (Gandolfi *et al.*, 2006), the success of the ovarian tissue cryopreservation procedures was evaluated using morphological and histological assessment. The use of histology alone is insufficient to assess the freezing-thawing success, as morphological analysis is not often correlated to the viability or developmental competence of the follicle (Santos *et al*., 2007). These results need to be validated by *in vitro* oocyte development and by fertilization. Faheem *et al.* (2011) isolated bovine oocytes for *in vitro* culture using the procedure of dissection of ovarian cortex into small fragments followed by puncture of the follicle from frozen-thawed ovarian tissue. They recovered a great number of good quality oocytes, which were successfully matured *in vitro* (maturation rate 73-80 %), fertilized and developed into *in vitro* produced embryos at morula and blastocyst stages.

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

The aim of ovarian tissue cryopreservation is to store primordial follicles located in the ovarian cortex, which represents the oocyte reserve. Cryopreservation of matured (metaphase II) oocytes gives poor results due to problems encountered during fertilization and embryo development. Following thawing, hardening of the *zona pellucida* associated with the exocytosis of premature cortical granules blocks the penetration of the spermatozoon. The freezing procedure can also damage the oocyte cytoskeleton (Vincent and Johnson, 1992). The freezing of germinal vesicle oocytes could be an alternative because there is no risk of aneuploidy, however hardening of the *zona pellucida* and damage to the cytoskeleton is still present (Tucker *et al.*, 1998). Limited success with the above described methods has focused research on cryopreservation of the immature oocytes contained in primordial follicles, which are located in the ovarian cortex. This method is more likely to be successful, because the oocyte is less differentiated, it possesses fewer organelles, the *zona pellucida* is yet absent and the cortical granules do not exist, so the follicle is less prone to ischaemia (Oktay *et al*., 1998).

Usually, to produce oocytes suitable for *in vitro* fertilization, the extended (about two weeks) follicle culture is needed, because the majority of oocytes in ovarian tissue slices are situated within primordial follicles and are, therefore, immature (Cotrvindt *et al.*, 1996b; Hartshorne, 1997). However, a population of antral follicles with good post-thaw survivability exists in these tissues that can provide source of oocytes for *in vitro* maturation (Nayudu and Osborn, 1992). Therefore, by selecting the ovarian fragments containing more antral follicles it is possible to avoid prolonged procedure of ovarian *in vitro* culture, results of which may be unexpected.

METHODOLOGY OF OVARIAN TISSUE CRYOPRESERVATION

Cryopreservation of ovarian tissue offers many advantages over mature oocytes or embryos to preserve female germline of endangered animals. Firstly, the ovary contains a large pool of oocytes enclosed in primordial follicles. Secondly, ovarian tissue can be collected from animals of almost all developmental age (adult, prepubertal and foetus) and status (alive or dead) (Cleary *et al*., 2001). Thirdly, primordial follicles are more resistant to cryodamage, because their oocytes have a relatively inactive metabolism, lack of metaphase spindle,

zona pellucida and cortical granules and low amount of lipids (Hovatta, 2005). Nevertheless, cryopreservation of ovarian tissue is still problematic and should be optimized to handle the diversity of cell types and tissue components (oocytes, granulosa cells, extracellular matrix) (Hovatta, 2005). The cryopreservation and transplantation of ovarian tissue is currently being investigated in various large animals, most notably in sheep and pigs (Posillico *et al.*, 2010).

Cryopreservation aims to maintain cell in a viable state for long periods of time. Cells can be cryopreserved using any of two approaches: conventional freezing or vitrification. Using conventional freezing protocols in domestic animals, promising results have been obtained after cryopreservation of ovarian tissue from cats, sheep, goats, cows, pigs, horses and rabbits (Santos *et al*., 2010). In conventional freezing, sophisticated and expensive programmable freezers are required to assist the cooling procedure. However, the use of freezing devices is not always available, especially when endangered animals are found dead in the nature, because transport of such ovaries to laboratories may result in cellular degeneration due to hypoxia caused by the delay between animal death and the time of cryopreservation of the ovarian tissue. Therefore, an alternative for cryopreservation in field conditions may be a vitrification.

Although the conventional slow freezing method using programmable device has been successfully used for a number of years, it has disadvantages; the most noticeable is intracellular ice formation and subsequent cell damage. Opposite to that, vitrification is a rapid cooling cryopreservation method that results in solidification without crystallization, thus avoiding cryodamage resulting from ice formation (Bahchi *et al*., 2008). The first critical step in vitrification is the cooling rate, which must exceed the solution's critical cooling rate (CCR). Water has an extremely high CCR, which makes vitrification of cellular water almost impossible in lab settings. Therefore, water needs to be replaced as much as possible with cryoprotectants (CPA) such as dimethylsulfoxide (DMSO) or ethylene glycol (EG), which have lower CCR. By achieving the adequate cooling rate, that exceeds the CCR of the sample at a given viscosity and dehydration level, cryodamage due to ice formation can be avoided, as the sample transforms immediately from an aqueous to a vitreous state (Courbiere *et al.*, 2006). The high rates of cooling and thawing necessary for vitrification are achieved with classic protocols in which a small volume of the specimen is loaded into a loading device along with the minimum necessary volume of the cryoprotectant. The specimen is either directly exposed to liquid nitrogen or is loaded into a closed, thin-walled device to achieve maximum heat transfer rates (Kuwayama, 2007).

Vitrification presents many advantages for cryobiology and could be a suitable approach for organs vitrification, given the complexity of tissues and vascular system (Lornage and Salle, 2007). Since vitrification aims to avoid ice formation, it should principally provide a better alternative to tissue preservation than slow freezing. However, this procedure requires extremely fast cooling rate and extracellular water replacement by permeating cryoprotectants, which may cause cytotoxic effect (Lornage *et al*., 2006). In order to reduce this deleterious influence, different ways can be used to reduce the CPA concentration, such as a combination of relatively low concentrations of different CPA's in order to obtain a vitrifiable concentration of total solutions and to diminish the specific toxicity (Vajta and Nagy, 2006), or the addition of non-permeable CPA's, like sera, sugars and polymers.

Al-Aghbari and Menino (2002) reported in their study on ovine ovarian tissue that vitrification of ovarian tissue using an ultra-rapid cooling technique (Dinnyes *et al*., 2000) appears to be a viable alternative to cryopreserving sheep oocytes by conventional freezing or other vitrifying methods. Their study confirmed that dropping ovarian tissues, equilibrated in a vitrification solution, directly onto solid surface cooled by liquid nitrogen, can be used for oocyte cryopreservation. *In vitro* survival of oocytes obtained from vitrified ovarian tissues was similar to the non-vitrified control, suggesting that the vitrification process and subsequent thawing did not obviously induce damage to the oocytes. Cryopreservation rates for vitrified oocytes and oocytes obtained from vitrified ovarian tissues in the mentioned study were higher compared to conventional freezing and vitrification techniques using slower cooling rates (Martino *et al*., 1996; Saunders and Parks, 1999).

This success in cryopreservation procedure may be attributed to several new steps used in the procedure of Dinnyes *et al.* (2000). First, a higher cooling rate was reached by direct contact with metal surface chilled with liquid nitrogen. Second, ovarian tissues were thawed quickly by dropping the vitrified samples directly into warm thawing solutions. Third, ovarian tissues were relatively small (0.5 cm × 0.5 cm) which allowed rapid temperature exchange during cooling-thawing procedures, as well as rapid flushing out of a cryoprotectant.

The oocyte has always been the most challenging specimen to cryopreserve because of its high sensitivity and intolerance to cryopreservation due to large cytoplasmic volume and intricate cellular structure (Kim, 2006). Primordial follicles (PMF), which comprise about 90 % of the follicular population of each ovary, are less cryosensitive than mature oocytes, embryos and maturing follicles, primarily due to their morphology - less cytoplasm and cytoplasmic components, no *zona pellucida*, fewer granulosa cells - as well as the low

metabolic rate associated with their inactivity (Amorim *et al*., 2003; Courbiere *et al*., 2009). Therefore, PMF represents a better chance of surviving cryopreservation than any growing stage follicles (Huang *et al*., 2008). Until successful *in vitro* maturation procedure can be developed, the PMF can be stored *in situ* in the ovarian tissue (Amorim *et al*., 2003; Jewgenow and Paris, 2006).

Ovarian tissue can be cryopreserved in various forms, such as fragments, slices, hemi-ovaries or whole ovaries with a vascular pedicle for future vascular anastomosis. The smaller forms - fragments, slices and isolated follicles may have a higher probability of being viable after cryopreservation, because the cryoprotectants can more entirely permeate the tissue and prevent damages due to ice formation. Whole ovaries tend to be more difficult to preserve due to their dense tissue structure, intricate vascular system and diversity of cell types, all of which results in poor heat transfer and uneven cooling rates (Courbiere *et al*., 2009; Possilico *et al*., 2010).

An alternative approach to harvesting and vitrifying oocytes would be to cryopreserve ovarian tissues instead of follicles. Dissection of the ovary into ovarian fragments followed by puncture of the follicles is considered an effective technique to get a high number of oocytes in excellent conditions (Faheem *et al.*, 2011). Therefore, attention must be given to preserve this yield of good oocytes.

In most of the studies on human (Huang *et al.*, 2008; Wang *et al*., 2008; Zhou *et al.*, 2010) and cattle (Celestino *et al*., 2008; Gandolfi *et al*., 2006), the success of the ovarian tissue cryopreservation procedures was evaluated using morphological and histological assessment. The use of histology alone is insufficient to assess the freezing-thawing success, as morphological analysis is not often correlated to the viability or developmental competence of the follicle (Santos *et al*., 2007). These results need to be validated by *in vitro* oocyte development and by fertilization.

Faheem *et al.* (2011) isolated bovine oocytes for *in vitro* culture using the procedure of dissection of ovarian cortex into small fragments followed by puncture of the follicle from frozen-thawed ovarian tissue. They recovered a great number of good quality oocytes, which were successfully *in vitro* matured (maturation rate 73-80 %), fertilized and developed into *in vitro* produced embryos of morula and blastocyst stage.

CONCLUSION

Cryopreservation of bovine ovaries or ovarian follicles represents the main source of female gametes in future. In case of serious damage to a cow (limb fractures and others), when it is necessary to slaughter the animal, the entire ovary or the ovarian cortical tissue which usually contains a plenty of ovarian preantral or antral follicles can be collected and frozen. After thawing in relevant laboratory conditions, the biological material may be processed *in vitro* as a source of oocytes for *in vitro* fertilization and subsequent *in vitro* embryo production. This strategy could enable accumulation and long-term storage (at ultra-low temperature) of preimplantation stage embryos from animals which are not alive anymore. Such embryos may form basis for establishment of the animal gene bank. Currently, successful cryopreservation of animal ovarian tissue is still a challenge and protocols should be optimized.

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