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Original Article

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Abstract

Semen cryopreservation alters sperm structure and biochemical properties, and subsequently impairs fertilizing ability. A combination of two different sugar molecules revealed an enhanced beneficial effect on frozen-thawed spermatozoa of various species. However, the report in ram has been scarce. The present study investigated the effect of different combinations of sugar supplementation on post-thawed ram semen qualities in a cryopreservative extender in the presence of a single sugar or a combination of sugars. Data showed that a combination of sucrose and trehalose significantly improved cryopreserved sperm motility, viability, longevity and acrosome integrity. Fertility rates following laparoscopic artificial insemination (LAI) using frozen-thawed semen (n = 35) with the sucrose and trehalose supplementation revealed the same values as those utilizing fresh semen (n = 32) (82.9 v 84.4%, respectively). Our findings suggest that the use of a Tris-Citric extender with the addition of sucrose combined with trehalose is an effective extender for ram semen cryopreservation. The optimization of concentrations and ratios between the combination of sucrose and trehalose supplemented to ram semen freezing extender should be further examined.

Keywords: cryopreservation, ram, semen quality, spermatozoa, sugar

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Introduction

Artificial insemination (AI) combined with semen cryopreservation is not only a technique of choice for ram industry, but it also prevents the spread of venereal or contagious diseases (Cognie, 1999; Richardson et al., 2011). Furthermore, it expands a variety of genetics in farm animals. However, the cryopreservation technique has shown detrimental effects on biochemical properties and functions of cryopreserved spermatozoa, i.e. motility, viability and fertilizing ability, all of which have an enormous impact on pregnancy rate (Labbe et al., 2001; Richardson et al., 2011).

Regarding semen cryopreservation, a freezing extender plays a central role in protection of spermatozoa toward cryoinjury, resulting in an enhanced post-thawed semen quality. The ram semen freezing extender is generally composed of Tris-(hydroxymethyl)-aminomethane (Tris) for acid-base buffer, citric acid for osmotic pressure balance, cryoprotective agent (CPA) for cryoinjury prevention during cryopreservation and, most importantly, saccharides (sugars) for supporting energy and protection the spermatozoa (Abdelhakeam et al., 1991; Salamon and Maxwell, 2000b; Paulenz et al., 2002; Mortimer and Maxwell, 2004; Câmara et al., 2011). Many studies reported the advanced effects of sugars in a semen freezing extender on the post-thawed viability of animal spermatozoa (Garcia and Graham, 1989; Molinia et al., 1994; Garde et al., 2008; Tonieto et al., 2010). Sugars are divided into monosaccharide, disaccharide and trisaccharide types. Each type has different cryoprotective properties to protect spermatozoa during freezing and thawing (Yildiz et al., 2000; Medeiros et al., 2002).

Since monosaccharide is found mainly in the seminal plasma of ram, it is therefore a necessary supplement for a semen freezing extender as an energy source and a protective agent for spermatozoa (Salamon and Maxwell, 2000b), while disaccharide (sucrose or trehalose) provides effective protection for phospholipid membrane of sperm head, like CPA. However, recent studies suggested that post-thawed sperm qualities were improved by a semen freezing extender supplemented with a combination of mono- and disaccharides such as trehalose and sucrose in mammal (Aisen et al., 2002; Aboagla and Terada, 2003; Farshad and Akhondzadeh, 2008). Focusing on buck semen, Farshad et al. (2009) showed that an addition of sucrose plus trehalose to a freezing extender improved semen quality when compared with that of an individual sugar. Naing et al. (2010) also found that a combination of sugars supplemented to a freezing extender provided improvement to buck sperm function, viability and quality following cryopreservation. Few studies proposed that the use of a combination of fructose and trehalose (FT) achieved a higher percentage of viable and intact spermatozoa in ram (Aisen et al., 2000; Aisen et al., 2002; Matsuoka et al., 2006). To our best knowledge, the effects of various combinations of sugars in a freezing extender on post-thawed ram sperm qualities and fertility have never been studied. Thus, the present study aimed to determine the effects of a single sugar and combinations of sugars supplemented to a freezing extender on post-thawed quality of Dorper breed ram semen. A fertility test was further carried out by laparoscopic artificial insemination (LAI) of cryopreserved semen with selected freezing extender in comparison with fresh semen.

Materials and Methods

All chemicals in the present study were purchased from Sigma-Aldrich Chemical Company (Sigma, St. Louis, MO, USA) unless stated otherwise.

Experiment 1. Effect of different sugar supplementation on post-thawed ram sperm quality

Experimental animal: The use of animals and research methodology of this study were approved by the Institutional Animal Care and Use Committee (IACUC), Chulalongkorn University (Approval No. 13310050). Three mature pure-bred Dorper rams (aged 2 to 3 years) were used as semen donors. They were housed in a semi-intensive system under preventive and clinical veterinary care, and fed on concentrate feed 0.4 kg/day/animal (12%protein) and *ad libitum* of grasses with free access to mineral blocks and water.

Preparation of basic and testing semen extenders: The basic semen freezing extender used in this study was Tris-citric acid (TC) solution (300 mM Tris, 95 mM citric acid), supplemented with 15% (v/v) hen's egg yolk, 5% (v/v) glycerol (Sigma, Steinheim, Germany) (Mortimer and Maxwell, 2004) and 0.5% (w/v) Equex STM paste (Nova Chemical Sales Inc, Scituate, USA). Testing extenders were prepared by adding one or two types of sugar into the TC solution for a final concentration of 30 mM. pH and osmolality of the extenders were adjusted to 6.8-7.0 and 400 mOsm/kg, respectively.

Semen collection and processing: Semen was collected from the rams by an artificial vagina (AV) twice a week as described by Moore (1985). Semen with acceptable motility (>70%), sperm concentration (>1,500 x 10^6 spermatozoa/ml) and normal morphology (>90%) was pooled and divided into aliquots. Each aliquot was diluted with one of the testing extenders. Concentration was adjusted to 320 x 10^6 spermatozoa/ml. The diluted semen was equilibrated at 4°C for 4 h. After equilibration, it was loaded into 0.25 ml French mini straws (Minitüb®, Landshut, Germany). The straws were placed horizontally, approximately 4 cm above liquid nitrogen levels for 10 min in a Styrofoam box, before being plunged into liquid nitrogen for storage. After 24 h storage, one straw of each testing extender was randomly selected and thawed in a 37°C water bath for 30 sec (Salamon and Maxwell, 1995).

Assessment of sperm quality: All semen was evaluated at five time points as follows: within 5 min (T0), 1 h (T1), 3 h (T3), 6 h (T6) and 9 h (T9) after thawing. The motility of post-thawed semen was evaluated by a computer-assisted sperm analyzer (CASA, Hamilton-Thorne Biosciences IVOS, Version 12.3, Bevery, MA, USA), at a setting recommended by the manufacturer. Assessment setup parameters were as follows: Frame

rate, 60 Hz; cell size (min/max), 12/80 μm²; and minimum curvilinear velocity (VCL), 10 /μm. Samples of frozen semen to be analyzed with CASA were thawed and warmed at 37°C. At least 500 sperm cells (6 fields) were selected and assessed for motility (Mo, %), average path velocity (VAP, μm/s), straight-line velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), straightness (STR, %) and linearity (LIN, %). Sperm viability (Via) was evaluated using eosin-aniline blue staining. One drop of semen was mixed with Asian-aniline blue stain and thinly smeared onto a glass slide. The slide was then observed by a light microscope, and total count was 200 spermatozoa. Live spermatozoa, which were colorless, were classified as positive result, whereas dead spermatozoa, which were red, were classified as negative result.

A fluorescein isothiocyanated peanut agglutinin (FITC–PNA) was used to evaluate acrosome integrity (Acro) (Grasa et al., 2006). Thawed semen was diluted with 1:5 phosphate buffered saline (PBS). Six μl of semen mixture was smeared onto a glass slide and sperm membrane was permeabilized with 95% ethanol for 30 sec. The slide was covered with FITC-PNA (100 μg/ml in PBS) solution and incubated in a moist chamber at 4°C for 30 min, then rinsed with 4°C distilled water before air drying. The stained slides were evaluated using an epifluorescent microscope (BX51; Olympus®, Shinjuku, Japan). At least 200 spermatozoa per sample were evaluated. A spermatozoon with intense bright green fluorescent of acrosomal cap was indicated as intact acrosome.

Experiment 2. Effect of sugar supplementation in extenders used in fresh and frozen semen on fertility rate after laparoscopic artificial insemination (LAI)

Experimental animals: A total of sixty-seven multiparous local-bred ewes between 2 to 4 years of age were selected and randomly divided into two groups: fresh and frozen semen groups. The animals were managed the same as the ram semen donor group, but were fed on 0.3 kg/day/animal concentrates containing at least 14% (w/w) protein and water ad libitum.

Estrus synchronization: Estrus-synchronization was performed by intravaginal progesterone implant, (CIDR-G® ,Eazi-Breed™ CIDR-G®, 0.3 g progesterone in silicone, Interag, Hamilton, New Zealand) for 10 d, with an injection of 300 IU eCG (Folligon®, Intervet Schering-Plough Animal Health, The Netherlands) at the time of progesterone removal. Standing estrus was detected by a teaser ram 2 times/day (am/pm). Laparoscopic artificial insemination was performed at 22-24 h after standing estrus.

Semen preparation process for artificial insemination: Frozen-thawed semen was selected from the best quality semen in experiment 1.2. In the fresh semen group, the ejaculated Dorper ram semen was diluted to 320 x 10^6 spermatozoa/ml, the same as the freezing semen extender but without glycerol and Equex STM® paste.

Laparoscopic artificial insemination: All ewes submitted to the operation were restricted from feed for 24 h and water for 12 h. They were anesthetized with xylazine HCl (0.025 mg/kg) combined with ketamine HCl (1.1 mg/kg) and given 0.04 mg/kg phenylbutazone as an analgesic. The LAI technique followed the procedure described by Ehling et al. (2003) and Anakkul et al. (2013a). The greater curvature of each uterine horn was pierced with an insemination needle and either fresh or frozen-thawed semen was deposited into the uterine lumen. The insemination dose was 40 x 10^6 spermatozoa/horn. The insemination was done under observation via a direct view 5-mm laparoscope (Schölly, Denzlingen, Germany) (Alfaris et al., 2012). Post-operative care included intramuscular injection of 20,000 IU/kg penicillin-streptomycin and wound dressing with antiseptic for 3 d.

Pregnancy diagnosis

Pregnancy was detected by a real time B-mode ultrasonography (transcutaneous probe: HS-2000, Honda Electronics Co., Ltd., Japan) 60 d after insemination.

Experimental design: Experiment 1 was designed to evaluate semen quality after freezing and thawing in different semen extenders supplemented with different types of sugar. The experiment was designed into 2 consecutive experiments and was repeated for 9 replicates. Details of the 2 consecutive experiments are as follows:

Experiment 1.1: To compare the post-thawed ram sperm quality among four types of single sugars. The testing extenders used in this part contained 30 mM of glucose (Glu), fructose (Fru), sucrose (Su) or trehalose (Tre).

Experiment 1.2: To examine the effects of combined sugar on the freezing ability of ram spermatozoa. The testing extenders used in this part contained 30 mM of mixed sugars by combining 15 mM of fructose, sucrose or trehalose. The extender containing 30 mM of the sugar selected from experiment 1.1 was used as a control.

Experiment 2 focused on fertility after the laparoscopic artificial insemination (LAI) using the frozen semen with selected extender according to its performance from experiment 1.2. The frozen semen was tested compared to the fresh semen in hormonal synchronized ewes. Pregnancy rate was recorded

Statistical analysis: All statistical analyses were carried out using Statistical Analysis System (SAS) package version 9.2 (SAS 9.2, SAS Institute Inc., Cary, NC, USA). In experiment 1, the statistical model was used to evaluate 1 fixed effect (sugar supplementation) while the replicate was set as a random effect. The data of viability, acrosome integrity and results of motility determined by CASA in each group were expressed as mean ± standard error (SEM). The data were tested for normality by the Shapiro-Wilk test and for homogeneity of variance using the Levene test. The quantitative data of treatments were analyzed by one-way ANOVA followed by the Turkey-Kramer test.

In experiment 2, the percentage of fertility rates was analyzed in each treatment with a categorical model (CATMOD procedure). The Chi-square test (χ^2) was employed to compare the fertility rates between

groups. Differences between experimental groups were considered to be statistically significant when $p< 0.05$.

Results

Experiment 1. Effect of different sugar supplementation on post-thawed ram sperm quality

Effect of sugar types: The effects of sugar supplementation on the semen freezing extender quality are presented in Table 1. The semen freezing extenders with fructose or sucrose treatments showed a significantly higher percentage of post-thawed sperm motility when compared with the glucose or trehalose supplementation groups (64.3% or 63.6% versus 54.7% or 55.9%, respectively; $p< 0.05$).

Effect of combined sugars: The basic semen freezing extender using sucrose and trehalose (ST) analyzed by CASA showed significantly higher motility than the other groups. Mean percentage of viable spermatozoa (Via) at 1, 3 and 6 h of the ST group was greater than the fructose combined with sucrose (FS) and fructose combined with trehalose (FT) groups ($p< 0.05$). At 9 h of incubation time, the ST group had higher ($p< 0.05$) live and intact acrosome spermatozoa than the other groups.

Average path velocity (VAP) at 6 h of storage was significantly reduced ($p< 0.05$) in the Fru treatment compared to all other treatments. Within the ST group, there tended to be a higher percentage of straight-line velocity (VSL) at all times; however, significant difference ($p< 0.05$) was observed at 6 h of storage. The mean percentage of curvilinear velocity (VCL), straightness (STR) and linearity (LIN) did not differ ($P > 0.05$) between the groups during storage, but mean percent of STR in the Fru group at 9 h of storage was significantly less ($p< 0.05$) than the ST group (Table 2).

Table 1 Mean percentage of total motility, viability and acrosome integrity for each sugar supplementation in post-thawed ram sperm

Extender	Total motility (%)	Viability (%)	Acrosome integrity (%)
Fructose	64.3 ± 7.9[a]	79.4 ± 11.0[a]	79.6 ± 6.0[a]
Glucose	54.6 ± 5.9[b]	72.3 ± 12.3[a]	74.3 ± 7.3[a]
Sucrose	63.6 ± 8.7[a]	76.1 ± 9.0[a]	78.3 ± 6.4[a]
Trehalose	55.9 ± 9.2[b]	71.8 ± 10.4[a]	75.1 ± 6.2[a]

Different letters within the same column represent a significant difference (Turkey-Kramer test, $p< 0.05$).

Experiment 2. Effect of combinations of sugar supplementation on fertility rate after LAI with fresh or frozen semen

After the trial of *in vitro* characteristics of post-thawed semen, the TC based extender in the ST group showed greater results than the other treatments. This formula was, therefore, used to compare fertility after LAI with fresh semen.

Of the 70 ewes, three ewes were excluded because they did not show estrus sign after estrus synchronization. Therefore, the results in this study were based on 67 ewes (Table 3). The average time interval between CIDR-G® withdrawal to estrus was 35.3 ± 10.5 h (range 23.4 to 60.0 h). The mean of insemination time after withdrawal of CIDR-G® did not differ between the fresh and frozen groups (57.0 versus 58.6 h; $p > 0.05$). At the timing of insemination, the average number of dominant follicles (diameter > 7 mm.) per ewe was 1.7 follicles.

Fifty-six of 67 ewes (83.6%) were confirmed pregnant after insemination using ultrasonography. There was no significant difference in pregnancy rate between the frozen and fresh semen (82.9 versus 84.4%) when using the LAI technique (Table 3).

Discussion

As its chief function, exogenous sugar in a semen freezing extender serves as an energy source for spermatozoa. In the present study, four different types of sugars were compared in the efficiency of ram semen cryopreservation. The authors found that these sugars affected the post-thawed semen qualities differently. The post-thawed ram semen qualities were increased in the fructose group more than the other groups. This result agrees with previous reports that fructose is the best of the supported extenders for semen cryopreservation in dogs and rams (Salamon and Maxwell, 2000a; Ponglowhapan et al., 2004). Therefore, it is suggested that the fructose concentration used in this study may act as sperm support (Fiser et al., 1987; Salamon and Maxwell, 2000a). In small ruminant seminal plasma, fructose is the primary substrate for glycolysis (Glover, 1956; Pellicer-Rubio et al., 1997). The end product of glycolysis pathway produces more ATP, which supports sperm motility. In freezing extender, fructose produces ATP better than glucose because glucose is converted to fructose before glycolysis. Therefore, the post-thawed sperm motility in the fructose group in this study was significantly higher than the glucose supplementation.

Sugars can help spermatozoa not only as an energy source, but also as a spermatozoa protective action within the cryopreservation process. As temperature drops, more ice crystals in a spermatozoa cell are formed and cause injury to the plasma membrane in the spermatozoa called "Cryoinjury". It induces cell stresses with osmotic pressure changes and leads to death of frozen cells (Watson, 2000; Pegg, 2010). Regarding plasma membrane, it is the outer structure of spermatozoa which acts as a protective barrier of the spermatozoa and can be destroyed by semen cryopreservation, resulting in detrimental effects on its functions, including sperm fertilizing process (sperm capacitation, acrosome reaction and sperm-oocyte fusion) (Parks and Graham, 1992). Destruction of the plasma membranes affects viability, longevity and fertility of post-thawed spermatozoa. From previous findings, monosaccharide (glucose or

fructose) can be permeable to the membrane because of its small molecular weight. However, many additive agents in semen freezing extenders, including glycerol and Equex STM paste, can also be effective in protecting sperm plasma membrane (Akourki et al., 2004; Anakkul et al., 2013b).

Disaccharides are capable of protecting the lipid bilayer of cryopreserved spermatozoa through the hydrogen bonding of molecules and, thus, prevent the growth of ice crystals (Uchida et al., 2007). In addition to the above mechanism, trehalose decreases the supply of free water molecules from the solution to spermatozoa and induces dehydration in the cell, reducing intracellular ice formation. Not only trehalose, but also sucrose can reduce ice crystal formation. However, the inhibitory effect of sucrose on ice crystal growth was lower than trehalose (Sussich et al., 2001).

Table 2 Effect of the sugar combination supplemented to freezing extender on sperm quality parameters analyzed by CASA in post-thawed semen stored for various time periods at 37°C

Parameters	Treatments	Storage time (h)				
		0	1	3	6	9
Mo (%)	Fru	64.3 ± 7.9[a]	54.2 ± 6.9[a]	45.4 ± 10.6[ab]	38.0 ± 9.7[a]	24.4 ± 11.0[a]
	FS	66.8 ± 7.2[a]	58.0 ± 8.2[a]	50.0 ± 5.7[a]	39.9 ± 9.0[a]	27.3 ± 9.2[a]
	FT	61.1 ± 9.4[a]	52.4 ± 9.1[a]	41.1 ± 15.2[b]	34.8 ± 9.6[a]	27.9 ± 8.3[a]
	ST	78.6 ± 5.0[b]	70.3 ± 6.5[b]	62.3 ± 8.0[c]	56.8 ± 7.5[b]	43.8 ± 6.9[b]
Via (%)	Fru	79.4 ± 11.0[ab]	73.8 ± 10.2[ac]	66.9 ±10.3[ac]	57.3 ±9.7[ab]	47.4 ±9.8[a]
	FS	79.2 ± 8.5[a]	69.9 ± 6.9[ab]	60.4 ± 7.3[ab]	56.6 ± 8.3[a]	47.2 ± 8.1[a]
	FT	73.8 ± 10.7[ab]	64.7 ± 11.1[b]	58.2 ± 6.5[b]	50.4 ± 5.6[a]	41.4 ± 9.7[a]
	ST	84.4 ± 5.5[b]	78.6 ± 7.0[c]	73.3 ± 7.1[c]	64.7 ± 6.2[b]	56.3 ± 7.6[b]
Acro (%)	Fru	79.6 ± 6.0[a]	76.4 ± 5.7[a]	75.0 ± 5.7[ab]	69.6 ± 7.4[ab]	65.7 ± 5.9[a]
	FS	79.0 ± 6.0[a]	75.9 ± 6.6[a]	72.6 ± 6.4[ab]	69.1 ± 6.6[ab]	65.4 ± 6.2[a]
	FT	77.9 ± 5.8[a]	73.9 ± 7.8[a]	69.6 ± 9.7[a]	67.3 ± 8.6[a]	63.6 ± 7.9[a]
	ST	82.3 ± 5.5[a]	79.7 ± 5.8[a]	77.4 ± 5.0[b]	75.1 ± 5.0[b]	73.0 ± 5.2[b]
VAP (µm/s)	Fru	93.5 ± 20.6[a]	79.7 ± 15.2[a]	77.5 ± 14.4[a]	62.8 ± 17.9[a]	60.8 ± 27.5[a]
	FS	91.7 ± 17.0[a]	90.8 ± 19.5[a]	89.4 ± 32.1[a]	65.0 ± 16.3[ab]	47.7 ± 20.1[a]
	FT	91.5 ± 19.5[a]	83.3 ± 15.8[a]	74.0 ± 20.4[a]	63.1 ± 16.9[ab]	49.8 ± 21.4[a]
	ST	96.8 ± 21.4[a]	85.6 ± 18.5[a]	83.2 ± 12.1[a]	81.7 ± 12.7[b]	61.1 ± 23.1[a]
VCL (µm/s)	Fru	168.9 ± 41.4[a]	159.1 ± 30.6[a]	141.0 ± 26.0[a]	114.6 ± 35.8[a]	103.4 ± 38.8[a]
	FS	166.2 ± 36.6[a]	149.7 ± 43.5[a]	135.5 ± 33.6[a]	108.0 ± 16.2[a]	86.7 ± 29.4[a]
	FT	140.0 ± 20.6[a]	135.9 ± 35.2[a]	124.6 ± 34.3[a]	112.8 ± 34.9[a]	87.1 ± 36.2[a]
	ST	158.8 ± 50.7[a]	152.7 ± 44.6[a]	128.4 ± 35.5[a]	112.0 ± 16.0[a]	92.8 ± 31.3[a]
VSL (µm/s)	Fru	63.8 ± 14.0[a]	51.1 ± 9.3[a]	49.5 ± 10.5[a]	41.8 ± 11.1[a]	33.4 ± 9.6[ab]
	FS	61.9 ± 10.6[a]	56.3 ± 10.0[ab]	55.9 ± 23.9[a]	43.7 ± 9.8[a]	28.0 ± 9.0[a]
	FT	60.9 ± 17.4[a]	54.2 ± 15.3[ab]	46.7 ± 9.2[a]	39.9 ± 8.9[a]	29.0 ± 10.4[a]
	ST	71.6 ± 20.1[a]	65.0 ± 19.4[b]	59.5 ± 13.8[a]	56.7 ± 14.0[b]	45.1 ± 13.7[b]
LIN (%)	Fru	37.1 ± 5.6[a]	35.8 ± 4.2[a]	35.8 ± 4.9[a]	35.2 ± 9.4[a]	34.0 ± 4.8[a]
	FS	36.9 ± 6.7[a]	37.0 ± 7.5[a]	38.0 ± 8.1[a]	38.2 ± 10.7[a]	37.4 ± 7.8[a]
	FT	39.0 ± 8.4[a]	41.7 ± 12.7[a]	33.1 ± 5.3[a]	38.7 ± 8.1[a]	39.4 ± 10.8[a]
	ST	33.8 ± 6.6[a]	40.2 ± 6.4[a]	37.4 ± 10.2[a]	40.8 ± 10.3[a]	40.2 ± 10.8[a]
STR (%)	Fru	62.6 ± 5.6[a]	63.4 ± 13.2[a]	62.9 ± 10.4[a]	64.1 ± 7.0[a]	55.1 ± 13.7[a]
	FS	66.3 ± 12.5[a]	67.0 ± 7.1[a]	64.1 ± 7.6[a]	70.0 ± 7.3[a]	63.4 ± 9.4[ab]
	FT	59.0 ± 11.5[a]	66.4 ± 8.9[a]	61.2 ± 4.8[a]	64.7 ± 9.3[a]	63.8 ± 14.7[ab]
	ST	65.2 ± 11.3[a]	70.2 ± 11.7[a]	65.4 ± 11.0[a]	71.0 ± 8.8[a]	66.2 ± 8.8[b]

Fru, Fructose; FS, Fructose + Sucrose; FT, Fructose + Trehalose; ST, Sucrose + Trehalose
Different letters indicate significant differences between data in the same column and parameter (Turkey-Kramer test, $p < 0.05$).

Table 3 Effect of LAI with fresh or frozen ram semen on pregnancy rates

Group	No. of ewes inseminated	No. of ewes pregnant at Day 60 (%)
Fresh semen	32	27/32 (84.4%)
Frozen semen	35	29/35 (82.9%)

Sucrose is generally found in plants, while trehalose is principally found in animals. Both of these disaccharides are capable of persisting in low temperatures (Crowe et al., 1988). Both sucrose and trehalose can be found in natural CPA, which contains fructose or glucose rings linked by the glycosidic bond. Acid hydrolysis can break this bond and convert sucrose into fructose and glucose. In the TC solution used in this experiment, sucrose was hydrolyzed to fructose and glucose monomers by citric acid (Del Pilar Buera et al., 1995). This may explain the results of semen quality showing no statistically significant difference between sucrose and fructose in this context. Trehalose is composed of two units of glucose, therefore, the post-thawed motility in this research is

lower than the fructose and sucrose groups, but similar to the glucose group.

Besides, many studies found that trehalose protected spermatozoa from damage by oxygen radicals (Aisen et al., 2005; Hu et al., 2010; Tuncer et al., 2013). The semen freezing extender containing trehalose enhanced the glutathione peroxidase (GSH-Px) and catalase (CAT) activity in post-thawed ram and goat semen (Bucak et al., 2007; Atessahin et al., 2008). These enzymes defend against reactive oxygen species (ROS) and toxic products of metabolism during the freeze-thaw process, thus showing a marked improvement in sperm motility and viability.

In the current study, the effect of sugar combination on the ability to maintain post-thawed sperm viability and longevity was investigated. The combination of sugars supplemented the ram semen freezing extender showed higher post-thawed semen qualities than the single sugar. Similar results were also reported in goat, human and monkey (Nagai et al., 1982; Aboagla and Terada, 2003; Si et al., 2006; Khalili et al., 2009; Naing et al., 2010). Our findings showed that over 9 h of incubation time, the ST treatment group provided higher proportion motile and viable spermatozoa than the other treatment groups. Khalili et al. (2009) also suggested that sucrose combined with trehalose was capable of improving the percentage of motility, viability and membrane integrity in post-thawed goat spermatozoa.

In this study, we further investigated the fertility rate of cryopreserved ram semen using the semen extender supplemented ST. The data showed an acceptable pregnancy rate at 84.4% after LAI of post-thawing ram semen with ST, which was similar to the fresh semen at 82.8%. The pregnancy results obtained in previous studies ranged between 40.5 to 98.2% (Halbert et al., 1990; Findlater et al., 1991; Gimenez-Diaz et al., 2012).

In summary, the data of this study showed that the combination of sugar supplementation in TC freezing extender proved to be effective cryopreservation for ram semen. This study suggests that sucrose plus trehalose (at the final concentration of 30 mM) is the preferable supplementation which can improve semen quality and provide a high successful pregnancy rate in ram. However, the optimized molarity and ratio of sucrose or trehalose are factors that need further investigation. In addition, the understanding of functionality and positional protection during the cryopreservation process of different sugar molecules may lead us to select appropriate sugar implementation in semen freezing extender with higher pregnancy rate.

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บทคัดย่อ

ผลกระทบของการใช้น้ำตาลร่วมกันในน้ำยาทำน้ำเชื้อแช่แข็งต่อคุณภาพน้ำเชื้อแกะแช่แข็งภายหลังการละลาย

สฤษฏ์วิชญ์ ปัญญาบริบาลบ์ จันทร์เพ็ญ สุวิมลธีระบุตร นวเพ็ญ ภูติกนิษฐ์
ธีรวัฒน์ สว่างจันทร์อุทัย ธีรวัฒน์ ธาราศานิต มงคล เตชะกำพุ*

การแช่แข็งน้ำเชื้อทำให้ตัวอสุจิสูญเสียโครงสร้างและคุณสมบัติบางประการอันจะส่งผลต่อการปฏิสนธิ การใช้น้ำตาลร่วมกัน 2 ชนิด มีผลเชิงบวกต่อคุณภาพหลังการละลายของน้ำเชื้อแช่แข็งในสัตว์หลายชนิด แต่พบการรายงานที่น้อยในแกะ การทดลองนี้มีวัตถุประสงค์เพื่อศึกษาผลของน้ำตาลแต่ละชนิดและการใช้น้ำตาลร่วมกันในน้ำยาทำน้ำเชื้อแช่แข็งต่อคุณภาพของน้ำเชื้อแกะแช่แข็ง จากการทดลองพบว่าการใช้น้ำตาลร่วมกันระหว่างซูโครสและทรีฮาโรสช่วยเพิ่มคุณภาพของน้ำเชื้อแช่แข็งหลังการละลายอย่างมีนัยสำคัญทางสถิติ ($p< 0.05$) อันได้แก่ เปอร์เซนต์การเคลื่อนที่ ความยืนยาวในการรอดชีวิต และคุณภาพของอะโครโซมของอสุจิ ส่วนอัตราการผสมติดของน้ำเชื้อแช่แข็งหลังการผสมเทียมด้วยเทคนิคส่องช่องท้องผ่านกล้อง (Laparoscopic artificial insemination) พบว่าให้อัตราการผสมติดไม่แตกต่างกันอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับกลุ่มที่ใช้น้ำเชื้อสด (82.9 และ 84.4 เปอร์เซนต์ ตามลำดับ) จากการทดลองนี้สรุปได้ว่า การใช้น้ำตาลร่วมกันระหว่างซูโครสและทรีฮาโรสในน้ำยาทำน้ำเชื้อแช่แข็งช่วยเพิ่มคุณภาพของน้ำเชื้อแช่แข็งในแกะได้ อย่างไรก็ตามสัดส่วนที่เหมาะสมของความเข้มข้นระหว่างน้ำตาลซูโครสและทรีฮาโรสที่ใช้ในน้ำยาทำน้ำเชื้อแช่แข็งจะต้องได้รับการศึกษาต่อไป

คำสำคัญ: การแช่แข็งน้ำเชื้อ พ่อแกะ คุณภาพน้ำเชื้อ อสุจิ น้ำตาล

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