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Original Article

Influence of aqueous extract of *Annona muricata* leaves in Tris-egg yolk extender on storage of spermatozoa from West African Dwarf goat (*Capra hircus*)

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ABSTRACT

Background: Because oxidative stress can induce decreased quality of caprine semen during the storage, there has been limitation for the use of stored semen in the assisted reproductive technologies. The present study, therefore, assesses the potential of *Annona muricata* (*A. muricata*) to reduce semen storage associated-damages.

Methods: Semen was collected by electro-ejaculation from ten bucks, and extended with Tris-egg yolk (TEY) supplemented with *A. muricata* leaf aqueous extract (SAE) at 20 (SAE20), 40 (SAE40), and 80 (SAE80) μg/mL. Sperm variables including motility, motion characteristics, viability, membrane functionality, and DNA integrity were assessed at different storage periods (6, 24, 48, and 72 hr). In addition, oxidative stress indicators in the extender supplemted with SAE were also assessed for each group.

Results: By adding SAE at 80 μg/mL in TEY, the storage of goat buck semen was improved, resulting in reduced loss of sperm motility, viability, DNA fragmentation, and membrane integrity during chilled storage at 4℃ for up to 72 hr. In addition, enrichment of TEY extender with SAE significantly ($p < 0.05$) reduced malondialdehyde, an indicator of oxidative stress, compared to the negative control.

Conclusions: Supplementation of SAE in TEY extender can reduce buck spermatozoa liquid storage associated damages due to oxidative stress.

Keywords: *Annona muricata*, buck semen, liquid semen storage, storage-associated damages, Tris-egg yolk extender

INTRODUCTION

The shortage of protein, particularly of animal origin, prevails in most developing countries. And the world food economy is increasingly being driven by the shift of diets towards animal-based products such as meat, milk, and dairy products (FAO, 2015). Unfortunately, in view of population growth projected to reach 9.6 billion by 2050 (UN, 2013), the deficit in animal-derived protein is likely to increase by 10-20% in Sub-Saharan African countries in the years ahead (FAO, 2015). In the face of this population growth, the improvement of farm animal productivity and consequently reproduction is critical.

Ruminant production plays an important role in food security. However, the rearing of large ruminants can be restricted by acute scarcity of feed and fodder (Kumar et al., 2010). Therefore, goat rearing may be used to overcome this. However, the poor production potential of goats during natural mating and their seasonal breeding habit can be a disadvantage, resulting in a seasonal availability of chevon (meat from goat) or kid (the young of a goat) in breeding sites whose main activity is production and sale of the latter. Environmental factors play an important role in animal evolution; in temperate regions, daily photoperiod and annual cycle of temperature are the main factors, while in tropical regions, annual cycle in rainfall, with the consequent cycles in food availability, are the more important ones (Vivien-Roels and Pévet, 1983). Furthermore, climate change with subsequent global warming is a source of heat stress for animals, moving body temperature from its resting state to conditions that are higher than animal's thermo-neutral zone (Maya-Soriano et al., 2013), negatively impacting animal reproduction.

Thus, seasonal breeding coupled with global warming can decrease goat production yield, which implies the need for boosting animal and food production through the rearing of genetically more productive animals. Considering the above mentioned, there is a need for more alternatives to guarantee an effective, efficient, and sustainable contribution of goat meat supply to improve food security. In goat farming, genetic improvement and palliation of seasonal breeding can be achieved by means of assisted reproductive technologies programs. This can only be possible with semen preservation of superior bucks using suitable extenders. Preservation of goat semen at refrigerated temperature is cheaper and more feasible than cryopreservation of semen at ultra-low temperature (Yimer et al., 2014). An affordable semen extender that can preserve spermatozoa characteristics is therefore worthwhile, as it will accelerate reproduction and kidding programs; it allows affordable transport of semen to moderately distant areas, and ensure the dissemination of genetically high-merit animals.

To preserve goat sperm, Tris-egg yolk-based extenders have been used for many years. Different egg yolk concentrations (2.5% to 20%) have been compared by various researchers (Ranjan et al., 2009; Priyadharsini et al., 2011; Kalyani et al., 2015; Gojen Singh et al., 2016). In an effort to minimize the adverse effects associated with sperm storage, several researchers have investigated the effects of antioxidants and other compounds (nanoparticles, royal jelly, and gelatin) as additives to semen extenders. In this regard, several authors have reported improvements in semen quality with the addition of enzymatic antioxidants (Silva et al., 2011), vitamins (Azawi and Hussein, 2013), fatty acids (Eslami et al., 2017), and amino acids (Akalin et al., 2016; Kaya et al., 2018). However, some of these additives have provided no improvement and even contradictory results when incorporated into semen extenders (Bucak et al., 2008; Mata-Campuzano et al., 2014)

Recently, studies have intensified to investigate the inclusion of natural antioxidant compounds to semen extenders. As these products are found naturally, they are easily accessible and it is believed that they could be more efficient than synthetic compounds. Furthermore, it is worth noting that two-thirds of the world's plant species have medicinal properties; in particular, many medicinal plants have proven interesting results in maintaining semen characteristics. Some interesting results of plasma membrane integrity, mobility, viability and DNA integrity have been documented with extracts of *Salvia rosmarinus* (Ros-Santaella and Pintus, 2021), *Psidium guajava* (Wurlina et al., 2020), and *Tribulus terrestris* (Ariyan et al., 2021). However, other natural compounds with potent activities, among which *Annona muricata* (*A. muricata*) also represent a rich source of bioactive compounds, with the ability to prevent or significantly reduce storage-associated sperm damages. Thus, the present study was initiated with a general objective to develop suitable extenders to preserve West African Dwarf buck semen at refrigerated temperature using natural plant extract, viz., *A. muri-*

cata aqueous extract, and egg yolk. More specifically, the present study evaluated the effects of enrichment of Tris-egg yolk extender with *A. muricata* leaf aqueous extract for sperm quality, oxidative stress indicators, and sperm DNA integrity.

MATERIALS AND METHODS

Study site

This study was conducted at the Teaching and Research Farm and the Laboratory of Physiology and Animal Health within the Department of Animal Science, Faculty of Agronomy and Agricultural Sciences at the University of Dschang. The university is located in the West region of Cameroon, which is part of the western highlands with a monomodal rainfall agroecological zone.

Annona muricata (Soursop) leaves harvesting and preparation of aqueous extract

A. muricata leaves were harvested from the same plant in the Western high lands of Cameroon, sun-dried, and mechanically milled. The resulting powder was used to prepare *A. muricata* leaf aqueous extract according to the procedure used and described by Iqbal et al. (2016) with minor modifications. For this, 10 g powdered *A. muricata* leaves was macerated in 100 mL each of distilled water, placed at room temperature for 48 hr in the dark. The soaked material was stirred every 6 hr. After 48 hr, the mixture was filtered using qualitative filter paper (Whatman 113V, England). The filtrate was evaporated to total dryness by vacuum distillation on a rotary evaporator at 45℃ and the resulting extract stored in dark at 4℃.

The *A. muricata* leaves were harvested in the Western Highlands of Cameroon, sun-dried, and mechanically milled. The resulting powder was used to prepare *A. muricata* leaf aqueous extract according to the procedure used and described by Iqbal et al. (2016) with minor modifications. For this, 10 g powdered *A. muricata* leaves was macerated in 100 mL each of distilled water, placed at room temperature for 48 hr in the dark. The soaked material was stirred every 6 hr. After 48 hr, the mixture was filtered using qualitative filter paper (Whatman 113V, England). The filtrate was evaporated to total dryness by vacuum distillation on a rotary evaporator at 45℃ and the resulting extract was stored in a dark place at 4℃. The extract was then weighed to calculate yield with the following formula:

*100

Animals and semen collection

10 healthy West African Dwarf bucks proven fertility, aged 2.5 to 3 years, weighing 27 ± 2 kg were used in this study. The animals were housed at the Teaching and Research Farm of the University of Dschang with an appropriate balanced diet. They were given commercial concentrate (0.5 kg/buck/day) and had *ad libitum* access to good quality hay, water, and mineral blocks. Ejaculates were collected twice a week for 6 consecutive weeks by electroejaculation. Immediately after collection, each ejaculate was transferred to a water bath maintained at 37℃ and instantly assessed for semen color, volume, concentration, and mass motility. Only ejaculates with a color score ≥ 3, volume ≥ 0.75 mL, concentration ≥ 2.5 × 10^9 sperms/mL, and mass motility score ≥ 3 were used in the study. Semen samples that met these characteristics were pooled to minimize individual variation and processed for extending.

Extender preparation and semen processing

The Tris-egg-yolk (TEY) extender consisted of 2.666 g Tris, 0.44 g glucose, 1.398 g citric acid in 100 mL distilled water, and egg yolk at a 12% (v/v) concentration. The pooled semen sample was divided into equal aliquots and extended at 37℃ using TEY supplemented with 0 μg/mL for the negative control (0), 0 μg/mL plus 0.3 g/dL of vitamin C, 100 IU/mL of penicillin, and 0.05 mg/mL of streptomycin for the positive control (0+), and supplementation of 20, 40, and 80 μg/mL of *A. muricata* leaf aqueous extract (SAE) for the SAE20, SAE40, and SAE80 groups, respectively, to reach a concentration of 200 × 10^6 sperms/mL. The extended semen samples were stored at 4℃ for 72 hr, and assessed for sperm quality, oxidative stress biomarkers, and DNA integrity at 6, 24, 48, and 72 hr post storage.

Assessment of sperm viability

Sperm viability was evaluated using eosin-nigrosin staining (Evans and Maxwell, 1987). Thin smears, prepared in duplicate, were created by mixing 10 μL semen (diluted to 25 × 10^6 sperms/mL with TEY extender) with 20 μL eosin-nigrosin (eosin-Y 1.67 g, nigrosin 10 g, and sodium citrate 2.9 g dissolved in 100 mL distilled water) on a warm slide (37℃) and immediately spreading with another slide. After air drying, viability was assessed by

counting a minimum of 200 sperms from 3-4 different fields with bright-field microscopy (400×). Spermatozoa showing partial or complete purple color were considered non-viable, while only spermatozoa showing white color, indicative of strict exclusion of the stain, were considered alive (Fig. 1).

Assessment of sperm functional membrane integrity

Sperm functional membrane integrity was evaluated based on the method described by Revell and Mrode (1994). A 20 μL aliquot of the extended semen sample was mixed with 200 μL pre-warmed (37℃) hypoosmotic solution (100 mOsm made by 9 g fructose and 4.9 g trisodium citrate per litre of distilled water) that was prepared daily and stored at 4℃. The mixture was incubated at 37℃ for 60 min. After incubation, the sample was gently mixed. Thin smears were prepared in duplicate by spreading a 15 μL drop of the treated mixture on a pre-warmed slide and allowing it to air-dry. A minimum of 200 spermatozoa were counted in 4-5 different microscopic fields at 400 × magnification. Spermatozoa with swollen or coiled tails were considered to have functional membranes, while those showing no swollen or coiled tails were considered to have defective plasma membranes (Fig. 2).

Assessment of sperm DNA integrity

Sperm DNA integrity was evaluated by the acridine orange (AO) test, as described by Tejada et al. (1984). The AO stains normal double-stranded DNA as green and denatured single-stranded DNA as red (Silva and Gadella, 2006). Thick smears were made on pre-cleaned slides and allowed to air-dry for 20 mins. The slides were fixed in Carnoys solution (3 parts methanol/1 part glacial acetic acid) for at least 2 hr and preferably overnight. The Carnoys solution was prepared daily. The slides were removed from the fixer and allowed to dry for 5 mins before staining. The stock solution consisted of 1 g AO in 1L distilled water. To prepare the staining solution, 10 mL stock solution was added to 40 mL 0.1 M citric acid and 2.5 mL 0.3 M $Na_2HP0_4 \cdot 7H_20$. All solutions were at room temperature. The final pH of the stain was 2.5, with a concentration of 0.19 mg/mL. The stock solution was stored in the dark at 4℃, and the AO stain was prepared daily. The slides were placed on a wire cradle, and 2-3 mL staining solution was spread over each slide for 5 mins. They were then gently rinsed under a stream of deionized water. Before the slide could dry, a coverslip was placed upon it. If allowed to dry, the slide was remoistened with one or two drops of distilled water. A paper towel was placed over the mounted slide, and any excess water was firmly squeezed out using a rubber roller. The coverslip was then sealed with nail polish. The slides were examined under a fluorescence microscope equipped with a digital camera and image analyzer computer software. The 200 spermatozoa per slide were assessed in 10 different optical areas to determine the percentage of spermatozoa with denatured DNA (Fig. 3).

Assessment of sperm motility

Sperm motility and motion characteristics were assessed by computer-assisted sperm analyzer (CASA) with a warmed stage at 37℃ (Sperm Analyze Vista, version V1.12 Maya, Guangzhou, China). The samples were diluted using TEY extender to 25 × 10^6 sperms/mL at 37℃. A semen sample (10 μL) was placed on a prewarmed CASA slide and covered with a cover slip. For each sample, 4-5 fields per drop were analyzed at magnification 200 ×

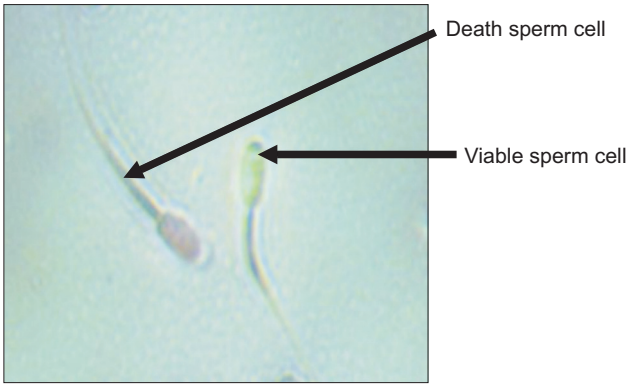

Fig. 1. Eosin-nigrosin staining to evaluate sperm viability.

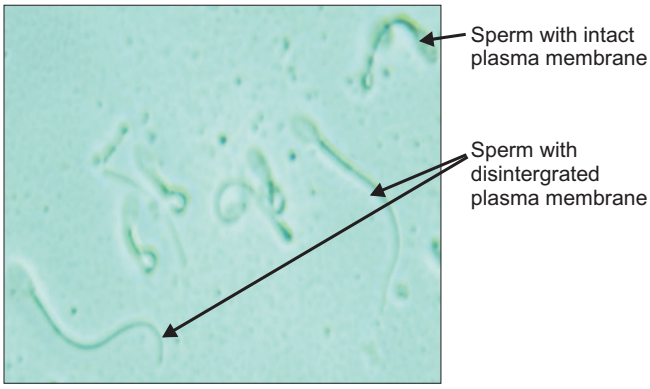

Fig. 2. Hypoosomtic test of sperm plama membrane intergrity.

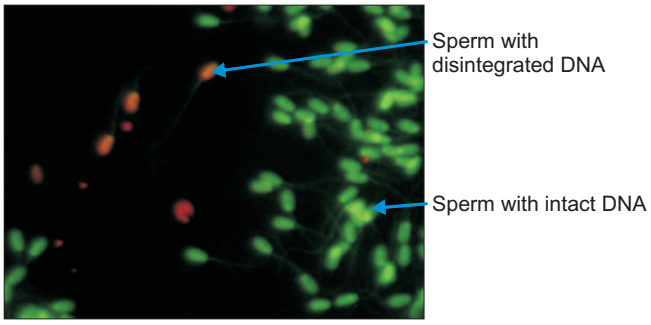

Fig. 3. Acridine orange (AO) staining. Sperm heads having intact DNA structure fluoresce green when AO binds to double-stranded DNA. Sperm heads having disintegrated DNA (single-stranded DNA) fluoresce a spectrum of colors ranging from orange, red to partially or entirely red.

and a minimum of 200 spermatozoa were evaluated as described by Eslami et al. (2017). The semen variables included in the analysis were total motility (TM, %), fast progressive motility (FPM, %), slow progressive motility (SPM, %), local motility (LM, %), curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s), average path velocity (VAP, μm/s), linearity (LIN, %), and straightness (STR, %).

Assesment of total peroxidases

The peroxidase activity was determined according to the method described by Habbu et al. (2008). A 0.5 mL of homogenate was added to 1 mL potassium iodide solution (10 mM) and 1 mL sodium acetate (40 mM) was added. The potassium iodide absorbance was read at 353 nm, which indicated the amount of peroxidase. Then, 20 μL H_2O_2 (15 mM) was added and the change in absorbance over 5 mins was recorded. Peroxidase activity units were expressed as the amount of enzyme required to change the optical density by one unit per min. The enzymatic activity of peroxidase was deduced by the Beer-Lambert law (Servais, 2004) as follows:

$$1.\ D.O. = \varepsilon.C.L$$

D.O. = optical density; ε = coefficient of molar extinction of peroxidases (11.3 M-1 cm-1); C = concentration in total peroxidases; L = length of the optical path (1 cm).

Assesment of lipid peroxidation

At the end of the storage period (72 h), semen samples were centrifuged at 550 g for 10 mins and the pellet was discarded. The supernatant was again centrifuged at 550 g for 10 mins and finally at 3,000 g for 30 mins. The complete supernatant, considered as the medium, was used to evaluate oxidant and antioxidant profiles. Lipid peroxidation was determined in extended semen sample by measuring the amount of malondialdehyde (MDA) in medium as per the procedure described by Kodjio et al. (2016). For this, equal volumes (500 μL) of 1% orthophosphoric acid solution and precipitating mixture (1% thiobarbituric acid + 1% acetic acid) were gently mixed with 100 μL of medium in glass tubes. Subsequently, tubes were incubated at 100℃ for 15 mins, cooled, and centrifuged at 1,000 g for 10 mins. Finally, the absorbance of the upper layer was read against the blank at 532 nm wavelength. The MDA levels were expressed in nmol/g of protein in the medium.

Assessment of superoxide dismutase activity

The superoxide dismutase (SOD) activity was evaluated according to the principle described by Misra and Fridovich (1972), which involved the auto-oxidation and illumination of adrenaline at 480 nm for 1.5 mins. One unit of total SOD activity was defined as the amount of protein that caused a 50% inhibition of adrenaline auto-oxidation. The total SOD activity was expressed as units per milligram (U/mg) of protein.

Assessment of catalase activity

The catalase activity (CAT) was evaluated according to the principle described by Sinha (1972). In the presence of hydrogen peroxide, dichromate in acetic acid is reduced to chromic acetate with the formation of perchromic acid as an unstable intermediate. The absorbance of the chromic acetate produced, which reflects the hydrogen peroxide not degraded by CAT in the sample, is then recorded at 570 nm. The concentration of hydrogen peroxide in the medium was determined using a standard curve developed for this purpose. The CAT activity was expressed in units per gram (U/g) of protein, where one unit is equivalent to the degradation of one mM of hydrogen peroxide per min.

Statistical analysis

The experiment was conducted with 6 replicates, and statistical analyses of the data were performed using R statistical package, version 4.2.0. One-way ANOVA was used to determine the difference among means at each time point. To evaluate changes in different variables over time, repeated measures ANOVA was used to reveal the effect of time in each group. If a significant differ-

ence was found, the Duncan test was used to separate the means. The results are reported as mean ± standard deviation of the mean, and values of $p < 0.05$ were considered statistically significant.

RESULTS

Effects of SAE on buck's sperm total motility, progressive motility, and local motility

The effects of SAE on sperm TM, FPM, SPM, and LM during storage are depicted in Table 1. There was no significant ($p > 0.05$) difference among treatments and storage time for TM, FPM, and LM. However, the highest TM was observed at 6 hr post preservation in all doses when compared to the control groups. Higher TM was also observed at SAE20 and SAE40 doses at 48 hr post preservation which approached that of the positive control groups. For FPM of semen samples treated with different concentrations of SAE, the SAE80 group showed higher values than other treatment groups which decreased with storage time. The percentage of SPM showed that there was a significant ($p < 0.05$) difference among treatments and storage time when compared to the control groups. Nevertheless, most treatment groups did not vary significantly ($p > 0.05$) with the positive control group but were significantly higher than the negative control group ($p < 0.05$), except for SAE20 at 24 hr and SAE40 at 72 hr, which instead approached the negative control group. The semen samples treated with SAE showed inferior LM in comparison to the negative control at each time point.

Simple interactions of time and doses of SAE on buck's sperm total motility, progressive motility, and local motility

Table 2 illustrates the effects of simple SAE interactions on sperm TM, FPM, SPM, and LM during storage. No significant differences were found among treatments ($p > 0.05$). The percentage of TM decreased significantly over

Table 1. Percentages of total and progressive and local motility of spermatozoa in buck semen extended in Tris-egg yolk supplemented with *Annona muricata* aqueous extract and stored at 4℃ for 72 hours

Parameter	Treatment	Storage duration (h)			
		6	24	48	72
%TM	0	80.56 ± 6.13	79.02 ± 8.39	74.58 ± 8.89	72.01 ± 14.08
	0+	82.88 ± 5.42	78.68 ± 12.27	81.38 ± 10.02	80.01 ± 10.02
	SAE20	82.26 ± 6.81	71.05 ± 18.80	81.93 ± 5.03	79.74 ± 12.63
	SAE40	83.62 ± 4.22	76.05 ± 14.88	82.45 ± 8.71	74.44 ± 7.34
	SAE80	82.87 ± 8.74	78.83 ± 17.32	78.30 ± 14.98	76.24 ± 7.22
FPM	0	3.81 ± 1.40	4.34 ± 1.65	1.60 ± 0.50	1.31 ± 069
	0+	7.61 ± 2.21	7.03 ± 3.74	6.87 ± 2.37	4.59 ± 1.40
	SAE20	5.02 ± 1.72	3.38 ± 1.37	2.66 ± 1.21	3.19 ± 1.48
	SAE40	5.98 ± 1.92	3.96 ± 1.92	3.71 ± 1.66	2.08 ± 0.73
	SAE80	7.21 ± 2.95	5.65 ± 1.34	4.72 ± 1.91	3.74 ± 1.52
SPM	0	19.94 ± 7.24^{aA}	20.83 ± 6.50^{abA}	12.55 ± 3.94^{bB}	14.81 ± 5.12^{bAB}
	0+	23.25 ± 6.92^{aA}	25.53 ± 9.66^{aA}	24.03 ± 3.99^{aA}	29.53 ± 8.22^{aA}
	SAE20	23.97 ± 6.78^{aA}	17.36 ± 7.19^{bB}	18.09 ± 5.01^{abAB}	19.93 ± 4.79^{bAB}
	SAE40	23.52 ± 4.07^{aA}	21.30 ± 7.97^{abA}	21.27 ± 8.43^{aA}	13.96 ± 4.37^{bB}
	SAE80	23.59 ± 8.85^{aA}	23.81 ± 6.83^{abA}	22.28 ± 8.42^{aA}	17.93 ± 6.46^{bA}
LM	0	56.81 ± 5.16	53.85 ± 5.95	60.43 ± 6.64	55.89 ± 13.16
	0+	52.03 ± 6.81	46.12 ± 8.94	50.48 ± 10.54	45.89 ± 17.43
	SAE20	53.26 ± 5.19	50.31 ± 17.89	60.02 ± 6.45	58.79 ± 9.78
	SAE40	54.11 ± 4.46	50.41 ± 12.53	57.47 ± 8.14	58.39 ± 5.52
	SAE80	52.07 ± 6.12	49.36 ± 16.67	51.30 ± 7.69	54.57 ± 7.74

h, hour; %, percentage; 0, neutral control group with 0 μg/mL of SAE; 0+, positive control group with 0 μg/mL of SAE plus 0.3 g/dL of vitamin C, 100 IU/mL of penicillin and 0.05 mg/mL of streptomycin; SAE20, SAE40, and SAE80 groups with 20, 40, and 80 μg of Soursop Aqueous extract per mL of extender; TM, total motility; FPM, fast progressive motility; SPM, slow progressive motility; LM, local motile; ^{a,b}Values with different superscripts indicate significant differences ($p < 0.05$) among treatments at each time point; ^{A,B}Values with different superscripts indicate significant differences ($p < 0.05$) among storage time points for each treatment.

Table 2. Simple interactions of time and doses of *Annona muricata* aqueous extract on total and progressive motility of spermatozoa in buck semen extended in Tris-egg yolk extender and stored at 4℃.

	TM (%)	FPM	SPM	LM
Doses				
0	76.54 ± 10.00	2.77 ± 1.75^{d}	17.00 ± 6.62	56.70 ± 8.38^{a}
0+	80.70 ± 9.67	6.52 ± 2.73^{a}	25.60 ± 7.61	48.60 ± 11.50^{c}
SAE20	78.70 ± 12.50	3.57 ± 1.66^{cd}	19.80 ± 6.36	55.60 ± 11.40^{ab}
SAE40	79.10 ± 10.10	3.93 ± 2.11^{c}	20.00 ± 7.26	55.10 ± 8.56^{ab}
SAE80	79.10 ± 12.50	5.33 ± 2.34^{b}	21.90 ± 7.78	51.80 ± 10.20^{ac}
Times				
6 h	82.40 ± 6.46^{a}	5.93 ± 2.46^{a}	22.90 ± 6.82	53.70 ± 5.66^{ab}
24 h	76.70 ± 14.50^{ab}	4.87 ± 2.49^{b}	21.80 ± 7.91	50.00 ± 12.9^{b}
48 h	79.70 ± 10.10^{b}	3.91 ± 2.42^{c}	19.60 ± 7.29	56.00 ± 8.85^{a}
72 h	76.50 ± 10.60^{b}	2.98 ± 1.66^{d}	19.20 ± 8.02	54.70 ± 12.00^{a}
p-value				
Doses	–	***	***	***
Times	*	***	**	*
Doses × times	–	*	**	–

h, hour; %, percentage; 0, neutral control group with 0 μg/mL of SAE; 0+, positive control group with 0 μg/mL of SAE plus 0.3 g/dL of vitamin C, 100 IU/mL of penicillin and 0.05 mg/mL of streptomycin; SAE20, SAE40, and SAE80 groups with 20, 40, and 80 μg of Soursop Aqueous extract per mL of extender; TM, total motility; FPM, fast progressive motility; SPM, slow progressive motility; LM, local motile; ^{a,b}Values with different superscripts indicate significant differences ($p < 0.05$) among storage time points for each treatment or significant differences ($p < 0.05$) among treatments at each time point; ***($p \leq 0.000$) = strongly significant; **($p \leq 0.001$) = moderately significant; *($p \leq 0.05$) = weakly significant; – ($p \geq 1$) = strongly insignificant.

time ($p < 0.05$). FPM of spermatozoa was significantly higher in SAE80 compared to the other treated samples, and it also decreased significantly from 6 to 72 hr ($p < 0.05$). No significant difference in SPM was observed for different treatments when compared to the control groups ($p > 0.05$). The change in time of storage also showed no significant difference ($p > 0.05$). The percentage of LM spermatozoa was significantly higher in SAE80 compared to the other treated samples but showed no significant difference with the positive control group ($p > 0.05$). A significant increase in LM was recorded over time.

Effects of SAE on buck's sperm motion characteristics

Table 3 presents data for the motion characteristics of spermatozoa. The general results revealed no significant ($p > 0.05$) difference among treatments at each time point when compared to the control groups. However, some important observations were recorded. Semen samples treated with SAE showed VAP values close to the positive control group but higher than the negative control. There was an exception of the VAP value of the negative control group at 48 hr which appeared to be higher than the respective samples at the same time point (Table 3). Changes of the sperm motion characteristics showed a decreasing trend over time of storage precisely for VCL, VSL, and VAP with minor exceptions like in VAP of the negative control at 48 hr post storage, which is highest. In all treatments motion characteristics were generally higher at 6 hr post preservation of semen but with no significant difference ($p > 0.05$). Considering each time point, the enrichment of TEY extender with SAE neither influenced significantly ($p < 0.05$) LIN nor STR. In the treatment groups the highest value for LIN was obtained from SAE80 which approaches those of the positive control group. A similar trend was registered for STR.

Simple interactions of time and doses of SAE on buck's sperm motion characteristics

The data on sperm motion characteristics summarized in Table 4 presented that semen samples treated with SAE showed no significant difference for VAP and VLC ($p > 0.05$). However, for VSL, there was a significant decrease in the treatment groups compared to the positive control group ($p < 0.05$). Nevertheless, semen samples treated with 80 μg/mL SAE showed higher VSL values than the negative control group ($p < 0.05$). Enrichment of TEY extender with SAE did not significantly influence LIN compared to the control groups except at the dose of 40 μg/mL, which

Table 3. Motion characteristics of spermatozoa in buck semen extended in Tris-egg yolk supplemented with *Annona muricata* aqueous extract and stored at 4c for 72 hours

Parameter	Treatment	Storage duration (h)			
		6	24	48	72
VAP	0	15.09 ± 2.45	15.32 ± 2.59	16.32 ± 4.70	10.73 ± 3.86
	0+	16.40 ± 3.16	15.61 ± 3.95	15.26 ± 2.84	15.98 ± 1.87
	SAE20	17.16 ± 3.27	13.59 ± 1.91	13.68 ± 3.33	12.61 ± 5.26
	SAE40	16.99 ± 1.69	12.94 ± 5.15	14.99 ± 2.65	12.59 ± 3.25
	SAE80	16.59 ± 4.09	15.63 ± 3.44	14.18 ± 4.97	13.69 ± 3.21
VCL	0	24.53 ± 4.39	25.20 ± 4.45	22.13 ± 4.79	17.19 ± 6.74
	0+	27.17 ± 5.97	20.00 ± 3.82	23.68 ± 5.39	24.99 ± 2.21
	SAE20	27.97 ± 5.40	20.84 ± 4.24	23.22 ± 4.61	19.93 ± 8.53
	SAE40	27.64 ± 3.11	21.28 ± 8.87	24.53 ± 4.41	20.53 ± 6.22
	SAE80	26.35 ± 7.39	24.94 ± 6.66	22.84 ± 8.31	21.85 ± 4.89
VSL	0	5.64 ± 1.19	5.39 ± 1.59	5.24 ± 2.20	4.11 ± 1.48
	0+	6.75 ± 0.78	6.28 ± 1.73	6.60 ± 0.81	6.72 ± 1.11
	SAE20	6.52 ± 1.22	4.48 ± 1.96	5.95 ± 2.48	5.09 ± 1.01
	SAE40	6.24 ± 0.81	5.11 ± 1.91	5.77 ± 1.19	4.61 ± 0.86
	SAE80	6.59 ± 1.26	5.87 ± 1.93	5.78 ± 1.34	5.25 ± 1.06
LIN	0	23.35 ± 2.29	25.17 ± 6.81	31.79 ± 9.01	25.19 ± 2.92
	0+	25.38 ± 3.04	25.38 ± 7.20	28.35 ± 2.73	27.38 ± 2.90
	SAE20	23.48 ± 3.48	23.57 ± 3.60	23.96 ± 2.35	26.39 ± 3.94
	SAE40	22.95 ± 2.01	25.20 ± 3.77	24.05 ± 3.09	23.79 ± 4.34
	SAE80	26.22 ± 6.91	27.39 ± 9.71	25.72 ± 3.29	24.05 ± 1.31
STR	0–	37.36 ± 3.24	39.09 ± 6.67	35.96 ± 6.02	35.47 ± 3.23
	0+	41.98 ± 3.78	40.26 ± 7.54	43.93 ± 2.97	42.79 ± 3.18
	SAE20	37.48 ± 4.11	37.76 ± 3.82	38.25 ± 2.72	40.92 ± 3.80
	SAE40	39.06 ± 6.73	40.64 ± 4.40	38.41 ± 3.47	37.61 ± 4.05
	SAE80	40.29 ± 5.24	41.62 ± 9.35	41.00 ± 4.06	38.54 ± 1.99

h, hour; %, percentage; 0, neutral control group with 0 μg/mL of SAE; 0+, positive control group with 0 μg/mL of SAE plus 0.3 g/dL of vitamin C, 100 IU/mL of penicillin and 0.05 mg/mL of streptomycin; SAE20, SAE40, and SAE80 groups with 20, 40, and 80 μg of Soursop Aqueous extract per mL of extender; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity; LIN, linearity; STR, straightness.

showed a significant decrease ($p < 0.05$). Furthermore, the addition of 80 μg/mL SAE in TEY extender significantly increased STR of buck's spermatozoa compared to the control groups and other treatment groups ($p < 0.05$). Motion characteristics (VAP, VLC, VSL, LIN) of buck spermatozoa significantly decreased with storage time, except for STR, which showed no significant difference as storage time increased ($p > 0.05$).

Effects of SAE on buck's sperm viability, plasma membrane and DNA integrity

Table 5 summarizes the analysis of sperm motility data. The addition of SAE to the TEY extender resulted in lower levels of cell death. Sperm samples treated with SAE80 showed no significant difference compared to the positive control at each time point except for 72 hr, in which SAE80 was significantly higher than the other treatment groups at the same time point ($p < 0.05$). Changes in motility over storage time were not significantly different at consecutive time points when comparing each treatment group to the positive control ($p > 0.05$). However, there was a significant decrease in motility in the negative control compared to the positive control and other treatment groups from 24 to 72 hr of storage ($p < 0.05$). Table 5 also presents the sperm plasma membrane integrity values after the addition of SAE to the TEY extender. The percentage of sperm with functional sperm membranes showed no significant difference among treatment groups compared to the positive control ($p > 0.05$). However, the values were higher than the negative control at each time point. No significant differences were observed among treatments for DNA integrity ($p > 0.05$). There was a trend

Table 4. Simple interactions of time and doses of *Annona muricata* aqueous extract on motion characteristics of spermatozoa in buck semen extended in Tris-egg yolk extender and stored at 4℃

	VAP	VCL	VSL	LIN	STR
Doses					
0	14.40 ± 4.03	22.30 ± 5.91	5.10 ± 1.70^{c}	26.40 ± 6.58^{a}	36.97 ± 5.05^{c}
0+	15.80 ± 2.96	24.00 ± 5.14	6.59 ± 1.15^{a}	26.60 ± 4.41^{a}	42.24 ± 4.76^{a}
SAE20	14.30 ± 3.91	23.00 ± 6.53	5.51 ± 1.87^{bc}	24.30 ± 3.48^{ab}	38.60 ± 3.77^{bc}
SAE40	14.40 ± 3.74	23.50 ± 6.48	5.43 ± 1.37^{bc}	24.00 ± 3.38^{b}	38.93 ± 4.77^{bc}
SAE80	15.00 ± 4.00	24.00 ± 6.89	5.87 ± 1.46^{b}	25.80 ± 6.10^{ab}	40.36 ± 5.71^{ab}
Times					
6 h	16.40 ± 3.00^{a}	26.70 ± 5.36^{a}	6.35 ± 1.10^{a}	24.27 ± 3.61	39.24 ± 6.54
24 h	14.60 ± 3.62^{b}	22.50 ± 6.09^{b}	5.43 ± 1.86^{ab}	25.34 ± 2.97	39.87 ± 4.91
48 h	14.90 ± 3.78^{b}	23.30 ± 5.52^{b}	5.87 ± 1.71^{bc}	26.78 ± 2.85	39.51 ± 4.74
72 h	13.10 ± 3.91^{c}	20.90 ± 6.38^{b}	5.15 ± 1.40^{c}	25.36 ± 2.00	39.06 ± 4.10
p-value					
Doses	•	–	***	*	***
Times	***	***	***	•	–
Doses × times	•	–	–	•	–

h, hour; %, percentage; 0, neutral control group with 0 μg/mL of SAE; 0+, positive control group with 0 μg/mL of SAE plus 0.3 g/dL of vitamin C, 100 IU/mL of penicillin and 0.05 mg/mL of streptomycin; SAE20, SAE40, and SAE80 groups with 20, 40, and 80 μg of Soursop Aqueous extract per mL of extender; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity; LIN, linearity; STR, straightness; ^{a,b,c}Values with different superscripts indicate significant differences ($p < 0.05$) among storage time points for each treatment or significant differences ($p < 0.05$) among treatments at each time point; ***($p \leq 0.000$) = strongly significant; *($p \leq 0.05$) = weakly significant;– ($p \geq 1$) = strongly insignificant; •($p \geq 0.1$) = weakly insignificant.

Table 5. Percentages of viable, intact plasma membrane and DNA of buck spermatozoa extended in Tris-egg yolk enriched with *Annona muricata* aqueous extract and stored at 4℃ for 72 hours

Parameter	Treatment	Storage duration (h)			
		6	24	48	72
Viability (%)	0	81.67 ± 6.38^{aA}	65.17 ± 7.19^{bB}	69.25 ± 6.75^{bB}	52.50 ± 11.95^{bC}
	0+	86.33 ± 5.42^{aA}	83.17 ± 4.32^{aAB}	77.67 ± 6.05^{abC}	74.67 ± 5.67^{aC}
	SAE20	82.17 ± 10.63^{aA}	80.17 ± 5.99^{aAB}	73.92 ± 3.84^{abBC}	70.25 ± 6.62^{aC}
	SAE40	82.00 ± 3.85^{aA}	80.00 ± 7.87^{aAB}	73.67 ± 6.19^{abB}	70.33 ± 6.31^{aC}
	SAE80	81.50 ± 7.23^{aA}	79.83 ± 4.36^{aA}	71.92 ± 3.09^{abB}	69.58 ± 5.62^{aB}
I-PLM (%)	0	81.00 ± 5.55	76.17 ± 9.79	75.75 ± 3.49	69.83 ± 6.40
	0+	85.08 ± 5.28	83.67 ± 4.55	81.00 ± 2.89	79.50 ± 6.03
	SAE20	82.67 ± 5.57	81.92 ± 2.87	80.08 ± 4.08	76.33 ± 3.61
	SAE40	83.17 ± 4.62	81.00 ± 13.07	79.50 ± 3.82	75.50 ± 2.51
	SAE80	84.50 ± 6.25	80.33 ± 4.68	80.33 ± 2.96	78.67 ± 4.93
I-DNA (%)	0	99.75 ± 0.27	99.75 ± 0.27	98.50 ± 1.76	94.58 ± 7.93
	0+	99.33 ± 0.75	99.42 ± 0.58	99.25 ± 1.17	99.00 ± 1.14
	SAE20	98.75 ± 1.04	98.42 ± 1.86	98.33 ± 2.96	99.75 ± 0.42
	SAE40	97.67 ± 3.67	97.75 ± 3.73	97.50 ± 3.58	99.17 ± 0.82
	SAE80	98.33 ± 1.25	99.83 ± 0.26	99.58 ± 0.58	99.58 ± 0.20

h, hour; %, percentage; 0, neutral control group with 0 μg/mL of SAE; 0+, positive control group with 0 μg/mL of SAE plus 0.3 g/dL of vitamin C, 100 IU/mL of penicillin and 0.05 mg/mL of streptomycin; SAE20, SAE40, and SAE80 groups with 20, 40, and 80 μg of Soursop Aqueous extract per mL of extender; I-PLM, Intact plasma membrane; I-DNA, Intact DNA. ^{a,b}Values with different superscripts indicate significant differences ($p < 0.05$) among treatments at each time point. ^{A,B,C}Values with different superscripts indicate significant differences ($p < 0.05$) among storage time points for each treatment.

towards higher percentages of DNA integrity with longer storage time.

Simple interactions of time and doses of SAE on buck's sperm viability, plasma membrane and DNA integrity

The addition of SAE in TEY extender did not show any significant difference ($p > 0.05$) on the viability, plasma membrane function, and DNA integrity of buck's spermatozoa compared to the positive control group. In contrast, there was a significant decrease ($p < 0.05$) in sperm plasma membrane integrity in the negative control group. Additionally, increasing the storage time up to 72 hr did not significantly affect ($p > 0.05$) the viability and DNA integrity of stored sperm samples. However, a significant decrease ($p > 0.05$) in plasma membrane integrity was observed as storage time increased (Table 6).

Effects of incorporating SAE in TEY extender on lipid peroxidation biomarkers content in buck's semen

As shown in Table 7, addition of SAE in TEY extender revealed no significant ($p > 0.05$) difference for evaluated lipid peroxidation biomarkers upon comparing the SAE treated groups and control groups.

Simple interactions of time and doses of SAE in TEY extender on oxidative stress biomarkers content in buck's semen

As shown in Table 8, CAT did not show any significant ($p > 0.05$) difference irrespective of the dose of SAE when compared with control groups. For total peroxidase (POX) and MDA, although there existed a significant ($p < 0.05$) difference between the negative control and positive control, there was no significant ($p > 0.05$) difference between the treated samples and the control groups. Addition of 40 and 80 μg/mL SAE significantly ($p < 0.05$) decreased SOD when compared to the positive control group. But there existed no significant ($p > 0.05$) difference between treatment groups and negative control. It was noted that the level of MDA did not vary significantly ($p > 0.05$) with time. However, POX, CAT and SOD significantly ($p < 0.05$) decrease with increase in time of storage.

DISCUSSION

During liquid *in vitro* sperm storage, there are many factors that can influence its quality, including duration of storage, temperature, and quality of extender (Johnson et al., 2000). Defects in semen quality during liquid pres-

Table 6. Simple interactions of time and doses of *Annona muricata* aqueous extract on viability, plasma membrane integrity and DNA integrity of spermatozoa in buck semen extended in Tris-egg yolk extender and stored at 4℃

	Viability (%)	I-PLM (%)	I-DNA (%)
Doses			
0	67.10 ± 13.20	75.69 ± 7.45^{b}	98.10 ± 4.37
0+	80.50 ± 6.86	82.31 ± 5.03^{a}	99.20 ± 0.89
SAE20	76.60 ± 8.30	80.71 ± 5.10^{a}	98.80 ± 1.80
SAE40	76.50 ± 8.30	79.69 ± 7.38^{a}	98.10 ± 3.10
SAE80	75.7 ± 7.15	80.50 ± 4.56^{a}	99.20 ± 0.96
Times			
6 h	82.70 ± 6.82	83.28 ± 5.29^{a}	98.80 ± 1.86
24 h	77.70 ± 8.62	80.62 ± 7.82^{ab}	99.00 ± 1.94
48 h	73.30 ± 5.73	79.33 ± 3.74^{b}	98.60 ± 2.26
72 h	67.50 ± 10.5	75.97 ± 5.73^{c}	98.40 ± 3.89
p-value			
Doses	***	***	•
Times	***	***	–
Doses × times	*	*	*

h, hour; %, percentage; 0, neutral control group with 0 μg/mL of SAE; 0+, positive control group with 0 μg/mL of SAE plus 0.3 g/dL of vitamin C, 100 IU/mL of penicillin and 0.05 mg/mL of streptomycin; SAE20, SAE40, and SAE80 groups with 20, 40, and 80 μg of Soursop Aqueous extract per mL of extender; I-PLM, intact plasma membrane; I-DNA, intact DNA; ^{A,B,C}Values with different superscripts indicate significant differences ($p < 0.05$) among treatments at each time point; ^{a,b}Values with different superscripts indicate significant differences ($p < 0.05$) among storage time points for each treatment; ***($p \leq 0.000$) = strongly significant; *($p \leq 0.05$) = weakly significant; –($p \geq 1$) = strongly insignificant; •($p \geq 0.1$) = weakly insignificant.

Table 7. Effects of *Annona muricata* aqueous extract on oxidative stress parameters following 72 h liquid storage at 4c of buck semen extended in Tris-egg yolk extender

Parameter	Treatment	Storage duration (h)			
		6	24	48	72
POX	0	30.16 ± 6.69	28.61 ± 7.87	27.02 ± 3.51	23.40 ± 6.55
	0+	37.96 ± 5.77	34.66 ± 10.07	30.05 ± 9.71	33.79 ± 6.29
	SAE20	31.89 ± 6.76	30.45 ± 2.61	29.97 ± 9.44	29.74 ± 5.23
	SAE40	33.97 ± 6.27	30.17 ± 5.92	28.71 ± 5.72	28.16 ± 5.23
	SAE80	35.39 ± 9.87	31.99 ± 6.79	28.59 ± 4.44	25.97 ± 4.55
MDA	0	1.85 ± 0.86	1.95 ± 0.37	2.04 ± 0.57	2.25 ± 0.83
	0+	1.21 ± 0.28	1.51 ± 0.42	1.33 ± 0.39	1.32 ± 0.34
	SAE20	1.31 ± 0.36	1.46 ± 0.66	1.56 ± 0.28	1.87 ± 0.64
	SAE40	1.34 ± 0.12	1.61 ± 0.49	1.60 ± 0.38	1.57 ± 0.29
	SAE80	1.32 ± 0.35	1.41 ± 0.37	1.44 ± 0.43	1.49 ± 0.35
CAT	0	15.09 ± 2.87	13.31 ± 1.19	11.59 ± 2.48	10.35 ± 1.54
	0+	16.37 ± 0.86	15.06 ± 4.26	14.28 ± 2.98	14.97 ± 2.19
	SAE20	15.19 ± 8.27	14.91 ± 2.89	12.98 ± 6.66	12.39 ± 2.71
	SAE40	15.69 ± 2.80	16.11 ± 1.83	13.75 ± 1.69	12.46 ± 5.58
	SAE80	16.28 ± 2.33	14.83 ± 8.15	14.76 ± 6.29	14.51 ± 2.61
SOD	0	2.19 ± 0.25	1.70 ± 0.44	1.73 ± 0.45	1.10 ± 0.31
	0+	2.39 ± 0.54	2.35 ± 0.56	2.14 ± 0.55	2.00 ± 0.33
	SAE20	2.10 ± 0.79	2.13 ± 0.72	1.80 ± 0.43	1.76 ± 0.65
	SAE40	2.12 ± 0.40	2.19 ± 0.58	1.48 ± 0.45	1.37 ± 0.57
	SAE80	2.09 ± 0.62	1.90 ± 0.29	1.75 ± 0.71	1.81 ± 0.47

h, hour; %, percentage; 0, neutral control group with 0 μg/mL of SAE; 0+, positive control group with 0 μg/mL of SAE plus 0.3 g/dL of vitamin C, 100 IU/mL of penicillin and 0.05 mg/mL of streptomycin; SAE20, SAE40, and SAE80 groups with 20, 40, and 80 μg of Soursop Aqueous extract per mL of extender; POX, peroxidase; MDA, malondialdehyde; CAT, catalase; SOD, superoxide dismutase.

Table 8. Simple interactions of time and doses of *Annona muricata* aqueous extract on oxidative stress biomarkers of spermatozoa in buck semen extended in Tris-egg yolk extender and stored at 4℃

	POX	MDA	CAT	SOD
Doses				
0	27.29 ± 6.46^{b}	2.02 ± 0.67^{a}	12.59 ± 2.70	1.68 ± 0.53^{b}
0+	34.12 ± 8.16^{a}	1.34 ± 0.36^{b}	15.17 ± 2.77	2.22 ± 0.49^{a}
SAE20	30.51 ± 6.12^{ab}	1.55 ± 0.52^{b}	13.87 ± 5.43	1.95 ± 0.64^{ab}
SAE40	30.25 ± 5.88^{ab}	1.53 ± 0.35^{b}	14.51 ± 3.48	1.79 ± 0.60^{b}
SAE80	30.49 ± 7.29^{ab}	1.41 ± 0.36^{b}	15.09 ± 5.12	1.89 ± 0.53^{b}
Times				
6 h	33.87 ± 7.24^{a}	1.40 ± 0.49	15.73 ± 3.99^{a}	2.18 ± 0.53^{a}
24 h	31.18 ± 6.90^{ab}	1.59 ± 0.49	14.85 ± 4.21^{ab}	2.06 ± 0.55^{a}
48 h	28.87 ± 6.64^{b}	1.59 ± 0.47	13.47 ± 4.34^{b}	1.78 ± 0.54^{b}
72 h	28.21 ± 6.32^{b}	1.70 ± 0.59	12.94 ± 3.45^{b}	1.61 ± 0.56^{b}
p-value				
Doses	*	***	•	**
Times	**	*	*	***
Doses × times	–	–	–	–

h, hour; %, percentage; 0, neutral control group with 0 μg/mL of SAE; 0+, positive control group with 0 μg/mL of SAE plus 0.3 g/dL of vitamin C, 100 IU/mL of penicillin and 0.05 mg/mL of streptomycin; SAE20, SAE40, and SAE80 groups with 20, 40, and 80 μg of Soursop Aqueous extract per mL of extender; POX, peroxidase; MDA, malondialdehyde; CAT, catalase; SOD, superoxide dismutase; ^{a,b}Values with different superscripts indicate significant differences ($p < 0.05$) among storage time points for each treatment or significant differences ($p < 0.05$) among treatments at each time point; ***($p \leq 0.000$) = strongly significant; **($p \leq 0.001$) = moderately significant; *($p \leq 0.05$) = weakly significant; –($p \geq 1$) = strongly insignificant; •($p \geq 0.1$) = weakly insignificant.

ervation continue to be a major hindrance to the efficient application of reproduction biotechnologies like artificial insemination (Pierrette et al., 2024). The results of the present study have demonstrated that as storage time increases, sperm quality decreases. However, the addition of SAE at suitable doses and varying with the duration of storage can effectively reduce the decline of TM, PM, VCL, VSL, VAP, percentages of viable spermatozoa, spermatozoa with functional plasma membrane, and DNA integrity of spermatozoa. SAE can reduce LPO and stimulate POX, SOD, and CAT activities during low temperature liquid storage.

This study showed that adding SAE to TEY semen extender can actually preserve sperm motility, morphology, and sperm motion characteristics VSL and VAP during chilled storage. The results from this study agree with many previous findings that show adding natural antioxidant compounds to semen extenders can preserve sperm during low temperature storage (Allai et al., 2016; Wen et al., 2019) and cryopreservation (Merati and Farshad, 2020; Pierrette et al., 2024), but it is different from another study by Taşdemir et al. (2020).

Motility is an essential sperm parameter for fertility (Kasimanickam et al., 2011). Mammalian spermatozoa have a long journey through the female reproductive tract to the oviduct, during which spermatozoa ultimately fertilize the oocytes. To accomplish this journey, sperm cells are equipped to overcome various obstacles, such as navigating the uterine cervix and penetrating the egg extracellular matrices (Ikawa et al., 2010). In artificial insemination procedures, sperm cells must also move within the female reproductive tract to reach the ovum. Effective semen storage requires a reversible decrease in motility and metabolic activity of sperm cells following chilling. However, exposure of sperm cells to artificial conditions amplifies the generation of reactive oxygen species (ROS), which normally arises as a consequence of aerobic conditions in which live sperm cells are involved (Agarwal and Prabakaran, 2005). As the ROS accumulate and reach a critical concentration, oxidative stress occurs and provokes an irreversible loss of motility, inhibition of fructolysis and respiration in sperm cells (Salamon and Maxwell, 2000), hence the decrease over time in sperm motility and motion characteristics over time of storage as was the case in this study. Also, motility, which is an energy-dependent parameter, is particularly associated to mitochondrial activity and therefore may also decrease as a consequence of insufficient supply of energy from mitochondria which impairment drives to adenosine triphosphate (ATP) depletion. It should be noted that sperm mitochondrion is particularly sensitive to cooling process and this sensitivity results in disturbance in ATP transport chain, consequently reducing sperm motility (Zarei et al., 2021).

All SAE-treated samples showed superior progressive motility parameters (FPM, SPM) compared to the negative control sample. Progressive motile sperm cells represent the sperm fraction that can effectively swim within the female reproductive tract to reach the oocyte after insemination. Therefore, by reducing the loss of progressive motility (PM), SAE may improve the fertilization rate of chilled semen. Enrichment of extenders with SAE at 80 μg/mL was beneficial for motion characteristics, even at 72 hr post-preservation, as no significant difference was observed between treated samples and the positive control group. The preservation of these sperm parameters can be attributed to the capacity of bioactive molecules in SAE to inhibit the generation and/or scavenge excess ROS.

Bioactive molecules found in SAE may inhibit the mitochondrial outer membrane enzyme monoamine oxidase that catalyzes the oxidative deamination of biogenic amines, producing a large amount of H_2O_2 that contributes to an increase in the steady state concentrations of reactive oxygen species within both the mitochondrial matrix and cytosol (Cadenas and Davies, 2000). In this way, SAE may ensure the scavenging balance of the ROS produced and consequently preserve the metabolic activity of sperm cells. It is well known that *A. muricata* is a rich source of bioactive ingredients, among which vitamin C and E are considered essential components of the sperm antioxidant defense system. Therefore, they are one of the major protectors against oxidative stress and lipid peroxidation (LPO) (Olatunji and Nwachukwu, 2022). SAE, thanks to the presence of these vitamins that are liposoluble, may have inhibited the peroxidation of PUFAs abundant in buck sperm plasma membrane.

Viability, as assessed by dye exclusion, allows for the discrimination of necrozoospermia from the total lack of motility associated with structural deficiencies in the tail zone (Chemes and Rawe, 2003). The results of this study demonstrate the beneficial influence of SAE, par-

ticularly at 40 and 80 μg/mL, on sperm viability during storage. Natural plants, such as Guava leaves (Psidium guajava) and green tea (Camellia sinensis), used as additives to semen extenders, have been shown to improve sperm viability during *in vitro* storage (Wurlina et al., 2020; Susilowati et al., 2021). During semen storage, an imbalance between antioxidant defense mechanisms and ROS above the detoxifying capacity of spermatozoa leads to peroxidative damage of membrane proteins, phospholipids, PUFAs (Kameni et al., 2021), and sperm DNA (Twigg et al., 1998), resulting in a loss of membrane and DNA integrity and subsequent cell death. Additionally, for Hashem and Eslami (2018), the decrease in spermatozoa viability during liquid storage may be associated with endogenous ROS production. When in excess, ROS can stimulate apoptosis through the release of cytochrome C. The improvement in sperm viability observed in this study can be attributed to the bioactivity of secondary metabolites such as reducing agents, free Quinones, and alkaloids, which have been shown to be the main components responsible for the antioxidant activity of *A. muricata* due to their strong free radical scavenging properties (Mut-Salud et al., 2016). The preservation of sperm viability observed in this study can be explained by the presence of reducing agents, free Quinones, alkaloids, and other chemicals in *A. muricata*, which likely neutralized the excess reactive oxygen species (ROS) generated, thereby reducing the detrimental effects of ROS on sperm membrane components and inhibiting lipid peroxidation (LPO). Additionally, SAE may have inhibited the release of cytochrome C from the mitochondria to the cytosol, which is the initial step in the apoptosis cascade (Silva, 2006).

The current study's results showed that SAE, particularly at a dose of 80 μg/mL, maintained sperm functional membrane integrity up to 72 hr after preservation. This observation can be linked to SAE's ability to inhibit free radical generation and its negative effects on phospholipid bilayer interactions and protein anchorage to the bilayer, thereby preventing the loss of the sperm plasma membrane's physiological function.

In the present study, no difference in DNA integrity was detected with regard to either the dose of SAE used in the extender or the different storage times, up to 72 hr. These results contradict those of López-Fernández et al. (2007) and Sampaio et al. (2020), who demonstrated that during cooling storage of spermatozoa in stallions, lipid peroxidation (LPO) in the sperm plasma membrane, released under the control of phospholipase A2 enzyme, can induce DNA damage (Twigg et al., 1998). These results can be justified by the fact that the addition of SAE inhibits the generation of free radicals susceptible to attacking and damaging DNA, similar to the case of green tea extract, which prevented DNA fragmentation during refrigerated storage of buck spermatozoa (Susilowati et al., 2021).

Investigations have shown an increase in the antioxidant capacity of semen and inhibition of LPO following the addition of antioxidants from natural sources to extenders during semen storage (Ros-Santaella and Pintus, 2021; Susilowati et al., 2021). In this experiment, enriching TEY diluent with SAE maintained POX, SOD, and CAT levels and decreased MDA production during cooling storage at 4℃. Under low LPO levels (non-toxic conditions), cells initiate maintenance and survival through intrinsic antioxidant defense systems or signaling pathway activation that upregulate protein antioxidants, resulting in an adaptive stress response. On the other hand, under medium to high LPO levels (toxic conditions), the magnitude of oxidative stress exceeds repair capacity, and cells undergo apoptosis or necrosis (Ayala et al., 2014). Therefore, by moderating LPO levels, as observed in this study, SAE may have created non-toxic conditions that favor an increase in enzymatic antioxidant activities and preservation of sperm quality.

CONCLUSION

Ultimately, SAE preserved the quality of goat buck semen during storage at 4℃ for 72 hr. By adding SAE at 80 μg/mL in TEY, the storage of goat buck semen was improved, resulting in reduced loss of sperm motility, viability, DNA fragmentation, and membrane integrity during chilled storage at 4℃ for up to 72 hr. Furthermore, SAE inhibited LPO and stimulated enzymatic antioxidants POX, SOD, and CAT at 72 hr of storage.

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