Effect of Adding Sugars in a Tris-Based Egg Yolk Extender on The Cryopreservation of Ram Spermatozoa

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Abstract: The aim of this study was to evaluate the effect adding sugars (trehalose, sucrose, fructose and glucose) in a tris-based egg yolk extender on ram spermatozoa characteristics prior and after cryopreservation. Ejaculates were collected from healthy rams using an artificial vagina. In experiment 1, semen samples were extended in four different extenders containing 62.5 mM glucose, 62.5 mM fructose, 35.00 mM sucrose and 35.00 mM trehalose. In experiment 2, semen samples were extended using a combination between of 35.00 mM trehalose with 62.5 mM fructose, 62.5 mM glucose or 35.00 mM sucrose compared to 62.5 mM fructose by itself (control). In experiment 3, glucose at the concentration of 62.5 mM was combined with four different concentrations of trehalose (35, 50, 75, 100, 150 mM). In experiment 1 the percentages of total sperm motility, progressive motility, live spermatozoa, abnormal spermatozoa and acrosome integrity were assessed prior and after cryopreservation. There were no significant differences among monosaccharide and disaccharides for all sperm characteristics evaluated either prior or after cryopreservation ($P > 0.05$). In experiment2, the combination of trehalose with sucrose, fructose or glucose, improved sperm characteristics after cryopreservation and the best results were obtained using a combination of trehalose with glucose ($P < 0.05$). In experiment 3, the addition of 100 mM trehalose to the freezing extender improved significantly ram sperm characteristics after cryopreservation. However, increasing trehalose concentration to 150 mM had a negative effect on ram sperm characteristics prior and after cryopreservation. In conclusion, using trehalose in treating ram sperm prior to cryopreservation would improve sperm cryosurvival. The present results indicated that successful ram sperm cryopreservation can be obtained using an extender containing a combination of trehalose and glucose.

Keywords: cryopreservation, ram, spermatozoa, sugars.

INTRODUCTION

The sheep industry has not been able to utilize many of the assisted reproductive technologies in general and AI in particular due to inefficiencies in collecting, freezing and inseminating frozen ram semen (Purdy *et al.*, 2010). Glycerol is the most commonly utilized penetrating cryoprotectant in extenders for frozen semen, but it is potentially cytotoxic (Holt, 2000; Watson, 2000). Thus, the search for alternative cryoprotectant solutions for frozen ram semen is justified. Sugar maintains the osmotic pressure of the diluents by inducing cell dehydration and less ice crystal formation in spermatozoa (Leibo and Songsasen, 2002; Purdy, 2006). Trehalose may be used as a cryoprotectant (Molinia *et al.*, 1994; Sánchez-Partida *et al.*, 1998), as it promotes cell dehydration, which reduces the negative effects of water flow through the sperm membrane during freezing (Yildiz *et al.*, 2000) and the formation of ice crystals (Aisen *et al.*, 2002, 2005). Trehalose also interacts with the membrane phospholipids and proteins, providing the membrane more flexibility against cryo-injuries (Aisen *et al.*, 2002; Bucak *et al.*, 2007).

The objective of this study was to evaluate the addition of monosaccharides and disacchrides in a tris based extender containing low glycerol concentration (3%). The effects of sugars addition were observed on ram sperm characteristics prior and after cryopreservation to optimize extender medium to reach optimal ram sperm characteristics after cryopreservation.

MATERIALS AND METHODS

Semen collection and handling:

Eight rams of local breeds (Ossimi and Rahmani) were housed at the Suez Canal University Farm (Ismailia - Egypt). Animals were fed a diet providing 100% of their nutritional needs, and provided water *ad libitum*. The rams were 24-48 months of age old and kept under semi-extensive conditions. Semen was collected from each ram twice a week, in different and at non-consecutive days (not everyday), using an artificial vagina. The initial percentages of motile sperm and the sperm concentration were determined for each ejaculate. Ejaculates (3 - 4 individual ejaculates) containing more than 80% motile sperm were pooled and considered as one sample. After collection, all semen samples were assessed for sperm concentration and percentage of motile spermatozoa. A total of 45 semen samples were used for cryopreservation. Fifteen semen samples were used in each experiment.

Semen extension and cryopreservation:

Ram semen was extended in a Tris-egg yolk diluent (TEY1: 250 mM Tris, 88.5 mM citric acid, and 20 % (v/v) egg yolk, and antibiotics: 500 IU penicillin, 5 mg streptomycin per ml) using a two-step dilution process. In the first step, semen was extended to the 100×10^6 sperm/ml using TEY1 diluent containing no cryoprotectant at 37°C. The spermatozoa were then cooled to 5°C over 2 h and an equal volume of TEY2 (TEY1 + 6% glycerol + sugar treatment) diluent was added, resulting in a final cryoprotectant concentration reduced to 50% of its initial and a final spermatozoal concentration of 50×10^6 sperm/ml according to Awad and Graham (2004).

Three experiments were conducted to examine the effect of sugars on sperm characteristics prior and after cryopreservation. In experiment 1, four different diluents of TEY2 were prepared by adding two monosaccharides (glucose, fructose) at 125 mM and two disaccharides (sucrose and trehalose) at the concentration of 70 mM respectively. In experiment 2, four different diluents of TEY2 were prepared by adding 125 mM fructose (control), 125 mM fructose + 70 mM trehalose, 125 mM glucose + 70 mM trehalose and 70 mM sucrose + 70 mM trehalose. In experiment 3, five different combinations were prepared by adding 125 mM glucose with 5 different concentrations of trehalose (70, 100, 150, 200, 300 mM).

Extended semen samples were equilibrated at 5°C for an additional 2 h prior to freezing. After final extension, semen was loaded into 0.25 ml French straws immediately, then, kept for additional 2 h at 5°C for equilibration. The straws were placed horizontally on an aluminum rack and frozen in liquid nitrogen vapor in Styrofoam box (outside dimensions: 39L x 30W x 28H cm; inside dimensions: 35L x 24W x 24H cm). The Styrofoam box was filled with liquid nitrogen (LN) partially. The tray of straws was held at 5 cm above liquid nitrogen level. Straws were left in liquid nitrogen vapor for 10 min before being plunged into liquid nitrogen (-196°C). Thawing was carried out in a water bath at 37°C for 60s.

Semen evaluation:

Immediately after thawing, the percentages of total motility, progressive motility, live spermatozoa, abnormal spermatozoa and acrosome integrity were evaluated. The percentage of motile spermatozoa in each semen sample was evaluated under a phase contrast microscope at 200× magnification by placing a drop of diluted semen on a slide and covered with a glass cover slip from three selected representative fields. The mean of the three successive estimations was recorded as final motility score. The percentages of live and dead spermatozoa and morphologically abnormal spermatozoa were assessed using nigrosin-eosin stain (Evans and Maxwell, 1987). The percentage of spermatozoa with intact acrosomes was determined by adding 50 microlitter of each sperm sample to 500 microlitter of a formalin citrate solution (96 ml of 2.9% sodium citrate with 4 ml of 37% formaldehyde added) and mixing carefully (Weitze, 1977). A small drop of the mixture was placed on a microscope slide and a total of 200 spermatozoa counted in at least three different fields for each sample, using differential interference contrast microscopy at 400× magnification.

Statistical analysis:

Percentage data were transformed using arcsine, and treatment differences, in the percentages of total motility, forward motility, live spermatozoa, abnormal spermatozoa, and acrosome integrity were measured by analysis of variance (SAS, 1985). Individual treatment means were separated using Student-Newman-Keuls multiple range test (SAS, 1985). Differences with values of $P < 0.05$ were considered to be statistically significant.

RESULTS

In experiment 1 (Table 1), sperm treated with trehalose, sucrose, fructose and glucose, exhibited no significant differences of all sperm characteristics evaluated either before or after cryopreservation ($P > 0.05$). In experiment 2 (Table 2), the combination of trehalose with sucrose, fructose or glucose, exhibited no significant differences on all sperm characteristics prior to cryopreservation compared to the addition of fructose by itself (Control). After cryopreservation, the combination of trehalose with glucose caused a significant increase ($P < 0.05$) in the total percentages of sperm motility, progressive motility and acrosome integrity, as well as in live spermatozoa. The combination of trehalose with fructose caused a significant increase ($P < 0.05$) in the percentage of total sperm motility only. There were no significant effects of any combination on the percentage of abnormal sperm. The best post-thaw sperm characteristics were obtained using a combination of trehalose with glucose (Table 2). In experiment 3 (Table 3), addition of 100 mM trehalose to tris-based egg yolk-glycerol extender showed the highest sperm characteristics after cryopreservation, but increasing trehalose concentration to 150 mM significantly decreased sperm characteristics prior to and after cryopreservation. After cryopreservation, 100 mM trehalose showed the least negative effect on sperm acrosome integrity and morphology, whereas 150 mM trehalose significantly decreased sperm acrosome integrity and morphology.

Table (1): Effect of adding sugars in a tris-based egg yolk extender on ram spermatozoa characteristics prior and following cryopreservation.

	Total motility%		Progressive motility%		Acrosome integrity%		Live spermatozoa%		Abnormal spermatozoa%	
	PC	FC	PC	FC	PC	FC	PC	FC	PC	FC
Trehalose	76.2[a]	35.4[a]	71.2[a]	25.9[a]	79.3[a]	26.7[a]	74.4[a]	37.4[a]	7.6[a]	34.3[a]
Sucrose	78.4[a]	33.6[a]	69.4[a]	23.6[a]	78.6[a]	28.9[a]	75.6[a]	34.9[a]	6.9[a]	35.1[a]
Glucose	77.0[a]	34.6[a]	68.6[a]	22.9[a]	78.4[a]	28.6[a]	76.2[a]	36.6[a]	6.5[a]	35.9[a]
Fructose	78.1[a]	32.8[a]	70.3[a]	23.4[a]	77.9[a]	30.6[a]	75.9[a]	34.8[a]	7.2[a]	36.4[a]
SEM	1.4	1.5	1.5	2.4	1.7	1.5	1.8	1.5	2.5	1.5

[a,b] Means with different superscripts within a column differ significantly ($P < 0.05$).
PC: prior cryopreservation, FC: following cryopreservation

Table (2): Effect of adding sugars combination in a tris-based egg yolk extender on ram spermatozoa characteristics prior and following cryopreservation.

	Total motility%		Progressive motility%		Acrosome integrity%		Live spermatozoa%		Abnormal spermatozoa%	
	PC	FC	PC	FC	PC	FC	PC	FC	PC	FC
F	77.9[a]	39.5[a]	68.4[a]	27.6[a]	84.5[a]	28.1[a]	82.9[a]	39.9[a]	11.2[a]	33.9[a]
FT	78.6[a]	46.9[b]	67.9[a]	30.2[a]	85.6[a]	27.9[a]	84.1[a]	42.1[a]	10.9[a]	34.1[a]
ST	79.2[a]	38.6[a]	69.1[a]	29.4[a]	86.4[a]	26.4[a]	83.5[a]	41.6[a]	09.8[a]	32.9[a]
GT	78.4[a]	44.8[b]	68.8[a]	35.9[b]	83.9[a]	36.2[b]	83.7[a]	47.6[b]	10.2[a]	33.1[a]
SEM	1.3	2.0	1.7	2.7	1.5	2.4	1.6	2.6	1.9	2.8

[a, b] Means with different superscripts within a column differ significantly ($P < 0.05$).
F = Fructose, FT = combination of fructose and trehalose, ST = combination of sucrose and trehalose, GT = combination of glucose and trehalose. PC: prior cryopreservation, FC: following cryopreservation.

Table (3): Effect of adding different concentrations of trehalose in a tris-based egg yolk extender on ram spermatozoa characteristics prior and following cryopreservation.

	Total motility%		Progressive motility%		Acrosome integrity%		Live spermatozoa%		Abnormal spermatozoa%	
	PC	FC	PC	FC	PC	FC	PC	FC	PC	FC
T1	81.1[a]	38.8[a]	68.6[a]	25.9[a]	82.9[a]	25.1[a]	84.2[a]	41.6[a]	8.9[a]	30.9[a]
T2	82.3[a]	40.6[a]	69.4[a]	26.6[a]	83.7[a]	27.3[a]	85.6[a]	43.2[a]	7.8[a]	32.2[a]
T3	81.9[a]	41.9[a]	67.9[a]	28.2[a]	81.6[a]	28.9[a]	87.1[a]	40.9[a]	9.2[a]	31.6[a]
T4	82.8[a]	54.2[b]	70.3[a]	42.1[b]	84.1[a]	41.9[b]	83.9[a]	56.9[b]	9.9[a]	23.2[b]
T5	46.4[b]	24.9[c]	32.9[b]	13.6[c]	37.2[b]	13.9[c]	45.3[b]	27.9[c]	27.2[b]	42.9[c]
SEM	2.1	2.7	1.9	2.8	2.8	2.3	2.4	2.3	2.1	2.7

[a, b, c] Means with different superscripts within a column differ significantly ($P < 0.05$).
T1 = 35 mM trehalose, T2 = 50 mM trehalose, T3 = 75 mM trehalose, T4 = 100 mM trehalose, T5 = 300 mM trehalose. PC: prior cryopreservation, FC: following cryopreservation.

DISCUSSION

In Experiment 1, there were no significant differences among monosaccharides or disaccharides prior to cryopreservation. Also all sperm characteristics evaluated after cryopreservation were not improved in extenders supplemented with either monosaccharides (glucose or fructose) or disaccharides (trehalose or sucrose). It seems that all sugars at the concentration tested in this experiment had no effect on improving ram sperm characteristics after cryopreservation. In the case of sperm morphology, there was an increase in the percentages of abnormal spermatozoa after cryopreservation. It has been demonstrated that a decrease in the number of morphologically normal sperm in ejaculates leads to reduced fertility (Chandler *et al.*, 1988; Gravance *et al.*, 1998). Therefore, the lower fertility of the cryopreserved ram semen samples may well be a result of a decrease in the number of normal sperm in these samples (Gravance *et al.*, 1997). In all treatments, the percentage of cells with acrosomes damaged by cryopreservation was greater than that with motility loss, consistent with previous reports (Celeghini *et al.*, 2008; Salamon and Maxwell, 1995) that frozen-thawed sperm cells with damaged membranes may remain motile, although they probably have substantially reduced fertilizing capacity. It is improbable that such sperm could penetrate the zona pellucida and fertilize an oocyte. Damage to acrosomal membranes of cryopreserved sperm is due to changes in temperature and osmolarity, which cause morphological alterations in the lipid organization and composition of the sperm membrane (Amann and Pickett, 1987). Sperm acrosome integrity is essential for cell survival and fertilizing ability (Mehmood *et al.*, 2009). Moreover for successful fertilization, a spermatozoon must maintain an intact acrosome up to the time it binds to zona pellucida of the oocyte and undergoes the acrosome reaction to release acrosomal enzymes (Graham and Moc'e, 2005). The presence of an acrosomal cap is important in the fertilization process and has been also highly related with fertility of frozen semen (Lindsay *et al.*, 2005).

In Experiment 2, it was observed that the combination of trehalose with other sugars reduced cryopreservation damage compared to fructose by itself (control). Fructose (Monosaccharide) was more permeable so that dehydration was not so effective (Lee *et al.*, 1989). These findings are in close agreement with the results obtained in ram (Aisen *et al.*, 2000, 2002), rabbit (Dalimata and Graham, 1997), boar (Hu *et al.*, 2009) and mouse (Storey *et al.*, 1998; Sztein *et al.*, 2001) spermatozoa when trehalose was added to the freezing extender, whereas no significant positive effect was observed in bull (Chen *et al.*, 1993; Foote *et al.*, 1992), stallion (Squires *et al.*, 2004) and Iberian red deer (Fernández-Santos *et al.*, 2007). Plasma membrane evaluation of cryopreserved ram sperm containing trehalose by ultramicroscopy also indicated that there was significant reduction in the membrane damage (Aisen *et al.*, 2000).

In Experiment 3, there was significant improvement in quality parameters of frozen ram spermatozoa in response to addition of trehalose to tris-based extender enhanced ram sperm characteristics after cryopreservation. The results clearly indicated that the cryoprotective capacity of trehalose varied depending

on concentration of supplementation in the extenders. Increasing trehalose concentration to 100 mM produced better sperm cryosurvival characteristics. Tonieto *et al.* (2010) concluded that extenders including 100 mM trehalose and 8% LDL (low density lipoprotein) can preserve post-thaw sperm motility and membrane integrity of frozen ram sperm as efficiently as extenders including traditional cryoprotectants, such as egg yolk and glycerol. This is also consistent with other studies (Hu *et al.*, 2009), showing that boar spermatozoa cryopreseved in 100 mM trehalose significantly improved sperm motility, membrane integrity and acrosome integrity.

The mechanism by which trehalose protects spermatozoa during cryopreservation has not been explained well. It is possible that trehalose may contribute to cellular dehydration before freezing. Therefore, it would lower incidence of intracellular ice formation and provide greater survival of spermatozoa (Nagase *et al.*, 1964; Purdy, 2006). In addition to its dehydration action, trehalose confers a specific cryoprotection exerted on the lipid bilayer (Bakas and Disalvo, 1991). For this reason, trehalose may be a better cryoprotectant than other disaccharides (Aisen *et al.*, 2005). Glycerol is frequently used in cryoprotectants to avoid macro-crystal formation from intracellular water. When trehalose is added to tris-based extender, Aisen *et al.* (2002) observed a synergic effect with glycerol on cell integrity, because of water sequestration to the extracellular side and membrane stabilization in a fluid state (Crowe *et al.*, 1989).

Trehalose has a protective action related both to osmotic effect and specific interactions with membrane phospholipids, rendering media hypertonic, causing cellular osmotic dehydration before freezing, and then decreasing the amount of cell injury by ice crystallization (Liu *et al.*, 1998; Molina et. al., 1994; Storey *et al.*, 1998). It is thought that this disaccharide can form hydrogen bonds with the polar head groups of phospholipids, binding to the membrane interface and thereby replacing water molecules. The presence of this sugar may render the membrane less vulnerable to the morphological changes that occur during the rapid reflux of water.

In conclusion, treating ram sperm with 100 mM trehalose as a non-penetrating cryoprotectant prior to cryopreservation benefits sperm cryosurvival in vitro. These results indicated also that improved ram sperm cryopreservation could be obtained using an extender containing a combination of trehalose and glucose.

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تأثير إضافة السكريات لمخفف الترس على صفات السائل المنوى للكباش

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الهدف من هذه الدراسة هو تقييم تأثير إضافة السكريات (تريها لوز– سكروز- فركتوز- جلكوز) لمخفف الترس على صفات السائل المنوى قبل وبعد الحفظ بالتجميد. تم جمع القذفات المنوية من الكباش بإستخدام المهبل الصناعى. فى التجربة الأولى تم تخفيف عينات السائل المنوى فى أربعة مخففات تحتوى على ٦٢,٥ ملى مول جلكوز أو ٦٢,٥ ملى مول فركتوز أو ٣٥ ملى مول سكروز أو ٣٥ ملى مول تريها لوز، وفى التجربة الثانية تم تخفيف عينات السائل المنوى بإستخدام الجمع بين ٣٥ ملى مول التريهالوز مع ٦٢,٥ ملى مول فركتوز أو ٦٢,٥ ملى مول جلكوز أو ٣٥ ملى مول سكروز مقارنة بمعاملة ٦٢,٥ ملى مول فركتوز منفرداً. وفى التجربة الثالثة تم إستخدام الجلكوز بتركيز ٦٢,٥ ملى مول مع تركيزات مختلفة من التريها لوز ٣٥ أو ٥٠ أو ٧٥ أو ١٠٠ أو ١٥٠ ملى مول.

فى التجربة الأولى تم تقدير الحركة الكلية والحركة التقدمية والحيوانات المنوية الحية والشاذة وسلامة الأكروسوم قبل وبعد التجميد ولم تكن هناك فروق معنوية بين السكريات الأحادية والثنائية التى درست قبل وبعد الحفظ بالتجميد. لكن الجمع بين التريهالوز والسكروز أو الفركتوز أو الجلكوز أدى إلى تحسن خصائص الحيوانات المنوية بعد الحفظ بالتجميد وأفضل النتائج التى وجدت كانت بين التريهالوز مع الجلكوز. وفى التجربة الثالثة أدى إضافة التريهالوز بتركيز ١٠٠ ملى مول لمخفف الترس إلى تحسين خصائص السائل المنوى للكباش بعد التجميد وعلى الرغم من ذلك وجد أن زيادة تركيز التريهالوز إلى ١٥٠ ملى مول له تأثير سلبى على خصائص الحيوانات المنوية سواء قبل أو بعد التجميد.

نستنتج من ذلك أن إستخدام التريهالوز قبل التجميد يمكن أن يساعد على حماية الحيوانات المنوية وأفضل النتائج كانت للمخففات التى تميزت بالجمع بين التريهالوز والجلكوز.