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Does *Lagenaria siceraria* seed oil-enriched extender regulate sperm quality, oxidant/antioxidant markers, and sperm mitochondrial enzymes in chilled diluted rabbit semen?

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Abstract

This study investigated the cryoprotective effects of *Lagenaria siceraria* seed oil (BG) on rabbit sperm quality during a 72-hour period of chilled storage at 4 °C. While a prevalent method for preserving rabbit semen, cryopreservation can elicit cold shock and other stressors, resulting in a decline in sperm quality. Thereafter, the researchers hypothesized that BG, potentially due to its antioxidant properties, could mitigate these detrimental effects. For the experiment, semen samples were diluted in extender and assigned to treatment groups receiving BG at concentrations of 0 (BG0), 100 (BG100), 200 (BG200), or 400 (BG400) µL/mL, followed by storage at 4 °C. Sperm quality parameters (motility, viability, membrane integrity, and morphology) were assessed at 24-, 48-, and 72-hour time points of storage. Results indicated a quadratic improvement in sperm motility, viability, and membrane integrity with the addition of 100 or 200 µL/mL of BG across all time points ($P < 0.01$). A quadratic relationship was observed between BG supplementation levels and the concentrations of GPx and SOD, indicating a dose-dependent increase. BG treatment at all concentrations led to elevated total antioxidant capacity (TAC) compared to the control, with peak TAC values at 200 and 400 µL/mL BG. Conversely, nitric oxide (NO) levels significantly decreased ($P < 0.001$) with increasing BG dosage. BG treatment significantly decreased malondialdehyde, H_2O_2, and protein carbonyl levels compared to the control ($P < 0.01$). Additionally, succinate dehydrogenase (SDH) and malate dehydrogenase (MDH) activities were significantly and quadratically improved at BG concentrations of 200 and 400 µL/mL relative to the 100 µL/mL concentration. In conclusion, supplementing rabbit semen extenders with BG significantly enhanced sperm quality during 72-hour chilled storage by attenuating oxidative stress, bolstering antioxidant capacity, and promoting mitochondrial enzyme activity. These findings suggest that BG is a promising additive for improving the preservation of chilled rabbit semen, potentially benefiting artificial insemination and rabbit breeding programs.

Keywords Chilled rabbit semen, Sperm quality, Mitochondria function, *Lagenaria siceraria* seed oil

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Introduction

Artificial insemination plays a vital role in implementing genetic improvement programs in rabbits. The use of chilled rabbit semen is a common practice in artificial insemination protocols within rabbit farms [1, 2]. While chilled rabbit semen can yield acceptable fertilization rates, ongoing research aims to optimize this method and alleviate the detrimental effects of cold stress [3]. Exposure to low temperatures can adversely impact sperm quality and their fertilizing capacity [4, 5]. This decline may be associated with reduced sperm motility, impaired functionality, and decreased viability [5], as well as mitochondrial dysfunction [6, 7]. An imbalance between antioxidant capacity and the production of reactive oxygen species (ROS) results in oxidative stress, which negatively affects cellular structure and function, ultimately leading to sperm dysfunction [3, 8–10]. The supplementation of chilled rabbit semen diluents with natural exogenous antioxidants represents a strategy to enable spermatozoa to counteract the negative effects of cold-stress-induced free radicals [3, 11]. Mitochondrial integrity is paramount for the production of high-quality, fertile spermatozoa, as it serves as the primary site of ATP synthesis via oxidative phosphorylation [12]. Succinate dehydrogenase (SDH), a crucial enzyme complex within this metabolic pathway, plays a vital role not only in ATP production but also in signaling mechanisms [13], pyrimidine and purine synthesis, epigenetic regulation, and fatty acid and amino acid metabolism in sperm [14]. Further, SDH deficiency disrupts oxidative phosphorylation, leading to impaired mitochondrial energy production and a consequent reduction in the ATP availability essential for sperm survival [15, 16]. This can result in spermatozoa of compromised quality and male infertility [16].

Endogenous and exogenous antioxidants are crucial for maintaining sperm quality and mitigating infertility [16]. They support mitochondrial function, enhance antioxidant capacity, and reduce oxidative stress, thereby contributing to improved reproductive health [7, 12, 17]. Phytochemical extracts derived from plants play a significant role in medicine. One such plant, bottle gourd (*Lagenaria siceraria*, BG), possesses a long history of traditional use. Belonging to the Cucurbitaceae family, it is extensively cultivated in tropical and subtropical regions [18].This plant is generally recognized as safe, exhibits nutritional value, and may offer potential therapeutic benefits.

Lagenaria siceraria seed oil exhibits a complex composition, including a variety of fatty acids such as palmitic acid and stearic acid [18], as well as the omega-9 fatty acid erucic acid, which contributes to its oxidative stability and, consequently, its nutritional and therapeutic potential [19]. Beyond fatty acids, the oil also contains a valuable array of vitamins, minerals, and amino acids. This diverse nutritional profile positions *Lagenaria siceraria* seed oil for versatile applications, encompassing culinary uses, pharmaceutical formulations, and dietary supplements [20]. The therapeutic properties of *Lagenaria siceraria* seed oil, including anti-inflammatory, antimicrobial, and antioxidant effects, are attributed to its high content of unsaturated fatty acids, phytosterols, polyphenols, vitamins, amino acids, and minerals [18, 21]. This complex nutrient profile acts synergistically to promote health [22].*Lagenaria siceraria* has been traditionally employed for wound healing [21], mitigating oxidative stress induced by carbon tetrachloride in mice [23]. While bottle gourd seed oil (BG) has exhibited various health benefits, its potential applications in animal reproduction, particularly concerning sperm preservation, remain largely unexplored. Therefore, this study investigates the effects of *Lagenaria siceraria* seed oil-enriched extenders on rabbit sperm quality during a 72-hour storage period at 4 °C. The investigation focuses on key indicators of oxidative homeostasis (oxidant/antioxidant markers), sperm quality parameters, and sperm energy metabolism (mitochondrial enzyme activity). This research aims to contribute valuable insights for optimizing short-term preservation strategies for rabbit semen, ultimately supporting advancements in rabbit breeding and production.

Materials and methods

Ethics and consent to participate

This study was reviewed and approved by the Institutional Animal Care and Use Committee (ZU-IACUC) of Zagazig university under Approval Number; IACUC/2/F/25/2023 in compliance with the ARRIVE guidelines. This also in accordance with the U.K. Animals (Scientific Procedures) Act, 1986, and associated guidelines, EU Directive 2010/63/EU for animal experiments, the National Research Council's Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978). The study was carried out at the Farm of Department of Animal Production, Faculty of Agriculture, Zagazig University, Egypt. We confirmed the owners' greed in using these animals as sperm donors.

GC/Mass of bottle gourd (*Lagenaria siceraria*) seed oil (BG)

Bottle gourd (*Lagenaria siceraria*) seed oil (BG) was obtained from AB Company (Mansoura, Egypt). The chemical composition of the BG was determined using gas chromatography-triple quadrupole mass spectrometry (GC-TSQ MS) on a Thermo Scientific system (Austin, TX, USA) equipped with a TG-5MS capillary column (30 m × 0.25 mm × 0.25 µm film thickness) [24]. The column oven temperature was programmed to increase from 60 °C to 250 °C at a rate of 5 °C/min, held at 250 °C for 2 min, and then increased to 300 °C at 30 °C/min. The

injector temperature was maintained at 270 °C. Helium was used as the carrier gas at a constant flow rate of 1 mL/min. Following a 4-minute solvent delay, 1 µL aliquots of diluted sample were automatically injected in split mode using an AS3000 autosampler coupled to the GC. Electron ionization (EI) mass spectra were acquired at 70 eV over the *m/z* range 50–650 in full scan mode. The ion source and transfer line temperatures were set at 200 °C and 280 °C, respectively. Component identification was performed by comparing the acquired mass spectra with the WILEY 09 and NIST14 mass spectral databases.

Animal and semen collection

A total twelve of proven fertile rabbit bucks (New Zealand breed) were sourced by the Rabbit Farm, Faculty of Agriculture, Zagazig University, utilized in this study. The age of bucks ranged from 12 to 14 months, with average body weight 3.10 ± 0.15 kg. These bucks were individually housed in galvanized wire cages (70 × 50 × 35 cm) under standard photoperiod conditions (12–14 h of light per day) within a naturally ventilated room maintained at a temperature range of 16–28 °C. The animals were provided with a commercially formulated rabbit diet (16.3% crude protein, 13.2% crude fiber, and 2600 kcal/kg digestible energy) and had *ad libitum* access to clean water.

Following appropriate training with teaser does, the bucks were employed for semen collection. For a two-week period, the bucks underwent training for semen collection using the artificial vagina (AV) method. Semen was collected from the rabbit bucks using an AV (maintained at 40–42 °C) mounted on a teaser doe. Immediately following collection, the gel plug was removed. The collected semen was then placed in a water bath at 37 °C for subsequent evaluation. All ejaculates were assessed for motility, concentration, and morphology using standard techniques [3, 11]. Only ejaculates meeting the following selection criteria were used for further experimentation: ≥70% progressive motility, a volume of 0.2 mL, a sperm concentration of $\geq 200 \times 10^6$ spermatozoa/mL, and ≥ 80% morphologically normal spermatozoa [10].

Extender Preparation and experimental groups

This experiment utilized a total of 50 qualifying ejaculates, with each buck contributing a minimum of five samples. All ejaculated semen samples underwent initial evaluation based on established standard criteria. Only samples meeting these criteria were included in this study. Following initial assessment, the ejaculates were pooled and diluted with a tris-citric acid-glucose (TCG) based extender to a final sperm concentration of 50×10^6 spermatozoa mL^{-1} [9]. The TCG extender was prepared with the following constituents: citric acid (79.76 mM), Tris (250.04 mM), streptomycin (75.00 IU), glucose (69.38 mM), and penicillin-G (166.20 IU) [25]. The osmolarity and pH of the extender were meticulously adjusted to 299 mOsm kg^{-1} and 7.14, respectively.

The pooled diluted semen was then divided into four treatment groups. The first aliquot was further diluted 1:10 in the TCG based extender and served as the control group (BG0). The 2nd, 3rd, and 4th treatment groups consisted of TCG extenders supplemented with *Lagenaria siceraria* seed oil (BG) at concentrations of 100 (BG100), 200 (BG200), and 400 (BG400) µL mL^{-1}, respectively. The extended semen treatments were stored at 4 °C for a duration of 72 h. Sperm motility (%), viability (%), abnormality (%), and membrane function integrity (MFI, %) were assessed at 24, 48, and 72 h of storage at 4 °C. Mitochondrial enzyme activity (in sperm cells), oxidative stress markers, and antioxidant markers were assessed in semen at the end of the 72-hour storage period.

Sperm motility assessment

Sperm motility was assessed subjectively using a phase-contrast microscope (400×, Olympus BX20, Tokyo, Japan) equipped with a heated stage maintained at 37 °C. A 10 µL aliquot of semen was placed on a pre-warmed (37 °C) microscope slide, covered with a coverslip, and immediately examined under the microscope.

Evaluation of sperm viability and abnormality

Thin, uniform smears were prepared by mixing a 10 µL aliquot of semen with 1% nigrosin-eosin stain (Sigma-Aldrich, St. Louis, MO, USA) on a pre-warmed glass slide. Following air-drying, the smears were examined using phase-contrast microscopy at 1000× under oil immersion to differentiate live spermatozoa (unstained heads) from dead spermatozoa (stained or partially stained heads) [26]. Using the same microscope, sperm morphology was also assessed. The percentages of sperm cells exhibiting abnormal tail morphology (coiled, broken, terminally coiled, or double tails) and abnormal head morphology (microcephalic, pear-shaped, round short, loose, or double heads) were recorded, following established methods.

Assessment of sperm membrane integrity

To assess sperm plasma membrane integrity, a 25 µL aliquot of semen was incubated in 475 µL of hypo-osmotic solution (100 mOsm kg^{-1}) at 35 °C for 15 min [17]. Following this osmotic challenge, a wet mount was prepared, and approximately 200 spermatozoa were evaluated at 400× using a bright-field microscope (Olympus Corporation, Hachioji, Tokyo, Japan). Sperm cells were categorized based on tail curling, which served as an indicator of plasma membrane integrity or damage. The

percentage of spermatozoa with intact membranes was then calculated.

Assessment of antioxidant status

Following 72 h of storage, treated semen samples were centrifuged at 6000 rpm for 10 min at 4 °C. The supernatant was then collected, and the total antioxidant capacity (TAC), superoxide dismutase (SOD), and glutathione peroxidase (GPx) activities were determined using commercially available kits (Bio diagnostic, Giza, Egypt) following the manufacturer's protocols. Absorbance was measured at 505 nm using a spectrophotometer. Malondialdehyde (MDA, MBS739495), protein carbonyl (PC, MBS1601647), nitric oxide (NO, MBS2540419), and hydrogen peroxide (H_2O_2, MBS822356) levels in the extender were quantified using commercially available kits (MyBioSource, San Diego, USA). All assays were performed according to the manufacturers' instructions. All assays were performed as described in the respective kit protocols, and measurements were conducted using a Spectro UV-Vis Auto UV-2602 spectrophotometer (USA).

Assessment of mitochondria enzymes in sperm

Succinate dehydrogenase (SDH) and malate dehydrogenase (MDH) activities were determined using ELISA kits provided by Nanjing Jiancheng Bioengineering Institute (Jiangsu, China), following the methods described in references [7, 27]. After 72 h of storage at 4 °C, treated semen samples were centrifuged at 6000 rpm for 10 min at 4 °C. Sperm specimens were then lysed ultrasonically (20 kHz, 750 W, 40% power, 5 cycles of 3 s on and 5 s off) and subsequently centrifuged at 10,000 rpm for 10 min at 4 °C. The resulting supernatants were added to a 96-well plate for the analysis of SDH and MDH activities using a microplate reader at wavelengths of 600 nm and 340 nm, respectively. MDH and SDH activities were expressed as milliunits per milligram of protein (mU/mg protein).

Statistical analysis

Data were entered into Microsoft Excel 365 and subsequently analyzed using IBM SPSS Statistics version 25. Orthogonal contrasts (linear and quadratic effects within one-way ANOVA) were performed on all data to assess treatment-related trends. Data visualization was performed using GraphPad Prism version 9 to generate violin plots. Statistical significance was defined as a p-value of less than 0.05 ($p < 0.05$). Results are presented as the mean ± standard error of the mean (SEM).

Results

GC/mass for *Lagenaria siceraria* seed oil

Gas chromatography-mass spectrometry (GC-MS) analysis of BG (as presented in Fig. 1; Table 1) revealed the presence of several compounds. The major constituents identified in BG oil included Octasiloxane, silicic acid, Hexasiloxane, Pentasiloxane, Benzofuran, 3-methyl-, Androst-9(11)-en-17-one, Benzoic acid, 1,2-Indandione, 3,3-dimethyl-, and 3-Phenyl-2-propyn-1-one.

Impacts on semen quality at 24 h

The total progressive motility, abnormality, viability, and membrane functionality after 24 h of preservation at 4 °C (Fig. 2). Adding BG at 100–200 µg/mL to the rabbit extender significantly improved total progressive motility compared to the BG400 and BG0 groups ($P < 0.001$; Fig. 2A). Further, BG400 had higher total progressive motility compared to the BG0 group ($P < 0.001$). Only a quadratic effect was observed in sperm abnormality after BG-enriched in rabbit extender, where BG100 and BG200 exhibited the lowest values of sperm abnormality (Fig. 2B, $P < 0.001$). Sperm viability was quadratically higher in chilled semen supplemented with 100–200 µg/mL of BG (Fig. 2C), while the BG400 and BG0 groups had similar results for sperm viability ($P > 0.05$). All BG groups showed greater MFI than the control (except for BG400, $P < 0.003$). It's interesting to note that BG

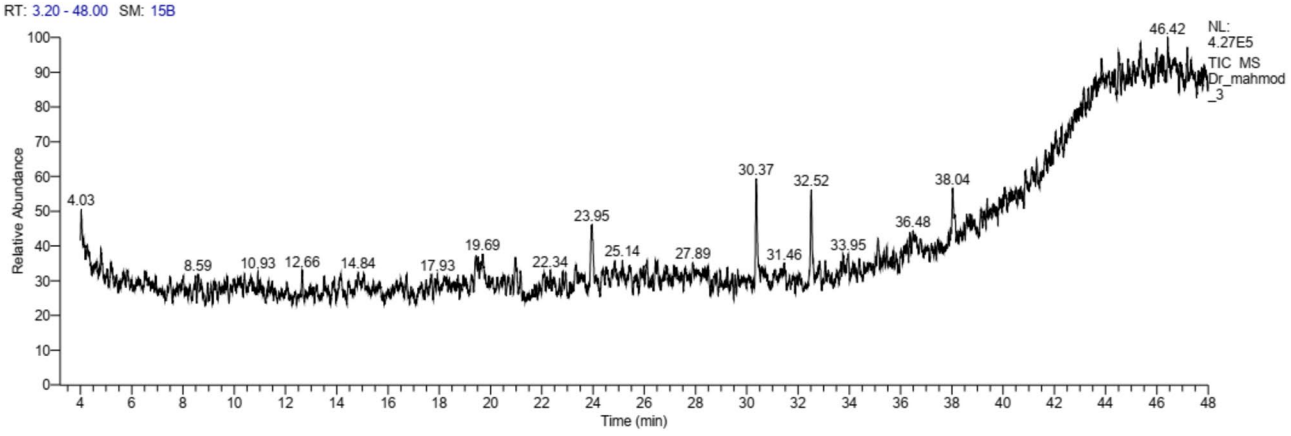

Fig. 1 The Gc/mass analysis of *Lagenaria siceraria* seed oil used in this experiment

Table 1 The main compounds identified in Lagenaria siceraria seed oil and its potential biological activities of these compounds based on the literature

RT	Compound	Area	Molecular Formula	Molecular Weigh	Biological activity	Reference
20.96	Benzofuran, 3-methyl-	1.5	C_9H_8O	132	Antioxidant and antimicrobial	[28]
20.96	1,2-Indandione, 3,3-dimethyl	1.5	$C_{11}H_{10}O_2$	174	Antioxidant, anticancer and anti-coagulant activities	[29]
23.95	Octasiloxane,	2.51	$C1_6H_{50}O_7Si_8$	578	Antioxidant and antimicrobial	[28]
23.95	Benzoic acid	2.51	$C_{14}H_{24}O_3Si_2$	296	Regulate sperm function	[30]
32.51	Androst-9(11)-en-17-one, 3-[(trimethylsilyl)oxy]-, O-methyloxime, (3à,5à)-	2.91	$C_{23}H_{39}NO_2Si$	389	Antioxidant effects	[31]
32.51	4 H-1-Benzopyran-4-one, 2-(2,6-dimethoxyphenyl) -5,6-dimethoxy	2.91	$C_{19}H_{18}O_6$	342	Antioxidant effects	
44.87	Prazepam	0.54	$C_{19}H_{17}ClN_2O$	324	Relieve anxiety and nervousness.	[22]
47.18	Pentasiloxane	0.82	$C_{16}H_{50}O_7Si_8$	578	Emollient and antioxidant agent	[32]
48.40	Cyclotetrasiloxane	0.90	$C_{14}H_{44}O_6Si_7$	504	Cold resistance agent, anti-aging and antioxidant effects	[33]
48.47	1-Monolinoleoylglycerol trimethylsilyl ethe	1.11	$C_{27}H_{54}O_4Si_2$	498	Anti-microbial effects	[34]
38.03	6-Amino-5-cyano-4-(5-cy ano-2,4-dimethyl-1 H-pyrr ol-3-yl)-2-methyl-4 H-pyra n-3-carboxylic acid ethyl ester	1.93	$C_{17}H_{18}N_4O_3$	326	Anti-fungal effects	[35]
38.03	1,4-Cyclohexadiene, 1,3,6-tris(trimethylsilyl)-	1.93	$C_{15}H_{32}Si_3$	296	Antioxidant effects	[36]
38.03	Hexasiloxane	1.31	$C_{12}H_{38}O_5Si_6$	430	Antimicrobial and Antioxidant effects	[28]
50.70	Octasiloxane	10.69	$C_{16}H_{50}O_7Si_8$	578	Antioxidant effects	[18]
48.61	Silicic acid	1.51	$C_{12}H_{38}O_5Si_6$	430	Antioxidant activity	[28]

at 100–200 µg/mL significantly promoted the MFI (Fig. 2D), sperm viability, and TPM than other groups in a quadratic effect, while BG at a high level (400 µg/mL) produced nearly similar results compared to the control group.

Effects on semen quality at 48 h

Preservation of rabbit semen at 4 °C for 48 h (Fig. 3) significantly enhanced total progressive motility (Fig. 3A), viability (Fig. 3C), and plasma membrane functionality (MFI, Fig. 3D) in semen supplemented with BG at concentrations of 100 or 200 µL/mL (BG100 and BG200 groups), demonstrating a quadratic dose-response. Conversely, sperm abnormalities (Fig. 3B) exhibited a quadratic decrease in chilled rabbit semen treated with these concentrations ($P<0.01$). These results indicate an optimal concentration range of 100–200 µL/mL of BG for enhancing sperm quality during chilled preservation of rabbit semen.

Effects on semen quality at 72 h

Total progressive motility showed a quadratic increase following the addition of 100 or 200 µL/mL of BG to the chilled rabbit semen extender after 72 h of preservation (Fig. 4A). The BG400 and control groups exhibited comparable total progressive motility ($P<0.01$). Furthermore, sperm abnormalities were significantly reduced in the BG100 group compared to the control (BG0) group (Fig. 4B, $P<0.05$), whereas higher concentrations (200 or 400 µL/mL) showed no significant difference compared to the control ($P>0.05$). Viability (Fig. 4C) and MFI (Fig. 4D) were lowest in the BG400 and control groups, respectively, compared to the other groups (quadratic effect; $P<0.001$). Consistently, the highest sperm viability and MFI were observed in the BG100 and BG200 groups compared to the other groups ($P<0.001$; quadratic effect).

Antioxidant capability

Supplementation with BG significantly increased GPx (Fig. 5A) and SOD (Fig. 5B) levels in a quadratic dose-dependent manner compared to the control group ($P<0.01$). All BG-treated groups exhibited higher TAC levels than the control (Fig. 5C), with maximum values observed in the BG200 and BG400 groups ($P<0.05$). Conversely, TAC levels were significantly lower in chilled rabbits' semen preserved with 100 µL/mL of BG compared to those preserved with 200 or 400 µL/mL of BG ($P<0.01$).

Oxidative stress markers

The addition of BG to chilled rabbit semen resulted in a significant dose-dependent decrease in nitric oxide (NO) levels (Fig. 6D, $P<0.001$). Malondialdehyde (MDA) (Fig. 6A), protein carbonyl (PC) (Fig. 6B), and hydrogen peroxide (H_2O_2) (Fig. 6C) levels were significantly decreased in BG-treated groups compared to the control group ($P<0.01$). For PC, the greatest reduction was

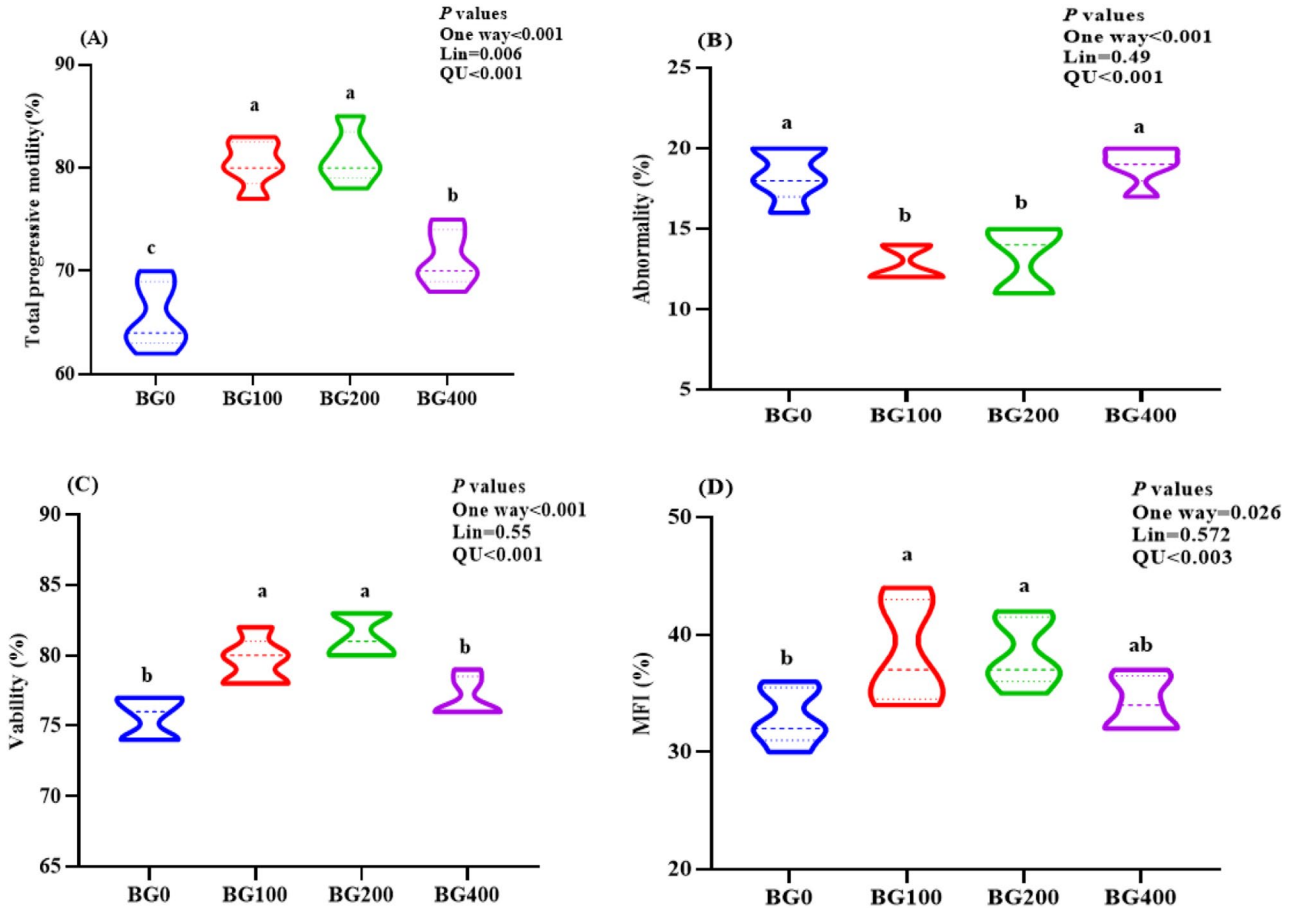

Fig. 2 Effects of varying concentrations of bottle gourd (*Lagenaria siceraria*) seed oil (0, 100, 200, and 400 µL/mL of extender), denoted as BG0, BG100, BG200, and BG400, respectively, on chilled rabbit semen stored at 4 °C for 24 h. The parameters evaluated were total progressive motility (Fi. 2 A), abnormalities (Fig. 2B), viability (Fig. 2C), and plasma membrane integrity (Fig. 2D). Within each parameter, bars with different superscript letters (**a**, **b**, **c**) indicate statistically significant differences between treatment groups ($P < 0.05$)

observed in the BG100 group (quadratic effect), while the greatest reduction in H_2O_2 was observed in the BG200 and BG400 groups (quadratic effect, $P < 0.01$). Overall, supplementing chilled rabbit semen extenders with 200 or 400 µL/mL of BG quadratically decreased the levels of these oxidative stress markers ($P < 0.001$).

Mitochondria enzymes

Mitochondrial enzyme activities, specifically malate dehydrogenase (MDH) and succinate dehydrogenase (SDH), were significantly enhanced by BG supplementation in chilled rabbit semen extenders (Fig. 7). The addition of 200 or 400 µL/mL of BG significantly increased SDH (Fig. 7A) and MDH (Fig. 7B) activities in a quadratic manner compared to the BG100 group ($P < 0.001$). Collectively, supplementing with 200 or 400 µL/mL of BG provided greater support for mitochondrial function compared to the 100 µL/mL group.

Discussion

This experiment demonstrates that supplementing chilled rabbit semen extender with BG significantly improves total progressive motility, sperm viability, and plasma membrane integrity, while reducing sperm abnormalities after 24, 48, and 72 h of storage at 4 °C. This improvement accompanied with a significant increase in antioxidant enzyme activities and total antioxidant capacity, along with enhanced mitochondrial enzyme activities and a reduction in oxidative stress markers in chilled rabbit semen after 72 h of storage at 4 °C.

Mitochondria are critical organelles in spermatozoa, functioning as both a primary source of energy required for motility and a potential source of oxidative stress [7, 12]. Maintaining a delicate balance between minimizing oxidative stress (OS) and maximizing energy production is therefore crucial for optimizing sperm motility and ultimately improving fertilization rates [16]. Cooled rabbit semen is a widely employed method for AI, often yielding higher fertility rates compared to cryopreserved semen [37]. Nevertheless, ongoing research efforts are

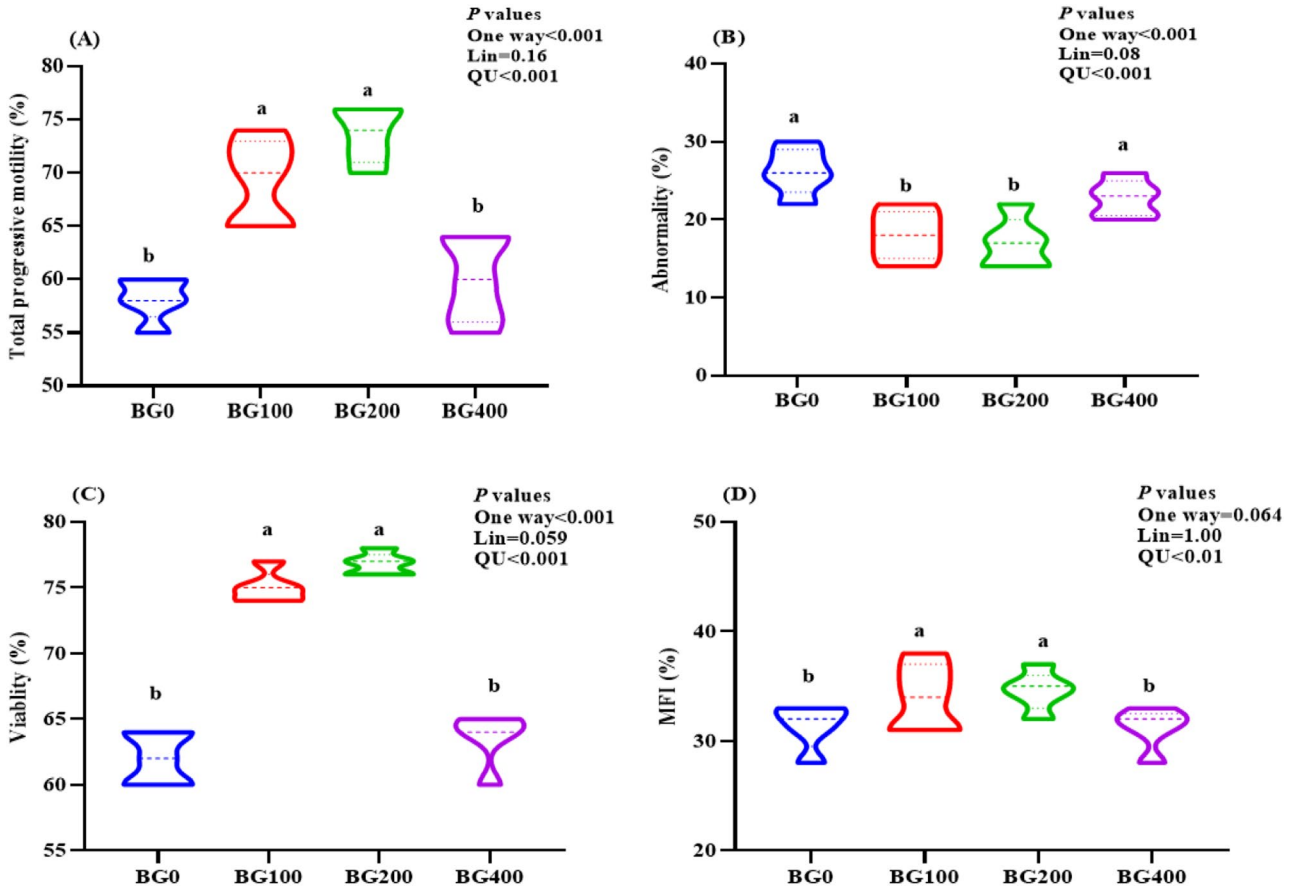

Fig. 3 Effects of varying concentrations of bottle gourd (*Lagenaria siceraria*) seed oil (0, 100, 200, and 400 µL/mL of extender), denoted as BG0, BG100, BG200, and BG400, respectively, on chilled rabbit semen stored at 4 °C for 48 h. The parameters were evaluated total progressive motility (Fig. 3A), abnormalities (Fig. 3B), viability (Fig. 3C), and plasma membrane integrity (Fig. 3D). Within each parameter, bars with different superscript letters ($^{a, b}$) indicate statistically significant differences between treatment groups ($P < 0.05$)

focused on optimizing the cooling process and mitigating oxidative stress during 4 °C preservation to extend semen viability and ultimately enhance fertility outcomes. The addition of 200 or 400 µL/mL of BG to rabbit sperm stored at 4 °C enhanced sperm motility over an extended preservation period of 72 h. These beneficial effects could be attributed to the bioactive properties of BG, which appear to enhance sperm resilience to cold stress. Bottle gourd seed oil is a rich source of fatty acids, phenolic compounds, and micro- and macronutrients [18].

Furthermore, GC-MS analysis detected octasiloxane in BG (Table 1), a compound reported to exhibit various robust biological activities [18, 38]. Consistent with our findings, a previous study [8] reported that supplementing chilled semen (preserved for 72 h at 5 °C) extender with taurine and glutathione significantly improved sperm motility and viability in rabbits. Similarly, lycopene has been shown to provide significant protection during rabbit semen storage at 5 °C for 48 h [11]. In rams, mitochonic acid 5 has been shown to notably improve sperm quality (motility, membrane integrity, and acrosomal integrity) during 4 °C storage by reducing oxidative stress and promoting ATP production and mitochondrial membrane potential [6].

The improvements observed with BG in our findings, including sperm motility, viability, and plasma membrane integrity, likely result from the antioxidant activity imparted to the chilled extender. This is evidenced by increased GPx, SOD, and TAC levels, which may mitigate cold-induced OS and consequently enhance sperm resilience. BG contains several active compounds with robust antioxidant capacity, such as Benzofuran and octasiloxane [28], and androst-9(11)-en-17-one [31]. Moreover, benzoic acid was detected in BG, and a positive association between GABA and benzoic acid has been observed in bull spermatozoa [30]. In vitro studies have shown that benzoic acid can increase glutamate efflux, a precursor in the synthesis of GABA via L-glutamate decarboxylation. Notably, benzoic acid abundance was higher in high-fertility bull sperm compared to low-fertility sires. Furthermore, a recent study in rats also found a positive correlation between benzoic acid abundance and sperm

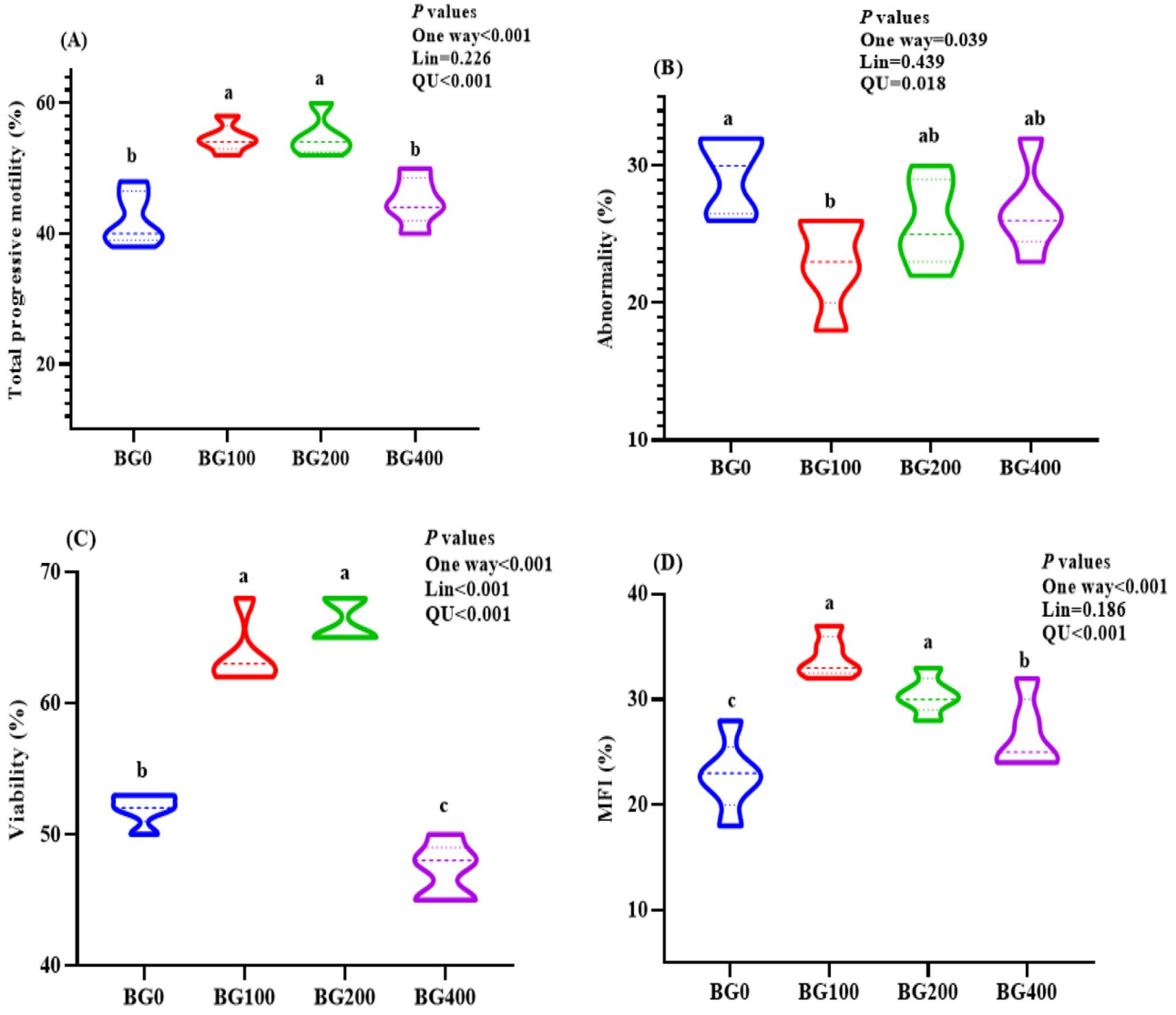

Fig. 4 Effects of varying concentrations of bottle gourd (*Lagenaria siceraria*) seed oil (0, 100, 200, and 400 µL/mL of extender), denoted as BG0, BG100, BG200, and BG400, respectively, on chilled rabbit semen stored at 4 °C for 72 h. The parameters assessed were total progressive motility (Fig. 4A), abnormalities (Fig. 4B), viability (Fig. 4C), and plasma membrane integrity (Fig. 4D). Within each parameter, bars with different superscript letters ($^{a, b, c}$) indicate statistically significant differences between treatment groups ($P < 0.05$)

count, suggesting a potential role for this compound in male fertility [39].

Cold shock, a phenomenon that impairs sperm membrane reorganization during capacitation by reducing permeability and damaging acrosomal membranes [6, 17], likely contributed to the decline in plasma membrane and acrosome integrity, and consequently sperm motility, observed in the control group after 72 h at 4 °C. This observation aligns with the findings of a study [25], demonstrating that Sericin supplementation improved the plasma membrane integrity in chilled rabbit semen. The protective effect of BG on sperm membranes may be attributed to its high content of fatty acids, which could potentially replace those damaged by cold stress.

Cold stress compromised sperm's antioxidant defenses by promoting oxidative stress. This imbalance arose from increased production of ROS coupled with reduced activity of antioxidant enzymes [37]. Incorporating vegetable oils into semen extenders may enhance sperm resistance to cold stress during preservation [40], potentially due to the specific physical and chemical properties of BG. In this study, we observed significant improvements in antioxidant indices (SOD, GPx and TAC) and reductions in oxidative stress markers, including MDA, PC, H_2O_2, and nitric oxide, in chilled rabbit semen, particularly at BG concentrations of 200 or 400 µL/mL. The low peroxide value of this oil indicates a low degree of oxidation, suggesting greater resistance to oxidative degradation [21].

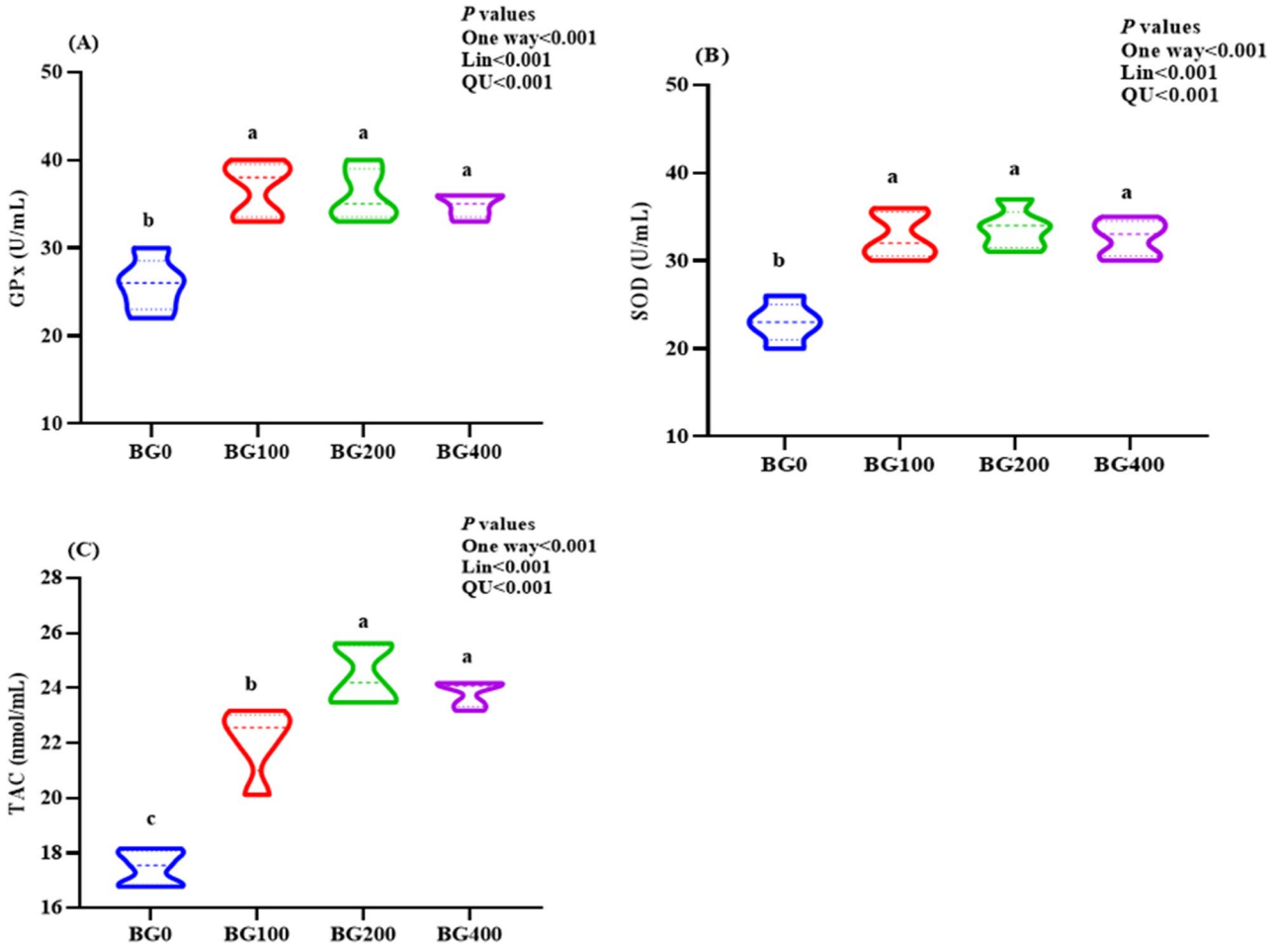

Fig. 5 Effects of varying concentrations of bottle gourd (*Lagenaria siceraria*) seed oil (0, 100, 200, and 400 µL/mL of extender), denoted as BG0, BG100, BG200, and BG400, respectively, on the antioxidant status of chilled rabbit semen stored at 4 °C for 72 h. The parameters evaluated were GPx (Fig. 5A), SOD (Fig. 5B) and TAC (Fig. 5C). Within each parameter, bars with different superscript letters (**a**, **b**, **c**) indicate statistically significant differences between treatment groups ($P < 0.05$)

The chemical composition of BG, detailed in Table 1, suggests its protective and robust antioxidant effects due to its diverse constituents. For instance, hexasiloxane, identified in BG, exhibits anticancer and antimicrobial activities, potentially attributed to its ability to scavenge oxidative stress [22].

Mitochondria, the powerhouses of cells, play a vital role in sperm function, and their health is closely linked to fertility outcomes in rabbits [3].This study demonstrates that the addition of BG significantly improved the activity of mitochondrial enzymes such as MDH and SDH in sperm, suggesting prolonged maintenance of sperm mitochondrial structure. This improvement may be attributed to the benzoic acid content of BG, which can regulate the sperm physiology by regulating energy production in mitochondria [41]. Benzoic acid, along with related compounds like ketoisocaproic acid and choline, may play a role in sperm metabolism. These compounds are involved in anabolic processes, which are essential for energy production and the synthesis of crucial components like phospholipids in sperm. These processes are vital for sperm motility, viability, and overall function. Consistent with these findings, a study [17] found that supplementing with L-carnitine significantly improved mitochondrial function in cryopreserved rabbit semen.

Elevated MDH and SDH activities, indicative of a highly active TCA cycle, suggest robust ATP generation in sperm. Supplementing boar semen freezing media with oleic or palmitic acid has been shown to significantly increase MDH and SDH activities [7], an effect accompanied by a significant improvement in ATP synthesis in the same study. In our study, we observed higher MDH and SDH levels in chilled rabbit semen supplemented with BG, along with a significant reduction in MDA, H_2o_2, PC, and nitric oxide. Several studies have reported similar results in ram semen using β-Nicotinamide mononucleotide [42] and mitochonic acid 5 [6].This suggests that the active compounds in BG may promote

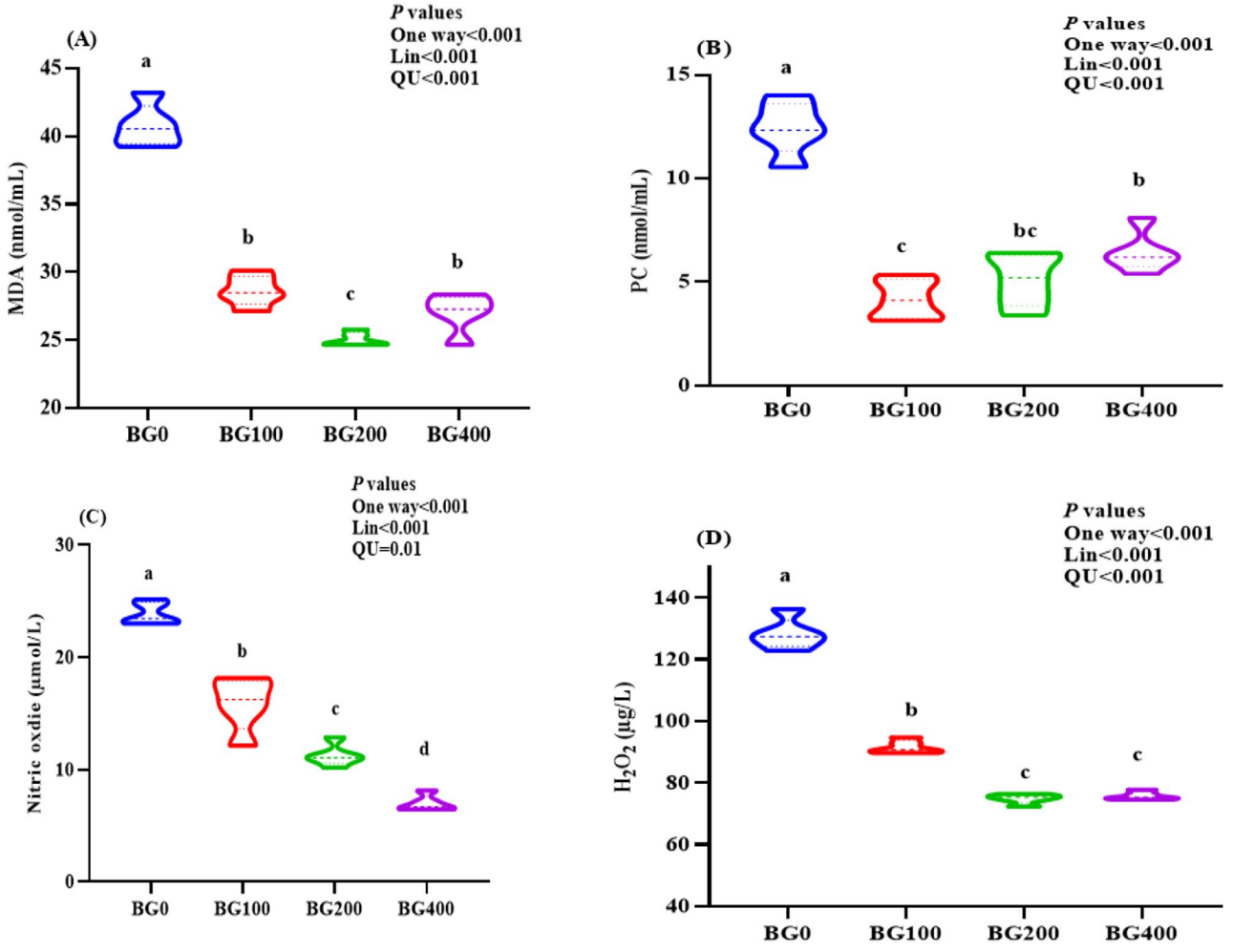

Fig. 6 Effects of varying concentrations of bottle gourd (*Lagenaria siceraria*) seed oil (0, 100, 200, and 400 µL/mL of extender), denoted as BG0, BG100, BG200, and BG400, respectively, on oxidative stress markers of chilled rabbit semen stored at 4 °C for 72 h. The parameters assessed were MDA, (Fig. 6**A**), PG (Fig. 6**B**), nitric oxide (Fig. 6**C**) and H_2O_2 (Fig. 6**D**). Within each parameter, bars with different superscript letters (**a**, **b**, **c**) indicate statistically significant differences between treatment groups ($P < 0.05$)

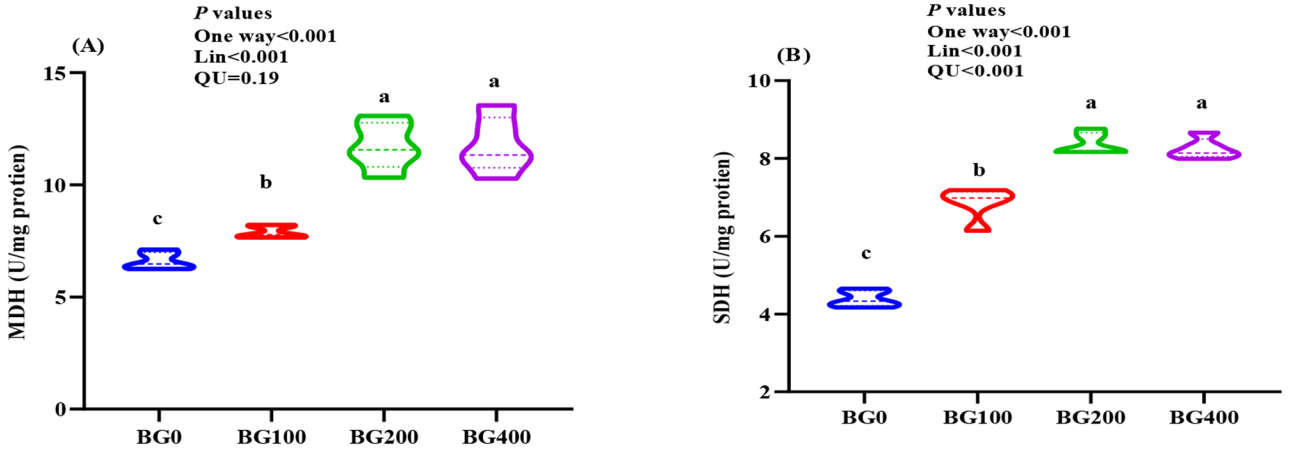

Fig. 7 Effects of varying concentrations of bottle gourd (*Lagenaria siceraria*) seed oil (0, 100, 200, and 400 µL/mL of extender), denoted as BG0, BG100, BG200, and BG400, respectively, on mitochondrial enzymes including MDH (Fig. 7A) and SDH (Fig. 7B) of chilled rabbit semen stored at 4 °C for 72 h. Within each parameter, bars with different superscript letters (**a**, **b**, **c**) indicate statistically significant differences between treatment groups ($P < 0.05$)

beta-oxidation, leading to increased levels of mitochondrial enzymes responsible for energy production, while simultaneously scavenging oxidative stress induced by the cooling process. Due to the limited resources for this study, we assessed lipid peroxidation, antioxidant enzyme activities, and mitochondrial enzyme activities solely at the experimental endpoint. Further experiments should be conducted, particularly employing metabolomics and proteomics assays, to enhance our understanding of the detrimental effects of short-term semen storage. This knowledge is crucial for developing novel protocols aimed at improving the sustainability and application of artificial insemination (AI) using chilled semen.

Conclusion

Low temperatures compromise rabbit sperm quality by impairing mitochondrial function, increasing oxidative stress, and diminishing antioxidant capacity. Supplementing the semen extender with 200 or 400 µL/mL of BG significantly improved sperm motility, plasma membrane integrity, mitochondrial enzyme activity, and antioxidant capacity, while significantly reducing oxidative stress markers (MDA, PC, H_2O_2, and nitric oxide). Overall, our findings suggest that BG can be used effectively to maintain rabbit sperm quality during storage at 4 °C. Further investigations are warranted to elucidate the optimal dose of this oil, particularly concerning its effects on underlying molecular pathways.

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Author contributions
Sameh Abdelnour, Asmaa M. Sheiha and Yasser H. A. Saber, performed experiments, designed the experiment, analysis the data, writing, management of animals, and laboratory analysis and reviewing the manuscript. Sameh A. Abdelnour, Ramya Ahmad Sindi, Mohammed A. Alfattah, writing, editing and revising the manuscript. Ehab El-Haroun: Revision and English Editing: All authors confirmed that this version is accepted for publication.

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Data availability
The data supporting the findings of this study will be made available upon reasonable request to the corresponding author.

Declarations

Ethics and consent to participate
This study was reviewed and approved by the Institutional Animal Care and Use Committee (ZU-IACUC) of Zagazig university under Approval Number; IACUC/2/F/25/2023 in compliance with the ARRIVE guidelines. This also in accordance with the U.K. Animals (Scientific Procedures) Act, 1986, and associated guidelines, EU Directive 2010/63/EU for animal experiments, the National Research Council's Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978). The study was carried out at the Farm of Department of Animal Production, Faculty of Agriculture, Zagazig University, Egypt. We confirmed the owners' greed in using these animals as sperm donors.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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