

## CRYOPRESERVATION OF RAM SPERM FROM AUTOHTONOUS BREEDS AS A METHOD FOR PRESERVING BIODIVERSITY

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### ABSTRACT

In the last few decades biodiversity of domestic animals has been declined. The importance of preserving indigenous breeds is reflected in the fact that they contain the original genetic code, are adaptable to adverse conditions and more resistant to disease. In this study we have investigated the possibility of cryopreservation of ram sperm as a method for preserving biodiversity. Cryopreservation of the Wallachian sheep gametes would allow easier exchange of genetic material between geographically distant locations and, thus, expand the genetic diversity of otherwise small and distant locations. The experiment was performed on rams of Wallachian sheep breed. Six semen samples from two rams were collected by electroejaculation. After collection and 60 or 120 min of incubation at 37 °C the sperm was analysed for motility by a computer-assisted sperm analyser. Collected sperm was diluted and frozen in straws using a rapid freezing method. The straws were thawed after one month. Results of this study showed that freezing influenced total and progressive motility compared to fresh sperm ( $P = 0.000$ ). Comparing progressive motility immediately after thawing, and 60 or 120 min after thawing we found statistically significant differences between both rams (Ram 1,  $P = 0.0359$ ; Ram 2,  $P = 0.0361$ ). In this experiment inter-male variability was not confirmed. For further analysis higher number of animals and other breeds need to be tested.

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

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### INTRODUCTION

Biodiversity preservation is a process of genetic conservation through renewal of degraded ecosystems and natural habitats and the preservation and recovery of breeds. Sustainable use represents utilization of biodiversity components that does not cause distortion of biodiversity but represents a rational use of natural resources and maintenance of the potential biodiversity (Stanivuk *et al.*, 2017).

In the past decades, the number of autochthonous sheep breeds has been declined.

The main factors affecting the number of autochthonous sheep breeds are low productivity and unprofitability of their production (Dragin *et al.*, 2017). Criteria of breed selection for conservation must be multiple and well and reasonably chosen. Criteria must respect the potential value of the breed that is the genetic constitution and eventually useful genes for future research at breeder discretion. The possibility of losing a breed is also one of the important criteria, because once lost genes or gene combinations can never be brought back in any way (Dragin *et al.*, 2015).

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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 (Kulikova *et al.*, 2018). In the last few decades, almost all farm animal breeds are experiencing a significant decrease of genetic diversity (Prentice and Anzar, 2011). Modern biotechnologies in reproduction allowed the production of large number of progeny from a single individual, as well as the use of effective methods of transport and long-term storage of sperm cells, oocytes and early embryos (Patterson and Silversides, 2003).

Gene banks are defined as systematic and organized collection, preservation and exploration of genetic material, by *in situ* (*in vivo*), or *ex situ* (*ex vivo*). The *in situ* method involves preservation of small herd of animal species, breed and lines (Wildt, 1999; Stančić, 1999). *In situ* method is common in rural area where a tradition of breeding autochthonous breeds of domestic animals is present. The disadvantages of *in situ* conservation are brought about by a lack of complete control over the many factors which influence the survival of individuals and therefore the genetic makeup of the conserved population (Henson, 1992; Furukawa *et al.*, 1998). The method *ex situ* involves long-term storage of gametes (sperm cells and oocytes) (Johnston and Lacy, 1995; Stančić, 2000; Stančić *et al.*, 2001; Stančić *et al.*, 2002; Stančić *et al.*, 2005; Stanković, 2012) or early embryos by cryopreservation technology (Stančić, 2004; Boettcher *et al.*, 2005; Pereira and Marques, 2008; Prentice and Anzar, 2011; Chrenek *et al.*, 2013) as well as by cryopreservation of testicular or ovarian tissue somatic cells (Andrabi and Maxwell, 2007; Pereira and Marques, 2008). The major advantage for *ex situ* conservation is the relative cost of collecting, freezing and storage of frozen material, as compared to maintaining large live population (Stančić *et al.*, 2013). Improvements in gamete cryopreservation methods may be useful for preserving valuable genetic stock in breeding programs (Anel *et al.*, 2006), and also for establishing a semen bank for sheep breeds with an increased risk of loss of genetic variability due to selection programs (Ehling *et al.*, 2006; Nel-Themaat *et al.*, 2006).

The natural resistance of spermatozoa to cryopreservation varies among species, breeds and even among individual animals of the same breed (Marco-Jiménez and Vicente, 2004). Sperm undergo changes during epididymal maturation and after the addition of seminal fluids (James *et al.*, 1999; Martinez-Pastor *et al.*, 2006; Yeung *et al.*, 2006; Tamayo-Canul *et al.*, 2011b), and these changes include variation in the plasma membrane of ram spermatozoa (Hammerstedt and Parks, 1987). These changes in plasma membrane leads to problems with cryopreservation of ram spermatozoa. Ram sperm is more sensitive to freezing than sperm of other species, and therefore, have a low pregnancy rates after cervical AI with frozen-thawed semen (Maxwell *et al.*, 1999). Frozen-thawed semen is used for intrauterine insemination, while fresh semen is used for cervical insemination (Maxwell *et al.*, 1999). Post-thaw sperm quality is reduced due to the occurrence of cold shock and osmotic stress during the freeze-thawing process (Salamon and Maxwell, 2000). The damage from freezing can be reduced by adding the extenders and cryoprotectants. Common extenders are the egg yolk and soybean lecithin. Optimal cryopreservation of epididymal spermatozoa following accidental death of ram and other endangered species would also markedly help to preserve biodiversity (Kaabi *et al.*, 2003; Hishinuma *et al.*, 2003; Martinez-Pastor *et al.*, 2009; Fernández-Santos *et al.*, 2006).

The success of cryopreservation of semen is determined by the rate of sperm dilution. Membrane destabilization and capacitation-like changes in spermatozoa can be linked to excessive dilution, and cryopreservation can have an additive effect on spermatozoa (Akçay *et al.*, 2012).

In our study we analysed fresh and frozen-thawed sperm for its quality from two Wallachian rams. The aim was to compare the effect of freezing and different times of incubation on sperm quality.

## MATERIAL AND METHODS

### Semen collection

Semen from two Wallachian sheep breed rams was collected by electroejaculation. The rectum was cleaned of faeces. A three electrode probes 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 volts, impulses remained at this level until the ejaculation was complete. After collection, semen was transported to a laboratory in a water bath at 37 °C.

### Sperm motility evaluation and cryopreservation

An aliquot taken from each fresh semen sample was used for motility analysis immediately after collection and following 60 or 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 Standard Count Analysis Chamber Leja (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 analysed for average concentration (CON;  $1 \times 10^9$ ) and percentage of total motility (TM; motility & gt;  $5 \mu\text{m}\cdot\text{s}^{-1}$ ) and progressively moving spermatozoa (PM; motility & gt;  $20 \mu\text{m}\cdot\text{s}^{-1}$ ). 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 minutes. 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  $\text{LN}_2$  (-196 °C) for storage.

After one month of storage in  $\text{LN}_2$ , 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 60 or 120 min of incubation at 37 °C, as stated above.

### Statistical analysis

Sperm motility between the two rams and between different times of incubation was compared by a Duncan test using Statistica 13.2 software (TIBCO Software Inc). Values at  $P \leq 0.05$  were considered as statistically significant.

## RESULTS AND DISCUSSION

Table 1 shows the results of total (TM) and progressive (PM) motility of thawed ram sperm, immediately after thawing. The results in the first table were obtained from the ejaculates from the ram located in the Slovak Republic (Ram no. 1).

From the attached table we can see that in each sample the progressive motility is less than the total motility, and that it decreases with increasing time interval.

**Table 1. Motility of thawed sperm at different time intervals, ram 1**

Time intervals	TM 00	PM 00	TM 60	PM 60	TM 120	PM 120
Sample 1	12.90 %	4.30 %	17.90 %	12.00 %	15.10 %	7.10 %
Sample 2	13.20 %	7.00 %	17.50 %	10.70 %	13.30 %	7.80 %
Sample 3	16.60 %	9.50 %	18.60 %	10.20 %	16.50 %	10.50 %
Sample 4	19.00 %	9.50 %	17.80 %	7.60 %	15.50 %	5.60 %
Sample 5	17.20 %	9.90 %	19.20 %	12.70 %	18.00 %	8.70 %
Sample 6	16.40 %	6.60 %	14.60 %	8.00 %	6.70 %	3.80 %

TM – Total motility, PM – Progressive motility

The natural resistance of spermatozoa to cryopreservation varies between breeds and even between individuals of the same breed (Marco-Jiménez and Vincente, 2004). Testing of thawed sperm from two rams of the Wallachian breed revealed the motility of the sperm and their suitability for cryopreservation. Cryopreservation process itself is affected by many factors. The composition of cryoprotectants and the rate of dilution before freezing maybe a key factor in sperm freezing (D'Alessandro, 2000).

Table 2 shows the results of total (TM) and progressive (PM) motility of thawed sperm from the ram located in Serbia (Ram no. 2). The total and progressive motility in this ram changed with the increasing time intervals.

Cryopreservation significantly affects the quality of thawed sperm by lowering sperm motility characteristics (Kubovičová *et al.* 2011). The maintenance of sperm function during freezing and thawing depends on several related factors including cooling rate, equilibration and freezing technique (Salamon and Maxwell, 2000; Bailey *et al.* 2000; Curry, 2000; Anel *et al.* 2006).

In Table 3, we compared the total (TM) and progressive (PM) motility of fresh and thawed sperm between two rams.

The results in the Table 3 showed high statistically significant differences between fresh and thawed sperm in both rams. Ram sperm are sensitive to extreme temperature changes during the freezing process (Salamon and Maxwell, 1995), what leads to sperm changes (Watson, 1995).

Based on the obtained results we can see that there are differences in progressive motility between different time intervals. By comparing progressive motility immediately after thawing, 60 or 120 min after thawing, we found statistically significant differences in both rams between 60 and 120 minutes after thawing. At each stage of the freezing cycle, which includes the process of ejaculate collection, dilution, equilibration, and freezing, sperm may lose the ability to fertilize normally (Watson, 1995).

These results show no statistically significant differences between ejaculates after thawing.

Kubovičová *et al.* (2011) investigated the motility of frozen-thawed sperm between two breeds of

**Table 2. Motility of thawed sperm at different time intervals, ram 2**

Time intervals	TM 00	PM 00	TM 60	PM 60	TM 120	PM 120
Sample 1	13.20 %	6.30 %	13.60 %	6.80 %	14.20 %	6.90 %
Sample 2	15.80 %	8.20 %	12.50 %	8.90 %	14.50 %	8.60 %
Sample 3	12.65 %	7.40 %	15.40 %	10.20 %	12.60 %	5.30 %
Sample 4	18.90 %	9.60 %	18.70 %	8.50 %	16.90 %	8.20 %
Sample 5	17.40 %	4.90 %	18.20 %	9.10 %	8.60 %	7.90 %
Sample 6	15.50 %	8.70 %	16.80 %	12.30 %	15.10 %	5.80 %

TM – Total motility, PM – Progressive motility

**Table 3. Comparison between ram 1 and ram 2 of total and progressive motility of thawed sperm at different time intervals**

Sperm motility at different time intervals	Frozen				Fresh			
	TM 60	PM 60	TM 120	PM 120	TM 60	PM 60	TM 120	PM 120
Ram 1	17.60 %	12.00 % <sup>ab</sup>	14.20 %	7.10 % <sup>ac</sup>	78.20 %	69.90 % <sup>b</sup>	78.60 %	69.70 % <sup>c</sup>
Ram 2	15.90 %	10.70 % <sup>ad</sup>	13.60 %	7.80 % <sup>ae</sup>	78.00 %	80.20 % <sup>d</sup>	75.10 %	76.10 % <sup>e</sup>

TM – Total motility, PM – Progressive motility, <sup>a,b,c,d,e</sup> – significant differences (P < 0.05) within a row.

sheep and also obtained significant differences. Total and progressive sperm motility after incubation was significantly reduced. Variability in the quality of thawed sperm in males of the same breed was noted (Waterhouse *et al.*, 2006; Lavara *et al.*, 2013; Sallem *et al.*, 2015; Kulíková *et al.*, 2017).

Kulíková *et al.* (2018) found no differences in the total and progressive motility of fresh sperm between two tested Wallachian breed rams. The quality of fresh sperm was similar, but thawed sperm showed a difference and thus confirmed various sensitivity to the cryopreservation procedure.

## CONCLUSION

The results of this study indicate that freezing significantly affects total and progressive sperm motility. No difference in total and progressive sperm motility was found between the two rams, but significant differences in sperm motility and progressive motility in both rams 60 and 120 min after thawing were observed in comparison with fresh semen. In conclusion, ram sperm is poorly susceptible to freezing, and it is necessary to perform a new research in order to improve the quality of frozen-thawed sperm.

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