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SPIRULINA (*Arthrospira Platensis*) EXTRACT PROMOTES MOTILITY, MICROSCOPIC, AND ANTIOXIDATIVE PARAMETERS OF RAM SEMEN DURING REFRIGERATED STORAGE

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Abstract: This study investigated the effect of spirulina ethanolic extract (SEE) on the quality of ram semen during low-temperature liquid storage and the relationship between sperm features. Ejaculates were collected from five *Djallonké* rams, pooled, extended with Tris-egg yolk (TEY) enriched with 0 (control), 20 (SEE20), 40 (SEE40), or 80 µg/mL (SEE80) of SEE to reach the concentration of 200×10^6 spz/mL, and stored at 4 °C for 72 h. Extended semen samples were assessed for total motility, progressive motility, sperm motion characteristics, viability, membrane integrity, and morphology at 6, 24, 48 and 72 h of storage. Moreover, malondialdehyde (MDA), nitric oxide (NO), superoxide dismutase, (SOD) and catalase (CAT) levels were measured at 72 h of storage. The enrichment of TEY with SEE at 40 and 80 µg/mL, improved sperm total motility at 48 and 72 h of storage ($P<0.05$). Also, all SEE-treated samples evidenced higher progressive motility in comparison to the control at 48 and 72 h ($P<0.05$). SEE80 group showed the highest percentages of viability (76.26±0.90%) and membrane integrity (58.19±1.50%); whereas, SEE40 demonstrated the lowest percentage of morphological abnormality (18.14±1.01%) at 72 h of storage. SEE did not influence NO levels; however, at 40 µg/mL, it reduced MDA concentration and improved SOD and CAT activities ($P < 0.05$). Total motility was positively correlated to progressive motility ($r=0.69$, $P<0.01$), viability ($r=0.91$, $P<0.01$), and membrane integrity ($r=0.49$, $P<0.05$); while, morphological abnormality was negatively correlated to the other sperm parameters. Furthermore, MDA was negatively correlated to total motility ($r=-0.91$, $P<0.01$), progressive motility ($r=-0.70$, $P<0.01$), and viability ($r=-0.91$, $P<0.01$), and positively correlated to morphological abnormality ($r=0.70$, $P<0.01$). An entirely opposite figure was recorded for SOD. Overall, the results indicated that SEE, especially at 40 µg/mL, can protect ram semen against liquid storage-associated damages. Furthermore, positive and negative correlations exist between semen parameters.

Keywords: Liquid storage, Ram semen, Spirulina, Semen quality

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1. Introduction

Liquid semen storage represents a practical and affordable alternative to freeze-thawing especially in developing countries where farmers are smallholders, mainly relying on extensive practices, and more prone to loss of production as a consequence of global warming. Liquid semen storage consists of the reduction of the metabolism of sperm cells thereby extending their shelf life. Coupled with artificial insemination (AI), this assisted reproductive tool allows the acceleration of lambing program and the dissemination of genetics of high merit, prevents disease transmission associated to natural mating (Baldassarre and Karatzas, 2004; Shi et al., 2020), and can therefore considerably improve food security thereby assisting with poverty alleviation and household income security.

However, like freeze-thawing process, liquid semen storage is associated to the decrease of sperm quality which arises as a consequence of oxidative stress (Gundogan et al., 2011). Indeed, following semen collection, sperm cells are exposed to *in vitro* conditions and inherent sources of stress (light, temperature, dilution, and pressure to name a few), leading to the overproduction of reactive oxygen species (ROS). Furthermore, the dilution process reduces semen antioxidant capacity (Bilodeau et al., 2000) in which superoxide dismutase (SOD) and catalase (CAT) play essential roles. Besides, ram spermatozoa are devoid of

synthesis ability as they loss their antioxidant-rich cytoplasm during the differentiation step of spermatogenesis, hence they cannot compensate the deficient antioxidant capacity (Bucak et al., 2007; Eslami et al., 2017). When ROS surpass the detoxifying capacity of semen, oxidative stress occurs. In excess, ROS attack membrane phospholipids, proteins, carbohydrates, DNA, and respiration process, leading to the loss of membrane functionality, DNA integrity, mitochondrial activity, motility, antioxidant activities, and increase of lipid peroxidation (LPO) (Peris-Frau et al., 2020; Kameni et al., 2021), hence the deleterious effects on sperm quality. Moreover, the abundance of polyunsaturated fatty acids (PUFAs) in cellular membrane makes ram spermatozoa particularly vulnerable to LPO, thus the accumulation of toxic products such as malondialdehyde (MDA) during semen storage (Eslami et al., 2017; Zarei et al., 2021). Therefore liquid semen storage suffers from insufficient antioxidant capacity to prevent the generation and/or scavenge excess ROS, maintain homeostasis, and ultimately prevent the loss of sperm quality.

To face this limitation, enrichment of semen extenders with antioxidant compounds from natural origin has shown to be effective in reducing storage-associated damages of semen and consequent gain in fertility after AI (Allai et al., 2016; Abadjieva et al., 2020).

Research directed towards the filamentous cyanobacterium blue-green alga *Arthrospira platensis* commonly known as spirulina, which is an abundant source of bioactive molecules, has provided evidence of its robust antioxidant capacity (Kannan et al., 2014) and its beneficial effect against oxidative stress (Karadeniz et al., 2008; Sorelle et al., 2020). Spirulina belongs to the substances that are listed by the US Food and Drug Administration under the category Generally Recognized as Safe and its typical composition as percentage dry weight can be summarized as: 50% -70% protein, 15 - 25% carbohydrates, 6 - 13% lipids, 4.2 - 6% nucleic acids, and 2.2 - 4.8% minerals (Belay, 2002). Additionally, spirulina possesses PUFAs and other potent antioxidants such as carotenoids, vitamins (B and E), spirulans, C-phycocyanin which is particularly accountable for the antioxidant activity, and allophycocyanin (Estrada et al., 2001). These chemicals makes this alga a rich source of biomolecules with strong antioxidant potential.

With this background, the objectives of the present study were to assess the effect of the supplementation of Tris-egg yolk (TEY) extender with different concentrations of spirulina ethanolic extract (SEE) on ram sperm motility, motion characteristics, viability, membrane integrity, and morphology of the *Djallonké* breed during liquid storage at 4 °C for different time intervals (6, 24, 48, and 72 h). Moreover, levels of MDA, an indicator of LPO, nitric oxide (NO), SOD, and CAT were evaluated at 72 h of storage. The correlations between sperm quality parameters, LPO, NO, SOD, and CAT were also investigated.

2. Materials and Methods

2.1. Preparation of The Spirulina Ethanolic Extract

Spirulina pellets were harvested on the shores of the Lake Chad, sun-dried, and mechanically milled. The resulting powder was used to prepare SEE, according to the procedure described by Baghshahi et al. (2014) with minor modifications. Briefly, 400 g of spirulina powder was added to 2 L of ethanol 96% and soaked at 4 °C in the dark. The soaked material was stirred every 12 h. After 72 h, 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 °C and the resulting extract stored in the dark at 4 °C.

2.2. Animals and Semen Collection

Five healthy Djallonké rams of proven fertility, aged 2.5 - 3 years, weighing 38±2 kg were used in this study. The animals were housed at the Teaching and Research Farm of the University of Dschang with appropriate balanced diet: commercial concentrate (0.5 kg/ram/day) and *ad libitum* access to good quality hay, water, and mineral blocks. Ejaculates were collected once a week for 6 consecutive weeks using electroejaculator. Immediately after collection, each ejaculate was transferred to a water bath (37 °C) and instantly assessed for semen colour, volume, concentration, and mass motility. Only ejaculates with colour score ≥ 3, volume ≥ 0.75 mL, concentration ≥ 2.5×10^9 spz/mL, and mass motility score ≥ 3 were included in the study. Semen samples that met the above-mentioned characteristics were pooled to minimize individual variation and processed for extending.

2.3. Extender Preparation and Semen Processing

The 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 12% (v/v). To prevent bacterial growth, penicillin and streptomycin (0.05 mg/mL) were added to freshly prepared TEY. Pooled semen sample was divided in equal aliquots and extended at 37 °C using TEY supplemented with 0 (control), 20 (SEE20), 40 (SEE40), or 80 µg/mL (SEE80) of SEE to reach the concentration of 200×10^6 spz/mL and stored at 4 °C for 72 h. Extended semen samples were assessed for sperm quality at 6, 24, 48 and 72 h of storage.

2.4. Semen Evaluation

2.4.1. Assessment of sperm motility

Sperm motility and motion characteristics were assessed by computer-assisted sperm analyser (CASA) with a warmed stage at 37 °C (Sperm Analyze Vista, version V1.12 Maya, Guangzhou, China).

The extended samples were further diluted using Tris-based extender without egg yolk to 25×10^6 spz/mL at 37 °C. A semen sample (10 µL) was placed on a warmed slide and covered with a cover slip. For each sample, 4 – 5 fields per drop were analysed at 200 × 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, %), progressive motility (PM, %), curvilinear velocity (VCL, µm/s), straight line

velocity (VSL, µm/s), average path velocity (VAP, µm/s), linearity (LIN, %), and straightness (STR, %).

2.4.2. Assessment of sperm viability and morphology

Sperm viability was evaluated with eosin-nigrosin staining (Evans and Maxwell, 1987). Thin smears, made in duplicate, were prepared by mixing 10 µl of semen (diluted at 25 × 10^6 spz/mL with Tris-based extender without egg yolk) with 20 µL of eosin-nigrosin (eosin-Y 1.67 g, nigrosin 10 g, sodium citrate 2.9 g, dissolved in 100 mL distilled water) on a warm slide (37 °C) and immediately spread with another slide. After air drying, the viability was assessed by counting a minimum of 200 cells from 3-4 different fields with bright-field microscopy (400×). Spermatozoa showing partial or complete purple colour were considered non-viable and only spermatozoa showing white colour, indicative of strict exclusion of the stain were considered to be alive.

The same slides were used to determine sperm morphology; with similar microscopic settings. A minimum of 200 sperm cells per slide were examined and morphological abnormalities included head, midpiece, and tail defects (Zarei et al., 2018).

2.4.3. Assessment of sperm functional membrane integrity

Sperm functional membrane integrity was assessed following the principle described by Revell and Mrode (1994). Extended semen sample (20 µL) was mixed with 200 µL of pre-warmed (37 °C) 100 mOsm hypoosmotic solution (9 g fructose, 4.9 g trisodium citrate per litre of distilled water) which was prepared daily and kept at 4 °C. The mixture was incubated at 37 °C for 60 min. After incubation, the sample was gently mixed. Smears were realized in duplicate. A drop (15 µL) of the treated mixture was smeared on a pre-warmed slide and air-dried. 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; whereas, sperm cells showing no swollen or coiled tails were considered to have defective plasma membranes.

2.4.4. Measurement of lipid peroxidation

At the end of the storage period (72 h), semen samples were centrifuged at 550 g for 10 min and the pellet was discarded. The supernatant was again centrifuged at 550 g for 10 min and finally at 3000 g for 30 min. The resulting 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 MDA in medium as per the procedure described by Kodjio et al. (2016). Briefly, 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 °C for 15 min, cooled, and centrifuged at 1000 g for 10 min. 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.

2.4.5. Measurement of nitric oxide

The NO content was evaluated according to the Griess reaction (Griess, 1879). An aliquot of medium (350 µL) was mixed with 350 µL of 1% sulphanilamide (prepared in 1% orthophosphoric acid) in glass tube and the mixture was incubated in the dark at room temperature for 5 min. Afterwards, 350 µL of 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride (prepared in 10 mM Tris) was added to the preparation which was again incubated for 5 min in similar conditions as previously. Finally, the absorbance of the preparation was read at 530 nm. NO amounts in the samples were calculated using a standard curve developed for this purpose and expressed in µmol/g of protein.

2.4.6. Assessment of superoxide dismutase activity

The SOD activity was evaluated according to the principle described by Misra and Fridovich (1972), assaying the auto-oxidation and illumination of adrenalin at 480 nm for 1.5 min. One unit total SOD activity was considered as the amount of protein causing 50% inhibition of adrenalin auto-oxidation. The total SOD activity was expressed as units per milligram (U/mg) of protein.

2.4.7. Assessment of catalase activity

The activity of CAT was evaluated according to the principle described by Sinha (1972). When heated 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, one unit being equivalent to one mM of hydrogen peroxide degraded per minute.

2.4.8. Measurement of total protein content

Total protein content was determined using Chronolab commercial kit, strictly following the manufacturer directives. The kit is based on the Biuret method. In alkaline medium, proteins form with copper salts, an intensive violet-blue complex with maximum absorbance at 540 nm. The intensity of the colour is proportional to the total protein concentration in the sample.

2.5. Statistical Analysis

The experiment was conducted in 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. Changes in different variables over time were evaluated using repeated measure ANOVA to reveal time effect in each group. In case of significant difference, the Tukey post hoc test was used to separate means. The results were reported as mean ± standard error of the mean and values of $P<0.05$ were considered statistically significant. Pearson correlations between parameters were evaluated by combining data for all treatments.

3. Results

3.1. Sperm Motility and Kinematics

The effects of SEE concentration and storage period on sperm motility and kinematic parameters are depicted in Table 1. Independently of the treatments, the total and progressive motility of ram sperm decreased steadily with increasing storage time. Semen samples treated with 40 and 80 µg/mL evidenced significantly (P<0.05) higher TM in comparison to the control sample at 48 and 72 h of refrigerated storage. Likewise, all SEE treated semen samples demonstrated higher PM in comparison to the control at 48 and 72 h of refrigerated storage (P<0.05). Kinematic parameters VCL, VSL, and VAP showed a decreasing trend over time. Both SEE20 and SEE80 treated semen samples showed significantly (P<0.05) higher VSL at 72 h and VAP at 24 h in comparison to the respective control. On the other hand, SEE neither influence LIN nor STR of semen samples, both parameters showed to be constant during storage (Table 2).

Table 1. Percentages of total and progressive motility of spermatozoa in ram semen stored at 4 °C for 72 h in Tris-egg yolk extender supplemented with spirulina ethanolic extract

Parameters	Treatments	Storage duration (h)			
		6	24	48	72
TM	Control	80.49 ± 1.88^{Aa}	76.49 ± 1.11^{Ba}	69.86 ± 1.37^{Bb}	66.53 ± 0.93^{Bb}
	SEE20	82.54 ± 1.67^{Aa}	79.73 ± 0.94^{ABab}	77.24 ± 1.19^{Ab}	70.96 ± 1.55^{ABc}
	SEE40	84.83 ± 1.16^{Aa}	78.97 ± 1.57^{ABb}	79.40 ± 1.43^{Ab}	71.79 ± 1.25^{Ac}
	SEE80	84.94 ± 1.06^{Aa}	82.83 ± 0.90^{Aa}	77.42 ± 1.14^{Ab}	74.62 ± 0.90^{Ab}
PM	Control	62.49 ± 1.26^{Ba}	60.55 ± 1.18^{Ba}	51.39± 0.92^{Bb}	43.94 ± 0.90^{Cc}
	SEE20	66.78 ± 1.18^{ABa}	63.15 ± 0.84^{ABab}	61.36 ± 1.24^{Ab}	53.72 ±0.71^{Ac}
	SEE40	66.33 ± 1.15^{ABa}	63.32 ± 2.63^{ABa}	60.64 ± 1.68^{Aa}	48.50 ± 1.28^{Bb}
	SEE80	69.38 ± 1.37^{Aa}	68.75 ± 1.29^{Aa}	60.82 ± 1.38^{Ab}	53.96 ± 0.96^{Ac}

TM= total motility, PM= progressive motility, SEE20, SEE40, and SEE80= 20, 40, and 80 µg of spirulina ethanolic extract per mL of extender, $^{A, B, C}$ values with different superscripts indicate significant differences (P<0.05) within groups at each time point. $^{a, b, c}$ values with different superscripts indicate significant differences (P<0.05) within groups over storage time.

Table 2. Kinematic parameters of spermatozoa in ram semen stored at 4 °C for 72 h in Tris-egg yolk extender supplemented with spirulina ethanolic extract

Parameters	Treatments	Storage duration (h)			
		6	24	48	72
VCL	Control	109.08 ± 2.88^{Aa}	97.70 ± 5.78^{Aa}	71.40 ± 3.79^{Ab}	62.46 ± 2.83^{Ab}
	SEE20	112.18 ± 3.45^{Aa}	100.88 ± 3.27^{Aa}	83.46 ± 3.76^{Ab}	72.73 ± 2.95^{Ab}
	SEE40	109.85 ± 3.27^{Aa}	97.55 ± 3.16^{Aa}	75.03 ± 3.27^{Ab}	68.23 ± 3.11^{Ab}
	SEE80	111.18 ± 4.55^{Aa}	99.89 ± 4.71^{Aa}	83.60 ± 2.89^{Ab}	74.46 ± 3.89^{Ab}
VSL	Control	25.20 ± 1.19^{Aa}	19.09 ± 1.28^{Ab}	17.41 ± 1.63^{Ab}	14.95 ± 1.31^{Bb}
	SEE20	23.65 ± 1.01^{Aa}	22.12 ± 1.01^{Aa}	20.10 ± 1.05^{Aa}	20.29 ± 1.01^{Aa}
	SEE40	23.05 ± 1.58^{Aa}	21.74 ± 1.63^{Aa}	20.98 ± 1.92^{Aa}	18.32 ± 1.38^{ABa}
	SEE80	24.52 ± 1.82^{Aa}	23.98 ± 1.42^{Aa}	22.24 ± 1.53^{Aa}	21.11 ± 1.21^{Aa}
VAP	Control	36.86 ± 1.03^{Aa}	28.26 ± 1.36^{Bb}	24.39 ± 0.98^{Ab}	19.90 ± 0.96^{Ac}
	SEE20	34.58 ± 1.34^{Aa}	30.36 ± 1.23^{Aba}	24.26 ± 1.46^{Ab}	22.18 ± 1.02^{Ab}
	SEE40	33.64 ± 1.65^{Aa}	30.32 ± 1.61^{ABab}	26.59 ± 1.87^{Abc}	22.45 ± 1.41^{Ac}
	SEE80	35.02 ± 1.53^{Aa}	33.84 ± 1.46^{Aa}	27.75 ± 1.51^{Ab}	24.11 ± 1.22^{Ab}
LIN	Control	26.34 ± 3.22^{Aa}	26.81± 2.78^{Aa}	22.62 ± 1.95^{Aa}	23.32 ± 1.48^{Aa}
	SEE20	27.45 ± 1.89^{Aa}	23.11 ± 1.62^{Aab}	21.26 ± 1.28^{Ab}	21.26 ± 1.16^{Ab}
	SEE40	26.99 ± 3.20^{Aa}	26.66 ± 2.84^{Aa}	22.01 ± 2.06^{Aa}	21.24 ± 1.82^{Aa}
	SEE80	27.96 ± 2.38^{Aa}	26.18 ± 2.12^{Aa}	25.09 ± 1.79^{Aa}	22.16 ± 1.26^{Aa}
STR	Control	67.85 ± 1.83^{Aa}	69.54 ± 1.41^{Aa}	65.23 ± 1.18^{Ba}	70.91 ± 1.67^{Aa}
	SEE20	68.85 ± 2.03^{Aa}	68.31 ± 2.43^{Aa}	69.98 ± 1.51^{ABa}	71.61 ± 1.58^{Aa}
	SEE40	68.65 ± 1.96^{Aa}	70.72 ± 1.86^{Aa}	71.50 ± 1.27^{Aa}	69.78 ± 1.37^{Aa}
	SEE80	69.61 ± 1.55^{Aa}	71.64 ± 0.92^{Aa}	69.63 ± 1.48^{ABa}	68.30 ± 1.26^{Aa}

VCL= curvilinear velocity (µm/s), VSL= straight line velocity (µm/s), VAP= average path velocity (µm/s), LIN= linearity (%), STR= straightness (%), SEE20, SEE40, and SEE80= 20, 40, and 80 µg of spirulina ethanolic extract per mL of extender, $^{A, B}$ values with different superscripts indicate significant differences (P<0.05) within groups at each time point, $^{a, b, c}$ values with different superscripts indicate significant differences (P < 0.05) within groups over storage time.

3.2. Sperm Viability and Morphology

Irrespective of the treatments, sperm viability decreased over time, with significantly (P<0.05) lower viability at 48 and 72 h compared to the corresponding values at 6 h (Table 3). However, enrichment of TEY extender with SEE preserved sperm viability at 6, 48, and 72 h of cooling storage where treated semen samples with SEE at 40 and 80 µg/mL demonstrated significantly higher viability in comparison to the control sample (P<0.05). The percentage of morphological abnormality gradually increased as the storage period progressed, especially in the control sample where values were significantly (P<0.05) higher from 24 h to 72 h in comparison to corresponding one at 6 h (Table 3). The incorporation of SEE at 20 and 40 µg/mL in Tris-based extender significantly (P<0.05) reduced sperm abnormality at 48 and 72 h, respectively.

3.3. Sperm Membrane Functional Integrity

As shown in Table 3, except at 48 h, the percentage of spermatozoa with functional membrane was significantly (P<0.05) higher in SEE80 group compared to the corresponding control values throughout the storage period; however, other SEE treated samples did not show any significant difference (P>0.05) compared to the control. Changes of spermatozoa membrane functionality over time revealed significant (P<0.05) decrease when comparing values for consecutive time points, especially in the control and SEE80 groups. Conversely, SEE20 and SEE40 groups revealed no difference in membrane functionality for consecutive time points.

3.4. Lipid Peroxidation and Nitric Oxide Content

As depicted in Figure 1a, only samples of SEE20 and SEE40 groups showed significantly (P<0.05) lower MDA level compared to the control. Conversely, no significant difference among treatment groups was observed for NO level (Figure 1b).

Table 3. Percentages of sperm viability, membrane functional integrity, and abnormal morphology in ram semen stored at 4 °C for 72 h in Tris-egg yolk extender supplemented with different concentrations of spirulina ethanolic extract

Parameters	Treatments	Storage duration (h)			
		6	24	48	72
Viability	Control	81.25 ± 1.11^{Ba}	78.62 ± 1.23^{Aab}	75.25 ± 0.94^{Bb}	70.22 ± 0.55^{Bc}
	SEE20	84.00 ± 1.07^{ABa}	82.37 ± 0.86^{Aab}	79.37 ± 1.16^{ABb}	73.78 ± 0.64^{Ac}
	SEE40	86.12 ± 1.38^{Aa}	82.12 ± 2.59^{Aa}	80.94 ± 1.93^{Aab}	74.37 ± 1.25^{Ab}
	SEE80	88.37 ± 1.25^{Aa}	84.51 ± 0.81^{Aab}	81.25 ± 1.11^{Ab}	76.26 ± 0.90^{Ac}
Membrane functional integrity	Control	63.99 ± 0.43^{Ba}	60.59 ± .080^{Bb}	56.89 ± 0.85^{Ac}	51.91 ± 1.15^{Bd}
	SEE20	65.43 ± 1.41^{Ba}	62.23 ± 1.73^{Bab}	59.82 ± 1.11^{Abc}	56.72 ± 1.06^{ABc}
	SEE40	62.89 ± 1.08^{Ba}	59.47 ± 0.92^{Ba}	57.61 ± 1.38^{Aab}	52.94 ± 2.36^{ABb}
	SEE80	73.21 ± 1.02^{Aa}	67.53 ± 1.58^{Ab}	60.86 ± 1.21^{Ac}	58.19 ± 1.50^{Ac}
Abnormal morphology	Control	9.69 ± 0.85^{Aa}	14.00 ± 0.79^{Ab}	18.19 ± 0.68^{ABc}	23.64 ± 0.58^{ABd}
	SEE20	10.94 ± 0.92^{Aa}	12.58 ± 0.62^{Aab}	15.69 ± 0.45^{Cb}	20.22 ± 1.15^{BCc}
	SEE40	11.98 ± 0.87^{Aa}	13.31 ± 0.94^{Aab}	16.02 ± 0.70^{BCbc}	18.14 ± 1.01^{Cc}
	SEE80	12.61 ± 0.96^{Aa}	14.56 ± 0.86^{Aa}	20.15 ± 0.69^{Ab}	27.02 ± 0.97^{Ac}

SEE20, SEE40, and SEE80= 20, 40, and 80 µg of spirulina ethanolic extract per mL of extender, $^{A, B, C}$ values with different superscripts indicate significant differences (P<0.05) within groups at each time point, $^{a, b, c, d}$ values with different superscripts indicate significant differences (P<0.05) within groups over storage time.

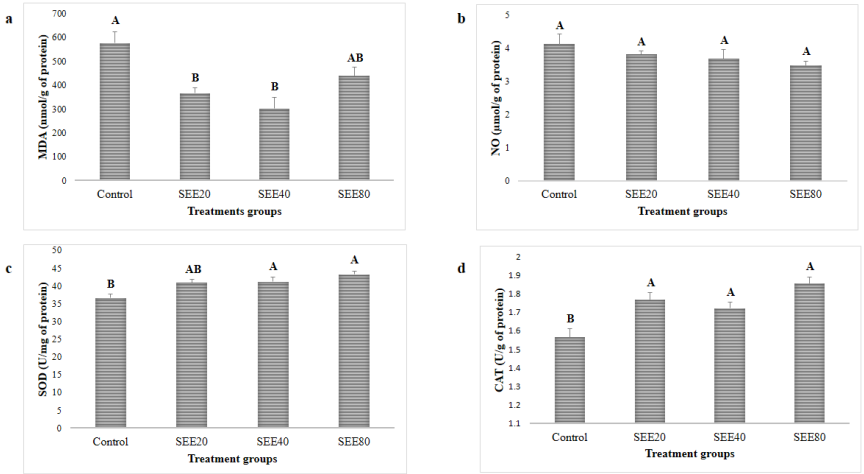

Figure 1. MDA (nmol/g of protein) (a), NO (µmol/g of protein), SOD (U/mg of protein), and CAT (U/g of protein) values after 72 h of liquid storage at 4 °C of ram semen extended in Tris-egg yolk supplemented with different concentrations of spirulina ethanolic extract (SEE20, SEE40, and SEE80: 20, 40, and 80 µg of spirulina ethanolic extract per mL of extender, $^{A, B}$ values with different superscripts indicate significant differences (P<0.05) among groups.

3.5. Superoxide Dismutase and Catalase Activities

Semen samples treated with SEE at 40 and 80 µg/mL showed significantly higher (P<0.05) SOD activity in comparison to the control (Figure 1c). Likewise, compared to the control, the enrichment of TEY with the different concentrations of SEE significantly increased (P<0.05) CAT activity (Figure 1d).

3.6 Correlations between ram Sperm Parameters after 72 h of Liquid Storage at 4 °C in Tris-Egg Yolk Extender

As evidenced in Table 4, sperm TM was positively correlated to PM (r= 0.69, P<0.01), VCL (r=0.62, P<0.01), VSL (r=0.49, P<0.05), viability (r=0.91, P<0.01), and membrane integrity (r=0.49, P<0.05) and negatively correlated to morphological abnormalities (r=-0.65, P<0.01). In the same way, PM was positively correlated to VCL (r=0.50, P<0.05), VSL (r=0.48, P<0.05), and viability (r=0.81, P<0.01) and negatively correlated to morphological abnormalities (r=-0.61, P<0.05). Viability was positively correlated to membrane integrity (r=0.48, P<0.05), whereas morphological abnormality was negatively correlated to viability and membrane integrity (r=- 0.68, r=-0.56, respectively, P<0.01).

3.7 Correlations of Sperm Parameters with Oxidative Stress Indicators after 72 h of Liquid Storage at 4 °C in Tris-egg Yolk Extender

The relationship of sperm parameters with oxidative stress indicators are summarized in Table 5. MDA was negatively correlated to TM (r=- 0.91, P<0.01), PM (r=-0.70, P<0.01), VCL (r=-0.49, P<0.05), VSL (r=-0.67, P<0.01), and viability (r=-0.91, P<0.01) and positively correlated to STR (r=0.45, P<0.05) and morphological abnormality (r=0.70, P<0.01). SOD was positively correlated to TM (r=0.66, P<0.01), PM (r=0.52, P<0.05), VSL (r=0.70, P<0.01), VAP (r=0.51, P<0.05), viability (r=0.55, P<0.05), and membrane integrity (r=0.52, P<0.05) and negatively correlated to morphological abnormality (r=0.74, P<0.01). Moreover CAT was positively correlated to membrane integrity (r=0.59, P<0.01).

Table 4. Correlations (r) among ram sperm parameters after 72 h of liquid storage at 4 °C of ram semen in Tris-egg yolk

	PM	VCL	VSL	VAP	LIN	STR	Viab	SFM	Abn
TM	0.69[a]	0.62[a]	0.49[b]	0.40	-0.24	-0.312	0.91[a]	0.49[b]	-0.65[a]
PM		0.50[b]	0.48[b]	0.24	-0.15	0.17	0.81[a]	0.44	-0.61[a]
VCL			0.31	0.58[a]	-0.32	-0.55[b]	0.64[a]	0.54[b]	-0.99[a]
VSL				0.42	0.48[b]	0.06	0.53[b]	0.32	-0.35
VAP					-0.004	-0.11	0.39	0.30	-0.57[a]
LIN						0.48[b]	-0.14	-0.18	0.33
STR							-0.37	-0.19	0.51[b]
Viab								0.48[b]	-0.68[a]
SFM									-0.56[a]

TM= total motility (%), PM= progressive motility (%), VCL= curvilinear velocity (µm/s), VSL= straight line velocity (µm/s), VAP= average path velocity (µm/s), LIN= linearity (%), STR= straightness (%), Viab= viability (%), SFM= spermatozoa with functional membrane (%), Abn= morphological abnormality (%), [a] correlation is significant at the 0.01 level, [b] correlation is significant at the 0.05 level.

Table 5. Correlations (r) of ram sperm parameters with oxidative stress indicators after 72 h of liquid storage at 4 °C of ram semen in Tris-egg yolk

Oxidative stress parameters	Sperm parameters									
	TM	PM	VCL	VSL	VAP	LIN	STR	Viab	SFM	Abn
MDA	-0.91[a]	-0.70[a]	-0.49[b]	-0.67[a]	-0.35	0.26	0.45[b]	-0.91[a]	-0.43	0.70[a]
NO	-0.21	-0.35	-0.19	-0.41	-0.35	0.13	0.27	-0.28	-0.12	0.42
SOD	0.66[a]	0.52[b]	0.36	0.70[a]	0.51[b]	-0.41	-0.37	0.55[b]	0.52[b]	-0.74[a]
CAT	0.38	0.14	0.04	0.26	0.30	-0.29	-0.18	0.33	0.59[a]	-0.25

TM= total motility (%), PM= progressive motility (%), VCL= curvilinear velocity (µm/s), VSL= straight line velocity (µm/s), VAP= average path velocity (µm/s), LIN= linearity (%), STR= straightness (%), Viab= viability(%), SFM= spermatozoa with functional membrane (%), Abn= morphological abnormality (%), MDA= malondialdehyde (nmol/g of protein), NO= nitric oxide (µmol/g of protein), SOD= superoxide dismutase (U/mg of protein), CAT= catalase (U/g of protein), [a] correlation is significant at the 0.01 level, [b] correlation is significant at the 0.05 level.

4. Discussion

The important loss of semen quality during liquid storage limits the usage of stored semen to short time interval and hence represent a drawback for efficient application of assisted reproductive techniques such as AI and consequent improvement of production. The results of the current study indicated that sperm quality parameters decline steadily throughout the storage period. However, SEE at appropriate concentration 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 spermatozoa with normal morphology. SEE can inhibit LPO, and stimulate SOD and CAT activities during low temperature liquid storage. Moreover, there were significant positive and negative correlations between sperm and oxidative stress parameters.

This study demonstrated that enrichment of TEY with SEE can enhance the preservation of sperm TM, PM, and sperm motion characteristics VSL and VAP during refrigerated storage. The results reported herein are consistent with previous findings regarding the supplementation of extenders with antioxidant compounds from natural origin during storage at low temperature (Allai et al., 2016; Wen et al., 2019) and cryopreservation (Merati and Farshad, 2020), but contrary to other (Taşdemir et al., 2020). The comparison of the results of sperm kinematics obtained from different experiments is challenging considering the variety of sperm concentrations in the samples and diluents used (Câmara et al., 2011).

Motility has been documented as one of the essential sperm parameters for fertility (Kasimanickam et al., 2011), especially in AI procedures that require sperm cells to move within the reproductive tract of the females to reach the ovum. Effective semen storage relies on reversible decrease in motility and metabolic activity of sperm cells following cooling at lower temperatures; however, exposure of sperm cells to artificial conditions amplifies the generation of ROS which normally arises as a consequence of aerobic conditions where live sperm cells are involved (Agarwal et al., 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 as observed in the present study. Additionally, motility, which is an energy-dependent function, 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. In fact, sperm mitochondrion is particularly sensitive to cooling process and this sensitivity results in disturbance in ATP transport with consequent reduction in motility (Zarei et al., 2021).

Interestingly, all SEE treated samples showed superior PM in comparison to the control sample from 48 h onwards. Progressive motile sperm cells represent the spermatozoa fraction that can effectively move within the female reproductive tract once insemination is performed. Therefore, by reducing the loss of PM, SEE may improve the fertilization rate of chilled semen. Enrichment of extenders with SEE at 80 µg/mL beneficially affected VSL and VAP respetively at 72 h and 24 h. The preservation of these sperm attributes can be ascribed to the capacity of the bioactive components present in SEE to inhibit the generation and/or scavenge ROS in excess. Particularly SEE bioactive components may inhibit the mitochondrial outer membrane enzyme monoamine oxidase that catalyses 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 species within both the mitochondrial, matrix and cytosol (Cadenas and Davies, 2000). In this way, SEE may restore the balance between the amounts of ROS produced and scavenged, and consequently preserve the metabolic activity of sperm cells. It is well known that spirulina is a rich source of bioactive ingredients among which vitamin E which is considered as an essential component of the sperm antioxidant defence system, hence one of the major protector against oxidative stress and LPO (Yousef et al., 2003). SEE, thanks to the presence of vitamin E which is liposoluble, may have inhibited the peroxidation of PUFAs abundant in ram sperm membrane.

Viability, as assessed by dye exclusion, allows to discriminate the necrozoospermia from the total lack of motility associated to structural deficiencies in the tail zone (Chemes and Rawe, 2003). The results of this study evidenced the beneficial influence of SEE especially at 40 and 80 µg/mL on sperm viability during storage. Natural herbs cladodes (*Opuntia ficus indica*) and green tea (*Camellia sinensis*) used as additives to semen extenders improved viability (Allai *et al*., 2016; Mehdipour *et al*., 2016). During semen storage, the accumulation of ROS above the detoxifying capacity of spermatozoa leads to peroxidative damage of membrane proteins, phospholipids, and PUFAs (Peris-Frau et al., 2020; Kameni et al., 2021), hence the loss of membrane integrity and subsequent cell death. This phenomenon is particularly prominent in ram because of the abundance of PUFAs in sperm plasma membrane (Bucak *et al*., 2007). The improvement of sperm viability in the present study may be essentially linked to the bioactivity of phycocyanin which has been documented as the compound mainly responsible for the antioxidant activity of spirulina thanks to its strong radical scavenging properties (Piñero Estrada et al., 2001). Phycocyanin and other chemicals present in spirulina may have scavenged the ROS generated in excess, hence reducing ROS detrimental action on sperm membrane constituents, inhibiting LPO, and ultimately preserving sperm viability as noticed in this investigation. Furthermore, SEE may have inhibited the release of cytochrome C from the mitochondria to the cytosol, release which is the initiation point of the apoptosis cascade (Silva, 2006). Besides improving the antioxidant defence of spermatozoa, SEE may have strengthen the levels of phosphoinositide-3 kinases which have been documented as potent stimulators of several anti-apoptotic effectors, hence preventing cell death (Oudit et al., 2004).

The results of the current work indicated that, SEE at 80 µg/mL improved sperm functional membrane integrity at 6, 24, and 72 h. This observation may be associated to

the capacity of SEE to inhibit the generation of free radicals and their negative action on lipid bilayer interactions and proteins' anchorage to the bilayer, ultimately preventing the loss of physiological function.

For satisfactory results following AI in small ruminants, the threshold of 15% has been suggested as the maximum critical percentage of sperm morphological defects (Rehman et al., 2013). In this study, as the storage was extended, the percentages of sperm morphological abnormalities increased. This result is in accordance with previous reports (Gundogan et al., 2011; Gheller et al., 2018). However, enrichment of extenders with SEE at the intermediate concentration (40 µg/mL) showed to be effective at reducing sperm morphological defects. While many studies have reported no effect following extender supplementation with antioxidant compounds on sperm morphology (Amini et al., 2019; Zarei et al., 2021), arguing that morphology is mainly related to spermatogenesis, others have highlighted positive effects (Allai et al., 2016; Rateb, 2018). It seems as the dynamics of sperm morphology expands beyond the scope of spermatogenesis, and morphology alteration might also be prevented thanks to the antioxidative potential of SEE. Moreover, the nature, chemical composition and incorporation level of the antioxidant compound coupled with the variety of methodologies used to assess sperm morphology may account for the discrepancy observed.

Recent investigations have evidenced the increase of the antioxidant capacity of semen and the inhibition of LPO following addition of antioxidants from natural origin to extenders during semen storage (Wen et al., 2019, Taşdemir et al., 2020). Likewise, the enrichment of TEY extender with SEE increased SOD and CAT levels and inhibited MDA production during cooling storage at 4°C. Under low LPO rates (sub-toxic conditions), cells initiate their maintenance and survival through intrinsic antioxidant defence systems or signalling pathways activation that up-regulate protein antioxidants resulting in an adaptive stress response. On the other hand, under medium or high LPO rates (toxic conditions), the magnitude of oxidative stress exceeds repair capacity, and the cells induce apoptosis or necrosis (Ayala et al., 2014). Therefore, by moderating the rate of LPO as observed in the present work, SEE may have created sub-toxic conditions that favour the increase of enzymatic antioxidant activities and the preservation of sperm quality.

The correlation analysis revealed that TM was positively related to PM, VCL, VSL, viability and sperm membrane functionality. Similar relations have been established in earlier reports (Câmara et al., 2011; Singh et al., 2014). The positive correlations among sperm quality parameters reported herein may be linked to the involvement of sperm membrane in the maintenance of these parameters. On the other hand and as reported by Gupta and Singh (2018), morphological abnormality was negatively correlated to other sperm quality parameters. Impaired morphology, especially at the level of the tail which is the spermatozoon structure in charge of locomotion, may lead to decrease motility. In addition, morphology impairment may alter normal metabolism with reduction of energy production and accumulation of toxic products, and subsequent loss of sperm motility, viability, and membrane integrity.

MDA was negatively correlated with sperm TM, PM, viability and positively correlated to morphological abnormality; whereas, the totally inverse figure was observed for the SOD. These results are consistent with previous findings (Kadirve et al., 2014). Alvarez and Storey (1992) reported that semen samples with highest viability after freeze-thawing were characterized by high SOD activity and a strong correlation between loss of SOD activity and loss of motility and membrane integrity, and concluded that the loss of sperm quality might be at least partly mediated by SOD. However, Lone et al. (2018) observed no correlation between the SOD and semen parameters following freeze-thawing of buffalo semen.

5. Conclusion

In conclusion, SEE at 40 µg/mL can improve ram semen storage by reducing the loss of sperm motility, viability, morphology, and membrane integrity during chilled storage at 4 °C for up to 72 h. Moreover, SEE can inhibit LPO and stimulate enzymatic antioxidant SOD and CAT at 72 h of storage. Significant positive and negative correlations exist between sperm and oxidative stress parameters. It will be worth to assess the underlying mechanism(s) of the SEE protection of semen during storage and conduct AI trial with SEE treated semen.

Author Contributions

Concept: S.L.K. (100%), Design: S.L.K. (100%), Supervision: S.L.K. (50%) and F.N. (50%), Data collection and/or processing: S.L.K. (25%), A.B.N.D. (25%), T.T.T. (25%) and F.D.T.B. (25%), Data analysis and/or interpretation: F.M. (100%), Literature search: A.B.N.D. (35%), T.T.T. (35%) and F.D.T.B. (30%), Writing: S.L.K. (100%), Critical review: S.L.K. (50%) and F.N. (50%), Submission and revision: S.L.K. (50%) and F.N. (50%). All authors reviewed and approved final version of the manuscript.

Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Consideration

The experimental procedures used in the present study were reviewed and validated by the local Animal Care and Ethics Committee of the Department of Animal Science, University of Dschang, in accordance with the Code of Ethics of the EU Directive 2010/63/EU for animal experiments as amended by Regulation (EU) 2019/1010.

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