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POST-THAW CHARACTERISTICS OF PINZGAU BULL SEMEN FOLLOWING LONG-TERM AND SHORT-TERM STORAGE

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ABSTRACT

The aim of the study was to examine sperm viability of Pinzgau bull insemination doses following long- or short-term storage. Insemination doses, provided by Slovak Biological Services Inc. (Lužianky, Slovak Republic), were slow-frozen and stored in containers with liquid nitrogen for 1 - 18 years. The sperm samples were divided, according to the length of storage, to the short-term (≤ 15 years) and long-term (> 15 years) groups. Post-thaw sperm assessment included total motility (CASA), dead/necrotic sperm occurrence (propidium iodide) and sperm morphology examination. No significant influence of storage length on the spermatozoa characteristics of Pinzgau bulls was noted. However, high inter-male variability in the susceptibility of Pinzgau bull sperm to cryodamages was found ($P \leq 0.05$). Our results suggest that bull´ individual variability should be taken into account when semen doses are to be stored as genetic resources for a future use.

Key words: Pinzgau cattle; sperm; storage; viability

INTRODUCTION

Preservation of genetic diversity of domestic animals is a global issue, which is important from a biological, economical and ethical standpoint (Prentice and Anzar, 2011). Intensive genetic selection, close range of production and reproduction traits of animals results in serious genetic diversity decline. Nowadays, number of farm animal breeds became extinct as a consequence of unilateral selection (Buerkle, 2007). Pinzgau breed is registered by the UN FAO (Food and Agriculture Organization, OSN) as threatened breed and it is classified as Animal Genetic Resource – AnGR since 1994 (Krupa *et al*., 2011). It represents dual purpose cattle, and is preferentially kept (bred) in the mountain regions of Slovakia (Kadlecik *et al*., 2004). The long-term national program for animal genetic resources protection can ensure minimization of extinction risk and support for sustainable utilization of local breeds (Tomka *et al.* 2013).

Cryopreservation of livestock semen has been used to improve the breeding of animals of genetic importance, and has contributed to the conservation of endangered species (Holt, 2000; Johnson *et al*., 2000). There has been a growing interest in the understanding of long-term storage effects on post-thaw survival of mammalian sperm (Yogev *et al*., 2010; Fraser *et al*., 2014). The issue is of practical importance for the establishment of cryobanks and its operation. However, freezing-thawing process and storage of samples may lead to decreased viability and fertilization ability of frozen insemination doses. Most of the damage to spermatozoa brought by cryopreservation is caused by production of reactive oxygen species (ROS) during freezing, that might alter

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sperm membrane fluidity and decrease the sperm function following cryopreservation (Chatterjee and Gagnon, 2001). There is also evidence that long period of storage may result in a loss of sperm surface proteins associated with bull fertility (Lessard *et al*., 2000).

Therefore, the aim of our study was to examine sperm viability of Pinzgau bull insemination doses following long- or short-term storage. The viability characteristics, including sperm morphology, motility and occurrence of dead/necrotic sperm were analyzed in post-thaw sperm samples.

MATERIAL AND METHODS

Semen collection and cryopreservation process

Commercially available sperm insemination doses from nine healthy Pinzgau bulls, used in this study, were supplied by the Slovak Biological Services Inc., Lužianky, Slovak Republic. Semen was collected using an artificial vagina. Only fresh semen with required quality (min 70 % of motile sperm and concentration 0.7 x 10^6 ml^{-1}) was used for insemination dose production. The semen samples were diluted in a Triladyl extender, loaded onto 0.25 ml straws and slowly frozen using a programmable freezing device. The straws were stored in containers with liquid nitrogen for 1 - 18 years. The 'long-term storage' (LT) group included a total of 8 samples (4 bulls, 2 samples each), which were thawed for analysis after 16 -18 years of storage. A second group designated as the 'short-term storage' group (ST), was composed of 10 samples that were donated by 5 bulls (2 samples each). These specimens were kept in the bank for routine inseminations and were cryostored for 1 - 15 years. The definition for long and short-term storage was decided arbitrarily, by splitting the accessible specimens into two groups with a comparable number of individuals.

Semen thawing and analysis

The straws were thawed in a water bath at 37 ± 1 °C for 1 min. For sperm total motility measure, computer assisted semen analysis (CASA; Sperm Vision™ 3.5) was used. Sperm samples were diluted in a saline. Each sample was analyzed at the time intervals of 0, 0.5 h or 2 h following thawing and incubation at 37 °C.

Fluorescence assay was performed immediately after thawing. Occurrence of dead/ necrotic sperm cells was detected with nuclear stain - propidium iodide (PI). Samples were incubated in a staining solution (5 μg.ml^{-1} of PI in saline) for 20 min in the dark and washed in a saline. Four μl of sperm suspension were mixed gently on a microslide with 4 μl of Vectashield mounting medium containing DAPI (H-1200, Vector Laboratories, Burlingame, CA, USA), a blue-fluorescent DNA stain, which marks nucleoplasma of all the sperm in sample. Samples were immediately observed under a Leica fluorescent microscope (Mikro Ltd, Bratislava, Slovak Republic) with respective bandwidth filters for red and blue fluorescence.

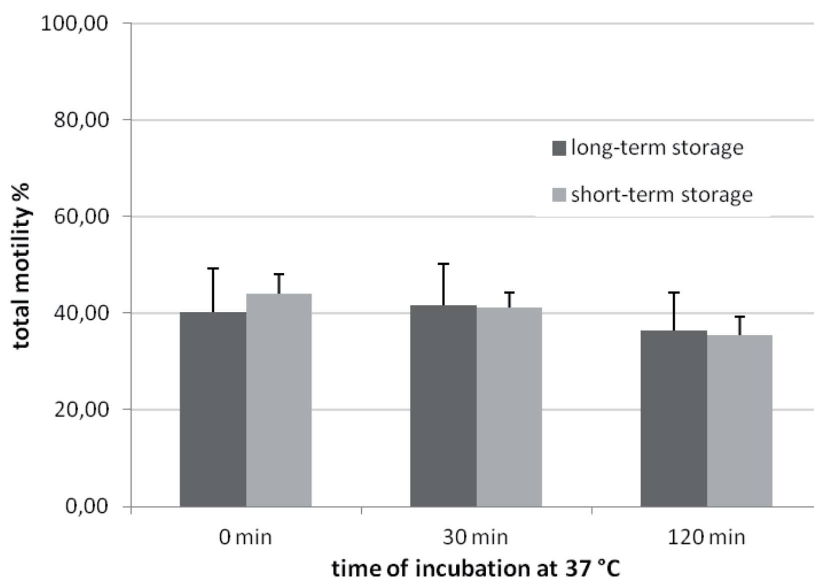

Fig. 1: Sperm total motility of frozen-thawed bull semen following long-term or short-term storage in liquid nitrogen

Assessment of pathological spermatozoa was performed by examining spermatozoa under a light microscope at 500x magnification, following sperm dilution/immobilization in distilled water. For each male a minimum of 400 spermatozoa were evaluated. The following changes in spermatozoa morphology were considered as pathological: separated tail (ST), knob-twisted tail (KT), torso tail (TT), rounded tail (RT), retention of cytoplasmic drop (RCD), broken tail (BT), small head (SH), large head (LH), acrosomal changes (ACH), other forms (OF) of pathological spermatozoa (teratogenic changes, club bag tumour, tail ball, etc.).

Statistical analysis

Statistical analysis was performed by One-Way ANOVA (Tukey test) for comparison of mean values using the SigmaPlot software. Differences at $P \leq 0.05$ were considered as statistically significant.

RESULTS

Cryopreserved semen used in this study was collected from sexually mature bulls that were of proven fertility and were undergoing regular semen collection for commercial artificial inseminations. The post-thaw motilities recorded for the males of LT or ST group were expressed as the means ± SE (standard errors of the mean). No significant difference in total motility between LT and ST groups measured at different time points after thawing and incubation at 37 °C was found (Fig. 1).

In both groups, post-thaw total motility was about 40 % and sperm were able to maintain this level around 30 % after 2h of incubation at 37 °C. The relatively large SEs in LT and ST groups (Fig. 1) may be explained by a high variability in mean values of post-thaw motility among the individual bulls (Tab. 1).

High level of inter-male variability was also found in percentage of necrotic sperm (Fig. 2). When the bulls were grouped according to length of storage, no difference in mean values between the LT and ST groups (31.16 ± 7.69 vs. 29.08 ± 3.96), respectively, was found. The percentage of morphologically abnormal sperm ranged from 10 to 33 % with no difference between the examined groups (17.21 ± 1.91 vs. 19.61 ± 3.99 %).

DISCUSSION

Long-term storage of cryopreserved sperm in LN_2 is of high importance for livestock breeding programs, gene bank establishment and maintenance of genetic diversity (Jalme *et al.*, 2003). Pinzgau cattle belong to the endangered breeds, due to radical decreasing of its population in Slovakia (Kadlecik *et al.*, 2004). Cryopreservation and long-term storage of gametes is potential option for genetic diversity preservation of Pinzgau breed. However, knowledge about the effect of long-term storage in liquid nitrogen on sperm functionality is insufficient. Therefore, this study examined differences in post-thaw viability characteristics of long- and short-term stored bull sperm. Generally sperm cryopreservation and thawing process can lead to sperm membrane structures damage, motility deterioration (Bailey *et al.*, 2000) and decline in sperm forward progression in the female reproductive tract, that might cause reduction in fertilization ability (Salamon and Maxwell, 2000). Usually, normalized percentage of motile sperm in frozen-thawed livestock semen is about 50 % of those in the fresh counterparts (Barbas and Mascarenhas, 2009). All the samples used in our study fulfilled the recommended criterion of at least 30 % motility for the production of insemination

Table 1: Frozen-thawed sperm total motility (TM) of individual bulls (n = 9) observed immediately after thawing

Total motility % (mean ± SE)			
long-term storage (> 15years)		short-term storage (≤ 15 years)	
LT1	40.00 ± 1.53^{b}	ST1	56.01 ± 1.36^{a}
LT2	43.42 ± 2.38^{b}	ST2	50.50 ± 1.52^{a}
LT3	60.82 ± 1.61^{a}	ST3	42.28 ± 1.61^{b}
LT4	16.65 ± 0.74^{d}	ST4	36.82 ± 2.59^{bc}
		ST5	34.39 ± 1.22^{c}

Different superscripts indicate significant differences (P < 0.05).

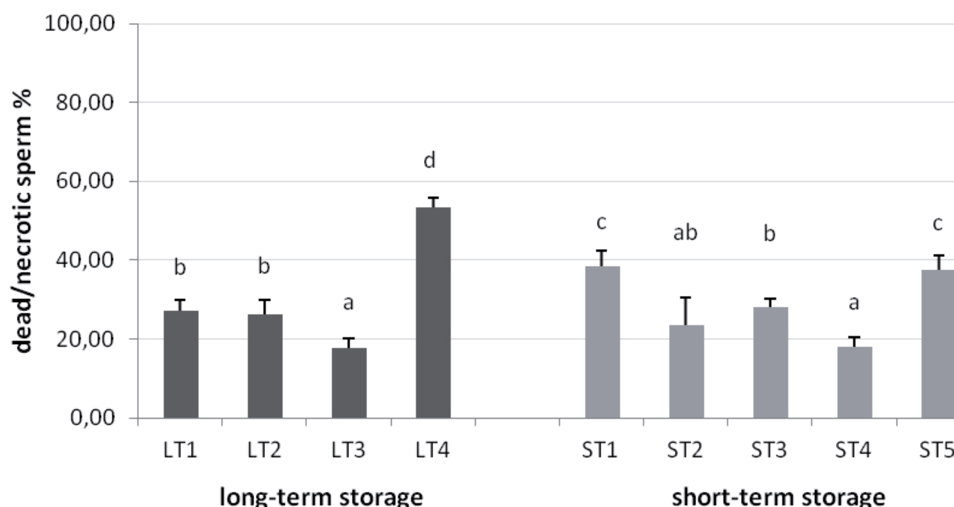

Fig. 2: Inter-male differences in the occurrence of dead/necrotic sperm (PI, %) in frozen-thawed bull semen after long-term or short-term storage. Different superscripts indicate significant differences (P < 0.05)

doses. No difference in total sperm motility either immediately after thawing or after incubation at 37 °C was found between long- and short-term stored groups. This finding is in accordance to the currently accepted cryobiological viewpoint, that there is no functional loss in case of proper storage at −196 °C in liquid nitrogen for indefinite periods of time (Clarke *et al*., 2006).

However, this argument might be discrepant with the results of Haugan *et al.* (2007), that dairy cow conception rates are negatively affected after semen prolonged cryostorage. The long-term storage was reported to be unfavourable compared to short-term storage concerning human sperm motility (Edelstein *et al.*, 2008), mitochondrial function and plasma membrane integrity of cryopreserved boar sperm (Fraser *et al*., 2014).

Our results did not show any substantial difference between sperm viability characteristics of long- or short-term stored bull sperm. The percentage of dead/necrotic sperm was almost similar in each group. Similarly, Edelstein *et al*. (2008) confirmed no difference in incidence of sperm with DNA damage between long- and short-term stored human sperm. In light of these contrary findings, there are still misdoubts in the effect of long-term storage in LN_2 on sperm viability and function. Our results have shown rather high inter-individual bull variability in motility and dead sperm occurrence. It was already stated, that differences in the ability of bull sperm to withstand the stresses of standard cryopreservation protocols and sperm viability are markedly depended on the impact of individual bulls (Kreysing *et al.*, 1997; Loomis and Graham, 2008).

In terms of morphological changes, most of the individuals examined demonstrated morphology in accordance to the commercial insemination dose standards (malformation rate ≤ 20 %). For two males this value was higher than 20 %. Deleterious effect of freezing-thawing procedure on sperm morphology, especially in the head and tail region, was confirmed by several authors in boars, ruminant and human (Garcıa-Herreros *et al.*, 2008; Hidalgo *et al.*, 2005; Connell *et al.*, 2002; Donnelly *et al.*, 2001; Hammadeh *et al.*, 1999). Abnormal bull sperm represented one of the more significant effects on bull fertility (Freneau *et al.*, 2010). Such spermatozoa caused decrease in embryonic development when were used for IVF (Walters *et al.*, 2005). Therefore, morphometry measurements seem to be sensitive biomarker related to sperm fertilization ability (Sailer *et al.*, 1996). In our study, abnormalities in long- and short-term stored sperm were localized mainly in the tail region, represented by minor defects like knob-twisted tail, coiled and rounded tail, and were generally regarded as a tertiary (post-ejaculation) defects, which usually occur after osmotic changes. Tail defects after cryopreservation have been previously reported in human, and plasma membrane destruction in this region has been suggested as the probable reason for these defects (Ozkavukcu *et al.*, 2008).

No effect of storage time on occurrence of sperm morphology abnormalities was proved in short-term and long-term groups, however high inter-male variability was observed. This might be related to season of semen collection or age of bulls (Soderquist *et al*., 1996; Brito *et al*., 2002).

CONCLUSION

In conclusion, our results show no difference in the effect of storage time on the Pinzgau bull spermatozoa characteristics. On the other hand, we have observed the high inter-male variability in the susceptibility of bull sperm to cryoinduced damage. Although, our study was performed on a limited number of animals, it can be suggested that individual differences are an important factor that should be taken into account when semen from individual bulls is to be stored for a long time period as a genetic resource.

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