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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 Fennel Powder

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Abstract

BUFFALO bull spermatozoa exhibited a higher degree of cryodamage compared to bull spermatozoa and in turn leading to reduced fertility rates following thawing. This poor freezability of buffalo bull spermatozoa can be attributed to distinct lipid composition of their plasma membrane compared to those of other species, rendering them more susceptible to damage during cryopreservation. Reproductive scientists endeavour to minimize this inevitable cryodamage through adding specific additives to the semen extender used for cryopreservation of buffalo bull spermatozoa. Fennel is a promising candidate of these additives, where the most potent antioxidants in fennel are rosmarinic, flavonoid and anethole. The present study aimed to investigate the possible effects of adding different concentrations of freeze-dried fennel powder on the post-thaw quality, enzymatic antioxidant activity, *in vivo* fertility of buffalo bull spermatozoa. Ejaculates were collected from seven healthy Egyptian buffalo bulls by artificial vagina twice weekly. Good-quality ejaculates were pooled and dispensed into four Aliquots; each aliquot was diluted with Tris-egg yolk citrate extender containing: 0 being as control (CTRL), 10 (FEN10, 20 (FEN20) and 30 (FEN30) µL/mL freeze-dried fennel powder, cooled, equilibrated, loaded in mini-straws before being frozen stored in liquid nitrogen at − 196°C. Frozen straws were thawed in a water bath at 37 °C for 30 s and examined for sperm motility, viability, plasma membrane integrity, acrosome integrity and DNA integrity. Seminal total protein, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) and malonaldehyde (MDA) levels were determined. The results revealed that FEN20 significantly ($P < 0.05$) improved the proportions of total motility, progressive motility, viability and plasma membrane integrity. Intriguingly, the control group had higher proportions of acrosomal membrane integrity and DNA integrity. There was no significant variation among the control and all treated groups in the total protein level and CAT. While SOD, GPX and MDA activity were significantly ($P < 0.05$) higher in FEN10. FEN10 yielded the greatest in vivo fertility rate compared to the control, FEN20, and FEN30 groups.

Keywords: Egyptian buffaloes; Sperm characteristics; Fennel; Enzymatic antioxidants; Frozen-thawed; In vivo fertility.

Introduction

Water buffaloes (*Bubalus bubalis*) are commercially and culturally important dairy animals throughout Asia, South America, and the Mediterranean region, particularly Egypt. The Arabs domesticated and introduced buffalo to Egypt and Italy in the seventh century [1]. According to FAO STAT (2023), the Egyptian buffalo is ranked fourth in global milk output and sixth in meat production, indicating a significant contribution to our national economy. This ranking emphasizes the Egyptian buffalo's perceived efficiency and production in relation to world agriculture.

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Artificial insemination (AI) is frequently used in cattle [2], humans [3], but less so in sheep [4], buffaloes [5], equines [6], deer [7], and other species [8]. Semen cryopreservation has enabled the fast development of reproductive technologies such as AI and embryo transfer. Advances in reproductive biotechnology, notably semen cryopreservation, have paved the way for more effective preservation of buffalo semen, which was previously thought to be troublesome. However, cryopreservation causes damage to all sperm compartments. Likewise, the varying degree of morphological, physiological, and biochemical changes in living spermatozoa after storage at ultra-low temperatures renders these cells inappropriate for optimal fertility [9]. During cryopreservation, sperm cells are subjected to both internal and external stressors, which cause membrane perturbations, lipid/protein reconfiguration, and osmotic alterations across the cell membranes [10].

Mahmoud *et al*. [11] found that the association between sperm characteristics and fertility has frequently proven inconsistent. Although there has been considerable advance in the field of semen cryopreservation; the conception rate using cryopreserved semen is approximately 33% [12]. This lower conception rate could be attributed to acrosomal damage [13], reduced motility [14], and altered plasma membrane integrity [14] of frozen-thawed buffalo spermatozoa. Khan *et al.* [15] showed that freezing and thawing of buffalo semen resulted in reduced motility, impaired plasma membrane integrity, a lower viable sperm count, and an increased percentage of apoptotic sperm cells.

Several studies have been conducted to explain this lower fertility of cryopreserved semen; however, cryopreservation-induced capacitation-like alterations in frozen thawed spermatozoa have lately gained investigations [16, 17]. Kadirval *et al.* [18] investigated the link between capacitation, cholesterol levels, membrane fluidity, and intracellular calcium in frozen-thawed buffalo spermatozoa.

Oxidants, such as reactive oxygen species (ROS), influence the post-thaw sperm quality. These chemicals are also created physiologically in living cells during respiration [19], as well as by defective or dead sperm and phagocytic cells of the male and female reproductive tracts [20]. ROS reduces sperm quality and makes it incapable to fertilize an oocyte [21]. Also, they have an adverse effect on sperm DNA and the formation of male pronuclei after fertilization [22]. Moreover, ROS have been demonstrated to impair motility, capacitation, and acrosome response of sperm cells, with inhibitory effects mediated mostly by lipid peroxidation of the sperm plasma membrane [22]. Sperm plasma membrane contains large levels of polyunsaturated fatty acids, which are sensitive to LPO caused by oxidative stress throughout freezing and thawing procedures. Further, the antioxidant system of SP and sperm cells is weakened during semen cryopreservation [23].

The most potent antioxidants in the fennel are rosmarinic acid, flavonoid, and anethole [24, 25]. Fennel extracts augmented ram sperm motility and viability [26]. MDA levels in boar semen decreased in fennel-treated groups [27]. According to Najafi *et al.* [26], the optimal concentration of fennel extract for ram semen cryopreservation was 10 µg/mL at a concentration of 200×10^6 sperm/mL. To the best of our knowledge, there is no published research on enriching buffalo semen extender with fennel. Thus, the current research aimed to investigate the possible effects of freeze-dried fennel powder on the post-thaw sperm quality, kinematics and in vivo fertility of buffalo bull spermatozoa. Also, the seminal level of total protein and the enzymatic antioxidants activities were determined.

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 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 fennel extract

Commercial fennel (*Foeniculum vulgare*) was soaked in deionized water to obtain on fennel extract according to Najafi *et al.* [26]. 10 g of fennel powder was stirred with 100 mL deionized water for 24 h. the aqua fennel was boiled for 15 min. Following boiling, the suspension was kept at room temperature (25°C) before being filtered to obtain clean extraction. A rotary evaporator was used to vacuum condense fennel (*Foeniculum vulgare*) extract [26] and the condensed fennel (*Foeniculum vulgare*) extract was freeze-dried for storage.

Semen extender preparation

The egg yolk extender was prepared 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) [28].

Semen collection and evaluation

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. Ejaculates were collected from bulls using an artificial vagina with inner sleeve temperature of 40 – 42 °C. The collected ejaculates were kept at warm water bath (35-37 °C) 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 > 800×10^6/mL sperm cell concentration were selected and pooled for cryopreservation [29]. The pooled ejaculates were dispensed into 4 aliquots. Each aliquot was diluted with Tris-egg yolk citric acid diluent enriched with different concentrations of fennel extract (*Foeniculum vulgare*) extract as follows: 1) 0 µL/mL as a control (CTRL), 2) 10 µL/mL (FEN10), 3) 20 µL/mL (FEN20), and 4) 30 µL/mL (FEN30) to a final concentration of 60 x 10^6 sperm/mL [29].

The four aliquots 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 diluted 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 [30]. It was ensured that straws were always immersed in LN_2 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 [30]. 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, and 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 [31].

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. [32]. A prewarmed hypo-osmotic solution (500 µL) of 150 mOsm/kg H_2O (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 [32].

Acrosomal membrane integrity

The acrosomal membrane integrity was evaluated with glutaraldehyde fixation method [33]. 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 5 mL of phosphate buffered saline (PBS) was used to wash the frozen-thawed semen. Following

centrifugation, the collected sperm pellet was re-suspended in 0.5 mL PBS following centrifugation. The sperm suspension was then glass smeared in a tiny 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 [34]. After fixation, the smears were stained for 5 minutes in a freshly prepared acridine orange (AO) 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 [35].

Biochemical evaluation of seminal plasma (SP)

Recovery of SP and measuring its total protein concentration (TPC) Immediately, after thawing SP was obtained by centrifugation at 12000 × g for 30 min in a cooling centrifuge at 4 °C. The supernatants (SP) were transferred into 0.6 mL centrifuge tubes, and centrifuged again to eliminate the remaining cells and debris to obtain on clear SP. Finally, SP was stored at −80 °C until analysis. The TPC (g/dL) of SP was determined using a hand-held Refractometer (ATAGO, Brix 0–32%, Japan) according to Marsh and Fingerhut. [36].

Enzymatic antioxidants and lipid peroxidation

On the analysis day, frozen-thawed straws were centrifuged at 3000 g for 15 min to separate seminal plasma (SP). The obtained SP was tested for total protein were measured using colorimetric techniques [3], enzymatic antioxidants including catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx), as well as malondialdehyde (MDA) as a LPO marker.

Catalase activity (CAT)

The activity of CAT was determined using a CAT kit (CA 2517, Biodiagnostic, Cairo, Egypt), as described by Aebi [37]. For this procedure, 100 µL of SP was required. In brief, CAT reacted with a particular amount of H_2O_2. A CAT inhibitor was used to stop the process after 1 min. A chromophore was produced when the residual H_2O_2 reacted with 4-aminophenazone and 3,5 dichloro-2-hydroxy benzene sulfonic. The absorbance rate of 510 nm was used to determine the CAT. SP CAT activity was reported in units of U/mL.

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 H_2O_2. 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 O_2 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 [38].

Glutathione peroxidase activity (GPX)

A GPX 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 GPX. When NADPH is present, oxidized glutathione (GSSG) quickly transform 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 CAT activity. The reaction was initiated by adding terta-butyl hydroperoxide (1.2 mM, 1 mL). NADPH utilization was determined colorimetrically at wave wavelength 340 nm every 5 s at 37 °C for 10 min [38].

Malondialdehyde content (MDA)

MDA levels were determined to assess LPO 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 [39].

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 CIDR-Synch protocol followed by a fixed time AI (FTAI) [40]. Each group of heifers (n =21) were 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 proportions of FEN are presented in (Table 1). FEN20 had the greater proportions of total motility (77.07 ± 2.51%), progressive motility (39.27 ± 2.15%), viability (85.2±1.24%), and intact plasma membrane (69.95 ± 1.92%). Intriguingly, the proportions of progressive motilities of all experimental groups were more or less the halved proportions of total motilities. In the same context, the control group had the greater proportion on intact acrosome (76.20 ± 1.24%) in comparison with FEN10 (58.20 ± 1.24%), FEN20 (47.20 ± 1.24%), and FEN30 (41.20 ± 1.24%) with significant difference among the three treatment groups as shown in (Table 1). The proportion of intact DNA was higher in the control (86.74 ± 0.79%) group and FEN10 (78.45 ± 2.09%) compared with FEN20 (76.45 ± 1.15%) and FEN30 (77.45 ± 1.15%) without significant difference between FEN20 and FEN30 (Table 1).

Sperm kinematics

Table (2) represents the effect of the added FEN powder extract on the sperm kinematics of frozen-thawed buffalo bull semen. The results obtained revealed that enriching the cryopreservation extender of buffalo bull semen with FEN improved the proportions of DAP, DSL, and BCF but it has no effect on the proportions of DCL and LIN or the value of VAP. Notably, FEN30 had the greater values for VCL (58.08 ± 1.81 µm/s) and VSL (58.08 ± 1.81 µm/s) compared with their counterparts in the control, FEN10 and FEN 20 groups. STR% of FEN10 (0.71 ± 0.01%) was higher that of FEN20 (0.71 ± 0.01%) and similar to those of FEN30 (0.73 ± 0.01%) and the control (0.73 ± 0.01%) groups. The low proportion of WOB was recorded in FEN10 (0.51 ± 0.01%) compared with other groups. Both control (3.42 ± 0.07%) and FEN30 (3.66 ± 0.13%) groups had higher % of ALH in comparison with FEN10 (2.42 ± 0.04%) and FEN20 (2.77 ± 0.20%) as shown in (Table 2).

Seminal levels of total protein, enzymatic antioxidants and lipid peroxidation marker

The levels of total protein, enzymatic antioxidants and the lipid peroxidation marker of frozen-thawed buffalo bull straws enriched with different concentrations of FEN powder extract are illustrated in (Table 3). The findings showed that neither total protein nor CAT are affected by any concentration of the added FEN powder extract. The SOD activity was higher ($P < 0.05$) in FEN10 (10.12 ± 0.50 U/mL) than the control (5.65 ± 0.72 U/mL), FEN20 (6.85 ± 0.52 U/mL) and FEN30 (6.68 ± 0.50 U/mL) groups. FEN10 (3.62 ± 0.89 mU/mL) and FEN20 (2.98 ± 0.44 mU/mL) had greater GPX activity in comparison with control (1.46 ± 0.41 mU/mL) and FEN30 (1.13 ± 0.28 mU/mL) as depicted in (Table 3). The MDA content was lower ($P < 0.05$) in FEN20 (21.50 ± 0.72 nmol/mL) and FEN30 (22.5 ± 0.72 nmol/mL) than the control (26.80 ± 0.78 nmol/mL) and FEN10 (29.20 ± 0.48 nmol/mL) groups.

In vivo fertility rate

The pregnancy rate following AI using our prepared frozen-thawed straws of buffalo-cows heifers subjected to estrus synchronization is presented in (Table 4). It was noticed that the pregnancy rate was significantly higher ($P < 0.05$) in FEN10 (66.66) than the control group (33.33) and FEN20 (33.33). While it was the lowest (5.55) in FEN30 compared with all experimental groups.

Discussion

Buffalo bull spermatozoa are more prone to freezing and thawing cryodamage than bull spermatozoa, which might be the reason for their low fertility rate [41]. The freezing and thawing procedures result in reduced post-thaw sperm motility, viability, and acrosome integrity [42]. However, several mechanisms have been proposed to explain frozen-thawed semen's lower fertility; cryopreservation-induced capacitation-like alterations assumption have lately gained more investigations [43, 44]. Buffalo spermatozoa have significant quantities of polyunsaturated fatty acids, which are subjected to lipid peroxidation generated by oxidative stress during freezing and thawing procedures [45]. MDA production in semen samples is directly proportional to the amount of polyunsaturated fatty acids in the sperm plasma membrane [46]. Oxidants, such ROS, influence the post-thaw sperm quality as ROS degrade sperm quality, rendering it incapable to fertilize an oocyte [21]. Further, they have a negative impact on sperm DNA, motility, capacitation, acrosome reaction and the subsequent production of male pronuclei following fertilization [47].

To our knowledge, only a few papers have addressed the effect of fennel on the post-thaw

quality of boar [27] and ram [26] semen only. Our study is unique to investigate the effect of freeze-dried fennel powder extract on the post-thaw quality and *in vivo* fertility of buffalo bull spermatozoa.

The presented study revealed that the concentration of FEN20 significantly improves post thaw sperm cell total motility, progressive motility, viability, and plasma membrane integrity. Our results come in concordance with Najafi *et al.* [26] who proven that the added FEN to ram semen extender improved several sperm parameters, including viability, motility, kinetics, plasma membrane integrity. However, Najafi *et al.* [26] found that the most suitable fennel extract concentration for the cryopreservation of ram semen was 10 µg/mL at a concentration of 200 × 10^6 sperm/mL; our result reported that FEN20 is the most effective fennel extract concentration added to buffalo extender at sperm cell concentration 15×10^6 sperm/mL.

This positive effect of the added fennel powder extract might be due to the presence of flavonoids, one of the main active components of fennel [25]. Flavonoids are able to interact with the lipid bilayer, but their antioxidant activity specific mechanism is still not well explained [48] due to their permeability across membranes and their membrane affinity, which depends on the degree of hydroxylation, the molecular configuration and the length of the side chain [49]. In the present study, FEN30 has deleterious effect on total motility, progressive motility, viability, plasma membrane integrity, and DNA integrity which might be due to one or more of the following possibilities: the first one, excessive levels of antioxidant in the extender can neutralize the oxidative stress and also affect the normal sperm function associated with ROS. Therefore, it is important to use the proper antioxidant with proper concentration [50, 51].The second possibility is the excessive removal of intracellular free radicals, or the third possibility attributed to the direct toxic effect [52].

Chromatin damage arises from oxidative stress during cryopreservation [53]. Herein, adding fennel to cryopreservation medium media in higher concentration (rather than FEN10) doesn't have significant effect on sperm DNA integrity although one of the main active components of fennel is rosmarinic acid [25]. On contrary, Yeni *et al.* [52] reported that adding rosmarinic acid in concentration of 25 and 50 µg/mL into semen extender exhibited favorable chromatin integrity. This discrepancy might be due to differences in animal species, breed, semen collection methods, evaluation methods, the used semen extender, the freezing protocol, and the source of rosmarinic acid.

The integrity of the sperm plasma membrane is critical for sperm cell survival and fertilization. Furthermore, for effective fertilization, the sperm cell must maintain an intact acrosome until it connects to the oocyte's zona pellucida and achieves the acrosome reaction, and releases acrosome enzymes [54]. Surprisingly, in the current study, the added fennel extract powder has an adverse dose-dependent effect on the acrosomal membrane integrity of frozen-thawed buffalo bull spermatozoa.

In our study, the added fennel has no effect on either seminal total protein concentration and CAT activity following cryopreservation. Seminal plasma proteins are important for sperm function and are known to be affected by freezing-thawing processes, but the addition of antioxidants like fennel extract aims to mitigate such damage, possibly maintaining protein integrity [55, 56]. And so, adding of fennel extract to cryopreserved semen sample aid in reduction of intracellular reactive oxygen species (ROS) post-thawing, indicating antioxidant protection that may help preserve protein function indirectly [57]. Studies on birds and mammals have shown that cryopreservation dramatically lowers CAT activity in seminal plasma [58]. However, fennel extract helps maintain the CAT level in post-thawed seminal plasma when added to cryopreserved medium.

The reduced freezability of buffalo bull spermatozoa can be related to different lipid compositions in their plasma membrane, making it more vulnerable to damage during cryopreservation [59] because their plasma membrane contains high levels of polyunsaturated fatty acids, particularly arachidonic and docosahexaenoic acids. This lipid content makes buffalo bull spermatozoa more susceptible to peroxidative damage caused by ROS, resulting in reduced sperm cell's functions [60]. In the current trial, only FEN10 has higher SOD, but both FEN10 and FEN20 have the higher GPX activity. Which is consistent with Torkamanpari *et al.* [57] who reported that supplementing semen cryopreservation media with fennel extract at a concentration of 10 mg/L for human sperm improves the antioxidant defense system, particularly by increasing SOD activity in seminal plasma. Similarly, Choi and Hwang. [61] found that feeding fennel extract to mice at a level of 200mg/kg body weight significantly increased plasma SOD and CAT activity. GPX is one of the most important antioxidant enzymes in sperm cells that transform peroxide (H_2O_2) and superoxide ($O_{2\text{-}}$) radicals into H_2O and $O_{2\text{-}}$ [62].

MDA serves as a biomarker of lipid peroxidation and implicated in male infertility [63]. In our report, FEN20 and FEN30 have the lowest MDA content than control and FEN10. Although, Najafi *et al.* [26] and Malo *et al.* [27] reported that adding of fennel extract to cryopreserved ram and boar semen, resulted in decreasing post thawed seminal plasma MDA level. On the other hand, Najafi *et al.* [26] found that the best fennel concentration is 10 µg/mL this come in opposite to our results.

In vivo fertility is the most effective approach for determining the success of an additive into the semen extender. Our results revealed that FEN10 had the highest conception rate compared to control, FEN20, and FEN30. These findings may be attributable to the fact that the most effective sperm quality parameters in sperm cell fertility are progressive motility, plasma membrane integrity, and acrosomal membrane integrity [64]. Furthermore, in our provided data, there was no significant variation in motility % and plasma membrane integrity between FEN10 and FEN20. In contrast, FEN10 had greater % of intact acrosome than FEN20, which improved the fertilizing ability of FEN10 over that of FEN20.

Conclusion

In conclusion, adding a medium concentration (FEN20) of fennel extract powder to buffalo bull semen cryopreservation extender bumped post-thaw sperm total motility, progressive motility, viability, intact plasma membrane, and decrease lipid peroxidation (MDA) levels. Surprisingly, incorporating a low concentration (FEN10) of fennel extract powder into the cryopreservation extension of buffalo bull semen increased post-thaw in vivo fertility. Therefore, further research is needed to assess the influence of fennel powder extract on sperm cell properties.

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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 fennel (n = 12).

Parameter (%)	CONT (0 µL/mL)	Fennel (µL/mL)		
		10	20	30
Total motility	67.70 ± 3.09^{c}	69.26 ± 1.73^{bc}	77.07 ± 2.51^{ab}	69.43 ± 1.75^{bc}
Progressive motility	33.43 ± 2.42^{ab}	22.79 ± 0.85^{d}	39.27 ± 2.15^{a}	30.91 ± 2.20^{bc}
Viability	67.16 ± 2.71^{b}	73.03 ± 1.26^{b}	85.20 ± 1.24^{a}	51.37 ± 1.32^{c}
Intact-plasma membrane	61.20 ± 0.62^{b}	66.95 ± 3.17^{ab}	69.95 ± 1.92^{a}	51.95 ± 2.02^{c}
Intact acrosome	76.20 ± 1.24^{a}	58.20 ± 1.24^{b}	47.20 ± 1.24^{c}	41.20 ± 1.24^{d}
Intact-DNA	86.74 ± 0.79^{a}	78.45 ± 2.09^{a}	76.45 ± 1.15^{b}	77.45 ± 1.15^{b}

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

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

Parameter	CONT (0 µL/mL)	Fennel (µL/mL)		
		10	20	30
DAP (%)	14.95 ± 0.14^{b}	16.49 ± 0.34^{a}	16.13 ± 0.53^{ab}	16.47 ± 0.43^{a}
DCL (%)	21.51 ± 0.20^{a}	24.02 ± 0.41^{a}	23.62 ± 1^{a}	24.16 ± 0.76^{a}
DSL (%)	10.77 ± 0.14^{b}	12.22 ± 0.22^{a}	11.49 ± 0.28^{ab}	12.06 ± 0.24^{a}
VAP (µm/s)	36.82 ± 0.36^{a}	37.15 ± 0.55^{a}	37.32 ± 1.56^{a}	39.71 ± 0.93^{a}
VCL (µm/s)	36.83 ± 0.35^{b}	37.14 ± 0.56^{b}	38.37 ± 1.37^{b}	58.08 ± 1.81^{a}
VSL (µm/s)	26.77 ± 0.32^{b}	27.62 ± 0.3^{b}	26.65 ± 0.93^{b}	58.08 ± 1.81^{a}
STR (%)	0.73 ± 0.01^{ab}	0.74 ± 0.01^{a}	0.71 ± 0.01^{b}	0.73 ± 0.01^{ab}
LIN (%)	0.51 ± 0.01^{a}	0.51 ± 0.01^{a}	0.49 ± 0.01^{a}	0.5 ± 0.02^{a}
WOB (%)	0.7 ± 0.01^{a}	0.51 ± 0.01^{b}	0.69 ± 0.01^{a}	0.68 ± 0.01^{a}
BCF (%)	15.26 ± 0.58^{c}	21.78 ± 0.88^{a}	20.47 ± 1.07^{ab}	18.91 ± 0.61^{ab}
ALH (%)	3.42 ± 0.07^{a}	2.42 ± 0.04^{b}	2.77 ± 0.2^{b}	3.66 ± 0.13^{a}

Within the same row means bearing one common superscript were non-significantly different at $P < 0.05$. CONT = Control, FEN = fennel, 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. Total protein, enzymatic antioxidants and lipid peroxidation marker (mean ± SEM) of frozen-thawed buffalo bull semen supplemented with different proportions of fennel (n = 12).

Parameter	CONT (0 µL/mL)	Fennel (µL/mL)		
		10	20	30
Total protein (g/dL)	14.05 ± 0.34[a]	14.22 ± 0.40[a]	14.13 ± 0.39[a]	13.95 ± 0.45[a]
SOD (U/mL)	5.65 ± 0.72[b]	10.12 ± 0.50[a]	6.85 ± 0.52[b]	6.68 ± 0.50[b]
CAT (mM/L)	31.38 ± 0.41[a]	30.13 ± 2.79[a]	33.26 ± 1.89[a]	30.06 ± 3.21[a]
GPX (mU/mL)	1.46 ± 0.41[b]	3.62 ± 0.89[a]	2.98 ± 0.44[a]	1.13 ± 0.28[b]
MDA (nmol/mL)	26.80 ± 0.78[a]	29.20 ± 0.48[a]	21.5 ± 0.72[b]	22.5 ± 0.72[b]

Within the same row means bearing one common superscript were non-significantly different at P < 0.05. CONT = Control, FEN = fennel, 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 fennel powder.

Parameter	CONT (0 µL/mL)	Fennel (µL/mL)		
		10	20	30
Inseminated buffalo-cows (n)	21	21	21	21
Pregnant buffalo-cows (n)	7	14	7	1
Pregnancy rate (%)	33.33[b]	66.66[a]	33.33[b]	5.55[c]
P - value				**< 0.0001**
Chi-square value				**27.0321**

CONT = Control, FEN = Fennel

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

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الملخص

أظهرت حيوانات منوية ثيران الجاموس درجة أعلى من التلف بالتبريد مقارنةً بحيوانات منوية الثيران الأخرى، مما أدى بدوره إلى انخفاض معدلات الخصوبة بعد الذوبان. ويُعزى ضعف قابلية حيوانات منوية ثيران الجاموس للتجميد إلى التركيب الدهني المتميز لغشاء البلازما مقارنةً بحيوانات الأنواع الأخرى، مما يجعلها أكثر عرضة للتلف أثناء الحفظ بالتبريد. ويسعى علماء الإنجاب إلى تقليل هذا التلف الحتمي بالتبريد من خلال إضافة إضافات خاصة إلى مُوسِّع السائل المنوي المستخدم لحفظ حيوانات منوية ثيران الجاموس بالتبريد. ويُعدّ الشمر خيارًا واعدًا لهذه الإضافات، حيث أن أقوى مضادات الأكسدة فيه هي الروزمارينيك والفلافونويد والأنيثول. هدفت هذه الدراسة إلى دراسة الآثار المحتملة لإضافة تراكيز مختلفة من مسحوق الشمر المجفف بالتجميد على جودة السائل المنوي بعد الذوبان، ونشاط مضادات الأكسدة الإنزيمية، وخصوبة الحيوانات المنوية لثيران الجاموس داخل الجسم الحي. جُمعت القذفات من سبعة ثيران جاموس مصرية سليمة باستخدام مهبل صناعي مرتين أسبوعيًا. جُمعت القذفات عالية الجودة ووُزعت في أربعة أجزاء؛ تم تخفيف كل جزء عليقي باستخدام موسّع سترات صفار البيض من تريس المحتوي على: 0 كعنصر تحكم (CTRL)، و10 (FEN10، و20 (FEN20) و30 (FEN30) ميكرولتر/مل من مسحوق الشمر المجفف بالتجميد، وتم تبريده وتوازنه وتحميله في مصاصات صغيرة قبل تجميده وتخزينه في النيتروجين السائل عند درجة حرارة -196 درجة مئوية. وتم إذابة المصاصات المجمدة في حمام مائي عند درجة حرارة 37 درجة مئوية لمدة 30 ثانية وفحصها من حيث حركة الحيوانات المنوية وقابليتها للحياة وسلامة الغشاء البلازمي وسلامة الأكروسوم وسلامة الحمض النووي. وتم تحديد مستويات البروتين الكلي في السائل المنوي، وفائق أكسيد ديسميوتاز (SOD)، والكاتالاز (CAT)، والغلوتاثيون بيروكسيديز (GPX) والمالونالديهايد (MDA). أظهرت النتائج أن FEN20 حسّن بشكل ملحوظ ($P < 0.05$) نسب الحركة الكلية، والحركة التقدمية، والحيوية، وسلامة الغشاء البلازمي. ومن المثير للاهتمام، أن المجموعة الضابطة أظهرت نسبًا أعلى لسلامة الغشاء الأكروسومي وسلامة الحمض النووي. ولم يُلاحظ أي اختلاف كبير بين المجموعة الضابطة وجميع المجموعات المعالجة في مستوى البروتين الكلي وCAT. بينما كان نشاط SOD وGPX وMDA أعلى بشكل ملحوظ ($P < 0.05$) في FEN10. وقد حقق FEN10 معدل خصوبة أعلى في الجسم الحي مقارنةً بمجموعات الضبط، وFEN20، وFEN30.

الكلمات الدالة: الجاموس المصري، خصائص الحيوانات المنوية، الشمر، مضادات الأكسدة الإنزيمية، التجميد والذوبان، الخصوبة في الجسم الحي.