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Amelioration of physical characteristics, antioxidant capacity, and fertilizing potential of cryopreserved buck goats' spermatozoa through *Amphora coffeaeformis* microalga extract

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ABSTRACT

This investigation consisted of two sequential experiments. The first one attempted to explore the effect of various levels of *Amphora coffeaeformis* microalga extract (ACME) supplementation to the semen medium on buck's sperm quality. Semen samples were collected with an artificial vagina from 6 fertile Damascus bucks and then were diluted with an extender containing ACME (2.5, 5, 7.5, 10, 12.5 µl/mL) or without (control). Aliquots of diluted semen were stored and maintained at 4°C for the subsequent 48 h during which sperm traits were assessed alongside total antioxidant capacity (TAC), alkaline phosphatase activity (ALP), reduction of the resazurin dye test (RRT), and sperm DNA fragmentation index using fluorescent imaging. In the second experiment, the optimum level of ACME supplementation was used to determine the fertilization ability of spermatozoa. The obtained results of the first experiment indicated clearly that level 12.5 µl/mL of ACME achieved higher ($P< 0.05$) values of progressive motility, live sperm, normal sperm, and intact acrosome at 48 h of chilled preservation than those of other experimental groups. Moreover, addition of 12.5 µl/mL ACME to the semen extender increased ($P<0.05$) TAC at T48 of the preservation period. Correspondingly, ACME supplementation at level of 12.5 µl/mL reduced the secondary sperm abnormalities at 48 h of chilled preservation. The results of DNA fragmentation index (DFI) improved ($P<0.05$) with level of ACME (12.5 µl/mL), compared with the other levels or control specimens at T48 h of storage period. Consequently, the optimum level of ACME (12.5 µl/mL) yielded higher ($P<0.05$) in fertilization rate compared to control. The emerged results accentuate the protective role of *Amphora coffeaeformis* microalga extract on cryopreserved spermatozoa. Furthermore, addition ACME at level of 12.5 µg/ mL to semen extender appeared to be the optimum level to express the valuable effects on the semen quality, antioxidant activities, DNA fragmentation index, and fertilizing potentials.

Introduction

Artificial insemination (AI) and in-vitro fertilization (IVF) are important tools used widely in sophisticated reproductive procedures in the livestock business to generate breeds with desirable qualities such as high productivity, disease resistance or high environmental adaptability (Câmara *et al*., 2011). As a result, developing cryopreserved sperm doses for commercial application purposes is crucial (Murphy *et al*., 2013). Despite many advantages of sperm cryopreservation, nevertheless, it has been reported to reduce sperm fertilizing capacity by 30-50% (Aliabadi *et al*., 2013), through some damage such as morphological and DNA damages, generally impaired sperm motility and viability (Lopes *et al*., 2021), in addition to increase programmed sperm death (Badr *et al*., 2010). During cryopreservation, the phospholipid cell membrane of goat sperm is more susceptible to lipid peroxidation than in other species because it is rich in polyunsaturated fatty acids (Garg *et al*., 2009). Moreover, sperm metabolic activity represents another source of reactive oxygen species (ROS) generation (Alvarez and Storey, 2005; Sanocka and Kurpisz, 2004). Naturally, seminal fluid contains an array of endogenous enzymatic (Calamera *et al*., 2003) and non-enzymatic antioxidants that act as free radical scavengers to protect spermatozoa (Meucci *et al*., 2003; Soren *et al*., 2016). However, sperm cells are deprived of these vital, naturally occurring antioxidants when semen is diluted for processing (Mostafa *et al*., 2009). Therefore, the inclusion of natural antioxidants in semen diluents has lately received a lot of interest in recent fertility-related practices (Badr *et al*., 2017). Based on chemical analyses by mass spectrometry in previous studies, several microalgae such as Chlorella sp., Spirulina sp., and Amphora sp. are receiving the attention as antioxidants because of their highly content of β-carotene, lutein, and phycocyanin besides a photosynthetic pigment with antioxidant and anti-inflammatory properties (Benedettia *et al*., 2004). The present study aimed to evaluate the protective effects of *A. coffeaeformis* microalga extract in terms of impact on the cryosurvival, oxidative status, and fertilization potential of buck sperm.

Materials and methods

Ethical approval

The Desert Research Center's Animal Care and Use Committee approved all procedures, which were carried out in accordance with ISO 9001: 2015 quality management regulations and the guidelines and regulations of the European Parliament's Animal Ethics Committee Institute for the protection of experimental animals (2010/ 63/ EU).

Animals

The current research was conducted at the Artificial Insemination Lab., the Desert Research Center's Mariout Research Station (Latitude 31° 00′ N, Longitude 29° 47′ E), Alexandria, Egypt. Six adult Damascus bucks, aged from 36 to 48 months with an average weight of 45.0±2.0 kg, were used during two months of breeding season (September- October 2023). During the experimental period, all bucks were kept in a walled stockyard and were fed a concentrate mixture according to their protein and calorie requirements (NRC, 2007). Hay, Egyptian clover (Trifolium alexandrinum), and fresh water were provided ad libitum. Bucks were clinically evaluated prior to the experiment, and no diseases or reproductive issues were discovered.

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Cooling storage medium (Semen extender)

Unless otherwise noted, all chemicals used in the semen extender were acquired from Company Panreac, Spain. As per a prior report (Daramola *et al*., 2017), the extender was composed of 3.8 g tris (hydroxymethyl aminomethane), 0.6 g glucose, 2.2 g citric acid, 0.028 g penicillin and 20% egg yolk in 80 mL of distilled water. After centrifugation (5,000 g for 10 min), the diluent was filtered (0.22 µm) to obtain a clear extender and was kept at 4°C for 24 hours following the semen collection.

Semen collection

Using an artificial vagina, 96 ejaculates (48 for each month) were collected from 6 bucks over the course of 8 collection sessions for each month. After collection, each raw ejaculate was transported to the laboratory, directly adjacent to the collection area, to evaluate sperm physical and morphometric traits. Oddly coloured or contaminated ejaculates were discarded.

Raw ejaculates assessment and pooling

Throughout the assessment, the raw ejaculates were maintained in a water bath at 37°C. The graded collecting tubes were used to record the ejaculate volume (mL) for every raw ejaculate. Analyses also included pH, sperm concentration (×10^6/mL), mass motility score (5= very motile, 0= immotile), progressive motility (%), viability (%), normal sperm (%) and intact acrosome (%). Table 1 shows the average values of the previously indicated criteria in raw-pooled ejaculates over the course of this investigation.

Preparation of Amphora coffeaeformis microalga extraction (ACME)

A. coffeaeformis microalga was obtained commercially from the Algal Biotechnology Unit, National Research Centre, Dokki, Giza, Egypt. The algal was washed with deionized water and centrifuged under cooling at 5000 g for 10 min to remove all accompanied nutrients, then air dried (Hassan *et al*., 2015). The air- dried *A. coffeaeformis* algae powder (10 g) was soaked in 100 ml of deionized water and shaken continuously for 24 h at room temperature. Then, it was centrifuged at 5000 g for 10 min and filtered to remove the cell debris. The extract was stored at 4°C until use (Chu *et al*., 2010).

Experimental design

The first experiment

In an attempt to designate the best level of *A. coffeaeformis* microalga extract (ACME) to boost oxidative status and liquid-chilled storage capacity of spermatozoa, 96 ejaculates were obtained from 6 bucks (16 each) during the experimental period. The ejaculates of each collection session were pooled and diluted (1:10) with the clarified base diluent. The diluted specimens were further split into six aliquots. The first aliquot served as a control (ACME-free) (Tr 1), whereas the other five aliquots were supplemented with 2.5 (Tr 2), 5 (Tr 3), 7.5 (Tr 4), 10 (Tr 5), or 12.5 (Tr 6) µl/mL of ACME. Directly after dilution (T0), all semen aliquots were stored in a cooling cabinet at 4°C for 48 h, to evaluate sperm morphometric and physical properties, along with total antioxidant capacity (TAC), alkaline phosphatase activity (ALP), and reduction of the resazurin dye test (RRT) indices at a 24-hour interval (T24, T48). Also, the ability of an optimal level of ACME to reduce sperm DNA fragmentation was evaluated after 48 h of preservation.

Semen assessment

At least 200 sperms were evaluated for each physical and morpho- functional parameter. Total sperm motility (%) was evaluated using a phase-contrast microscope (Leica Inc., Wetzlar, Germany) at 400× magnification, according to Penitente-Filho *et al.* (2018). On the other hand, sperm viability (%) was assessed by mixing and smearing 10 µL of semen with 5 µL of eosin-nigrosin stain (Sigma-Aldrich, USA), and the smeared slides were evaluated by a phase-contrast microscope (Leica) at 1000x magnification (Björndahl *et al*., 2003). By Romanowski's triple-stain method (DIFF-QUICK III, Vertex, Cairo, Egypt), primary and secondary sperm abnormalities, as well as acrosomal cap integrity were recorded (Brouland *et al*., 1995). Smears preparation and staining processes were performed as per the manufacturer's instructions, then, were evaluated by a phase-contrast microscope at 1,000× magnification with an oil immersion lens at 1000x magnification (Leica), according to Rateb (2018).

Assessment of enzymatic activity and oxidative stress indices

A sample (2 mL) of each semen group was aspirated and centrifuged (1,000 g for 10 min) at times parallel to those of sperm assessment (T0, T24, and T48) and stored at -20°C until it is assessed for total antioxidant capacity (TAC), alkaline phosphatase (ALP) activity, and the reduction of the resazurin dye test (RRT) by colorimetric kits (Biodiagnostic, Cairo, Egypt) according to Belfield and Goldberg (1971); Ohkawa *et al.* (1979) and Reddy and Bordekar (1999), respectively, using spectrophotometer (Techcomp, UV2300II).

Evaluation of sperm DNA fragmentation

The magnitude of DNA fragmentation in all semen groups treated with levels of ACME or control by utilizing kits from Halomax® (Halotech® DNA SL Corp., Madrid, Spain), by fluorescent imaging as a direct indicator for sperm fertilization potential at 48 h, the rate of sperm DNA fragmentation was evaluated post 48 h from the time of preservation at 4ºC as previously described by Abd El-Hamid (2023). The sperm DNA fragmentation index (DFI %) was calculated by dividing the fraction of DNA-fragmented spermatozoa by the total number of sperm (Yang *et al*., 2019).

The second experiment

Based on the results of experiment 1, optimal level of *Amphora coffeaeformis* microalga extract supplementation was further used in to assess the fertilizing rate by artificial insemination AI at 48 h. of chilled preservation.

Table 1. Mean value of physical characteristics of raw pooled buck's semen (mean±SEM)

Parameter	Value	Parameter (%)	Value
Volume (ml)	1.46±0.24	Progressive motility	89.33±1.77
pH	6.30±0.04	Live sperm	91.16±1.20
Sperm concentration (×10^6 /ml)	2277.83±183.13	Normal sperm	86.83±0.64
Mass motility score (5-0) *	3.83±0.38	Intact acrosome	86.33±0.87

*Mass motility score: 5 = highly motile; 0 = immotile.

Insemination trial

A total of 56 doe of Damascus goats were allocated into three groups: the first group (G1, n = 21) was naturally inseminated by mature fertile bucks. The second group (G2, n = 18) was artificially inseminated with cryopreserved semen. The third group (G3, n = 17) was artificially inseminated by the optimum dose of cryopreserved semen of ACME. Estrus was synchronized in normal cyclic goats, as mentioned by Rateb (2006). In brief, each female received double intramuscular doses of cloprostenol acetate PGF2α (Estrumate, Schering-Plough Animal Health, Germany), 125 µg each and 10 days apart, thought 72 h after the 2nd PGF2α injection. Ovaries were scanned using 5.0–7.5 MHz linear transducer attached with ultrasound unit (Medison, Korea) to detect mature follicles (Ø ≥ 5 mm) along with Rateb *et al*. (2019). Immediately before being artificially inseminated, each female showed a mature graafian follicle with estrous behavior received an intramuscular dose (4 mL) of OH oxytocin hormone (Oxytocin, Bimeda, USA). Artificial insemination was done using the above-mentioned semen groups by the modified plastic inoculation catheter in the uterine body twice (AM and PM) for each doe. On day 30 after insemination, the conception rate was checked using a 7.5–10 MHz linear-array transducer attached to a Medison, Korea, ultrasound unit, according to Suguna *et al*. (2008) (Figures 1& 2).

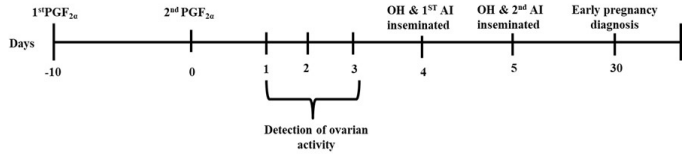

Fig. 1. Synchronization and Artificial insemination protocols of Damascus doe goats. PGF2α, each doe received 125 µg cloprostenol acetate intramuscularly, OH, each doe was treated with 4 mL oxytocin hormone intramuscularly.

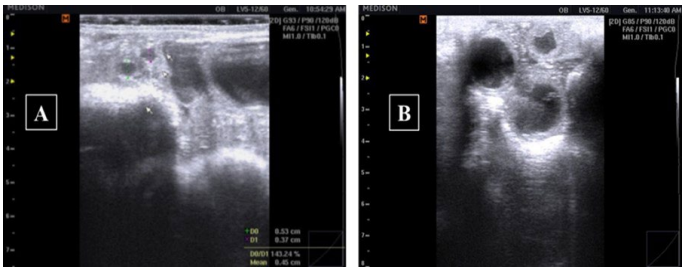

Fig. 2. [A] Ultrasonography monitoring of mature follicles (Ø ≥ 5 mm) in Damascus goat does before performing artificial insemination, [B] early diagnosis of pregnancy in goat does at day 30 following artificially insemination.

Statistical analysis

The Shapiro-Wilk test was used to determine whether the data were normally distributed or not. Mean values of pooled (raw) sperm physical and morphological properties throughout the experimental period were obtained by a simple Student's t test. Repeated measures analysis of variance (ANOVA) was used to examine changes in the physical characteristics of sperm and oxidative stress indices throughout the period of chilled storage (T0- T48) in order to identify the fixed effects of treatment levels, time (T0, T24, and T48), and treatment-by-time interaction. Duncan's post-hoc test at 5% significance was used to identify the differences among means. A chi-square of independence was used to compare sperm DNA fragmentation at T48 of chilled storage between levels. The same procedure was used to compare the conception rate among different groups after 30 days of females being inseminated. The data were analyzed using the IBM-SPSS statistics program (IBM-SPSS, 2013). The data are expressed as means±standard error (SEM).

Results

The first experiment

The obtained results showed that all levels of *A. coffeaeformis* microalga extract (ACME) and time of chilled storage enhanced (P<0.05) all sperm physical properties on treated specimens except for primary sperm abnormalities (Figure 3). At 48 h of chilled preservation, the highest (P<0.05) percentages of sperm progressive motility were recorded in Tr6 (12.5 µl/mL), Tr5 (10 µl/mL) and Tr4 (7.5 µl/mL) specimens with values being 70.00±4.18, 62.00±2.54 and 57.00±3.00, respectively. The lowest percentage was recorded in control specimens (Tr 1) (42.50±2.88%) (Figure 3).

In the meantime, the Tr6 specimens recorded the highest (P<0.05) percentage of live sperm (77.66±4.33%), and then it gradually decreased as treatment levels decreased in other specimens and unsurprisingly the lowest (P<0.05) percentage was observed in the control specimens (51.40±0.87%). Furthermore, the highest (P<0.05) percentage of normal sperm was also recorded in Tr6 specimens (84.80±1.06%), followed by other treated specimens Tr4 (77.00±1.00%), Tr5; (75.60±1.12%), Tr3 (73.00±1.48%), and Tr2 (71.20±2.70%), compared with control (Tr1) specimens (58.00±1.26%) (Figure 3).

Concerning secondary sperm abnormalities, the results demonstrated that Tr6 (12.5 µl/mL) specimens had the lowest (P<0.05) percent of secondary sperm abnormalities (13.40±1.07%), followed by other treated specimens; Tr4 (20.80±0.86%), Tr5 (22.40±1.12%), Tr3 (25.00±1.67%), and Tr2 (26.80±2.31%), while the highest value was recorded in the control specimen (36.00±1.76%) at T48 of chilled storage. However, the percentage of primary sperm abnormalities was not affected (Figure 3).

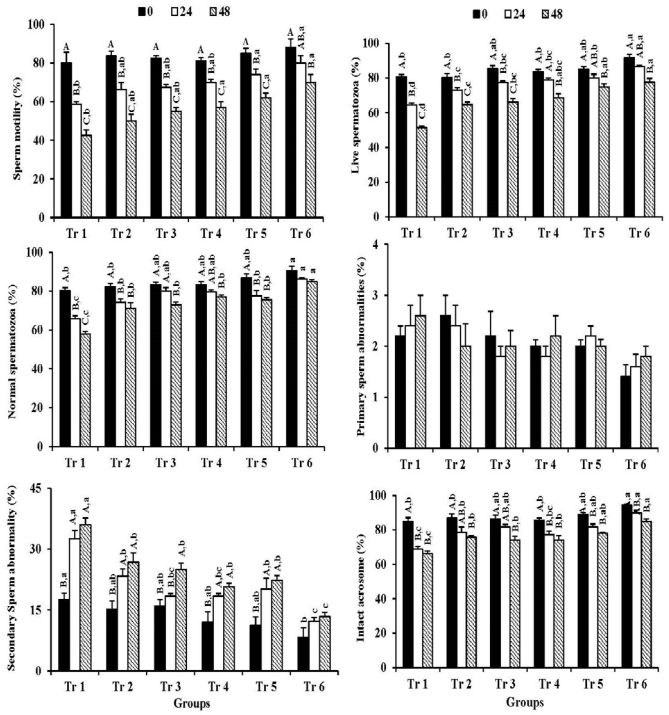

Fig.3. Effect of incorporating different doses of ACME into the diluent on characteristics of buck sperm throughout 48h of chilled preservation period at 4°C (mean±SEM).
[a-c] letters within each groups differ significantly (P<0.05).
[A-B] letters for effect of time within each group differ significantly (P<0.05).

Our results of intact acrosome showed the same trend of previous semen properties where the highest (P<0.05) mean percent was recorded in the Tr6 (12.5 µl/mL of ACME) with value being 85.00±1.41% compared to all other treated groups, while the lowest percent was recorded in the control specimens (66.20±1.56%) at T48 of chilled storage (Figure 3).

Regarding TAC concentrations throughout chilled storage (T0- T48),

the present results demonstrated that the higher (P<0.05) concentrations of TAC were recorded in the Tr6 and Tr5 specimens (0.90±0.01 and 0.86±0.03 mM/L, in order). The lowest concentration was recorded in the control group (0.66±0.07 mM/L) at T48 of the preservation period (Table 1). Contrarily, alkaline phosphatase ALP activity or RRT value were not affected by levels of *Amphora coffeaeformis* microalga extract (ACME) throughout preservation time (T0- T48) at 4ºC (Table 2).

DNA fragmentation magnitude (DFI %) after 48 h of chilled preservation

The fluorescent assessment of sperm nucleoids demonstrated that, following 48 h. of chilled storage, the lowest (P<0.05) sperm DNA fragmentation index was recorded in Tr6 (12.5 µl/mL, ACME) specimens (12.72%), while the highest percent was recorded in the control (Tr1) specimens (47.14%) (Figures 4& 5).

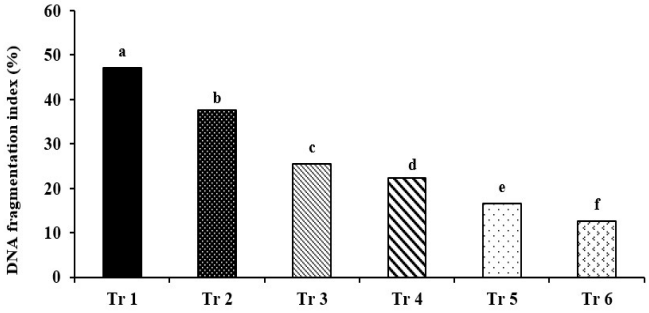

Fig. 4. Effect of the various levels of ACME into the sperm medium on DNA fragmentation index at 48 h of liquid-chilled storage at 4°C.
[a-f]letters within each groups differ significantly (P<0.05).

The second experiment

Insemination trial

The current results declared that adding ACME improved the fertilization ability where the conception rate of doe goats that were artificially inseminated with semen specimens treated with 12.5 l/mL of ACME exceed (P<0.05) that of the untreated semen specimens after 48 h of chilled preservation by 100% with values being 76.47 vs. 38.88%. However, the highest percentage of conception rate was recorded in naturally inseminated doe goats (90.00%) than other doe groups artificially inseminated (Table 3).

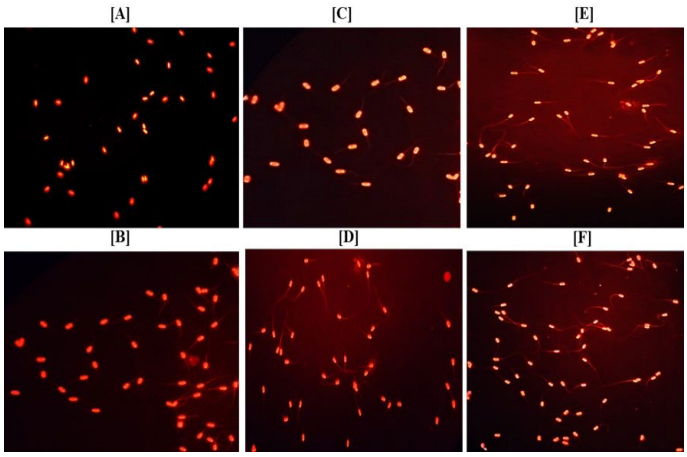

Fig. 5. Fluorescence imaging. [A], shows sperm nucleoids with a large and spotty halo of chromatin dispersion (i.e., fragmented DNA) of cryopreserved buck sperm nucleoids from control semen specimens. [B-F] Fluorescence imaging shows cryopreserved buck sperm nucleoids (non-fragmented DNA) of semen specimens treated with [B, 2.5], [C, 5], [D, 7.5], [E, 10] and [F, 12.5 µl/mL] of ACME respectively.

Discussion

Oxidative stress (OS) is a condition associated with an increasing rate of spermatozoa membrane damage induced by the formation of either reactive oxygen species (ROS) or reactive nitrogen species (RNS) during cryopreservation (Vemo *et al.*, 2018), which exposes sperm to lipid peroxidation (LP). This process destroys the lipid matrix of the plasma membrane (Ortega Ferrusola *et al.*, 2009), damages the sperm DNA, reduces sperm motility, and fertilization rate (Ball *et al.*, 2008; Aitken, 2017). Although sperm cells are protected by an antioxidant system in seminal

Table 2. Effect of incorporating different doses of ACME into the diluent on total antioxidant capacity (TAC), alkaline phosphatase activity (ALP) and reduction test (RRT) throughout 48 h of liquid-chilled storage at 4°C.

Items	Time (h)	Levels of treatments					
		Tr 1 (0 µL/mL)	Tr 2 (2.5 µL/mL)	Tr 3 (5 µL/mL)	Tr 4 (7.5 µL/mL)	Tr 5 (10 µL/mL)	Tr 6 (12.5 µL/mL)
TAC	0	0.810±0.04^{b}	0.92±0.01^{A,a}	0.93±0.01^{a}	0.95±0.01^{A,a}	0.92±0.01^{a}	0.93±0.01^{A,a}
	24	0.83±0.13	0.90±0.03^{A}	0.88±0.02	0.88±0.01^{B}	0.90±0.01	0.91±0.01^{AB}
	48	0.66±0.07^{b}	0.77±0.02^{B,ab}	0.81±0.04^{ab}	0.85±0.01^{B,ab}	0.86±0.03^{a}	0.90±0.01^{B,a}
ALP	0	126.15±1.55	101.32±17.03	110.81±8.16	113.67±11.83	105.25±13.66	99.78±17.58
	24	124.57±8.06	92.09±33.54	125.85±8.21	88.67±36.96	125.65±8.06	126.06±8.21
	48	101.90±14.52	108.61±11.76	117.09±5.65	100.42±5.39	86.53±8.01	103.41±12.18
RRT	0	2.75±0.22	2.65±0.12	2.84±0.21	2.59±0.19	2.86±0.16^{B}	2.90±0.19
	24	2.73±0.06	2.98±0.11	2.72±0.02	2.77±0.13	2.49±0.10^{AB}	3.11±0.30
	48	2.84±0.07	2.87±0.33	2.51±0.20	3.04±0.09	3.05±0.07^{A}	3.03±0.03

[a-b] letters for effect of time within each group differ significantly (P < 0.05). [A-b] within each time differ significantly (P< 0.05).

Table 3. Conception rates of goats following 30 days of artificial insemination with treated or non-treated semen vs. natural mating.

Treatment	Inseminated goats (n)	Conceived goats (n)	Conception rate (%)
Natural	21	18	90.00^{a}
Control (AI)	18	7	38.88^{c}
ACME (12.5µl/mL)	17	13	76.47^{b}

[a-c] Values in the same row with different superscript letters differ significantly (P< 0.05).

plasma, membranes and cytoplasm, this system is partially eliminated and severely altered during dilution and cryopreservation (Ros-Santaella and Pintus, 2021). Mara *et al.* (2007) also pointed out that exposure to light during handling of the semen may be a limiting factor in its conservation because it might cause ROS generation, which can harm sperm. The study of novel natural ingredients with antioxidant properties is interesting for treating male infertility. It reduces the impact of OS and controls physiological sperm functions (Ford, 2004). *Amphora coffeaeformis* microalgae are still under study, it has recently been reported to exhibit powerful antioxidant (Elsaman *et al*., 2023).

The current study revealed that, adding 12.5 µl/mL of *Amphora coffeaeformis* microalgae extract ACME had powerful potential for maintaining sperm characteristics, improving both of total antioxidant capacity and sperm redox capacity besides, decreasing DNA fragmentation index (DFI) post-exposure to the stress of processing and preservation. These results are in synergy with those reported previously where Badr *et al.* (2017). *Amphora coffeaeformis* microalgae extract (ACME) has been reported to contain vital compounds, such as chlorophylls, carotenoids (astaxanthin and canthaxanthin) (El-Sayed *et al.* 2018), flavonoids (Elsaman *et al*., 2023) and essential fatty acids, such as oleic acid (OA), linoleic acid (LA), γ-linolenic acid (GLA), α-linolenic acid (ALA), arachidonic acid (ACA), docosahexaenoic acid (DHA) (Beekrum and Amonsou, 2023). These fatty acids have been reported to be involved in lipid membrane composition of sperm (Yuan *et al*., 2023) and have the ability to target reproductive tissues altering reproductive function and fertility because they promote the creation of microdomains of flexibility, fluidity, fusogenicity, and permeability (Wassall and Stillwell, 2009). Moreover, these fatty acids are also regarded as the major determinants of sperm mobility characteristics, cold sensitivity, viability, and membrane integrity (Aksoy *et al*., 2006). From another point of view, the bioactive compounds such as phenolic compounds: Catechin, chlorogenic acid, caffeic acid, p-coumaric acid, cinamic acid hexadecanoic acid and polyphenols (Salim *et al*., 2019), vitamins including B1, B2, B9, C and E (Mekkawy *et al*., 2020; Hassan *et al*., 2021) and minerals like Ca, P, Zn and Se (Beekrum and Amonsou, 2023) have the ability to break up the radical chain reaction by transforming free radicals into stable products since they are potent electron donors (Lobo *et al*., 2010). furthermore, coffeaeformis microalgae extract is rich in essential amino acids (Beekrum and Amonsou, 2023) and protein-tyrosine kinase (PTKs) (Buhmann *et al*., 2014) which play various biological roles in spermatogenic cells, membrane protein formation, and their supporting cells (Ijiri *et al*., 2012). By quantitative polymerase chain reaction, expressions (Src, Lyn, and Hck) were detected for the correlation several families of PTKs with the cytoplasm in spermatocytes, the acrosomal region (Lawson *et al*., 2008), and the entire flagellum during the spermatogenic process, which supports the motility and biological functions of sperm (Goupil *et al*., 2011). There are some theories proposed to explain the mechanism of *A. coffeaeformis* microalgae on protection of cryopreserved spermatozoa. Bilodeau *et al.* (2000) suggested that beneficial effects may be due to its antioxidant activities, which scavenge reactive oxygen species (ROS) and shield sperm cells from harmful oxygen metabolites that cause lipid peroxidation of the sperm plasma membrane during cryopreservation. Our results supported the *A. coffeaeformis* microalgae extract's antioxidant properties and its critical function in increasing total antioxidant capacity. Antonio Marcos *et al.* (2018) reported that adding C-phycocyanin, an extracted chemical from microalgae to the boar semen medium may be able to regulate the production of intracellular ROS, which may have a beneficial effect on an increase in the percentage of progressive motility. Moreover, Wang *et al.* (2018) recorded that the beneficial effect of *A. coffeaeformis* microalgae may be due to the fact that it contains some active compounds that have anti-inflammatory properties, such as phytol and 2,6-dimethyl-4[3H]-quinazolinone. From another point of view, Qasem *et al.* (2016) and Abdel-Haleem *et al.* (2024) reported that the *A. coffeaeformis* microalgae extract has antimicrobial activities against Gram-negative bacteria (*Salmonella enterica* and *Klebsiella pneumoniae*) and Gram-positive bacteria (Micrococcus luteus and Staphylococcus aureus), and these are very problematic bacteria in AI centers (Hensel *et al*., 2021). It also has a role in combating toxicity factors such as monosodium glutamate salts widely used (Yousof *et al*., 2021), thus could possibly be used as a protective agent to reduce the microbial load, especially with the use of egg yolk extenders, and as antitoxic agent resulting from used chemical compounds in semen diluents (Namula *et al*., 2019; Kamal *et al*., 2022), hence improving the physical and functional sperm traits of spermatozoa during and after exposure to the stress of processing and preservation.

According to our findings, the higher TAC was recorded with adding 12.5 µl/mL of ACME at 48 h. of chilling storage could be explained by preserving the enzyme from leakage in extracellular fluid throughout the cooling stage (Badr *et al*., 2017), where it was reported total antioxidant capacity value (61.79 mg gallic g−1) in *Amphora coffeaeformis* extract (El-Sayed *et al*., 2018). While the increased RRT value at the same level may be attributed to the closely related improvement of sperm motility and acrosomal integrity (Robert, 1999) (Figure 3). Several of the ingredient's bioactivities for this *A. coffeaeformis* microalga extract such as docosahexaenoic, chlorogenic, caffeic, and quinic acids have antioxidant and anti-inflammatory properties, hence scavenging high levels of ROS, which cause DNA single and double strand breaks (Kothari *et al*., 2010) and the formation of 8-oxoguanine, resulting in ionizing radiation damage (Zribi *et al*., 2011) and defective chromatin packaging (Ward, 2009), hence inhibiting DNA damage (Naveed *et al*., 2018). This is in consonance with the DFI data reported in the present results.

Regarding the results of conception rate, unsurprisingly, the naturally mated doe goats were higher by 231% than doe groups that those artificially inseminated control group. These results are in synergy with those reported previously in goats (Fonseca *et al*., 2005; Duriˇci´c *et al*., 2012; Agossou and Koluman, 2018), which reported that the pregnancy rates for naturally mated goats were higher than those of artificially inseminated goats. On the other hand, ACME had a positive effect on conception rate where a higher conception rate in artificially inseminated doe goats with cryopreserved semen supplemented with ACME compared with artificially inseminated goats with control semen specimens. The improvement in conception rate with cryopreserved semen supplemented with ACME by 100% compared to untreated semen may be attributed to the valuable effects of ACME on sperm motility, viability, acrosomal integrity, antioxidant activities, and DFI reduction of the cryopreserved spermatozoa that would eventually enhance the fertilizing potentials of the cryopreserved spermatozoa (Allai *et al*., 2016; Mizera *et al*., 2019). These improvements in semen characteristics due to ACME supplantation minimized the huge gap in conception rate between natural and untreated artificial inseminations which might be an avenue to eliminate the damage of sperm might occur during cryopreservation.

Conclusion

Results demonstrated that supplementation of ACME (12.5 µl/mL) in semen extender had beneficial effects on cryopreserved bucks' spermatozoa in a dose-dependent manner. These effects were an improvement in sperm motility, viability, normal sperm, secondary abnormalities, acrosome integrity, total antioxidant capacity (TAC), and reduced DNA fragmentation index (DFI) at T48 h of the preservation period. The study also showed the beneficial effects of optimal level of ACME (12.5 µl/mL) when incorporated into cryopreservation medium on improvement of fertilization ability.

Conflict of interest

There are no disclosed conflicts of interest for any of the authors.

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