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# Cryopreserved Sperm Quality in Tsigai Sheep: Implications for Biodiversity Protection

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## Abstract

The objective of our research was to conduct a comparative analysis of cryopreserved sperm quality parameters of Tsigai (TS) sheep breed. Ejaculates (n=12) from TS sheep rams (n = 2) were collected using electro-ejaculation. Samples were prepared from suitable ejaculates, ensuring at least 70% progressive motility. These samples were then equilibrated in Triladyl® diluent and subjected to automated freezing. Before freezing and after thawing, the sperm samples underwent assessments including motility (CASA), viability and apoptosis (DRAQ7/Yo-Pro-1), mitochondrial activity (MitoTracker), capacitation status (FLUO4), and acrosomal status (PNA). The results revealed significant differences ($P < 0.05$) in total motility between fresh (91.25±0.80 %) and frozen/thawed (F/T) (41.14±12.04 %) samples, as well as progressive motility between fresh (89.83±1.85 %) and frozen/thawed (33.57±8.25 %) groups. Moreover, significant differences ($P<0.05$) were also observed in the proportion of apoptotic spermatozoa (2.26±0.60 %), dead (7.40±1.72 %), and mitochondrial active spermatozoa (84.52±5.60 %) in fresh samples compared to F/T samples (12.34±0.80 %, 46.59±2.82 %, 47.71±4.04 %). Our findings indicate that the selected cryopreservation protocol is relatively sufficient for the cryoconservation of sperm from Tsigai sheep breeds, since up to 50% of F/T sperm were motile and live. This has significant implications for biodiversity protection and simplifies the establishment of an animal genetic resources gene bank.

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Keywords: Ram, spermatozoa, CASA, flow cytometry

## Introduction

Protecting biodiversity is a critical challenge facing us today. Local animal breeds, representing a crucial component of cultural heritage, play a vital role in the biodiversity of individual countries and regions. Cryopreservation of sperm emerges as a powerful tool for conserving animal genetic resources through the establishment of gene banks housing long-term stored genetic material. However, the cryopreservation of ram sperm encounters various obstacles, including the potential impact of individuality and breed characteristics on susceptibility to damage induced by low temperatures. In our research, we focus on investigating these differences among individuals raised in Central Europe. Sperm cryopreservation is a crucial biotechnological tool for enhancing breeding programs in various species, including small ruminants. Recent efforts have been dedicated to optimizing the cryopreservation process for ram sperm to enhance reproductive performance (SILVA et al., 2011). While frozen-thawed ram sperm is valuable for genetic distribution, its application in artificial insemination often results in low pregnancy rates. Nevertheless, cryopreservation plays a vital role in the conservation of animal genetic resources and biodiversity (JIMÉNEZ-RABADÁN et al., 2016), enabling the long-term storage of biological materials for future use or research. Transporting genetic resources in a frozen state offers ethical and logistical advantages over live animal transport (RASPA et al., 2017).

Sheep farming plays a vital role in Slovak agriculture, with approximately 230,000 ewes in total, of which 105,000 are used for milking purposes. While the breeding programs for dairy sheep in Slovakia and other countries have historically focused on milk yield (ORAVCOVÁ et al., 2005; SMULDERS et al., 2007), there is a growing emphasis on functional traits in recent years. This shift is driven by increased production costs compared to milk prices, consumer demand for safe and high-quality food, and societal concerns for animal welfare (BARILLET, 2007). In addition to milk production, lamb production contributes significantly to the income of dairy sheep farmers, accounting for approximately 25 to 35% in Slovakia and 30 to 60% in Mediterranean countries (FAO, 2008). Therefore, enhancing ewe reproductive performance, lamb survival, and lamb growth is crucial for modern sheep breeding programs (LEGARRA et al., 2007).

The Tsigai (TS) sheep breed is a domesticated breed primarily found in Central and Eastern Europe, particularly in countries such as Hungary, Romania, Serbia, Slovakia, and Ukraine. The Tsigai breed is among the oldest native breeds, traditionally raised in regions situated between 500 to 800 meters above sea level, predominantly in semi-extensive mountain farming systems. breed is known for its multi-purpose characteristics. According to Performance Testing Slovakia (PS SR,

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2009), there are approximately 19,500 TS ewes, with 8,400 ewes from 25 breeding flocks, undergoing performance testing.

Predictions for climate change suggest a range of potential impacts, such as increasing global temperatures, changes in precipitation patterns, more frequent extreme weather events, and alterations in ecosystems and biodiversity. These changes are anticipated to profoundly affect human societies, economies, and the environment. Given these challenges, it is crucial to safeguard genetic material as a form of "insurance" in case the breed faces endangerment. Ultimately, the primary objective of this study was to evaluate and cryopreserve TS ram sperm for inclusion in an animal gene bank.

## Material and methods

Clinically healthy, sexually mature males (n=2) and 6 years old of TS were used in this experiment. The sperm collection process was conducted using electro-ejaculation equipment (Electro-ejaculator, Minitube, Tiefenbach, Germany). Initially, a voltage of 0.5 V was applied, which was gradually increased in each series of pulses, separated by 2-second breaks, until reaching a maximum value of 7 V. Once the voltage reached 7 V, the pulses were maintained at this level until ejaculation was complete. To alleviate stress, rams were administered xylazine at a dosage of 0.2 mg/kg (Xylariem 2% average unit volume (a.u.v.), Riemser Arzneimittel GmbH, Greifswald, Germany) if deemed necessary. Following collection, the sperm samples were transported to the laboratory in a collection vessel submerged in a water bath maintained at 30 °C. Upon arrival at the laboratory, semen was promptly evaluated for volume, concentration, motility (initial assessment). Heterospermic samples were prepared by blending suitable ejaculates, ensuring at least 70 % progressive motility (PM).

### *Diluent Preparation and Freezing Process*

Dilution of Triladyl® was performed on the day of collection. Raw Triladyl® was mixed with deionized water in a 1:3 ratio, followed by the addition of egg yolk to achieve a final concentration of 10 % v/v. The mixture was filtered, heterospermic sample was diluted with the prepared extender at a ratio of 1:10 (semen:extender). Dilution was carried out slowly with gentle rotations at room temperature (RT). The diluted semen was then transferred to 250 μL straws, sealed, and equilibrated in a refrigerator at 4 °C for 6 hours.

After equilibration, straws were placed in an automated freezing box (IceCube, Minitube) pre-cooled to +4 °C. The freezing program initiated automatically upon closing the lid, subjecting the samples to a temperature profile of −10 °C (120 s), −80 °C (450 s), −120 °C (100 s), and −140 °C (180 s) plunged into liquid nitrogen

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(−196 °C). After one week, samples were thawed in a water bath at 42 °C for 15 seconds and then transferred for sperm analysis.

***Sperm motility***

The motility and sperm movement were analysed by CASA (SpermVisionTM software, Minitube, Tiefenbach, Germany) with light microscope (at the 200× magnification; AxioScope A1, Carl Zeiss Slovakia, Bratislava, Slovakia) and Makler counting chamber (Microptic, Barcelona, Spain). Samples were diluted by saline (0.9% NaCl; Braun, Nuaille, Germany) at ratio 1:20 (v/v). A drop of diluted semen (10 μL) was transferred to a counting chamber and analysed with manufacturer's pre-set parameters for rams. We mainly focused on total (TM) and progressive motility (PM).

***Flow cytometry***

*Viability and apoptosis*

For the detection of apoptotic-like changes in ram spermatozoa, YO-PRO-1 nuclear green dye (Thermo Fisher Scientific, Waltham, MA, USA) was utilized. Semen samples containing 1 × 106 spermatozoa were diluted in 500 μL of phosphate-buffered saline (PBS) and then incubated with 0.5 μL of YO-PRO-1, achieving a final concentration of 100 nM. This incubation was carried out for 15 minutes in the dark at room temperature (RT). Subsequently, the samples were washed in PBS through centrifugation at 600× g for 5 minutes at 20 °C. Afterward, the samples were stained with ready-to-use DRAQ7 dye as previously described and analysed using a flow cytometer. The proportion of spermatozoa positive for YO-PRO-1, indicated by YO-PRO-1+/DRAQ7− and YO-PRO-1+/DRAQ7+, was considered as the proportion of apoptotic-like spermatozoa.

*Acrosome status*

To assess the integrity of the acrosome, PNA (peanut agglutinin) fluorescent probes was used.

One μL of PNA working solution (Alexa Fluor 488 conjugate; Thermo Fisher Scientific, Waltham, MA, USA) was incubated with 1 × 106 spermatozoa diluted in 200 μL of PBS for 15 minutes in the dark at RT. The PNA working solution was prepared at a concentration of 0.5 mg/mL by dissolving the protein (1 mg/mL) in 2 mL of deionized water. After the incubation period, the samples were washed by centrifugation at 600× g for 5 minutes at 20 °C, stained with ready-to-use DRAQ7 dye as previously described, and then analysed by flow cytometer. The proportion (%) of spermatozoa positive for PNA, indicated by PNA+/DRAQ7− and PNA+/DRAQ7+, was considered as the proportion of acrosome-damaged spermatozoa.

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*Sperm Capacitation Status*

To assess the capacitation status of ram spermatozoa, FLUO-4 AM, a specific Ca2+ green fluorescent probe (FLUO-4; Thermo Fisher Scientific, Waltham, MA, USA), was utilized. FLUO-4 dye (at a final concentration of 100 nM) was incubated with 1 × 106 spermatozoa diluted in 500 µL of PBS for 20 minutes in the dark at 37 °C. After the incubation period, the samples were washed by centrifugation at 600× g for 5 minutes at 20 °C, stained with ready-to-use DRAQ7 dye as described previously, and then analysed by flow cytometer. The proportion (%) of spermatozoa positive for FLUO-4, indicated by FLUO-4+/DRAQ7− and FLUO-4+/DRAQ7+, was considered as the proportion of capacitated spermatozoa.

*Mitochondrial Activity*

The activity of mitochondria was assessed by MitoTracker® Green FM fluorescent dye (MT Green; Thermo Fisher Scientific, Waltham, MA, USA). In summary, 1 × 106 spermatozoa diluted in 500 µL of PBS were incubated with MT Green dye at a final concentration of 300 nM in the dark at 37 °C for 10 minutes. Following incubation, the samples were washed by centrifugation at 600× g for 5 minutes at 20 °C, stained with ready-to-use DRAQ7 dye as described earlier, and then analysed by flow cytometer. The proportion (%) of spermatozoa positive for MT Green, indicated by MT Green+/DRAQ7−, was considered as the proportion of spermatozoa with high mitochondrial membrane potential (MMP).

Stained sample aliquots were promptly analysed using flow cytometry employing a FACSCalibur instrument (BD Biosciences, San Jose, CA, USA). This instrument was equipped with a 488 nm argon ion laser and a red diode (635 nm) laser. The acquired fluorescent signals were processed by Cell Quest ProTM software (BD Biosciences, San Jose, CA, USA), utilizing a 530/30 nm bandpass filter for the green FL1 channel and a 670 nm long-pass filter for the red FL3 channel. Each sample underwent analysis for a minimum of 10,000 events (spermatozoa) activity.

***Statistical Analysis***

Experiments with cryopreservation of ram spermatozoa were performed four times. For data normality distribution, the Shapiro–Wilk test was used. Subsequently, acquired data were evaluated by one-way ANOVA (Tukey method) using GraphPad Prism version 9.0.0 for Windows (GraphPad Software, San Diego, CA, USA). The data are represented as means ± SD. Differences at $P < 0.05$ were considered statistically significant.

## Results and discussion

Preserving the genetic diversity of farm animal species is a crucial endeavour to safeguard domestic biodiversity and ensure their adaptation to evolving

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environmental conditions, unforeseen breeding mishaps, or disease outbreaks (JOOST and BRUFORD, 2015). Many indigenous or endangered breeds with limited population sizes may preserve their genetic material through cryopreserved reproductive cells (RAKHA et al., 2016; SVORADOVÁ et al., 2018). However, cryopreservation of ram sperm faces challenges compared to other livestock species like bulls, rabbits, and horses, owing to its heightened susceptibility to freezing and thawing procedures and the sperm's low cryotolerance (GÁSPÁRDY et al., 2020). Moreover, variations in frozen sperm quality among small ruminants suggest breed-specific disparities in sperm vulnerability to cryopreservation methods (BARBAS and MASCARENHAS, 2009). Notably, native and purebred breeds are often deemed more robust and healthier than crossbreeds (KUMARESAN et al., 2021). Therefore, it's imperative to explore potential variances in the properties and quality of cryopreserved sperm across different breeds. Evaluating sperm characteristics is crucial for assessing the fertilization potential of male livestock. In our study, we noted a decrease in total motility after freezing and thawing. Similarly, progressive motility also showed a decline compared to the results obtained with fresh sperm (Table 1).

**Table 1. Motility parameters of fresh and frozen/thawed Tsigai ram sperm**

| Parameter | Fresh | Frozen/thawed |
|---|---|---|
| Total Motility (%) | 91.25±0.80 | 41.14±12.04* |
| Progressive motility (%) | 89.83±1.85 | 33.57±8.25* |

*Significant differences compared to the control at $P < 0.05$

GALARAZA et al. (2019), in their study utilizing three different protocols for automated freezing of Merino ram sperm, reported a decrease in total motility from 87.8 % in fresh to 61.4 % in thawed sperm and in progressive motility from 34.8 % in fresh to 27.2 % in thawed sperm. Although the decrease in progressive motility was slightly lower, it may be attributed to the initially lower value of progressive motility in fresh semen. In another investigation (PERIS-FRAU et al., 2019), Manchega ram semen was diluted with Biladyl® extender (Minitube) and frozen using a programmable biofreezer (Planer Kryo 10 Series III, Planer PLC, Sunbury-on-Thames, UK), resulting in a 49.1% relative decrease in total motility and 41.8 % in progressive motility. This indicates a similar obtained results compared to our findings, which may mean different breeds of rams used.

Sperm viability assessment utilized a range of fluorescent probes. Yo-Pro-1 was utilized to identify apoptotic-like ram sperm in conjunction with the DRAQ7 marker for dead sperm. FLUO4, a marker detecting sperm capacitation, was also evaluated. Additionally, PNA and MitoTracker were employed to assess acrosomal status and mitochondrial activity, respectively. To ensure high fertility levels, it's crucial to have a low percentage of damaged sperm, as only non-damaged ones can effectively

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fertilize an oocyte. All parameters evaluated through flow cytometry revealed higher significant $P < 0.05$ proportions of apoptotic and dead sperm and significant lower mitochondrial activity of sperm in frozen/thawed group compared to fresh (Table 2). However, no significant difference was found in the proportion of sperm with damaged acrosome in compared groups.

**Table 2. Flow cytometric parameters of fresh and frozen/thawed Tsigai ram sperm**

| Parameter | Fresh | Frozen/thawed |
|---|---|---|
| Apoptotic spermatozoa (%) | 2.26±0.60 | 12.34±0.80* |
| Dead spermatozoa (%) | 7.40±1.72 | 46.59±2.82* |
| Capacitated spermatozoa (%) | 1.42±0.60 | 3.23±0.06 |
| Acrosome damaged spermatozoa (%) | 3.52±1.30 | 4.55±2.95 |
| Mitochondrial membrane potential (%) | 84.52±5.60 | 47.71±4.04* |

*Significant differences compared to the control at $P < 0.05$

## Conclusion and recommendation

Our findings in the recent study indicate that the cryopreservation protocol of VOZAF et al. (2021) is also effective for preserving sperm from Tsigai ram breed. Parameters such as motility, viability, capacitation, acrosome status and mitochondrial activity significantly differ between fresh and frozen/thawed group. However, this decline is not dramatic, and the samples can be stored in a gene bank and used for artificial insemination when needed. These results have implications for safeguarding livestock biodiversity by potentially reintroducing native sheep breeds and storing male gametes as long-term animal genetic resources.

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