Archives of Veterinary Science **ISSN 2317-6822**
v.19, n.2, p.69-77, 2014 www.ser.ufpr.br/veterinary

TREHALOSE AND A CALCIUM CHELATOR FOR RAM SEMEN CRYOPRESERVATION

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ABSTRACT: Twenty semen samples from ten rams of the Santa Inês breed were cryopreserved with the goal of verifying the effect of trehalose addition (100mOsmol) (group 1 - TRIS+TRE) to a Tris-egg yolk-glycerol extender (group 2 - TRIS), associated or not to the calcium chelator EDTA (group 3 - TRIS+TRE+EDTA), on the post-thaw sperm viability. After evaluation, the diluted semen samples were cooled at 5°C and frozen in liquid nitrogen steam. After thawing, the kinetic sperm parameters were analyzed by a computer analyses system (CASA). The sperm viability was observed by using the multiple sperm parameter staining with propidium iodide (PMI, plasmatic membrane integrity), JC-1 (MMP, mitochondrial membrane potential) and FITC-PSA (ACI, acrosomal integrity). The statistical analysis was performed using the SAS software, applying the MEANS procedure for descriptive analysis and the GLM procedure with the Student-Newman-Keuls test (SNK) for comparison of sperm parameters among different groups (with $P<0.05$). The percentage of total and progressive motility post-thaw were the greatest ($P<0.05$) for the TRIS group in relation to trehalose ones. The PMI, MMP, and ACI rates (%) did not present differences ($P>0.05$) among the groups. It can be concluded that the trehalose addition to the TRIS extender, associated or not to EDTA, had detrimental effect on cryosurvival ram sperm.
Key Words: *Ovis aries,* frozen semen, hyperosmotic extender

TREALOSE E UM QUELANTE DE CÁLCIO PARA A CRIOPRESERVAÇÃO DO SEMEN OVINO

RESUMO: Vinte amostras de semen de dez carneiros da raça Santa Inês foram criopreservadas com o objetivo de verificar o efeito da adição de trealose (100mOsmol) (grupo 1 – TRIS+TRE) ao diluidor Tris-gema de ovo-glicerol (grupo 2 – TRIS), associado ou não ao quelante de cálcio EDTA (grupo 3 – TRIS+TRE+EDTA), sobre a viabilidade espermática pós-descongelação. Após avaliação, as amostras diluídas foram resfriadas à 5oC e congeladas em vapor de nitrogênio líquido. Após descongelação, os parâmetros de cinética espermática foram analizados por um sistema computadorizado (CASA). A viabilidade espermática foi observada através da coloração para avaliação multi-paramétrica com iodeto de propídio (MPI, membrana plasmática íntegra), JC-1 (PMM, potencial de membrana mitocondrial) e FITC-PSA (IAC, integridade acrossomal). A análise estatística foi realizada através do programa estatístico SAS, aplicando o procedimento MEANS para a análise descritiva e o procedimento GLM com o teste de Student-Newman-Keuls (SNK) para a comparação dos parâmentros espermáticos entre os diferentes grupos (com $P<0,05$). O percentual de motilidade total e progressiva pós-descongelaçào foram maiores ($P<0,05$) para o grupo TRIS em relação aos grupos com trealose. A taxas (%) de MPI, PMM e IAC não apresentaram diferenças ($P>0,05$) entre os grupos. Pode-se concluir que a adição da trealose, associada ou não ao EDTA, proporcionou efeito negativo para criopreservação do sêmen ovino.
Palavras-chave: Ovis aries, sêmen congelado, diluidor hiperosmótico

Recebido em 08/03/2013
Aprovado em 26/12/2013

INTRODUCTION

Trehalose (TRE) is a disaccharide presenting a great cryoprotectant activity because of its dehydrating property and interaction with cell membranes, exerting antioxidant effects and thus minimizing the ram sperm damage by cryopreservation (Aisen *et al.*, 2005). The TRE can be used in extenders and good indexes of viability *in vitro* and *in vivo* fertility has been observed by Aisen *et al.* (2002) at the concentration of 100mOsmol/L.

Previous studies have shown significant synergisms between EDTA and trehalose, resulting in benefits on the motility rates and preservation of the sperm morphology after thawing (Bakas and Disalvo, 1991; Aisen *et al.*, 2002).

The cryopreservation of semen promotes elevated intracellular calcium levels, resulting in cell dysfunction and death (Amann and Pickett, 1987). To minimize the calcium deleterious effect during the semen freezing process from different species, the use of ethylene-diamine-tetra-acetate-disodium (EDTA-Na) in freezing extender media has been tested since 1970's (Martin *et al.*, 1979; Bittencourt *et al.*, 2004; Aisen *et al.*, 2005). The EDTA main function is to chelate the extracellular calcium, reducing its influx to the intracellular environment, which minimizes the deleterious effect of calcium on the sperm (Amann and Pickett, 1987).

A study in cattle and buffaloes shows a 10-12% higher spermatic motility rate after thawing when the extender contained 0.1% EDTA in its composition compared to extender with no EDTA (Dhami and Shani, 1993). Similar results were observed in goats, where the spermatic motility after thawing was 10% higher in the extender with EDTA (0.1%) in its composition (Bittencourt *et al.*, 2004).

Thus, the aim of this study was to evaluate the effect of the TRE addition (100mOsmol) to a Tris-egg yolk-glycerol (TRIS)-based extender with and without EDTA on ram sperm viability after thawing.

MATERIAL AND METHODS

The Tris-egg yolk (TRIS) solution (containing – 199,7 mM Tris-hydroxymethyl-aminomethane, 71,4 mM monohydrated citric acid, 69,4 mM glucose, 20% (v/v) egg yolk, 0,5% (v/v) sodium dodecyl sulfate, 6% (v/v) glycerol, 134 mg/L gentamicin sulphate and distilled water – pH 6.6 and osmolarity of 351 mOsmol/Kg) (Bittencourt *et al.*, 2008) was the extender used as a basis for three experimental groups: Group 1: TRIS, Group 2: TRIS+TRE, Group 3: TRIS+TRE+EDTA. To TRIS+TRE was added 100 mOsmol/L of TRE and to TRIS+TRE+EDTA, besides TRE, was added 0.1% of EDTA.

Twenty semen samples from 10 adult rams of the Santa Ines breed (purebred), selected after clinical and andrological examination were used in this experiment. Only ejaculates which met the following criteria were used: volume of 0.5–2 ml; minimum sperm concentration of 2.1×10^9 sperm/mL; motility of 70%. Soon after the collect by artificial vagina, which was performed on alternate days, the semen of each ram was sent to laboratory for processing.

For each sample, the fresh semen was kept in a water bath at 35^oC temperature and the volume, color and turbidity visually evaluated. The subjective total and progressive motility, vigor, turbulence, and concentration were assessed using a phase-contrast microscope (×100 magnification). Also, semen aliquots were withdrawn for evaluating sperm defects through phase-contrast microscopy (×1000 magnification).

Following initial assessments and sperm concentration determination, a

total of 1mL of the three extenders were added slowly to pre-warmed tubes, containing each one 320 x 10^6 spermatozoa with progressive motility. This comprised four insemination doses of 80 x 10^6 of spermatozoa with progressive motility/0.25 mL for each experimental group. Subsequently, samples were submitted to cooling at 5°C (0.47°C/min), equilibrium time (total of 2h), and then frozen in liquid nitrogen steam.

The thawing process (two straws per group) was done in a water bath, at 37°C for 50 seconds (Lucidi *et al.*, 2001), 90 days after freezing the samples.

An aliquot of 120 µL of X-CELL medium (IMV, L'Aigle, France), previously warmed to 37°C, was added slowly to 5 µL of pos-thawed semen (2 X 10^6 spermatozoa) from the different experimental groups (final concentration of 16.0 x 10^6 spermatozoa /mL) and kept at this temperature for five minutes. This X-CELL protocol was used for its proven effectiveness as a clarifying medium for the thawed ram semen evaluation (Azevedo *et al.*, 2006; Maia *et al.*, 2009; Sicherle *et al.*, 2011). Then 10 µL of this mix were transferred to a Makler's chamber (Sefi-Medical Instruments, Haifa, Israel) and the spermatic kinetics was assessed through the semen analyzer model IVOS 12 (Hamilton Thorn Biosciences, Beverly, MA, USA), with at least four different counting fields and 300 cells. The characteristics evaluated were: percentage of total motility (TM), progressive motility (PM), and rapid spermatozoa (RAP) as well as straight line velocity (VSL, µm/s), track velocity (VCL, µm/s), smoothed path velocity (VAP, µm/s), amplitude of lateral head displacement (ALH, µm), beat cross frequency (BCF, Hz), linearity (ratio VSL/VCL), and straightness (ratio VSL/VAP).

The CASA setup parameters used were frames acquired = 30 Hz; frames per second = 60; minimum contrast = 46; minimum cell size = 10 pix; threshold straightness = 80.0%; low VAP cut off = 10.0 µ/s; low VSL cut off = 0.0 µ/s; non-motile head size = 5 pix; non-motile head intensity = 55.

Analysis of sperm viability was performed after supravital staining sperm with eosin dye (Barth and Oko, 1989) and through epifluorescence microscopy by associating the fluorescent probes propidium iodide - PI (2 µl) (PMI, plasma membrane integrity); 5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbocyanine iodide - JC-1 (2 µl) (MMP, mitochondrial membrane potential) and fluorescein isothiocyanate-conjugated *Pisum sativum* agglutinin - FITC-PSA (25 µL) (ACI, acrosomal integrity) according to Celeghini *et al.* (2007), with modifications. An aliquot of 120µL of X-CELL medium (IMV, L'Aigle, France), previously warmed to 37°C, was added slowly to 5 µL of pos-thawed semen (2 X 10^6 spermatozoa) from the different experimental groups (final concentration of 16.0 x 10^6 spermatozoa /mL). It was then placed into a warmed microcentrifuge tube and 2 µL PI (0.5 mg/mL in saline solution), 2 µL JC-1 (153 µM in DMSO) and 25 µL FITC-PSA (100 µg/mL in PBS/sodium azide) solutions added. After incubation at 37°C for 10 min, a drop of the sample was placed on a slide, covered with a coverslip and evaluated immediately by epifluorescent microscopy (Leica DMLB, Leica Microsystems, Leitz, Germany) using a filter I3 (excitation BP 450–490 nm, suppression LP 515 nm) at 1000 x magnification.

From each treatment, 100 sperm per slide were evaluated and classified into 8 categories (Celeghini *et al.*, 2007): C1-intact plasma membrane, intact acrosome and high mitochondrial membrane potential; C2-intact plasma membrane, intact acrosome and low mitochondrial membrane potential; C3-

intact plasma membrane, damaged acrosome and high mitochondrial membrane potential; C4-intact plasma membrane, damaged acrosome and low mitochondrial membrane potential; C5-damaged plasma membrane, intact acrosome and high mitochondrial membrane potential; C6-damaged plasma membrane, intact acrosome and low mitochondrial membrane potential; C7-damaged plasma membrane, damaged acrosome and high mitochondrial membrane potential and C8-damaged plasma membrane, damaged acrosome and low mitochondrial membrane potential. The sperm in categories C1-C4 were all with plasma membrane integrity (PMI). Sperm classified in categories C1, C2, C5 e C6 were grouped together in one category named acrosomal integrity (ACI). The same was done regarding mitochondrial membrane potential. The categories C1, C3, C5 e C7 were grouped in the high mitochondrial membrane potential category (MMP).

The experiment was conducted as a completely randomized design. For statistical analysis of the sperm characteristics, the Statistical Analysis System package, SAS (1996) was used, applying the MEANS procedure for descriptive analysis and the GLM procedure with the Student-Newman-Keuls test (SNK) for comparison of sperm parameters among different groups at 5% of significance level. All the percentage data were transformed to arcsine, before statistical analyses. Re-transformed data are reported as the mean±S.E.M.

RESULTS

Sperm kinetics

The computerized analysis of sperm kinetics (Tab. 1) showed that the addition of trehalose to the freezing medium negatively influenced (P<0.05) most of the parameters studied, except the BCF, STR, and LIN.

It was also found that the addition of EDTA to the medium containing trehalose (TRIS + TRE + EDTA) did not improve the rates of post-thaw sperm kinetics (Table 1). Again, as observed with the TRIS + TRE, the averages of the TM, PM, VAP, VSL, VCL, ALH, and RAP values were lower than the medium with no TRE (TRIS).

Table 1. Means of the sperm kinetic parameters after thawing, obtained by computerized analysis, in the different extenders evaluated (TRIS, TRIS + TRE, and TRIS + TRE + EDTA).

PARAMETERS	TREATMENT		
	TRIS ± SEM	TRIS+TRE ± SEM	TRIS+TRE+EDTA ±SEM
TM (%)	73.60 ± 11.16[a]	59.05 ± 15.25[b]	55.50 ± 13.31[b]
PM (%)	52.55 ± 09.74[a]	34.40 ± 20.96[b]	33.30 ± 16.97[b]
VAP (µm/s)	149.06 ± 15.55[a]	97.46 ± 27.48[b]	101.88 ± 21.75[b]
VSL (µm/s)	132.43 ± 15.93[a]	88.60 ± 27.41[b]	92.69 ± 22.01[b]
VCL (µm/s)	219.55 ± 22.92[a]	141.70 ± 23.19[b]	146.06 ± 16.96[b]
ALH (µm)	06.59 ± 00.86[a]	04.75 ± 00.53[b]	04.74 ± 00.65[b]
BCF (Hz)	42.16 ± 03.12[a]	42.03 ± 02.94[a]	41.39 ± 03.39[a]
STR (%)	85.35 ± 03.21[a]	87.00 ± 03.93[a]	87.00 ± 04.26[a]
LIN (%)	59.70 ± 06.61[a]	60.60 ± 09.97[a]	61.85 ± 10.01[a]
RAP (%)	63.00 ± 11.53[a]	37.90 ± 22.35[b]	37.05 ± 18.32[b]

? Total motility (TM), progressive motility (PM), straight line velocity (VSL), track velocity (VCL), smoothed path velocity (VAP), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), straightness (STR), linearity (LIN) and rapid spermatozoid (RAP).
? TRIS: Tris medium without EDTA or trehalose, TRI+TRE: Tris medium added of trehalose and TRIS+TRE+EDTA: Tris medium added of trehalose and EDTA.
Values followed by different letters in the same row differ by SNK test (P <0.05).

Sperm membranes viability

The results of the sperm viability parameters are in the Table 2. The results of PMI behaved similarly to those observed by the TM, PM, VAP, VSL, VCL, ALH, and RAP, which were significantly higher for the TRIS extender, compared to other groups, with TRE or TRE and EDTA additives.

Table 2. Means of the sperm viability parameters after thawing, obtained by epifluorescence microscopy (PMI, ACI, and MMP) or supravital staining (EOS), in the different extenders evaluated (TRIS, TRIS + TRE, and TRIS + TRE + EDTA).

PARAMETERS	TREATMENT		
	TRIS ± SEM	TRIS+TRE ± SEM	TRIS+TRE+EDTA ± SEM
PMI	35.80 ± 16.14[a]	27.43 ± 12.69[b]	23.78 ± 09.52[b]
ACI	73. 28 ± 14.70[a]	72.76 ± 13.28[a]	65.55 ± 10.33[a]
MMP	41.79 ± 32.35[a]	41.68 ± 28.96[a]	33.94 ± 31.14[a]
EOS	58.43 ± 12.94[a]	56.20 ± 11.78[a]	53.15 ± 10.53[a]

? Plasma membrane integrity (PMI), acrosomal integrity (ACI) and mitochondrial membrane potential (MMP)
? TRIS: Tris medium without EDTA or trehalose, TRI + TRE: Tris medium added of trehalose and TRIS+TRE+EDTA: Tris medium added of trehalose and EDTA.
Values followed by different lowercase letters in the same column differ by SNK test (P <0.05).

The use of TRE did not affect (P>0.05) the levels of EOS in the extenders tested, as observed to the ACI and MMP rates. And the EDTA addition to the extender in the presence of TRE did not improve (P> 0.05) the PMI, neither the levels of ACI, MMP and EOS.

DISCUSSION

Sperm kinetics

In this study the trehalose addition negatively influenced (P<0.05) most of the kinetic parameters evaluated. The most probable explanation for this finding is that the glycerol concentration of 6% has caused cell toxicity when added to a media of high osmolarity (TRIS +TRE and TRIS+TRE+EDTA) as reported by Becker (2004). The glycerol concentration between 6 and 8 % was maintained in basis extender as recommended for semen frozen by the slow conventional method and using hypertonic diluents (Salamon and Maxwell, 2000).

These results contradict the reports of Aisen *et al.* (2002), which showed that the use of a trehalose-based hypertonic extender increased the viability of ram semen after thawing. In their study, the TRE at 100 mOsmol/L was the concentration with the best results compared to 50, 200 and 400mOsmol. When added to the isotonic extender (Tris-yolk-glycerol), it enabled 35% increase in sperm motility after thawing compared to frozen semen using extender with no TRE. However, it is important to emphasize that the study by Aisen *et al.* (2002) used glycerol at 3% in the basic isotonic medium.

Some differences in the extender composition may explain the differences found between the results. In the study by Aisen *et al.* (2002), a extender medium with 10% egg yolk and 3% glycerol was used, half of that used in this work, which may have given to the extender lower cryoprotectant capacity. Similar fact was reported by Jafaroghili *et al.* (2011), whose the sperm motility was higher (P<0.05) with medium supplemented with 100 mM trehalose (46.8%) compared to the control group without the disaccharide trehalose and with other concentrations (50 and 70mM); however, despite the authors use levels of glycerol next to the used in this study (5% *versus* 6%), the percentage of egg yolk was only 5%, lower than recommended for freezing semen of ruminants. Thus, the addition of TRE to the extender in their works contributed to raise the trehalose cryoprotectant activity, improving (P<0.05) rates of post-thawing motility. Probably the isosmotic extender medium used in this study, with appropriate levels of glycerol and egg yolk, has already brought the conditions for adequate cryoprotection to the spermatozoa such that the TRE addition did not turn out any beneficial effect during preservation and had detrimental effect on sperm kinetics. Similar findings were described by CIRIT *et al.* (2013), which sperm velocity (um/s) after thawing and thermoresistance test were lower in the medium with trehalose.

A hypothesis that may explain the negative effect of TRE on sperm kinetics may be related to the density of the hypertonic extender medium, which may influence sperm displacement, reducing the movement (ALH) and movement speeds (VAP, VSL, VCL). Supporting this statement, Mortimer (1997) reported that increasing the density of the surrounding medium, results in decreased amplitude of spermatozoa' flagellar wave. Moreover, Amirat *et al.* (2004) reported higher values for VCL and VAP in extender containing low-density lipoprotein, compared to medium with egg yolk. Therefore, the outcomes from sperm kinetics evaluations are somehow impaired in

hypertonic media compared to the most commonly used isotonic media. Thus, these differences may not represent important sperm changes, such as those related to the plasma membrane or to organelles, especially mitochondria, which are responsible for energy metabolism and are one of the reasons of the observed reduction in the sperm kinetics parameters. There is a need to use complementary techniques that may support this conclusion such as the evaluation of plasma membrane integrity and mitochondrial potential, which were used in this work.

Bakas and Disalvo (1991) and Aisen *et al.* (2000) showed that trehalose cryoprotectant activity could be increased when used in conjunction with EDTA because of the synergism between these substances. Aisen *et al.* (2000) observed that the addition of TRE (100mOsmol) associated with EDTA (0.15%) increased ($P < 0.05$) the sperm motility rate (59%) compared to medium without additives (48.8%). According to these authors, the Ca^{++} competes with TRE, inhibiting its stabilizing effect on the sperm membrane, and by adding EDTA to the extender, it sequesters the Ca^{++} from the extracellular surrounding, leaving the TRE free to play its cryoprotectant role. Addition of EDTA to the extender with the TRE did not improve the rates of sperm kinetics in the present work.

These discrepancies on the effectiveness of the EDTA use for sperm cryopreservation observed between both studies are related to the occurrence of some phenomena. Among these, the main factor would be the interaction between the extender components, because not always the calcium removal from the extracellular medium is desirable. In a study to evaluate the effect of TRE and EDTA combination (Aisen *et al.* 2005), they showed that the extender plus EDTA, at removing calcium from the extracellular medium, inhibited the antioxidant properties of trehalose, suggesting that a positive effect of the interaction between trehalose and calcium exists on the polar region of the sperm membrane phospholipids.

Sperm membranes viability

Differently to this study, other reports showed that TRE addition to medium improves the rates of maintenance of plasma membrane integrity. Abgoala and Terada (2003) observed that the complete replacement of the Tris-egg yolk medium with a solution of TRE (375mOsmol) significantly increased the rates of membrane fluidity, improving the rate of freezing of goat semen. Hu *et al.* (2009) working with pigs, obtained better maintenance level of the PMI (hypoosmotic test) with 100mOsmol TRE compared to isotonic medium and the other test groups after adding different concentrations of TRE (25 to 200mOsmol/Kg) to isotonic medium. Likewise, and using the same method, Aisen *et al.* (2002) found an improvement of ram spermatozoa PMI and birth rates 2.5 times higher with a hyperosmotic medium supplemented with 100mOsmol of TRE in relation to the isosmotic medium without TRE. Later, using the same experimental groups (isosmotic medium vs. 100mOsmol TRE), Aisen *et al.* (2005) and Jafaroghili *et al.* (2011) found higher levels of PMI with extender plus TRE compared to with no TRE. Importantly, the isosmotic extender used in the studies cited were different from the one used in the present study, which had half (10%) and quarter (5%) of the egg yolk percentage, respectively, and thus it may have provided less sperm protection and justified the lower freezability among the extenders used.

Additionally, the negative effect of GLY at higher concentrations in hyperosmotic medium was previously

reported by Becker (2004) who observed higher (P<0.05) levels of PMI in goat semen frozen in hyperosmotic medium containing sucrose at 375mOsmol and 1.7 or 3.4% GLY, compared to 6.8% GLY (similar to the 6% used in this study).

Bakas and Disalvo (1991) supported the hypothesis that Ca^{++}, by competing with the TRE for the same binding sites in the plasma membrane, inhibits its cryoprotectant activity. Thus, the cryoprotectant activity of TRE could be increased when it is used with EDTA, because it chelates the extracellular Ca^{++}, leaving the TRE free to fully play its cryoprotectant role (Aisen *et al.,* 2000). However, the synergism between the TRE and EDTA was not demonstrated in this study, where EDTA addition to the extender did not improve (P>0.05) the PMI, neither the levels of ACI and MMP. Aisen *et al.* (2005) reported that the cell percentage with PMI obtained by electron microscopy and hypoosmotic test were lower for the extender with TRE + EDTA (72.6% vs. 40% and 64% vs. 40.3%, respectively). The authors justified the results stating that EDTA, by removing the Ca^{++} from the extracellular surrounding, inhibited the antioxidant properties of trehalose, which is favored by interaction with Ca^{++}.

The lowest numerical values of ACI (P>0.05) observed with TRIS+EDTA may be related to the sum of two negative factors already discussed in aforementioned sections, not only the cytotoxicity imposed by the combination of GLY6% in hyperosmotic medium, but also the detrimental effect of EDTA, when associated with the extender with TRE.

The addition of TRE did not affect the levels of ACI in the extenders tested. Similar reports were made by Aisen *et al.* (2000); Aisen *et al.* (2002) and Valente *et al.* (2010), using phase contrast microscopy, observed no differences in the percentages of cells with acrosomal damage after freezing them in hyperosmotic medium with TRE or in isotonic medium. These findings disagree with those reported by Hu *et al.* (2009), who using FITC-PNA detected higher levels of ACI in frozen sperm with different concentrations of TRE (25 to 200mOsmol) than in medium without TRE.

The high levels of calcium are related with the occurrence of changes in the sperm acrosome and play an important role in the process of acrosome reaction (Roldan *et al.* 1994), which can be inhibited in the presence of EDTA that chelate the Ca^{++} in the extracellular environment by preventing its influx. However, the addition of EDTA to the tested freezing extenders did not affect (P>0.05) the rates of ACI. Bittencourt *et al.* (2008) using phase contrast microscopy, also found no improvements in the rates of acrosomal integrity with 0.1% EDTA in the freezing medium of goat semen, although it has achieved a reduction in total sperm defects. Likewise, Aisen *et al.* (2000) obtained similar rates of acrosome injury in frozen sperm using medium with or without EDTA and only found positive effect of EDTA on the acrosomal integrity, when associated with the TRE.

The differences obtained between the works may be related to several factors like the extender composition, as discussed earlier. The quality and rate of the cooling process may also influence the efficacy of EDTA use, since the increased influx of Ca^{++} into the cell is maximized as the temperature reduces (Robertson and Watson, 1986). Probably, appropriate cooling rates that minimize the thermal stress by the cold can prevent the exacerbated influx of Ca^{++}, so that addition of the EDTA has no positive effect.

As observed for EOS, there was no significant effect of treatment on the MMP parameter. And the addition of

TRE did not improve (P>0.05) the rates of MMP, as observed to the PMI and ACI. Similarly, the association TRE+EDTA had no effect (P>0.05) on the MMP, which was also not expected, since the influx of intracellular calcium derived from the semen cooling process promotes the activation of phospholipases, generating the hydrolysis of spermatozoa membrane phospholipids. Both the alterations to phospholipids and the formation of free fatty acids cause increased permeability and injury to the mitochondrial membrane (Amann and Pickett, 1987).

The addition of EDTA to freezing extender should minimize this process, improving the levels of MMP, because it would sequester the calcium extracellular and then preventing its influx. The justifications previously used for the lacking of EDTA effect in the extenders tested here are also suitable for the levels of MMP.

Similar to ACI and PMI, the lowest numerical values (P>0.05) of MMP occurred for semen frozen with TRIS+TRE+EDTA, reinforcing the low freezing capacity afforded by the combination of its components at the used concentrations used.

CONCLUSION

Thus the addition of trehalose to the Tris-egg yolk medium, alone or combined with EDTA did not improve the ovine sperm viability indexes; instead it negatively influenced the parameters of sperm kinetics.

ACKNOWLEDGEMENTS

Financial support: Foundation for Research Support of São Paulo State - FAPESP; the authors wish to thank Frederico Ozanan Papa for his help with the laboratory work.

INFORMATIVE NOTES

The experiment received approval from the Ethics Committee for Animal Experimentation of the São Paulo State University (Brazil, CEUA 006/2008).

REFERENCES

ABGOALA, E.M., TERADA, T. Trehalose-enhanced fluidity of the goat sperm membrane and its protection during freezing. **Biology of Reproduction**, v.69, n.4, p.1245-1250, 2003.

AISEN, E.G.; ALVAREZ, H.L.; VENTURINO A. *et al.* Effect of trehalose and EDTA on cryoprotective action of ram semen diluents. **Theriogenology**, v.53, n.5, p.1053-61, 2000.

AISEN, E.G., VENTURINO, A., MEDINA, V. Cryopreservation and post-thawed fertility of ram semen frozen in different trehalose concentrations. **Theriogenology**, v.57, n.7, p.1801-1808, 2002.

AISEN, E.G., QUINTANA M, MEDINA V, *et al.* Ultramicroscopic and biochemical changes in ram spermatozoa cryopreserved with trehalose-based hypertonic extenders. **Cryobiology**, v.50, n.3, p.239-249, 2005.

AMANN, R.P.; PICKETT, B.W. Principle of cryopreservation and a review of stallion spermatozoa. **Equine Veterinary Science**, v.7, n.3, p.145-174, 1987.

AMIRAT, L., TAINTURIER, D., JEANNAAU, L., *et al.* Bull semen in vitro fertility after cryopreservation using egg yolk LDL. A comparison with Optidyl®, a commercial egg yolk extender. **Theriogenology**, v.61, p.895-907, 2004.

AZEVEDO, H.C., BICUDO, S.D.; SOUSA, D.B. *et al.* 2006. Simultaneous evaluation of plasma membrane integrity, acrosome reaction and mitochondrial membrane potential in ram spermatozoa by a combination of fluorescent probes. In**: Abstracts of the 7th International Ruminant Reproduction Symposium**, 2006, Wellington. New Zealand. Wellington: IRRS. pp .19.

BAKAS, L.S.; DISALVO, E.A. Effect of Ca2+ on the cryoprotective action of trehalose. **Cryobiology**, n.28, p.347-353, 1991.

BARTH, A.D.; OKO, R.J. **Abnormal morphology of bovine spermatozoa**. Ames, IA: Iowa State University, 1989. 285p.

BECKER, S.C. **Tolerance limits of goat spermatozoa to hyperosmotic sucrose solutions and survival rate after cryopreservation in extenders containing sucrose or trehalose and reduced concentrations of permeant cryoprotectants**. 2004. Botucatu, 128f. Tese (Doutorado em Reprodução Animal. Curso de Pós-Graduação em Reprodução Animal, Universidade Estadual Paulista Júlio de Mesquita Filho, Campus Botucatu.

BITTENCOURT, R.F.; RIBEIRO FILHO, A. de L.; SANTOS, A.D.F. *et al.* Freezing goat semen with glycerol and ethylene glycol as cryoprotective agents. **Brazilian Animal Science**, v.5, n.1, p.27-32, 2004.

BITTENCOURT, R.F., RIBEIRO FILHO, A. DE L., ALVES, S.G.G. *et al.* Effects of a calcium chelator, a detergent and the soybean lecithin on the quality of the frozen-thawed goat semen. **Brazilian Journal of Veterinary Research and Animal Science**, v. 45, n.4, p. 305-312, 2008.

CELEGHINI, E.C.C., ARRUDA, R.P., ANDRADE, A.F. *et al.* 2007.Practical techniques for simultaneous fluorimetric assessment of plasma, acrosomal and mitochondrial membranes. **Reproduction in Domestic Animals**, v.42, p.479-488, 2007.

CIRIT, U.; BAGIS, H.; DEMIR, K. *et al.* Comparasion of cryoprotective effects of iodixional, trehalose and cysteamine on ram semen. **Animal Reproduction Science**, v.139, n.1-4, p.38-44, 2013.

DHAMI, A.J.; SAHNI, K.L.S.O. Effect of extenders, additives and deep freezing on the leakage of lactic dehydrogenase from cattle and buffalo spermatozoa. **Indian Journal of Animal Sciences**, v.63, n.3, p.251-56, 1993.

HU, J.H.; LI, Q.W.; JIANG, Z.L. *et al.* Effects of different extenders on DNA integrity of boar spermatozoa following freezing–thawing, **Cryobiology**, v.57, p.257–262, 2009.

JAFAROGHLI, M.; KHALILI, B.; FARSHAD, A. *et al.* The effect of supplementation of cryopreservation diluents with sugars on the post-thawing fertility of ram semen. **Small Ruminant Research**, v.96, n1, p.58-63, 2011.

LUCIDI, P.; BARBONI, B.; MATTIOLI, M. Ram-induced ovulation to improve artificial insemination efficiency with frozen semen in sheep. **Theriogenology**, v.55, n.9, p.1797-1805, 2001.

MAIA, M.S.; BICUDO, S.D., AZEVEDO H.C. *et al.* Motility and viability of ram sperm cryopreserved in a Tris-egg yolk extender supplemented with anti-oxidants. **Small Ruminant Research**, v.85, n.2-3, p.85-90, 2009.

MARTIN, J.C., KLUG, E., GUNZEL, A.R. Centrifugation of stallion semen and storage in large volumes straws. **Journal of Reproduction Fertility,** Suppl, v.27, p.47-51, 1979.

MORTIMER, T. A critical review of the physiological importance and analysis of sperm movement in mammals. **Human Reproduction Update**, v.5, p.403-439, 1997.

ROBERTSON, L., WATSON, P.F. Calcium transport in diluted or cooled ram semen. **Journal Reproduction Fertility**, v.77, n.1, p.177-85, 1986.

ROLDAN, E.R.S., MURASE, T., SHI, Q.X. Exocytosis in spermatozoa in response to progesterone and zona pellucida. **Science,** n.266, p.1578-1581, 1994.

SALAMON, S.; MAXWELL, W.M.C. Storage of ram semen. **Animal Reproduction Science**, v.62, n.1-3, p.77-111, 2000.

SAS, 1996: SAS/STAC Software. SAS Inst. Inc., Cary, NC.

SICHERLE, C.C.; MAIA, M.S.; BICUDO, S.D. *et al.* Lipid peroxidation and generation of hydrogen peroxide in frozen-thawed ram semen supplemented with catalase or Trolox. **Small Ruminant Research**, v.95, n.2-3, p.144-149, 2011.

VALENTE, S.S.; PEREIRA, R.M.; BAPTISTA, M.C. *et al.* In vitro and in vivo fertility of ram semen cryopreserved in different extenders. **Animal Reproduction Science**, v.117, n.1, p.74-77, 2010.