Original article

The effect of two packaging systems on the post-thaw characteristics of canine sperm

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Abstract

The aim of this study was to compare the effect of different packaging systems on some parameters of cryopreserved canine spermatozoa. The experimental material consisted of the sperm-rich fractions of ejaculates collected from four Beagle dogs. Semen samples for cryopreservation were stored in 0.25 ml plastic straws and two aluminum tubes with a total volume of 5.0 ml. Semen was frozen in static nitrogen vapor for 10 minutes (0.25 ml straws) or 15 and 20 minutes (aluminum tubes). Post-thaw assessments involved the determination of sperm motility parameters using a computer assisted sperm analyzer (CASA), sperm plasma membrane integrity (SPMI), mitochondrial membrane potential (MMP) and acrosome integrity (normal apical ridge, NAR). Regardless of the packaging system applied, no significant differences in total sperm motility (TMOT) or selected kinematic parameters were observed after freezing-thawing. However, spermatozoa frozen in 0.25 mL straws were characterized by improved functionality, in particular mitochondrial function, after thawing. The results indicate that large quantities of canine semen can be frozen in aluminum tubes. Further studies are required, however, to evaluate different freezing and thawing rates of aluminum tubes.

Key words: dog, semen, freezing, straws, aluminum tubes

Introduction

The effectiveness of canine semen cryopreservation is determined by various parameters, including extender composition, type of cryoprotectant, freezing and thawing rates, and semen packaging method (Nothling et al. 2005). Canine semen intended for cryopreservation is most often stored in 0.5 ml or 0.25 ml straws (Farstad 2010). Pellets and minitubes are less frequently used to store frozen semen (Niżański and Dubiel 2003). Methods for storing large volumes of semen do not have many practical applications in the cryopreservation of canine semen because the frozen sperm-rich fraction of ejaculate has relatively low volume (Niżański and Dubiel 2003). In some cases, large-volume semen samples with optimal sperm cell concentrations contribute to the effectiveness of artificial insemination in female dogs (Ivanova-Kicheva et al. 1997).

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Unlike in other animal species (Tischner 1979, Strzeżek et al. 1985, Ali and Tischner 1988, Fraser and Strzeżek 2007), there is a lack of information regarding the comparison of the quality of post-thaw canine semen following cryopreservation in plastic straws and aluminum tubes. Furthermore, there is only one study which compared the quality of post-thaw canine semen when cryopreserved in straws and aluminum tubes (Kosiniak et al. 1992). The objective of this study was to compare the effect of different packaging systems on some parameters of cryopreserved canine spermatozoa.

Materials and Methods

Semen collection and initial evaluation

Sperm-rich fractions of ejaculates were collected from four Beagle dogs (aged 2-6 years), once weekly over a period of 5 weeks. The dogs were housed in individual pens and fed commercial canine food. Water was available ad libitum. Animal experiments were carried out in accordance with the guidelines set out by the Local Ethics Committee.

A preliminary assessment of ejaculates involved an evaluation of sperm motility by the CASA method and determination of sperm cell concentrations by the cytometric method in a hemocytometer. Semen samples with TMOT higher than 80% and sperm counts higher than 200×10^6 sperm cells per 1 ml of semen were qualified for further analyses.

Freezing-thawing procedure

After individual evaluation, semen was pooled and centrifuged (700 x g, 5 min). Seminal plasma was removed, semen sediments were diluted with standard Tris-citrate-fructose (TCF) extender (Rota et al. 1995) to a concentration of 200×10^6 spermatozoa/ml and left to stand for 30 minutes at room temperature (approx. 22ºC). In next step, the samples were cooled at 5ºC for 60 minutes. After chilling of the semen, a second dilution (1:1) was performed with an extender containing 5.0 mL TCF, 4.0 mL hen egg yolk, 0.8 mL glycerol and 0.2 mL Orvus Es Paste, to obtain a concentration of 100×10^6 spermatozoa/ml. The final concentrations of hen egg yolk, glycerol and Orvus Es Paste in diluted semen samples were determined at 20%, 4% and 1%, respectively. Equilibration time at 5ºC was 15 minutes. Semen was loaded in 0.25 ml plastic straws (Minitüb, Germany) and two aluminum tubes with a total volume of 5.0 ml (Opakomet, Poland), pre-cooled to 5ºC. Semen samples were frozen

in a closed styrofoam box by placing filled straws and aluminum tubes on a rack suspended 4 cm above the surface of liquid nitrogen. Freezing time was 10 minutes for semen stored in straws (S) and: a) 15 minutes for semen stored in the first tube (T15), b) 20 minutes for semen stored in the second tube (T20). Frozen straws and tubes were placed in a container with liquid nitrogen (LN_2). Straws were thawed in a water bath at 70ºC for 5 s (Nöthling et al. 2005). Aluminum tubes were thawed at 65ºC for 12-15 s (Ivanova-Kicheva et al. 1997). Thawed samples were subjected to a qualitative analysis.

Post-thaw evaluation of sperm parameters

Sperm motility characteristics were evaluated using the Hamilton-Thorne Sperm Analyzer IVOS version 12.3 (Hamilton-Thorne Biosciences, USA). Software settings for the semen analyzer were recommended by the manufacturer for canine sperm analyses: frame acquired – 30, frame rate – 60 Hz, minimum cell contrast – 75, minimum cell size – 6 pixels, straightness threshold – 75%, path velocity threshold – 100 µm/s, low VAP cut-off – 9.9 ,m/s, low VSL cut-off – 20 µm/s, static size gates – 0.80-4.93, static intensity gates – 0.49-1.68, static elongation gates – 22-84. To accurately determine sperm motility parameters in the presence of the egg yolk (yolk globules can be misidentified as immobile sperm), sperm samples were stained with fluorescent IDENT stain (Hamilton Thorne Biosciences, Beverly, MA, USA) before analysis, and UV excitation was used (Niżański 2006). A droplet of approximately 5 µl was placed in a Makler counting chamber (Sefi-Medical Instruments Ltd., Haifa, Israel) at 37ºC, and six fields were examined per sample. The following sperm motility parameters were determined by the IVOS analyzer: total motility (TMOT, %), progressive motility (PMOT, %), average path velocity (VAP, µm/s), straight line velocity (VSL, µm/s), curvilinear line velocity (VCL, µm/s), amplitude of lateral head displacement (ALH, µm), beat cross frequency (BCF, Hz), straightness (STR %) and linearity (LIN, %).

Sperm plasma membrane integrity (SPMI) was assessed by the dual fluorescent staining technique described by other authors (Garner and Johnson 1995), using SYBR-14 and propidium iodide, PI (PI, Live/Dead Sperm Viability Kit; Molecular Probes, OR, USA). Aliquots (10 µL) of stained sperm samples were examined under an epifluorescence microscope (Olympus CH 30, Tokyo, Japan). For each aliquot, approximately 200 sperm cells were classified as spermatozoa with intact or damaged plasma membrane.

Table 1. The influence of packaging system and freezing time on the motility parameters of frozen-thawed canine spermatozoa.

Packaging system	Time (min)	TMOT (%)	PMOT (%)	Kinematic parameters						
				VAP (%)	VSL (%)	VCL (%)	ALH (%)	BCF (%)	STR (%)	LIN (%)
0.25 ml straws	10	55.36±4.86	29.55±3.03	105.10±2.48[a]	92.45±2.58[a]	148.09±3.06[a]	5.88±0.15[ab]	13.30±1.51	87.55±0.93	64.73±1.21
5.0 ml aluminum tubes	15	54.67±3.09	20.58±2.69[b]	90.27±2.24[b]	81.09±1.92[b]	125.20±3.51[b]	5.63±0.14[a]	16.19±1.08	89.73±0.68	67.36±1.08
	20	56.57±3.64	20.57±1.43[b]	93.57±0.97[b]	82.57±0.75[b]	130.86±1.65[b]	6.16±0.09[b]	15.26±1.78	88.29±0.52	65.86±1.42

The results are presented as mean values (± S.E.M). Values with different superscripts (a, b) are significant at p<0.05, within columns.
Time – time of incubation above the surface of LN_2, TMOT – percentage of total motile sperm, PMOT – percentage of progressively motile sperm, VAP – average path velocity, VSL – straight line velocity, VCL – curvilinear line velocity, ALH – amplitude of lateral head displacement, BCF – beat cross frequency, STR – straightness, LIN – linearity.

Table 2. The effect of packaging system and time of sample incubation in nitrogen vapor on selected functionality indicators of frozen-thawed canine spermatozoa.

Packaging system	Time (min)	Indicators of sperm functionality		
		SPMI (%)	MMP (%)	NAR (%)
0.25 ml straws	10	49.22 ± 1.23[a]	45.12 ± 0.82[a]	89.05 ± 1.23[a]
5.0 ml aluminum tubes	15	45.38 ± 0.84[b]	39.75 ± 1.86[b]	83.67 ± 1.56[b]
	20	47.00 ± 1.29[ab]	40.52 ± 2.02[b]	86.32 ± 1.97[ab]

The results are presented as mean values (± S.E.M). Values with different superscripts (a, b) are significant at p<0.05, within columns.
Time – time of incubation above the surface of LN_2, SPMI – sperm plasma membrane integrity, MMP – sperm mitochondrial membrane potential, NAR – spermatozoa with normal acrosomal ridges.

Mitochondrial membrane potential (MMP) was assessed in sperm aliquots using a dual fluorescent staining technique with 5,5',6,6'-tetrachloro-1,1',3,3'--tetraethylbenzimidazolylcarbo-cyanineiodide (JC-1) (1 mg/ml DMSO) (Molecular Probes, Eugene, OR, USA) and PI (10 µl of PI solution in 0.5 mg/ml PBS), according to a previously described method (Thomas et al. 1998). Aliquots (10 µl) of stained sperm samples were examined under an epifluorescence microscope (Olympus CH 30, Tokyo, Japan). Sperm cells displaying only orange-red fluorescence at the mid-piece region were considered viable spermatozoa with high MMP.

The percentage of spermatozoa with normal acrosomal ridges (NAR) was assessed using the Giemsa staining method described by Watson (1975). Approximately 200 sperm cells per slide were examined under a light microscope at 1000 × magnification and were classified as spermatozoa with NAR acrosome or damaged apical ridge acrosome.

Statistical analysis

All values were expressed as the means ± standard error of the mean (SEM). The data were analyzed by ANOVA and Duncan's multiple comparison test using the Statistica software package (StatSoft Incorporation, Tulsa, OK, USA). Differences between means were regarded as significant at p≤0.05.

Results

No significant differences (p>0.05) in TMOT values of post-thaw sperm were observed between semen samples frozen in 0.25 ml straws (S) and aluminum tubes (T15 and T 20) (Table 1). However, PMOT values were significantly higher (p<0.05) in semen samples frozen in S. Samples cryopreserved in 0.25 ml straws were also characterized by the highest velocity parameters (VAP, VSL, VCL). The applied cryopreservation method did not affect the values of BCF, STR or LIN. Prolonged freezing time in aluminum tubes (from 15 to 20 minutes) had no significant effect on the majority of sperm motility parameters, excluding ALH.

After thawing, no significant differences (p>0.05) in SPMI or NAR were observed between semen samples cryopreserved in 0.25 ml straws and in aluminum tubes with prolonged freezing time in the vapor phase of liquid nitrogen (T20) (Table 2). Spermatozoa frozen in 0.25 ml straws were characterized by higher MMP values. The lowest values of SPMI,

MMP and NAR were shown by semen samples cryo-reserved in aluminum tubes and cooled in liquid nitrogen vapor for 15 min (T15). However, samples cryoreserved in aluminum tubes and cooled in nitrogen vapor for 15 minutes and 20 minutes (T15 and T20) did not differ significantly (p>0.05) in their values of SPMI, MMP or NAR.

Discussion

There is a general scarcity of published studies into the cryopreservation of large quantities of canine semen. Also, there is a lack of comparative studies regarding the quality of canine semen following cryopreservation in straws and aluminum tubes. Thomas et al. (1993) demonstrated that larger quantities of canine semen can be frozen in 2.5 ml straws that had been previously used to cryopreserve boar semen. However, higher sperm motility values were reported in semen frozen in pellets and 0.5 ml straws. Kosiniak et al. (1992) confirmed, for the first time, that cryopreservation of semen in 0.5 ml straws yielded higher percentage of post-thaw sperm motility (40-50%) compared with semen samples cryopreserved in 1.0 ml aluminum tubes (30-40%). Our studies did not show marked significant differences in post-thaw motility of canine spermatozoa between semen samples cryopreserved in 0.25 ml straws and 5.0 ml aluminum tubes. However, semen samples cryopreserved in 0.25 ml straws exhibited significantly higher percentage of spermatozoa with progressive motility (PMOT) than those cryopreserved in 5.0 ml aluminum tubes. In a study by Ivanova-Kitcheva et al. (1997), canine semen cryopreserved in the Tris-citrate-fructose extender in 5.0 ml aluminum tubes was characterized by a higher percentage of motile spermatozoa after thawing (25.9±1.76) than semen frozen in pellets (20.45±1.71). In our study, the percentage of motile sperm, determined by CASA, exceeded 50%, which could be attributed to different methods of assessment of sperm motility and semen handling procedures in the cryopreservation protocol. Ivanova-Kitcheva et al. (1997) added glycerol-containing extender directly to semen and equilibrated semen samples for 180 minutes. Glycerol is the most popular cryoprotectant for the cryopreservation of canine semen, but it exerts a toxic effect on spermatozoa, in particular at higher temperatures (Martins-Bessa et al. 2006). In most semen cryopreservation protocols, glycerol is added at 32-37°C or at room temperature (Peña et al. 1998, Rota et al. 1998, Silva et al. 2006). In our study, the contact between sperm and glycerol was limited by adding glycerol at 5°C and applying the cryoprotectant at a low final concentration of 4% in the semen-extender mixture for cryopreservation. The

adopted procedure probably reduced glycerol's toxic effect on sperm motility and other parameters of cryopreserved spermatozoa. Rapid freezing rates of sperm stored in small packaging (such as straws) also lower glycerol concentrations in the extender (Niżański and Dubiel 2003). The applied cryoprotectant prevents cell damage resulting from low freezing rates (Peña et al. 2006). The freezing process is probably slower in aluminum tubes which are characterized by larger volume than straws. According to other authors, boar and canine semen stored in aluminum tubes should be cooled in static nitrogen vapor for minimum 15 minutes (Ivanova-Kicheva et al. 1997, Strzeżek et al. 1985). In our study, prolonged freezing time in aluminum tubes (from 15 to 20 minutes) had no significant influence on the quality of thawed semen.

Rapid freezing combined with rapid temperature increase during thawing improve semen quality (Rota et al. 1998, Bielas et al. 2010). In the present study, semen samples were rapidly frozen by placing packaged samples 4 cm above the surface of liquid nitrogen. This procedure is consistent with the classical method proposed by Anderson (1972) where semen is frozen in nitrogen vapor at -140°C. Straws were thawed at high temperature over a short period of time (70°C/5 sec) (Nothling et al. 2005). Aluminum tubes were also exposed to high temperature (65°C) in accordance with the protocol developed by Ivanova-Kitcheva et al. (1997). During rapid freezing-thawing, the applied semen storage method has a significant effect on post-thaw semen quality. Straws, minitubes and pellets have a high surface-to-volume ratio, which permits rapid cooling of the entire sample during freezing and limits temperature fluctuations during water crystallization (Bielas et al. 2003, Niżański and Dubiel 2003). As a result, the temperature inside the sample responds rapidly to changes in ambient temperature, and the degree of overcooling is minimal in comparison with larger packaging. In general, post-thaw semen quality is significantly higher when semen is stored in packaging with a smaller volume and a flat or/and round cross-section. Such packaging has a high surface-to-volume ratio, which speeds up heat transfer between its contents and the surroundings (Ericsson et al. 2002, Bielas et al. 2010). Aluminum tubes are flat and have a large surface, therefore, heat transfer is high during freezing. Aluminum tubes also store larger quantities of semen, and significant differences could exist between freezing and thawing rates in the central and peripheral sections of the tube. The above hypothesis seems to validate the results of our study where semen samples cryopreserved in aluminum tubes were characterized by somewhat lower average values of selected quality parameters in comparison

with the samples stored in straws. The use of programmable freezers is the optimal solution for achieving uniform freezing conditions for semen frozen in larger packaging (Niżański and Dubiel 2003). Programmable freezers minimize temperature fluctuations inside the packaging in comparison with semen samples frozen over the surface of liquid nitrogen (Schäfer-Somi et al. 2006).

Our findings indicate that canine semen can be frozen in larger quantities by introducing certain modifications to the cryopreservation protocol, in particular the time of tube incubation in nitrogen vapor.

Acknowledgements

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