Sperm storage of wild cervid species: comparison of available cryoprotectants

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Abstract

We compared four diluents (Andromed, dimethylacetamide, dimethyl sulphoxide, Triladyl) used for semen cryopreservation in wild ungulates from the family *Cervidae*, i.e. roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*), and fallow deer (*Dama dama*). Epididymal sperm samples were collected and analysed immediately using Computer Assisted Sperm Analysis with the module for concentration and motility determination and, after equilibration, frozen and placed in liquid nitrogen for one month. After thawing, the samples were again subjected to the same examination procedure. Influence of cryopreservation and choice of the cryoprotectant was assessed by monitoring sperm concentration, average head area (HA), average velocity and progressivity (VAP), beat frequency (BF), total motility (MO), total progressive motility (PR), circular tracks (CT) and mucous penetration (MP). We observed no significant differences in sperm concentration between cryoprotective diluents or fresh and post-thaw samples in all species. All motility indicators (MO, PR, VAP, BF, CT) were influenced by the treatment but did not differ significantly between diluents used in red and roe deer. In fallow deer, commercial diluents (Andromed, Triladyl) resulted in better sperm survival than the alternatives (dimethyl sulphoxide, dimethylacetamide). Only HA showed significant differences ($P < 0.001$) in all species based on the diluent, with no effect of treatment. In contrast, MP was influenced by both the diluent and the cryopreservation process in roe deer and, partly, fallow deer. In future studies, we suggest expanding both the members of the *Cervidae* family examined and the sample size. Knowledge how to optimise cryopreservation protocols for different mammalian species has implications for conservation reproductive medicine of endangered wildlife.

Wild deer reproduction, semen collection, cryopreservation, sperm quality

Long-term storage of mammalian semen, synchronisation of oestrus, artificial insemination and other assisted reproduction methods are common strategies used in livestock and pet reproduction programmes and are increasingly playing an important role in conservation programmes aimed at endangered species (Cseh and Szolti 2005). Currently, such methods are rather infrequently used in wild animals, including farmed game species (Jabbour et al. 1993). Owing to interspecific variability in the reproductive biology of game species males (differences in ejaculate composition, male germ cell dimensions, permeability of sperm membranes to water and solutes, etc.), it is necessary to design species-specific cryopreservation protocols. Sperm survival during cryopreservation will be influenced by the method used, with the quality of ejaculate decreasing during all important methodological phases, i.e. cooling, freezing and thawing. The degree of sperm resistance to cryopreservation is given not only by species specificity but also by the type and concentration of the cryoprotectant used. Moreover, the ratio of other components included in the freezing extender will also have an important effect on the success of long-term ejaculate storage (Prieto-Pablos et al. 2016). For example, Berk et al. (2016) have shown that a higher content of antioxidants can have a favourable effect on sperm viability

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during the cryopreservation process. Commonly used commercial freezing extenders for ruminants are divided into several basic groups, i.e. egg yolk cryoprotectants (low-density lipo-protein protection), soybean or liposome-based cryoprotectants, and alternative cryoprotectants such as dimethyl sulphoxide (DMSO) and dimethylacetamide (DMA) (Tibary and Manar 2018).

Red deer (*Cervus elaphus*), European fallow deer (*Dama dama*), and roe deer (*Capreolus capreolus*) are common game animal species in Europe. Nevertheless, these species differ significantly in their behavioural adaptations (Langbein and Thirgood 1989), including their reproductive biology. The red deer rutting season, for example, culminates in late September and early October, while the reproductive activity of both young and adult roe deer is generally restricted to May to September (Blottner et al. 1996), with the second rut occurring in December of only marginal importance (Goeritz et al. 2003) and that of fallow deer culminating in October (Apollonio and Di Vittorio 2004). Species-specific variations in mating behaviour most probably contribute to differences in basic semen characteristics (Veldhuizen 1994).

While there have been several studies on sperm survival during cryopreservation in individual species (Jabbour et al. 1993; Martinez-Pastor et al. 2009; Prieto-Pablos et al. 2016), a comparison of different cryoprotectants across multiple species is lacking. In line with this, the aim of this study was to compare four diluents used for semen cryopreservation in ruminants represented by three species of wild cervids. The information obtained will help optimise cryopreservation protocols for selected game species and will be useful in developing methodologies for other endangered wildlife.

Materials and Methods

Animals under study

Adult male red deer (n = 3), fallow deer (n = 8), and roe deer (n = 6) originating from the game enclosure and/or free hunting grounds of the Nový Jičín Teaching Farm, University of Veterinary Sciences Brno, Czech Republic, were killed by hunters during the peak of their rutting seasons, i.e. red deer in September and October, fallow deer in October and November, roe deer in June and July. As all animals were three to six years old, they were expected to have high quality sperm (Garde at al. 1998).

Semen collection and quality assessment

Semen samples were collected from the testicles (caput epididymis) of each animal shortly after it was killed, using a Discovery Comfort 1-250 µl innovative pipette (BIOTECH, Prague, Czech Republic) after the incision into the dorsal part of caput epididymis, and then diluted immediately with one of the four extenders. The average volume of a semen sample collected from epididymis was 5 µl. Diluent to sample volume ratio was 20:1. During sampling, every effort was made to avoid contact with water and exposure to direct sunlight and/or temperatures outside the range of 19–21 °C. Immediately after collection, fresh semen characteristics were determined using the Sperm Class Analyser–Computer Assisted Sperm Analysis system (SCA CASA; MICROPTIC S.L., Barcelona, Spain) fitted with a concentration and motility determination module. A Nikon eclipse E200LED MV R camera (Nikon Corporation, Tokyo, Japan) equipped with a Nikon 10 × 0.25 Ph 1 BM WD 7.0 lens and a camera acA 1300-200uc Basler c-Mount (Basler A.G., Mannheim, Germany) was then used to visualise the sperm cells. Sperm concentration and motility parameters were measured in Leja SC 20-01-08-B-CE 8 x 2 µl disposable counting chambers (Cryo Tech s.r.o., Liběchov, Czech Republic).

The fresh and post-thaw (see below) semen characteristics assessed included sperm concentration (CONC; $\times 10^6$/ml), average sperm head area (HA; µm), average velocity and progressivity (VAP; %), beat frequency (BCF; Hz), total motility (MO; %), spermatozoa showing total progressive motility (PR; %), circular tracks (CT; %) and mucous penetration (MP; %). Progressive motility refers to spermatozoa that are mostly swimming in a straight line or in very large circles.

Freezing and post-thaw procedure

The four media used to cryoprotect samples collected from all deer species were Andromed (Minitüb Slovakia s.r.o. Čeľadice, Slovakia) containing aqua bidest, fructose, glycerol, citric acid, buffers, phospholipids, spectinomycin, lincomycin, tylosin, gentamicin, Triladyl (Minitüb Slovakia s.r.o. Čeľadice, Slovakia) containing aqua bidest, glycerol, TRIS, citric acid, fructose, spectinomycin, linkomycin, tylosin, gentamicin, DMSO (Thermo Fisher Scientific, Brno, Czech Republic) and DMA (Thermo Fisher Scientific). The commercial cryoprotectants (i.e. Andromed and Triladyl) were diluted according

to the manufacturers' instructions, while Dulbecco's modified eagle's medium (DMEM; Thermo Fisher Scientific) was used to produce a final concentration of 8% for both the DMSO and DMA cryoprotectants. Before dilution, all media were heated to 37 °C in an Eppendorf ThermoMixer C thermoblock (Thermo Fisher Scientific). After dilution, the samples were placed in clear 0.25 ml Ministraws (Minitüb Slovakia s.r.o.) and equilibrated at 22 °C for 30 min, followed by cooling at 5 °C for 10 min in a Thermobox LBT 168 (ATS CZ s.r.o, České Budějovice, Czech Republic), after which the sample was inserted into liquid nitrogen vapor (−80 °C) for 10 min (Prieto-Pablos et al. 2016). Finally, the samples were inserted into liquid nitrogen and stored for one month. Immediately after thawing in a water bath at 37 °C for 10 min, the samples were examined for quality characteristics as described above.

Statistical analysis

All semen samples were treated with all four cryoprotective media except for fallow deer samples, where just two of eight samples were treated with DMA. Additionally, nine samples were excluded from the analysis due to missing data from the post-thaw assessment (Andromed – two fallow deer, Triladyl – one fallow deer and two roe deer, DMSO – one fallow deer and one roe deer, and DMA – two roe deer). Normality was tested for each species' data subset using the Kolmogorov-Smirnov and Shapiro-Wilk tests. Four sperm parameters (CONC, HA, VAP and BCF) displayed normal distribution and were not transformed, while the four motility parameters (MO, PR, CT and MP) were arcsine transformed to represent the inverse sine of the square root of the proportion, the results of transformation being expressed in radians. Differences between fresh pre-freezing vs. post-thaw assessment values and cryoprotective diluents were tested separately for each deer species using factorial ANOVA with Bonferroni correction, with the probability value set at $P = 0.006$. The interaction between both parameters was included into the model.

Results

There were no significant differences in CONC between fresh and post-thaw samples or between the cryoprotective diluents used in all species (Plate V, Fig. 1). The morphological parameter HA was significantly different in all deer species depending on the cryoprotective diluent used, but was not altered by treatment (i.e. fresh pre-freezing vs. post-thaw; Table 1). On the other hand, all motility parameters (MO, PR, VAP, BF, CT) were influenced by treatment, but did not differ significantly between the diluent used. In red deer and roe deer, the final progressive motility parameters in the post-thaw period were comparable for all diluents used. In fallow deer, however, the used commercial diluents, i.e. Andromed and Triladyl, proved to be more suitable for long-term sperm storage than the alternative diluents DMSO and DMA (Plate VI, Fig. 2). While MP was significantly influenced by both the diluent used and the cryopreservation process in roe deer, and partly also in fallow deer, choice of the diluent had no effect on MP in red deer (Plate VII, Fig. 3).

Table 1. Factorial ANOVA of sperm quality indicators with Bonferroni correction.

	Cervus elaphus			*Dama dama*			*Capreolus capreolus*		
Indicator	Diluent	Treatment	Interaction	Diluent	Treatment	Interaction	Diluent	Treatment	Interaction
CONC	0.106	0.536	0.806	0.093	0.186	0.511	0.940	0.537	0.971
HA	< 0.001*	0.091	0.013	< 0.001*	0.294	0.556	< 0.001*	0.021	0.319*
VAP	0.141	< 0.001*	0.182	0.109	< 0.001*	0.002*	0.419	< 0.001*	0.002*
BF	0.534	< 0.001*	0.092	0.139	< 0.001*	< 0.001*	0.411	< 0.001*	0.003*
PR	0.184	< 0.001*	0.047	0.144	< 0.001*	< 0.001*	0.085	< 0.001*	0.002*
MO	0.418	< 0.001*	0.014	0.065	< 0.001*	< 0.001*	0.056	< 0.001*	0.005*
CT	0.651	< 0.001*	0.037	0.287	< 0.001*	0.005*	0.012	< 0.001*	0.021
MP	0.333	< 0.001*	0.186	0.114	< 0.001*	0.001*	0.003*	< 0.001*	0.001*

CONC – sperm concentration; HA – average of head area; VAP – average of velocity and progressivity; BF – beat frequency; MO – percentage of total motility; PR – percentage of spermatozoa showing total progressive motility; CT – percentage of circular tracks; MP – percentage of mucous penetration.
Significant differences are marked with an asterisk.

Discussion

While many methodological procedures have been designed for freezing the male reproductive cells of free-living ruminants (Pintus and Ros-Santaella 2014), this study was conducted to compare the use of different cryoprotective diluents for freezing the sperm of wild ungulates, strictly adhering to a uniform methodology. Under normal circumstances, the concentration of sperm in fresh semen of fallow deer and red deer differs, with typical mean values of 90×10^6/ml and 620×10^6/ml, respectively (Hernández-Souza et al. 2014). While the concentration of spermatozoa before freezing and after thawing might be expected to differ due to possible fragmentation of cells during the freezing phase (Magyar et al. 1989; Ros-Santaella et al. 2019), our results indicated that all the cryoprotectants used were sufficient to preserve the integrity of deer spermatozoa during freezing. Sperm differences can also be observed at the cellular morphology level, with sperm HA, for example, being approximately 30 μm^2 in red deer (Esteso et al. 2003), 33 μm^2 in fallow deer, and 43 μm^2 in roe deer (Andraszek et al. 2014; Ros-Santaella et al. 2019). Our results indicated notable changes in sperm HA for some of the diluents used, suggesting partial morphological damage to the sperm, particularly the integrity of the acrosome (Alsaadoon et al. 2021) or the phospholipid membrane itself (Favoretto et al. 2012).

The major changes observed during sperm cryopreservation were in motility parameters (see also Boveda et al. 2018), especially in fallow deer. In their cryopreservation study focused on red deer sperm, Martinez-Pastor et al. (2009) noted that there was even a difference between commercial diluents in favour of Triladyl, though this was not confirmed in our study. In the former case, the higher concentration of antioxidants in Andromed may have played a role, in that they increase the sperm's ability to cope with damage from reactive oxygen species during freezing (Ansari et al. 2017). In red and roe deer, differences in motility indicators were also present when comparing different diluents, with Andromed showing the best results, though the differences were non-significant, possibly due to the small number of samples. Differences in fallow deer sperm motility indicators may have been due to differences in sperm morphology, size and/or resistance of the sperm cytoplasmic membrane (Ros-Santaella et al. 2019). The lowest motility indicators were observed in all species when DMA was used, though again the differences were non-significant. Although DMSO appeared to show the highest cytotoxicity (see also Fernández-Santos et al. 2005; Awan et al. 2020), it is likely that DMA also exerts partially cytotoxic effects. Measurements of MP are largely concerned with the ability of sperm to fertilise an oocyte, and such measurements rely on a combination of species-specific values (Van der Horst 2021). Mucous penetration is a factor used to evaluate the sperm fertilization ability (Hamano et al. 2001). Thus, the significant differences observed in roe deer and fallow deer MP (Table 1) point to a violation of one of the mechanisms required for fertilisation of the oocyte. As such, it can be assumed that the ability of the cell to resist steric obstruction and adhesion was impaired by the cryopreservation process. In this regard, red deer sperm appear to be more resistant to sudden changes in temperature during cryopreservation and thawing.

In conclusion, our results confirm that all the cryoprotective diluents examined may be used for long-term storage of semen of free-living ungulates from the family *Cervidae*, i.e. red deer, fallow deer, and roe deer. Commercial diluents (Andromed, Triladyl) proved to be more suitable for fallow deer than the alternative cryoprotectants examined (DMSO, DMA), particularly as regards preservation of motility parameters (MO, PR, VAP, BF, CT). The concentration of spermatozoa following the freezing and thawing process did not change in any species, whichever diluent was used. For red deer and roe deer, most of the observed indicators showed no significant differences in relation to the diluent choice,

while red deer sperm appeared to be the most resistant to sudden changes in temperature during cryopreservation and thawing.

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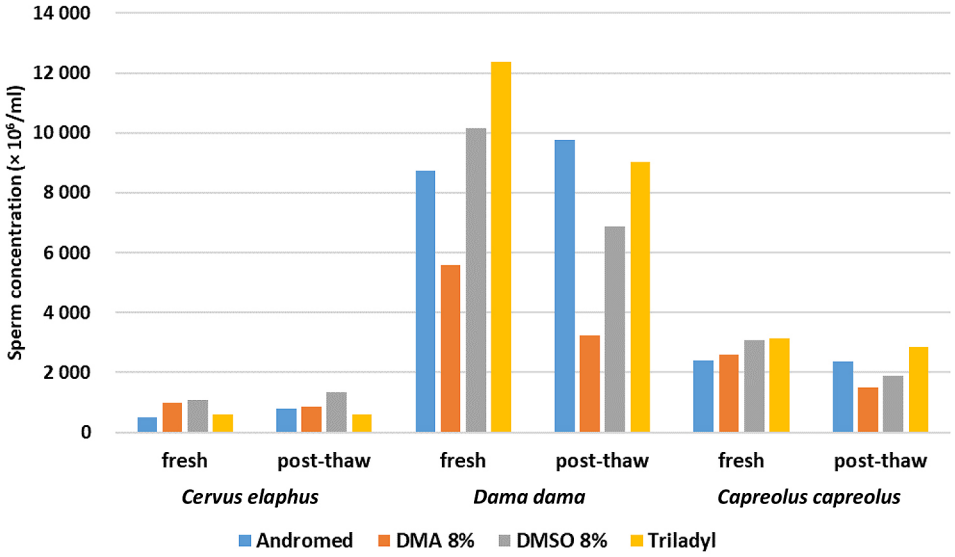

Fig. 1. Sperm concentration in red, fallow and roe deer.

Post-thaw – after storage in liquid nitrogen for one month; Andromed – soybean lecithin based cryoprotectans; DMA – dimethylacetamide; DMSO – dimethylsulphoxide; Triladyl – egg yolk cryoprotective medium.

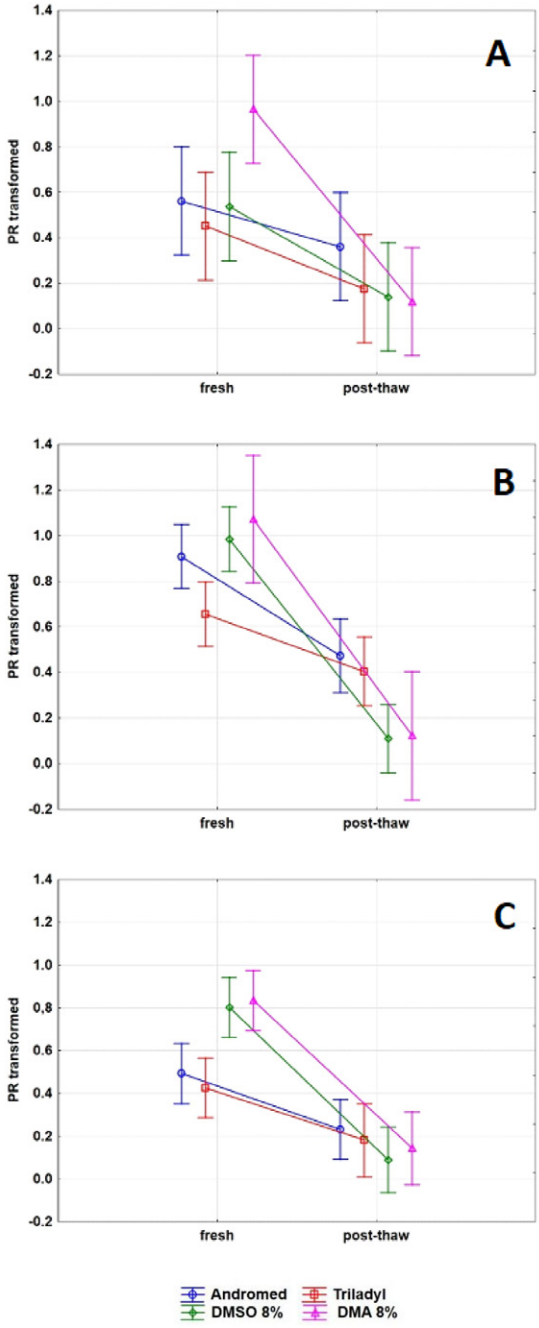

Fig. 2. Sperm progressive motility (PR) in red, fallow, and roe deer. A – *Cervus elaphus,* B – *Dama dama*, C – *Capreolus capreolus*.

Progressive motility refers to spermatozoa that are swimming mostly in a straight line or in very large circles. Example showing a difference between game species in the post-thaw active motility indicator of sperm.

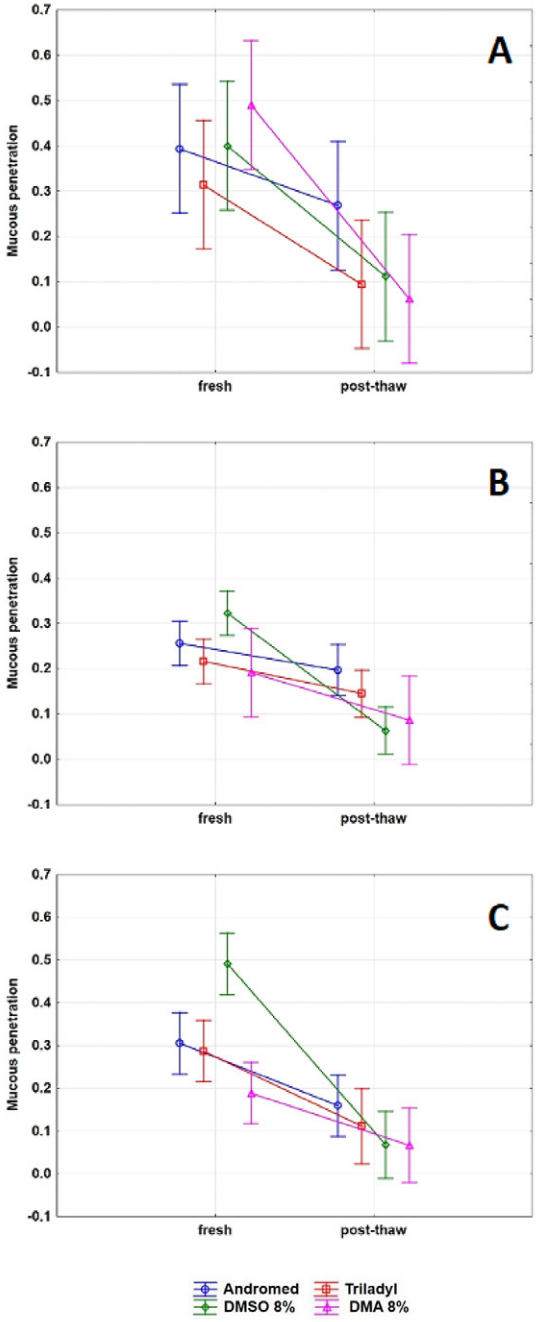

Fig . 3. Sperm mucous penetration in red, fallow, and roe deer. A – *Cervus elaphus*, B – *Dama dama*, C – *Capreolus capreolus*.

Mucous penetration is the ability of sperm to penetrate cervical mucus. Example showing a difference between game species in the post-thaw indicator of sperm.