Replacement of salamon with shotor diluent and egg yolk with low density lipoprotein for chilled storage of ram semen

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Summary

The present study investigated the possibility of replacing salamon with modified shotor diluent (MSD) and egg yolk (EY) with low density lipoprotein (LDL) for chilled storage of ram semen. Good quality semen (>80% progressive forward motility (PFM) of sperm) from 3 fertile rams was collected using an artificial vagina and pooled for each experiment. Low density lipoprotein was extracted from fresh EY. In experiment 1, semen was divided into 2 fractions and extended in MSD or salamon. In experiment 2, semen was assigned into 5 fractions and extended in MSD supplemented with 12 and 15% EY or 3, 5 and 8% LDL. In experiment 3, semen was divided into 2 fractions and extended in MSD supplemented with 12% EY or 5% LDL. Viability of sperm was assessed at times 0 (immediately after semen dilution), 2 or 4 (at 4°C) and up to 72 h after semen dilution. Data was analyzed using General Linear Model (GLM) procedure, including repeated measures. In experiment 1, the viability of sperm was similar in two diluents ($P>0.05$). In experiment 2, PFM of sperm was similar among groups at the time of dilution ($P>0.05$); but remained elevated in 5 and 8% LDL compared to other groups afterward ($P<0.05$). In experiment 3, PFM of sperm was superior at 48 and 72 h after dilution in 5% LDL compared to 12% EY ($P<0.05$). In conclusion, MSD supplemented with 5% LDL is a suitable diluent for ram fresh semen preserved at 4°C for 72 h.

Key words: Ram, Semen preservation, Low density lipoprotein, Diluent

Introduction

Tris-egg yolk (EY) extenders have been widely used for preservation of ram semen (Salamon and Maxwell, 1995). Whole EY of avian species is a protectant for maintaining the sperm viability during cooling process (Manjunath *et al*., 2002; Bergeron *et al*., 2004; Su *et al*., 2008; Kulaksiz *et al*., 2010; Gholami *et al*., 2012). However, whole EY could inhibit respiration, diminish motility of sperm, interfere with biochemical assays, metabolic investigations and computer assisted sperm analysis (Pace and Graham, 1974; Ahmad and Foote, 1986; Bousseau *et al*., 1998; Moussa *et al*., 2002). Moreover, it may vary widely in its composition from one batch to another (Canisso Igor *et al*., 2008). Therefore, in the last few years, there have been increasing demands to replace whole EY with its cooling protective components in semen extenders.

Low density lipoproteins (LDLs) are the major cooling component of EY (Pace and Graham, 1974; Graham and Foote, 1987; Moussa *et al*., 2002; Amirat *et al*., 2004; Bergeron and Manjunath, 2006). Low density lipoprotein extracted from EY was used for cryopreservation of bull (Moussa *et al*., 2002; Amirat *et al*., 2004; Vera-Munoz *et al*., 2009; Hu *et al*., 2010) dog (Bencharif *et al*., 2008; Varela Junior *et al*., 2009), boar (Jiang *et al*., 2007; Yamauchi *et al*., 2009), buck (Ahmad *et al*., 2008) and ram semen (Tonieto *et al*., 2010). In the latter study, conducted to freeze ram semen, there was no experiment to demonstrate the best concentration of LDL for cryopreservation of ram semen. The purpose of this study was to investigate the possibility of replacing salamon with modified shotor diluent (MSD) and to find out the best concentrations of LDL for chilled storage of ram semen.

Materials and Methods

Experimental animals and location

During breeding season, three fertile Shal rams (3-5 years of age), belonging to Veterinary Medicine Research Institute, University of Tehran (longitude: 35°34′ N, latitude: 51°29′ E, altitude: 1047 m) were used for this study.

Materials

All materials were from Merck Co., Darmstadt, Germany, unless otherwise stated. Salamon diluent consists of tris (3.634 g), citric acid (1.99 g), fructose (0.5 g), penicillin G sodium (100000 IU, Jaber Ibn Hayyan Pharmaceutical Co., Tehran, Iran) and streptomycin sulfate (100 mg, Jaber Ibn Hayyann), in 100 ml of deionized water (Evans and Maxwell, 1987). Modified shotor diluent consists of tris (2.36 g), citric acid (1.3 g), glucose (1.2 g), fructose (1.2 g) and similar antibiotics, in 100 ml of deionized water (Niasari-Naslaji *et al*., 2006).

Osmolality and pH of semen and extenders were measured using Osmo-meter (Model 13, Roebling, France) and pH-meter (Model CG 822, Schott-Geräte, GmbH, Germany), respectively.

Semen collection, processing, and evaluation

Semen was collected with artificial vagina and kept at 37°C. Good quality semen (progressive motility of ≥80%) was used and pooled for the experiments. Semen was extended at the ratio of 1:1 and cooled to 4°C. Viability of sperm including: progressive forward motility (PFM), live percentage and plasma membrane integrity (PMI) was evaluated up to 72 h after incubating at 4°C. Each experiment was replicated at least 4 times. To evaluate sperm viability, the semen was further diluted to 50×10^6/ml.

In order to determine PFM of sperm, 10 µl of diluted semen was placed on the microscope slide (76.2 × 24.5 mm, Pearl, China) covered with a coverslip (24 mm × 24 mm, Menzel-Glaser, Germany) and examined under a phase contrast microscope (Olympus BX51, Japan) at magnification of ×400. The microscope was equipped with warm stage, set at 37°C. The percentage PFM was assessed on 200 sperm, subjectively (Comhaire and Vermeulen, 1995).

Plasma membrane integrity was assessed by hypo-osmotic swelling (HOS) test consisting of fructose (0.9% w/v) and sodium citrate solution (0.49% w/v, 100 mOsm/kg), which was prepared on a daily basis and kept at 4°C (Correa and Zavos, 1994). Diluted semen (20 µl) was added to the pre-warmed (37°C) HOS solution (200 µl) in a test tube and then incubated for 60 min at 37°C. Hypo-osmotic swelling test was undertaken under a phase contrast microscope at a magnification of ×400, counting 200 sperms, to determine the percentage of sperm with curled/swollen tail.

To assess the live percentage of sperm, diluted semen (20 µl) was added to eosin B-fast green (10 µl), smeared and air dried immediately. The percentage of live sperm was determined in a total number of 200 sperm with a phase contrast microscope (×1000).

LDL extraction from egg yolk

To extract LDL, fresh hen eggs (*Gallus gallus domesticus*) were manually broken and albumen was discarded (Moussa *et al*., 2002). Egg yolk was collected into a container positioned in an ice bath. Egg yolk was diluted with an equal volume of a 0.17 M NaCl solution and stirred for 1 h at 4°C. It was then centrifuged (L5-L65 ultracentrifuge, Beckman, USA) at 10,000 × g for 45 min at 4°C, to separate plasma from granules (sediment). Centrifugation was repeated to remove the granules completely. Ammonium sulfate (40%) was added to the plasma and stirred for 1 h at 4°C followed by centrifugation at 10,000 × g for 45 min at 4°C. The supernatant was dialyzed (Cut off: 10 kDa, Sigma-Aldrich, St. Louis, MO, USA) against deionized water for at least 16 h, and then centrifuged at 10,000 × g for 45 min at 4°C. The floating residue, rich in LDL, was collected. Dry matter was determined after desiccating at 104°C for 48 h and expressed as a dry matter per 100 g fresh sample.

Experimental design

Experiment 1

Each fraction of pooled semen was extended in MSD and salamon containing 24 and 14% EY, respectively. The final EY concentrations were 12 and 7% for MSD and salamon, respectively. The diluted semen reached to 4°C by 2 h. Viability of sperm was assessed immediately after collection (time 0), after reaching to 4°C and at 24, 48 and 72 h.

Experiment 2

Each fraction of pooled semen was extended in MSD containing 24 or 30% EY or 6, 10 and 16% LDL. The final EY and LDL concentrations were 12 and 15% for EY groups and 3, 5, 8% for LDL groups, respectively. The diluted semen reached to 4°C by 4 h. Viability of sperm was assessed immediately after collection (time 0), after reaching to 4°C and at 8, 24 and 32 h after collection.

Experiment 3

Each fraction of pooled semen was extended in MSD containing 24% EY or 10% LDL. The final EY and LDL concentrations were 12 and 5%, respectively. The diluted semen reached 4°C by 2 h. Viability of sperm was assessed immediately after collection (time 0), and after reaching 4°C and at 24, 48 and 72 h after collection.

Statistical analysis

All percentage data were subjected to arcsine transformation. Changes in viability parameters were analyzed using General Linear Model (GLM) procedure, including Least Square Means in the model, in SAS (2005). Data were presented as means ± SEM.

Results

Osmolality and pH of raw semen and extenders were illustrated in Table 1.

Experiment 1

Viability of sperm was similar at any given time during the experiment in salamon and MSD (P>0.05,

Table 1: Osmolality (mOsm/kg) and pH of MSD (tris: 2.36 g, citric acid: 1.3 g, glucose: 1.2 g, and fructose: 1.2 g) supplemented with different concentrations of EY (12 and 15%) and LDL (3, 5 and 8%)

Ingredients	Egg yolk (%)		Low density lipoprotein (%)		
	12	15	3	5	8
Osmolality	321 ± 3.02	322 ± 3.00	320 ± 2.16	318 ± 2.01	314 ± 1.99
pH	6.86 ± 0.01	6.83 ± 0.01	7.07 ± 0.02	6.96 ± 0.05	6.94 ± 0.03

Figs. 1A-C). The patterns of sperm viability over time were also similar between extenders (P>0.05, Figs. 1A-C).

Experiment 2

At time 0, PFM was similar for all treatment groups (P>0.05, Fig. 2A). Since specimen reached 4°C, PFM remained elevated for 5 and 8% LDL compared to other experimental groups (P<0.05, Fig. 2A). At any given time, PMI did not differ between 5 and 8% LDL and remained greater than 15% EY (P>0.05, Fig. 2B). Live percentage of sperm was similar among groups at any given time (P>0.05, Fig. 2C).

Experiment 3

There was no difference in PFM between 12 and 5% LDL until 48 h incubation at 4°C (P>0.05, Fig. 3A). At times 48 and 72, PFM of 5% LDL was greater than 12% EY (P<0.05, Fig. 3A). At any given time, PMI and live percentage of sperm did not differ between 5% LDL and 12% EY (P>0.05, Figs. 3B-C).

Discussion

The first experiment indicated that MSD and salamon are similar for short term preservation of ram semen. The osmolality of fresh ram semen (324 mOsm/kg) was similar to that of MSD (321 mOsm/kg), but lower than that in salamon (362 mOsm/kg). Modified shotor diluent has two energy sources (glucose and fructose). Fructose is the only simple carbohydrate present in ram semen, but sperm can also metabolize glucose and mannose when they are included in the diluent (Salamon and Maxwell, 2000). Fresh ram semen was slightly acidic compared to both extenders.

Modified shotor diluent supplemented with 5 and 8% LDL had better PFM compared to 3% LDL, 12 and 15% EY, and remained superior in PMI compared with 15% EY throughout the experiment (experiment 2). The superiority of 5% LDL to 12% EY, as far as PFM concerns, was further elaborated up to 72 h incubation at 4°C (experiment 3). The reduction of PMI over time occurred earlier (at 2 h) in 12% EY compared with 5% LDL (at 24 h, experiment 3). Low density lipoprotein provided better protection than EY in cryopreservation of bull (8%, Moussa *et al*., 2002; Amirat *et al*., 2004; Vera-Munoz *et al*., 2009; Hu *et al*., 2010), dog (8%, Bencharif *et al*., 2008; Varela Junior *et al*., 2009), boar (6%, Jiang *et al*., 2007; Yamauchi *et al*., 2009) and buck (9%, Ahmad *et al*., 2008) sperm. Tonieto *et al*. (2010) used 8% LDL for the cryopreservation of ram semen without explaining the rationale for using such concentration. In the present study, using varying concentrations of LDL (3, 5 and 8%), 5% LDL has been found to be sufficient for chilled storage of ram semen. To the best of our knowledge, there is no study to explain the appropriate concentration of LDL for short or long term preservation of ram semen.

Plasma membrane is a stable and metabolically inert structure (Flesch and Gadella, 2000). Any damage to plasma membrane during the cooling or freezing process could be irreversible. Protein, phospholipid, cholesterol and other components of the plasma membrane of sperm could not be newly synthesized (Flesch and Gadella, 2000). Low density lipoprotein may prevent the damage

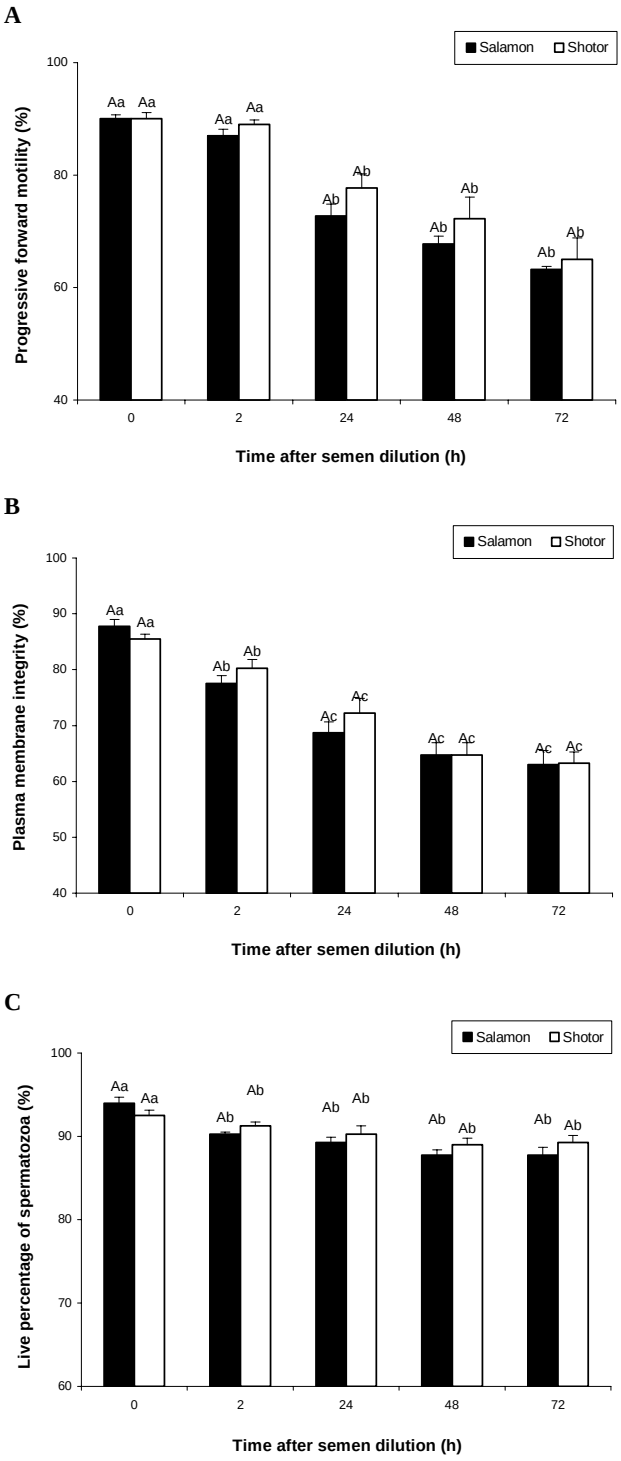

Fig. 1: Effect of tris-based EY diluents: salamon and modified shotor on the PFM of sperm (A), PMI of sperm (B) and live percentage of spermatozoa (C) of ram semen preserved at 4ºC for 72 h. [abc] Values with different superscripts indicate the difference over time within experimental groups (P<0.05). There was no significant difference between groups at any particular time (P>0.05)

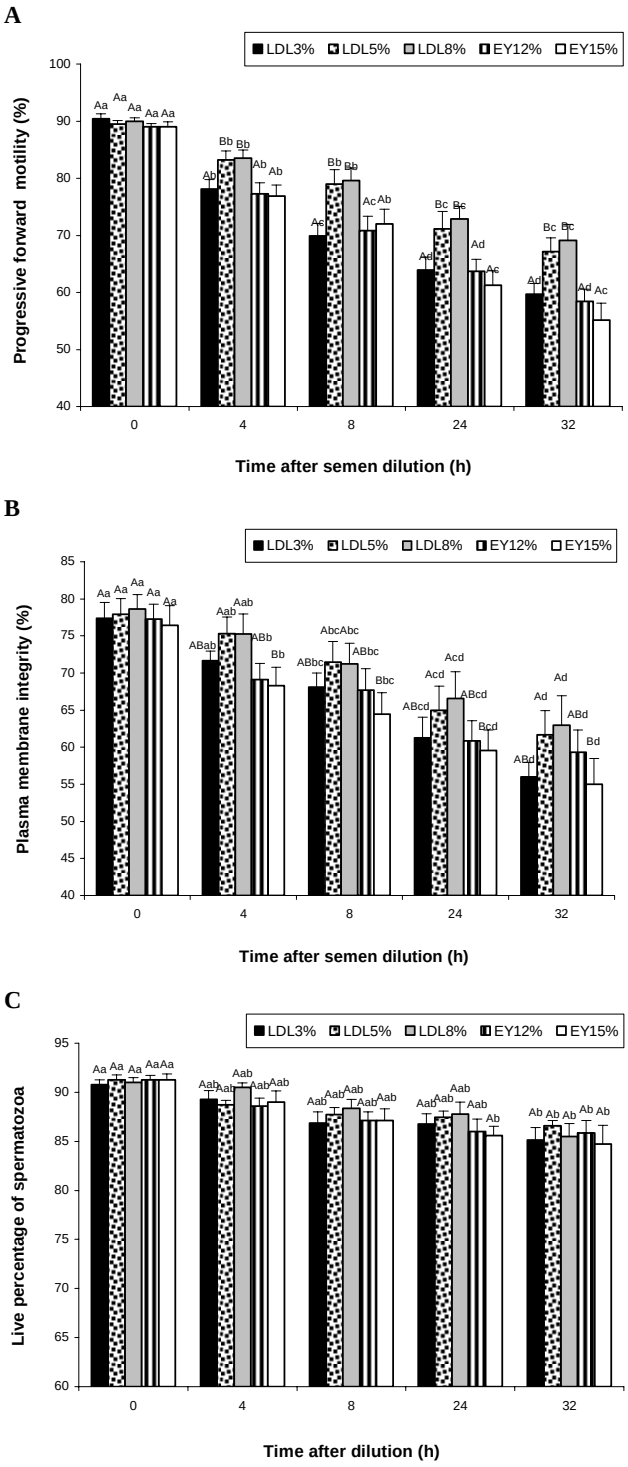

Fig. 2: Effect of different concentrations of LDL (3, 5 and 8%) and EY (12 and 15%) on the PFM of sperm (A), PMI of sperm (B) and live percentage of spermatozoa (C) of ram semen preserved at 4ºC for 32 h. [abcd] Values with different superscripts indicate the difference over time within experimental groups (P<0.05). [AB] Values with different superscripts at any particular time among experimental groups differ (P<0.05).

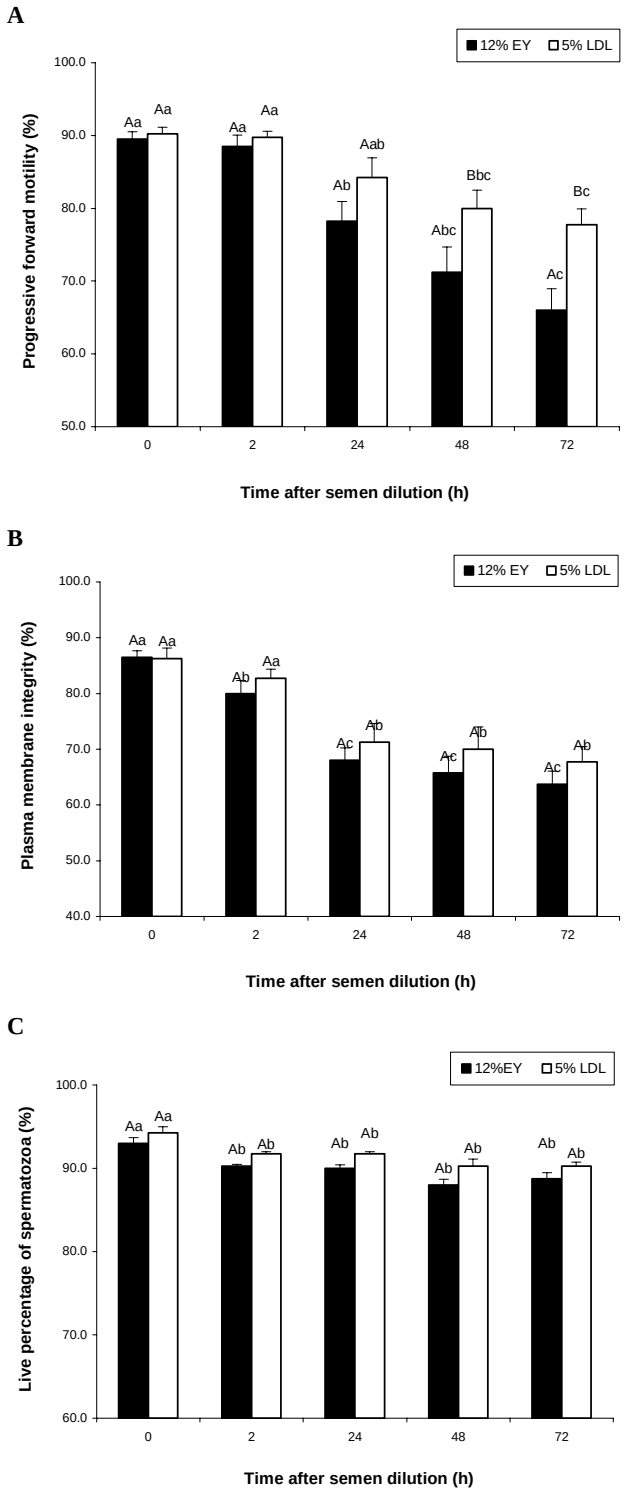

Fig. 3: Effect of MSD supplemented with LDL (5%) and EY (12%) on the PFM of sperm (A), PMI of sperm (B) and live percentage of sperm (C) of ram semen preserved at 4°C for 72 h. [abc] Values with different superscripts indicate the difference over time within experimental groups (P<0.05). [AB] Values with different superscripts at any particular time between groups differ (P<0.05)

to plasma membrane of sperm during preservation. Many theories have been suggested for the protective mechanisms of LDL. Vishwanath *et al*. (1992) proposed that cationic peptides in the seminal plasma might have detrimental effects on plasma membrane. Some of the seminal plasma proteins (Bovine seminal plasma proteins and their homologues in different species such as RSP proteins in ram) are known to be detrimental factors in the preservation of semen (Manjunath and

Therien, 2002; Manjunath *et al*., 2002; Bergeron *et al*., 2004; Bergeron *et al*., 2005; Bergeron and Manjunath, 2006). These proteins, immediately after ejaculation, bind to the plasma membrane and continuously induce cholesterol and phospholipid efflux followed by plasma membrane injuries subsequent with the decline in sperm resistance to cold shock and freezing. If rapidly after ejaculation the semen is diluted with extender supplemented with EY or LDL, LDL sequestrates most of the BSP proteins that are present in semen. This could result in minimal alteration of the sperm plasma membrane and allow better sperm storage (Bergeron *et al*., 2004; Bergeron and Manjunath, 2006). In addition, LDL has a role as a molecular chaperone (unpublished data). Molecular chaperones are a group of structurally diverse and mechanistically distinct proteins that either promote folding polypeptides or prevent the aggregation of other proteins (Sukyeong and Francis, 2005). We hypothesized that LDL inhibits the binding of BSP proteins to phospholipids of sperm membrane and prevent them from misfolding or unfolding.

The protective effect of LDL through binding with seminal plasma proteins may depend on the concentrations of these proteins in seminal plasma. The concentration of seminal plasma proteins in bull, ram, goat, stallion and boar were 65, 30, 20, 20 and 1.1%, respectively (Bergeron and Manjunath, 2006). Although the protective effect of LDL on PMI of bull semen may be explained by binding of LDL to these proteins due to high concentrations of these proteins, this may not be considered as the main mechanism of LDL in the species with low concentrations of BSP homologues. Moreover, in case of stallion, the seminal plasma is usually discarded by centrifugation during the first stage of semen processing. Therefore, it does not appear that the protective mechanism of LDL explained for bull semen could be extended to stallion semen. Regardless, EY still has a protective effect on preserving stallion semen (Jasko *et al*., 1992; Vidament *et al*., 2000).

Another protective effect of LDL may be explained by stabilizing the plasma membrane of sperm (Watson, 1975, 1981; Foulkes, 1977; MacDonald and Foulkes, 1981). It was proposed that phospholipids from LDL could replace some phospholipids on sperm membrane, subsequent with the reduction in phase transition temperature of plasma membrane, the temperature at which lipids change from the fluid to the crystalline state. This, in turn, could decrease the susceptibility of the sperm to the cold shock during cooling process (Foulks, 1977; Graham and Foot, 1987).

It was hypothesized that, LDL is responsible for gelatin formation during freeze-thaw process of EY occurred at -6°C. Lipid-protein interactions are disrupted following freezing and triglycerides and phospholipids are liberated in the medium and, apoproteins form a gel (Moussa *et al*., 2002). Subsequently, the gelatin formation of LDL could form a protective film against ice crystal formation during freezing process (Moussa *et al*., 2002). Quinn *et al*. (1980) suggested that after LDL disruption, phospholipids could form a protective film at the surface of sperm membranes. Neither of these two suggested mechanisms could explain the protective effects of LDL during the cooling process at which the temperature is only reduced to 4°C and the whole structure of LDL is still intact. Additionally, it was observed that addition of frozen LDL to extender did not provide the protective effect for sperm during cooling process (unpublished data). Moreover, the removal of protein-rich fraction from the surface of LDL reduced its ability to associate with the cell (Watson, 1981). It was also observed that LDL frozen at -20 and -80°C or freeze dried LDL reduced the solubility of LDL in the tris-base extender (unpublished data). The solubility of LDL is high (Anton *et al*., 2003) and it seems that apoproteins of LDL are responsible for solving the lipid and binding to the cell membrane (Watson, 1981). Therefore, the whole structure of this nano particle is necessary in the protection of sperm.

In conclusion, MSD is a suitable alternative extender and 5% is an optimum concentration of LDL for chilled storage of ram semen for 72 h at 4°C.

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