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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

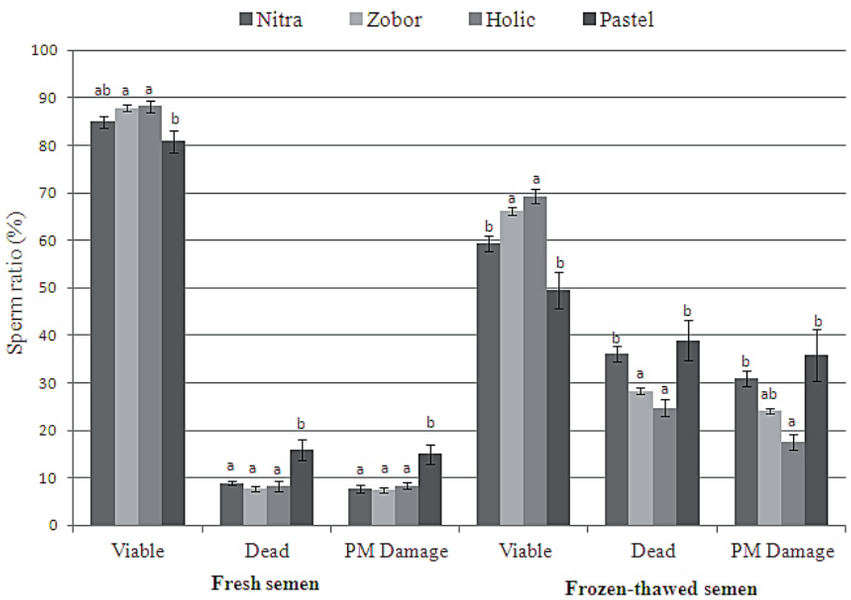
Frozen	TM	PM	VAP	VCL	VSL	STR	LIN	ALH	BCF
Nitra	38.4 ± 1.1^{ab}	25.7 ± 1.9^{a}	58.8 ± 2.1	121.7 ± 6.3	40.9 ± 3.1	0.69 ± 0.02	0.32 ± 0.02	5.1 ± 0.2	22.3 ± 0.8
Zobor	40.3 ± 2.2^{ab}	26.7 ± 2.1^{a}	54.1 ± 3.3	109.2 ± 5.4	35.1 ± 1.3	0.63 ± 0.02	0.32 ± 0.01	4.8 ± 0.2	19.7 ± 0.8
Holic	44.8 ± 2.6^{a}	32.9 ± 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.

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REFERENCES

AUERBACH, A. B. – NORINSKI, R. – HO, W. – LOSOS, K. – GUO, Q. – CHATTERJEE, S. – JOYNER, A. L. 2003. Strain-dependent differences in the efficiency of transgenic mouse production. *Transgenic Research*, vol. 12, 2003, p. 59–69.

CARNEIRO, M. – AFONSO, A. – GERALDES, A. – GARREAU, H. – BOLET, G. – BOUCHER, S. – TIRCAZES, A. – QUENEY, G. – NACHMAN, M. W. – FERAND, N. 2011. The genetic structure of domestic rabbits. *Molecular Biology and Evolution*, 2011, vol. 28 (6), p. 1801–1816.

KULÍKOVÁ, B. – DI IORIO, M. – KUBOVIČOVÁ, E. – KUŽELOVÁ, L. – IAFFALDANO, N. – CHRENEK, P. 2014. The cryoprotective effect of Ficoll on rabbit spermatozoa quality. *Zygote*, vol. 23 (5), 2014, p. 785–794.

KULÍKOVÁ, B. – ORAVCOVÁ, M. – BALÁŽI, A. – SUPUKA, P. – CRENEK, P. 2017. Factors affecting storage of Slovak native rabbit semen in the gene bank. *Zygote*, vol. 24, 2017, p. 1–9.

LAVARA, R. – DAVIV, I. – MOCÉ, E. – BASELGA, M. – VICENTE, J.S. 2013. Environmental and male variation factors of freezability in rabbit semen. *Theriogenology*, vol. 79, 2013, p. 582–589.

LONG, J. A. 2006. Avian semen cryopreservation: what are the biological challenges? *Poultry Science*, vol. 85, 2006, p. 232–236.

MOCÉ, E. – LAVARA, R. – VICENTE, J. S. 2005. Influence of donor male on the fertility of frozen-thawed rabbit sperm after artificial insemination of females of different genotypes. *Reproduction in Domestic Animals*, vol. 40, 2005, p. 516–521.

MOCÉ, E. – VICENTE, J. S. 2009. Rabbit sperm cryopreservation: a review. *Animal Reproduction Science*, vol. 110, 2009, p. 1–24.

MOCÉ, E. – VICENTE, J. S. – LAVARA, R. 2003. Effect of freezing–thawing protocols on the performance of semen from three rabbit lines after artificial insemination. *Theriogenology*, vol. 60, 2003, p. 115–123.

SAFFA, H. M. – VICENTE, J. S. – LAVARA, R. – VIUDES DE CASTRO, M. P. 2008. Semen evaluation of two selected lines of rabbit bucks. *World Rabbit Science*, vol. 16, 2008, p. 141–148.

SELLEM, E. – BROEKHUIJSE, M. L. W. J. – CHEVRIER, L. – CAMUGLI, S. – SCHMITT, E. – SCHIBER, L. – KOENEN, E. P. C. 2015. Use of combinations of *in vitro* quality assessments to predict fertility of bovine semen. *Theriogenology*, vol. 84, 2015, p. 1447–1454.

SUPUKA, P. – SUPUKOVÁ, A. – ZIGO, F. – PAVĽAK, A. – MAŽENSKÝ, D. 2012. Slovenské národné plemená králikov. Králik ako produkčné a modelové zviera: *XXV. vedecká konferencia „Aktuálne smery v chove brojlerových králikov“*, 2012, p. 157–159, ISBN 978-80-89418-21-3.

THURSTON, L. M. – WATSON, P. F. – HOLT, W. V. 2002. Semen cryopreservation: a genetic explanation for species and individual variation? *Cryoletters*, vol. 23, 2002, p. 255–262.

WATERHOUSE, K. E. – HOFMO, P. O. – TVERDAL, A. – MILLER, R. R. 2006. Within and between breed differences in freezing tolerance and plasma membrane fatty acid composition of boar sperm. *Reproduction*, vol. 131, 2006, p. 887–894.