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In vitro evaluation of canine spermatozoa cryopreserved in different extenders

[*Avaliação* in vitro *do sêmen canino criopreservado em diferentes diluidores de congelação]*

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ABSTRACT

The efficacy of three extenders, tris-egg yolk-5% ethylene glycol (T1), lactose-egg yolk-5% ethylene glycol (T2) and lactose-egg yolk-5% dimethyl formamide (T3) on preserving the viability of post-thawing canine spermatozoa was evaluated. Three ejaculates per dog were obtained of five animals. The semen was packaged in 0.5ml straws and cooled to 4°C for 120min. The straws were frozen 4cm above the nitrogen level for 15min and thawed in water-bath at 37°C for 60sec and at 75°C for 7sec. Progressive motility and vigour were evaluated immediately after thawing (time 0) and at 30, 60, 90 and 120min. Structural and functional integrity of plasma membrane of the spermatozoa were evaluated, respectively, by fluorescent staining probes and hypoosmotic swelling test. Lactose-egg yolk based extenders showed better cryoprotectant capability and dimethyl formamide was an alternative cryoprotectant agent for dog sperm cells.

Keywords: dog, semen, cryopreservation, ethylene glycol, dimethyl formamide

RESUMO

Avaliou-se a eficácia de três diluidores, tris-gema com 5% de etileno glycol (T1), lactose-gema com 5% de etileno glicol (T2) e lactose-gema com 5% de dimetil-formamida (T3) na criopreservação do sêmen de cães. Foram obtidos três ejaculados por cão de um total de cinco animais. O sêmen foi envasado em palhetas de 0,5ml e resfriado até 4°C por 120min. As palhetas foram congeladas 4cm acima do nitrogênio líquido por 15min e descongeladas em banho-maria a 37°C por 60seg e 75°C por 7seg. A motilidade progressiva e o vigor foram avaliados imediatamente após a descongelação (tempo 0) e aos 30, 60, 90 e 120min. A integridade estrutural e funcional da membrana plasmática do espermatozóide foi avaliada, respectivamente, por meio da coloração de fluorescência e pelo teste hiposmótico. Os diluidores à base de lactose gema foram mais eficazes em preservar a viabilidade espermática pós-descongelação e a dimetil-formamida é um crioprotetor eficaz para espermatozóides de cães.

Palavras-chave: cão, sêmen, criopreservação, etileno glicol, dimetil-formamida

INTRODUCTION

There is an increasing demand for conservation of canine semen among the breeders of valuable dogs. Cryopreservation is an important procedure as it facilitates a wide use of ejaculates of stud dogs, regardless of time and location.

The success of the preservation of spermatozoa by cooling, freezing and thawing is dependent

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upon a series of steps aimed at reducing damage to the cell and securing adequate longevity *in vitro* and *in vivo* (Fastad, 1996). Many protocols have been described for canine semen cryopreservation, and differences are linked to cooling rates and equilibration time (Strom et al., 1997; Peña and Linde-Forsberg, 2000) composition of the extenders (Peña et al., 2003) and nature of the cryoprotectants (Olar et al., 1989).

Most of the recent researches have used glycine-egg yolk, skimmed milk, lactose-egg yolk (Olar et al., 1989) and tris-egg yolk (Peña and Linde-Forsberg, 2000; Peña et al., 2003). Also, several companies have developed their own buffers (Silva and Verstegen, 1995; Nothling et al., 1995; Strom et al., 1997).

Glycerol is the most widely cryoprotectant agent used for freezing canine spermatozoa. However, the toxicity of glycerol (Fary, 1986; England, 1993) is leading to the need to elect other alternatives. Penetrating cryprotectants such as dimethyl sulphoxido (DMSO) (Olar, 1989) and ethylene glycol (Vannucchi et al., 1999) have been used for canine sperm preservation. Recent studies used some forms of amides, including methyl and dimethyl formamide (DMF) and acetamide as cryoagent for equine spermatozoa preservation (Hanada and Nagase, 1980; Snoek, 2003; Squires et al., 2004). No reports were found indicating the use of amides on cryopreservation of canine spermatozoa.

Cryopreservation of canine sperm with the available techniques still reduces substantially the survival of spermatozoa in the bitch's genital tract from seven days (Doak et al., 1967) to 12h or less (Concannon and Battista, 1989), which is particularly critical in this species due to the long period of heat and receptivity. The high individual variability of the quality of post-thawed semen and the short lifespan of thawed spermatozoa *in vitro* indicate that the current cryopreservation methods are not optimal, which may partially account for reduced conception rates obtained after artificial insemination (AI) with frozen semen (Peña et al., 2003).

The aim of this study was to study the efficacy of three extenders on preserving the viability of post-thawing canine spermatozoa.

MATERIALS AND METHODS

Five dogs were used being two Shar-Pei, one Retriever of Labrador, one Basset-Hound and one crossbred, 2 to 8 year-old. After extragonadal reserve sperm renewal, three ejaculates per dog were obtained with an interval of 72h. Immediately post-collection, seminal analysis included concentration and sperm progressive motility (PM), vigour (V) and morphology. Only ejaculates showing ≥80% PM, vigour ≥4 and ≥70% of morphologically normal sperm cells were included in the study.

Semen was initially diluted 1:1 in glucose-EDTA extender (Martin et al., 1979), divided into three aliquots and centrifuged at 655g for 6min. The supernatant was discarded and each sperm pellet was diluted in one of the three freezing extenders (Table 1). The extenders were: tris-egg yolk-5% ethylene glycol (T1), lactose-egg yolk-5% ethylene glycol (T2) and lactose-egg yolk-5% dimethyl formamide (T3). Final dilution was performed in order to obtain $100x10^6$ spermatozoa/ml. Samples were packaged in 0.5ml straws and placed in a refrigerated styrofoam box to cool down to 5°C in 60 minutes; additional 60 minutes at 5°C completed equilibration time. Straws were frozen 4cm above the surface of liquid nitrogen (LN_2) for 15min (Bueno, 2000).

The thawing regimens were: 37°C for 1min (Silva et al., 1998) and 75°C for 7sec followed by a minimum of 1min in 37°C water-bath (Strom Holst et al., 2000). A 200µl aliquot of thawed semen was placed in a 1.5ml plastic tube and incubated at 37°C. Sperm PM and V were evaluated immediately after thawing (time 0) and at 30, 60, 90 and 120min.

Functional integrity of sperm membrane of the tail was assessed immediately after thawing by the hypo-osmotic swelling test (HOS-test) (Kumi-Diaka, 1993) using a 60-mosmol fructose solution. Samples were processed according to Jeyendran et al. (1984). Two hundreds cells were counted at 1000X magnification and the percentage of curling/swelling tails was determined according to Melo and Henry (1999).

Structural integrity of sperm head membranes was assessed immediately after thawing using

6-carboxifluorescein diacetate (C-FDA) and propide iodide (PI) (Harrison and Vickers, 1990). Hundred sperm cells per sample were counted at 400X magnification. Cells were classified as *intact* – showing green fluorescence of the C-FDA due to plasma and acrosomal membranes integrity, and *damaged* - showing red fluorescent of the PI due to damaged plasma and acrosomal membranes.

Table 1. Composition of freezing extenders used for canine semen cryopreservation

	Tris5%EG(T1)	Lac5%EG*(T2)	Lac5%DMF*(T3)
Tris	3.025g	-	-
Citric acid, monohydrate	1.7g	-	-
Fructose	1.25g	-	-
Lactose 11%	-	50ml	50ml
Glucose-EDTA	-	25ml	25ml
Egg yolk	20ml	20ml	20ml
Ethylene glycol	5ml	5ml	
Dimethyl formamide	-	-	5ml
Streptomycin sulphate	0.10g	-	-
Equex STM Paste**	-	0.5ml	0.5ml
Distilled water	to 100ml	-	-
pH	6.86	6.91	6.97
Osmolarity	1220	1365	1289

*Martin et al. (1979) modified

**Nova Chemical Sales, Scituate, Inc., MA, USA.

Results are presented as means and standard deviation. Analysis of variance was used to evaluate differences between extenders and the effect of thawing regimens. Means obtained for different parameters (progressive motility and integrity of sperm membranes) were compared by the Students't test ($P<0.05$), while vigour was analyzed by Kruskall-Wallis test (Sampaio, 1998). All analyses were performed using the Statistical Package for Social Science Program (Statistical…,1999).

An independent two-sample t-test was used to compare results of the thermalresistance test. For dependent samples, it was used the paired t command. The commercial software Minitab version 13 was used for this analysis.

RESULTS

Parameters of post-thawed sperm cells are shown in Table 2. Immediately post-thawing, the effect of thawing regimen within extenders was observed only for PM in T2 and for HOS in T1. Significantly higher PM in T2 and lower HOS response in T1 samples were observed when both were thawed at 37°C ($P<0.05$).

Table 2. Parameters of sperm cells of immediately post-thawed dog semen cryopreserved in three extenders and thawed at two temperatures

Freezing extender	TR	PM(%)	NOR(%)	INT(%)	HOS(%)
Tris5%EG (T1)	1	34.8±7.4bA	84.4±8.9aA	49.7±4.7bA	55.5±7.0aB
	2	28.7±6.6bA	88.4±7.0aA	52.5±8.7bA	62.4±8.6aA
Lac5%EG (T2)	1	37.7±8.4abA	78.0±5.2aA	67.6±4.1aA	58.8±7.8aA
	2	28.5±10.4bB	75.6±6.9bA	70.8±7.2aA	65.0±9.6aA
Lac5%DMF (T3)	1	45.5±11.3aA	65.3±14.2aA	56.7±7.5bA	49.2±6.6aA
	2	43.3±6.4aA	69.5±11.2bA	56.5±5.3bA	51.2±7.9bA

PM = progressive motility; NOR=morphologically normal spermatozoa; INT=normal structural integrity of plasma and acrossomal membranes; HOS=functional integrity of plasma membrane; TR = thawing regimen: 1 - 37°C for 1min; 2 - 75°C for 7sec followed by a minimum of 1min in 37°C water-bath. Lac=lactose; EG=ethylene glycol; DMF=dimethyl formamide

Means with different capital letters between TR and different lower case letters between freezing extenders differ, $P<0.05$ (Student t-test).

Samples thawed at 37°C showed lower PM in T1 compared to T3 (P<0.05); higher proportion of sperm with normal structure of plasmatic and acrosomal membranes was found in T2 (P<0.05) and no effect was observed for the HOS response. Using 75°C as thawing temperature, PM was higher in T3 (P<0.05), higher normal sperm morphology was found in T1, higher proportion of sperm cells had normal structure of the plasmatic and acrosomal membranes in T2 (P<0.05) and lower proportion of sperm cells reacted to the HOS in T3 (P<0.05).

PM values during incubation at 37ºC are shown in Table 3. Drop of PM was faster in samples frozen in T1 when samples were thawed at 37ºC (P<0.05). A similar trend was observed when thawing temperature was 75ºC.

Table 3. Percentage of progressively motile spermatozoa during incubation at 37ºC of canine semen cryopreserved in three extenders and thawed at two temperatures

Thawing rate: 37ºC for 1min	Incubation time(min)				
Freezing extender	0	30	60	90	120
Tris5%EG (T1)	34.8±7.4b	11.82±5.08b	3.32±4.25b	1.32±2.17b	0b
Lac5%EG (T2)	37.3±8.4ab	20.66±5.21a	15.98±7.51a	7.98±5.07a	2.88±2.83a
Lac5%DMF (T3)	45.5±11.3a	21.16±5.09a	10.98±6.31a	5.3±3.63a	3.78±3.0a
Thawing rate: 75ºC for 7sec	Incubation time(min)				
Freezing extender	0	30	60	90	120
Tris5%EG (T1)	28.7±6.6b	10.87±6.37b	5.66±6.62b	3.64±4.46b	1.66±2.35b
Lac5%EG (T2)	28.5±10.4b	19.1±9.39ab	12.64±7.4ab	10.32±8.09ab	3.98±3.25a
Lac5%DMF (T3)	43.3±6.4a	27.76±10.90a	19.94±9.32a	13.32±6.78a	7.32±4.34a

Lac = lactose; EG = ethylene glycol; DMF = dimethyl formamide.
Column values with different letters differ (P<0.05) for freezing extenders.

DISCUSSION

The use of several *in vitro* tests showed that each extender had different capability of sperm preservation depending on the evaluated parameter and they were not similarly better or worst for a given extender. Sperm motility immediately post-thawing was higher in semen diluted in T3, a higher percentage of morphologically normal sperm cells was found in T1, the percentage of sperm cell maintaining the integrity of the plasma and acrosome membranes was higher in T2, while lower percentage of cells reacted to the hipoosmotic test in T3 when thawed at 75ºC. Also, the integrity of the plasmatic and acrosomal membranes and functional integrity of the tail membrane seemed to be, immediately after thawing, better preserved than the motility. In other studies, low correlation were found between these parameters of sperm cells post-thawing (Snoeck, 2003). Better HOS–test response than motility after thaw has also been observed in equine spermatozoa (Neild et al., 1999). According to Melo and Henry (1999), it is possible to find spermatozoa with functional plasma membrane integrity, but without motility. These spermatozoa could maintain the potential to acquire motility inside the female reproductive tract. This fact is supported by the statements of Silva and Verstegen (1995), who tested the efficacy of three extenders using artificial insemination and showed higher fertility index for samples that presented the lowest percentage of motility after thawing. In the present study, the results demonstrate that, when thawed at 75°C, T3 showed the lowest number of spermatozoa with functional plasma membrane integrity when compared to the other two treatments, despite the higher progressive motility post-thawing. It is imperative, in order to adequately rank the cryoprotectant potential of extenders, to study the correlation between each evaluated parameter and fertility. It would be interesting to know if functional and/or structural integrity of the plasma membrane immediately

after thawing have a higher correlation than motility with the fertilizing capacity of dog spermatozoa. Considering the four parameters together, immediately post-thawing, it is not evident which extender has a better cryoprotectant potential.

A discrete interaction between extenders and thawing rate could be observed in the present study. Lower ($P<0.05$) post-thawing motility was observed in T2 and higher ($P<0.05$) percentage of sperm cell reacting to the HOS-test was observed in T1 extender when the samples were thawed at 75ºC as compared to 37ºC. Other parameters for all three extenders did not vary according to thawing temperature. This indicates that efficiency of post-thawing cell survival for a given extender may be improved if thawing temperature is adjusted.

Considering the thermalresistance test, it can be seen that lactose based extenders, independently of the cryoagent used, showed better sperm longevity than the TRIS extender using ethylene glycol as cryoagent. This was particularly seen when samples were thawed at 37ºC. Using 75ºC, T3 also showed a better performance than T1, while post-thawed longevity of sperm cells frozen in T2 showed intermediate values. Better longevity performance of lactose based extenders may reflect, in one side, an initial better post-thawing motility (T3) and, on the other side, a better protection of sperm membranes (T2). These results indicate that the use of several parameters to evaluate post-thawing sperm cells in vitro may help in predicting viability. As far as longevity is concerned it seemed that sperm morphology (T1) had low predicting value. Molinia et al. (1994) suggested that extenders with superior motility immediately after thawing were likely to sustain the motile cell population longer, while those with lower motility were likely to have a higher incidence of cell mortality. However, Strom et al. (1997) suggested that a higher initial motility leads to a quicker consumption and depletion of ATP and increase in metabolic waste products, which could contribute to a quicker decrease in motility. The results of the present study indicate that spermatozoa with a high initial motility do not necessarily have a low longevity, since a longer longevity was found when T3 was used, which showed the best PM immediately post-thawing.

Several compounds varied between T1 and T2/T3 extenders. In previous studies Peña and Linde-Forsberg (2000) and Peña et al. (2003) showed a beneficial effect of the addition of Equex STM paste to Tris-egg yolk freezing extender.

The active compound in Equex STM paste is the sodium dodecyl sulphate (SDS), a water-soluble, anionic detergent that probably exerts its action by reducing lipid phase transitions and/or protecting the function of membranes by controlling the calcium influx (Strom Holst et al., 2000; Peña et al., 2003).

Ethylene diamine tetra-acetate (EDTA) was also present in the lactose-egg yolk based extenders (T2 and T3), but not in the Tris-egg yolk (T1). EDTA acts as a chelator limiting the movement of divalent ions across the plasma membrane by binding calcium and magnesium resulting in lower intracellular calcium concentrations (Graham, 1996). It prevents or delays the progress of capacitation-like changes in thawed spermatozoa, prolonging their post-thawing longevity (Pena et al., 2003),

The integrity of plasma membrane of spermatozoa is essential for cell viability, as its selective permeability maintains intracellular metabolic activities, pH and ionic composition (Strom et al., 1997). An important way in which a cryoprotectant stabilizes cells during freezing involves interactions between cryoprotectant and cell membranes (Strom Holst et al., 2000). Limited studies are available on the use of ethylene glycol (EG) as a cryoprotectant for freezing canine semen. Vanucchi et al. (2002) reported a progressive motility of 62.9% for frozen semen using 5% EG as a cryoprotectant. Soares et al. (2002) obtained similar results with semen which was frozen in extenders containing 0.25 or 0.5M of EG. These data showed that T2 preserved better the structural integrity of the plasma membrane than the two other treatments. The association of lactose-egg yolk extender with a cryoprotectant of low toxicity as EG, which has high permeability due to its low molecular weight (Vannucchi, 1999) is likely to better stabilize the structure of the plasma

membranes. Based on the results of the present study and preliminary data from two above mentioned studies, a larger trial using EG as the cryoprotectant should be conduct.

There is a lack of information concerning the effects of amides as cryoprotectants for freezing canine semen. Studies in other species showed that amides and particularly DMF showed a cryoprotectant effect on sperm cells (Hanada and Nagase, 1980; Alvarenga et al., 2000; Medeiros et al., 2002; Squires et al., 2004). In the present study, 5% DMF extender showed a better capacity of preserving motility post-thawing compared to the other two extenders. On the other hand, it is not known whether the lower capability of DMF to preserve the integrity of plasma membranes of spermatozoa when compared to the ethylene glycol (T2) is related to the concentration used or to a more effective protective effect of the membranes by the ethylene glycol. The 5% DMF concentration used in this study was chosen because most extenders currently using DMF as cryoagent for semen in other species contain a concentration varying between 2% to 6% (Hanada and Nagase, 1980; Squires et al., 2004).

Due to differences in the composition between T1 and T2/T3 extenders, it makes difficult, to identify which compound or association of compounds could have brought improvements as shown using T2 and T3 extenders.

It can be concluded that the association of lactose-egg yolk with 5% EG (T2) and lactose-egg yolk with 5% DMF (T3) improves the post-thawing viability of dog semen when compared with tris-egg yolk 5% EG (T1). It has also been demonstrated that the cryoprotectant agent DMF is an alternative cryoagent for canine semen cryopreservation.

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