

## DILUTION FACTOR AFFECTS THE ABILITY OF RAM SPERM TO SURVIVE CRYOPRESERVATION: SHORT COMMUNICATION

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### ABSTRACT

In this preliminary study, the fresh and frozen-thawed sperm from the original Valachian sheep breed were analysed for its quality. Semen was collected from two rams by electroejaculation and used for motility analysis using computer-assisted sperm analysis immediately after collection and following 60 and 120 min of incubation at 37 °C, or for rapid freezing. Semen was equilibrated at 15 °C for 20 min and diluted with a commercial diluent OviXcell (IMV Technologies) supplemented with 100 mM trehalose to a ratio of 1:1 (DR 1:1 group) or 1:2 (DR 1:2 group) (v:v). After 90 min of equilibration at 5 °C, the straws were suspended horizontally in liquid nitrogen vapours for 10 min before being plunged into a liquid nitrogen for storage. After one month, the straws were thawed by immersing into a water bath at 38 °C for 60 s. Sperm motility analysis was done immediately after thawing and after 30 and 60 min of incubation at 37 °C, as stated above. Our results showed that the dilution ratio has considerable effect on ram sperm survivability. Higher percentage of total motility and progressive movement ( $P \leq 0.05$ ) was found in the DR 1:2 when compared to the DR 1:1 group. In addition, our results confirmed inter-male variability in the susceptibility to the cryopreservation process. Nevertheless, results of this study are of preliminary character. Experiments using higher number of samples (individuals) as well as other methodology approaches need to be tested in order to find the best procedure for semen cryopreservation of Slovak national sheep breeds.

**Key words:** ram; sperm; cryopreservation; dilution

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### INTRODUCTION

Generally, animal gene banks play an important role in agricultural production globally for the present and the future, and in sustaining the most of production systems and community livelihoods. The situation with animal genetic resources in the Slovak Republic is not satisfactory due to the fact that livestock semen doses stored in the gene bank are originated only from several Slovak breeds (Chrenek *et al.*, 2017). Therefore, if there is an opportunity

to obtain biological material from valuable breeds, it is desirable to optimize specific cryopreservation process. The original Valachian sheep has the important functions such as maintaining natural landscape and agrotourism, and its historical value has been recognized (Oravcová and Krupa, 2011).

In order to optimize ram sperm cryopreservation in our conditions, we aimed to analyse whether different semen dilution ratios with freezing solution affects the post-thaw quality of Valachian sheep semen.

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Received: January 11, 2018

Accepted: January 25, 2018

## MATERIAL AND METHODS

### Semen collection

Semen was collected by electroejaculation from two original Valachian sheep breed rams (PV1; PV2). The rectum was cleaned of faeces. A three electrode probe 1" for ram and boar with diameter of 2.54 cm and length of approximately 16 cm, connected to a power source that allowed voltage and amperage control, were used (Minitube Electro-ejaculator). The EE regime (automatic mode, type of curve 2 – the power output is linearly increased from 0.5 Volt to 7 Volt) consisted of consecutive series of 2 s pulses of similar voltage, each separated by 2 s break. The initial voltage was 0.5 V, which was increased in each series until maximum of 7 V. Upon reaching a voltage of 7 V, impulses remained at this level until the ejaculation was complete. After collection, the semen was transported to the laboratory in a water bath at 37 °C.

### Sperm quality evaluation and cryopreservation

An aliquot taken from each fresh semen sample was used for motility analysis immediately after collection and following 60 and 120 min of incubation at 37 °C. Semen was diluted in a saline (0.9 % NaCl; Braun, Germany) at the ratio of 1:20, immediately placed into a Leja Standard Count Analysis Chamber (depth of 20 microns; MiniTüb, Tiefenbach, Germany) and evaluated under a Zeiss AxioScope A1 microscope using the CASA system (Sperm Vision™; MiniTübe, Tiefenbach, Germany). For each sample and repetition, seven microscopic view fields were analyzed for average concentration (CON;  $1 \times 10^9$ ) and percentage of total motility (TM; motility  $> 5 \mu\text{m}\cdot\text{s}^{-1}$ ) and progressively moving spermatozoa (PM; motility  $> 20 \mu\text{m}\cdot\text{s}^{-1}$ ). The rest of the semen samples were used for cryopreservation.

Semen was frozen using a rapid freezing method. Individual semen samples were cooled down to 15 °C for 20 min to minimize cold-shock damage. After cooling, an aliquot of semen was diluted in a commercial diluent (OviXcell, IMV Technologies, France) enriched with 100 mM trehalose (Sigma Aldrich, Germany) to a ratio of 1:1 or 1:2 (v:v). Thereafter, the semen was loaded into 0.25 ml plastic straws and equilibrated at 5 °C for 90 min. The straws were suspended horizontally in liquid nitrogen vapours (LNV) 5 cm above the liquid nitrogen ( $\text{LN}_2$ ) level for 10 min (-125 to -130 °C) before being plunged into a  $\text{LN}_2$  (-196 °C) for storage. After one month of storage the straws were thawed by immersing into a water bath at 38 °C for 60 s. Sperm motility analysis was done immediately after thawing and after 30 and 60 min of incubation at 37 °C, as stated above.

### Statistical analysis

Sperm quality between the two dilution ratios and between the two rams was compared by a t-test using a Sigma Plot software (Systat Software Inc., Germany). Values at  $P \leq 0.05$  were considered as statistically significant.

## RESULTS AND DISCUSSION

In our study, the fresh and frozen-thawed sperm from two males of original Valachian sheep were analysed for its quality. We aimed to compare the effect of different dilution ratios during the cryopreservation process on individual sperm motility. No difference in CON, TM and PM of fresh semen was found between the two males tested (Table 1).

**Table 1. Concentration and motility of fresh sperm from the two Valachian sheep rams**

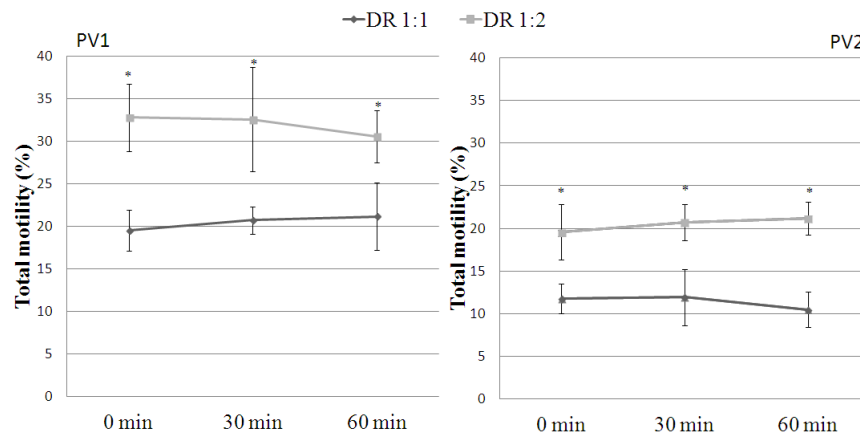
Ram	CON ( $\times 10^9$ )	TM00 (%)	PM00 (%)	TM60 (%)	PM60 (%)	TM120 (%)	PM120 (%)
PV1	1.655 $\pm$ 0.2	78.9 $\pm$ 3.1	74.5 $\pm$ 2.4	78.2 $\pm$ 6.1	74.7 $\pm$ 4.6	78.6 $\pm$ 3.9	75.5 $\pm$ 4.6
PV2	1.725 $\pm$ 0.2	75.2 $\pm$ 2.7	69.9 $\pm$ 2.4	72.4 $\pm$ 1.6	69.4 $\pm$ 2.1	77.4 $\pm$ 2.8	73.5 $\pm$ 2.7

VOL – volume; CON-concentration; TM – total motility; PM – progressive movement

Using the frozen-thawed semen, the dilution ratio has considerable effect on ram sperm survivability. Total motility was higher ( $P \leq 0.05$ ) in semen diluted to a ratio of 1:2 (DR 1:2) when compared to DR 1:1 in both males (PV1; PV2) and at each time point post-thaw (Figure 1). The same trend was noticed in progressive movement (PM; Table 2). It was already shown that sperm concentration at freezing affects post-thaw quality of ram sperm (Alvarez *et al.*, 2012).

Moreover, our results confirmed inter-male variability in the susceptibility to the cryopreservation process. Although the fresh semen quality was similar between PV1 and PV2, frozen-thawed semen showed difference ( $P \leq 0.05$ ) in TM and

PM between the two males (Figure 1; Table 2). Variability in post-thaw quality among males of the same breed has been reported for several species (Waterhouse *et al.*, 2006; Lavara *et al.*, 2013; Sellem *et al.*, 2015; Kulíková *et al.*, 2017). Therefore, in order to make a ram semen collection for later use, each individual sample needs to be tested before storage. Nevertheless, results of this study are of preliminary character. Experiments on higher number of samples (individuals) as well as other methodology approaches (different media, equilibration times, addition of other cryoprotectants) need to be tested in order to find the best procedure for semen cryopreservation of Slovak national sheep breeds.



DR – dilution ratio; PV1, PV2 – two individual males;  
\* means difference ( $P \leq 0.05$ ) between DR 1:1 and DR 1:2

**Figure 1. Differences in total motility between two dilution ratios used**

**Table 2. Differences in sperm post-thaw progressive movement between two dilution ratios and between two individual rams**

Ram	PV1			PV2		
Group	PM00	PM30	PM60	PM00	PM30	PM60
DR 1:1	7.8 ± 2.2 <sup>b</sup>	10.2 ± 2.1 <sup>b</sup>	7.3 ± 2.4 <sup>b</sup>	6.0 ± 1.4 <sup>b</sup>	7.6 ± 2.6	6.4 ± 0.9 <sup>b</sup>
DR 1:2	21.2 ± 3.4 <sup>a*</sup>	26.8 ± 4.5 <sup>a*</sup>	22.2 ± 3.7 <sup>a*</sup>	11.7 ± 2.6 <sup>a*</sup>	11.9 ± 1.9 <sup>*</sup>	12.6 ± 1.6 <sup>a*</sup>

PM-progressive movement at 0, 30 or 60 min of incubation at 37 °C; DR – dilution ratio; <sup>a</sup> vs <sup>b</sup> means difference ( $P \leq 0.05$ ) between dilution ratios; \* means difference ( $P \leq 0.05$ ) between rams (PV1; PV2) at specific time of incubation

## ACKNOWLEDGEMENTS

This research was supported by the Slovak Research and Development Agency (grant no. APVV-14-0043) and by VEGA (1/02611/15). The authors thank to Mr. Milan Dobiáš for technical assistance with the semen collection.

## REFERENCES

- ALVAREZ, M. – TAMAYO-CANUL, J. – ANEL, E. – BOIXO, J.C. – MATA-CAMPUZANO, M. – MARTINEZ-PASTOR, F. – ANEL, L. – de PAZ, P. 2012. Sperm concentration at freezing affects post-thaw quality and fertility of ram sperm. *Theriogenology*, 2012, vol. 77, p. 1111–1118.
- CHRENEK, P. – KUBOVICOVA, E. – MAKAREVICH, A. 2017. Current situation in the gene bank of animal genetic resources in Slovakia: a review. *Slovak Journal of Animal Science*, 2017, vol. 50 (4), p. 135–148.
- KULÍKOVÁ, B. – ORAVCOVÁ, M. – BALÁŽI, A. – SUPUKA, P. – CRENEK, P. 2017. Factors affecting storage of Slovak native rabbit semen in the gene bank. *Zygote*, 2017, vol. 24, p. 1–9.
- LAVARA, R. – DAVID, I. – MOCÉ, E. – BASELGA, M. – VICENTE, J.S. 2013. Environmental and male variation factors of freezability in rabbit semen. *Theriogenology*, 2013, vol. 79, p. 582–589.
- ORAVCOVÁ, M. – KRUPA, E. 2011. Pedigree analysis of the former Valachian sheep. *Slovak Journal of Animal Science*, 2011, vol. 44 (1), p. 6-12.
- 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*, 2015, vol. 84, p. 1447–1454.
- 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*, 2006, vol. 131, p. 887–894.