The Cryoprotective Effect on Frozen-thawed Boar Semen of Egg Yolk Low Density Lipoproteins

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ABSTRACT : In order to protect the spermatozoa against cold shock, hen egg yolk is widely used as a cryoprotective agent in semen freezing extenders for domestic animals. The protective action of yolk is largely presumed to be due to low density lipoproteins (LDL). The effects of LDL on sperm quality of bull and northern pike (Esox lucius) after freezing-thawing have been reported, but no study has been made to evaluate the effect of LDL on boar sperm motility and other characteristics. The experiment was carried out to investigate the effect of LDL on the freezing of boar sperm in 0.25 ml straws. The aim was to evaluate the quality of boar spermatozoa cryopreserved in the presence of LDL. Motility of semen cryopreserved in LDL was analyzed and compared to semen cryopreserved with Tris-citric acid-glucose (TCG) and Tris-citric acid-fructose (TCF), two basic freezing extenders containing egg yolk. Similarly, acrosome and plasma membrane integrity were also evaluated and compared to semen cryopreserved with TCG and TCF. Analysis of sperm quality after freeze-thaw showed that the motility, acrosome and plasma membrane integrity were improved with LDL in the extender, as compared to the TCG and TCF. The highest post-thaw integrity of acrosome and plasma membrane and motility were obtained with 9% LDL (w/v). Consequently, the optimum LDL concentration in the extender was 9%. It is also suggested that the concentration of LDL addition is important for the effect on boar sperm protection during freezing and thawing. The percentage of motile spermatozoa was significantly higher after freezing in 9% LDL than in TCG and TCF 54.4% versus 30.4% and 30.1% ($p<0.05$), respectively. The integrity of acrosome and plasma membrane were also significantly higher at 70.3% and 50.5% respectively with semen frozen in 9% LDL extender compared to TCG at 37.8% and 30.3% and TCF at 36.4% and 29.9%, respectively ($p<0.05$),. In conclusion, we propose that extender containing LDL extracted from hen egg yolk could be used as a cryoprotective media with a better efficiency than TCG and TCF. LDL improved boar semen quality, allowing better spermatozoa motility, acrosome and plasma membrane integrity after the freeze-thaw process. Furthermore, we found out that the extender with 9% LDL concentration significantly enhanced motility, acrosome and plasma membrane integrity of boar sperm after freezing and thawing. ***(Asian-Aust. J. Anim. Sci. 2006. Vol 19, No. 4 : 486-494)***

Key Words : Boar Semen, Cryopreservation, Yolk LDL, Sperm Motility, Acrosome Integrity, Plasma Membrane Integrity

INTRODUCTION

Artificial insemination (AI) is an important tool for distribution of the genetic potential of males, the aim of sperm freezing is the production of a bank of sperm cells to be used for it. In cattle, AI is the reproductive biotechnology which has been widely used for genetic improvement, and most of the semen has been frozen. Overall conception rate to AI in cattle in different countries varies from 20 to 65 percent (Kumar et al., 2004). However, various biochemical and anatomical compartments in the spermatozoa cells may be altered during freeze-thaw process, and the current methods for cryopreservation of boar spermatozoa are unsatisfactory. They have poorer motility, acrosomal morphology and viability than fresh sperm (Zhou et al., 2004). The resulting of low fecundity rates (40-50%) and low litter size have made frozen boar semen impractical for the commercial swine producer (Gillmore et al., 1998; Cheon et al., 2002; Yi et al., 2004a, b). Consequently, the first goal of sperm-freezing protocols, including the use of extenders, the cooling and warming processes, is to prevent the formation of lethal intracellular ice crystal and to improve sperm motility, and to reduce acrosome and plasma membrane damage during and after cryopreservation.

The most popular diluents for boar sperm freezing were glucose and egg yolk (Polge et al., 1970); Tris, fructose, EDTA, citric acid, glucose and egg yolk (Visser and Salamon, 1974); Tris, glycine, citric acid, glucose and egg yolk (Obando et al., 1984). The use of hen egg yolk in domestic animals semen dilution was first reported for its beneficial effect for low-temperature storage of bull semen. Egg yolk provides an excellent protection for mammalian spermatozoa in resisting against cold shock during the freeze-thaw process (Bogart and Mayer, 1950) and is widely used in semen extenders.

Egg yolk was usually used at the concentration of 20%

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(v/v) in mammalian semen extenders. Laboratory studies revealed that this concentration make results difficult to standardize and interfered with biochemical assays and metabolic investigations. Whereas, it could be overcome by removing some components in the egg yolk by centrifugation (Polge et al., 1970). Furthermore, Pace et al. (1974), and Watson and Martin (1975) observed that some substances in yolk inhibit respiration of spermatozoa or diminish their motility, acrosome and plasma membrane integrity. Consequently, it could be of great benefit to remove them, and add only the cryoprotectant agent, after extraction, to the extender, rather than complete egg yolk. Because of the presence of some detrimental substances in yolk, there have been increasing demands to replace whole egg yolk by the cryoprotective fraction in semen extenders in recent years.

The precise mechanism by which egg yolk aids in the protection of spermatozoa during the freeze-thaw process is not clearly established. Many investigations attempt to find out which component in egg yolk provides sperm cells protection with the aim to prepare chemically defined extender. Pace et al. (1974) had purified egg yolk using ultracentrifugation and observed that the low-density fraction had a cryoprotective action. Numerous authors (Polge et al., 1970; Evans et al., 1973; Watson and Martin, 1975) then have proposed that low-density fraction of egg yolk, mainly composed of low density lipoproteins (LDL), could be largely responsible for the resistance against cold shock and for the improvement of sperm motility, acrosome and plasma membrane integrity after freezing and thawing process. Subsequently, Demianowicz and Strezek (1996) and Moussa et al. (2002) confirmed that LDL have a cryoprotective action in the egg yolk. Polge et al. (1970), and Graham and Foote (1987) suggested that LDL could adhere to cell membranes during the freeze-thaw process, thus preserving spermatozoa membranes. Moreover, Moussa et al. (2002) obtained better results in terms of motility and movement characteristics when replacing whole egg yolk by 8% (w/v) LDL in bull semen freezing.

Cryopreservation of spermatozoa offers an effective means for long-tern storage of important genetic material of domestic animals. This method can eliminate the difficulty associated with transporting animals or fresh semen over long distances or extended periods of time. However, AI and *in vitro* fertilization (IVF) demand higher quality spermatozoa. The ability of fertility of spermatozoa after cryopreservation is an important factor of high-pregnancy rates in mammalian after insemination, and it being associated with the quality of sperm after freeze-thaw process. Motility is a primary parameter of evaluation of sperm quality, but no correlation has been established between spermatozoa motility and fertility of mammalian (Andersson et al., 1992; Stalhammar et al., 1994). Consequently, the evaluation of only spermatozoa motility is an insufficient predictor of fertility in many species (Comizzoli et al., 2001; Roth et al., 1998). Parameters like AI and IVF, FITC-labeled peanut agglutinin (FITC-PNA), hypo-osmotic swelling test (HOST), and the integrity of acrosome and membrane could be associated to the motility results. Acrosome function is essential for the fertilizing ability of sperm no matter what it is fresh semen or frozen semen, because acrosomal enzymes allow them to reach the oocyte plasma membrane (Hammadeh et al., 2001). Damage of the acrosomes has been reported to be associated with a lower fertilizing capacity. Therefore, proper assessment of spermatozoa quality is of utmost importance for frozen semen of boar. Coomassie blue staining, Fluorescein isothiocyanate-peanut aggfutinin (FITC-PNA), hypoosmotic swelling test (HOST), SYBR-14 and propidium iodide (PI) staining have been applied for assessing the membrane integrity and acrosome status of the spermatozoa (Fazeli et al., 1997; Garcia-Lopez et al., 1996; Larson and Miller, 1999; Rodriguez-Martinez et al., 1997).

Until now, some studies have reported results of LDL effects on bull and northern pike (Esox lucius) semen motility, but no authors evaluated the boar spermatozoa quality after freezing in LDL. The objective of the present study was to obtain information about the cryoprotective effect of LDL in boar semen extender instead of egg yolk in the freezing in 0.25 ml straws. The experiments in freeze-thaw treatment of boar semen were performed. We assessed concentrations of LDL of 6, 7, 8, 9 and 10%, and compared boar spermatozoa quality after the freeze-thaw process with TCG and TCF extenders containing whole egg yolk or with that of extenders containing LDL. Semen motility was evaluated after cryopreservation in TCG and TCF, and compared to semen cryopreserved in LDL. Also, the integrity of spermatozoa acrosome and plasma membrane were checked by hypo-osmotic swelling test (HOST) and fluorescein isothiocyanate-peanut aggfutinin (FITC-PNA), respectively.

MATERIAL AND METHODS

LDL extraction

Fresh eggs were collected from one flock of hens which received the same standard diet. After the disinfection with 75% ethanol, eggs were manually broken and yolks were separated from the albumen. All egg yolks were carefully rolled on a filter paper to remove chalazae and traces of albumen adhering to the vitellin membrane. The vitellin membrane was then disrupted with a scalpel blade. Yolk was collected in a beaker which is cooled in iced water.

LDL were extracted from egg yolks according to the method described by Moussa et al. (2002). Egg yolk was

diluted twice or three times (w/w) with an isotonic saline solution (0.17M NaCl) (w/w) and stirred for 1h before centrifugation at 10,000×g for 45 min at 4°C. The supematant (plasma) was separated from the sediment (granules). To avoid contamination with granules completely, the plasma was centrifuged again. Plasma was then mixed with 40% ammonium sulfate to precipitate livetins. After 1 h of stirring in refrigeratory at 4°C, the mixture was centrifuged at 10,000×g for 45 min to separate a supernatant from sediment. The sediment was discarded and the supematant was then dialyzed about 12 h against distilled water in order to eliminate ammonium sulfate. After complete ammonium sulfate elimination, the solution was again centrifuged at 10,000×g for 45 min at 4°C and the floating residue, rich in LDL, was collected. The purity of LDL was 97%.

Semen collection

Semen was collected from sixteen mature Duroc boars once weekly by the glove-hand technique and filtered through 4 layers of sterile cotton gauze to remove the gel particles, and then transferred into a insulated vacuum bottle. The semen samples of the sperm-rich fractions were assessed for volume, sperm concentration and percentage of motile spermatozoa, and quality of movements. The sperm-rich fractions of ejaculates with greater than 80% motile sperm were used. Semen from each boar was processed independently and subjected to each treatment.

Extender preparation

The basic freezing diluent used in this study was TCG and TCF. They were also cryoprotective extenders of control and composed as follow: TCG (in g/100 ml sterile nonpyrogenic water): Tris 2.42, citric acid 1.48, glucose 1.10, and TCF (in g/100 ml sterile nonpyrogenic water): Tris 2.42, citric acid 1.48, fructose 1.10. The cooling extenders of TCG control were composed of 2.42 g Tris, 1.48 g citric acid, 1.10 g glucose, 25 mg gentamicine, 50,000 IU penicillin and 20 ml egg yolk for 100 ml sterile nonpyrogenic water. The cooling extenders of TCF control were composed of 2.42 g Tris, 1.48 g citric acid, 1.10 g fructose, 25 mg gentamicine, 50,000 IU penicillin and 20 ml egg yolk for 100ml sterile nonpyrogenic water. For the cooling extenders of treatments, that differs only with TCG by the replacement of 20% egg yolk by LDL. In order to determine the optimum LDL concentration, we used 6-10% (w/v) LDL concentrations: 6, 7, 8, 9 and 10%. The freezing extenders of control and treatment were composed of the cooling extender and 9% (v/v) glycerol, respectively.

Frozen semen processing

The semen was processed and frozen by the straw freezing method. The fresh collected semen was randomly divided and transferred into 15 ml pre-warmed tubes, and held for 30 min at room temperature, and subsequently centrifuged at room temperature for 10 min at 500×g to remove the seminal plasma. After elimination of seminal plasma, about 12 ml pre-warmed addition of BTS solution (The recipes for BTS (in g/L): glucose 37.0, EDTA 1.25, sodium citrate 6.0, sodium bicarbonate 1.25, and potassium chloride 0.75) was added to all of the above tubes, respectively. The tubes were wrapped by 12 to 15 layers of sterile gauze, and the sperm suspension were slowly cooled to 17°C, and subsequently centrifuged at 17°C for 10 min at 800×g. The supernatant was then removed.

The concentrated semen was diluted with the cooling extenders of control and treatment to obtain 1.5×10^9 spermatozoa/ml, respectively. The diluted semen was gently mixed, and all of the tubes were wrapped by 12 to 15 layers of sterile gauze. They were slowly cooled to 5°C, and equilibrated for 1.5 to 3 h.

The semen was further diluted (2:1, two parts semen to one part extender) with different freezing extender respectively. The final sperm concentration was 1.0×10^9 spermatozoa/ml, and the final glycerol concentration was 3%. Straws (0.25 ml) were immediately filled and sealed manually, using metallic sealing balls. The straws were horizontally placed on an aluminum rack and maintained at 5°C for 2 to 3 h. The freezing programme had the following steps: from +5°C to -5°C with 1°C/min by programmable freezing device (Mini Digitcool 1400, IMV, France). All of the straws were then placed in contact with nitrogen vapor for 15 min, about 3 to 4 cm (-120°C)above the nitrogen liquid level, and then immersed into the liquid nitrogen (-196°C) for storage.

Sperm quality evaluation

Sperm motility : For each extender, four straws were thawed separately by immersion in a water bath at 37°C for 45 s. Immediately, all of the thawed samples were then transferred into a plastic tubes containing 9.5 ml of BTS solution and 0.5 ml of the relevant cooling extender (pre-warmed). After 5min incubation at 37°C, 10 μl aliquots were transferred onto glass slides and cover-slips were applied. Sperm motility was assessed by determining the percentage of spermatozoa showing any movement of the flagellum. The percentage of motile sperm was estimated at 37°C by light microscope at 400×. At least 300 spermatozoa were counted per slide.

FITC-PNA staining : Acrosome integrity was evaluated by the FITC-labeled peanut agglutinin (FITC-PNA). After thawing, semen was transferred into a plastic tube containing 2 ml 3% PVP (The solution prepared by mixing 3 g PVP in 100 ml of PBS), and centrifuged at room temperature for 3 min at 800×g, and the supernatant

Table 1. Effect of yolk LDL addition in extenders, instead of egg yolk, on the motility of boar spermatozoa after the freeze-thawing process

Semen extender	Total spz.	Spz. linear motile	Spz. linear motile (%)
TCG	2,900	882	30.4±1.2[e]
TCF	2,900	873	30.1±0.9[e]
LDL 6%	2,900	1,088	37.5±0.6[d]
LDL 7%	2,900	1,351	46.6±0.6[c]
LDL 8%	2,900	1,467	50.6±0.3[b]
LDL 9%	2,900	1,578	54.4±0.7[a]
LDL 10%	2,900	937	32.3±0.6[e]

Data with different letters in the same row differ significantly at p<0.05.

solution was poured off. The sediment (sperm) was diluted with PBS solution (37°C) to obtain 1-2×10^6 spermatozoa/ml. Aliquots (30 μl) of sperm were then used to prepare smears on microscope slides. After air-drying, sperm smears were fixed with absolute methanol for 10 min at room temperature. Thirty microliters FITC-labeled peanut agglutinin (FITC-PNA) solution (100 μg/ml) in PBS were 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 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 an epifluorescence microscope (LEIKA DM-IRB linked up to a Nikon digital camera DXM). All treatments were replicated 6 times with 6 ejaculates from the same boar. All samples were coded before evaluation, and were evaluated by one observer. The whole acrosome was visualized with strong green fluorescence under a fluorescence microscope and was scored as acrosome-intact sperm cells. The percentage of fluorescent acrosome-intact spermatozoa was counted in at least 300 sperm cells 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.

Hypoosmotic swelling test (HOST) : Membrane integrity was evaluated by the hypo-osmotic swelling test (HOST). The straws were thawed in a water bath at 37°C for 45 s. The assay was performed by mixing 50μl of the semen with 1 mL of hypo-osmotic solution prepared by mixing 7.35 g sodium citrate $2H_2O$ and 13.51 g fructose in 1 L of distilled water. After incubation for 30 min at 37°C (Garcia-Lopez et al., 1996), sperm swelling was assessed by placing 15 μl 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× magnification, and viable spermatozoa had coiled tails after HOST. 300 spermatozoa per slide were observed. The spermatozoa

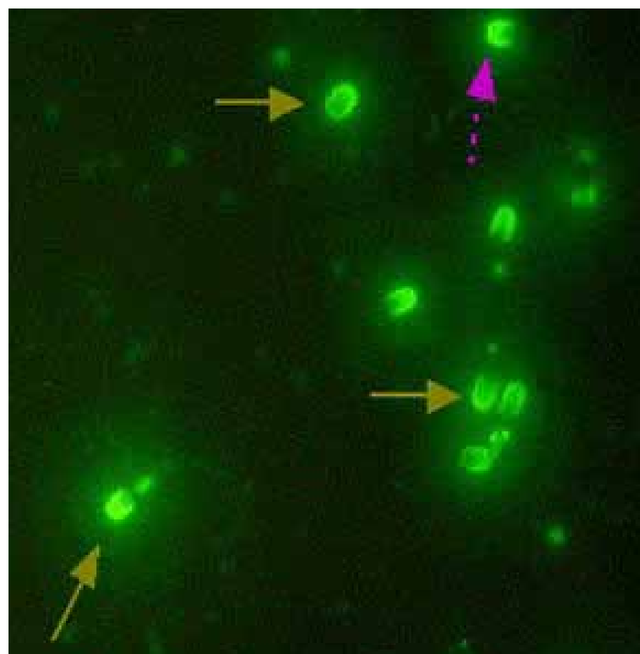

Figure 1. Fluorescent patterns of spermatozoa stained with FITC-labeled peanut agglutinin (FITC-PNA) (spermatozoa observed here were frozen-thawed in 9% LDL extender). ⟶ intact acrosome - - - -► partially damaged acrosome.

were classified as positive or negative based on the presence or absence of coiled tail.

All reagents for these experiments used Sigma Products of USA.

Statistical analysis

The results of sperm motility were expressed as mean±SEM. The statistical significance of the data concerning the effect of LDL solutions on the motility of frozen-thawed semen was determined by analysis of variance. The percentages of spermatozoa showing different staining in individual experiments were compared mean values using Duncan's multiple range test by ANOVA procedure, when the F-value was significant (p<0.05). The correlation among the above-mentioned methods was evaluated by linear regression analysis. The results of control were analyzed with the Student's t-test (matched series). Statistical analyses were performed with the SPSS11.0 statistical Package. Values of p<0.05 were considered statistically significant.

RESULTS

Semen motility

When the concentrations of LDL was 6 to 9% (w/v), the motility of the freeze-thaw treated sperm were significantly higher (p<0.05) in these extenders as compared to control extender (TCG and TCF containing 20% (v/v) egg yolk) (Table 1).

Table 2. Evaluation and comparison of acrosome integrity among semen cryopreserved in LDL, TCG and TCF extenders

Semen extender	Total spz.	Spz. intact acrosome	Spz. intact acrosom (%)
TCG	2,400	907	37.8±1.1[d]
TCF	2,400	874	36.4±0.9[d]
LDL 6%	2,400	1,529	63.7±1.4[c]
LDL 7%	2,400	1,565	65.2±1.3[c]
LDL 8%	2,400	1,642	68.4±1.2[ab]
LDL 9%	2,400	1,687	70.3±1.1[a]
LDL 10%	2,400	1,567	65.3±1.3[bc]

Data with different letters in the same row differ significantly at p<0.05.

Five LDL concentrations were tested: 6, 7, 8, 9 and 10% (w/v). We observed that LDL concentrations between 6 and 9% corresponded to an enhancement of motility. Concerning percentage of motile sperm, when LDL concentration increased above 10%, there was a sharp drop. The experiment was conducted to determine more precisely optimal concentration (between 8 and 9%, w/v) of LDL in the extender, and sperm motility exceed 50%. Furthermore, we observed that 9% LDL offers the best results. At this concentration, percentage of spermatozoa motility reaches 54.4% versus 30.4% for TCG extender and 30.1% for TCF extender containing egg yolk. No significant difference was observed for sperm motility between TCG and TCF extenders (p>0.05).

These results suggest that in our experimental conditions, LDL extender afford better protection than egg yolk during the freeze-thaw process and that the most efficient concentration is 9% LDL (w/v).

Acrosome integrity

After exposure of permeabilized spermatozoa to the FITC-labeled peanut agglutinin (FITC-PNA), two types of spermatozoa were present: those with a stained acrosomes (Figure 1), and those unstained group (without acrosomes). Acrosomal integrity is thus maintained after freezing either in control and treatment. The percentage of acrosome-intact spermatozoa is shown in Table 2.

Analysis revealed that acrosome integrity was significantly higher in the extenders supplemented with 6 and 10% LDL than in control extender (TCG and TCF)(p<0.05). When LDL concentrations increased from 6% to 9%, the acrosome integrity corresponded to an enhancement. We observed that 9% LDL offers the best results, and 70.3% of the spermatozoa remained acrosome-intact. It was almost twice as much compared to the control. When LDL concentration increased above 10%, the integrity of acrosome decreased. No significant difference was observed for acrosome integrity between TCG and TCF (p>0.05).

These results suggest that LDL solutions afford better protection than egg yolk during the freeze-thaw process and that the most efficient concentration is between 8 and 9% LDL.

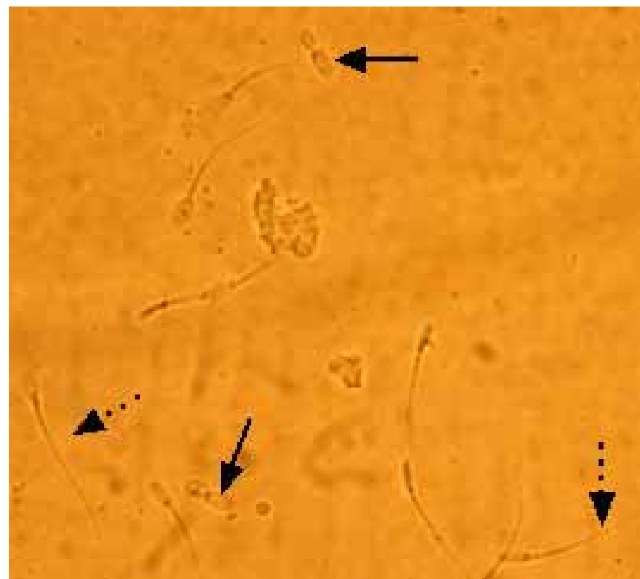

Figure 2. Swollen patterns of spermatozoa observed with hypoosmotic swelling test (HOST), react spermatozoa with a curved tails (spermatozoa observed here were frozen-thawed in 9% LDL extender). ⟶ motile sperm of curved tails ----→ dead sperm of straight tails.

Membrane integrity

After incubation of frozen-thawed spermatozoa in a hypo-osmotic solution, sperm cells with a swollen tails were considered as spermatozoa with an intact plasma membrane (Figure 2). We observed a significant difference on the membrane integrity between semen cryopreserved of the two groups of control and treatment, the percentages of swollen spermatozoa being significantly higher in LDL extenders (between 6% and 9%)than in those in TCG and TCF extender (p<0.05). Similarly, we observed that LDL concentrations between 6 and 9% corresponded to an enhancement of membrane integrity. However, when LDL concentration increased above 10%, the percentages of coiled tail spermatozoa significantly decreased. We observed that the plasma membrane integrity of the spermatozoa in extender supplemented with 9% LDL was the best results based on HOST, with 50.8% of tail coiled cells. Concerning the percentages of swollen spermatozoa, no significant difference was observed between semen cryopreserved with 10% LDL and semen cryopreserved with TCG and TCF. This result showed that plasma membrane integrity was maintained after semen cryopreservation with extender containing LDL (between 6% and 9%) instead of total egg yolk (Table 3). However, The optimal supplement of LDL in extender is 9% (w/v).

Similarly, at this concentration, percentage of swollen

Table 3. Effect of semen extender on the percentage of swollen spermatozoa subjected to the HOS test after freezing-thawing

Semen extender	Total spz.	Swollen spz.	Swollen spz. (%)
TCG	2,700	818	30.3±0.8[d]
TCF	2,700	807	29.9±0.9[d]
LDL6%	2,700	986	36.5±1.2[c]
LDL 7%	2,700	1,201	44.5±1.2[b]
LDL 8%	2,700	1,226	45.4±1.1[b]
LDL 9%	2,700	1,372	50.8±1.3[a]
LDL 10%	2,700	826	30.6±1.0[d]

Data with different letters in the same row differ significantly at p<0.05.

spermatozoa reaches 50.8% versus 30.3% for TCG and 29.9% for TCF extender containing egg yolk, respectively. Our results show that LDL solutions afford better protection than egg yolk during the freeze-thaw process and that the optimal concentration of LDL in the extender is 9% (w/v).

Correlation coefficient

The correlation coefficients among different tests (sperm motility; acrosome integrity; membrane integrity) are summarized in Table 4. The correlation among 3 tests was high (ranging from 0.503 to 0.965). All of the correlations among the 3 tests were significant (p<0.01).

Table 4. Correlation coefficients among different methods for testing boar sperm quality after freezing-thawing

Term	Sperm motility	FITC-PNA procedure	HOST procedure
Sperm motility	-	0.545	0.965
FITC-PNA procedure	0.545	-	0.503
HOST procedure	0.965	0.503	-

HOST: hypoosmotic swelling test.
PNA-FITC: fluorescein isothiocyanate-peanut agglutinin.

DISCUSSION

The mechanism by LDL used instead of egg yolk in the protection of spermatozoa during the freeze-thaw process

The total solids of hen egg yolk are made up of about two-thirds of LDL and other components. The structure of the LDL is based on a triglyceride core surrounded by a film of proteins and phospholipids having their polar residues in contact with the aqueous phase (Cook et al., 1969). Although the respective precise role of lipids and apoproteins on the cryoprotective action of LDL were not clearly established, the previous experiments affirmed that the LDL fraction was responsible for the cryoprotective effect of egg yolk during freeze-thaw processing of mammalian spermatozoa (Polge et al., 1970; Evans et al., 1973; Demianowicz and Strezek, 1996; Trimeche et al., 1996).

Concerning the protective mechanism of egg yolk with spermatozoa during the freeze-thaw process, many recent advances had been achieved. It is known that yolk undergoes gelation when it is subjected to a freeze-thaw process and that LDL are responsible for this gelation, while the other constituents of yolk do not directly participate (Tsutsui, 1988). The primary stage of this gelation is the disruption of the LDL structure. This disruption is favored by dehydration caused by the freeze-thaw process. Because of the formation of lethal intracellular ice crystal during freeze-thaw process, the sperm quality was reduced, as compared to the fresh semen.

LDL are composed of 85-90% lipids and 10-15% proteins. The interactions of lipid and protein are disrupted under freezing, but interactions between proteins are increased. Consequently, LDL are disrupted under the freezing and thawing. Triglycerides and phospholipids are liberated in the medium, and apoproteins form a gel. Furthermore, phospholipids could form a protective film at the surface of spermatozoa membranes after disruption of LDL. Graham and Foote (1987) and Trimeche et al. (1996) proposed that phospholipids from LDL could replace some phospholipids of spermatozoa membrane. Moreover, Graham and Foote (1987) observed that phosphatidylserine alone or in combination with phosphatidylcholine is the most effective phospholipid for spermatozoa protection.

We can also propose that gelation of LDL apoproteins around the spermatozoa membrane could form a protective film against the formation of lethal intracellular ice crystal during freezing, and that the protection could be ensured by fixation of the LDL on the spermatozoa membrane.

The optimal concentrationof LDL in the extender was 9% (w/v) in our study, which was very similar to 8% described by Moussa et al. (2002). Furthermore, the TCG and TCF extenders then contain 6-7% (w/v) of LDL according to the definiens of Moussa et al. (2002), which was very near 8% and a slight lower than that of 9%. We suggest that this result could be related to the difference of species.

It is known that some components in egg yolk bring a negative role to the cryoprotective effect of LDL. Pace and Graham (1974) reported, using LDL in bull semen extenders, that granules of yolk had a harmful effect on spermatozoa post-thaw motility. Watson and Martin (1975), in another study with ram spermatozoa also corroborated this assessment. Demianowicz and Strezek (1996) separated yolk into two lipoproteins: LDL and high density lipoproteins (HDL). They observed that if LDL provided better protection of boar spermatozoa than yolk, HDL decreased significantly the spermatozoa motility compared to yolk, and pointed that it was due to the granules contained in HDL. It could be hypothesized that HDL have could a detrimental effect on its cryoprotective properties.

Moreover, the increase of LDL concentration in the

extender above 10% leads to a decrease in spermatozoa performance after freeze-thaw in this study, and the similar results were also reported by Moussa et al. (2002). This latter observation could be related to the granules. An hypothesis could be advanced that this effect is due to the increase of LDL concentration leads to aggregation of granules. In addition, another possibility could be that the increase of LDL concentration also leads to HDL aggregation. Thus the inactivation of the effect of aggregated LDL and HDL engender for all of them contain granules, and the granules had a harmful effect on the sperm quality after the freezing and thawing.

The spermatozoa quality after freezing and thawing

To date, there are no reports of the effects of LDL used instead of egg yolk in boar semen freezing. Three methods have been developed to detect the spermatozoa quality after freezing and thawing in present study. Our results show clearly that, under our conditions, LDL can replace egg yolk in extender with better results in terms of motility, and acrosome and membrane integrity of boar spermatozoa. Furthermore, the optimum concentration of LDL has been determined to be 9% (w/v).

The sperm motility score for semen diluted by extender containing 9% LDL was 54.4% in our study, which was close to 57.3% described by Moussa et al. (2002). The HOST score for semen diluted by extender containing 9% LDL was 50.8% in this experiment, which was better than that described by Lamia Amirat et al. (2004). The FITC-PNA score for semen diluted by extender containing 9% LDL was 70.3%.

The percentages of post-thaw motilities were higher in LDL extender than in TCG and TCF. Extenders differ in composition. TCG and TCF extenders contain 20% (v/v) egg yolk, and egg yolk contains 50% dry matter, 66% of which are LDL. Consequently, it means that TCG and TCF extenders naturally contain 6-7% (w/v) LDL. This concentration is slight lower than that in our LDL extender. This suggests that egg yolk could contain some deleterious components which are potent to reduce semen motility (Moussa et al., 2002).

Spermatozoa are unable to fertilize the egg immediately after ejaculation in mammalian, and they must acquire the capacity to fertilize "capacitation" in the female reproductive tract (Yanagimachi, 1994). Binding to the zona pellucida stimulates the spermatozoa to undergo acrosome reaction (AR) in which the outer acrosomal membranes fuses with the overlying plasma membrane, this physiological exocytotic event results in the release of hydrolytic enzymes which are essential for the fertilization process. Spermatozoa which acrosome have induced loss spontaneously after ejaculation or by physical damages are unable to fertilize eggs. Acrosomal integrity is a necessary further indicator of potential sperm function to fertilize the oocytes at Al. Therefore, the evaluation of acrosome damages after the freeze-thaw process is a very important parameter to consider. To evaluate acrosomal status the binding of many lectins, proteins which interact with the glycoconjugates of the acrosomal membranes have been proposed. They include peanut agglutinin, concanavalin A, arachis hypogaea agglutinin and ricinus communis agglutinin. Presently, we used the FITC-PNA binding test. It shows an affinity for terminal α-D-glucosyl and α-D-mannosyl residues of glycoproteins mostly present in the sperm acrosome membranes of mammalian.

The feasibility of using FITC-PNA for studying the acrosome reaction in boar spermatozoa has been demonstrated previously by Vazquez et al. (1993). At the level of fluorescence microscopy, the signal representing PNA binding is mainly limited to the acrosomal cap of boar spermatozoa. Thus, FITC-PNA can be used as a reliable probe for detecting acrosome reactions in boar spermatozoa (Fazeli et al., 1997). Microscopic observation, showed that spermatozoa frozen in LDL extender displayed a green fluorescence in their acrosomal region, this fluorescence is an indicator of the presence of the acrosome. There were 70.3% acrosome-intact spermatozoa in semen diluted by extender containing 9% LDL in our study, which decreased significantly as compared with fresh semen. Acrosomal loss may occur because of a degenerative process following the death of the spermatozoa (Fazeli et al., 1997).

We used the hypo-osmotic swelling test (HOST) as a predictor of an intact plasma membrane in this study. HOST is based on the principle that spermatozoa swelled in hypotonic media display coiled flagella when their plasma membrane remains intact. Water flows throw the membrane and enters the cell, thus re-establishing equilibrium between the extra cellular and intracellular fluid compartments. The volume of cell increases and the membrane area expands, causing the flagellum to coil. Spermatozoa with damaged plasma membranes do not inflate and no swelling or curling of tails occurs (Jeyendran et al., 1984; Mladenovic et al., 1995; Hossain et al., 1998). In the mouse, the percentage of coiled tail spermatozoa was highly correlated with the percentage of acrosome reactions and zona penetrations (r = 0.9)(Jeyendran et al., 1984). Infertile males being associated with a result of less than 50% (Check et al., 1989). The osmolality of the HOS solutions must be sufficiently low to produce the highest effect without lysis of the sperm membrane. The osmolality of our study is 150 mOsm/kg H_2O, which yields optimized results with frozen-thawed spermatozoa (Correa et al., 1994; Revell and Mrode, 1994; Rota et al., 2000). The best result of HOST score for our experiment was 50.8%, which show that LDL solutions afford better protection than egg yolk during the freeze-thaw process.

However, the suitability of the HOST as a predictive tool for IVF of mammalian have not yet been established (Rota et al., 2000). The ability of the plasma membrane of bull spermatozoa to swell without disruption in hypotonic solutions does not always correlate with the fertility of the samples investigated, but it represent an easy method of high replication (Clarke and Johnson, 1987). These species specific differences could be related to different membrane elasticities, maximal swell volumes, water permeabilities, cell geometry (Cotirtens and Rety, 2001). In this study the HOST score of each group was lower than the percentage of moti1e sperm cells, which was also reported by Vazquez et al. (1993). The difference may be due to the fact that some spermatozoa with membrane damage remain motile.

The correlation among sperm motility, acrosome integrity and membrane integrity

The results of present study indicate a significant correlation among sperm motility, FITC-PNA and HOST methods used to evaluate semen quality after freeze-thaw process. Sperm swelling in response to hypo-osmotic conditions results in the appearance of a coiled tail. The HOST procedure was a sensitive, reproducible test for assessing the functional integrity of boar sperm membranes after incubation under hypo-osmotic stress conditions, and it may be a useful tool for detecting populations of less viable spermatozoa when used in conjunction with another type of membrane integrity test (Vazquez and Martinez, 1997). Sperm motility was highly correlated with HOST (r = 0.965), suggesting that plasma membrane integrity is related to sperm motility. The correlation coefficients between sperm motility and acrosome integrity, and plasma membrane integrity and acrosome integrity are 0.545 and 0.503, respectively.

All 3 tests were used to assess sperm viability, the close correlation among the tests may suggest that any one of them would be an equally effective diagnostic indicator of boar sperm quality after freezing and thawing.

In conclusion, our results show clearly that, under our conditions, LDL possess remarkable cryoprotective properties for freeze-thaw boar spermatozoa, and can replace whole egg yolk in extender with better results in terms of motility (54.4%), acrosome integrity (70.3%) and plasma membrane integrity (50.8%). Furthermore, the optimum LDL concentration in boar semen extender has been determined to be 9% (w/v).

Freezing boar semen in LDL extender offers a high number of functional spermatozoa available for AI and IVF, as demonstrated by the significant correlations among outcomes with sperm motility, FITC-PNA and HOST. Furthermore, it can improve spermatozoa quality and allows to reduce the number of spermatozoa in the straws. Moreover, LDL extender is interesting because it has the advantage to be chemically defined, and does not interfere with microscopic observations and the field of vision under microscopic is extraordinary clear, which is not the case with TCG and TCF containing whole egg yolk.

More research is needed to evaluate and understand the respective precise mechanism of lipids and apoproteins of protecting spermatozoa in freeze-thaw process from LDL. Moreover, the new method and technology is also needed to develop and isolate the antagonistic agent contained in egg yolk.

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