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Research Article

Effect of Bioxcell® and Triladyl® Extenders and Removal of Seminal Plasma on Equilibrated and Cryopreserved Semen from South African Unimproved Indigenous Bucks

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Abstract

The objectives of the study were to evaluate the effect of two extenders (Triladyl® and Bioxcell®) and the removal of seminal plasma on indigenous buck's semen. Semen was collected from six indigenous bucks using an electro-ejaculator. Raw semen was pooled and randomly allocated into six groups as follows: (i) Raw non-washed, (ii) Raw washed, (iii) Triladyl®-washed, (iv) Triladyl®-non-washed, (v) Bioxcell®-washed and (vi) Bioxcell®-non-washed. Both the Triladyl® and Bioxcell® washed semen samples groups were diluted (1:4 v/v) with Phosphate Buffered Saline (PBS) then centrifuged at 1500x g for ten min and seminal plasma was removed. The groups were analysed for spermatozoa motility rates using Computer-Aided Sperm Analysis (CASA). The spermatozoa viability was assessed using Eosin-Nigrosin, acrosome integrity using Spermac, chromatin structure using Acridine Orange, and mitochondria using JC-1 staining solutions. Semen samples were diluted (1:4 v/v) as follows: Triladyl® (washed and non-washed) or Bioxcell® (washed and non-washed) and then equilibrated at 5ºC for 2 hours. Equilibrated semen samples in 0.25 mL French straws were placed 5 cm above a Liquid Nitrogen (LN_2) vapour for 10 min, and stored for one month. Frozen semen straws per treatment group were thawed at 37ºC for 30 seconds. Significant differences among the mean values of semen parameters were determined by Tukey's test using ANOVA, GLM procedure of SAS version 12.1 of 2010. The spermatozoa progressive motility rate in non-washed semen extended with Bioxcell® was significantly higher (89.6±7.5[a]) compared with that of non-washed Triladyl®, washed Bioxcell® and Triladyl® ($P<0.05$). Live spermatozoa percentage in washed semen extended with Triladyl® extender was reduced (27.7±17.1) significantly compared with the other groups ($P<0.05$). There was a lower percentage of spermatozoa with high mitochondrial membrane potential in non-washed and washed semen extended with Bioxcell® (39.5±23.2 and 37.9±28.6, respectively) compared with that of non-washed and washed semen extended with Triladyl® ($P>0.05$). The spermatozoa progressive motility rate in non-washed semen extended with Bioxcell® (58.5±10.0) extender was significantly higher compared with that of the other groups ($P<0.05$). There was a higher live and normal spermatozoa percentage in non-washed semen extended with Bioxcell® (45.7±21.2) compared with that of the other groups ($P<0.05$). In conclusion, Washing of seminal plasma in semen extended with Triladyl® was not essential, as it lowered viability, progressive motility and chromatin membrane integrity prior and post-cryopreservation. However, Bioxcell® extender was found to be more suitable for preserving spermatozoa during equilibration and freezing/thawing process of non-washed and washed buck semen.

Keywords: Indigenous Bucks; Seminal Plasma; Membrane Integrity; Bioxcell®; Triladyl®

Introduction

Goats are prolific and require low inputs for a moderate level of production. They tend to reach maturity early and are profitable to keep [1]. Also, goats are tolerant of different parasites and diseases, non-selective browsers and able to survive on the poorest vegetation [2]. Most South African indigenous goats are kept in rural areas and used for milk, hides, and meat production [1]. However, there is limited information regarding their reproductive status [2], semen quality and their tolerance to cryopreservation process [3]. This is mainly because they have received little attention from researchers/scientist in the past. However, there is recent interest from commercial farmers and this may be due to their hardiness and adaptability to the local harsh environmental conditions as well as their exceptional

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capacity to produce and reproduce efficiently under poor nutritional conditions [3].

Furthermore, there is a need to conserve their genetic materials. Genetic resource banks are normally utilised with reproductive technologies for the conservation of endangered species [4]. Semen collection and storage is essential in controlled breeding programs of many endangered species [5]. The cryopreservation of gametes is vital because it would allow researchers to support a genome resource bank for this breed for an indefinite period [6]. Cryopreservation of goat spermatozoa also extends the male reproductive life of a goat germline [7].

Frozen-thawed goat semen may be utilised for Artificial Insemination (AI) to enhance improvement of livestock as breeders mostly make use of genetically superior goats [2]. But, cryopreservation of goat semen remains a challenge due to poor post-thaw sperm motility and viability recovery percentages. This method includes temperature reduction, cellular dehydration, eventual freezing and subsequent thawing. Cryopreservation has been shown to stop all cellular activities, restarting its normal metabolic functions, following thawing. Nevertheless, semen cryopreservation generally induces the formation of intracellular ice crystals, osmotic and chilling injury, which causes sperm cell damage, cytoplasmic fracture, or some effects on the cytoskeleton or the genome-related structures [8-10]. These eventually impair spermatozoa transport and decrease the survival rate in the female reproductive tract, thus reducing fertility [10].

Phospholipase A2 activity of Egg Yolk Coagulating Enzyme (EYCE) catalyses the hydrolysis of egg yolk Phosphatidylcholine (PC) into fatty acids and Lysophosphatidylcholine (LPC). The LPC has a toxic effect on buck spermatozoa when it is extended with egg-yolk-based extenders by acting like a detergent on biomembrane, resulting in a loss of motility, membrane integrity and consequently low fertility rate [11,12]. Among the factors affecting the freezing ability of spermatozoa, supplementation of the freezing medium with different types of cryoprotectant plays a vital role in minimising the physical and chemical stresses occurring during cryopreservation procedure [13].

Hence, it is of utmost importance to develop and modify protocols to improve cryopreservation protocols to preserve indigenous germplasm. Seminal plasma plays an important role in spermatozoa survival during the cryopreservation process. This is because the causes of reduced spermatozoa motility are related to seminal plasma enzymes [11]. Goat semen is currently washed to eliminate the seminal plasma from the spermatozoa prior to dilution with standards extenders containing egg yolk [14]. Currently, soy-lecithin-based extenders are used to avoid problems encountered when egg-yolk-based extenders are used to process goat semen [15]. The present study focused on the effect of extenders (egg-yolk-based extender and soy-lecithin-based extender) and the removal of seminal plasma on South African indigenous goat semen parameters.

Materials and Methods

Chemicals

The extenders used in this study were Bioxcell® (IMV, L'Aigle and France) and Triladyl® (Minitüb, Germany). The stains used were Spermac® stain (Stain enterprises, South Africa), Acridine Orange (AO), Phosphate Buffered Saline (PBS) and Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich, St Louis, MO, USA), JC-1 stain (Molecular probesTM, Eugene. Oregon. USA), and Eosin-Negrosin (Merck Millipores corporation, South Africa).

Experimental bucks, semen collection and processing

The study was conducted at the University Of Venda Centre Of Excellence in Animal Assisted Reproduction (CEAAR). Semen samples were collected twice a week (spring to early summer) from six unimproved indigenous bucks (3 to 4 years of age). They were kept in pens and maintained on ewe and lamb pellets with water provided ad libitum throughout the experiment. Semen was collected with the aid of an electro-ejaculator into pre-warmed (37°C) 15 mL graduated plastic tubes and immediately placed in a thermo flask with water at 37°C. The collected buck semen samples were then transported to the laboratory for further analysis.

The buck semen samples were pooled to eliminate individual differences between the samples. Pooled semen was randomly allocated into six aliquots, namely (i) Raw non-washed, (ii) Raw washed, (iii) Triladyl®-washed semen, (iv) Triladyl®-non-washed semen, (v) Bioxcell®-washed semen and (vi) Bioxcell®-non-washed washed. Triladyl® and Bioxcell® washed semen groups were mixed with PBS at a ratio of 1:4 v/v. Semen samples were then centrifuged at 1500x g for 10 min. Following centrifugation, seminal plasma was removed using 1mL sterile plastic hand pipette, leaving only the spermatozoa pellets.

Preparations of semen extenders

Preparation of Bioxcell® extender: One bottle of Bioxcell® (100mL) was diluted with 400mL of distilled water for a final extended, ready to use volume of 500mL. It was then aliquoted into 15mL graduated tubes and stored in the refrigerator (-20°C) until use.

Preparation of Triladyl® extender: The chicken egg yolk was separated from the albumen by passing the egg yolk from one-half of the shell to the other to get rid of the albumin [7]. The egg yolk was placed in a gauze swab and an 18-gauge needle was used to punch the egg yolk to separate the egg yolk from its membrane and some contents of albumin to the 15 mL graduated tube. Triladyl® concentrate was added slowly to the water and the mixture was added to egg yolk. The mixture was stirred and filtered using a sterile filter funnel. The final mixture of Triladyl® extender was kept in 15 mL graduated tubes, sealed with Parafilm and stored in the refrigerator (at 5°C).

Semen extension, equilibration, freezing and thawing

Semen samples were extended with pre-warmed (37°C) Bioxcell® or Triladyl® at a ratio of 1:4 v/v (semen to extender). Rapid cooling was done by placing the tubes in a beaker with cool water at room temperature (25°C). After 10 min, the beaker was transferred into the refrigerator and equilibrated at 5°C for two hours [7]. After equilibration, the semen samples were loaded into 0.25 mL French straws and sealed with polyvinyl powder per treatment group. The straws were frozen in LN_2 (-196°C), 5 cm above a liquid nitrogen surface for 10 min and plunged into LN_2 and stored for a month.

Semen evaluation

Evaluation of spermatozoa motility: Spermatozoa motility rate

was analysed using the Computer-Aided Sperm Analyser (CASA) system, version 5.4, (Microptic SL, Barcelona, Spain) [7]. Five hundred microliters (μL) of PBS plus 20 μL of raw semen were mixed in an Eppendorf tube using a hand pipette. A drop (2 μL) of semen sample was placed on a pre-warmed (37°C) microscopic glass slide and mounted with a cover of a microscopic glass slip and examined under a CASA microscope. Four fields per sample were captured under 10X magnification with a Ph1 phase contrast.

Evaluation of spermatozoa viability (live/dead): Spermatozoa viability was analysed using an Eosin-Nigrosin staining solution [16]. A drop of 20 μL Eosin and a drop of 10 μL Nigrosin were placed on the one end of the pre-warmed (37°C) microscopic glass slide using a hand pipette. Then 10 μL of semen was placed on the stains and they were gently mixed together with the tip of the hand pipette and then a smear was made using a pipette tip (to avoid breakage of spermatozoa during the smear) at 37°C. The slide was placed on the slide warmer at 120°C to allow the smear to dry fast. Dried microscopic slides were analysed for viability using the CASA microscope at 60X magnification with oil immersion and 300 spermatozoa were counted/slide. The live spermatozoa are unstained and the dead spermatozoa are stained.

Evaluation of spermatozoa acrosome membrane damage: Spermatozoa acrosome membrane damage was analysed by staining spermatozoa with Spermac® stain [16]. A drop of 15 μL of semen was placed on a microscopic glass slide using a hand pipette. A smear was made by gently pulling semen from one end to the other end using a hand pipette tip and allowed to air dry for about 10 min on a warm plate at 37°C. The absorbent paper was spread inside the staining tray to absorb excess stain during the staining procedure and the smeared slide was placed on the staining tray. The smeared slide was fixed with the Spermac® fixative by placing fixative drops on the smeared slide until it was covered with the fixative using a hand pipette. After 1 min, the microscopic slide was then placed vertically on absorbent paper to drain excess fixative into the staining tray. The slide was then washed gently by dipping it seven times in a glass beaker with distilled water to remove the stain. The fixed slide was placed on the staining tray with absorbent paper and stained with stain A by placing stain drops on the fixed slide until it was covered with stain A using the hand pipette for 1 min. The stained slide was then drained by holding the slide vertical on its side and washed by dipping the slide seven times in a glass beaker with distilled water. The slide was placed vertically on absorbent paper to drain excess water. The same procedures were repeated with stains B and C. The slides were air dried for 15 min and then observed under a light microscope (60X magnification) using oil immersion and 300 spermatozoa per stained slide were counted. The acrosome stained dark green and nucleus stained red.

Acridine Orange (AO) staining procedure for evaluation of sperm chromatin structure: The AO staining method was from a protocol previously described by [17]. The raw semen sample was washed three times in PBS by centrifuging at 1500x g for 10 min. Fifteen microliter drops of the spermatozoa pellets were smeared on a microscopic slide using a hand pipette tip and allowed to air-dry for 10 min. The smears were fixed for 1 hr in ethanol-acetone (1:1) at 4°C and allowed to air-dry for 10 min. The microscopic slides were then stained for 7 min with AO (0.19 mg/mL) at room temperature in the dark. The slides were gently rinsed with distilled water and air-dried for 10 min, then viewed under a UV light inverted microscope at 40X magnification (Nikon eclipse TI, Narishige Co., Ltd. USA). Three hundred spermatozoa per stained slide were counted.

JC-1 staining procedure for evaluation of sperm mitochondrial membrane potential: The JC-1 stain was used to analyse the spermatozoa mitochondrial membrane potential [18]. Five mg of JC-1 was dissolved in 5 mL of DMSO. Semen was diluted with PBS (1:4 v/v) and centrifuged at 1500 x g for 10 min and the fluid was discarded. Ten microliters of JC-1 staining solution was diluted in pre-warmed 100 μl of PBS in an Eppendorf tube and placed in a water bath at 37°C. The spermatozoa pellets were diluted with PBS and 5 μL of PBS with spermatozoa were mixed with 5 μl of the JC-1 staining solution in an Eppendorf tube and incubated for 10 minutes at 37°C. Incubated spermatozoa were washed by mixing with 40 μL of PBS using a hand pipette. Two microliters of PBS plus spermatozoa was placed on a slide and covered with a coverslip. Spermatozoa were observed immediately under a UV light inverted microscope at 40X magnification (Nikon eclipse TI, Narishige Co., Ltd. USA). Three hundred spermatozoa per stained slide were counted. The spermatozoa mitochondria with high membrane potential appeared reddish and mitochondria with low membrane potential appeared greenish.

Statistical analysis

The data were Analysed by Analysis of Variance (ANOVA) for a 3 X 2 factorial design in a completely randomized design using the General Linear Model (GLM) procedure of SAS version 12.1 of 2010. A significant difference ($P<0.05$) among the mean values of semen parameters were determined by Tukey's test method.

Results

Sperm motility, viability, acrosome, chromatin and mitochondrial membrane potential of equilibrated semen

The spermatozoa total motility rate in non-washed semen extended with Bioxcell® was significantly higher (97.8±2.3) compared with that of non-washed Triladyl® and washed Bioxcell® extended semen ($P<0.05$) (Table 1). The spermatozoa progressive motility rate in non-washed semen extended with Bioxcell® was significantly higher (89.6±7.5a) compared with that of non-washed Triladyl®, washed Bioxcell® and Triladyl® ($P<0.05$). Live spermatozoa percentage in washed semen extended with Triladyl® extender was reduced (27.7±17.1) significantly compared with the other groups ($P<0.05$). The percentage of spermatozoa acrosome integrity in non-washed semen extended with Bioxcell® (71.1±13.8) extender was not significantly different from the other groups ($P<0.05$). The spermatozoa chromatin integrity was lower in washed semen extended with Triladyl® (46.3±9.4) compared with non-washed semen extended with Bioxcell® and Triladyl® extenders ($P>0.05$). There was a lower percentage of spermatozoa with high mitochondrial membrane potential in non-washed and washed semen extended with Bioxcell® (39.5±23.2 and 37.9±28.6, respectively) compared with that of non-washed and washed semen extended with Triladyl® ($P>0.05$).

Sperm motility, viability, acrosome, chromatin and mitochondrial membrane potential of frozen-thawed semen

The spermatozoa total motility rate in non-washed semen

Table 1: Effect of extenders and seminal plasma on equilibrated spermatozoa parameters (mean ± SE).

Treatment	Total motility (%)	Progressive motility (%)	Viability Live & normal (%)	Normal acrosome (%)	Normal chromatin (%)	$\Delta\psi m^{high}$ (%)
Raw NW	98.9±1.2^{a}	92.9±4.7^{a}	71.7±12.3^{a}	62.8±13.7^{a}	82.2±16.5^{a}	83.4±14.2^{a}
Biox NW 2 h	97.8±2.3^{a}	89.6±7.5^{a}	73.1±14.0^{a}	71.1±13.8^{a}	79.8±21.9^{ab}	39.5±23.2^{c}
Tril NW 2 h	90.5±7.5^{c}	77.0±10.8^{b}	60.7±22.8^{ab}	60.1±15.9^{a}	67.5±24.4^{abc}	51.2±31.4^{bc}
Raw W	98.2±2.7^{a}	91.4±7.9^{a}	60.7±22.9^{ab}	68.4±19.0^{a}	65.1±17.5^{bc}	65.0±20.8^{ab}
Biox W 2 h	92.5±4.6^{bc}	78.8±6.4^{b}	48.0±28.8^{b}	71.3±22.7^{a}	56.8±13.6^{cd}	37.9±28.6^{c}
Tril W 2 h	94.9±5.5^{ab}	78.6±10.8^{b}	27.7±17.1^{c}	70.1±13.4^{a}	46.3±9.4^{d}	59.1±33.1^{bc}

Different superscripts (a, b, c and d) within the same column indicate significant differences among the means (P<0.05).
Raw NW: Raw Non-Washed; Biox NW 2 h: Bioxcell® Non-Washed 2 hours; Tril NW 2 h: Triladyl® Non-Washed 2 hours; Raw W: Raw Washed; Biox W 2 h: Bioxcell® Washed 2 hours; Tril W 2 h: Triladyl® Washed 2 hours, $\Delta\psi m^{high}$: High Mitochondrial Membrane Potential.

Table 2: Effect of extenders and seminal plasma on frozen-thawed spermatozoa parameters (mean ± SE).

Treatment	Total motility (%)	Progressive motility (%)	Viability Live & normal (%)	Normal acrosome (%)	Normal chromatin (%)	$\Delta\psi m^{high}$ (%)
Raw NW	98.9±1.2^{a}	92.9±4.7^{a}	71.7±12.3^{a}	62.8±13.7^{ab}	79.9±15.6^{a}	83.4±14.2^{a}
Biox NW FT	85.0±3.4^{b}	58.5±10.0^{b}	45.7±21.2^{b}	50.4±23.5^{b}	63.7±9.1^{ab}	49.8±20.1^{c}
Tril NW FT	73.9±13.8^{c}	45.4±11.2^{c}	24.5±22.2^{c}	55.6±22.1^{ab}	51.2±18.7^{bc}	68.7±26.8^{ab}
Raw w	98.3±2.7^{a}	91.4±7.9^{a}	60.7±22.9^{ab}	68.4±19.0^{a}	65.1±17.5^{ab}	65.0±20.8^{cb}
Biox W FT	68.2±13.5^{cd}	47.0±15.7^{c}	5.2±4.9^{d}	65.4±11.4^{ab}	48.7±21.3^{bc}	65.3±14.9^{cb}
Tril W FT	63.1±15.1^{d}	39.9±13.1^{c}	6.9±8.6^{d}	65.5±15.4^{ab}	37.9±24.3^{c}	78.4±16.8^{ab}

Different superscripts (a, b, c and d) within the same column indicate significant differences among means (P<0.05).
Raw NW: Raw Non-Washed; Biox NW 2 h: Bioxcell® Non-Washed 2 hours; Tril NW 2 h: Triladyl® Non-Washed 2 hours; Raw W: Raw Washed; Biox W 2 h: Bioxcell® Washed 2 hours; Tril W 2 h: Triladyl® Washed 2 hours, $\Delta\psi m^{high}$: High Mitochondrial Membrane Potential.

extended with Bioxcell® (85.0±3.4) extender was significantly higher compared with that of the other groups (P<0.05) (Table 2). The spermatozoa progressive motility rate in non-washed semen extended with Bioxcell® (58.5±10.0) extender was significantly higher compared with that of the other groups (P<0.05). There was a higher live and normal spermatozoa percentage in non-washed semen extended with Bioxcell® (45.7±21.2) compared with that of the other groups (P<0.05). For spermatozoa acrosome and chromatin integrity there was no significant difference in non-washed and washed semen extended with Bioxcell® and Triladyl® extenders (P>0.05). There was a lower percentage of spermatozoa with high mitochondrial membrane potential in non-washed and washed semen extended with Bioxcell® (49.8±20.1 and 65.3±14.9, respectively) compared with that of non-washed and washed semen extended with Triladyl® (P<0.05).

Discussion

Egg yolk coagulating enzyme in egg yolk containing extenders and bulbourethral gland secretion in buck semen causes a detrimental effect on the quality of a spermatozoa during dilution, freezing and thawing Khalifa [19]. The current study demonstrated that the removal of seminal plasma in indigenous goat semen increased the percentage of motile spermatozoa in equilibrated and frozen-thawed samples in both the extenders. The results of spermatozoa total motility of non-washed and washed raw semen were better than the total motility of raw semen recorded by Ramukhithi [2] and Roof et al., [20]. Progressive motility percentage of non-washed and washed raw semen were much better than spermatozoa progressive motility obtained by Ramukhithi [2] and Vidal et al., [15]. It was concluded that these differences could be attributed to the individual buck and environment, semen collection facilities available and seasonal effect on spermatozoa quality (Camara et al., [21]; Van Staden [22]).

The results for spermatozoa total motility rate of equilibrated non-washed semen samples extended with Bioxcell® were higher than those extended with Triladyl®. In this study, the results of total spermatozoa concur with what previous studies indicated, that soy-lecithin-based extender had higher results than the egg-yolk-based extender [19]. The findings of [23] disagree with findings of the present study which might be due to the high concentration of unsaturated fatty acids present in the spermatozoa membrane, which in the presence of ROS are highly susceptible to lipid peroxidation that disrupts cells and causes a reduction in motility [24,25]. In the present study, washing of semen prior to extension with a Bioxcell® is not necessary as non-washed semen extended with Bioxcell® shown to have higher spermatozoa total motility than the other groups. However, it was necessary to wash semen samples before extending with Triladyl® as it yields higher total motility results than Bioxcell washed and Triladyl non-washed semen samples.

The lowest spermatozoa live and normal percentage were observed in equilibrated semen samples that were washed and extended with Triladyl®. These are caused by the non-enzymatic antioxidants (ascorbic acid, α-tocopherol, taurine, and albumin) lost with the removal of seminal plasma [24]. Nevertheless, Bioxcell® extender seemed to compensate for the loss of non-enzymatic antioxidants because it has been supplemented with taurine and the results for washed semen samples extended with Bioxcell® was statistically higher than the Triladyl® extended semen for live and normal spermatozoa percentages.

There is a relationship between the spermatozoa abnormalities and fertilisation ability of preserved semen. Normal acrosome is important for acrosome reaction, which is needed for fertilisation to take place [26]. There was no significant difference (P>0.05) in the percentage spermatozoa with normal acrosome of non-washed and washed semen samples extended with Bioxcell® and Triladyl®. But,

with no significant difference (P>0.05) between the four treatment groups, there was a decrease in the percentage spermatozoa with normal acrosome of non-washed semen samples that were extended with Triladyl®, compared to the other treatment groups. There was also a decrease in percentage spermatozoa with normal chromatin of washed semen samples extended with Triladyl®, compared to the other treatment groups. The percentage of spermatozoa high mitochondrial membrane potential of non-washed and washed semen samples extended with Bioxcell® were significantly lower (P<0.05) than the non-washed and washed semen samples extended with Triladyl®. There was a high percentage of abnormal spermatozoa in the current study, which concur with what [27] and [28] reported during spring season.

It is necessary to balance the equilibration periods with the glycerol for the protective properties of glycerol to take an effect without any unnecessary loss of spermatozoa prior to cryopreservation Van Staden, [22]. Both extenders used in the present study had glycerol and the equilibration time used was 2 hours prior to cryopreservation. This maintained the spermatozoa' motility parameters, viability, morphology, and membrane integrity. The findings of the present study, concur with the findings of previous studies which indicated that a short duration equilibration of 2 hours at 5°C with 7% glycerol resulted in optimum preservation of goat spermatozoa total and progressive motility, plasma and acrosomal membrane integrity during cryopreservation [29,30].

The diluents types, freezing rate, glycerol levels and glycerol equilibration time interact with the thawing procedure, which in turn affect the post-thaw fertilising ability [31]. The results of frozen-thawed semen on spermatozoa total motility of washed semen samples extended with Bioxcell® and Triladyl® were higher and disagree with results obtained by Ramukhithi [2], who reported lower frozen-thawed washed semen's recovery rate for South African unimproved indigenous goats and Boer goats. The results for washed semen samples extended with Bioxcell® extender were comparable to the results obtained by [25]. The results reported by [25] for semen that was centrifuged and extended with Triladyl® extender (31.5%) were lower than the results obtained in the current study. Frozen-thawed progressive motility of semen samples that were not washed, extended with Bioxcell® was significantly greater than other groups. These results concur with the results reported by [25].

The results of pre-treatment (removal and non-removal of seminal plasma) are not constant in different studies. In some studies, the removal of seminal plasma was essential for maximising spermatozoa motility of frozen-thawed goat semen [32]. However, other authors reported that the presence of seminal plasma was beneficial for frozen-thawed spermatozoa motility of goat semen [2]. In the present study, the removal and non-removal of seminal plasma maintained frozen-thawed motility in both extenders (Bioxcell® and Triladyl®). This concurred with what was reported by [33,25] reported progressive motility of frozen-thawed semen samples extended with Bioxcell® to be 22.3% and with Triladyl® to be 7.0%. Jimenez-Rabadan et al., [34] and Kurien et al., [35] also found similar results on frozen-thawed semen extended with Biladyl® (45.3%) and Triladyl® 32.5%, which were also higher. [14] reported post-thaw motility of 38% in Angora buck semen when using Bioxcell®. Sundararaman and Edwin [29] considered frozen-thawed percentage spermatozoa' progressive motility of 40% and above to be good and acceptable. Therefore, the results for frozen-thawed progressive spermatozoa motility in this study are acceptable.

The composition of the extender and suitable cryoprotectants are important factors for successful semen cryopreservation [15,33]. The removal of the seminal plasma by washing is beneficial when the spermatozoa are frozen in a diluent containing egg yolk. The toxicity of egg yolk coagulating enzyme is influenced by season of the year [13]. Bulbourethral glands increase their activity by high plasma concentrations of prolactin and produce more phospholipase A2 enzyme during the non-breeding season [36]. The current study was conducted during the spring months (September, October and November) and the beginning of summer (December) and the percentage of post-thawing spermatozoa motility was high during spring months. This concurred with [13], who reported a higher percentage of post-thaw spermatozoa motility as well as recovery rates during autumn months (September, October and November) than during the other months of the year. Although there was a significant effect on interactions in this study, Bioxcell® extender did not show an advantage over Triladyl® extender as both similarly maintained spermatozoa motility. This may be due to the natural constituent of egg yolk (phospholipids, cholesterol and low-density lipoproteins) in Triladyl® extender which is equally effective in protecting spermatozoa against oxidative stress as the forfeited antioxidants found in Bioxcell® extender [33].

The live and normal spermatozoa percentage were greatly affected (P<0.05) following freezing and thawing in both extenders compared to equilibrated semen in both extenders. The thermal stress and cryoinjury that occur on the spermatozoa membrane during cryopreservation processes and after thawing caused spermatozoa death in the current study. [35,38] also reported that freezing and thawing causes more damage than other stages of cryopreservation to the spermatozoa. Seminal plasma removal did not improve the live and normal spermatozoa percentages in both the extenders in this study. This may be because the protective effects of natural antioxidants present in the seminal plasma were available in low concentrations as seminal plasma was removed and spermatozoa become more susceptible during cryopreservation [38].

Acrosome intactness is a pre-requisite for fertilisation, though the correlation between acrosome status and fertility vary [39]. Spermatozoa' normal acrosome percentage was high in frozen-thawed non-washed and washed semen samples extended with Triladyl®. This was not evident in what [40] and El-kon et al., [41], reported about the effects the egg yolk added on extender has on acrosome integrity and the post-thaw viability of ejaculated spermatozoa in goats.

Chromatin structure was reported to have been affected by the freeze-thawing process [32,42]. [42] reported that the mean percentage of human spermatozoa with damaged DNA content was higher in semen frozen in liquid nitrogen vapour than in a controlled biological freezer. Similarly, the buck semen in the present study was frozen in liquid nitrogen vapour and the freeze-thawing process affected the chromatin structure. From the results in the present study, there was lower spermatozoa membrane integrity and low chromatin integrity. Spermatozoa membrane integrity can be directly related to ROS production. However, it should not be a direct

indicator of DNA integrity, as DNA fragmentation can be observed even in spermatozoa with normal morphology [43]. The antioxidants present in the seminal plasma were said to be an essential form of protection (prevention, interception, and repair) to spermatozoa against ROS [44]. In the present study, removal of seminal plasma showed lowered membrane integrity and viability of frozen-thawed semen. However, it was not evident to all spermatozoa membranes. This might be because of the antioxidants present in the extenders (Bioxcell® and Triladyl®) that provided protection in replacement to the antioxidant that is present in the removed seminal plasma.

Low viscosity of lecithin extenders protects and improves mitochondrial kinematics during cryopreservation better than egg yolk containing extenders [19]. This is not evident in the current study, as it showed a higher percentage of high mitochondrial membrane potential of frozen-thawed non-washed and washed semen samples extended with Triladyl®. Mitochondria are localised in the mid-piece area and provide the spermatozoa mid-piece and the spermatozoa head with the Adenosine Triphosphate (ATP) required for maintenance processes of membranes [45,46]. In the present study, motility and mitochondrial membrane potential were the least affected parameters. This is because the mitochondrial membrane potential is a good indicator of spermatozoa motility dysfunction Mansour [47].

Conclusion and Recommendation

Though there was a reduction in the percentage of progressive motility rate in non-washed and washed semen extended with Bioxcell® and Triladyl®, they still maintained acceptable motility (40%) post-thaw. The study also demonstrated that viability reduced drastically with or without washing of seminal plasma in both Triladyl® and Bioxcell® extended semen post-cryopreservation. Washing of seminal plasma in semen extended with Triladyl® was not essential, as it lowered viability, progressive motility and chromatin membrane integrity prior and post-cryopreservation. Bioxcell® extender reduced the high mitochondrial membrane potential in non-washed and washed semen prior and post-cryopreservation. The freezing-thawing process did reduce the indigenous buck semen parameters irrespective of non-washing and washing of seminal plasma. However, Bioxcell® extender was found to be more suitable for preserving spermatozoa during equilibration and freezing/thawing process of non-washed and washed buck semen. Therefore, additional studies should be conducted following the success of the present study to determine spermatozoa's fertilising ability both *in vivo* and *in vitro* using South African unimproved indigenous bucks.

Authors' Contributions

LP, ML, LR, NC, PVM, TL, and DM conceive of the study, carried out the experiments, the study's design, coordination and drafted the manuscript. DM and TL assisted with the interpretation of its results. All authors read and approved the final manuscript.

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