Cryopreservation of Boar Semen by Egg Yolk-Based Extenders Containing Lactose or Fructose is Better Than Sorbitol

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ABSTRACT. The present study determined the effect of different types of sugars (lactose, fructose, glucose and sorbitol) used in egg yolk-based extender on the post-thawed boar semen quality. Twenty-two ejaculates from 6 fertility-proven Yorkshire boars were cryopreserved by liquid nitrogen vapor method. Sperm motility, viability, acrosome integrity and intact functional plasma membrane were determined at 0, 2 and 4 hr after thawing. It was found that the lactose-based extender resulted in a higher percentage of post-thawed sperm motility, viability, intact acrosome and functional plasma membrane than sorbitol-based extender ($P<0.05$) and fructose-based extender yielded a higher post-thawed sperm motility and viability than sorbitol-based extender ($P<0.05$). It could be concluded that sorbitol was not an effective sugar for the cryopreservation in boar semen.

KEY WORDS: energy, sperm freezing, sugar, swine.

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Boar semen cryopreservation greatly facilitates the distribution of valuable and desirable genes because the frozen semen can be transported over a long distance; and a stock of frozen semen enables the use of boar that is dead or ill [3]. However, the use of frozen-thawed (FT) boar semen is limited due to the low fertility outcomes compared to the extended fresh semen [9]. During cryopreservation, both physical and chemical factors including the rapid change in temperature or thermal stress, oxidative stress and osmotic stress lead to the damage of the sperm plasma membrane [19]. In general, the egg yolk-based freezing extender of boar semen consists of cryoprotectant, sugar, buffer and antibiotic [16]. Glycerol is the most common cryoprotectant used in the egg yolk-based semen extender for cryopreservation of the boar semen [14] and egg yolk is regarded as a non-permeable cryoprotectant. Sugar is mainly an energy source of the boar sperm. Furthermore, it protects the boar sperm from dehydration and intracellular ice formation during the semen freezing process [27]. Different types of sugar, e.g., trehalose, lactose, fructose, have been used in the freezing extender of boar semen [15,18].

Recently, special interest on researches concerning with the energy utilization of the boar sperm have been noticed [13, 15, 18, 20]. In general, the boar sperm is able to utilize a variety of substrates to obtain energy. Generally, monosaccharides are more common energy source used in extended fresh boar semen than disaccharides [20]. However, lactose (disaccharide) is commonly used for the cryo-

preservation of boar semen by many researchers [8, 9, 16, 17]. In our previous studies [10, 17] lactose was used for sperm cryopreservation, while other monosaccharides such as glucose, fructose and sorbitol have never been examined. The effect of type of sugar supplementation in the freezing extenders on the post-thawed semen quality has been reported in the dog [29], buck [22], bull [28], ram [1] and stallion [24]. The stallion's cryopreserved sperms utilize sorbitol in the semen extender better than fructose and glucose [24]. In the boar, information associated with the effect of sugar types used in egg yolk-based semen extender on the FT semen qualities is limited. Therefore, the objective of the present study was to determine the effect of different sugar types (lactose, fructose, glucose and sorbitol) used in egg yolk-based semen extender on the sperm motility, sperm viability, acrosome integrity and functional plasma membrane integrity of the cryopreserved boar semen.

Six fertility-proven Yorkshire boar age between 1–3 years old used for routine artificial insemination (AI) in a commercial herd were used in the experiment. The boars were raised in individual pens in an evaporative cooling house. Water was *ad libitum* provided via water nipples. Feed were given approximately 3 kg/day/head (twice a day) with a rice-corn-soybean-fishmeal base containing 15% crude protein. In total, 22 ejaculates of semen were collected using the glove-handed method. Only sperm-rich fraction was collected and diluted (1:1 v/v) with a semen extender (Modena™, Swine Genetics International, Ltd., Iowa, U.S.A.) before transportation. Semen volume, pH, sperm concentration, sperm motility, viability and sperm morphology were evaluated. Only ejaculate with $\geq 70\%$ sperm motility and $\geq 80\%$ normal morphology was used for cryopreservation.

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After transportation at 15°C for 120 min, diluted semen was centrifuged at $800 \times g$ for 10 min; and supernatant was discarded. The sperm pellet was resuspended with freezing medium I [80% v/v sugar solution (321 mM in water) and 20% v/v egg yolk] to a concentration of 1.5×10^9 sperm/ml. At this step, the semen was divided into 4 groups according to different sugar compositions of the freezing medium, i.e., lactose, fructose, glucose and sorbitol. The osmolality of these solutions were measured using micro-osmometer (The FISKE® Micro-Osmometer model 210, Norwood, Massachusetts, U.S.A.) and adjusted to 350 ± 10 mOsm/kg. After cooling at 5 °C for 90 min, two parts of the semen were diluted with one part of freezing medium II (89.5% of freezing medium I, 9% glycerol and 1.5% Equex-STM®, Nova chemical sales Inc, MA, U.S.A.) to the final concentration of 1.0×10^9 sperm/ml. The processed semen was loaded into 0.5 ml straws (Bio-Vet, Z.I. Le Berdoulet, France) and sealed with PVC powder. The straws were frozen by placing in contact with nitrogen vapour about 3 cm above the liquid nitrogen level for 20 min in an styrofoam box; and were plunged into liquid nitrogen (−196°C) for storage. Two weeks later, thawing was done at 50°C for 12 sec [26]. Immediately after thawing, the semen was diluted (1:4) with a Modena™ extender. Post-thawed sperm quality was evaluated after incubation in a 37°C waterbath at 0, 2 and 4 hr after thawing.

Sperm concentration was assessed by direct cell count using a Bürker haemocytometer (Boeco, Humburg, Germany) [4]. The individual motility of both fresh and FT sperm were evaluated at 37°C under a phase contrast microscope at $200 \times$ and $400 \times$ magnification. The percentage of sperm viability was determined by SYBR-14/Ethidiumhomodimer-1 (EthD-1) (Fertilight®, Sperm Viability Kit, Molecular Probes Europe, Leiden, The Netherlands). This technique was standardized in our previous study [10]. Briefly, 10 μ l of diluted semen were mixed with 2.7 μ l of the working solution of SYBR-14 (1:100 v/v in dimethyl sulfoxide) and 10 μ l of EthD-1. After incubation at 37°C for 20 min, 200 sperm were assessed ($\times 1,000$) under fluorescent microscope. Sperm were classified into two types; live sperm stained green with SYBR-14 and dead sperm stained red with EthD-1. Acrosome integrity was assessed using fluorescein isothiocyanate-labeled peanut (*Arachis hypogaea*) agglutinin (FITC-PNA) staining. Ten μ l of the diluted semen were mixed with 10 μ l of EthD-1; and were

incubated at 37°C for 15 min. Five μ l of the mixture was smeared on a glass slide and fixed with 95% ethanol for 30 sec. Fifty μ l of FITC-PNA (dilute FITC-PNA with PBS 1:10 v/v) was spreaded over the slide and incubated in a moist chamber at 4°C for 30 min. After incubation, the specimen was rinsed with cold PBS and air dried. In total, 200 sperm were assessed under fluorescent microscope at $1,000 \times$ magnification and classified as intact acrosome, reacted acrosome and loose acrosome [2]. The functional integrity of the sperm plasma membrane was assessed using a short hypo-osmotic swelling test (sHOST) [23]. Sperm were incubated, at 38°C for 30 min, with 75 mOsm/kg a hypo-osmotic solution [0.368% (w/v) Na-citrate, 0.675% (w/v) fructose (Merck, Darmstadt, Germany)] and, then, were fixed in hypo-osmotic solution plus 5% formaldehyde (Merck, Darmstadt, Germany), for later assessment. In total, 200 sperm were assessed under a phase contrast microscope at $400 \times$ magnification. The coil-tailed (sHOST positive-) sperm found following incubation were functional intact plasma membrane.

The statistical analysis was performed using the Statistical Analysis Systems version 9.0 (SAS Institute Inc., 1996, Cary, N.C., U.S.A.). Data were presented as mean $\pm$ SEM. Normality of the data was evaluated using the UNIVARI-ATE procedure option NORMAL PLOT. Sperm motility, viability, acrosome integrity (arcsine transformation) were analyzed by using General Linear Mixed Model (MIXED) procedure of the SAS. The model included sugar types and times after thawing as a fixed effect; and included boar and number of ejaculation nested within boar and time as random effects. Least-squares means were obtained from each class of the factors; and were compared by using least significant different test (LSD) with Tukey-Kramer adjustment for multiple comparisons. Statistically significant difference was defined as $P < 0.05$.

On average, volume, concentration, individual motility, viability and plasma membrane integrity of fresh boar semen were 130.8 ± 6.4 ml, $504.9 \pm 12.7 \times 10^6$ sperm/ml, $84.4 \pm 0.9\%$, $81.4 \pm 0.9\%$ and $73.4 \pm 1.2\%$, respectively. For the FT boar sperm, it was found that lactose- and fructose-based freezing extenders yielded a significantly higher post-thawed sperm motility ($P < 0.05$) than the sorbitol-based freezing extender (Table 1). Individual motility of the FT sperm in the glucose-based extenders did not differ significantly from other extenders at any time (Fig. 1). Sperm

Table 1. Means $\pm$ SEM of individual motility (%), sperm viability (%), acrosome integrity (%) and sHost (%) of frozen-thawed boar sperm among 4 different types of sugar (22 ejaculates per group) at 0 hr after thawing

Sperm parameters	Frozen-thawed sperm			
	Lactose	Fructose	Glucose	Sorbitol
Individual motility (%)	32.5 ± 2.2^a	31.6 ± 3.4^a	$27.5 \pm 2.8^{a,b}$	19.8 ± 2.5^b
Sperm viability (%)	38.1 ± 9.8^a	38.8 ± 2.6^a	$34.8 \pm 2.4^{a,b}$	27.4 ± 2.3^b
Intact acrosome (%)	46.1 ± 2.2^a	$42.3 \pm 2.8^{a,b}$	$41.4 \pm 2.2^{a,b}$	36.3 ± 2.5^b
sHost (%) ¹	21.8 ± 1.4^a	20.5 ± 2.8^a	17.7 ± 2.1^a	11.4 ± 1.3^b

¹ sHost=functional integrity of sperm plasma membrane. a,b) Values with different letters within the same row were significantly different ($P < 0.01$).

Fig. 1. Means $\pm$ SEM of individual motility, sperm viability, intact acrosome of frozen-thawed boar sperm with 4 types of sugar, i.e., lactose, fructose, glucose and sorbitol after thawing at 0, 2 and 4 hr (22 ejaculates per group); a,b values with different letters within the same line are significantly different ($P<0.05$).

viability in the fructose- and lactose-based extenders were higher than sorbitol-based freezing extender ($P<0.05$). Sperm viability in the glucose-based freezing extender did not differ significantly from other groups (Table 1).

At 2 and 4 hr after thawing, no significant difference on the sperm viability was observed among the treatment groups (Fig. 1). The percentage of sperm with intact acrosome was higher in the fructose- than the sorbitol-based freezing extender both at 0 hr and 2 hr after thawing ($P<0.05$). Likewise, the lactose-based freezing extender had a higher intact acrosome than that in sorbitol-based freezing extender at 0 hr, 2 hr and 4 hr ($P<0.01$) (Fig. 1). Intact acrosome of the FT sperm did not differ significantly among lactose-, fructose- and glucose-based extenders at any time after thawing ($P>0.05$) (Fig. 1). The functional

integrity of the sperm plasma membrane (sHost) in the sorbitol-based freezing extender was significantly lower than lactose-, fructose- and glucose-based freezing extenders (Table 1). The functional integrity of the sperm plasma membrane did not differ significantly among lactose-, fructose- and glucose-based extenders ($P>0.05$) (Table 1).

In general, sugar acted as a non-permeable cryoprotectant and minimized osmotic stress during freezing and thawing processes [15]. The present study demonstrated that different sugars used in the freezing extender significantly influenced the post-thawed boar semen quality. Studies on the effect of sugar types used in the boar semen freezing extender have been limited; and the results have still been controversial [12, 18]. In the present study, sorbitol was less effective than lactose and fructose for protecting sperm during cryopreservation. The exact mechanisms for this have not been fully revealed. However, the boar sperm utilized glucose, fructose and sorbitol through glycolysis in different ways; resulting in a different rates of cell phosphorylation which was closely related to extracellular L-lactate formation. Glucose was the most lactatogenic substance, while sorbitol induced the lowest lactatogenic rate [20]. These findings indicated that the energy utilization of boar sperm involved with several complex pathways. Besides, it has been clearly demonstrated that different sugars involved with different pathways of the energy utilization [20]. Furthermore, disaccharides may lead to the formation of a hydrogen bond between hydroxyl groups of sugars. Membrane phospholipid, in turn, stabilized membrane lipid bilayer and maintained sperm membrane integrity [25]. In addition, the effect of sugar concentration used in the semen extender was reported [11]. Corcuera *et al.* [11] found that the use of 12% lactose-based extender (osmolality 360 mOsm/kg) resulted in a higher post-thawed sperm motility and normal apical ridge than the use of 11% and 14% lactose extenders. These studies revealed that both type and concentration of sugar used in the extender were important to the cryopreservation of boar semen.

Sorbitol is an alcoholic sugar which can penetrate sperm plasma membrane [7]. In the present study, sorbitol was not effective for boar sperm cryopreservation. Molinia *et al.* [21] found that the supplementation of sorbitol improved the post-thawed motility of ram sperm in extender without glycerol, but impaired the post-thawed sperm motility in extender containing glycerol. Recently, Pojprasath *et al.* [24] demonstrated that the addition of sorbitol in freezing extender improved the post-thawed motility and viability of stallion sperm. However, the mechanism by which sorbitol could protect the ram and stallion sperm during cryopreservation was unknown. However, in fresh semen, it was found that the sperm metabolism, in relation to extracellular substrates, e.g., fructose, glucose, mannose, sorbitol and acetaldehyde, differ among species [5]. Furthermore, it was demonstrated that a highly active dehydrogenase could be obtained from the ram's, but not from the boar's sperm [6]. These findings indicated that the boar sperm may lack of some enzymes and/or coenzymes that were important to the

sorbitol metabolism. Thus, they could not utilize sorbitol like in the ram's and the stallion's sperm.

In summary, the different types of sugar supplemented into the freezing extenders had different capabilities of protecting sperm during cryopreservation. This suggested that either lactose or fructose was recommended to be used for boar semen cryopreservation. However, sorbitol was not an effective sugar for the cryopreservation of boar semen.

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