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Green tea extract supplementation optimizes sperm quality, DNA integrity, and antioxidant activity of cryopreserved buffalo bull (*Bubalus bubalis*) semen

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Abstract

Background and Aim: Freezing and thawing processes increase the susceptibility of spermatozoa to oxidative stress, which negatively impacts sperm cells functions and reduces its potential fertilizing capacity. The current study aimed to explore the ameliorative effects of various proportions of GTE on post-thawing buffalo bull spermiograms, DNA integrity, and antioxidant activities.

Methods: Phytochemical analysis of GTE was done by high-performance liquid chromatography (HPLC). Seventy-two ejaculates were collected from fertile buffalo bulls weekly (n = 12) for 6 weeks, diluted in a tris-based extender supplemented with GTE (0%, 0.1%, 0.5%, 0.75%, 1.0%, and 2.0%) and assessed for sperm motilitiy characterestics, acrosome, plasma membrane and DNA integrities, as well as for the activities of antioxidant enzymes (superoxide dismutase, glutathione reduced, catalase, and total antioxidant activity) and lipid peroxidation (MDA).

Results and conclusion: Phytochemical analysis of GTE by high-performance liquid chromatography (HPLC) indicated the presence of five high polyphenol content with high accounting for epigallocatechin-3 gallate and epicatechin. Computer-assisted sperm analysis (CASA) showed the highest total and progressive motility values, and wobble coefficient (WOB) was obtained with 0.75% GTE. At the same time, the distance of average path (DAP) was enhanced with 2.0% GTE compared to the control. Incorporation of 0.75% GTE significantly ($P< 0.001$) increased acrosome and plasma membrane integrities, and reduced the percentage of DNA-damage, tail length, and tail moment of frozen-thawed buffalo bull spermatozoa. Activities of antioxidant enzymes (superoxide dismutase, glutathione reduced, catalase, and total antioxidant activity) improved with 0.75% GTE, whereas lipid peroxidation (MDA) was reduced significantly ($P< 0.001$) with 0.5%, 0.75%, and 1.0% GTE compared to control. In conclusion, the addition of 0.75% GTE in buffalo bull semen diluted with a tris-based extender ameliorated the cryodamage influence on sperm parameters as well as reduced DNA damage with optimum antioxidant capacity.

Keywords: Buffalo bull; Cryopreservation; Green tea; HPLC; Sperm kinetics.

1. Introduction

Sperm cryopreservation effectively preserves male fertility in humans and animals (Sharma, 2011). The storage of frozen semen can maintain the motility and viability of spermatozoa in a controlled environment (Morrell et al., 2018). However, the freezing process may cause physical damage to spermatozoa and result in a reduction in the number of viable sperm (Kaka et al., 2016; Ugur et al., 2019).

The freezing and thawing processes increase the susceptibility of spermatozoa to oxidative stress, which negatively impacts sperm cells functions and reduces sperm motility, in addition to the integrity of the acrosome, plasma membrane, and DNA in buffaloes (Ahmed et al., 2019). Additionally, the level of essential antioxidants protecting spermatozoa against the reactive oxygen species ROS decreases during the freezing process (Sikka, 2004). Therefore, the balance between the endogenous antioxidants and the excessive production of ROS is impaired (Wathes et al., 2007).

The generation of ROS in ruminant semen following the freezing-

thawing process primarily results from activating L-amino acid oxidase in cryopreserved or dead sperm (Peris-Frau et al., 2020). This activation leads to lipid peroxidation (LPO) (Kumaresan et al., 2006). More specifically, LPO occurs due to the interaction between ROS molecules—such as superoxide, hydroxyl radicals, nitric oxide, and hydrogen peroxide—and the lipid components of the plasma membrane (Gundogan et al., 2010).

The concentration of polyunsaturated fatty acids (PUFAs) in the plasma membranes makes buffalo spermatozoa more susceptible to cryodamage and oxidative stress (Tvrdá et al., 2016). Consequently, the structural and functional integrity of buffalo spermatozoa are significantly compromised during the freezing-thawing process (Gundogan et al., 2010).

A low concentration of ROS is essential for sperm capacitation and acrosomal reactions, both of which are regulated by redox processes (free radical regulation) that allow sperm to fertilize an ovum (Aitken, 2017). However, elevated levels of ROS can have a genotoxic effect, leading to decreased sperm motility, impaired DNA integrity, and reduced antioxidant capacity. These effects ultimately result in lower sperm quality in buffalo bulls (Ahmed et al., 2020a).

Supplementation with exogenous antioxidants is an effective strategy to mitigate excess ROS during freezing and thawing protocols (Ahmed et al., 2019). Recently, plant extracts have gained popularity as natural antioxidants in reproductive technologies (Avdatek et al., 2018). Green tea extract (GTE) is rich in natural antioxidants, including polyphenols (Rahman et al., 2018), vitamins C and E, and essential minerals like zinc and selenium (Faridullah et al., 2015). The polyphenols in green tea, particularly epigallocatechin-3-gallate (EGCG), not only scavenge free radicals and chelate metal ions through reactions with hydrogen molecules but also enhance the antioxidant capacity (Hosen et al., 2015; Plaza Dávila et al., 2015).

Additionally, GTE provides strong antioxidant activity (Wu et al., 2013) that reduces ROS production and helps alleviate protein carbonylation, LPO, and DNA damage in buffalo spermatozoa (Martin-Hidalgo et al., 2019; Tvrda et al., 2019). Therefore, GTE is proposed as a protective agent for spermatozoa against oxidative stress, potentially improving the post-thaw quality of buffalo spermatozoa (Ahmed et al., 2020b; Feuillie et al., 2014).

This study aimed to investigate the antioxidant activity of GTE and its effects of on the structure and function of frozen and thawed buffalo bull semen, and focusing on DNA integrity, antioxidant activity, and oxidative stress.

2. Materials and methods

This study was conducted at the Animal Reproduction Research Institute (ARRI) within the Agriculture Research Center (ARC), in collaboration with the Department of Theriogenology, Faculty of Veterinary Medicine, at Benha University, Egypt. All experiments were supervised by the Ethics Committee of the Faculty of Veterinary Medicine, Benha University (BUFVTM 04–02-23).

2.1. Green tea methanol extract preparation.

The methanolic extraction of GTE was carried out following a standardized protocol described by Chan et al. (2007) and Gale et al. (2015). At room temperature (25 °C), green tea leaves were ground into a fine powder, and eight grams of this powder were soaked in 400 mL methanol for 24 hours. After the soaking period, the mixture was centrifuged at 1500 rpm for 30 minutes. The supernatant was carefully separated, filtered through filter paper (8~10 µm), and stored at −20 °C until used.

2.2. Green tea extracts polyphenols detection using high-performance liquid chromatography (HPLC).

Analysis was conducted using the Agilent 1260 series. The separation was performed using a Zorbax Eclipse Plus C8 column (with an inner diameter of 4.6 mm x 250 mm in length, and a particle size of 5 µm). The mobile phase was composed of water and 0.05% trifluoroacetic acid in acetonitrile, referred to as phase A, and was delivered at a flow rate of 0.9 mL/minute. The mobile phase was arranged in a linear gradient, exactly as follows: 0 minute (82% A); 0–1 minute (82% A); 1–11 minutes (75% A); 11–18 minutes (60% A); 18–22 minutes (82% A); and 22–24 minutes (82% A). The multi-wavelength detector was set to examine at a wavelength of 280 nm. The entire injection process was completed within this framework (5 µL for every sample solution). The column's temperature was maintained at 40 °C (Khoddami et al., 2013).

2.3. Determination of green tea extract (GTE) antioxidant activity by 2,2-diphenyl picryl hydrazyl (DPPH) radical scavenging method.

The *in vitro* antioxidant activity of the GTE was estimated by using the bleaching of a purple-colored methanolic solution of 2,2-diphenyl picryl hydrazyl (DPPH) (Gulluce et al., 2004). Various amounts of extract (40, 80, 120, 160, and 200 µg) were added to 1 mL of a 0.004% (2 mM) methanolic DPPH solution. The mixture was incubated for 30 minutes at room temperature, the absorbance was measured at a wavelength of 517 nm by a spectrophotometer, and a blank was used as a reference. Inhibition of the free radical DPPH (%) was measured using this equation according to Gulluce et al. (2004). Antioxidant activity (AA) = (AC – AS) / AC x 100. AC: Control reaction absorbance. AS: Testing sample absorbance.

2.4. Semen collection and cryopreservation.

Twelve healthy, mature Egyptian buffalo bulls, aged 5 to 6 years, were used for semen collection from January to March 2023. These bulls were housed individually at the Animal Reproduction Research Institute's (ARRI) farm in Giza, Egypt under an ideal housing, nourishment, and lighting system. An ejaculate was collected weekly from each bull for six weeks using a pre-warmed bovine artificial vagina (Neustadt/Aisch, Müller, Nürnberg, Germany). Semen samples with motility greater than 70%, viability greater than 75%, and concentration exceeding (800×10^6 sperm/mL) were pooled. The pooled semen was diluted with a Tris-based egg yolk extender with a pH of 6.8. tris extender was composed of 375 mM Tris, 41.6 mM glucose, 124 mM citric acid, 20% (v/v) egg yolk, 7% (v/v) glycerol, 25 mg gentamicin, and 500 IU/mL penicillin, all dissolved in 100 mL of deionized water) (Shokry et al., 2024). The chemicals used were sourced from Sigma-Aldrich (Burlington, Massachusetts, USA).

The diluted semen with a dilution rate of a proximal 1:5 was divided into six equal aliquots, to which the GTE was added at the proportions of 0%, 0.1%, 0.5%, 0.75%, 1.0 %, and 2.0 % to reach a final concentration of 20×10^6 sperm/straw. In a cold cabinet (IMV, France), the diluted semen samples were cooled to 5 °C within 2 hr. After cooling, the samples were equilibrated in a cold cabinet at 5 °C for 4 hrs then were filled into 0.25 mL polyvinyl straws (IMV, France). Straws were then arranged horizontally on a chilled rack and subjected to liquid nitrogen (LN_2) vapors for 15 minutes. Finally, the straws were immersed in LN_2 tanks at −196 °C (Khalifa, 2001).

2.5. Post-thaw semen quality

Frozen straws (6 straws/treatment/week) were placed in a water bath at 37 °C for 30 seconds and then examined for sperm kinematics using CASA, acrosomal integrity, and plasma membrane integrity were evaluated.

2.5.1. Post-thaw sperm motion characteristics, velocity, and kinematics.

Computer-assisted sperm analysis (CASA; CEROS II, version 1.10; Hamilton Thorn Beverly) is used to evaluate sperm motility and kinematics (Naz et al., 2018). A total of 200 individual spermatozoa were analyzed for their motion characteristics and CASA motility [total motility (TM), progressive motility (PM), distance of average path (DAP), distance curved line (DCL), distance straight line (DSL), average path velocity (VAP), curved liner velocity (VCL), straight liner velocity (VSL), straightness (STR), linearity (LIN), wobble (WOB), and beat cross frequency (BCF)] for each semen sample.

2.5.2. Post-thaw sperm acrosomal integrity

Silver nitrate staining was used to determine the acrosome integrity of post-thawed buffalo bull spermatozoa according to, Chinoy et al. (1992). A thin semen film was fixed using 70% and 95% ethyl alcohol for 2 minutes per concentration. Subsequently, it was stained with silver nitrate dye for two hours in an incubator heated to 65 °C and humidity 100%. After staining, the semen films were rinsed with distilled water and allowed to dry at room temperature. A light microscope at 100× (Olympus-Bx50) was used to evaluate the acrosomal membrane integrity.

2.5.3. Post-thaw sperm plasma membrane integrity

The plasma membrane integrity was evaluated by the hypo-osmotic swelling test (HOST) according to **Akhter et al. (2008)**. Briefly, 50 µL of frozen-thawed semen sample was incubated with 500 µL of hypo-osmotic swelling solution (0.735 g sodium citrate and 1.35 g fructose dissolved in 100 mL of distilled water, (osmolality, 190 mOsmol/L) (Gonotec Osmomate 030 D Osmometer, Germany). The mixture was incubated at 37 °C for 30 minutes. The slides were examined under the phase contrast microscope at 400× (Olympus Bx40, Japan). The percentage of spermatozoa exhibiting inflated and curled tails was registered as affirmative to the intact plasma membrane.

2.5.4. Post-thaw sperm DNA integrity

To assess DNA integrity, 6 straws/treatment per week were thawed in a water bath at 37 °C, then pooled and subsequently centrifuged at 1500 rpm for 15 minutes. The sperm pellets were recovered to be assessed by the neutral COMET test (Boe-Hansen et al., 2005). The slides were examined under a fluorescence microscope (Olympus), Japan, at a magnification of 400×. DNA fragmentation was assessed using the TriTek Comet-Score, image analysis (Ver. 1.5, USA), which evaluates the following parameters: percentage of DNA fragmentation, tail length in micrometers (The length of migrated DNA from the nuclear core in the comet's head's leading edge to the tail's leading edge), percentage of tail DNA (the fraction of total DNA present in the tail of migrated DNA), and tail moment is a measure that combines tail length and migrated DNA quantity (measured by multiplying tail length by the DNA percentage in the tail (Fraser, 2004). DNA-damage was recognized by a fragmented DNA tail, resulting in a ‘comet' pattern. In contrast, sperm heads that did not exhibit this comet pattern were not considered damaged.

2.5.5. Post-thaw antioxidant biochemical

In a water bath at 37 °C, six straws/treatment per week were thawed, pooled together, and centrifuged at 1500 rpm for 15 minutes. Seminal plasma was collected and analyzed for various parameters, including superoxide dismutase (SOD), glutathione reduced (GSH), catalase (CAT), total antioxidant capacity (TAC), and malondialdehyde (MDA), as described elsewhere by operating commercial kits (Bio Diagnostic, Cairo, Egypt) (SD 25 21, GR 25 11, CA 25 17, TA 25 13, and MDA 25 29, respectively) at the following wavelengths: 560 nm for SOD (Campos-Shimada et al., 2020), 510 nm for GSH (Ognjanović et al., 2008), 405 nm for CAT (Bendou et al., 2021), 505 nm for TAC (Koracevic et al., 2001), and 534 nm for MDA (Partyka et al., 2012).

2.6. Statistical analysis

The data were presented as mean ± SEM. The data were evaluated using the Shapiro-Wilk test and were found to follow a normal distribution. SPSS software (IBM® SPSS® Statistics, version 25) was used to analyse the data using one-way ANOVA followed by Duncan's multiple range test to compare means between groups.). The significance level for statistical testing was marked at $P< 0.05$.

3. Results

1. Analysis of polyphenols content and in vitro antioxidant activity of GTE

An efficient HPLC analysis of green tea extract clearly shows the polyphenol content (Fig. 1A**)**, with the highest area accounting for epigallocatechin-3 gallate content (77.1%) and epicatechin (12.70%). The other components were catechin (1.07%), coumaric acid (9.51%), gallic acid (5.52%), coffeic acid (1.05%), syringic acid (0.28%), rutin (0.38%), ellagic acid (1.31%), vanillin (0.01%), naringenin (0.04%), rosmarinic acid (0.16%), daidzein (0.16%), querctin (0.05%), cinnamic acid (0.07%), kaempferol (0.06%), and hesperetin (0.11%). The *in vitro* antioxidant capacity of GTE increased gradually as its concentration increased ($y = 0.1551x + 39.533$), and the IC_{50} of DPPH for the corresponding GTE was 67.5 µg/mL, as seen in Fig.1B.

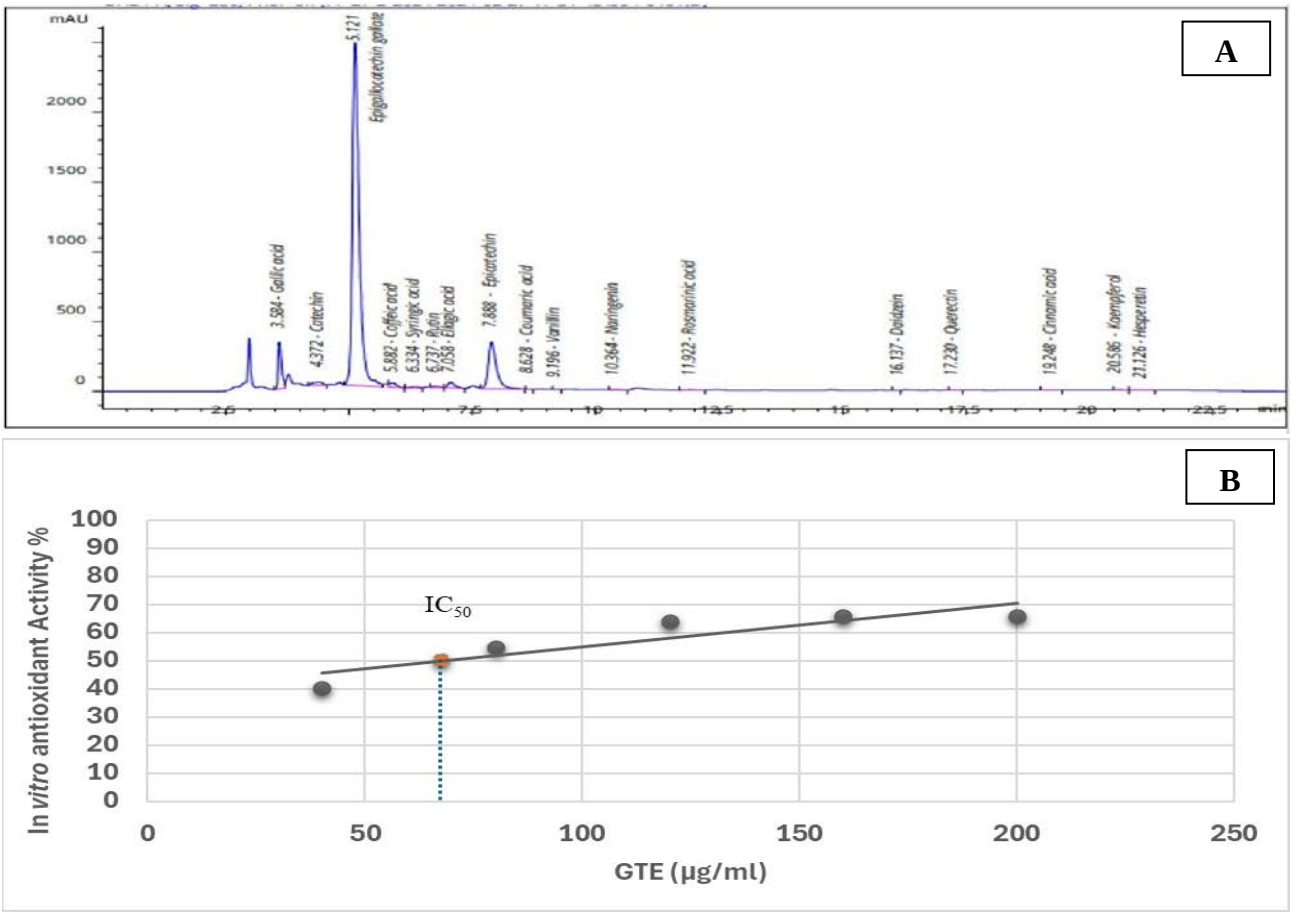

Figure 1. Green tea extract (GTE) polyphenol content and antioxidant activity. A: GTE's chromatogram; B: GTE's DPPH radical scavenging activity in *vitro*. The dashed line refers to half maximum inhibitory concentration (IC_{50}) of GTE.

3.2. Effect of GTE on motion kinetics, acrosomal and plasma membrane integrities of buffalo bull frozen-thawed semen.

Data in Table 1 showed that when GTE was added to buffalo bull cryo-diluent media at concentrations of (0.1–2.0%) enhanced TM and VAP, while PM increased at concentrations (0.1–1.0%) (P< 0.001). Compared to the control group WOB was significantly (P< 0.01) higher at concentrations (0.1–0.75%). Similarly, the addition of 2% GTE to the buffalo bull semen extender significantly (P< 0.001) improved DAP. The acrosomal and plasma membrane integrities are significantly (P< 0.001) increased by the addition of GTE at a concentration of 0.75%.

3.3. Effect of GTE on DNA damage of buffalo bull spermatozoa after freezing-thawing.

Adding (0.1–2.0%) GTE to buffalo bull semen extender significantly (P< 0.001) reduced post-thawed sperm DNA fragmentation, tail length, and tail moment compared to the control, especially at a concentration of 0.75% of GTE which showed the lowest value of DNA-damage, tail length, and tail moment (Table 2 and Fig. 2).

3.4. Effect of GTE on antioxidant defense mechanism and LPO of buffalo bull spermatozoa after freezing-thawing

Table 3 showed a marked (P< 0.001) elevation in seminal plasma antioxidants following GTE supplementation in buffalo semen extender. Involvement of 0.1–2% GTE on buffalo semen extender increased activity of SOD. Furthermore, the inclusion of GTE at a dose of 0.75% enhanced the activities of GSH, CAT, and TAC compared to the control group and other doses of GTE. On the other hand, the addition of GTE at doses of 0.5%, 0.75%, and 1% results in a low MDA level (P< 0.001) as shown in Table 3.

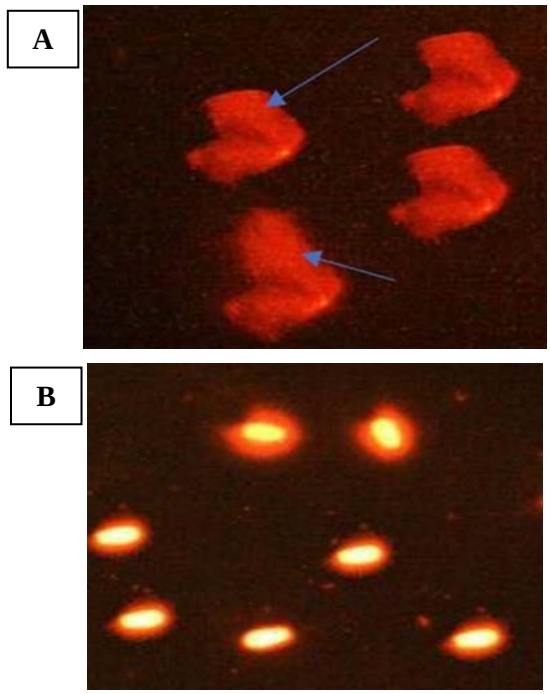

Figure 2: Post-thawed buffalo spermatozoa after the single cell gel electrophoresis (Comet assay). A: Control; B: 0.75% green tea extract. Blue arrows show comet tail.

Table 1. Effect of GTE on motion kinetics, acrosomal and plasma membrane integrities of buffalo bull spermatozoa after freezing-thawing

Parameter	Control	Dose of GTE					P value
		0.1%	0.5%	0.75%	1.0%	2.0%	
TM (%)	46.06±2.08^{b}	61.59±3.61^{a}	63.12±2.67^{a}	66.18±1.60^{a}	63.61±4.90^{a}	57.75±4.72^{a}	0.005
PM (%)	28.13±2.42^{d}	42.66 ±0.81^{c}	43.17±0.73^{c}	57.32±0.60^{a}	48.22±0.95^{b}	31.72±1.20^{d}	0.0001
DAP (µm)	15.33 ± 0.41^{b}	16.07 ±0.44^{b}	16.56±0.03^{ab}	16.04±0.29^{b}	15.37±0.34^{b}	17.83±0.78^{a}	0.004
DCL (µm)	25.04±1.46	24.46±1.02	24.42±0.72	24.66±0.07	25.59±0.40	27.21±0.95	0.30
DSL (µm)	11.73±0.44	11.27±0.30	11.45±0.20	12.75±1.30	10.68±0.04	12.03±0.69	0.33
VAP (µm/s)	34.94±1.66^{d}	39.91±0.23^{ab}	38.32±0.79^{bc}	38.12±0.64^{bc}	36.98±1.22^{C}	41.83±0.58^{a}	0.0001
VCL (µm/s)	56.98±3.71	59.47±1.35	57.43±2.62	58.03±1.66	59.79±1.82	64.02±1.49	0.40
VSL (µm/s)	26.8±1.19	28.32±0.10	26.87±0.28	29.95±2.38	25.17±0.62	28.36±1.00	0.12
STR (%)	0.77±0.03	0.70±0.01	0.70±0.01	0.78±0.07	0.70±0.01	0.67±0.02	0.14
LIN (%)	0.47±0.01^{b}	0.48±0.01^{ab}	0.48±0.02^{ab}	0.56±0.06^{a}	0.42±0.00^{b}	0.44±0.03^{b}	0.04
WOB (%)	0.62±0.03^{bc}	0.67±0.01^{a}	0.68±0.02^{a}	0.70±0.01^{a}	0.60±0.01^{c}	0.65±0.02^{abc}	0.01
BCF (Hz)	20.73 ±1.89	19.88±1.40	19.85±1.31	15.16±1.35	18.89±1.16	17.92±1.48	0.12
Acrosome integrity (%)	74.50 ± 1.89^{b}	78.83 ±3.13^{ab}	79.50±1.78^{ab}	85.17±2.07^{a}	77.60±3.31^{ab}	77.60±2.47^{b}	0.001
Membrane integrity (%)	37.00 ± 2.05^{c}	45.50 ±1.98^{b}	51.67±3.37^{b}	59.50±3.32^{a}	50.60±3.78^{b}	43.57±1.39^{bc}	0.001

TM, total motility; PM, progressive motility; DAP, distance of average path; DCL, distance curved line; DSL, distance straight line; VAP, average path velocity; VCL, curved liner velocity; VSL, straight liner velocity; STR, straightness; LIN, linearity; WOB, wobble; and BCF, beat cross frequency. Data presented as Mean ± SEM. Values carrying different superscript letters within the same row are significantly different at P< 0.05.

Table 2 Effect of GTE on DNA damage of buffalo bull spermatozoa after freezing-thawing.

Dose of GTE	DNA damage (%)	Tail length (µm)	Tail DNA (%)	Tail moment
Control	19.32 ± 0.66^{a}	16.62 ± 0.92^{a}	12.40 ± 1.40	2.08 ± 0.17^{a}
0.1 %	13.16 ± 0.58^{c}	13.20 ± 0.76^{bc}	10.58 ± 1.43	1.44 ± 0.27^{b}
0.5 %	12.48 ± 0.24^{cd}	11.27 ± 1.00^{cd}	9.85 ± 0.67	1.08 ± 0.03^{bc}
0.75 %	10.67 ± 0.37^{d}	8.99 ± 0.98^{d}	10.78 ± 1.19	0.78 ± 0.08^{c}
1.0 %	14.13 ± 0.47^{bc}	14.12 ± 1.55^{b}	8.67 ± 0.61	1.24 ± 0.14^{bc}
2.0 %	15.38 ± 1.09 ^{b}	10.94 ± 0.60^{cd}	10.95 ± 0.95	1.16 ± 0.13^{bc}
P value	0.0001	0.0001	0.29	0.0001

Data presented as Mean ± SEM. Values carrying different superscript letters within the same column are significantly different at P< 0.05. DNA damage % is the change in DNA composition. Tail length refers to the length of migrated DNA. Tail DNA percentage indicates the trailing damaged DNA streak. Tail moment is calculated by multiplying the tail length by the tail DNA percentage.

Table 3. Effect of GTE on antioxidant defence mechanism and lipid peroxidation of buffalo bull spermatozoa after freezing-thawing.

Dose of GTE	SOD (U/ml)	GSH(U/L)	CAT (U/L)	TAC (mmol/ml)	MDA (nmol/ml)
Control	17.44 ± 0.91^{d}	3.11 ± 0.20^{c}	1.85 ± 0.08^{c}	0.27 ± 0.03^{b}	12.94 ± 1.20^{a}
0.1%	21.26 ± 0.80^{c}	3.22 ± 0.27^{c}	2.42 ± 0.28^{bc}	0.24 ± 0.02^{bc}	10.53 ± 0.65^{ab}
0.5%	25.93 ± 1.02^{b}	4.84 ± 0.63^{b}	3.48 ± 0.18^{ab}	0.24 ± 0.02^{bc}	10.08 ± 0.61^{b}
0.75%	27.78 ± 0.86^{b}	6.35 ± 0.29^{a}	4.41 ± 0.54^{a}	0.37 ± 0.03^{a}	8.58 ± 0.65^{bc}
1.0%	30.48 ± 0.42^{a}	5.03 ± 0.57^{b}	3.56 ± 0.57^{a}	0.30 ± 0.00^{b}	7.39 ± 0.96^{c}
2.0%	20.98 ± 0.57^{c}	3.42 ± 0.33^{c}	2.40 ± 0.20^{bc}	0.19 ± 0.01^{c}	11.39 ± 0.47^{ab}
P value	0.0001	0.0001	0.0001	0.0001	0.001

Data presented as Mean ± SEM. Values carrying different superscripts letters within the same column are significantly different at P< 0.05. SOD, GSH, CAT, TAC, and MDA referred to superoxide dismutase, Glutathione reduced, catalase, total antioxidant capacity, and malondialdehyde, respectively.

4. Discussion

The endogenous protective antioxidant mechanism in sperm is responsible for shielding spermatozoa from the detrimental effects of LPO during cryopreservation and the subsequent thawing and post-thawing steps (Nichi et al., 2006). However, the antioxidant activity of these cells decreases significantly after cryogenic preservation (Bilodeau et al., 2000). Additionally, there is a primary lack of sperm cytoplasmic antioxidants to scavenge free radicals effectively. This, combined with disruption in the antioxidant system and ROS balance during the freezing regime, creates an unfavorable scenario for the characteristic features of buffalo bull spermatozoa, which contain high levels of PUFA (Aitken, 1995).

To avoid these risks, various natural antioxidants have been incorporated into the cryo-diluent media, including GTE, which is rich in (EGCG) and other polyphenols such as epicatechin-3-gallate (ECG), epi-gallocatechin (EGC), and epicatechin (EC) (Shivaprasad and Khanam, 2006; Rahman et al., 2018).

The results of the present study confirmed the potent antioxidant activity of GTE on buffalo semen. Adding GTE to the tri–base extender during the processing of frozen buffalo bull semen improved the characteristics of post-thaw buffalo semen. These findings were identical to those of Khan et al. (2017), who reported improved progressive motility and membrane integrity at 0.75% GTE in *Achai* bulls. Previous studies in dogs (Wittayarat et al., 2013), bulls (Ali et al., 2014), buffalo bulls (Ahmed et al., 2020a), humans (Alqawasmeh et al., 2021), and bucks (Mustofa et al., 2021) recorded a similar enhancement in cryopreserved semen quality after GTE supplementation in semen extenders.

The estimation of CASA motion characteristics serves as a reliable indicator of buffalo sperm fertility (Ahmed et al., 2016). In our investigation, sperm PM, TM, and WOB were greatly enhanced at 0.75% GTE. Additionally, GTE showed the highest value of DAP and VAP at a proportion of 2%. These findings were parallel with those reported by Tvrda et al. (2019) and Ahmed et al. (2020b).

The significant impact on post-thaw motility parameters may be attributed to the green tea polyphenols and antioxidant potentials of GTE, which eliminate reactive oxygen and nitrogen species and promote chelation (Frei and Higdon, 2003; Park and Yu, 2017; and Ahmed et al., 2020b). However, it is interesting to note that the addition of 0.1 mg/mL GTE to the extender of cow bull (İnanç et al., 2019) and 50 µg/mL GTE to the dog extender (Bucci et al., 2019) did not change the motility characteristics of spermatozoa. This lack of effect could be attributed to differences animal species, the dose of GTE, or variations in the setting of the CASA software.

Furthermore, undamaged acrosome and plasma membrane functionality are considered relevant indicators of the fertilization potential of buffalo bull spermatozoa to fertilize successfully (Ahmed et al., 2016; and Ahmed et al., 2020b). The current study noted that acrosome and plasma membrane integrities were significantly improved in cryo-diluent solutions fortified with 0.75% GTE compared to the control. Similar findings were reported in studies involving bulls (Khan et al. 2017; Tvrda et al. 2019), buffalo bulls (Ahmed et al., 2020 a&b), Simmental bulls (Susilowati et al., 2021), and kacang bucks (Mustofa et al., 2021). The positive effects of GTE are likely due to its flavonoid contents, which catalyze bilayers of lipids and enhance the functioning of membranes, besides the antioxidant action exhibited by polyphenol groups (Roychoudhury et al., 2017).

In contrast, adding GTE to the extender of ram (Gil et al., 2010), boars (Gale et al., 2015), dogs (Bucci et al., 2019), and bulls (İnanç et al., 2019) semen didn't improve the post-thaw sperm motility, acrosomal integrity, or plasma membrane integrity. This disparity in results could

be attributed to some factors, such as the species or the dose of GTE added to the extender, the density of egg yolk used in the extender, or the degree of stability of catechins, which can vary with environmental conditions like temperature, pH concentration, and oxygen levels (İnanç et al., 2019).

Several studies have reported a considerable increase in the fragmentation of DNA after cryopreservation (Donnelly et al., 2001; Nekoonam et al., 2016). In our investigation, GTE at a dose of 0.75% was associated with a marked decrease in fragmented DNA, percentage of tail length, and tail moment in comparison with control and other doses. This result is supported by the findings of İnanç et al. (2019) and Ahmed et al. (2020b), who suggested that GTE's phenolic compound and antioxidant properties contribute to lowering LPO and improving DNA integrity.

SOD, GSH, and CAT are the key antioxidants implicated in the protection of sperm cells against ROS (Bansal and Bilaspuri, 2011). However, the transfer of these enzymes to another section of the sperm was hampered due to the restricted volume of sperm cytoplasm (Yildiz et al., 2007). Besides, the reduced antioxidant capacity of SOD and GSH in ram and bull spermatozoa following cryopreservation may explain why frozen-thawed sperm is more susceptible to LPO and oxidative damage (Marti et al., 2008).

In the present study, SOD levels increased at doses of 0.1%–2% GTE when compared with the control. This result is consistent with a recent study on buffalo bull conducted by Ahmed et al. (2020b) . In the meantime, a significant increase in post-thaw GSH and CAT was recorded in the extender supplemented with 0.75% GTE. Furthermore, the inclusion of GTE at a dose of 0.75% improved post-thaw TAC. At doses of 0.5%, 0.75%, and 1%, there was also a notable reduction in MDA levels when compared to the control.

These findings are parallel with Malo et al. (2010) in boar, Dias et al. (2014) in stallion, Mehdipour et al. (2016) in ram, and Ahmed et al. (2020a), who confirmed the same results in buffalo bull at doses of 0.5%, 0.75%, and 1%. Meanwhile, El-Seadawy et al. (2017) declared that adding pomegranate peel methanolic extract, which is rich in GTE, to rabbit semen reduced LPO and elevated antioxidant activity. Zhao et al. (2009) discovered a significant negative relationship between Rhodiala sacra extract, which is rich in polyphenols, and MDA levels in frozen-thawed boar semen.

In this regard, Kameni et al. (2021) demonstrated that GSH can maintain sperm motility, motion parameters, and membrane integrity during chilled ram semen storage, and according to Câmara et al. (2011), CAT has a positive effect in mitigating the harmful impact of chilling on both motility and viability during ram sperm liquid storage. This could support our favorable notion of GTE's anti-oxidative effect on buffalo bull semen parameters.

Conclusion

GTE supplementation at a proportion of 0.75% is the most effective and protective dose helped to improve post-thawed buffalo bull kinematics parameters, semen quality, and DNA integrity linked to the elevated levels of activity of antioxidant enzymes, and reduced levels of MDA due to GTE's antioxidant effect in reducing oxidative stress and enhancing the endogenous defense system..

Conflict of interest

The authors declare that they have no conflict of interest.

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