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The Post Thaw Quality, Antioxidant Activity, and In vivo Fertility of Frozen-Thawed Buffalo Bull Semen Frozen-Stored in the Presence of Different Concentrations of Freeze-Dried Clove Powder

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Abstract

Artificial insemination (AI) is a critical reproductive technology in the buffalo industry, offering numerous benefits for improving genetic quality by selective breeding and faster genetic progress. The plasma membrane of buffalo bull spermatozoa is rich in polyunsaturated fatty acids and is susceptible to lipid peroxidation during freezing and thawing processes. The addition of herbal extracts to buffalo bull semen extenders has been explored as a potential method to improve semen quality and fertility. The most potent antioxidants in clove bud extract are phenolic compounds, particularly eugenol, which is the major active component. This study aims to minimize the damaging effect of oxidative stress during freezing-thawing procedures, in turn improving the post-thaw quality and in vivo fertility of buffalo bull spermatozoa. Ejaculates were collected from seven healthy Egyptian buffalo bull by artificial vagina twice weekly. Good-quality ejaculates were pooled and dispensed into 4 Aliquots; Each aliquot was diluted with Tris-egg yolk citric acid diluent containing different concentrations of clove buds (Syzygium aromaticum) extract as follows: 1) 0 µg/mL as a control (CTRL), 2) 5 µg/mL (CLOV5), 3) 10 µg/mL (CLOV10), and 4) 15 µg/mL (CLOV15). Diluted semen samples were equilibrated at 4 °C for 4 hours, loaded into French mini straws, sealed, frozen stored in liquid nitrogen. Frozen straws were thawed and examined for sperm characteristics; also, enzymatic antioxidants (SOD, CAT, GPX) as well as the lipid peroxidation marker (MDA) were determined. A total of 84 (21 females/group) mature and healthy buffalo-cows were selected, exposed to estrus synchronization, and inseminated by the prepared frozen-thawed straws to calculate their in vivo fertility rates. The results revealed that CLOV15 and CLOV10 have higher ($P < 0.05$) proportions of all post-thaw sperm characteristics, and also, CLOV15 yielded the greatest pregnancy rate, 66.66%, in comparison with CLOV5 (33.33%) and CLOV10 (11.11%). Niether SOD nor CAT affected by the clove addition whereas the GPX activity improved in CLOV10 compared with other group. In the same way, MDA content was lower in the higher concentration of clove compared with the low concentration and control groups. In conclusion, enriching cryopreservation extender with 15µg/ml clove buds powder improved almost all sperm characteristics. On the other side, CLOV10 and CLOV5 improved the enzymatic antioxidant, and only CLOV15 improved the in vivo fertility rate of frozen-thawed buffalo bull semen.

Keywords: Buffalo bulls, Sperm characteristics, Clove, SOD, Freezing, Thawing, In vivo fertility.

Introduction

The Egyptian buffalo is a cornerstone of Egypt's agricultural sector, contributing significantly to dairy and meat production, rural livelihoods, and cultural traditions. With proper investment in breeding, nutrition, and veterinary care, the Egyptian buffalo industry has the potential further to enhance food

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security and economic development in the country [1, 2]. The Egyptian buffalo industry faces challenges such as low genetic potential, decreasing reproductive performance, inadequate veterinary services, and limited access to modern farming technologies. There is significant potential for improving productivity through better breeding programs, improved nutrition, and enhanced veterinary care [3].

Artificial insemination (AI) is a critical reproductive technology in the buffalo industry, offering numerous benefits for improving genetic quality by selective breeding and faster genetic improvement. [4], productivity through higher milk yield and better meat production [5] and overcoming reproductive challenges, as buffaloes often exhibit silent or weak estrus signs, making natural mating less effective than AI. AI, combined with proper estrus synchronization protocols, improves reproductive efficiency. In the same way, fixed timed AI (FTAI) following estrus/ and or ovulation synchronization could overcome the seasonal breeding patterns of buffaloes, enabling year-round breeding and calving [6].

However, frozen semen is a valuable tool for genetic improvement and reproductive management in buffaloes. It has many drawbacks, such as reduced sperm quality, low fertility rates, and high cost compared with fresh semen. Moreover, buffalo bull spermatozoa are more prone to cryodamage due to their plasma membrane having a higher phospholipid content compared to bull sperm [7] and Buffalo sperm have lower intrinsic antioxidant capacity compared to bull sperm, making them more susceptible to oxidative stress during cryopreservation [8]. The plasma membrane of buffalo bull spermatozoa is rich in polyunsaturated fatty acids and is susceptible to lipid peroxidation during freezing and thawing processes [9]. The production of malondialdehyde (MDA) in semen is directly related to the amount of polyunsaturated fatty acids in the sperm plasma membrane [10]. Thus, MDA serves as a biomarker of oxidative stress and is implicated in male infertility [11].

Addition of herbal extracts to buffalo bull semen extender has been explored as a potential method to improve the post-thaw semen quality, and fertility. Herbal extracts are rich in antioxidants, which minimize oxidative stress and thus improve sperm plasma integrity during storage. Many herbal extracts, such as those extracted from clove buds [12, 13], green tea [14] and ginseng [15] can protect sperm from oxidative damage caused by reactive oxygen species (ROS). The most potent antioxidant extracted from the clove bud are phenolic compounds, particularly eugenol [12], the major active component. Other contributing compounds include eugenol acetate [16], gallic acids [17], and phenolic acids [18]. Eugenol may reduce ROS (peroxide and nascent oxygen) levels, decreasing MDA production and DNA damage in sperm cells [12]. Clove bud has been employed as an additive to different types of cryopreservation extenders of ram semen [13]. This study aimed to minimize the inevitable oxidative stress of freezing and thawing procedures by using different concentrations of clove bud extract in an attempt to improve the post-thaw quality and in vivo fertility of frozen-thawed buffalo bull semen.

Material and Methods

Unless otherwise stated, the chemicals were high purity and obtained from Sigma-Aldrich Chemical Co., USA.

Experimental animals

Seven healthy, mature (4 – 7 years old) Egyptian buffalo (Bubalus bubalis) bulls kept at the International Livestock Management Training Center in Sakha, Kafrelsheikh, Egypt, were used to collect semen samples from November 2024 until the end of January 2025.

Eighty-four healthy and mature heifers free from any congenital anomalies were selected and used for an in vivo fertility experiment. The animals were kept at the Animal Production Research Station, Mahallet-Mousa, Kafrelsheikh (latitude 31°06′ N and longitude 30°56′ E), Egypt.

Preparation of clove buds' extract

A modified procedure was used to grind and extract 50 g of dried clove buds [13]. Briefly, the clove buds were soaked in 1 Liter of 80% ethanol at 4 °C in the dark for 72 h, stirred every 12 h with a sterile glass rod, and finally sieved with filter paper (Whatman, England No. 4 paper filter). A rotary evaporator was used to combine the collected extracts and vacuum-condense them at 35 °C. Finally, a freeze-drier (OSK 2139; Japan) was used to lyophilize the condensed extract [13].

Semen extender preparation

The egg yolk extender was prepared by using egg yolk (20 % v/v), Tris (3.025 % w/v), citric acid (1.675 % w/v), fructose (1 % w/v), penicillin (1000 IU/mL, Sigma-Aldrich, St. Louis, MO, USA), and streptomycin (1000 g/mL, Sigma-Aldrich, St. Louis, MO, USA) plus glycerol (6.4 % v/v, Thermo Fisher Scientific, Mumbai, India) [19, 20].

Semen collection and evaluation

Ejaculates were collected from bulls using an artificial vagina with inner sleeve temperature of 40 – 42 °C. The collected ejaculates were kept at 37 °C in a water bath and examined macroscopically (volume, color, odor, consistency, and hygienic quality) and microscopically (sperm motility, morphology, and concentration) to select good-quality ejaculates for cryopreservation.

Semen dilution and cryopreservation

Ejaculates with 3–5 mass motility, >70% initial individual motility, <15% abnormal morphology, and > 800×106/mL sperm cell concentration were selected and pooled for cryopreservation [21]. The pooled ejaculates were dispensed into 4 aliquots. Each aliquot was diluted with Tris-egg yolk citric acid diluent containing different concentrations of clove buds (*Syzygium aromaticum*) extract as follows: 1) 0 µg/mL as a control (CTRL), 2) 5 µg/mL (CLOV5), 3) 10 µg/mL (CLOV10), and 4) 15 µg/mL (CLOV15) to a final concentration of 60 x 106 sperm/mL [21].

The four aliquots were gradually cooled to 4 °C and were kept for 5 h for equilibration with glycerol. Immediately following equilibration, pre-cooled mini straws (0.25 mL; IMV, L'Aigle, France) were filled with equilibrated semen using a filling machine, and the straws were sealed with a sealing machine. The straws were exposed to liquid nitrogen vapor (−120 - −140 °C) in a Thermo box for 10 min before being dipped into a liquid nitrogen (- 196 °C, IMV, L'Aigle, France) tank for storage [22]. It was ensured that straws were always immersed in LN2 by periodically replenishing the LN_2. Frozen straws were stored in LN_2 for at least 7 days before evaluation.

Post-thaw semen quality

Following each freezing trial, frozen straws were thawed in a water bath for 30 s at 37 °C [22]. Frozen-thawed straws were examined for the following:

Sperm motility and kinematics

Sperm cell motility and kinematics were determined by using A computer-aided sperm motion analyzer (CASA; Hamilton Thorne, Inc., Beverly, MA, USA) system. A clean, pre-warmed Makler counting chamber (37 °C) was loaded with 5 µL of diluted (1:10) frozen-thawed semen, covered, and analyzed at 200× magnification. Five randomly selected fields for each sample were observed. At least 200 sperm cells were examined to determine the total motility (%), progressive motility (%), average path velocity (VAP, µm/s), straight linear velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), straightness (STR, %), linearity (LIN, %), wobble coefficient (WOB, %), beat cross frequency (BCF%), and average lateral head displacement (ALH%).

Sperm viability

A semen smear stained with eosin-nigrosin was used to determine the proportion of viable spermatozoa. A phase-contrast microscope was used to observe (400×) at least 200 sperm cells. Viable sperm cells had white sperm heads, whereas those with partially or completely pink-stained sperm heads were considered dead [23].

Plasma membrane integrity

The functional integrity of sperm plasma membrane was determined by the hypo-osmotic swelling test (HOST), as reported by Revell and Mrode. [24]. A prewarmed hypo-osmotic solution (500 µL) of 150 mOsm/kg H2O (0.735 g sodium citrate, 1.351 g fructose dissolved in 100 mL Milli-Q water) was mixed with 50 µL of frozen-thawed semen and incubated at 37 °C for at least 30 min. Following incubation, sperm tail curling patterns were counted by spotting 2 µL of a well-mixed sample onto a prewarmed (38 °C) clean glass slide, covered by a prewarmed clean coverslip (18 x 18 mm), and observed under 400× magnification of a phase-contrast microscope. In total, 200 spermatozoa were carefully examined to determine the proportion of spermatozoa with curling or swelling of their tails (% HOST positive). To determine the true percentage of HOST-positive spermatozoa, subtract the proportion of spermatozoa with abnormal tail morphology from the proportion of HOST-positive spermatozoa [24].

Acrosomal membrane integrity

The acrosomal membrane integrity was evaluated with the glutaraldehyde fixation method [25]. Briefly, 5 µL of frozen-thawed semen was diluted with 50 µL of 0.16 M NaCl, and sperm cells were fixed with 1% (v/v) glutaraldehyde by mixing the diluted semen with an equal volume of 2% (v/v) glutaraldehyde/0.165 M sodium cacodylate buffer (pH 7.3 at 25 °C) at room temperature for 30 min. After incubation, sperm cells were examined under a phase-contrast microscope (1000×) for acrosome integrity (% intact-acrosome), where spermatozoa showing a dense, thick apical ridge on the head were considered intact-acrosome; in total 200 spermatozoa were examined.

DNA fragmentation index

A five mL of phosphate-buffered saline (PBS) was used to wash the frozen-thawed semen. Following centrifugation, the collected sperm pellet was resuspended in 0.5 mL PBS following centrifugation. The sperm suspension was then glass-smear in an aliquot (50 µL). Three smears for each sample were prepared, allowed to air dry, and then fixed overnight in Carnoy's solution (methanol/acetic acid, 3:1) according to the methodology of Liu and Baker. [26]. After fixation, the smears were stained for 5 min in a freshly prepared acridine orange stain in a dark room. The stained smears were visualized under a fluorescence microscope (Leitz, Germany; excitation of 450-490 nm) where sperm cells with normal DNA content or intact chromatin fluoresced green, while sperm with aberrant DNA content fluoresced in a spectrum that ranged from yellow green to red [27].

Biochemical evaluation of seminal plasma (SP)

Glutathione peroxidase activity

A glutathione peroxidase kit (GP 2425, Biodiagnostic, Cairo, Egypt) was used to quantify the activity of GPX. GPX in semen samples was determined by measuring NADPH consumption. The reaction between reduced glutathione (GSH) and hydrogen peroxide is initiated by glutathione reductase (GSSGr) and glutathione peroxidase (GPX). When NADPH is present, oxidized glutathione (GSSG) quickly transforms into GSH. The GPX activity was estimated by mixing GSH (1 mM, 1 mL), GSSRr (0.25 U/mL, 0.2 mL), NADPH (0.12 mM, 0.01 mL), sodium azide (0.25 mM, 0.2 mL), and SP (1 mL). EDTA (6.3 mM, pH 7.5) and phosphate buffer (143 nM) were utilized to dissolve NADPH. GSH was dissolved in 5% metaphosphoric acid. Sodium azide was used to inhibit catalase activity. The reaction was initiated by adding tert-butyl hydroperoxide (1.2 mM, 1 mL). NADPH utilization was determined calorimetrically at wave wavelength of 340 nm every 5 s at 37 °C for 10 min [28].

Superoxide dismutase activity (SOD)

SOD activity (U/mL) was measured using SOD test kits (SD 2521, Biodiagnostic, Cairo, Egypt). Semen samples have SOD activity that competes with cytochrome C, reducing superoxide free radicals to H2O2. Absorbance was measured every 5 s at 25 °C. The assay mixture included 1 mL of SP, 84 mL of a solution containing cytochrome C (1 mM), xanthine (50 mM), and 16 mL of xanthine oxidase diluted in sodium phosphate/EDTA buffer at 50 and 100 mM (pH 7.8). Xanthine oxidase activity was optimized to produce the most O2 while reducing cytochrome C levels. The rate of cytochrome C reduction was determined at 0.025 units of absorbance/min at 550 wavelengths, with one unit of total SOD activity accounting for 50% of the value [28].

Catalase activity (CAT)

The activity of CAT was determined using a CAT kit (CA 2517, Biodiagnostic, Cairo, Egypt), as described by Aebi. [29] For this procedure, 100 µL of SP was required. In brief, CAT reacted with a particular amount of H2O2. A catalase inhibitor was used to stop the process after 1 min. A chromophore was produced when the residual H2O2 reacted with 4-aminophenazone and 3,5-dichloro-2-hydroxybenzene sulfonic acid. The absorbance rate of 510 nm was used to determine the CAT. SP CAT activity was reported in units of U/mL.

Malondialdehyde content (MDA)

MDA levels were determined to assess lipid peroxidation using commercial kits (MD 2529, Biodiagnostic, Cairo, Egypt). Thiobarbituric acid was mixed with 200 µL from each concentration, incubated for 30 min at 95 °C, then cooled and centrifuged at 200 g for 5 min. The supernatants were collected, and MDA levels were quantified using colorimetry at 534 nm optical density and reported as nmol/mL [30].

In vivo fertility

In total, 84 healthy mature heifers free from congenital anomalies, following ultrasound scanning of their genitalia, were selected and randomly enrolled into 4 groups. All heifers were treated with the CIDR-Synch protocol followed by a fixed time AI (FTAI: [31]). Each group of heifers (n =21) was inseminated with one of the 4 groups of prepared frozen-thawed straws supplemented with different concentrations of the clove bud's extract powder. Pregnancy was confirmed on day 45 post-insemination via trans-rectal ultrasound scanning utilizing Mindray M5 portable ultrasound with linear array transducer while the frequency is adjusted at 3.5 MHz. The in vivo fertility rate for each group was calculated from the following equation: The in vivo fertility rate = No of pregnant heifers/No of inseminated heifers x 100.

Statistical analysis

Data were analyzed using GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, USA, www.graphpad.com) with repeated measures of one-way analysis of variance (ANOVA) and Tukey's multiple comparison test. The data were checked for normal distribution using the Shapiro-Wilk and Kolmogorov-Smirnov normality tests. The data are presented as mean ± SEM, and $P < 0.05$ was considered statistically significant. The results of the in vivo fertility rate were analyzed using the Chi-square statistical model.

Results

Sperm characteristics

Sperm characteristics of buffalo-bull semen frozen-stored in the presence of different concentrations of clove are presented in Table 1. Frozen-stored spermatozoa in the presence of 15 µL/mL CLOV had a greater ($P < 0.05$) proportion of total (84.51 ± 1.40) and progressive (45.94 ± 2.51) motilities as compared to the control, CLOV10, and CLOV5. All concentrations of the CLOV have higher numerical proportions of sperm viability compared with the control group, but without a significant difference, as shown in Table 1. The proportion of plasma membrane integrity (true HOST-positive) in CLOV15 (70.95 ± 2.07) was higher ($P < 0.05$) than that of the control (61.20 ± 0.22) group and similar to those of CLOV10 (65.20 ± 0.73) and CLOV5 (63.70 ± 0.92). Unexpectedly, the acrosomal membrane integrity was greater in the control group (76.20 ± 0.84) than in all treatment groups. The proportion of DNA integrity was higher in CLOV10 (91.41 ± 0.86) and CLOV5 (91.20 ± 0.84) than that of CLOV15 (86.24 ± 0.43) and was similar to that of the control, as shown in Table 1.

Sperm kinematics

The obtained data presenting the effect of different concentrations of clove buds extract powder on the sperm kinematics of frozen-thawed buffalo bull semen revealed that CLOV5 showed higher ($P < 0.05$) proportions of DAP (17.35 ± 0.40%), DCL (26.10 ± 0.59%), DSL (11.90 ± 0.19%), and higher value of VAP (42.57 ± 0.96 µm/s) in comparison with the control group and CLOV15 (Table 2). All the proportions of DAP, DCL, and DSL of CLOV10 were similar to those of CLOV5 and higher than those of CLOV15, as shown in Table 2.

It is worth noting that the CLOV15 group revealed that higher ($P < 0.05$) values of VCL (50.95 ± 2.17 µm/s) and proportion of STR (0.79 ± 0.01%) and ALH (3.85 ± 0.04%) than the control and other treatment groups. VSL, LIN, and WOB were similar ($P \geq 0.05$) among all treatments and the control group. As shown in (Table 2), the proportion of BCF was higher ($P < 0.05$) in CLOV5 (19.13 ± 0.38%) and CLOV10 (19.62 ± 0.52%) in comparison with the control (15.26 ± 0.58%) group and that of the latter was similar with that of CLOV15 (17.77 ± 0.55%) treatment group.

Enzymatic antioxidants and lipid peroxidation markers of SP

Table (3) shows the enzymatic antioxidants and the MDA lipid peroxidation marker of frozen-thawed buffalo bull semen in the presence of different concentrations of the clove powder. The activity of SOD and CAT was similar among all treatments and the control group. The activity of GPX was greater ($P < 0.05$) in CLOV10 (4.27 ± 0.16) in comparison with its counterparts of the control (2.46 ± 0.11) and CLOV15 (2.59 ± 0.06), but it was similar to that of CLOV5 (3.98 ± 0.10). (Table 3) shows that the lowest ($P < 0.05$) MDA levels were found in CLOV15 (21.50 ± 0.67 nmol/mL), then it increased in control (26.80 ± 0.28 nmol/mL), and became higher ($P < 0.05$) in CLOV5 (30.00 ± 0.22 nmol/mL) and CLOV10 (30.70 ± 0.27 nmol/mL) treatment groups.

In vivo fertility

Table (4) shows the effects of various concentrations of the clove powder on the in vivo fertility of frozen-thawed buffalo bull semen. It was noticed that frozen-thawed straws enriched with 15 µL/mL clove powder (CLOV15) yielded the highest ($P < 0.001$, $x2 = 27.032$) pregnancy rate (66.66%), whereas those enriched with 10 µL/mL clove powder (CLOV10) yielded the lowest (11.11%) pregnancy rate. The pregnancy rate of 5 µL/mL clove powder (CLOV5) enriched frozen-thawed straws (33.33%) was identical to that of the control group (33.33%) (Table 4).

Discussion

Buffalo bull spermatozoa are more susceptible to cryodamage because of their unique membrane composition, which has a higher cholesterol-to-phospholipid ratio than bull spermatozoa. This limits the success of AI in the buffalo industry owing to the low sperm characteristics with a subsequent low fertility rate and higher processing costs of frozen-thawed buffalo bull spermatozoa [32]. Additionally, buffalo bull spermatozoa are more vulnerable to oxidative stress during cryopreservation due to their lower intrinsic antioxidant capacity when compared to bull spermatozoa [8].

During freezing and thawing, buffalo bull sperm plasma membrane, which is rich in polyunsaturated fatty acids (PUFA), is vulnerable to lipid peroxidation [9]. The quantity of PUFA in the sperm plasma membrane has a direct correlation with the production of MDA [10]. MDA impairs the composition and function of the sperm plasma membranes in bovine and human semen [33]. Accordingly, MDA is linked to Egyptian buffalo bull fertility [34] and acts as a biomarker of oxidative stress [35].

Buffalo sperm motility [36], viability [37], plasma membrane integrity [38], acrosomal integrity [39], and DNA fragmentation [40] are all adversely affected by oxidative stress during the freezing-thawing procedures. To the best of our knowledge, our study is unique in adding freeze-dried clove bud extract into the cryopreservation extender of buffalo bull semen in an attempt to enhance its post-thaw quality and in vivo fertility. Our study revealed that adding 15 and 10 µL/mL semen extender improved the proportion of post-thaw total and progressive motilities in comparison with the control and CLOV5 groups. This improvement in post-thaw sperm motility might be attributed to the antioxidant effect of the clove bud extract, which is dose-dependent. As a result, higher concentration (CLOV15) was positively effective on sperm cell motility. This finding is in line with [13], who recorded that the lower and medium concentration of clove buds' extracts (35 and 75 µg/mL), when added to ram semen diluent, have a beneficial effect on sperm cells. Further, we noticed an upper layer of fat globules over the semen samples, which increased their viscosity and resulted in mechanical interfere with sperm cell motility in accordance with Hirai et al. [41]. The improved functional integrity of sperm plasma membrane by increasing the concentration of clove in semen extender is mainly attributed to the Clove bud extract it its main component is eugenol [40]. It was previously reported that this terpenoid may be incorporated into the membrane due to its hydrophobic nature [42] and may inhibit free radical attack by decreasing lipid peroxidation [43]. May increase the fluidity of the sperm plasma membrane, in turn reducing the physical and functional integrity of the sperm plasma membrane [44]. On the other

hand, the DNA integrity was improved by adding different clove concentrations of CLOV5, CLOV10, and CLOV15, which backed up the findings of Kumar et al. [45] Who reported that the antioxidant activity of clove bud extract protects sperm DNA from oxidative damage, and in turn reduces DNA fragmentation.

Comparing the plasma membrane with the nuclear membrane, it indicates that the main component of the plasma membrane is phospholipids [46]. Especially, in all species the highest concentration of Filipin-sterol (primarily cholesterol) complexes is found in the plasma membrane overlying the acrosome and shows a slight increase during maturation whereas the main component of nuclear membrane is protein [47]. Also, according to previous research [48] That reported that the clove bud contains one of the main active compounds is eugenol, which exists with free hydroxyls in its structure and can act as a strong donor of hydrogen atoms or electrons when it encounters free radicals, resulting in sturdy antioxidant activity. Thus, the higher concentrations of clove negatively affect the acrosomal membrane and induce acrosomal reaction, while sperm cell DNA is considered protected as the nuclear membrane is mainly protein in nature [47].

In the same way, the main antioxidant activity of eugenol presents in the clove [49], expected that low level of clove (CLOV5) would improve the antioxidant activity of SOD, CAT, GPX and reduce the content of MDA while the higher level of clove as (CLOV10 and CLOV15) would induce the deleterious effect of clove. Our obtained results were partially inconsistent with this assumption, because incorporation of clove buds extract powder failed to improve the SOD and CAT activities. Moreover, CLOV10 succeeded in improving the GPX activity, but CLOV5 revealed a higher numerical value of GPX activity without a significant difference. Further, CLOV5 and CLOV10 increased the seminal content of MDA. This discrepancy with previous literature might be attributed to the different animal species and breeds, semen collection method, and evaluation method. The novelty of our study is that CLOV15 yielded a higher in vivo fertility rate (66.66%) in comparison with the control, CLOV5, and CLOV10. It has higher functional plasma membrane integrity with lower acrosomal integrity and increased DNA fragmentation requires future investigation to confirm this possibility.

Conclusion

In conclusion, adding a higher concentration (CLOV15) of clove buds' powder in the cryopreservation extender of buffalo bull semen improved post-thaw sperm motility and kinematics, lipid peroxidation (MDA) content, and also, in vivo fertility. So, Further investigations are warranted to determine the effect of increasing the clove powder extract on sperm cell characteristics.

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Declaration of Conflict of Interest

The authors declared no conflicts of interest in the publication of this research article.

Ethical of approval

The Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine at Kafrelsheikh University, Egypt, allowed approval (KFS-IACUC/245/2025) for the present study involving the sampling.

TABLE 1. Sperm characteristics (mean ± SEM) of frozen-thawed buffalo bull semen supplemented with different proportions of clove (n = 12).

Parameter (%)	CONT	CLOV (µg/mL)		
		5	10	15
Total motility	67.7 ± 2.69^{c}	67.54 ± 0.85^{c}	76.99 ± 1.09^{ab}	84.51 ± 1.40^{a}
Progressive motility	33.46 ± 2.02^{b}	19.95 ± 0.61^{c}	36.62 ± 1.16^{b}	45.94 ± 2.51^{a}
Viability	67.16 ± 2.31^{b}	70.53 ± 0.73^{b}	69.83 ± 0.18^{b}	72.2 ± 0.84^{b}
Plasma membrane integrity	61.20 ± 0.22^{b}	63.70 ± 0.92^{ab}	65.2 ± 0.73^{ab}	70.95 ± 2.07^{a}
Acrosomal integrity	76.20 ± 0.84^{a}	70.20 ± 0.84^{b}	66.28 ± 0.88^{b}	69.20 ± 0.84^{b}
DNA integrity	83.74 ± 0.39^{ab}	91.20 ± 0.84^{a}	91.41 ± 0.86^{a}	86.24 ± 0.43^{b}

*Within the same row means bearing one common superscript were non-significantly different at $P < 0.05$. CONT = Control, CLOV = Clove.

TABLE 2. Sperm Kinematics (mean ± SEM) of frozen-thawed buffalo bull semen supplemented with different proportions of Clove (n = 12).

Parameter	CONT	CLOV (µg/mL)		
		5	10	15
VAP (µm/s)	36.82 ± 0.36^{bc}	42.57 ± 0.96^{a}	38.87 ± 0.55^{b}	34.16 ± 0.72^{c}
VCL (µm/s)	36.83 ± 0.35^{c}	42.58 ± 0.97^{b}	38.87 ± 0.55^{bc}	50.95 ± 2.17^{a}
VSL (µm/s)	26.77 ± 0.32^{a}	29.89 ± 0.55^{a}	28.16 ± 0.52^{a}	27.00 ± 0.35^{a}
STR (%)	0.73 ± 0.01^{b}	0.69 ± 0.01^{c}	0.72 ± 0.01^{bc}	0.79 ± 0.01^{a}
LIN (%)	0.51 ± 0.01^{a}	0.47 ± 0.01^{a}	0.41 ± 0.08^{a}	0.53 ± 0.02^{a}
WOB (%)	0.70 ± 0.01^{a}	0.67 ± 0.01^{a}	0.68 ± 0.01^{a}	0.67 ± 0.02^{a}
ALH (%)	3.42 ± 0.07^{bc}	3.81 ± 0.05^{ab}	3.24 ± 0.05^{c}	3.85 ± 0.04^{a}
BCF (%)	15.26 ± 0.58^{b}	19.13 ± 0.38^{a}	19.62 ± 0.52^{a}	17.77 ± 0.55^{ab}
DAP (%)	14.95 ± 0.14^{b}	17.35 ± 0.40^{a}	16.19 ± 0.20^{ab}	13.00 ± 0.29^{c}
DCL (%)	21.51 ± 0.20^{bc}	26.10 ± 0.59^{a}	23.81 ± 0.40^{ab}	19.52 ± 0.79^{c}
DSL (%)	10.77 ± 0.14^{c}	11.90 ± 0.19^{a}	11.65 ± 0.20^{ab}	10.12 ± 0.13^{c}

*Within the same row means bearing one common superscript were non-significantly different at P < 0.05. CONT = Control, CLOV = Clove, DAP = Average path distance, DCL = Curved line distance, DSL = Straight line distance, VAP = Average path velocity, VCL = Curvilinear velocity, VSL = Straight linear velocity, STR = Straightness, LIN = Linearity, WOB = Wobble, BCF = Beat cross frequency, ALH = Average lateral head displacement.

TABLE 3. Enzymatic antioxidants and lipid peroxidation marker (mean ± SEM) of frozen-thawed buffalo bull semen supplemented with different concentrations of Clove (n = 12).

Parameter	CONT	CLOV (µg/mL)		
		5	10	15
SOD (U/mL)	5.65± 0.32^{a}	7.19 ± 0.17^{a}	7.09 ± 0.25^{a}	6.78 ± 0.45^{a}
CAT (U/L)	31.38 ± 0.21^{a}	32.80 ± 1.76^{a}	30.52 ± 1.27^{a}	32.20 ± 1.12^{a}
GPX (mU/mL)	2.46 ± 0.11^{b}	3.98 ± 0.10^{ab}	4.27 ± 0.16^{a}	2.59 ± 0.06^{b}
MDA (nmol/mL)	26.80 ± 0.28^{c}	30.00 ± 0.22^{a}	30.70 ± 0.27^{a}	21.50 ± 0.67^{b}

*Within the same row means bearing one common superscript were non-significantly different at P < 0.05. CONT = Control, CLOV = Clove, SOD = Superoxide dismutase, CAT = Catalase enzyme, GPx = Glutathione peroxidase, MDA = Malondialdehyde.

TABLE 4. In vivo fertility of frozen-thawed buffalo bull straws supplemented with different proportions of freeze-dried clove powder.

Parameter (%)	CONT	CLOV (µg/mL)		
		5	10	15
Inseminated buffalo-cows (n)	21	21	21	21
Pregnant buffalo-cows (n)	7	7	2	14
Pregnancy rate (%)	33.33^{b}	33.33^{b}	11.11^{c}	66.66^{a}
P - value				**< 0.0001**
Chi-square value				**27.0321**

CONT = Control, CLOV = Clove

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جودة السائل المنوي لثور الجاموس المجمد والمذاب بعد إذابته، ونشاط مضادات الأكسدة، والخصوبة داخل الجسم، في وجود تركيزات مختلفة من مسحوق القرنفل المجفف بالتجميد

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الملخص

يُعد التلقيح الاصطناعي (AI) تقنية تكاثرية بالغة الأهمية في صناعة الجاموس، إذ يوفر فوائد عديدة لتحسين الجودة الوراثية من خلال التهجين الانتقائي وتسريع التطور الوراثي. الغشاء البلازمي للحيوانات المنوية لثيران الجاموس غني بالأحماض الدهنية المتعددة غير المشبعة، وهو عرضة لأكسدة الدهون أثناء عمليتي التجميد والذوبان. وقد بُحثت إضافة مستخلصات عشبية إلى موسعات السائل المنوي لثيران الجاموس كطريقة محتملة لتحسين جودة السائل المنوي والخصوبة. وتُعد المركبات الفينولية، وخاصةً الأوجينول، المكون النشط الرئيسي، من أقوى مضادات الأكسدة في مستخلص براعم القرنفل. تهدف هذه الدراسة إلى تقليل التأثير الضار للإجهاد التأكسدي أثناء عمليات التجميد والذوبان، مما يُحسّن بدوره جودة الحيوانات المنوية لثيران الجاموس بعد الذوبان وخصوبة الحيوانات المنوية في الجسم الحي. جُمعت القذفات من سبعة ثيران جاموس مصري سليمة باستخدام مهبل صناعي مرتين أسبوعيًا. جُمعت القذفات عالية الجودة ووُزِّعت في أربعة أجزاء؛ خُفِّفت كل جزء بمُخفِّف حمض الستريك من صفار بيض تريس، والذي يحتوي على تركيزات مختلفة من مستخلص براعم القرنفل (السيزيجيوم العطري) على النحو التالي: ١) ٠ ميكروغرام/مل كعينة ضابطة (CTRL)، ٢) ٥ ميكروغرام/مل (CLOV5)، ٣) ١٠ ميكروغرام/مل (CLOV10)، و٤) ١٥ ميكروغرام/مل (CLOV15). وُضِعت عينات السائل المنوي المخففة في درجة حرارة ٤ درجات مئوية لمدة ٤ ساعات، ووُضِعت في ماصات فرنسية صغيرة، وأُغلِقَت، وخُزِّنت مُجمَّدة في النيتروجين السائل. أُذيبت الماصات المُجمَّدة وفُحِصَت لخصائص الحيوانات المنوية. كما تم تحديد مضادات الأكسدة الإنزيمية (SOD، CAT، GPX) بالإضافة إلى مؤشر بيروكسيد الدهون (MDA). تم اختيار 84 جاموسه ناضجة وصحية (21 أنثى/مجموعة)، وعرضت لتزامن الشبق، ثم أُلقحت باستخدام السائل المُجمد والمذاب المُجهز لحساب معدلات الخصوبة لديها داخل الجسم الحي. أظهرت النتائج أن CLOV15 و CLOV10 لديهما نسب أعلى ($P < 0.05$) من جميع خصائص الحيوانات المنوية بعد الذوبان، وأيضًا، حقق CLOV15 معدل حمل أعلى بنسبة 66.66٪ بالمقارنة مع 33.33) CLOV5٪) و 11.11) CLOV10٪). لم يتأثر SOD ولا CAT بإضافة القرنفل بينما تحسن نشاط GPX في CLOV10 مقارنة بالمجموعة الأخرى. وبنفس الطريقة، كان محتوى MDA أقل في التركيز العالي للقرنفل مقارنة بالتركيز المنخفض ومجموعات الضبط. وفي الختام، أدى إثراء موسعات التجميد بمسحوق براعم القرنفل بتركيز 15 ميكرولتر / مل إلى تحسين خصائص الحيوانات المنوية تقريبًا. من ناحية أخرى، حسّن CLOV10 و CLOV5 مضادات الأكسدة الأنزيمية وحسن CLOV15 فقط معدل الخصوبة في الجسم الحي لسائل المنوي المجمد المذاب للثيران الجاموسية.

الكلمات الدالة: ثيران الجاموس , خصائص الحيوانات المنوية , القرنفل ,SOD , التجميد , إذابة الجليد , الخصوبة في الجسم الحي .