Indian Journal of Animal Sciences **86** (4): 427–430, April 2016/Short Communication
https://doi.org/10.56093/ijans.v86i4.57867

Effect of low density lipoprotein on replacement of egg yolk in liquid preservation of mithun semen

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Received: 17 July 2015; Accepted: 13 August 2015

Key words: Liquid storage, Low density lipoprotein, Mithun, Semen

Egg yolk from hen is a widely used component in semen preservation extenders for different animal species and it protects the sperm against cold shock damages and improves sperm fertilizing ability (Bogart and Mayer 1950). But due to the presence of high density lipoprotein, anti-cryoprotective agents, high level of Ca^{2+} and steroid hormones and its precursor molecules leads to many adverse effects on the SQPs (Akhter *et al*. 2011). Instead of whole EY, LDL extracted from EY has improved the freezability and fertility in many species at different concentration viz. porcine (9%, Jiang *et al*. 2007), ovine (8%, Tonieto *et al*. 2010), bovine (8%, Hu *et al*. 2011) and bubaline (10%, Akhter *et al*. 2011). Further, perusal of literatures revealed no information on LDL as a replacement for EY on the maintenance of SQPs, CASA and biochemical parameters in mithun semen preservation. Therefore, the present study was designed to evaluate the LDL as a substitute for EY in TCG extender in liquid storage of mithun semen.

Apparently healthy mithun bulls (10) of 4 - 6 years of age were selected from the herd, ICAR-NRC on Mithun, Jharnapani, Nagaland, India. Experimental animals were maintained as per the farm schedule. A total of 25 good quality ejaculates were collected through rectal massage method. The extraction of LDL from egg yolk was done as per the method developed by Moussa *et al*. (2002). The egg shell was cleaned with 70% alcohol and paper towel. It was broken manually and egg white was separated from egg yolk. The egg yolk was placed on a filter (Whatman circle filters) and carefully rolled the yolk on the filter to remove the remaining albumin (egg white) and the chalazas. A separate filter was folded into half two times to form a pointy tip and the vitelline (yolk) membrane was punctured with the filter tip; allowed the yolk to drain into a beaker while holding the membrane in the filter. The yolk was diluted @ 1:2 (w/w) with isotonic saline (0.17 M sodium chloride). The solution was stirred with magnetic stirrer for 1 h at room temperature. The solution was centrifuged at 10000 × g for 45 min at 10°C. The supernatant was recovered and granule (pellet) was discarded. The supernatant was centrifuged at 10000 × g for 45 min at 10°C. The yolk plasma (supernatant) was recovered and the granules (pellet) were discarded. Precipitation of livetins was done at 4°C. The yolk plasma was mixed with ammonium sulphate (40% saturated solution). The mixture was stirred by use of magnetic stirrer for 1 h at 4°C. Then the mixture was centrifuged at 10000 × g for 45 min at 4°C and finally the livetins (pellet) was discarded and the supernatant was kept. The supernatant was filled into a cellulose dialysis membrane and the ends were closed tightly. Dialysis was done with distilled water for a period of 21 h and water was changed frequently. The solution was centrifuged at 10000 × g for 45 min at 4°C and the LDL-rich floating residue (top layer) was collected. The extender used in this study contained standard TCG with 20% EY as control group (group 1) and different concentrations of LDL such as 8 or 10% (w/v) in place of EY in group 2 or 3, respectively. Different concentrations of LDL was selected on the basis of earlier experiments conducted in other bovine species. Diluted semen samples were cooled from 37 °C to 5°C in a cold cabinet and maintained at 5°C for 10 h. The SQPs such as sperm motility, viability and total sperm abnormality, acrosomal and plasma membrane integrity, nuclear integrity and vanguard distance travelled by sperm in the estrus bovine cervical mucus were determined with standard procedure. CASA parameters were assessed by Hamilton thorne sperm analyser. Lipid peroxidation level was measured by determining the MDA production as per the modified method of Suleiman *et al*. (1996). Biochemical parameters such as total cholesterol (TCH) and intracellular enzymes such as aspartate amino transferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH) activity and total antioxidant capacity (TAC) were estimated by using commercially available diagnostic kits. The results

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were analysed statistically with one way analysis of variance (ANOVA), followed by the Tukey's post hoc test to determine significant differences between the three experimental groups using the SPSS/PC computer and differences with values of $P < 0.05$ were considered to be statistically significant.

Variations in SQPs in response to addition of either EY or two differing concentrations of LDL in extender following liquid storage (5ºC) have been presented in Fig. 1. Averaged over time, mean total sperm abnormalities were 12.20 ± 2.38, 9.53 ± 1.41 and 10.62 ± 1.69% for groups 1–3 respectively. Similarly, the corresponding value for vanguard distance travelled by sperm in the cervical mucus was 18.44 ± 1.87, 21.88 ± 1.99 and 19.16 ± 1.84 mm/h, respectively. Results revealed a significant ($P<0.05$) improvement in SQPs and CASA parameters (Fig. 2) and parallel decrease in non-viable or abnormal spermatozoa and acrosome or nuclear abnormalities in ejaculates mixed with 8% LDL. Similarly, the extender containing 8% LDL significantly ($P<0.01$) lowered AST, ALT and LDH activity as compared to other experimental groups. TCH (Fig. 4) content of spermatozoa and TAC (Fig. 3) showed significant ($P<0.05$) improvement with simultaneous decrease in MDA production. Overall, 8% LDL contained extender (group 2) significantly ($P<0.05$) improved the SQPs, CASA and biochemical parameters than other treatment (group 3) and control (group 1) groups. Inclusion of LDL at 8% level protected the structures and functions of spermatozoa efficiently. In recent times, several authors (Moussa *et al*. 2002, Jiang *et al*. 2007, Tonieto *et al*. 2010, Akhter *et al*. 2011) reported that LDL is far less aggressive to mammalian sperm cells incubated at 4°C, a duration compatible with most classical freezing protocols and improves the structural and functional parameters of spermatozoa.

In the present study, addition of LDL has showed improvement in SQPs and CASA parameters with reduction in total sperm abnormalities (Fig. 2). Optimum level of LDL in the extender has improved the intactness of normal acrosome and integrity of the plasmalemma of spermatozoa in mithun as in earlier research work reported (Jiang *et al.* 2007, Hu *et al*. 2011). In ultrastructural studies of spermatozoa following addition of LDL, Amirat *et al.* (2005) reported that after 24 h of storage at 4°C, 31% of cells showed a swollen acrosome while very few displayed plasma membrane ruptures (0.97%) and 63% of spermatozoa remained intact. Acrosomal integrity of spermatozoa is protected by LDL in two ways. Firstly, the association of LDL with bovine seminal plasma (BSP) proteins protects sperm by preventing the binding of BSP on surface of spermatozoa intrinsically (Manjunath *et al*. 2002). Secondly, the lipids from LDL can associate with the sperm acrosomal membrane and thus it preserves the integrity of the acrosome during sperm preservation (Nauc and Manjunath 2000). In the present study, the intactness of acrosome was protected significantly in 8% LDL than EY contained extender because the LDL contained extender

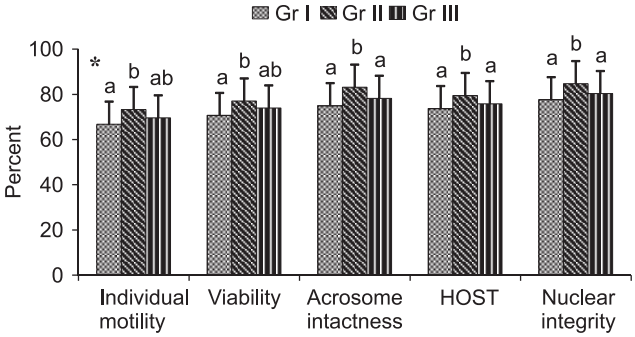

Fig. 1. Effect of Low density lipoprotein (LDL) supplementation on SQPs of mithun spermatozoa preserved at 5°C (* indicates P<0.05).

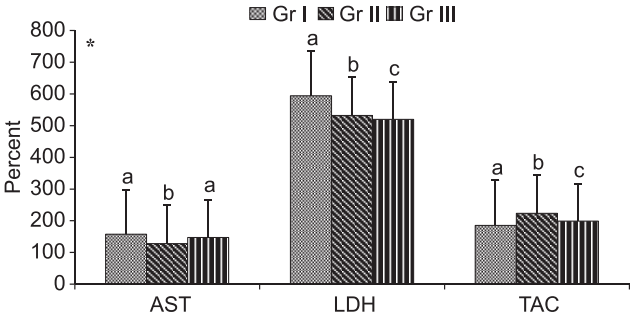

Fig. 3. Effect of Low density lipoprotein (LDL) supplementation on AST, LDH and TAC of mithun semen preserved at 5°C (* indicates P<0.05).

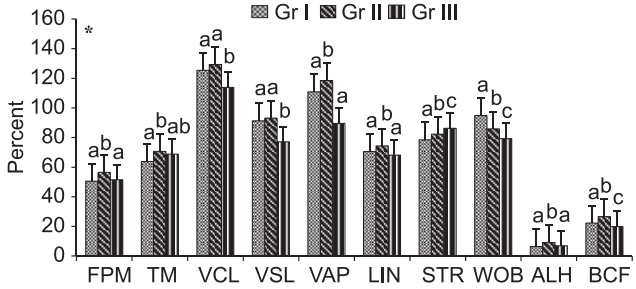

Fig. 2. Effect of Low density lipoprotein (LDL) supplementation on CASA parameters of mithun spermatozoa preserved at 5°C (* indicates P<0.05).

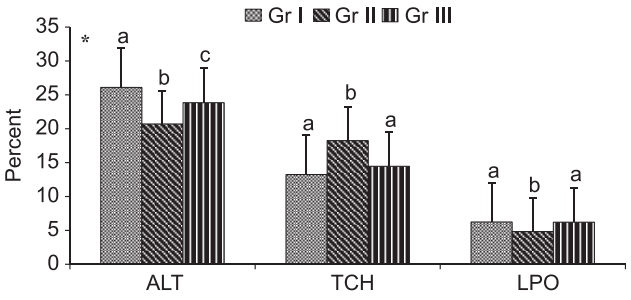

Fig. 4. Effect of Low density lipoprotein (LDL) supplementation on ALT, TCH and LPO (MDA) of mithun semen preserved at 5°C (* indicates P<0.05).

has less progesterone and its precursors molecules due to the presence of filtering effect of the dialysis membrane. During preservation, LDL is disrupted and phospholipids are liberated into the medium, which could form a protective film at the surface of sperm membranes (Cookson *et al*. 1984). Other workers (Hu *et al*. 2011) demonstrated that LDL is responsible for the gelation process in preservation of spermatozoa. Bergeron *et al*. (2004) suggested that LDL could adhere to cell membranes during the preservation process and preserve the membrane integrity of sperm.

Higher TCH and TAC content and simultaneous decrease in leakage of intracellular enzymes and MDA production were observed in the present study. Acrosomal destructions following efflux of cholesterol could result from intrusion of Ca^{2+}, which is present in high concentrations in EY and rapidly enters the cells when the temperature is below 30°C (White 1993). Along with phospholipids, cholesterol is necessary for cell physical integrity and ensures fluidity of the cell membrane and plays a special role in the sperm function because its release from the sperm membrane initiates the key step in the process of capacitation and acrosome reaction that is crucial for fertilization (Witte and Schafer-Somi 2007). In agreement with present finding, several studies have demonstrated that cholesterol influx reduces spontaneous acrosome reaction whereas efflux of cholesterol leads to increase in acrosomal reaction of spermatozoa implying predisposition to cryoinjury (Srivastava *et al*. 2013). In the present report, higher TCH content of spermatozoa in 8% LDL group than other two groups indicated greater stability of sperm membranes. Manjunath *et al*. (2002) explained that the principal mechanism by which LDL protects the spermatozoa is via the sequestration of BSP proteins, responsible for membrane cholesterol efflux.

Mammalian sperm membrane has high poly unsaturated fatty acids and it renders the sperm cells susceptible to lipid peroxidation during processing (Perumal *et al*. 2011a, 2011b). Lipid peroxidation ultimately leads to the impairment of sperm function, membrane integrity and damage of sperm DNA and lowered fertility (Maxwell and Stojanov 1996). Aitken and Clarkson (1987) reported the axoneme and mitochondria in sperms may be damaged by a high level of reactive oxygen species and Malondialdehyde. Results of the present study indicated that LDL can serve as a good antioxidant in liquid semen storage. As in the present studies, others (Moussa *et al.* 2002, Hu *et al*. 2011) have reported lower MDA production following addition of LDL in semen extender. The intracellular enzymes such as AST and ALT levels in seminal plasma are very important for sperm metabolism as well as sperm functions, providing energy for survival, motility and fertility of spermatozoa. In the present study, AST and ALT levels were lowered in semen preserved at 8% LDL as it stabilises the membrane integrity of acrosome, plasma, mitochondria and flagella of the sperm. The LDL protects integrity of spermatozoa by sequestration of free-floating BSP proteins responsible for efflux of cholesterol causing cell membrane disruption (Nauc and Manjunath 2000). Thus, LDL mediated protection of intracellular enzymes results from greater stability of spermatozoa plasma membrane. As with previous reports, LDL fraction not only acts as a membrane protector but also plays an important role in reduction of DNA damage in sperm (Jiang *et al*. 2007). As increase in the concentration of LDL above the optimum level, the semen quality parameters were decreased significantly due to the detrimental changes in the osmotic pressure of the extender and caused high fluidity of plasma membrane, making sperm more prone to acrosome and plasma membrane damages (Moussa *et al*. 2002, Hu *et al*. 2011).

The search for an additive, which can greatly minimize or alleviate the stress and damages caused in the process of preservation of modulated semen is the ongoing research. LDL is one such molecule which has shown promise recently. In the present investigation, the LDL (8%) contained extender guaranteed higher cell viability and less physical and oxidative damages than EY or LDL @ 10%. Thus, TCG extender with LDL @8% can be used advantageously for storage of mithun spermatozoa for short durations for breeding purposes.

SUMMARY

This investigation explored the use of low density lipoproteins (LDL) from egg yolk (EY) in replacement EY on semen quality parameters (SQPs), velocity and motility parameters by Computer Assisted Sperm Analyzer (CASA) and biochemical parameters following liquid storage (5°C) of mithun semen. A total of 25 ejaculates collected from ten healthy mithun bulls and diluted with the standard tris citrate glycerol (TCG) extender were splited into three equal aliquots: Group 1, Control; EY, Group 2 and Group 3 contained 8 and 10% (w/v on dry weight basis) LDL, respectively. SQPs, CASA and biochemical parameters were evaluated following incubation of semen at 5°C. Results revealed a significant ($P<0.05$) improvement in these parameters in group 2 as compared to group 1 and 3. It was concluded that addition of 8% LDL (w/v) holds a clear advantage over EY or 10% LDL in preservation of mithun semen at liquid storage.

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