

Original Research Paper

Study the Effect of Extracted Low-Density Lipoprotein from Egg Yolk on the Cryopreservation of Nguni Bull Semen

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Abstract: The mechanism on how egg yolk (EY) helps sperm to survive the low temperature outside the animal body during cryopreservation and thawing process is not yet known. It is usually believed that the Low-Density Lipoprotein (LDL) is the major component that provides protection. The present study was undertaken to assess the cryo-protective effect of different concentrations (6, 8, 10 and 12%) of LDL and 20% EY (control) on sperm motility, morphological parameters and cleavage rate of the cows ovaries following freezing and thawing. Semen was collected from five Nguni bulls aged 2-4 years of known and proven fertility with an aid of electro-ejaculated and immediately transported to the laboratory. The LDL was extracted from the chicken egg yolk and further processed. The collected semen samples were evaluated for microscopic sperm motility and morphological parameters. Thereafter, the semen samples were diluted with different concentrations (6, 8, 10 and 12%) of LDL and 20% EY (control) extender supplemented with 12% Glycerol as a cryoprotectant. Semen samples were loaded into 0.25 straws, placed into a controlled rate programmable freezer and stored in liquid nitrogen tank. Following semen thawing, sperm motility, morphological were evaluated. Furthermore, *in vitro* fertilization (IVF) was conducted on cow's oocytes to test the fertilizing ability of semen diluted with LDL on the cleavage rate. Data was analyzed with the aid of ANOVA. Furthermore, treatment means were compared with the Least Significance Difference (LSD) test. The percentage of motility parameters and percentage of live sperm with intact plasma membrane, acrosome membrane and DNA integrity were significantly higher ($P<0.05$) in semen diluted with 20% EY (control) and 8% compared to semen diluted with (6, 10 and 12% LDL). Cleavage rates were significantly higher ($P<0.05$) higher in extender containing 20% EY (control) and 8% LDL. In conclusion, 8% LDL extender can be used as an alternative to 20% EY extender in preserving the sperm motility rate, percentage of live sperm, intact plasma membrane, acrosome, DNA integrity and fertilizing ability of frozen-thawed Nguni bull semen.

Keywords: Cryopreservation, DNA Integrity, Egg Yolk, Low-Density Lipoprotein, *In vitro* Fertilization

Introduction

The indigenous Nguni cattle breed is adapted to South African (SA) environment; it is hardy with superior genetic material. The indigenous Nguni cattle breed is multi-coloured or uncolored (brown, grey, red, white, black cream and dun). The Nguni cattle have traits that make them favourable for resource-poor cattle farmers of

SA. It is further tolerant to nematodes, ticks and tick-borne diseases, has good temperament, good walking ability and can be in good condition even during winter season (Spickett *et al.*, 1989; Muchenje, 2007; Ndlovu, 2007). These characteristics make the Nguni breed to be conserved through utilization. The Nguni cattle gene pool is almost on the verge of extinction due to crossbreeding with other exotic genotypes. This leads to the need to

cryopreserve its genetic material. Cryopreservation is the freezing of cells or tissues at sub-zero temperatures, typically -196°C , until future use. Cryopreservation has been practiced as a routine technique to preserve bovine sperm for artificial insemination (Watson *et al.*, 1995). This technique results in the loss of 40-50% of viable sperm during the freeze-thaw process and little improvement over the last decades has been reported (Prathalingam *et al.*, 2006). Cold shock, osmotic stress, ice crystal formation or oxidative damage is the main causes of sperm cryo-injury which lead to the loss of sperm viability and fertility (Mazur, 1984).

This is mainly due to freeze-thawing process which exposes the semen to thermal shock that causes a detrimental effect to the structure, biochemical and functional damage leading to a reduction of sperm motility, membrane integrity, damage to DNA, membrane damage and ultimately a reduction in the fertilizing ability of sperm (Salamon and Maxwell, 1995; Tekin *et al.*, 2006). The establishment of a cryoprotectant such as egg yolk (EY) added a new era in semen cryopreservation. The EY serve as the most effective cryoprotective agent of freezing extender that protects the sperm against cold shock (Bergeron *et al.*, 2005). The EY has been successfully used as a cryoprotectant for cryopreservation of bull semen. Higher survival and fertility rates of bull sperm with 20% egg yolk have been reported. Despite the successes, there are reservation for its use due to possible risks of contamination and loss of semen quality (Thun *et al.*, 2002). Others have suggested that the components of EY, like the Low-Density Lipoprotein (LDL), have a better cryoprotective effect than EY (Bencharif *et al.*, 2008). In particular, the LDL has been implicated as the main EY component leading to improved semen quality following cryopreservation (Amirat *et al.*, 2004; Bencharif *et al.*, 2008). During the freeze-thawing process, LDL forms a protective cover at the surface of the sperm membrane (Bergeron *et al.*, 2004). The LDL interact specifically with Bovine Seminal Plasma (BSP) proteins and decreased the binding of the major proteins of BSP to the sperm, preventing lipid efflux from the sperm membrane. The LDL may offer protection to sperm by reducing the deleterious effect of seminal plasma proteins on sperm membranes. achieved acceptable sperm motility results when the EY was substituted with the 8% (w/v) LDL in frozen-thawed bull semen. Therefore, the objectives of this study were to evaluate the effect of different concentrations of LDL on sperm motility, viability, membrane integrity, acrosome integrity and DNA integrity and to determine the fertilizing potential of the semen diluted with LDL on the cleavage rate.

Materials and Methods

Study Site

The study was conducted at the Agricultural Research Council-Irene at Germplasm Conservation and Reproductive Biotechnology laboratory. The area is located at $25^{\circ}53' 59.6''$ south latitude and $28^{\circ}12' 51.6''$ east longitude in Pretoria, SA and situated on the Highveld with altitude of 1525 m above sea level. The experimental procedures were approved by the Tshwane University of Technology (AREC2015/10/004) and the Agricultural Research Council Ethics Committee (APIEC15/041).

Nguni Bulls Management

The Nguni bulls ($n = 5$), aged between two to four years were utilized for semen collection. All bulls were maintained on natural pastures and water was provided *ad libitum*. Semen was collected twice per week from five Nguni bulls for five weeks with the aid of an electro-ejaculator into a 15 mL Falcon tube during the natural breeding season and ten replicates were done in this experiment after collection, the semen samples were kept in thermoflask that contained warm water at 37°C and transported to the laboratory for macro and microscopically semen analysis.

Extraction of Low-Density Lipoprotein

The LDL was extracted according to the technique defined by. Fresh eggs were collected and broken manually by separating the egg yolk from the albumin. Egg yolk was diluted three times with an isotonic saline solution (0.17 M NaCl) and stirred using a magnetic stirrer for 1 h before being centrifuged at $10,000 \times g$ for 45 min at 4°C . The plasma (supernatant) was collected and diluted mixed 40% ammonium sulphate (1:1 ratio) to precipitate livetins. After 1 h of stirring at 4°C , the mixture was centrifuged at $10,000 \times g$ for 45 min to separate the plasma from the granules. The granules were discarded and the plasma was dialyzed for 12 h against distilled water in order to remove ammonium sulphate. After the complete removal of ammonium sulphate, the mixture was centrifuge again at $10,000 \times g$ for 45 min at 4°C and the floating residue, which is rich in LDL, was collected.

Bulls Semen Evaluation

Briefly, 5 μL of diluted semen was pipetted on a microscope slide and covered with a warm glass slide (76-26-1 mm, Germany) over the microscope warm plate (Omron) adjusted at 37°C . The sperm motility rates were evaluated before and after freeze-thawing by using a Sperm Class Analyzer[®] system (Microptic, Spain) at the magnification of $\times 10$ (Nikon, China). The sperm morphology was recorded after staining the semen samples with Eosin-nigrosin, Acridine-Orange, FITC-PSA and host solution on a microscope slide. The stained-glass

slide was placed on the microscope table for evaluation. Before evaluation, a drop of immersion oil was poured on the stained-glass slide, a fluorescent microscope (Olympus, Corporation BX 51FT, Tokyo, Japan) was used at x 100 magnification and a total of 200 sperm were counted per slide.

Bulls Semen Dilution, Freezing and Thawing

Freshly collected semen was divided into five equal segments. One segment was diluted (1:1) with the egg yolk citrate extender, without a cryoprotectant (fraction A) and stored at 5°C for 2 h. Subsequently, a second (fraction B) dilution (1:2) with egg yolk citrate extender containing 12% of Glycerol (GLY) was further equilibrated for another 2 h at 5°C. The other four equal segments were diluted (1:1) with different concentration 6, 8, 10, 12% of LDL, without a cryoprotectant (fraction A) and stored at 5°C for 2 h. Subsequently, a second (fraction B) dilution (1:2) with different concentration 6, 8, 10, 12% of LDL containing 12% of GLY was further equilibrated for 2 h at 5°C. Following the 2 h of equilibration with fraction B, the semen samples were loaded into 0.25 mL polyvinyl chloride straws, sealed and placed horizontally into a controlled rate programmable freezer from 5 to -5°C at 0.008°C min and from -4 to -130°C at 6°C min (Seshoka *et al.*, 2012). When the target temperature of -130°C was reached, the frozen straws of the semen samples were plunged directly into the liquid nitrogen tank (-196°C) for storage. A week after storing the frozen semen sample in liquid nitrogen tank, the frozen straws were thawed in a water bath at 37°C for 1 min. Thawed semen samples were evaluated for sperm motility traits using Sperm Class Analyzer® system and morphological traits using a fluoresce microscope.

Evaluation of Live and Dead Sperm from Nguni Bulls

The live and dead sperm of the fresh and frozen-thawed semen were determined using eosin/nigrosine swelling (Onderstepoort, Faculty of Veterinary Science Pharmacy, SA) and 5 µL of raw semen was mixed with 20 µL of Eosin-Nigrosin swelling. However, 7 µL of the mixture was smeared on a glass slide. The stained slides were allowed to dry at room temperature before being placed on a microscope table and a drop of immersion oil was placed on the slide to evaluate live and dead sperm using a fluorescent microscope (Olympus, BX 51FT, Tokyo, Japan) under × 100 magnification. Live and dead sperm were determined by evaluating the percentage of sperm that absorbed the swelling, those ones that absorbed the swelling were considered as dead, while the ones that did not absorbed the swelling were considered live and 200 sperm were counted per bull, per replicate.

Evaluation of Sperm Membrane Integrity

Sperm membrane integrity was evaluated by using a hypo-osmotic swelling test before and after freezing of the Nguni semen samples. A semen volume of 0.1 mL was mixed with 1 mL of hypo-osmotic swelling (8.71 g/L of fructose and 4.47 g/L of sodium chloride, pH 8.05) (Sigma - Aldrich, SA) (Pty) (Ltd) and incubated for 1 h at 37°C (Naing *et al.*, 2010). Following incubation, a drop of diluted semen was placed on a glass slide, covered with a cover slip and analysed under a phase contrast microscope (x 100). A total number of 200 sperm were analysed from each slide. Sperm with a swollen and coiled tail were recorded as intact (Naing *et al.*, 2010).

Evaluation of Sperm Acrosome Integrity

The Nguni bull sperm acrosome integrity was determined using isothiocyano-fluoresceinated Pisum sativum agglutinin (FITC-PSA) (Partyka *et al.*, 2010). Briefly, 20 µL of the diluted semen was suspended in 500 µL of Phosphate Buffer Saline (PBS) and centrifuged for 10 min at 100 xg, the supernatant was discarded. The sperm pellet was re-suspended in 250 µL of PBS. One drop of the re-suspended semen was smeared on a glass slide and air dried; this was followed by fixing the air-dried slide with acetone for 10 min at 4°C. The smears of sperm suspensions were mounted with FITC-PSA swelling (50 µg/mL in PBS swelling) and kept in a dark room for 30 min. The slide was then rinsed with a PBS swelling and examined under a fluorescence microscope. A total of 200 sperm were evaluated from each stained glass slide for acrosome integrity.

Evaluation of Sperm DNA Integrity

The sperm DNA integrity was determined by using a method described by Tejada *et al.* (1984). Acridine Orange (Sigma-Aldrich, St Louis, MO, USA) was used to analyse the DNA integrity of the Nguni bull sperm. Briefly, 20 µL of the fresh and frozen-thawed semen was transferred to a 15 mL conical tube and washed three times with PBS using the centrifugation machine. A drop of the sperm pellet was smeared on a glass slide using a pipette tip to avoid breakage of the sperm and allowed to air-dry for ten minutes. The smeared glass slide was then fixed for 1 h in ethanol-aceton (1:1) inside a plastic container lid at 4°C and allowed to air-dry for few minutes. The glass slide was then stained with Acridine-Orange (0.19 mg/m) for 7 min at the room temperature in the dark. The stained glass slide was gently rinsed with distilled water and air dried, then viewed under a fluorescence microscope (Olympus, BX 51FT, Tokyo, Japan) at × 100 magnification under oil immersion. A total number of 200 sperm were evaluated by using a fluorescence microscope (Olympus, BX 51FT, Tokyo, Japan) under × 100 magnification. Sperm with an intact (double stranded) DNA fluorescence green, while sperm with a damaged

DNA fluorescence red or yellow-orange to red and these sperm were counted as sperm with a single stranded DNA.

In Vitro Fertilization of Oocytes

Matured oocytes were placed in the fertilization (Bracket and Oliphant IVF) medium. Frozen- thawed semen diluted with 8% low-density lipoprotein and 20% egg was used. Semen was thawed in a water bath at 37°C for 1 min. Semen was washed twice by centrifugation (800 g for 8 min) in a pre-warmed Bracket and Oliphant washing medium. Thereafter, the supernatant was removed with a serological pipette. For fertilization, 50 μ L of the diluted bull semen solution with a sperm concentration of approximately 1×10^6 /mL was utilized to inseminate each 50 μ L droplets (final volume of 100 μ L). Subsequently, oocytes together with sperm were incubated for 18 h at 38.5°C in 5% CO₂ in air. Following *in vitro* fertilization, presumptive zygotes were washed by vortexing for 1 min and 30 sec, in order to remove the cumulus complex. The presumptive zygotes were washed three times with a pre-warmed tissue culture medium. Subsequently, presumptive zygote were transferred to a pre-warmed 50 μ L droplets of synthetic oviduct fluid medium in a petri dish which was covered with mineral oil and incubated for two days at 38.5°C in 5% CO₂ and N₂. The cleavage rate was determined after 48 h of culture by recording the number of oocytes that developed into a two- and four-cell stage.

Statistical Analysis

The experiment was replicated five times for each treatment group. The results were expressed as mean values \pm standard error. The comparison of the percentage of motile sperm was performed by utilizing the t-test. The average values of the percentage of sperm with different solution in each experiment, the live and dead spermatozoa, plasma membrane integrity, acrosome integrity and DNA integrity were compared by using a Duncan's multiple range test by ANOVA procedure, once the F-value was significant ($P < 0.05$). All statistical evaluations were performed using Statistical Product and Services Solutions (SPSS 11.5 for Windows; SPSS, Chicago, IL, U.S.A).

Results and Discussion

The results of the effect of LDL on sperm motility parameters of fresh and frozen-thawed Nguni semen are presented in Table 1. For all the sperm motility parameters measured, both 10 and 12% LDL were worst substitutes for egg yolk. The highest sperm total motility was recorded with 8 LDL (54.8 \pm 4.3) followed by the diluent containing 6% LDL (41.5 \pm 5.2) while 20% egg yolk was 63.9 \pm 3.6. For progressive motility and rapid motility, no significant differences were found between 6 LDL, 8% LDL and 20% egg yolk.

The results of the effect of different concentration of LDL on live and dead sperm percentage (Means \pm SEM) of frozen thawed sperm are presented in Table 2. There was no statistical difference ($P > 0.05$) observed in the percentage of live sperm following freezing and thawing of Nguni bull semen diluted with 6% (64.9 \pm 2.4) LDL and 8% (65.5 \pm 2.1) LDL. The frozen-thawed semen sample diluted with 10% (40.2 \pm 5.3) LDL and 12% (27.0 \pm 5.5) LDL had the lowest percentage of live sperm, while the semen diluted with 20% (77.3 \pm 5.9) EY had a statistical ($P < 0.05$) higher percentage of live sperm compared to the semen diluted with 6% (64.9 \pm 2.4), 8% (65.5 \pm 2.1), 10% (40.2 \pm 5.3), and 12% (27.0 \pm 5.5) LDL. Therefore, no statistical difference ($P > 0.05$) observed on the percentage of dead sperm diluted with 6% (34.5 \pm 2.1) and 8% (34.9 \pm 2.4) LDL following freezing and thawing. There was a significant difference ($P < 0.05$) observed in the frozen-thawed percentage of dead sperm diluted with 10% (59.9 \pm 5.5) and 12% (72.9 \pm 5.5) LDL. Semen diluted with 12% (72.9 \pm 5.5) LDL had a high percentage of dead sperm. The frozen-thawed semen samples diluted with 20% (27.6 \pm 5.9) EY had the lower percentage of dead sperm compared to the frozen thawed semen diluted with (6, 8, 10 and 12%) LDL.

The results of the effect of different concentration on sperm plasma membrane integrity percentage (Means \pm SEM) of frozen-thawed Nguni bull semen are presented in Table 3. The highest percentage of sperm having an intact plasma membrane integrity was recorded in the semen diluted with 8% (66.0 \pm 2.8) LDL followed by the semen diluted with 6% (63.3 \pm 1.9) LDL, the semen diluted with 10% (38.8 \pm 3.9) and 12% (27.6 \pm 3.4) LDL had a lower percentage of intact plasma membrane integrity. The semen diluted with 20% (71.5 \pm 4.7) control had a significantly ($P < 0.05$) higher percentage of sperm having an intact plasma membrane compared to the semen diluted with (6, 8, 10 and 12%) LDL. However, 12% LDL had the highest percentage (72.3 \pm 3.4) of damaged sperm plasma membrane integrity followed by the semen diluted with 10% (61.2 \pm 4.0) of LDL.

The results of the effect of different concentrations of LDL on the sperm acrosome integrity percentage (Means \pm SEM) of frozen-thawed Nguni bull semen are presented in Table 4. The analysis revealed that there was no significant difference in the acrosome integrity diluted with 6% (64.0 \pm 2.0) and 8% (65.8 \pm 2.5) LDL ($P > 0.05$). The semen diluted with 10 (38.9 \pm 3.9) and 12% (25.8 \pm 4.3) LDL had low percentage of intact acrosome integrity. Semen diluted with 20% (73.1 \pm 4.3) egg yolk had a significantly ($P < 0.05$) high percentage of sperm with an intact acrosome, while the semen diluted with 12% (74.5 \pm 4.1) had a high percentage of sperm with damaged acrosome integrity followed by the semen diluted with 10% (61.0 \pm 4.0) LDL. These suggest that semen diluted with LDL afford better protection to the sperm acrosome integrity compared to the semen diluted with (6, 10 and 12%) LDL during the freeze-thawing process and that the most efficient concentration is 8% LDL.

The results of the effect of different concentrations of LDL on the sperm acrosome integrity percentage (Means \pm SEM) of frozen-thawed Nguni bull semen are presented in Table 5. The results show no significant ($P>0.05$) difference between the semen diluted with 6% (64.4 \pm 2.4) and 8% (66.2 \pm 2.4) LDL. There was a significant ($P<0.05$) difference observed between semen diluted with 10% (49.8 \pm 14.3) and 12% (27.8 \pm 2.9) with the semen diluted with 12% (27.8 \pm 2.9) LDL having a high percentage of sperm with an intact DNA integrity. Semen diluted with 20% egg yolk (control) had a significantly ($P<0.05$) higher percentage of sperm with an intact DNA integrity compared to the semen diluted with (6, 8, 10 and 12%) LDL. There was no significant ($P>0.05$) difference observed between the semen diluted with 6% (35.3 \pm 2.4) and 8% (33.7 \pm 2.4) LDL on the percentage of sperm with a damaged DNA integrity. There was a significant ($P<0.05$) difference between the semen diluted with 10% (50.1 \pm 14.3)

and 12% (72.1 \pm 2.9) LDL on the percentage of sperm with a damaged DNA integrity, although the semen diluted with 12% (72.1 \pm 2.9) LDL had a high percentage of sperm with a damaged DNA integrity. These results suggest that 8% LDL can offer better protection to the sperm DNA integrity during the freeze-thawing process.

The percentage of cleaved embryos was significantly different ($P<0.05$) between the semen diluted with 8% LDL (21.2 \pm 3.5) and 20% EY (25.8 \pm 2.4) (Table 6). There was a significant difference ($P<0.05$) on the embryonic development rates from 2-cell on the semen diluted with 8% LDL and 20% EY (12.8 \pm 1.9 and 12.8 \pm 1.9, respectively), with the semen diluted with 20% EY having the highest percentage of embryos that develop into a 2-cell stage. At the 4-cell stage, there was also a significant difference ($P<0.05$) observed between the semen diluted with 8% LDL and 20% EY (7.1 \pm 1.5 and 10.4 \pm 1.2, respectively).

Table 1: The effect of different LDL concentrations on the sperm motility parameters of the frozen-thawed Nguni bull semen (Means \pm SEM)

Treatments	Total motility (%)	Progressive motility (%)	Non-progressive motility (%)	Rapid (%)	Medium (%)	Slow (%)	Static (%)
Raw semen	94.4 \pm 6 ^a	34.3 \pm 15 ^a	60.0 \pm 18.0 ^a	39.6 \pm 22.7 ^a	37.2 \pm 19.9 ^a	17.8 \pm 15.8 ^c	5.6 \pm 6.7 ^d
6% LDL	41.2 \pm 5.2 ^d	9.0 \pm 5.3 ^b	32.1 \pm 5.2 ^d	2.1 \pm 2.6 ^b	12.0 \pm 7.2 ^d	26.1 \pm 8.3 ^b	59.5 \pm 13.3 ^b
8% LDL	54.8 \pm 4.3 ^c	12.4 \pm 6.4 ^b	42.6 \pm 6.5 ^c	3.8 \pm 3.6 ^b	17.8 \pm 9.8 ^{bc}	41.5 \pm 17.6 ^b	6.6 \pm 22.0 ^c
10% LDL	28.0 \pm 6.5 ^e	4.5 \pm 2.7 ^d	23.4 \pm 5.6 ^e	3.0 \pm 3.6 ^b	6.8 \pm 3.1 ^{de}	18.2 \pm 6.5 ^c	1.8 \pm 6.5 ^b
12% LDL	21.2 \pm 7.3 ^f	3.7 \pm 3.3 ^d	18.0 \pm 6.4 ^f	0.6 \pm 1.6 ^c	3.8 \pm 3.3 ^d	20.4 \pm 15.1 ^b	7.4 \pm 9.9 ^a
20% EY	63.9 \pm 3.6 ^b	10.6 \pm 7.7 ^b	53.3 \pm 7.8 ^b	5.0 \pm 4.8 ^b	18.2 \pm 11.4 ^b	44.5 \pm 11.3 ^a	32.2 \pm 8.3 ^c

^{a-f} values with different superscript within column differ significantly at $P<0.05$

LDL = Low-density lipoprotein; EY= Egg yolk (control)

Table 2: The effect of different LDL concentrations on percentage of live and dead sperm of the frozen-thawed Nguni bull semen (Means \pm SEM)

Treatments	Live sperm (%)	Dead sperm (%)
Raw semen	91.4 \pm 3.1 ^a	8.5 \pm 3.1 ^e
6% LDL	64.9 \pm 2.4 ^c	34.5 \pm 2.1 ^c
8% LDL	65.5 \pm 2.1 ^c	34.9 \pm 2.4 ^c
10% LDL	40.2 \pm 5.3 ^d	59.9 \pm 5.5 ^b
12% LDL	27.0 \pm 5.5 ^e	72.9 \pm 5.5 ^a
20% EY	77.3 \pm 5.9 ^b	27.6 \pm 5.9 ^d

^{a-e} values with different superscripts within column differ significantly at $P<0.05$

LDL = Low-density lipoprotein; EY = Egg yolk (control)

Table 3: The effect of different LDL concentration on the sperm plasma membrane integrity of the frozen-thawed Nguni bull semen (Means \pm SEM)

Treatments	Intact plasma membrane integrity (%)	Damaged plasma membrane (%)
Raw semen	91.9 \pm 2.9 ^a	10.2 \pm 4.1 ^f
6% LDL	63.3 \pm 1.9 ^d	36.6 \pm 1.9 ^c
8% LDL	66.0 \pm 2.8 ^c	33.9 \pm 2.8 ^d
10% LDL	38.8 \pm 3.9 ^e	61.2 \pm 4.0 ^b
12% LDL	27.6 \pm 3.4 ^f	72.3 \pm 3.4 ^a
2% EY	71.5 \pm 4.7 ^b	28.4 \pm 4.7 ^e

^{a-f} values with different superscripts within column differ significantly at $P<0.05$

LDL = Low-density lipoprotein; EY = Egg yolk (control)

Table 4: The effect of different concentrations of LDL on the sperm acrosome integrity percentage (Means \pm SEM) of frozen-thawed Nguni bull semen

Treatments	Intact acrosome (%)	Damaged acrosome (%)
Raw semen	88.0 \pm 3.3 ^a	11.9 \pm 3.4 ^c
6% LDL	64.0 \pm 2.0 ^c	35.9 \pm 2.0 ^c
8% LDL	65.8 \pm 2.5 ^c	34.1 \pm 2.5 ^c
10% LDL	38.9 \pm 3.9 ^d	61.0 \pm 4.0 ^b
12% LDL	25.8 \pm 4.3 ^e	74.5 \pm 4.1 ^a
20% EY	73.1 \pm 4.3 ^b	27.2 \pm 4.1 ^d

^{a-e} values with different superscripts with column differ significantly at $P < 0.05$

LDL= Low-density lipoprotein; EY = Egg yolk (control)

Table 5: The effect of different concentrations of LDL on the DNA sperm integrity of the frozen-thawed Nguni bull semen (Means \pm SEM)

Treatments	Intact DNA (%)	Damaged DNA (%)
Raw semen	89.0 \pm 4.6 ^a	11.9 \pm 3.4 ^c
6% LDL	64.4 \pm 2.4 ^c	35.9 \pm 2.0 ^c
8% LDL	66.2 \pm 2.4 ^c	33.7 \pm 2.4 ^c
10% LDL	49.8 \pm 14.3 ^d	50.1 \pm 14.3 ^b
12% LDL	27.8 \pm 2.9 ^e	72.1 \pm 2.9 ^a
20% EY	76.0 \pm 7.1 ^b	23.9 \pm 7.1 ^d

^{a-e} values with different superscripts within column differ significantly at $P < 0.05$

LDL = Low-density lipoprotein; EY = Egg yolk (control)

Table 6: Fertilization rate following using frozen-thawed Nguni bull semen diluted with 8 LDL and 20% EYC

Treatments	Maturation (%; n)	Total cleavage (%; n)	2-cell stage (%; n)	4-cell stage (%; n)
8% LDL	32.1 \pm 7.1 ^a (223)	21.2 \pm 3.5 ^b (149)	12.8 \pm 1.9 ^b (90)	7.1 \pm 1.5 ^b (50)
20% EY	34.4 \pm 4.9 ^a (241)	25.8 \pm 2.4 ^a (170)	17.0 \pm 2.3 ^a (119)	10.4 \pm 1.2 ^a (73)

^{a,b} values with different superscripts with column differ significantly at $P < 0.05$

LDL = Low-density lipoprotein; EY = Egg yolk

This study was conducted to evaluate the effect of different concentrations of extracted LDL from chicken egg yolk on the cryopreservation of Nguni bull semen and fertilizing ability of semen cryopreserved with LDL. There was a statistical difference ($P < 0.05$) on the total sperm motility of the frozen-thawed Nguni bull semen diluted with 20% EY compared to the frozen-thawed semen diluted with (6, 8, 10 and 12%) LDL. Semen diluted with 20% EY resulted in higher sperm total motility (63.9) as compared to the semen diluted with different concentrations of LDL. According to Yunsheng *et al.* (2008), semen samples diluted with 9% gave better results when compared with 20% EY. This finding were in agreement with who reported that the ideal LDL concentration for freezing caprine semen was 8%. This is also an ideal concentration for bull semen following freezing. The findings of our research were in agreement with Toniet *et al.* (2010) who stated that semen diluted with 8% LDL is able to offer more protection to ram sperm motility following freezing and Bencharif *et al.* (2008) witnessed that LDL offered better cryoprotectant action to boar semen as compared to egg yolk. These observations could be related to the granules as the increase of LDL concentration result in an aggregation of granules. Furthermore, an increase of LDL concentration could result to HDL aggregation. Semen diluted with 20%

EY preserved the frozen-thawed semen better than the semen diluted with different LDL concentration and this may be due to the components that are found in the egg yolk, which may contribute to sperm post-thaw survival. For example, egg yolk is rich in various antioxidants such as vitamin E and A and lutein as well as several amino acids that play a major role in the preservation of sperm membrane. However, there was a statistical difference observed ($P < 0.05$) on the semen diluted with 20% EY extender when compared to the semen diluted with different concentrations (6, 8, 10 and 12%) of LDL. El-Sharawy *et al.* (2012) reported a lower percentage of live sperm in a semen diluted with 20% of EY and other semen diluted with (4, 6 and 15% LDL). Semen diluted with 20% EY resulted in a high percentage of live sperm compared to the semen diluted with different concentrations of LDL. Therefore, no statistical difference ($P > 0.05$) observed in the percentage of live sperm diluted with 6 (64.9%) LDL and 8% (65.5%) LDL. The semen sample diluted with 10 (40.2 \pm 5.3) LDL and 12% (27.0 \pm 5.5) LDL had the lowest percentage of live sperm compared to the semen diluted with 6 and 8% LDL. However, semen diluted with 12% LDL had the highest percentage of dead sperm. The semen diluted with 20% EY preserved the frozen-thawed Nguni bull semen better than the semen diluted with different LDL concentrations. Furthermore, 8 LDL had an

intact plasma membrane integrity than 6, 10 and 12% on thawed Nguni bull semen. Amirat *et al.* (2004) reported that LDL did not cause more injury to bull sperm following freezing and thawing as compared to EY. Bencharif *et al.* (2008) reported a better cryoprotectant action of plasma membrane integrity for dog sperm diluted with LDL compared to EY. This can be described by Bergeron *et al.* (2004) and Moustacas *et al.* (2011), who reported that LDL get attached to the plasma membrane, repairing the lost caused by natural outflow and thereby increasing the stability of the membrane, protecting the sperm from cold shock.

However, semen diluted with 20% EY resulted in a higher percentage of sperm with intact plasma membrane compared to semen diluted with LDL concentrations. According to Amirat *et al.* (2004), semen diluted with 20% EY gave a higher percentage of sperm with a damaged plasma membrane compared to the semen diluted with LDL. Semen diluted with 12% LDL had a higher percentage of sperm with a damaged plasma membrane. An increase in the LDL concentration above 8% resulted in a higher percentage of sperm with a damaged plasma membrane and this may be due to the aggregation of HDL and granules that have a negative effect on sperm viability (Hu *et al.*, 2010). Moreover, there was a significant difference ($P < 0.05$) on the percentage of sperm with an intact acrosome integrity diluted with 20% EY. Semen diluted with 20% (73.1±4.3) EY had a highest percentage of sperm with an intact acrosome compared to semen diluted with (6, 8, 10 and 12%) LDL. These results contradict with the results obtained by who find out that extenders containing LDL provided better protection to the acrosome than the extender containing 20% EY ($P < 0.05$). Hu *et al.* (2010) also reported a higher percentage of sperm with an intact acrosome integrity following freezing and thawing of bull semen when the semen extender contained 8% LDL compared to the whole egg yolk 20%. These results are in agreement with (Moreno *et al.*, 2013) who reported that LDL provided better protection to the acrosome, possibly by a direct action through the exchange or repair of acrosome membrane phospholipids because the extender has lower progesterone content compared to egg yolk. There was no significant difference ($P < 0.05$) on percentage of sperm with an intact acrosome when semen was diluted with 6 LDL (64.0±2.0) and 8% LDL (65.8±2.5). However, semen diluted with 12% LDL (74.5±4.1) had the highest percentage of sperm with a damage acrosome. These results contradict with (Bencharif *et al.*, 2008) who reported that the extender containing 12% LDL provided better protection of the acrosome integrity and it was due to a direct action through the exchange or repair of acrosomal membrane phospholipids. Therefore, semen samples diluted with 20% LDL preserved the Nguni bull semen DNA integrity

better than the semen diluted with different LDL concentrations. Frozen-thawed Nguni bull semen diluted with 20% EY (control) had a significantly ($P < 0.05$) high percentage of sperm with an intact DNA integrity compared to the semen diluted with 6, 8, 10 and 12% LDL. These results contradict with Hu *et al.* (2010) who obtained better DNA integrity when the ovine semen was diluted with 8% LDL as opposed to the semen diluted with 20% EY. No significant difference ($P < 0.05$) was observed on the semen diluted with 6 and 8% LDL on the percentage of sperm with an intact DNA integrity. There was a significant difference ($P > 0.05$) observed between semen diluted with 10% (49.8±14.3) and 12% (27.8±2.9) LDL with the semen diluted with 12% (27.8±2.9) LDL having a high percentage of sperm with a damaged DNA integrity. Furthermore, there was a higher percentage of matured oocytes and cleavage rate on the semen diluted with 20% EY compared to the diluted with 8% LDL. However, these results contradict to the results obtained by Amirat *et al.* (2004) who obtained a higher cleavage rate with frozen-thawed semen diluted with 8% LDL than with 20% EY extender. These findings are in agreement with Marco-Jiménez *et al.* (2004) who showed that LDL extender was more effective in ovine sperm following freezing and resulted in higher fertilization rate as compared to egg yolk. In bulls, (Amirat *et al.*, 2004) obtained higher cleavage rate when semen was diluted with LDL compared to semen diluted with EY extender but no difference was witnessed on the blastocyst rate between semen diluted with LDL and EY extenders. Our results are not in agreement with the results obtained by Akhter *et al.* (2011), who obtained higher fertilization rates when buffalo semen was diluted with 1% LDL as compared with the control 20% EY.

Conclusion

Frozen-thawed Nguni bull semen diluted with 20% EY resulted in a higher post-thawed semen motility, viability, sperm plasma membrane, acrosome, DNA integrity and fertilization rate compared to the frozen-thawed semen cryopreserved with 6, 8, 10 and 12% LDL. However, better results were also obtained when semen was cryopreserved with 8% of LDL compared to other LDL concentrations. The inclusion of 8% LDL in the semen diluent improved the frozen-thawed quality of the Nguni bull semen in terms of the post-thawed semen motility, viability, sperm plasma membrane, acrosome and DNA integrity. Semen cryopreserved with 8% of LDL can be used for *in vitro* fertilization compared to other semen diluted with 6, 10 and 12% LDL. However, semen cryopreserved with 20% EY extender resulted in a higher cryopreserved with 8% LDL can also be used as an alternative to 20% EY extender in the cryopreservation and *in vitro* embryo production of cattle ovaries.

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Authors Contributions

Mduduzi Tshabalala, Masindi Lottus Mphaphathi, Khathutshelo Agree Nephawe, Cyril Pilane and Tshimangadzo: Lucky nedambale were in charge of the project design, data collection and writing of the manuscript.

Conflict of Interest

All authors have received and approved this final version of the manuscript. There is no conflict of interest in our submission.

Ethics

The experiment was evaluated and approved by the Agricultural Research Council Ethics Committee under the Cryo-gene bank program and by the Tshwane University of Technology Ethics Committee under the project number AREC2015/10/004.

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