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EFFECTS OF SUGAR TYPES IN SEMEN EXTENDER ON SPERM QUALITY AND LONGEVITY OF FROZEN GOAT SEMEN

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ABSTRACT

Supplementation of beneficial substances in semen extender is one of crucial ways to maintain the optimal sperm quality of frozen semen. The addition of sugar to semen extender has an advantageous effect and can be used as an energy source. The aim of this study was to evaluate the effects of different sugar types in semen extenders on sperm quality and longevity after cryopreservation. Semen samples were obtained from healthy Boer goats (*Capra aegagrus hircus*) twice a week for four weeks. Individual sample was initially evaluated for quality. For each day of semen collection, good quality semen samples were pooled, divided into three groups, and cryopreserved in Tris-base extenders supplemented with different three sugar types: glucose, fructose, and trehalose. All samples were promptly frozen for further study. The samples were analyzed for motility, viability, membrane integrity, and sperm morphology after thawing and incubated at 1, 2, and 3 h. The results showed that total motility, progressive motility, viability, and membrane integrity of sperms in extender supplemented with trehalose were significantly higher than those in extenders supplemented with glucose and fructose ($p<0.05$). This suggested that trehalose can be potentially used as a cryoprotectant in a semen extender to preserve the optimal quality of motility, viability, and membrane integrity of goat sperms.

Key words: cryopreservation, sugar, semen, evaluation, artificial insemination

INTRODUCTION

The development of goat production is an important role in increasing the economical return for goat producers. Artificial insemination technique (AI) is extensively applied to improve goat breeding programs and genetic selection. Frozen goat semen is one of the necessary resources to be used with AI. Spermatozoa that has undergone freezing and thawing processes is confronted with numerous biochemical and physiological changes which leads to reduced sperm quality. This phenomenon directly affects the fertilization rate. Although supplementation with cryoprotectants of both the egg yolk and glycerol in semen extenders assists to optimize sperm quality after cryopreservation, the application of AI with frozen goat semen to the animal could possibly result in low pregnancy rates (Ritar and Salamon, 1982, Tuli and Holtz 1994).

The addition of sugar to semen extender has a beneficial effect. Sugar can be used as an energy source utilized by spermatozoa through glycolysis and oxidative phosphorylation to support sperm viability and motility (Ponglowhapan et al. 2004). Additionally, there are some advantages of

non-penetrate sugars used as cryoprotectants to maintain the optimal osmotic pressure outside the spermatozoa which can induce cell dehydration and keep less ice crystal formation within the cell (Purdy 2006). Regarding membrane reorganization, when sugar was interacted with the phospholipids in the plasma membrane, sperms from frozen semen added with sugar-cryoprotected semen extenders showed better membrane stabilization and higher survival rates after being cryopreserved (Aboagla and Terada 2003).

Monosaccharides, glucose and fructose, were first considered to be supplemental in semen extenders which showed satisfactory experimental results that were better than those of sorbitol supplementation in boar semen (Chanapiwat et al. 2012). Several studies suggest that the addition of different sugar types in semen extenders have strong effects on the spermatozoa during the process of cryopreservation and clearly improve the after-thawed sperm quality, especially when trehalose, known as a natural alpha-linked disaccharide, had been used in semen extenders for goats (Aboagla and Terada 2003), rams (Aisen et al. 2002) and boars (Malo et al. 2010). However, some previous studies showed that different sugar types (i.e. glucose, fructose, sucrose, and/or trehalose) added to semen extenders had no significantly different effects on after-thawed sperm quality of canines (Ponglowhapan et al. 2004), or Boer goats (Naing et al. 2010).

Although disaccharides efficiently enhance sperm motility and other characteristics after cryopreservation, longevity of after-thawed spermatozoa is still unclear. This study sought to evaluate the roles of different sugar types: glucose, fructose, and trehalose, in semen extenders for goat sperm quality and longevity after cryopreservation.

MATERIALS AND METHODS

Animals and semen collection

The semen samples were obtained from two healthy mature Boer goats, three years of age. These bucks were housed in a semi-intensive system in Songkhla Artificial Insemination and Biotechnology Research Center, Department of Livestock Development, Songkhla, Thailand. The bucks were fed daily with a blend of one kilogram of concentrate (12% crude protein) and *ad libitum* of fresh grasses, water, and supplemented with mixed macro- and micro- mineral block (NaCl 99.511%, Mg 0.2%, Zn 0.0081%, Cu 0.022%, I 0.01%, Se 0.001%). Ejaculated semen and spermatozoa were collected by using an artificial vagina twice a week for four weeks. For each time of semen collection, individual sample was initially evaluated for quality. Only semen samples having a concentration of > 2.5×10^9 sperms/ml, > 70% initial motility, and > 85% sperm with normal morphology were selected and subsequently processed for the cryopreservation method. The concentrations of sperm were determined by using a hemocytometer (WHO 2010). After the evaluation of optimal quality, two semen samples were then pooled each day of semen collection, and cryopreserved in different Tris-base extenders supplemented with three different sugar types: glucose, fructose, and trehalose (5 samples/sugar type/semen collection). All samples were promptly frozen for further study.

Extender preparation

All chemicals were reagent grade. The extender consisted of 250 mM Tris (hydroxyl methyl aminomethane), 90 mM citric acid, 18% (v/v) egg yolk and 8% (v/v) glycerol, antibiotics (500 IU penicillin and 5 mg streptomycin) and supplemented with one of three different sugar types (70 mM glucose, 70 mM fructose, and 70 mM trehalose), respectively.

Freezing and thawing procedures

Having passed an initial evaluation for each time of semen collection, two semen samples from two boars were pooled and divided into three parts. Each part of fresh semen was then diluted with normal saline as a washing solution at 1:19. The fresh semen sample (0.5 ml) and washing

solution (9.5 ml) were placed into 10 ml centrifuge tube and then centrifuged at 1500 x g for 10 min at room temperature to separate seminal plasma. The supernatant was removed by using micropipette. Subsequently, pellet semen was diluted with a semen extender consisting of one of three different sugar types by adjusting the final concentration at 150 x 10^6 sperms/straw (600 x 10^6 sperms/ml) immediately and incubated at room temperature for 5 min. The concentrations of sperm were determined by using a hemocytometer (WHO 2010). The extended semen was then placed in a cooling chamber at 4ºC and kept for 4 h. The diluted sperm was loaded into 0.25 ml plastic straws, placed on an aluminum rack and frozen by placing the straws in a foam box 5 cm above the surface of liquid nitrogen (-120ºC) for 13 min. The frozen straw semen was immersed into the liquid nitrogen. The thawing process was done in a 37ºC water bath for the evaluation of sperm quality and longevity.

Semen evaluation

A total of one hundred and twenty frozen semen samples from the extenders containing three different sugar types (40 samples/sugar type) were determined at four different incubating times (10 samples/sugar type/incubation time). Frozen semen samples were evaluated after thawing (0 h), 1, 2, and 3 h after incubating in a water bath at 37ºC.

Assessment of fresh semen

After collecting semen from an artificial vagina, the semen sample was analyzed immediately for quality in terms of volume, mass movement, and total motility. The measurement of semen volume was conducted by using graduated pipette. The mass movement and total motility of fresh semen was evaluated by placing a drop of fresh semen on a rectangle piece of glass slide and observed under a phase-contrast microscope at 200x magnification.

Assessment of sperm motility

The motility of frozen and then thawed semen was evaluated by computer-assisted sperm analysis (CASA) (IVOS model 12.3, Hamilton-Thorne Biosciences, Beverly, MA, USA). The setting was conducted in accordance with the guidance for goat sperm sample analysis [frame rate per sec: 60 Hz, No. of frame: 45, cell detection (minimum contrast: 70 and minimum cell size: 5 pix), defaults (cell size: 10 pix and cell intensity: 80), progressive cell (path velocity: 50 µ/s and straightness: 80%), slow cell (VAP cutoff: 20 µ/s and VSL cutoff: 5.0 µ/s), static gates (minimum: 0.2 and maximum: 1.92), static size gates (minimum: 0.6 and maximum: 4.32), static elongation gates (minimum: 7 and maximum: 91)]. The post-thawed sperm were analyzed for total motility (MOT, %), progressive motility (PMOT, %), average path velocity (VAP, µm/s), straight-line velocity (VSL, µm/s) and curve-line velocity (VCL, µm/s). For each evaluation, a 5 µl sperm sample was placed in 2x-cell chamber slide which was placed on the thermo stable plate heated to 37°C prior to the analysis. The semen samples from the extenders containing three different sugar types at four different incubating times (0, 1, 2, and 3 h after incubation) were analyzed for sperm motility. The basic evaluation was conducted by selecting five fields per chamber slide for motility study and more than eight hundred spermatozoa were accepted to be analyzed.

Assessment of sperm viability

The viability of the spermatozoa was evaluated by staining with nigrosin-eosin to check the live and dead sperm cells. The dead sperm cells showed the red or dark pink heads (membrane-damaged), but the alive spermatozoa showed the white or light pink heads (membrane-intact) (WHO 2010). The examination was performed using a bright-field microscope (Model CX31RBSFA, Olympus, Tokyo, Japan) at 1000x magnification. A total of two hundred spermatozoa were counted for each sample (Peterson et al. 2007).

Assessment of sperm morphology

The morphology of spermatozoa was evaluated after nigrosin-eosin staining and then examined under a light microscope (Model CX31RBSFA, Olympus, Tokyo, Japan) at 1000x

magnification. A total of two hundred spermatozoa were counted to determine for both normal and abnormal morphology analysis. The abnormal morphology of spermatozoa was categorized according to Barth and Oko (1989). The abnormal characteristics of sperm were defined in two parts; head and tail, i.e. abnormal head (narrow head, narrow at base, pear shape, abaxial, tailless head, giant, round, small, broad, and double heads) and abnormal tail (bent tail, pathogenic mid-piece, tail coiled, and double tails).

Assessment of membrane integrity

The membrane functional integrity was evaluated by the hypo-osmotic swelling test (HOST). A hypo-osmotic solution (HOS) was made by diluting 8.72 g of fructose and 4.74 g of sodium citrate dehydrate in 1 liter of distilled water with an adjusted final pH of 8.05. Subsequently, 10 µl of semen sample was added into 1 ml of HOS solution and the mixed solution was incubated at 37ºC for 60 min. After incubation, one drop of the diluted semen was placed on a glass slide, covered with a cover slide, and evaluated under a phase contrast microscope (400x) (Model CX31RBSFA, Olympus, Tokyo, Japan). At least two hundred spermatozoa were randomly counted per slide and swollen spermatozoa were evaluated by observing changes in the shape of the cell and identified by coiling the tail. Under hypo-osmotic stress condition, live cells were differentiated by evidence of swelling of the sperm tail (WHO 2010). The percentage of swollen tail spermatozoa (number of swollen tail spermatozoa per 200 sperm x 100) was calculated (Naing et al. 2010).

Experimental design and analysis

Results were analyzed using a one-way analysis of variance, followed by Duncan's new multiple range test to determine significant differences in all parameters between groups using SAS statistical software (SAS version. 9.0 Cary, N.C., USA) at the significance level of $p<0.05$.

RESULTS AND DISCUSSION

Effects on sperm motility and velocity characteristics

The characteristics of motility and velocity of post-thawed spermatozoa after evaluation with CASA were presented in Table 1. The analysis results indicated that both total motility and progressive motility of sperms were significantly higher in the group of semen extender supplemented with trehalose ($p<0.05$) when compared with those in the groups of semen extenders supplemented with glucose or fructose after thawing and incubating for 3 h. There was no significant difference in any of the velocity characteristics (VAP, VSL, and VCL) of post-thawed spermatozoa at 0 h between the groups of glucose and fructose, but those characteristics of spermatozoa from glucose and fructose groups were still significantly higher than those in the group of trehalose ($p<0.05$). In contrast, when considered in other times after incubation (1, 2, and 3 h after thawing), VAP, VSL, and VCL of spermatozoa in trehalose-added extender were significantly higher than those in glucose and fructose-added extenders ($p<0.05$).

Effects on viability, hypo-osmotic swelling test, and normal morphology of goat spermatozoa

Sperm qualities in terms of viability, membrane integrity, and normal spermatozoa were presented in Table 2. After thawing and incubation, the percentages of viability and membrane integrity of sperms were significantly higher in semen extenders supplemented with trehalose than those in extenders supplemented with glucose and fructose at 0, 1, 2, and 3 h after incubation ($p<0.05$). However, there was no significant difference in percentage of normal spermatozoa from semen extenders containing glucose and fructose at 0, 1, 2, and 3 h after incubation. The percentages of sperm normality from semen extenders which consisted of trehalose were significantly lower than those from extender which consisted of glucose and fructose ($p<0.05$) at 0, 1, 2, and 3 h after incubation. Spermatozoa from the cryopreservation process could be damaged during a rapid reduction in temperature. This activity changes the concentration of semen extender, structure, and biochemical substances of the spermatozoa (Medeiros et al. 2002). The occurrence of ice crystal

formation causes a change in the lipid bilayer and function of the permeable-membrane of the spermatozoa. This leads to a rapid influx of calcium ions and induces the capacitation reaction. Therefore, the manner of the fusion action between the plasma membrane and the acrosome membrane occurs and induces a change in sperm structure. In addition, the biochemical reactions are changed by releasing glutamic-oxaloacetic-transaminase (GOT), inhibiting hyaluronidase enzyme and acrosin, reducing the modulation of adenosine triphosphate and adenosine diphosphate synthesis, and decreasing acrosomal proteolytic activity (Purdy 2006, Watson 2000, Barbas and Mascarenhas 2009). As a result, sperms that have undergone the cryopreservation process have an increase in mortality, and a decrease of motility and viability which results in an unsuccessful fertilization when actually applied for cervical artificial insemination.

The results of the present study showed that sperm from extender containing trehalose as sugar component had significantly greater MOT, PMOT, viability and membrane integrity at 0 h and 1, 2, and 3 h after incubation (Table 1). Similar effects were found and reported in other species such as pigs (Malo et al. 2010, Fernández et al. 2012), goats (Aboagla and Terada 2003, Khalili et al. 2009), rams (Aisen et al. 2002), buffaloes (Shiva Shankar Reddy et al. 2010) and dogs (Yildiz et al. 2000, Yamashiro et al. 2007) which showed that sperms diluted with semen extender supplemented with trehalose had both better survival and motility compared with those from other sugar-added extenders or non-added trehalose extender.

Generally, trehalose, a non-penetrate sugar, has an important role in reducing cell damage from the freezing process. We have known that trehalose has been used as a cryoprotectant in the freeze-drying process to maintain organic substance (Crowe and Crowe 2000). The properties of trehalose give it the ability to protect the damage of sperm cells from the freezing process and trehalose presents the greater potential for sperm cell protection than other monosaccharides. This action creates osmotic pressure outside the cell during a reduction in temperature and induces cell dehydration which causes less intracellular ice crystal formation (Purdy 2006). In addition, trehalose has a specific interaction with the phospholipid membrane that being the binding between hydroxyl group of trehalose and the phospholipid polar head group with a hydrogen bond. This action aids in the binding of water onto the cell membrane (Aisen et al. 2002). Reorganization of the lipid and protein of a sperm cell enhances the sperm membrane fluidity before freezing and sperms develop a cryodamage tolerance prior to freezing (Aboagla and Terada 2003).

The results of this study showed that glucose and fructose added to semen extenders increased VAP, VSL and VCL at post-thawing (Table 1). This can be explained by the fact that a monosaccharide supports the characteristics of sperm movement in cool storage and cryopreservation (Ponglowhaphan et al. 2004, Naing et al. 2010). After incubation in a water bath for 1, 2 and 3 h, the results showed that sperms from the trehalose group had better movement characteristics of MOT and PMOT than those from the glucose and fructose groups (Table 1). This is probably due to the effect of cryodamage and the production of reactive oxygen species (ROS) from dead sperm which induces a decrease in VAP, VSL and VCL of sperms from frozen semen which consisted of extenders supplemented with glucose and fructose after incubation in a water bath (Brinsko et al. 2003).

In the present study, the percentage of normal spermatozoa was impacted after thawing and incubation. It was found that trehalose as a disaccharide added to a semen extender more affected normal morphology of the spermatozoa compared with glucose and fructose added to semen extenders (Table 2). Although the present result is contradictory with other report which showed that sugar types have no significant difference in spermatozoa morphology of Boar goat (Naing et al. 2010), this consequence is possible to occur due to the extenders supplemented with disaccharide which have more osmotic pressure effects compared with the extenders supplemented with monosaccharide and possibly cause cell dehydration. The acceptable criterion for normal morphology of spermatozoa after-thawing (0 h) in all three sugar types was >80% (Table 2) with the reason that

the acceptable level is still able to be used for AI when compared with the previous study (Dorado et al. 2007).

Table 1. Effects of different sugar types in semen extenders on total motility (MOT), progressive motility (PMOT), average path-velocity (VAP), straight-line velocity (VSL) and curve-line velocity (VCL) of frozen-thawed goat sperms after incubating for 0, 1, 2 and 3 h.

	Sugar types in semen extenders		
Parameters	**Glucose**	**Fructose**	**Trehalose**
0 h			
MOT (%)	33.00 ± 5.87^{b}	31.2 ± 3.37^{b}	41.80 ± 0.97^{a}
PMOT (%)	13.20 ± 1.77^{b}	11.0 ± 0.89^{b}	17.00 ± 1.34^{a}
VAP (µm/s)	62.84 ± 1.89^{a}	62.54 ± 1.23^{a}	59.18 ± 0.90^{b}
VSL (µm/s)	52.36 ± 0.90^{a}	51.00 ± 1.08^{a}	47.78 ± 0.83^{b}
VCL (µm/s)	108.48 ± 2.74^{a}	105.92 ± 2.08^{a}	99.92 ± 1.04^{b}
1 h after incubation			
MOT (%)	29.60 ± 1.21^{b}	24.20 ± 1.46^{b}	39.20 ± 0.86^{a}
PMOT (%)	10.20 ± 0.86^{b}	9.80 ± 1.50^{b}	14.00 ± 0.32^{a}
VAP (µm/s)	53.68 ± 1.02^{b}	52.12 ± 0.32^{b}	58.36 ± 0.76^{a}
VSL (µm/s)	41.88 ± 0.78^{b}	41.04 ± 0.68^{b}	46.16 ± 1.18^{a}
VCL (µm/s)	91.56 ± 0.87^{b}	89.72 ± 0.59^{b}	97.72 ± 1.18^{a}
2 h after incubation			
MOT (%)	23.00 ± 2.55^{b}	19.6 ± 3.14^{b}	35.80 ± 2.87^{a}
PMOT (%)	4.20 ± 1.24^{b}	3.40 ± 0.68^{b}	11.40 ± 0.81^{a}
VAP (µm/s)	49.68 ± 1.04^{b}	45.28 ± 1.73^{b}	55.34 ± 2.52^{a}
VSL (µm/s)	36.36 ± 1.83^{b}	35.60 ± 1.35^{b}	45.22 ± 2.85^{a}
VCL (µm/s)	82.54 ± 1.68^{b}	81.00 ± 2.28^{b}	96.86 ± 5.24^{a}
3 h after incubation			
MOT (%)	15.80 ± 1.24^{b}	12.8 ± 0.73^{b}	31.40 ± 2.38^{a}
PMOT (%)	2.00 ± 1.12^{b}	1.6 ± 2.44^{b}	8.60 ± 1.03^{a}
VAP (µm/s)	45.74 ± 1.84^{b}	43.80 ± 2.32^{b}	50.60 ± 0.43^{a}
VSL (µm/s)	35.36 ± 1.83^{b}	34.58 ± 2.97^{b}	39.18 ± 0.48^{a}
VCL (µm/s)	78.84 ± 2.41^{b}	78.60 ± 2.86^{b}	86.54 ± 0.77^{a}

Mean ± SEM (Standard error of mean).
^{a,b} different alphabetical superscripts within the same row represent a significant difference ($p < 0.05$).

Table 2. Effects of different sugar types in semen extenders on viability, hypo-osmotic swelling test (HOST) and normal morphology of frozen-thawed goat sperms after incubating for 0, 1, 2, and 3 h.

	Sugar types in semen extenders		
Parameters	**Glucose**	**Fructose**	**Trehalose**
0 h			
Viability (%)	36.40 ± 2.40[b]	34.60 ± 1.65[b]	44.40 ± 1.36[a]
HOST (%)	38.30 ± 1.23[b]	29.70 ± 1.62[b]	48.30 ± 5.72[a]
Normality (%)	88.00 ± 0.44[a]	86.60 ± 1.07[a]	81.80 ± 1.85[b]
1 h after incubation			
Viability (%)	31.40 ± 2.18[b]	33.10 ± 1.10[b]	43.00 ± 1.10[a]
HOST (%)	28.50 ± 2.03[b]	25.70 ± 1.98[b]	39.60 ± 0.91[a]
Normality (%)	84.60 ± 1.28[a]	82.60 ± 1.22[a]	76.60 ± 0.50[b]
2 h after incubation			
Viability (%)	25.90 ± 0.56[b]	20.80 ± 3.10[b]	37.80 ± 1.59[a]
HOST (%)	21.20 ± 0.96[b]	17.80 ± 1.65[b]	30.80 ± 2.44[a]
Normality (%)	78.60 ± 1.56[a]	83.00 ± 1.07[a]	72.80 ± 1.28[b]
3 h after incubation			
Viability (%)	17.00 ± 0.76[b]	17.20 ± 0.80[b]	27.20 ± 1.39[a]
HOST (%)	20.20 ± 1.53[b]	16.30 ± 1.16[b]	24.40 ± 0.94[a]
Normality (%)	76.00 ± 1.65[a]	76.80 ± 0.80[a]	67.20 ± 2.03[b]

Mean ± SEM (Standard error of mean).
[a,b] different alphabetical superscripts within the same row represent a significant difference ($p < 0.05$).

Effect on morphology of goat sperm

For the present study, the results of sperm morphology were presented in terms of normal morphology, abnormal head, and abnormal tail (Table 3). The significantly higher ratio of normal morphology were observed in sperms from semen extenders supplemented with glucose and fructose compared with those from semen extender supplemented with trehalose at 0, 1, 2, and 3 h after incubation ($p<0.05$). The percentages of abnormal head of sperm in semen extender which consisted of trehalose were significantly higher than those in semen extenders which consisted of glucose and fructose ($p<0.05$). There were no significant differences in the ratio of abnormal tail of sperms among sugar-added extenders.

Table 3. Effects of different sugar types in semen extenders on sperm morphology of frozen-thawed goat sperms after incubation for 0, 1, 2, and 3 h.

Parameters	**Sugar types in semen extenders**		
(%)	**Glucose**	**Fructose**	**Trehalose**
0 h			
Normal morphology	88.00[a]	86.60[a]	81.80[b]
Abnormal head	9.60[b]	12.40[ab]	15.00[a]
Abnormal tail	2.40	1.00	3.20
1 h after incubation			
Normal morphology	84.60[a]	82.60[a]	76.60[b]
Abnormal head	14.20[b]	15.60[b]	21.20[a]
Abnormal tail	1.20	1.80	2.20
2 h after incubation			
Normal morphology	78.60[a]	83.00[a]	72.80[b]
Abnormal head	17.00[b]	13.40[b]	24.20[a]
Abnormal tail	4.40	3.60	3.00
3 h after incubation			
Normal morphology	76.00[a]	76.80[a]	67.20[b]
Abnormal head	19.60[b]	19.20[b]	30.20[a]
Abnormal tail	4.40	4.00	2.60

[a,b] different alphabetical superscripts within the same row represent a significant difference ($p < 0.05$).

CONCLUSIONS

In the process of cryopreservation, the addition of trehalose to semen extender could affect spermatozoa performance in the manner of motility and viability. Sperms from frozen semen in Tris-base extender supplemented with trehalose showed better total motility, progressive motility, viability and membrane integrity of sperms compared with those in extenders supplemented with glucose and fructose at post-thawing and incubated at 37°C for up to 3 h. It is suggested that trehalose can be potentially used as a cryoprotectant in semen extender to preserve the optimal quality of motility, viability, and membrane integrity of goat sperms.

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