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Influence of tannin-rich extract from commercial *Acacia mearnsii* and gallocatechin on ovine cryopreserved semen viability

Mohammed S. Liman[a,b], Abubeker Hassen[c], Mario P. Smuts[a], Ahmed D.A. Biraima[d], Peter Sutovsky[e], Lyndy J. McGaw[f] and Dietmar E. Holm[a]

[a]Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, Pretoria, South Africa; [b]Department of Range Management and Grazing Reserve, Niger State Livestock and Fisheries Institute, Ministry of Livestock and Fisheries, Minna, Nigeria; [c]Department of Animal and Wildlife Sciences, Faculty of Natural and Agricultural Science, University of Pretoria, Pretoria, South Africa; [d]Department of Meat Production, Faculty of Animal Production, University of Khartoum, Khartoum-North, Sudan; [e]Division of Animal Sciences, and Department of Obstetrics, Gynecology and Women's Health, University of Missouri, Columbia, MO, USA; [f]Department of Paraclinical Sciences, Faculty of Veterinary Science, Phytomedicine Programme, University of Pretoria, Pretoria, South Africa

ABSTRACT

The objective of this study was to evaluate the effect of a tannin-rich extract from commercial *Acacia mearnsii* (MTE_0), and gallocatechin, a flavonoid compound derived from *Acacia mearnsii*, on the long-term viability and motility of cryopreserved ovine semen. Six fresh ejaculates obtained from six adult merino rams twice per week for three weeks were allocated to five aliquots (0, 12.5, 25, 50, and 100 µM gallocatechin added into the Optidyl™ extender) before cooling and cryopreservation. Effects of MTE_0 and gallocatechin on post-thawed motility characteristics were analyzed using computer-assisted semen analysis (CASA), and viability (LIVE/DEAD® kit, Molecular Invitrogen, Waltham, MA), oxidative stress (2,7-dichlorodihydrofluorescein diacetate (H2DCFDA, Thermo Fisher®, Waltham, MA)) for reactive oxygen species (ROS), mitochondrial membrane potential (JC-1 MitoTracker, Molecular Invitrogen, Waltham, MA), acrosomal integrity (lectin PNA), and capacitation using merocyanine 540 (M540) and YO-PRO-1 dyes in flow cytometry. Data were analyzed using one-way ANOVA (IBM SPSS 21.0 for Windows, Armonk, NY). Gallocatechin at 25 µM positively affected ($p \leq .001$) kinematic parameters including average path velocity (VAP), progressive velocity (VSL), and beat cross frequency (BCF) of cryopreserved semen. Similarly, gallocatechin at 25 µM^{-} improved sperm motility (live 21.99 ± 2.06%), reduced ROS levels (26.45 ± 1.10%), and mitigated premature capacitation (viable and stable 20.08 ± 1.48%) compared to other treatments. Gallocatechin addition to semen resulted in a significant ($p \leq .001$) positive effect compared with the MTE_0 extract. It is concluded that gallocatechin inclusion at 25 µM significantly reduces semen deterioration following cryopreservation. This study is the first to introduce gallocatechin as an efficient antioxidant additive to ovine semen to improve its quality during storage. Our findings will help improve post-thaw ovine semen quality and longevity. Future studies to elucidate the mechanism of anti-oxidative stress action of gallocatechin and its derivatives on semen motility and longevity are recommended.

Abbreviations: AEC: Animal Ethics Committee; AI: artificial insemination; ALH: amplitude of lateral head displacement; ANOVA: analysis of variance; ATE: *Acacia mearnsii* commercial extract; AV: artificial vagina; BCF: beat cross-frequency; BPI: base peak ion; CASA: computer semen analyzer; CID: collision-induced dissociation; CTL: control; Da: Dalton; DIA: data independent acquisition; DNA: deoxyribonucleic acid; DMSO: dimethyl sulfoxide; EGCG: epigallocatechin-3-gallate; ESI: electrospray ionization; FC500: flow cytometer; FITC: fluorescein isothiocyanate; FL1: 525 mM blue laser; FL3: 620 mM, blue laser; FWHM: full width at half maximum; Galcat: purified gallocatechin; GSH: glutathione peroxidase; h: hour; H2DCFDA: 2′,7′-dichlorodihydrofluorescein diacetate; LBW: live body weight; LIN %: percent linearity; LMM: linear mixed model; MDA: malondialdehyde; mL: milliliter; MP: membrane potential; MTE_0: a tannin-rich extract from ATE extraction with ethanol and fractionated with an Amberlite packed column; MW: molecular weight; NA: not applicable; PBS: phosphate-buffered saline; PI: propidium iodide; PNA: *Arachis hypogaea* (peanut agglutinin); PPM: parts per million; PRG %: percent progressive motility; RMRD, SA: Red Meat Research and Development, South

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CONTACT Dietmar E. Holm dietmar.holm@up.ac.za Faculty of Veterinary Science, University of Pretoria, Room 2-16 Production Animal Studies Building, Onderstepoort Campus, Private Bag X04, Onderstepoort 0110, South Africa

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Africa; ROS: reactive oxygen species; RT: room temperature; SE: standard error; STR: straight line velocity; TM %: percent total motility; V: voltage; VAP: average path velocity; VCL: curvilinear velocity; VSL: progressive velocity; UPLCMS: ultra-performance liquid chromatography coupled with high-resolution mass spectrometry.

Introduction

Cryopreservation of ram semen is a subject of focused research due to the growing market demand for mutton coupled with the need to improve sperm quality for artificial insemination (AI) while reducing disease spread and controlling genotype losses (Clément et al. 2012). Spermatozoa of mammals have natural occurring antioxidants superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase, as well as some non-enzymatic antioxidants such as methionine, ascorbic acid and α-tocopherol but due to dilution during cryopreservation they tend to decrease (Bucak et al. 2010). The biosynthetic capacity of sperm is limited (Aitken and Fisher 1994) and the concentration of antioxidants present in the semen could be reduced due to the dilution, consequently decreasing the beneficial effect of the endogenous antioxidant defense. Thus, the addition of antioxidants, even in small concentrations, with very good preservation techniques improves the sperm function during storing with good capacitation/fertilization in the female reproductive tract.

Efficient cryopreservation requires experience and knowledge of the changes that occur during the process of freezing, as addressed by a previous study (Ramón et al. 2013). Cryopreserved ram semen has a short life span and markedly reduced sperm quality post-thawing due to atypical plasma membrane composition (Watson 1995, 2000). The polyunsaturated to saturated fatty acid ratio of the spermatozoal membrane is relatively high, with a lower cholesterol to phospholipid molar ratio, which increases susceptibility to oxidative damage (Najafi et al. 2014). Membrane lipid oxidation impairs the spermatozoal function, resulting in loss of motility and mitochondrial activity, increased deoxyribonucleic acid (DNA) damage, and lack of activation of apoptotic pathways (Aitken et al. 1998; Aitken 2020). Fertility problems may be derived from various factors like semen collection processes, infections, constituents of semen extenders, cryopreservation processes, and specialized skills required for intra-uterine insemination. In the same way, conception rates depend on the quality of semen, which is generally low post-thawing, and sperm capacitation; additionally, the fertilization process is hindered by sub-lethal dysfunction of spermatozoa (Watson 2000).

The selection of animals with good quality semen for cryopreservation and AI technologies is a critical step toward improving fertility levels of frozen-thawed semen (Roca et al. 2015; Yeste 2016). Despite some animal species having satisfactory fertility test results of fresh-stored/liquid semen, the frozen-thawed semen may not meet acceptable fertilization results in commercial AI programs (Roca et al. 2015; Yeste 2015; Vašíček et al. 2022). Accumulated evidence indicates that inherent male progeny variability in semen cryopreservation is one of the factors responsible for the marked differences in sperm cryo survival (Fraser et al. 2008; Roca et al. 2015; Yeste 2015; Yeste 2016). However, survival of spermatozoa following cryopreservation of ram semen is dependent on reversible metabolic activity of spermatozoa (Bucak et al. 2010). This could be achieved by provision of an effective environment for steady cooling of ram semen, with a focus on the development of extenders that maintain membrane integrity and high motility, as well as the ability to capacitate, prevent oxidative stress, and minimize the generation of reactive oxygen species (ROS). Antioxidant substances could reduce the impact of oxidative stress and thereby improve the quality of semen post-thaw as reviewed previously (Liman et al. 2022). Three previous studies have reported on the effect of adding tannin extract to semen. In 2018, it was reported that the ethanol extract of a commercial oenological tannin (*Quercus robur*, toasted oak wood, Tan'Activ®) has a biological effect at 10 µg/mL concentration (Spinaci et al. 2018). The extract stimulated an increase ($p < .001$) in *in vitro* swine sperm capacitation and also increased ($p < .001$) oocyte fertilization rate (Spinaci et al. 2018). However, at a concentration of 100 µg/mL, the opposite effect was recorded for both sperm capacitation (B pattern) and fertilizing ability associated with higher sperm viability (Spinaci et al. 2018). Similarly, in a different study, 5% crude tannin was added to semen of Bali cattle (Fitriyah et al. 2017) and chilled for a period of 14 days, after which it increased ($p < .001$) motility and viability, with a decrease in abnormal semen morphology (Fitriyah et al. 2017). Subsequently, 2.5% crude tannin added to liquid semen of Etawa crossbred goats was reported to improve ($p < .001$) the sperm viability, while at 20% crude tannin, it reduced the proportion of viable spermatozoa (Putranti et al. 2012).

In our published review (Liman et al. 2022), it was suggested that addition of *Acacia mearnsii* tannin-rich extracts, extract fractions or purified/compounds to semen may improve the quality and viability of semen intended for cryopreservation (Liman et al. 2022). Furthermore, a recent review indicated that using trace minerals could be beneficial to maintain the fine balance of ROS to improve the spermatozoa of post-thawed semen (Ferreira et al. 2022). However, the biological effects have not yet been sufficiently verified for potential application in biological samples such as semen.

This study is the first to investigate the effects of an extract of *Acacia mearnsii* tannin (MTE_0), and a flavonoid compound (gallocatechin), previously indicated as one of the potentially useful antioxidant compounds derived from *Acacia mearnsii*, on the viability and motility of cryopreserved merino ram semen.

Results

Gallocatechin significantly enhanced the cryopreserved sperm kinematic characteristics, especially including the parameters of average path velocity (VAP), progressive velocity (VSL), and beat cross frequency (BCF) ($p \leq .001$ for all the three). There were no significant differences between the gallocatechin treatments and control for other CASA parameters (progressive, rapid motility, curvilinear motility, and lateral displacement velocity (ALH)). However, we recorded a decreasing trend for gallocatechin total motility (TM), straight line velocity and linearity (LIN) (Figure 1).

For the TM, we recorded significant decrease as a trend ($p \leq .01$) for % motile spermatozoa as gallocatechin concentration increases from 12.5 µM (50.62 ± 3.30%) compared with the control, and then progressive motility (PRG) decreases as the concentration increased. In contrast, VAP and VSL increased significantly ($p \leq .001$) as the concentration increased. Similarly, the BCF significantly ($p \leq .001$) increased at gallocatechin concentrations of 50 µM and 100 µM compared with the control. The STR and LIN as a trend ($p \leq .01$) increased at 12.5 µM and 50 µM gallocatechin concentrations, respectively, during the cryopreservation (Supplemental Table 1).

The gallocatechin concentration of 25 µM significantly improved sperm viability (live 21.99 ± 2.06%), with a rise of ROS at 25 µM (26.45 ± 1.10%), 50 µM (26.03 ± 1.13%), and 100 µM (26.84 ± 0.72%) compared with the control. The capacitation status significantly ($p \leq .001$) remained without change at 25 µM (viable and stable 20.08 ± 1.48%) compared with control (Figure 2A) (Supplemental Table 2).

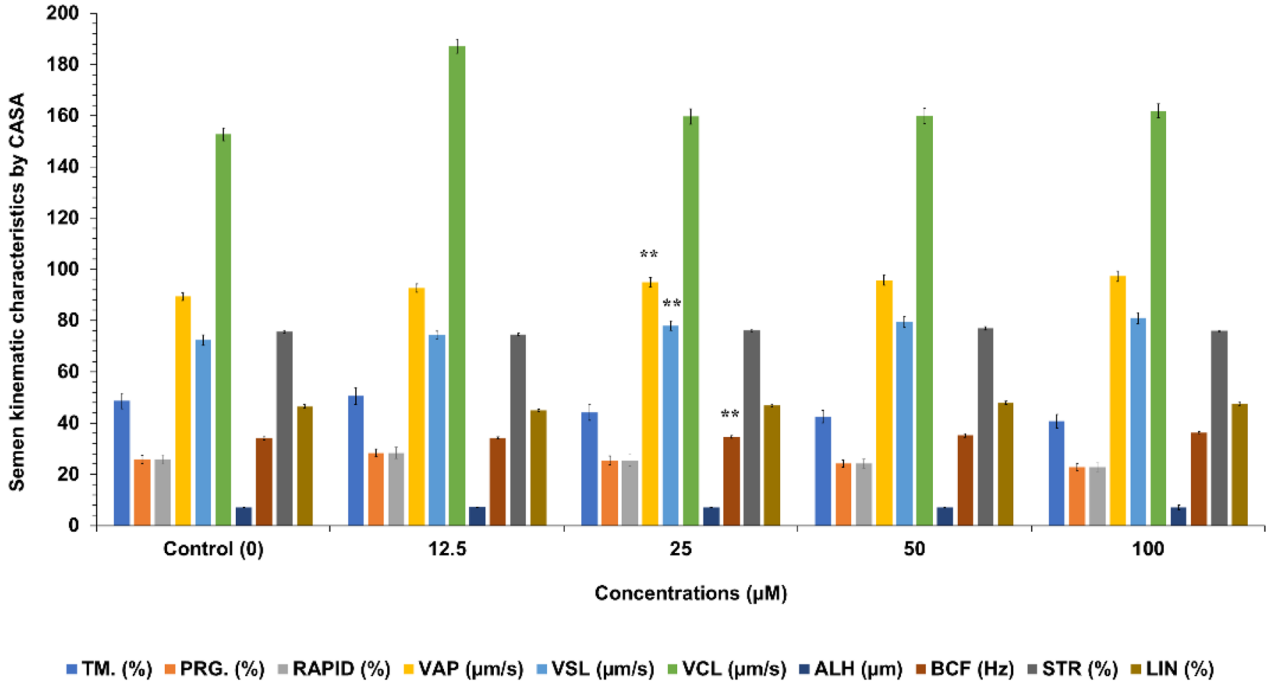

Figure 1. Motility kinematic parameters on the effect of gallocatechin on post-thaw cryopreserved ovine semen (mean ± SE) by CASA. **The kinematic characteristics positively ($p \leq .001$) affect at 25 µM compared to control. TM: total motility; PRG: progressive motility; VAP: average path velocity; VSL: progressive velocity; VCL: curvilinear velocity; ALH: lateral displacement velocity; BCF: beat cross frequency; STR: straight-line velocity; LIN: linearity.

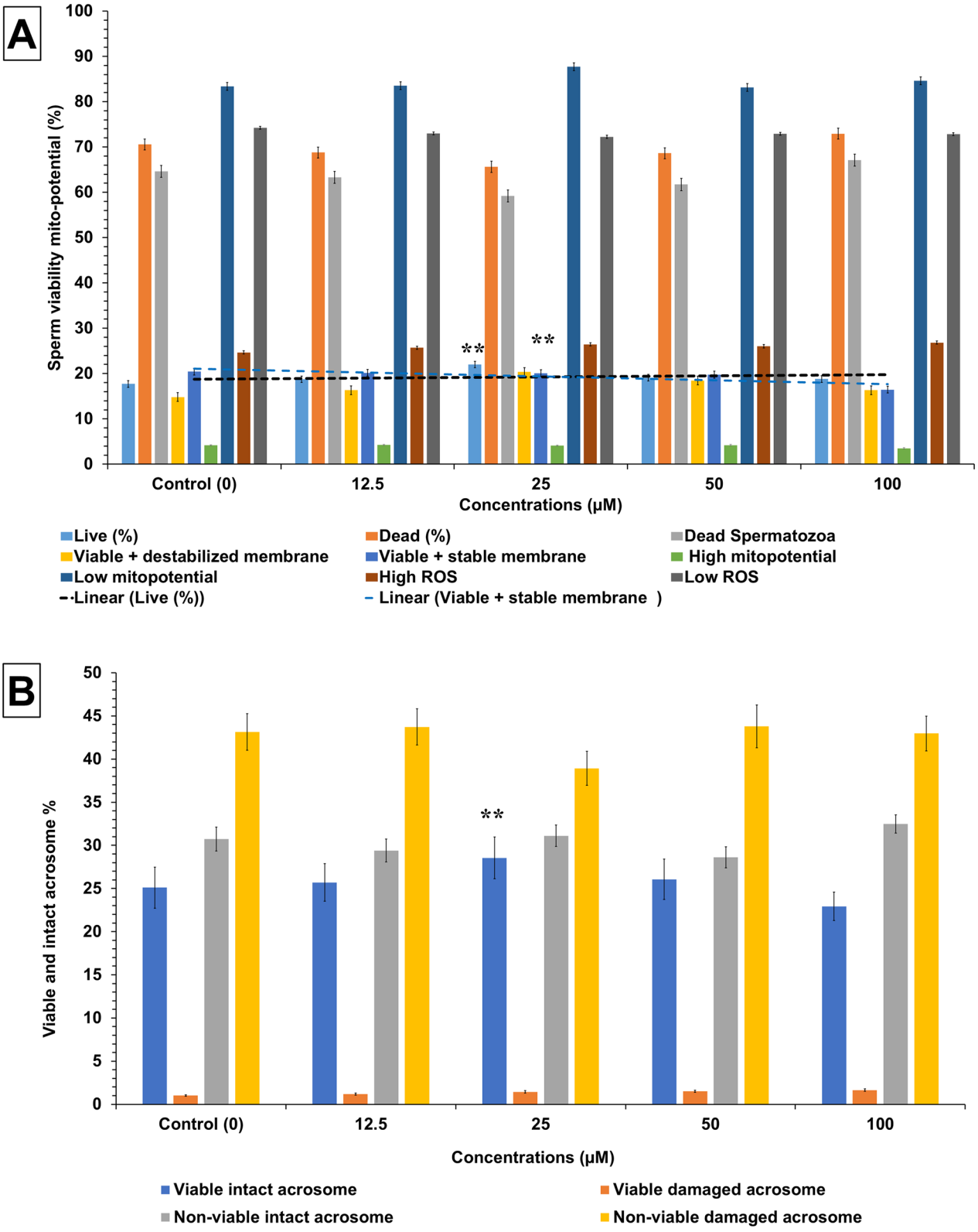

Figure 2. Viability parameters on the effect of gallocatechin on post-thaw cryopreserved ovine sperm (mean ± SE) by flow cytometry. (A) **The viability mito-potential positively ($p \leq .001$) affects semen at 25 µM concentration compared to control and others. (B) **The acrosomal integrity positively ($p \leq .001$) affects semen at 25 µM compared to control.

The acrosomal integrity (viable and intact 28.54 ± 2.42%) was improved at 25 μM compared to the control (Figure 2B) (Supplemental Table 3).

The percentage of live spermatozoa were significantly ($p \leq .001$) improved at 25 μM (21.99 ± 2.06%) compared with other concentrations, and equally reduced dead spermatozoa at the same concentration (25 μM) compared with others. Nonetheless, the capacitation status (viable and stable) was only significantly ($p \leq .001$) changed at 100 μM compared to other concentrations. Gallocatechin addition to semen resulted in a significant ($p \leq .001$) positive effect compared with the MTE_0 extract. Therefore, we can presume that a gallocatechin concentration of 25 μM addition into the extender could improve post-thaw fertility and longevity of cryopreserved ovine semen.

Discussion

Based on accumulated evidence, the utilization of plant extracts/compounds for various purposes is recommended due to their potent antioxidation and other useful biological properties (Clément et al. 2012; Ali and Banana 2020; Sobeh et al. 2020; Wurlina et al. 2020; Ahmed et al. 2021; Rad et al. 2021; Ros-Santaella and Pintus 2021; Liman et al. 2022). The alcoholic extract of a medicinal plant, *Bauhinia forficata*, which contains flavonoid compounds (such as gallocatechin) at 0.1 mL per 10 g body weight per day, lowered the malondialdehyde (MDA) levels in the epididymis of adult Wistar male rats (Sampaio et al. 2019). A previous report on the effect of supplementation of commercial tannin-rich extract on the reproductive performance of the South African Mutton Merino rams at 0.03 g/kg BW/day of non-encapsulated tannin (TE) and 0.06 g/kg BW/day of encapsulated tannin extract (ETE) for 16 weeks, improved the testicular length, semen volume, and concentration (Ahmed et al. 2021). Additionally, it was also reported to have an effect on the reduction in the percentage of sperm abnormality, and on reduction in PRG (Ahmed et al. 2021). However, when the concentration of tannin extracts was increased, it resulted in the reduction of sperm volume and increased morphological abnormal sperm (Ahmed et al. 2021).

Therefore, improved reproductive performance of rams with TE and ETE was the motivation of this study. Up until the current study of the addition of commercial (*Acacia mearnsii*) tannin-rich extract (MTE_0) and gallocatechin compound, no report or data were found in the literature relevant to this study. Gallocatechin (305.0661 (−3.6 ppm) *m/z*, $[M-H]^-$) is the only tentatively identified compound in the current study with its observed mass consistent with previous profiling of commercial *Acacia mearnsii* bark (Venter et al. 2012). Furthermore, it was reported that tannins with greater molecular weight and with higher hydroxyl groups in their structure achieve greater antioxidation action (Fraga-Corral et al. 2020). Above and beyond, the degree of polymerization of the tannins is highly related to the levels of antioxidation activity (Fraga-Corral et al. 2020). The gallocatechin in this study has high polymerization and a high number of hydroxyl groups, and thus has greater antioxidation properties (Fraga-Corral et al. 2020). Moreover, gallocatechin, a flavanol, is capable of antimicrobial activity since it has a tri-hydroxyl B ring which results in greater inhibition of bacteria than those with a di-hydroxyl group B ring, as reviewed previously (Fraga-Corral et al. 2020). It was also reported that tannins have varied bioactivities such as antioxidation, antimicrobial, anthelmintic, antiviral, and anti-inflammatory and this has been confirmed in various *in vitro* and *in vivo* studies (Fraga-Corral et al. 2020). The complete mechanism of the effects exerted on animal tissues is still unknown (Fraga-Corral et al. 2020).

Reports in the past have postulated that kinematic characteristics measured by gated CASA, as sampled in the present study (Supplemental Table 4), are reliable fertility indicators. The most commonly evaluated parameters include VAP, VCL, VSL, ALH, BCF, and LIN (Antończyk et al. 2010). In comparison, assessing semen motility and viability using a microscope, while somewhat valuable, is grossly inadequate (Martínez-Pastor et al. 2010). Therefore, to obtain accurate viability and motility data from semen samples, CASA and flow cytometry with gated scatter diagrams (Figure 3) are the preferred methods, despite their high instrumentation costs (Vašíček et al. 2022). This agrees with data obtained when antioxidants are added to preserve stored sperm motility in a dose-dependent manner (Taşdemir et al. 2020).

In the preliminary study, four distinct fractions were isolated (MTE_0, MTE_1, MTE_2, and MTE_3) from a tannin-rich commercial *Acacia mearnsii* extract (ATE). The MTE_0 fraction contained sucrose and at least seven other compounds: (1) gallocatechin, (2) glucosyringic acid, (3) gallocatechin-(4α→8)-catechin, (4) fisetinidol-(4α→8)-catechin, (5) arecatannin A1, (6) fisetinidol-(4α→6′)-fisetinidol-(4α→6)-gallocatechin, and (7) fisetinidol-(4α→8)-catechin-(6→4α)-fisetinidol. Furthermore, in the preliminary study, the fraction MTE_0 had the best antioxidant activity (unpublished). Similarly, in a pilot trial the MTE_0 fraction recorded similar activity to the control when added

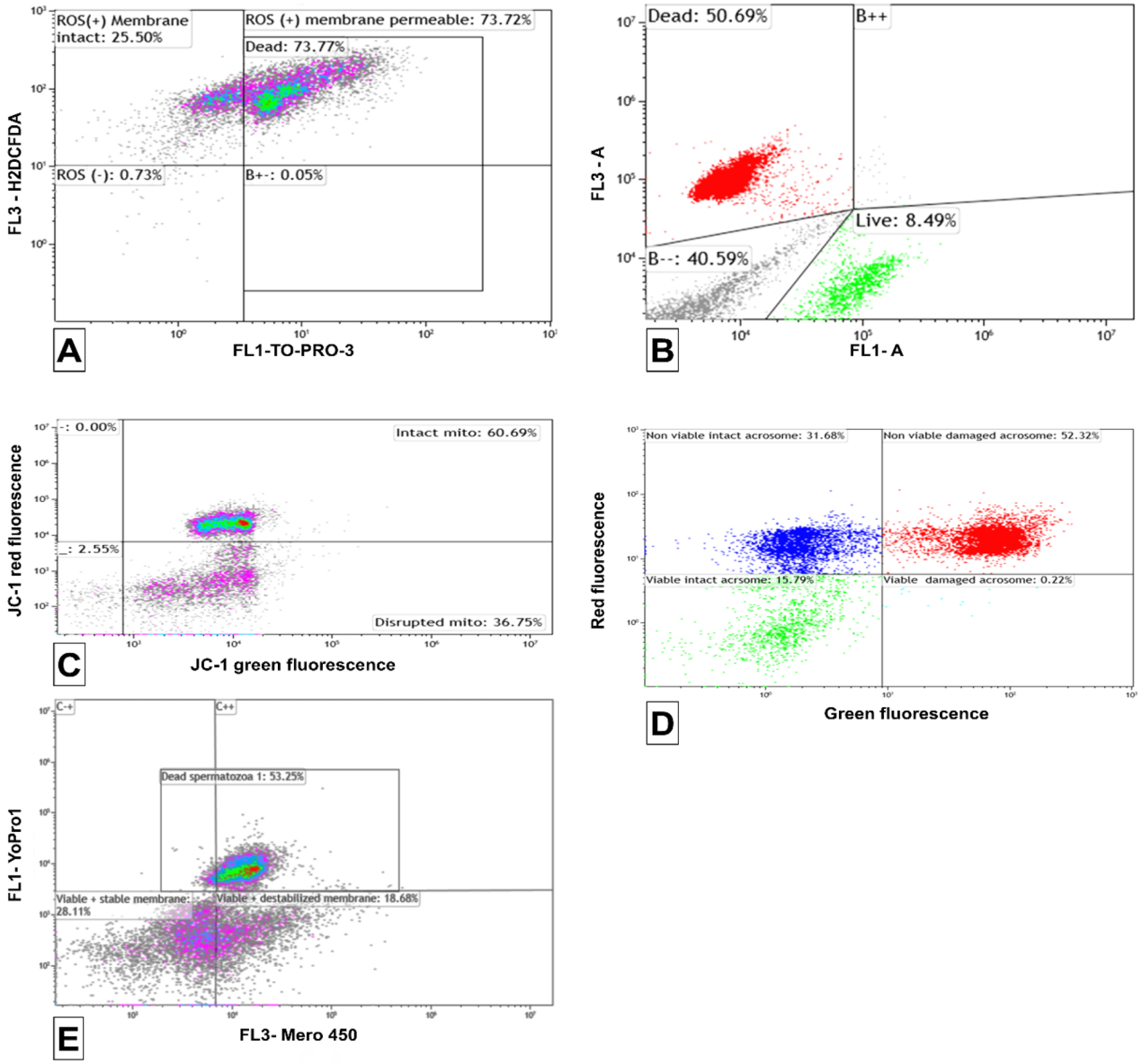

Figure 3. Representative of the flow cytometric scatter figure of the post-thaw ovine semen viability and integrity parameters for the % average ± SDE gated. (A) ROS measured by FL3-H2DCFDA and FL1-TO-PRO-3 dyes represent percentages of spermatozoa with intact plasma membrane, those with membrane improved and those dead. (B) Live/dead sperm assay; red dots indicate dead spermatozoa, green are live spermatozoa, and cellular debris is shown as black dots. (C) Green vs. red JC-1 fluorescence differentiates between high and low mito-potential. (D) Green histogram dots indicate viable spermatozoa with intact acrosomes, blue and red dots indicate non-viable spermatozoa with intact acrosomes and non-viable spermatozoa with damaged acrosomes. (E) High fluorescence of Merocyanine 450 represents viable, capacitated spermatozoa with intact plasma membrane, and those with weakly stained heads are considered non-capacitated.

to semen (Supplemental Tables S1 and S2). Thus, in the current study, only the results concerning the effect of gallocatechin on ovine cryopreserved semen were reported. Gallocatechin recorded significant interaction ($p \leq .001$) with the cryopreserved ovine semen, more than the MTE_0 fraction used in this study. Future studies could include quantifying gallocatechin and other flavonoids in the fraction to detect the correlations with antioxidant efficacy. The antioxidant potential in a similar study was demonstrated in crude alcoholic and aqueous extracts of *A. mearnsii* (Olajuyigbe and Afolayan 2011), with the former containing the highest total flavonoids in the form of gallic acid equivalents (Olajuyigbe and Afolayan 2011). Chen et al. (2018) profiled proanthocyanidins (PACs) from *Acacia mearnsii* bark in a forest farm and identified gallocatechin and catechin as having antioxidative and biological activities with the

potential to be used as a functional drug application in lowering blood glucose levels (Chen et al. 2018). A recent report recommended the use of tannin compounds in semen destined for cryopreservation due to their ability to bring about metal ion chelation, protein precipitation and biological antioxidation (Ros-Santaella and Pintus 2021). While this might be a useful future application of ATE and/or its flavonoid constituents, care should be taken to not trigger reductive stress in spermatozoa through the excessive removal of ROS (Sadeghi et al. 2023). Furthermore, the metal ion chelation properties could have deleterious effects, for instance, when the zinc (Zn) homeostasis is altered it could affect sperm capacitation, as was demonstrated in porcine semen (Zigo et al. 2022). In a recent report, Quinoa seed extracts (QSEs, *Chenopodium quinoa* Wild.) comprised complex compounds (Khalil et al. 2024) like the ATE. QSE was added into a freezing medium at 500–1000 µg/mL, and improved the post-thaw ram semen quality/cryo-damage (Khalil et al. 2024). Similarly, in the present study, QSE improved the PRG and viability, and maintained the plasma integrity of sperm (Khalil et al. 2024). Furthermore, QSE recorded improvement of the sperm velocity, and motion of post-thaw ram semen (Khalil et al. 2024). Linalool is a compound found in QSE and supposedly improved the post-thaw sperm quality in rams due to its ability to counter oxidative stress and reduce the mitochondrial dysfunction in neurons (Sabogal-Guáqueta et al. 2019). In another study, epigallocatechin 3-gallate (EGCG) added at 0.6 mg/L to cryopreserved bovine semen had a protective effect, as it increased ($p < .05$) the TM, with an additional improvement in kinematic properties such as VAP, straightness, curvilinear velocity (VCL), lateral displacement amplitude, and BCF (Li et al. 2022). EGCG also increased ($p < .05$) the spermatozoa's natural antioxidant enzymes CAT, GSH-Px, and SODs levels, and reduced ROS with the MDA in bovine semen (Li et al. 2022). In the current study, we recorded the increase in VAP, VSL, and BCH at 25 µM in the cryopreserved semen. In yet another recent study, resveratrol, a polyphenol from grape skin and seeds at 50–75 µM significantly ($p < .05$) improved ram semen motility, enhanced the acrosome, plasma membrane integrity, antioxidant capacity of mitochondrial membrane potentials (MPs), and reduced oxidative damage to sperm DNA compared to the control (Zhu et al. 2023). It was postulated to confer its antioxidation activities by enhancing the activities of SOD, CAT, GSH-Px, and other enzymes, and also by scavenging free radicals and inhibiting lipid peroxidation (Al-Mutary et al. 2020).

The limitation of this study is the absence of trial replication, which would require substantial time and resources. It is reassuring, however, that the benefits of semen supplementation with gallocatechin were observed in this study with cryopreserved semen of as many as six different rams. Given the low cost and wide availability of highly pure research grade gallocatechin, the authors are hopeful that other research groups and possibly even stakeholders in commercial studs will test this approach in rams and other food animal species.

Conclusions

Gallocatechin at 25 µM added to Optidyl™ semen extender significantly improved the motility and viability of cryopreserved ovine semen. Further studies to understand the mechanism of action of gallocatechin and other flavonoid compounds are recommended to improve the ovine sperm quality and longevity of the cryopreserved semen used for AI.

Materials and methods

Experimental location

Semen samples were collected in September 2022 at an authorized commercial semen station (RAMSEM, Bloemfontein, South Africa, 29° 05.70′S, 026° 20.42′E).

Animals and management

Six adult (3 ± 5 years) merino rams with LBW of 145, 123, 134, 129, 89 and 140 kg respectively were used for semen collection.

Rams were kept in individual pens and fed a concentrate mix with free access to mineral licks and lucerne hay, and water was supplied *ad libitum*.

Experimental design

The fresh ejaculates from the six adult merino rams were collected during a three-week sampling period, two collections per ram per week (Haneef et al. 2016). The semen was allocated to five aliquots (2 × control, concentrations of 12.5, 25, 50, and 100 µM) of MTE_0 and gallocatechin added into semen extender (Optidyl™, Biovet, Saint-Geours-de-Maremne, France) before cooling and cryopreservation.

The MTE_0 extract was obtained from fractionation of the ATE bark water extract of commercial tannin-rich *Acacia mearnsii*. The gallocatechin flavonoid compound [2S,3R (-2-(3,4,5-trihydr oxyphenyl)-3,4-dihydro-1(2H)-benzopyran-3,5,7-triol]

was purchased from Sigma-Aldrich Co. (St. Louis, MO) (catalogue no. G6657-5MG; ≥98% HPLC pure; mass 306.27 Da). Gallocatechin at 0.1% w/v was used in this study at 5 mg in 10 mL (1.632546 mM in pure phosphate-buffered saline, PBS), based on pilot studies with a range of concentrations.

Semen collection and processing

Immediately after semen collection, ejaculate volume was determined using a graduated conical glass tube, and values reflecting the TM and percentage of live spermatozoa were determined using a phase contrast microscope (Nikon, Centurion, South Africa).

The concentration of the fresh semen was determined spectrophotometrically (SDM6; Minitube®, Verona, WI), and the ejaculates were transferred into a water bath at 37 °C. One milliliter of fresh semen, with 2.3×10^9 spermatozoa mL^{-1}, and 60% live spermatozoa, with TM of 3–4 on a 0–5 scale (scale 0 lack of movement; scale 1 there is visible slight movement of distinctive spermatozoa; scale 2 a visible distinct local movement without waves; scale 3 evident wave motion, scale 4 wave motion without whiplash; and scale 5 very fast strong waves with whiplash at the edge of the droplets) (Chenoweth 2015; Peter et al. 2021) were used for the experiment. Semen was extended to a final concentration of 13×10^6 spermatozoa mL^{-1} with tris-based Optidyl™ extender (Biovet, Saint-Geours-de-Maremne, France) used as the base at the ram semen station.

The ejaculates were collected from six adult merino rams by using artificial vagina (AV) as described in a previous study (Sobeh et al. 2020; Li et al. 2022).

As a routine procedure at the semen station, the extender was freshly mixed prior to the collection for the day and kept at 37 °C in a water bath. The extender used was 100 mL of Optidyl™ comprising 150 mL deionized water, tris-diluent, ionized egg yolk, glycerol, and antibiotics (penicillin, streptomycin, spectinomycin, and lincomycin).

For each aliquot, the calculated volume was subtracted from extended semen volume and replaced with gallocatechin (concentration of 0, 12.5, 25, 50, and 100 µM) before cooling and cryopreservation. The volume of extended ejaculate required for each aliquot was 2 mL of semen to obtain the required concentration in each tube (Falcon™ 15 mL conical centrifuge tubes, Thermo Fisher, Waltham, MA). This was kept in a cold room (5 °C) and gradually cooled to 4 °C for 3 h.

The aliquots of the extended semen were loaded into eight Cassou straws (0.25 mL) using the filling and sealing machine (IMV, MPP Uno, 3017/0000, Minitube). Loaded straws were kept in a cold room for 3 h to gradually reduce the temperature from 10 °C to 4 °C. These aliquots were color coded, and labeled with ram number, date of collection, and labeled tannin ram semen. No color was used for control aliquots (0 µM), yellow (12.5 µM), green (25 µM), orange (50 µM), and blue (100 µM). The sealed straws were kept in horizontal position on the trays, moved into the computerized freezing machine (ice cube 14 M Minitube), and frozen at a pre-programmed rate of −3 °C min^{-1} from +4 °C to 1 °C; −3 °C min^{-1} from −10 °C, −8 °C to −17 °C, −15 °C to −25 °C, −25 °C to −100 °C and −20 °C min^{-1} from −100 °C to −130 °C. The sealed Cassou straws were plunged into liquid nitrogen and stored in the dewar flask until analysis.

Analyses

Sperm motility

The cryopreserved semen was thawed in a water bath at 37 °C for 30 s. After warming, the tubes and straws were dried with paper towel, sampled and analyzed for motility by using computer-assisted semen analysis (CASA; IVOS version 12: Hamilton-Thorne Biosciences, Beverly, MA) (Vašíček et al. 2022). The CASA was gated and optimized to ovine semen with capturing 60 frames per second, 30 frames to acquire the motility characteristics, with magnification setting of 100× objective, stage maintained at 38 °C and 15 fields captured in triplicate per aliquot. Ten milliliters of the thawed semen were loaded onto prewarmed (38 °C) chamber slides (20 mm; Leja 4D products B.V., Nieuw-Vennep, The Netherlands) of the CASA (Liman et al. 2021).

Individual parameters were recorded, printed, and stored electronically for further analysis later. The motility characteristics evaluated included TM (%), PRG (%), VAP (µm/s), BCF (Hz), VCL (µm/s), ALH (µm/s), LIN (%), VSL (µm/s), and straight-line velocity (STR, µm/s) as defined previously (Castellini et al. 2011; Van der Horst 2020).

Sperm viability and structural integrity

The viability and structural integrity were analyzed using multiple flow cytometry assays (live/dead), oxidative stress, acrosomal integrity, mitochondrial MP, and capacitation integrity on an FC 500 cytometer (Beckman Coulter, Brea, CA) (Rodriguez-Martinez and Barth 2007).

For the cryopreserved semen aliquots, Cassou straws were thawed in a water bath at 37 °C for 30 s and analyzed for sperm viability and integrity assays

(live/dead, oxidative stress, acrosomal integrity, MP, and capacitation status).

Prior to the analysis, the instrument was gated, and optimized to minimize the background noise and improve repeatability of results (Hossain et al. 2011). The PBS solution was added to all chilled and cryopreserved-thawed semen aliquots according to kit manufacturers' specification, and analyses were done in triplicate using the dedicated flow cytometry software (Kaluza 1.2; Beckman Coulter Inc., Brea, CA).

Oxidative stress (ROS)

The stock solution for the oxidative stress assay was prepared by dissolving 250 mg of 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA), in 2.5 mL DMSO and aliquoting it in 50 µL vials for storage at −20 °C. The TO-PRO-3 dye stock was prepared as a 25 mM solution in a 500 µL tube, and diluted 1/1000 in PBS to obtain a 1 µM working solution. The H2CFDA (green fluorescence) and TO-PRO-3 (red fluorescence) working solutions were warmed up and maintained at room temperature (RT) for at least 20 min prior to sperm labeling. A final concentration of 5 µM H2CFDA in 470 µL of PBS was mixed with 10 mM (final) TO-PRO-3 dye and 15 µL sperm sample. A positive control sample that contained 200 mM peroxide added before the incubation was included for analysis. The test samples and controls were mixed with dyes and incubated for 30 min. at 37 °C in the dark and were analyzed in the flow cytometer immediately after staining using fluorescein isothiocyanate (FITC) – FL1 (525 mM, blue laser) and FL3 (620 mM, blue laser) within 30 min. Resultant scatter diagrams of dye-induced fluorescence were recorded.

Live/dead sperm assay

Master mix was prepared using a final concentration of 100 nM SYBR-14 mixed with PI to a final concentration of 1.2 µM. A total of 15 µL of semen were added to 485 µL of the mix, and the samples vortexed and incubated at 37 °C for 15 min before being measured on the FC500 flow cytometer.

Mitochondrial membrane potential activity

The final working concentration of Mito Probe™ JC-1 from the Assay Kit for Flow Cytometry (Invitrogen, Waltham, MA) was optimized according to manufacturer's guidelines to 2 µM. The diluted JC-1 dye (in 485 µL) was mixed with 15 µL sperm and incubated for 30 min at 37 °C, before the sample was run on the FC500 flow cytometer by using FL1 and FL4 (675 nM, red laser) channels.

Acrosome integrity

A stock solution of *Arachis hypogaea* lectin (peanut agglutinin/PNA-Alexa Fluor™ 488 conjugate) was prepared by dissolving 0.5 mg/mL in deionized water, as described (Van Wilgen et al. 2011). Prior to staining, the stock solution was warmed to RT for at least 20 min and mixed to ensure that the DMSO was dissolved. One microliter of stock solution was added to 1×10^6 spermatozoa diluted in 200 µL of PBS, also supplemented with 1 µL PI. The samples were incubated for 15 min in the dark at RT, washed with PBS, and pelleted at $600 \times g$ for 5 min at 20 °C. The pellet was resuspended in 500 µL of PBS and analyzed by flow cytometry (FL1 and FL3 channels).

Capacitation status

The capacitation status was assessed by using merocyanine 540 (M540) and YO-PRO-1 as described previously (Watson 1995). Briefly, 485 µL of stock solution containing 2.7 µM of M540, 25 nM YO-PRO-1 dye, 25 mg/mL polyvinyl alcohol, and 25 mg/mL polyvinylpyrrolidone was mixed with 15 µL semen. After adding the semen to the solution, the samples were incubated for 30 min at 37 °C in the dark. Semen samples were analyzed within 30 min after staining using a flow cytometer (FL1 channel for green YO-PRO-1 dye and FL3 for orange M540.

Statistical analysis

The data for the cryopreserved semen viability and motility characteristics were analyzed using the linear mixed model (LMM) procedure in IBM SPSS 21.0 (Armonk, NY), with treatment (concentrations of 0, 12.5, 25, 50, and 100 µM) as fixed effect and ram ID and time (weeks) as random effects.

The significant interaction means were separated by LSD test at a 5% significance level. Data were expressed as means ± standard error (SE).

Ethics approval

The experimental procedure was approved by the University of Pretoria Animal Ethics Committee (AEC 193-19).

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Disclosure statement

No potential conflict of interest was reported by the author(s).

Authors' contributions

Conceptualization: A.H., D.E.H., M.S.L.; Methodology: A.H., L.J.M., D.E.H., M.S.L.; Validation: M.S.L.; A.H., D.E.H., L.J.M., P.S.; Formal analysis: M.S.L., A.D.A.B., M.S.; Investigation: M.S.L., A.D.A.B., M.S.; Resources: L.J.M., D.E.H., A.H.; Data curation: D.E.H., M.S.L., A.D.A.B., M.S.; Writing – original draft preparation: M.S.L.; Writing – review and editing: M.S.L., D.E.H., A.D.A.B., M.S., A.H., L.J.M.; P.S.; Visualization: L.J.M.; Supervision: D.E.H., L.J.M., A.H.; Project administration: D.E.H.; Funding acquisition: M.S.L., D.E.H., L.J.M. All authors have read and agreed to the published version of the manuscript.

Data availability statement

Data are available on request from the corresponding author.

ORCID

Mohammed S. Liman http://orcid.org/0000-0002-1671-1224
Abubeker Hassen http://orcid.org/0000-0002-8240-3414
Mario Smuts http://orcid.org/0000-0003-3358-3241
Ahmed Dayain Abdalla Biraima http://orcid.org/0000-0003-2728-9900
Peter Sutovsky http://orcid.org/0000-0002-2643-3360
Lyndy J. McGaw http://orcid.org/0000-0001-8447-0613
Dietmar E. Holm http://orcid.org/0000-0002-9340-6573

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