Indian Journal of Animal Sciences **87** (11): 1340–1344, November 2017/Article

Cryoprotective effect of low-density lipoproteins on post thaw semen quality in Hariana bull

MUKUL ANAND[1], SARVAJEET YADAV[2], VIJAY SINGH[3], SHALINI VASWANI[4] and PANKAJ K SHUKLA[5]

DUVASU, Mathura, Uttar Pradesh 281 001 India

Received: 3 March 2017; Accepted: 9 May 2017

ABSTRACT

The study aimed to evaluate the cryoprotective effect of low-density lipoprotein in Hariana bull semen. Four adult Hariana bull (3–4 years) after thorough andrological examination were selected for experiment. Semen was collected from each bull twice a week; total of 32 ejaculates (8 from each bull) were collected during the experiment. Collected sample from each bull was divided into 5 equal aliquots. Four aliquots were diluted separately with a Tris-based extender containing different concentration of low-density lipoproteins viz. 7% (T-1), 8% (T-2), 9% (T–3) and 10% (T- 4) as treatment while routinely used extender containing 20% hen egg yolk was used as control (C) to achieve final concentration of 125 million spermatozoa/ml. The diluted samples were cryopreserved and evaluated after 7 days. A significantly higher value of percent live sperms, sperm positive for hypo osmotic swelling test, sperms with intact acrosome and sperms exhibiting pattern F (uncapacitated sperm) were significantly higher in T-3 as compared to other treatment groups and control. The kinematic characters exhibited by sperm i.e forward progression %, VCL (µm/sec), VSL (µm/sec), VAP (µm/sec), Lin (%), Str (%), BCF (Hz) and ALH (µm) were significantly higher in T-3 as compared to other treatment group and control. In conclusion, extender containing 9% low density lipoproteins has better capacity to maintain the sperm character in post thaw Hariana bull semen and can be a preferred concentration to be utilized for cryopreservation with improved conception rate.

Key words: CASA, Cryopreservation, Hariana bull, Low-density lipoproteins, Sperm characteristics

Cryopreservation and artificial insemination (AI) are 2 assisted reproductive techniques utilized to dissipate superior germplasm (Anand *et al.* 2015). They together provide opportunity to prepare multiple doses from a single ejaculate for its wide dissipation and prolonged use. Cryopreservation involves semen collection and its dilution with desirable extender (Ray *et al.* 2015). Extender contains ingredient that impart cryoprotection to sperm during the freeze thawing process. Egg yolk is the most commonly used cryoprotectant in TRIS based extender. But, egg yolk in extender has been reported to inhibit respiration in sperm cell (Amirat *et al.* 2005), diminish their motility (Abouelezz *et al.* 2015), affects acrosomal integrity (Chaudhari *et al.* 2015), capacitation of spermatozoa (Leahy and Gadella 2011) and bacterial contamination (Crespilho *et al.* 2012). Replacement of egg yolk with low-density lipoproteins (LDL) can improve post thaw semen quality and conception rate. Hence, the present experiment was designed to evaluate and optimize the LDL concentration in semen extender utilized for cryopreservation of Hariana bull semen.

Present address: [1]Assistant Professor (drmukulanandvet @gmail.com), [2]Professor and Head (yadavsarvajeet24 @gmail.com), Department of Veterinary Physiology; [3]Associate Professor (vijaysohlot@yahoo.co.in), Department of Animal Reproduction and Gynecology; [4]Assistant Professor (shalini_vet @yahoo.com), Department of Animal Nutrition; [5]Professor and Head (pksmathura@yahoo.co.in), Department of Poultry Science, College of Veterinary Sciences and Animal Husbandry.

MATERIALS AND METHODS

Animal and semen collection: Healthy Hariana bulls (4) of similar age (3–4 years) and weight (400–450 kg) maintained at Dairy Demonstration Farm, DUVASU, Mathura were selected as semen donor. The semen was collected twice a week from each bull using artificial vagina. A total of 32 ejaculates were collected (8 from each bull) during the experiment. Semen collected from each bull was initially evaluated and samples > 80% live spermatozoa, >3.5 mass motility and < 5% sperm abnormality were selected for experiment.

Sampling: Collected sample was divided into 5 equal aliquots. Each aliquot was diluted separately with a Tris-based extender containing different concentration of low-density lipoproteins. Routinely used extender containing 20% hen egg yolk was used as control (C) while hen egg yolk was replaced with variable concentrations of low-density lipoproteins, viz. 7% (T-1), 8% (T-2), 9% (T-3) and 10% (T- 4) in treatment groups to achieve final concentration of 125 million spermatozoa/ml. The diluted samples were equilibrated (at 4°C for 4 h), loaded in precooled 0.25 ml

mini French straws and cryopreserved in liquid nitrogen using slow freezing technique (Baracaldo *et al.* 2007)

Semen analysis: The freezed samples after its storage in liquid nitrogen for 7 days were subjected to thawing process at 37°C for 40 sec and evaluated for different seminal attributes. Following parameters were evaluated in post thaw semen.

Percent live spermatozoa: Percent live sperms were counted using differential staining technique (Hancock 1952). A drop of thawed semen sample was mixed with 4–5 drops of stain (eosin-negrosine stain) in cavity block maintained at 37°C. Smear was prepared by placing a small drop of stained sample on clean grease free glass slide and air dried. A total of 200 sperm were evaluated for percent viable sperm in different treatment groups under phase contrast microscope (100× objective). The unstained sperm were counted as live while those that stained pink were counted dead.

Hypo-osmotic swelling test (HOST): The percent of sperms responding to hypo-osmotic solution were evaluated using hypo-osmotic swelling test (HOST) (Jeyendran *et al.* 1984). Sample (10 μl) was mixed with hypo-osmotic solution (150 mOsm/kg) and incubated at 37°C for 1 h. A total of 200 sperm were evaluated for their response to hypo-osmotic solution as evident through curled tail under phase contrast microscope (40× objective).

Capacitation like changes using chlortetracycline (CTC) dye: The capacitation like changes exhibited by spermatozoa was studied using CTC dye. Three different patterns, viz. F (uncapacitated sperm), B (capacitated sperm with intact acrosome) and AR (acrosome-reacted sperm) were recorded during the experiment (Collin *et al.* 2000). Fifteen microlitres of CTC solution (750 μM CTC-HCl, 130 mM NaCl, 5 mM L-cysteine, 20 mM Tris acid (pH 7.8)) was mixed with 10 μl of sperm suspension. 0.3 μl of 12.5% glutaraldehyde in 2.5 M Tris base was added as a fixative. Three different fluorescence patterns for capacitation like changes were observed using a Nikon Eclipse TE 2000-S microscope with phase contrast and epifluorescence optics under blue-violet illumination (excitation at 400–440 nm and emission at 470 nm).

Computer assisted semen analysis (CASA): Path velocities and motion characters exhibited by sperm cells in different treatment groups was evaluated using computer assisted semen analysis (Biovis CASA 2000, Version 4.6, India). A small drop (4 μl) of semen was placed in the makler sperm counting chamber and observed under negative phase contrast at 10× objective. A total of 3–6 fields with 250–300 sperms were counted for each sample analyzed. Setting of the CASA system used for semen analysis is presented in Table 1.

Statistical analysis: Statistical analysis was performed using Statistical Package for Social Science (SPSS® Version 20.0 for Windows®, SPSS Inc., Chicago, USA). The means were compared using Analysis of Variance, Duncan's multiple range test and presented as mean ± standard error (SE) at the significance level of P<0.01 or P<0.05.

Table 1. Settings of the CASA system

Parameters for sperm tracking	Range
Frames (per sec)	60
Number of frames acquired (per sec)	61
Max velocity (for tracking, μm/s)	150 motility min
Curvilinear velocity (VCL, μm/s)	>25 motility min
Average path velocity (VAP, μm/s)	>10 motility min
Straight-line velocity (VSL, μm/s)	>1 min
Track length (% of frames)	51
Aspect	0–99,999
Area	2–20
Axis (major)	4–20
Axis (minor)	2–10
Compactness	0–50
Perimeter ratio	0–99,999
Minimum cell size on major axis	20
Minimum cell size on min axis	10
Magnification	×10 phase
Calibration (pixels/unit)	1.905 μ
Y (pixels/unit)	1.905 μ
Size of image	1280 × 960

RESULTS AND DISCUSSION

The observed mean (±SE) values of different physical seminal attributes evaluated during the experiment are presented in Table 2. A significantly (P<0.01) higher value of percent live sperms, sperm positive for hypo-osmotic swelling test and sperms with intact acrosome were observed in T-3 as compared to other treatment groups and control. Cryopreservation involves several steps, such as dilution, cooling, freezing and thawing. These processes induce cold shock and cryoinjuries that increases sperm susceptibility to lipid peroxidation (Bucak *et al.* 2008). Sperm biological membranes exhibit membrane transition phase behaviour that result in loss of selective permeability and integrity of the plasma membrane (Morris *et al.* 1987). Low density lipoproteins that impart cryoprotection during the freeze-thawing process is reported to form a gel-like protective film on the spermatozoa that protects the lipid–protein complex of cell membranes and thereby safeguards the spermatozoa (Akhter *et al.* 2011). Significantly (P<0.01) higher values recorded in T-3 may be attributed to better cryoprotective capacity of 9% low density lipoproteins to form protective film, decrease the phase transition temperature of sperm plasma membrane and reducing the formation of ice crystals (Moussa *et al.* 2002) compared to other treatments and control. Effect of LDL concentration on capacitation like changes was studied using chloretetracycline (CTC) dye and three different patterns were evaluated. The sperms exhibiting pattern F (uncapacitated sperm) were significantly (P<0.01) higher while those exhibiting pattern AR (acrosome reacted sperm) were lowest (P<0.01) in T-3. Capacitation involves membrane destabilization in female reproductive tract. Although beneficial, but premature capacitation reduces the sperm capacity to bind with female gamete at the time of fertilization. Cold shock, cryoinjuries and osmotic stress

Table 2. Physical attributes exhibited by sperm in semen diluted with extender containing low-density lipoproteins during cryopreservation (N-32)

Treatment	Live percent (%)	HOST (%)	Acrosomal integrity (%)	Pattern F (%)	Pattern B (%)	Pattern AR (%)
Control (Egg yolk- 20%)	$46.17^{C}\pm0.50$	$42.75^{C}\pm0.47$	$40.42^{C}\pm0.57$	$25.92^{CD}\pm0.50$	$34.92^{B}\pm0.71$	$39.17^{AB}\pm0.76$
Treatment-1 (7% LDL)	$45.83^{C}\pm0.92$	$42.67^{C}\pm0.79$	$39.50^{C}\pm1.01$	$24.00^{D}\pm0.85$	$34.50^{B}\pm0.92$	$41.50^{A}\pm0.65$
Treatment-2 (8% LDL)	$50.00^{B}\pm0.68$	$46.17^{B}\pm0.90$	$43.83^{B}\pm0.70$	$29.50^{B}\pm0.93$	$38.00^{A}\pm0.65$	$32.50^{C}\pm1.33$
Treatment-3 (9% LDL)	$58.00^{A}\pm1.10$	$54.25^{A}\pm0.99$	$53.00^{A}\pm1.03$	$34.00^{A}\pm0.74$	$37.92^{A}\pm0.95$	$28.08^{D}\pm1.09$
Treatment-4 (10% LDL)	$46.33^{C}\pm0.76$	$42.67^{C}\pm0.58$	$41.33^{C}\pm0.74$	$26.58^{C}\pm0.63$	$36.08^{AB}\pm0.76$	$37.33^{B}\pm0.94$

Mean values marked with the capital letter show difference at ($P\leq0.01$). Different superscripts (A, B) within columns differ significantly. N, Number of samples analyzed; Pattern F- uncapacitated sperm; Pattern B, capacitated sperm with intact acrosome; Pattern AR, acrosome-reacted sperm.

Table 3. Path velocity and motion characters exhibited by sperm in semen diluted with extender containing low-density lipoprotein during cryopreservation (N-32)

Treatment	Progressive motiliy (%)	VCL (μm/sec)	VAP (μm/sec)	VSL (μm/sec)	LIN (%)	STR (%)	BCF (Hz)	ALH (μm)
Control	40.50^{C}	105.00^{B}	47.00^{B}	38.50^{C}	35.22^{b}	75.33^{b}	18.71^{AB}	3.72^{B}
(Egg yolk- 20%)	±0.61	±0.86	±0.68	±1.34	±1.24	±2.37	±0.67	±0.13
Treatment-1	39.83^{C}	86.33^{D}	36.33^{D}	30.00^{E}	34.45^{b}	75.08^{b}	16.28^{C}	3.01^{C}
(7% LDL)	±0.90	±1.74	±1.11	±0.89	±1.01	±1.72	±0.92	±0.16
Treatment-2	43.58^{B}	106.00^{B}	47.67^{B}	41.33^{B}	38.82^{a}	81.20^{a}	20.20^{A}	3.45^{BC}
(8% LDL)	±0.81	±0.81	±0.80	±0.88	±1.23	±1.69	±0.71	±0.16
Treatment-3	52.58^{A}	119.50^{A}	55.50^{A}	45.83^{A}	36.98^{ab}	76.48^{ab}	19.45^{AB}	4.48^{A}
(9% LDL)	±0.99	±2.63	±1.17	±0.79	±0.74	±0.99	±0.62	±0.28
Treatment-4	40.75^{C}	96.00^{C}	41.83^{C}	34.16^{D}	33.81^{b}	71.48^{b}	17.93^{BC}	3.52^{BC}
(10% LDL)	±0.79	±1.50	±0.70	±0.30	±0.81	±1.50	±0.52	±0.15

Mean values marked with the capital letter show difference at ($P\leq0.01$) and small letter at ($P\leq 0.05$). Different superscripts (A,B) within columns differ significantly. N, Number of samples analyzed; VCL, curvilinear velocity; VAP, average path velocity; VSL, straight line velocity; LIN, linearity; STR, straightness; BCF, beat cross frequency; ALH, amplitude of lateral head displacement.

during freezing induce lipid peroxidation that results in premature capacitation and reduce the survival of spermatozoa (Naresh and Atreja 2015). Low-density lipoprotein fraction has been reported to have the highest protective ability during cryopreservation (Neves *et al.* 2014). Low-density lipoproteins provide protection by associating with sperm membrane (Bellin *et al.* 1998). It prevents the loss of membrane phospholipids and cholesterol which increases the sperm capacity to maintain membrane structure and prevents premature capacitation (Parks and Graham 1992). In bull, LDL prevents seminal plasma protein (BSP-A1/A2, BSPA3 and BSP-30-kDa) to bind with sperm surface that triggers cholesterol and phospholipids efflux from the sperm membrane (Vera Munoz *et al.* 2009) thus reducing premature capacitation. The higher ($P<0.01$) values of sperm with uncapacitated state in T-3 may be the attributed to LDL capacity to bind seminal proteins and stabilize sperm plasma membrane that maintain uncapacitated state of sperm acrosome during the cryopreservative process.

The kinematic characters exhibited by sperm cell are presented in Table 3. The sperm exhibiting forward progression were significantly ($P<0.01$) higher in T-3 as compared to other treatment groups and control. Energy production system together with the flagellar apparatus is largely responsible for sperm progression in the fluid medium (Pereira *et al.* 2017). Any disturbance in the sperm plasma membrane integrity greatly influence the physiological processes in sperm cell that inturn affect energy production and its utilization for sperm progression resulting in reduced motility (Gadella and Luna 2014). The results when evaluated together with the physical seminal attributes depicted that low density lipoproteins at 9% level in T-3 has better capacity to maintain the sperm plasma membrane thus resulting in higher proportion of sperm with forward progression. Progression of sperm in fluid medium is the cumulative effect of its curvelinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP) (Anand *et al.* 2016). Path velocities together with the motion characters i.e linearty (Lin%), straightness (Str%), beat cross frequency (BCF), and amplitude of lateral displacement (ALH) depict the path adopted by sperm. These kinematic

characters have a positive correlation with sperm travelling time and its transport to fertilization site (García Vázquez *et al.* 2016). During the experiment, significantly higher values of different path velocities was recorded in T-3 as compared to other treatment group and control. Semen dilution affects the sperm motility and influence the way in which the spermatozoa travel in fluid medium (Peitz 1988). Sperm in suspension interacts with its surrounding fluid medium that comprise of seminal secretion and extender. The extender although provide the environment for sperm survival, yet make the medium viscous affecting sperm movements (Rittem *et al.* 2012). The net progression of sperm in diluted semen is cumulative effect of sperm interaction with its surrounding environment (Garner *et al.* 1997). The medium that imparts protection against the cold shock, cryoinjuries, ATP utilization, gas exchange, and ionic balance and osmotic stress, thus maintains the membrane characters of sperm exhibit better progression. The low-density lipoproteins in T-3 recorded better capacity to withstand temperature related injuries, protect plasma membrane and maintained sperm characters, thus recorded a better sperm kinematic in post thaw semen. In conclusion, extender containing 9% low density lipoproteins has better capacity to maintain the sperm character in post thaw Hariana bull semen and can be a preferred concentration to be utilized for semen cryopreservation with improved conception rate.

ACKNOWLEDGEMENT

The authors are thankful to Vice Chancellor, DUVASU and Dean, College of Veterinary Sciences and Animal Husbandry for providing funds and facilities to pursue this research work.

REFERENCES

Abouelezz F M, Castano C, Toledano-Diaz A, Esteso M C, Lopez-Sebastian A, Campo J L and Santiago- Moreno J. 2015. Sperm-egg penetration assay assessment of the contraceptive effects of glycerol and egg yolk in rooster sperm diluents. *Theriogenology* **83**: 1541–47.

Akhter S, Ansari M S, Rakha B A, Ullah N, Andrabi S M H and Khalid M. 2011. *In vitro* evaluation of liquid-stored buffalo semen at 5ºC diluted in soya lecithin based extender (Bioxcell), tris-citric egg yolk, skim milk and egg yolk-citrate extenders. *Reproduction in Domestic Animal* **46**: 45–49.

Amirat L, Anton M, Tainturier D, Chatagnon G, Battut I and Courtens J. 2005. Modifications of bull spermatozoa induced by three extenders: Biociphos, low density lipoprotein and Triladyl, before, during and after freezing and thawing. *Reproduction* **129**: 535–43.

Anand M, Yadav, Vaswani S and Shukla P K. 2015. Low density lipoprotein (LDL) as cryoprotectant in semen extender: A new approach. *Asian Journal of Animal Science* **10**: 211–19.

Baracaldo M I, Barth A D and Bertrand W. 2007. Steps for freezing bovine semen: From semen collection to the liquid nitrogen tank. *IVIS Reviews in Veterinary Medicine.* International Veterinary Information Service, Ithaca, NY.

Bellin M E, Oyarzo J N, Hawkins H E, Zhang H, Smith R G, Forrest D W, Sprott L R and Ax R L. 1998. Fertility-associated antigen on bull sperm indicates fertility potential. *Journal of Animal Science* **76**: 2032–39.

Bucak M N, Atessahin A and Yuce A. 2008. Effect of antioxidant and oxidative stress parameters on ram semen after the freeze-thawing process. *Small Ruminant Research* **75**: 128–34.

Chaudhari D V, Dhami A J, Hadiya K K and Patel J A. 2015. Relative efficacy of egg yolk and soya milk-based extenders for cryopreservation (–196°C) of buffalo semen. *Veterinary World* **8**: 239–44.

Collin S, Sirard M, Dufour M and Bailey J. 2000. Sperm calcium levels and chlortetracycline fluorescence patterns are related to the *in vivo* fertility of cryopreserved bovine semen. *Journal of Andrology* **21**: 938–43.

Crespilho A M, Sa´ Filho M F, Dell'Aqua Jr. J A, Nichi M, Monteiro G A, Avanzi B R, Martins A and Papa F O. 2012. Comparison of *in vitro* and *in vivo* fertilizing potential of bovine semen frozen in egg yolk or new lecithin based extenders. *Livestock Science* **149**: 1–6.

Gadella B M and Luna C. 2014. Cell biology and functional dynamics of the mammalian sperm surface. *Theriogenology* **81**: 74–84.

García Vázquez F A, Gadea J, Carmen Matas C and Holt W V. 2016. Importance of sperm morphology during sperm transport and fertilization in mammals. *Asian Journal of Andrology* **18**: 844–50.

Garner D L, Thomas C A and Allen C H. 1997. Effect of semen dilution on bovine sperm viability as determined by dual-DNA staining and flow cytometry. *Journal of Andrology* **18**: 324–31.

Hancock J L. 1952. The morphology of bull spermatozoa. *Journal of Experimental Biology* **29**: 445–53.

Ikawa M, Inoue N, Benham A M and Okabe M. 2010. Fertilization: a sperm's journey to and interaction with the oocyte. *Journal of Clinical Investigation* **120**: 984–94.

Jeyendran R S, Van H H, Perez-Pelaez M, Crabo B G and Zanevald L J D. 1984. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. *Journal of Reproduction and Fertility* **70**: 219–28.

Leahy T and Gadella B. 2016. Capacitation and capacitation-like sperm surface changes induced by handling boar semen. *Reproduction in Domestic Animals* **46**: 7–13.

Morris G J and Clark A. 1987. Cell at low temperatures. (Eds) Grout B W W and Morris G J. *The Effects of Low Temperatures on Biological Systems*. pp. 72–119. Edward Arnold, London.

Moussa M, Martinet V, Trimeche A, Tainturier D and Anton M. 2002. Low density lipoproteins extracted from hen egg yolk by an easy method: cryoprotective effect on frozen thawed bull semen. *Theriogenology* **57**: 1695–706.

Naresh S and Atreja S K. 2015. The protein tyrosine phosphorylation during *in vitro* capacitation and cryopreservation of mammalian spermatozoa. *Cryobiology* **70**: 211–16.

Neves M M, Heneine L G D and Henry M. 2014. Cryoprotection effectiveness of low concentrations of natural and lyophilized LDL (low density lipoproteins) on canine spermatozoa. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia* **66**: 769–77.

Parks J E and Graham J K. 1992. Effects of cryopreservation procedures on sperm membranes. *Theriogenology* **38**: 210–23.

Pereira R, Sa R, Barros A and Sousa M. 2017. Major regulatory mechanisms involved in sperm motility. *Asian Journal of*

Andrology **19**: 5–14.

Ray Kalyani R, Gojen S L and Bidhan S. 2015. Cryopreservation of Black Bengal buck semen by Tris-based extenders containing different levels of egg-yolk. *Annals of Veterinary and Animal Science* **2**: 47–54.

Rittem S, Thongthainun D, Tipkantha W, Siriaroonrat B and Thongtip N. 2012. Effects of semen extender on motility and movement patterns of frozen-thawed eld's deer (*Cervus eldii*) spermatozoa. *Thai Journal of Veterinary Medicine* **42**: 527–32.

Vera Munoz O, Amirat-Briand L, Diaz T, Vasquez L, Schmidt E, Desherces S, Anton M, Bencharif D and Tainturier D. 2009. Effect of semen dilution to low-sperm number per dose on motility and functionality of cryopreserved bovine spermatozoa using low-density lipoproteins (LDL) extender: comparison to Triladyl® and Bioxcell®. *Theriogenology* **71**: 895–900.