

Full Length Research Paper

The cryoprotective effects of soybean lecithin on boar spermatozoa quality

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Soybean lecithin has been attracted increasing attention and has been used to replace egg yolk in the cryopreservation of domestic animal semen. However, its effects on freezing boar spermatozoa have never been evaluated. In the present study, semen was collected from five Duroc boars and frozen-thawed in extender with different concentrations of soybean lecithin (3, 6, 9 and 12%) and 20% egg yolk. Semen parameters including sperm motion characteristics (the percentage of total motile sperm and motility), plasma membrane integrity and acrosome integrity were assessed with a computer-aided semen analysis (CASA) system, hypoosmotic swelling test (HOST) and fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) method, respectively. The result best result was obtained for the extender supplemented with 6% soybean lecithin, with values of 59.7% for the percentage of total motile sperm (TM%), 44.3% for motility, 45.3% for plasma membrane integrity and 61.9% for acrosome integrity. TM%, motility, acrosome integrity and plasma membrane integrity in the extender containing 6% soybean lecithin were significantly higher than that of other concentrations of soybean lecithin and 20% egg yolk ($P < 0.05$). However, the percentages of TM, acrosome integrity and plasma membrane integrity decreased with the increasing concentration of soybean lecithin in extender. In summary, the effect of soybean lecithin on spermatozoa quality was superior and the effective concentration of soybean lecithin in extender was 6% (w/v). Soybean lecithin might replace egg yolk in extender in the cryopreservation of boar semen.

Key words: Boar semen, cryopreservation, soybean lecithin, motility, acrosome integrity.

INTRODUCTION

Freezing of boar spermatozoa has been studied since 1956. However, current methods and freezing extenders for the cryopreservation of boar spermatozoa are unsatisfactory. Artificial insemination (AI) using frozen-thawed semen in the swine industry has been limited due to the low fertility and litter size (Watson, 2000) compared to AI with liquid semen (Gerrits et al., 2005). Different to other domestic animals, boar sperm is extremely vulnerable to cold shock by sudden cooling and this is thought as one of the most important factors which limit the development of freezing of boar spermatozoa (Parks and Lynch, 1992).

Egg yolk has been used for providing protection against cold shock in the cryopreservation of mammalian semen for over half a century. Previous studies had confirmed that boar semen could acquire a resistance to cold shock with the addition of 20% egg yolk (Westendorf et al., 1975; Johnson et al., 2000). In the recent years, low density lipoprotein (LDL) extracted from egg yolk has been found to have better effects on protecting boar spermatozoa against cold shock compared with egg yolk (Jiang et al., 2007). However, some researchers considered that those animal-originated ingredients might introduce risks of microbial contamination during AI-procedure of domestic animals (Bousseau et al., 1998). As one of phospholipids, lecithin (or phosphatidylcholine) is distributed widely in plants and it plays an important role in the regulation of the physiological function of animal cells bio-membrane (Voet and Voet, 1995). Soy-

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bean lecithin from soybean has similar ingredients to egg yolk used for protection of animal spermatozoa from cold shock in semen cryopreservation (Thun et al., 2002; Aires et al., 2003). It has been suggested that soybean lecithin may play a better protective role for spermatozoa than that of egg yolk or other materials during the freeze-thawing process and therefore reduce the risk of introducing bacterial and mycoplasma into freezing extenders (Fukui et al., 2008). Soybean lecithin is also used as an excellent oil-in-water emulsifier in confectionery industry (Trotta et al., 2002).

However, to date, there is no report on the effects of soybean lecithin supplementation as a cryoprotectant in the cryopreservation of boar semen. The objective of the present study was to investigate the effects of soybean lecithin in different concentrations (3, 6, 9 and 12%) on sperm motion characteristics including percentage of total motile sperm, linear motile sperm and plasma membrane integrity and acrosome integrity compared with 20% egg yolk on boar sperm cryopreservation.

MATERIALS AND METHODS

Chemical reagents

Soybean lecithin (Unicorn Co.Ltd., Beijing, China) was further purified (Wu and Wang, 2004). In brief, after 1 h of soybean lecithin-ethanol mixing, the mixture was centrifuged (950 × g) and separated into two phases, soybean lecithin was obtained by drying the upper phase. Other reagents used were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Semen collection

Semen was collected by the glove-hand technique from five Duroc boars with proven fertility (2 to 4-yr-old). Immediately after collection, filtered sperm-rich fractions were pooled and transferred into an insulated vacuum bottle. Only the sperm-rich fractions with more than 70% motile and 80% morphologically normal sperm were used. A total of 40 ejaculates (8 ejaculates every boar) were used.

Experimental design

Extenders with different concentrations of soybean lecithin and 0.05% Tween-80 (Sigma Aldrich) were treated with an ultrasonic cleaning machine for 3 h at 50°C. In this study, the control group was egg yolk extender (20%, v/v).

Extender preparation

BTS solution (glucose 0.027 M, EDTA 0.004 M, sodium citrate 0.023 M, sodium bicarbonate 0.014 M and potassium chloride 0.010 M in 1 l distilled water filtered through a 0.22 µm- filter) (Pursel and Johnson, 1975) and Tris-citric acid-glucose extender (Ritar and Salamon, 1982) (TCG, Tris 0.199 M, citric acid 0.077 M, glucose 0.061 M, 0.25% gentamicin and penicillin 500, 000 IU in 1 l distilled water filtering through a 0.22 µm- filter) were used in the present study. The cooling extender contained TCG (80%, v/v) and egg yolk (20%, v/v) or TCG extender added different concentration of soybean lecithin, each supernatant was obtained after centrifugation (5000 × g, 10 min). The freezing extender composed of the

cooling extenders (91%, v/v) and glycerol (9%, v/v).

Semen freezing and thawing processes

The semen was divided into 15 ml graduated tubes, held for 30 min at room temperature (RT) and subsequently centrifuged at 800 g for 10 min. The sperm pellet was resuspended in pre-warmed BTS to 15 ml and then cooled slowly from RT to 17°C within 4 h, subsequently centrifuged at 800 g for 10 min at 17°C and the supernatant was removed.

The concentrated semen was diluted with the cooling extender to a final concentration of 1.5×10^9 spermatozoa/ml. The diluted semen was gently mixed and slowly cooled to 5°C for 3 h. The semen samples were further diluted (2:1, v/v) with the freezing extender. The final spermatozoa and glycerol concentrations were 1×10^9 spermatozoa/ml and 3%, respectively. The 0.25 ml straws (IMV, Orsay, France) were immediately filled and sealed manually by sealing the ends of the straws with warming hemostat. The freezing programmer was as following: from + 5 °C to - 5°C with 1 °C/min-change by a programmable freezing device (Mini Digitcool 1400, IMV, France). Then the straws (ten straws every treatment) were placed on an aluminum rack above liquid nitrogen (LN). The straws were situated 3 cm above the LN and kept this level for 3 min before being immersed in the LN (- 196°C) for storage.

Thawing of semen was performed at 37°C for 30s after storage for 2 days. All samples were diluted with BTS (1:1, v/v) for assessment of sperm parameters.

Sperm quality evaluation

Motion parameters evaluations

Thawed semen was held in a water bath at 38°C for 20 min before analysis; the sperm concentration used for motility analysis was approximately 50×10^6 /ml. Sperm motion characteristics were evaluated with a computer-aided sperm analysis (CASA) system (Semen Analysis Windows XP version; Zoneking Softronics Co., Ltd., Beijing, China). The parameters of the CASA program were: 10 µm/s as a velocity limit for immobile objects, 25 µm/s as a velocity limit for local motile objects and 25 µm for the maximum radius of circles. Sperm deviating less than 10% from a straight line were designated linearly motile.

After incubation and mixture, 10 µl of semen was placed into a Makler counting chamber (10 µm deep). The sample was observed at least eight predetermined fields (sequences) were imaged and the analysis was carried out with a digital video recording. Motion parameters including proportions of total motile (TM) and motility were determined.

Assessment of acrosome integrity

Acrosome integrity was evaluated by the fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA, L7381, Sigma-Aldrich) method (Zeng et al., 2001). Briefly, after thawing, semen was transferred into a plastic tube containing 2 ml 3% polyvinylpyrrolidone (PVP, 3 g PVP in 100 ml PBS) and centrifuged at 800 × g for 3 min at RT and the supernatant solution was poured off. The sediment was diluted with PBS solution (37°C) to obtain $1 - 2 \times 10^6$ spermatozoa/ml. Aliquots (30 µl) of semen were used to prepare smears on microscope slides. Sperm smears were fixed with absolute methanol for 10 min at RT after air drying. 30 µl FITC-PNA solution (100 µg/ml) in PBS was spread over each slide. The slides were then incubated in a dark and moist chamber for 30 min at 37°C. After incubation, the slides were then rinsed with PBS, air dried and then mounted with 10 µl of antifade solution to preserve

Table 1. Effects of soybean lecithin on motion parameter, plasma membrane integrity and acrosome integrity of frozen-thawed boar sperm (mean \pm S.D.).

Item	EY 20%	Soybean lecithin			
		3%	6%	9%	12%
TM (%)	51.9 \pm 1.34 ^a	54.9 \pm 0.44 ^a	59.7 \pm 5.80 ^b	53.7 \pm 0.78 ^a	51.0 \pm 1.73 ^a
Motility (%)	40.6 \pm 0.82 ^{be}	42.3 \pm 0.82 ^c	44.3 \pm 1.21 ^d	42.1 \pm 0.75 ^{bc}	39.8 \pm 0.75 ^e
PI (%)	36.0 \pm 1.01 ^{be}	39.0 \pm 1.32 ^c	45.3 \pm 1.06 ^d	37.9 \pm 1.22 ^{bc}	34.2 \pm 2.08 ^e
AI (%)	37.7 \pm 0.77 ^b	55.9 \pm 1.32 ^c	61.9 \pm 1.27 ^d	54.5 \pm 2.95 ^c	37.2 \pm 1.52 ^b

Within a row, values without a common superscript (a - e) differ ($P < 0.05$).

TM: total motile sperm; PI: plasma membrane integrity; AI: acrosome integrity.

fluorescence. A coverslip was then applied and the edges were sealed with colorless nail polish.

The acrosome status of the spermatozoa was examined and photographed using a fluorescence microscope (Nikon TE2000, Nikon Optical Co. Ltd., Tokyo, Japan). All treatments were replicated 6 times with pooled ejaculates from the five boars. The whole acrosome was visualized with strong green fluorescence under a fluorescence microscope and was scored as acrosome-intact sperm. The percentage of acrosome-intact spermatozoa was counted in at least 300 spermatozoa per slide. Spermatozoa were observed according to the following patterns: 1) positive spermatozoa with a selective staining of the whole acrosome; 2) negative spermatozoa with no staining.

Assessment of plasma membrane integrity

Plasma membrane integrity was evaluated by the hypoosmotic swelling test method (HOST) (Pérez-Llano et al., 2003). In brief, the straws were thawed in a water bath at 37°C for 45 s. The assay was performed by mixing 50 μ l of the semen with 1 ml of hypoosmotic solution (7.35 g sodium citrate \cdot 2 \cdot H₂O and 13.51 g fructose in 1 l of distilled water). After incubation for 30 min at 37°C (García-López et al., 1996), sperm swelling was assessed by placing 15 μ l of well-mixed sample on a warm slide (37°C) which was covered with a cover glass before being observed under light microscopy at 400 \times magnification. Spermatozoa with intact plasma membrane had coiled tails after HOST. Three hundred spermatozoa per slide were observed. The spermatozoa were classified as positive or negative based on the presence or absence of coiled tail (García-López et al., 1996; Vazquez et al., 1997; Ollero et al., 1998).

Statistical analysis

The data were presented as means \pm S.D. data. A one-way analysis of variance (ANOVA) and the LSD multiple range test were used to compare mean values of individual treatments when the F-value was significant ($P < 0.05$). All analyses were performed using Statistical Product (SPSS 11.5 for Windows; SPSS, Chicago, USA).

RESULTS

Effects of different treatments on motion characteristics

The motion characteristics of frozen-thawed boar sperm were evaluated by CASA in present study (Table 1).

When the concentrations of soybean lecithin was 6% (w/v), the percentages of TM and sperm motility were higher than those of sperm in 20% egg yolk and other concentrations of soybean lecithin extenders ($P < 0.05$). In addition, the soybean lecithin extender appeared to have a lower viscosity compared to egg yolk extender. We also observed that the viscosity of soybean lecithin extender increased with its concentration.

Effects of different treatments on sperm acrosome integrity

The sperm acrosome integrity was evaluated by FITC-PNA method under fluorescence microscope. The percentage of sperm acrosome integrity of 6% soybean lecithin was higher than that of other concentrations of soybean lecithin and 20% egg yolk ($P < 0.05$, Table 1). However, the percentage of sperm acrosome integrity in soybean lecithin extenders decreased with the increasing concentration of soybean lecithin in extender.

Effects of different treatment on sperm plasma membrane integrity

In the present study, we used the hypoosmotic swelling test (HOST) as a predictor of plasma membrane for frozen-thawed boar sperm. Significant sperm plasma membrane integrity difference was observed for sperm plasma membrane integrity between 20% egg yolk extender and soybean lecithin. The sperm plasma membrane integrity in 6% soybean lecithin was better than that in other concentrations of soybean lecithin as well as 20% egg yolk (Table 1; $P < 0.05$). We also observed that the percentage of sperm plasma membrane integrity in soybean lecithin extenders decreased with the increasing concentration of soybean lecithin in extender ($P < 0.05$; Table 1).

DISCUSSION

The sperm motion characteristics, plasma membrane integrity and acrosome integrity were used as predictors

for quality of frozen-thawed boar spermatozoa. In the present study, freezing extender supplemented with 6% soybean lecithin could provide better cryoprotective action for boar spermatozoa during cryopreservation compared to others concentrations and the control group (20% egg yolk). This was similar to previous reports in the cryopreservation of sheep (Fukui et al., 2008) and bovine semen (Amirat et al., 2005; Gil et al., 2000).

Some researchers also reported that the cryoprotective effect of soybean lecithin extenders on freezing bovine sperm was similar or slightly inferior to that of 20% egg yolk extender (Thun et al., 2002; Aires et al., 2003). However, in our study, the effect of 6% soybean lecithin on the sperm motion characteristics, plasma membrane integrity and acrosome integrity was superior to 20% egg yolk. We inferred that the reasons were possibly as follows: firstly, egg yolk provided poor protection for boar spermatozoa during cryopreservation unlike for bovine sperm (Benson et al., 1967; Bathgate et al., 2006) so the protective effects of soybean lecithin on frozen boar sperm was better than those of egg yolk. Secondly, higher viscosity and the presence of particulate debris in extenders were thought as advantageous factors impair the sperm fertilizing ability (Vishwanath and Shannon, 2000; van Wagendonk-de Leeuw et al., 2000), so soybean lecithin extender could play a protective role for sperm during cryopreservation due to its low viscosity and less debris. Thirdly, optimum concentration of soybean lecithin might play a protective role for spermatozoa membrane during the freeze-thawing process. In addition, the spermatozoa might be able to swim more easily in freezing extenders containing soybean lecithin than in other extenders, which would lead to better sperm motion characteristics.

The precise mechanism by which soybean lecithin protects spermatozoa during the freeze-thawing process remains unclear. We inferred that it was similar to that of LDL. It was generally accepted that cold shock and cryo-damage might impair the physiological function of spermatozoa membrane due to the change of the lipid composition (such as saturated hydrocarbon, phospholipids, etc) of its bilayer and the fluidity of the plasma membrane during the freeze-thawing process (Johnson et al., 2000; Pettitt and Buhr, 1998; De Leeuw et al., 1990). To our knowledge, there are mainly two hypotheses to explain its protective mechanism in the cryopreservation of spermatozoa. As one of important compositions of bio-membrane of sperm of mammals, phospholipids were affirmed to play an important role in adjusting the physiological function of bio-membranes and entering the cell to decrease the freezing point of crystal by replacing plasmalogens to relieve mechanical damage for bio-membrane of spermatozoa (Graham and Foote, 1987; Waterhouse et al., 2006; Giraud et al., 2000). A few authors agreeing with this opinion believed that lecithin of egg yolk and soybean lecithin might reduce the cholesterol/ phospholipids ratio of sperm cell membranes by permeating into the sperm membrane, so capacitation-

like changes during the freezing process were restrained to improve the fertilizing ability of frozen-thawed spermatozoa (Gamzu et al., 1997; Davis, 1981). In addition, phospholipids might replace some phospholipids of the sperm membrane to maintain its structure and function (Graham and Foote, 1987; Trimeche et al., 1997). However, another hypothesis was also widely accepted by many researchers. They believed that phospholipids from egg yolk or soybean lecithin could not enter into sperm membrane to change the concentration of phospholipids in bio-membrane, but it might integrate with sperm membrane to form a protective film against the formation of lethal intracellular ice crystal and protect the sperm membrane from mechanical damage during the freeze-thawing process (Quinn et al., 1980; Simpson et al., 1987). We also observed that soybean lecithin micro-particles were relatively bigger than spermatozoa under microscope. Thereby, we speculated that it was almost impossible that soybean lecithin microparticles entered into sperm membrane and prevented sperm bio-membrane against cryodamage and we preferred to agree with the latter opinion.

In a way, soybean lecithin is also thought as a better emulsifier (Trotta et al., 2002; Rydhag and Wilton, 1981). It seems logical to hypothesize that soybean lecithin in freezing extender might promote cryoprotectants to distribute uniformly and reduce the its local concentration, which led to relieve the toxicity of cryoprotectants on boar spermatozoa during the freeze-thawing process. In addition, we inferred that soybean lecithin might maintain the ratio of lipid composition of the sperm membrane by interacting with detrimental particles of seminal plasma which is similar to LDL (Bergeron and Manjunath, 2006; Manjunath et al., 2007). However, these hypotheses should be further confirmed.

Our results showed that soybean lecithin-supplementation could lead to a greater cryoprotective capacity while freezing-thawing boar spermatozoa. It also greatly improved boar sperm motility, membrane integrity, acrosome integrity parameters. The effects of soybean lecithin on spermatozoa quality were superior and the optimum concentration in boar semen extender was determined to be 6% (w/v). We suggested that soybean lecithin might replace egg yolk in extender in the cryopreservation of boar semen and should be explored as a novel potential cryoprotectant.

To our knowledge, this study was the first study to evaluate the effects of soybean lecithin on freezing-thawing boar semen. Further research is required to obtain more information about soybean lecithin and to explain the protective mechanism during the freezing- thawing process.

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