

Effects of co-supplementation of trehalose with fetuin and taxifolin in freezing extender on quality parameters of bull spermatozoa following freeze-thaw process

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ABSTRACT

Glycerol is commonly used as a cryoprotectant in bovine sperm cryopreservation; however, its cytotoxic effects can negatively impact sperm quality. This study aimed to reduce glycerol concentration in semen extenders by supplementing with natural antioxidants (taxifolin and fetuin) and the disaccharide trehalose. Twelve ejaculates were collected from three bulls and initially evaluated, then diluted in a tris-egg yolk (TEY) extender containing either 7 % or 4 % glycerol. The control group received 7 % glycerol (TEY), while experimental groups included G7F (TEY + fetuin), G7T (TEY + taxifolin), G7M (TEY + fetuin + taxifolin), and 4 % glycerol groups supplemented with 50 mM trehalose: G4 (TEY + trehalose), G4F (TEY + fetuin), G4T (TEY + taxifolin), and G4M (TEY + fetuin + taxifolin). Post-thaw evaluations included assessments of sperm motility, plasma membrane integrity, acrosome integrity, mitochondrial membrane potential (MMP), DNA fragmentation, and oxidative stress markers. The G4F and G4T groups exhibited significantly higher total and progressive motility, viability, plasma membrane integrity, and MMP compared to other groups ($p < 0.05$). Furthermore, these groups showed significantly lower malondialdehyde (MDA) levels, indicating reduced lipid peroxidation and oxidative stress ($p < 0.05$). In conclusion, co-supplementation of 50 mM trehalose with 50 μ M taxifolin and 5 mg/mL fetuin in a cryopreservation extender containing 4 % glycerol may enhance the post-thaw quality of bull spermatozoa.

1. Introduction

Assisted reproductive techniques, such as artificial insemination (AI), are crucial to preserve valuable genetics in animals [1]. Sperm cryopreservation is a useful storage technique that is very important for AI in bull breeding [2]. Despite the significant advantages of sperm freezing, the freeze-thaw process induces irreversible changes in the structure and function of sperm [3].

During cryopreservation, sperm cells are exposed to multiple stress-inducing factors including ice crystal formation, osmotic shock, dehydration, and oxidative stress caused by free radical production [4,3]. These

reactive oxygen species (ROS) disrupt sperm motility, viability, and DNA integrity, ultimately leading to cellular apoptosis [5]. In recent decades, significant research efforts have focused on mitigating freeze-thaw damage through various approaches [6]. These include optimization of extender composition and evaluation of different cryopreservation techniques (slow freezing, rapid freezing, and vitrification) [7].

A standard freezing medium typically contains cryoprotective agents, energy substrates, protein sources, pH buffers, and various additives including antioxidants, fatty acids, and antibiotics [8]. To mitigate sperm damage caused by temperature fluctuations during freezing and thawing, specific chemical compounds must be incorporated into

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the extender. These substances, known as cryoprotectants, minimize ice crystal formation during cryopreservation [9].

Glycerol is the most widely used cryoprotectant in sperm freezing for both animals and humans [10,11]. The incorporation of glycerol into the freezing medium generates an osmotic pressure differential across the plasma membrane, driving the efflux of intracellular water molecules via aquaporin-mediated passive transport [12]. However, its application is limited due to toxic and osmotic effects on the sperm plasma membrane and metabolic functions [13]. Furthermore, elevated glycerol concentrations can compromise sperm functionality and impair fertilization capacity, thereby diminishing reproductive efficiency when incorporated into semen extenders across multiple animal species [14].

Based on previous studies, natural antioxidants have been incorporated into freezing extenders alongside glycerol to reduce the required glycerol concentration [15]. Trehalose, a disaccharide compound, provides protection against osmotic stress and interacts specifically with membrane phospholipids [16]. Additionally, trehalose induces hypertonic conditions and facilitates osmotic dehydration prior to cryopreservation, thereby reducing intracellular ice crystal formation and associated cellular damage [16]. In our previous studies, trehalose was utilized to attenuate the cytotoxicity of glycerol and to preserve sperm quality under low-glycerol freeze–thaw conditions.

In addition to trehalose, fetuin—a predominant glycoprotein in fetal calf serum—exhibits both cryoprotective and antioxidant properties, and has been proposed as a potential substitute or adjunct to glycerol in semen cryopreservation protocols [17]. Moreover, taxifolin, a naturally occurring plant flavonoid, possesses notable antioxidant and antimicrobial activities, further supporting its use in improving cryosurvival of spermatozoa [18,19]. The incorporation of such bioactive compounds into semen extenders has demonstrated potential to enhance post-thaw sperm quality by mitigating oxidative stress and cellular damage during the freeze–thaw process [20]. Therefore, this study aimed to assess whether the combined supplementation of trehalose, fetuin, and taxifolin in a low-glycerol semen extender enhances post-thaw sperm quality. Specifically, the objective was to elucidate their potential synergistic effects on preserving sperm functional integrity during cryopreservation.

2. Materials and methods

2.1. Sample collection and ethics

This study was approved by the Ethics Committee of Urmia University of Medical Sciences (Approval No. IR.UMSU.AEC.1401.019). A total of twelve ejaculates were collected from three Simmental bulls with an average age of four years at the Jabal Breeding Center, located in West Azerbaijan, Iran, using an artificial vagina. The ejaculates were pooled to minimize individual variation and subsequently divided into experimental groups. Following collection, semen quality parameters including volume, sperm concentration, and total motility were assessed. Only samples meeting the following criteria were included: volume between 2 and 6 mL, sperm concentration of $\geq 1 \times 10^9$ sperm/mL, and progressive motility exceeding 70 %.

2.2. Semen processing and cryopreservation

Following the initial analysis, semen samples were diluted in a Tris based extender containing Tris (2.66 g), citric acid (1.47 g), glucose (63 mg), and 20 % egg yolk in 100 mL of solution. The sperm concentration was adjusted to 100×10^6 sperm/mL, and the samples were assigned to eight experimental groups as follows: Control (CNT): based extender + 7 % glycerol; G7F: based extender + 7 % glycerol + 5 mg/mL fetuin; G7T: based extender + 7 % glycerol + 50 μ M taxifolin; G7M: based extender + 7 % glycerol + 5 mg/mL fetuin + 50 μ M taxifolin; G4: based extender + 4 % glycerol + 50 mM trehalose; G4F: based extender + 4 % glycerol + 50 mM trehalose + 5 mg/mL fetuin; G4T: based extender + 4

% glycerol + 50 mM trehalose + 50 μ M taxifolin; and G4M: based extender + 4 % glycerol + 50 mM trehalose + 5 mg/mL fetuin + 50 μ M taxifolin.

For cryopreservation, the diluted semen samples were loaded into 0.5 mL straws at a final concentration of 25×10^6 sperm/mL and frozen using a programmable cell freezer (Digitcool; IMV Technologies, France). The freezing protocol began at 15 °C, with the temperature gradually reduced to –15 °C over 7 min, then rapidly decreased to –150 °C over the next 6 min. Straws were then plunged into liquid nitrogen (–196 °C) and stored for two weeks. Thawing was performed in a 37 °C water bath for 40 s before post-thaw analysis. All evaluations were conducted by a single trained researcher to maintain consistency. A blinded assessment protocol was implemented, with samples randomly coded to conceal group identities during evaluation [21].

2.3. Sperm motility

Total and progressive sperm motility were assessed using a light microscope at 40 \times magnification. A 10 μ L aliquot of extended semen was placed on a pre-warmed microscope slide and covered with a coverslip. For each sample, motility was evaluated in at least five randomly selected microscopic fields, with a total of approximately 200 sperm cells analyzed per sample.

2.4. Sperm viability

Sperm viability was assessed using the LIVE/DEAD Viability Kit (L-7011, Molecular Probes, USA) following the manufacturer's instructions. A SYBR-14 working solution was prepared by diluting SYBR-14 in buffer (1:50; containing 10 mM HEPES, 150 mM NaCl, 10 % BSA, pH 7.4). A 50 μ L aliquot of sperm suspension ($\sim 2 \times 10^6$ spermatozoa/mL) was mixed with 1 μ L of the SYBR-14 working solution (final concentration: 100 nM) and incubated at 37 °C for 10 min in the dark. Subsequently, 1 μ L of propidium iodide (PI) solution was added, followed by a 5-min incubation under the same conditions. To immobilize the sperm, 10 μ L of Hancock's solution (containing 150 mL of 37 % formalin, 10 g sodium salicylate, 5 g sodium bicarbonate, and distilled water up to 1 L) was added. Next, 7 μ L of the stained suspension was placed on a microscope slide and examined using a fluorescence microscope (BX51, Olympus, Japan; excitation: 450–490 nm, emission: 520 nm) at 1000 \times magnification. A total of 200 spermatozoa were evaluated per slide; live sperm displayed green fluorescence (SYBR-14), while dead sperm fluoresced red (PI).

2.5. Sperm plasma membrane integrity

The hypo-osmotic swelling (HOS) test was conducted to evaluate the functional integrity of the sperm plasma membrane. For this assay, 30 μ L of semen was mixed with 300 μ L of a hypo-osmotic solution (prepared by dissolving 13.5 g of fructose and 7.35 g of sodium citrate in 1 L of distilled water; 100 mOsm/kg) and incubated at 37 °C for 60 min. After gentle mixing, a smear was prepared and examined under a phase-contrast microscope at 100 \times magnification. Spermatozoa were categorized based on tail morphology: those with curled (swollen) tails were considered membrane-intact, while those with straight tails were considered membrane-compromised. A total of 200 sperm cells were evaluated per sample across at least five randomly selected microscopic fields [22].

2.6. Mitochondrial membrane potential (MMP)

Mitochondrial membrane potential (MMP) was assessed using the JC-1 fluorescent dye. Initially, thawed sperm samples were centrifuged at 3000 rpm for 5 min. The supernatant was discarded, and the resulting sperm pellets were resuspended in PBS to a final concentration of approximately 2×10^6 spermatozoa/mL. For staining, 200 μ L of the

sperm suspension was incubated with 10 μ L of JC-1 working solution (prepared from a 200 μ M stock dissolved in DMSO) at 37 °C for 40 min in the dark. Following incubation, sperm motility was arrested by the addition of 10 μ L of Hancock's solution. Subsequently, 2.5 μ L of the stained suspension was placed on a microscope slide, covered with a coverslip, and examined under a fluorescence microscope (Olympus BX51, Japan) at 1000 \times magnification. Mitochondrial activity was evaluated based on the fluorescence pattern in the sperm midpiece: sperm exhibiting bright yellow to orange fluorescence were classified as having high MMP, while those displaying green fluorescence were considered to have low MMP. A minimum of 200 spermatozoa were analyzed per sample.

2.7. Acrosome integrity

Acrosome integrity was evaluated following the method described by Thys et al. [23], with minor modifications. Sperm samples were first centrifuged at 3000 rpm for 5 min, and the supernatant was discarded. The resulting pellet was resuspended in 100 μ L of 96 % ethanol to a final concentration of approximately 2×10^6 spermatozoa/mL and incubated at room temperature for 30 min for fixation. Subsequently, 10 μ L of the fixed sperm suspension was mixed with 30 μ L of fluorescein isothiocyanate-conjugated *Pisum sativum* agglutinin (FITC-PSA) on a glass slide and incubated for 20 min at room temperature in the dark. After incubation, the slides were rinsed thoroughly with distilled water (10 washes), air-dried, and mounted with glycerol. A total of 200 spermatozoa were assessed per slide under a fluorescence microscope (Olympus BX51) at 100 \times magnification. Sperm exhibiting uniform green fluorescence over the acrosomal region were classified as having intact acrosomes, while those lacking fluorescence in this region were considered acrosome-damaged.

2.8. Evaluation of DNA fragmentation

Sperm DNA fragmentation was assessed using acridine orange (AO) staining. This technique exploits the differential fluorescence of AO, which emits green when bound to double-stranded (intact) DNA and red or yellow when bound to single-stranded (denatured) DNA. Sperm smears were initially fixed in Carnoy's fixative (3:1 methanol:acetic acid) for 2 h at room temperature, followed by air drying for 5 min. The slides were then stained in an acridine orange solution (1 mg/mL prepared in distilled water) for 5 min. The staining solution was stored at 4 °C in the dark to preserve stability. After staining, a total of 200 spermatozoa per sample were evaluated under a fluorescence microscope (Olympus BX51) at 1000 \times magnification. Sperm heads with intact DNA displayed green fluorescence, whereas those with fragmented DNA showed red or yellow fluorescence [24].

2.9. Oxidative stress assessment

2.9.1. Malondialdehyde (MDA)

Oxidative stress was evaluated by quantifying malondialdehyde (MDA) levels as an indicator of lipid peroxidation. MDA concentration was measured according to the manufacturer's protocol using the Nalondi™ Lipid Peroxidation Assay Kit (Navand Salamat, Iran). The absorbance of the supernatant was recorded at 523 nm using a spectrophotometer (Thermo Fisher Scientific, USA). MDA levels were calculated based on a standard curve and expressed as nmol/mL [25].

2.9.2. Glutathione peroxidase (GPx)

Glutathione peroxidase (GPx) activity in post-thawed semen was determined using the Nagpix™ Glutathione Peroxidase Activity Assay Kit (CAT NS-15083; Navand Salamat, Urmia, Iran), following the manufacturer's instructions. GPx activity was expressed in milliunits per milliliter (mU/mL) [25].

2.10. Statistical analysis

The data were tested for normality using the Shapiro–Wilk test and for homogeneity of variances using Levene's test. A nested analysis of variance (ANOVA) was performed on sperm quality parameters using SPSS software (version 18.0). Post hoc comparisons between groups were conducted using Duncan's multiple range test. Differences were considered statistically significant at $P \leq 0.05$. Results are presented as mean \pm standard error of the mean (SEM). GraphPad Prism (version 8.0; USA) was used for graphical visualization.

3. Results

3.1. Semen motility parameters

The percentages of total and progressive sperm motility are presented in Fig. 1A and B, respectively. Significantly higher values for both parameters were observed in the G4T and G4F groups compared to the control and other treatment groups ($P < 0.05$). However, no significant difference was found between the G4T and G4F groups. The lowest percentages of total and progressive motility were recorded in the G4 group relative to the control and all treated groups.

3.2. Sperm viability and plasma membrane integrity

Data on sperm viability and plasma membrane integrity are presented in Fig. 1. As shown in Fig. 1C, the G4T group exhibited a significantly higher percentage of viable sperm compared to the control and G7M groups ($P < 0.05$). No significant differences were observed among the remaining treatment groups ($P > 0.05$). SYBR Green staining of sperm cells is illustrated in Fig. 2A. Regarding plasma membrane integrity (Fig. 1D), statistically significant differences were detected between the G4F and G4T groups compared to the control group. An image representing the hypo-osmotic swelling (HOS) test is shown in Fig. 2B.

3.3. Mitochondrial membrane potential and acrosome integrity

Mitochondrial membrane potential (MMP) and acrosome integrity results are presented in Fig. 3. As shown in Fig. 3A, the G4T group exhibited the highest percentage of sperm with high MMP compared to other treatment groups ($P < 0.05$). JC-1 staining of sperm cells is illustrated in Fig. 2C. In terms of acrosome integrity (Fig. 3B), no significant differences were observed among the treatment groups ($P > 0.05$). Representative FITC-PSA staining of bull spermatozoa is shown in Fig. 2D.

3.4. DNA fragmentation

The percentage of DNA fragmentation in the treatment groups following the freeze–thaw process is shown in Fig. 4A. No significant differences were observed among the treatment groups. Representative images of sperm stained with acridine orange are presented in Fig. 4B.

3.5. GPx and MDA results

The antioxidant status results are presented in Fig. 5A–B. No significant differences in glutathione peroxidase (GPx) activity were observed among the treatment groups. However, malondialdehyde (MDA) levels were significantly lower in the G4F and G4T groups compared to the other treatment groups ($P < 0.05$).

4. Discussion

Spermatozoa undergo a series of physical and biochemical alterations during the freezing and thawing process [26]. Cryopreservation

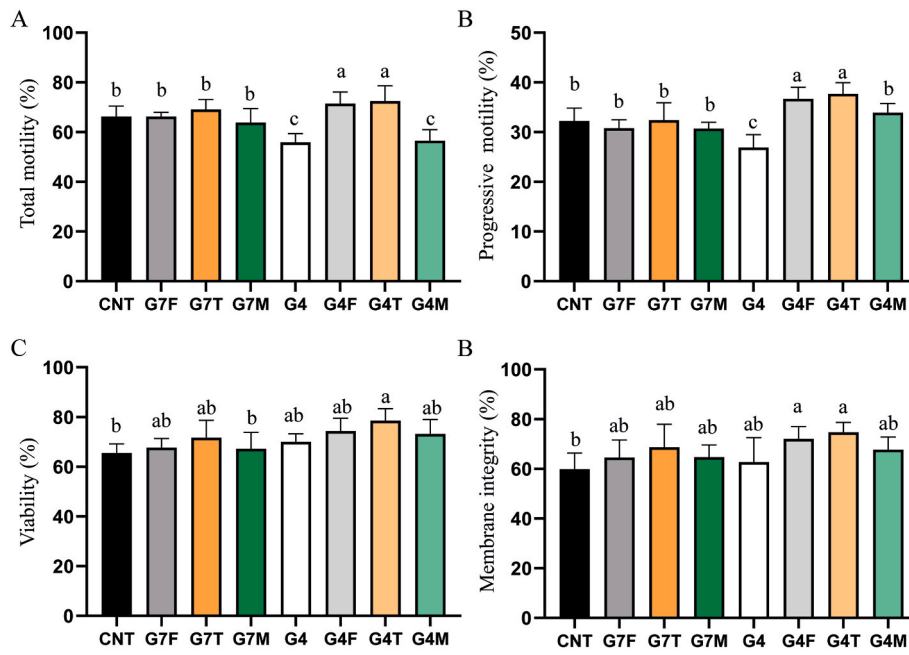


Fig. 1. Results of post thawed bull sperm motion parameter, viability and membrane integrity following supplementation of trehalose, fetuin and taxifolin (A) total motility, (B) progressive motility, (C) viability, and (D) plasma membrane integrity. Data are expressed as means \pm SEM. Different letters (a, b, c) in each bar indicate statistically significant differences ($P < 0.05$).

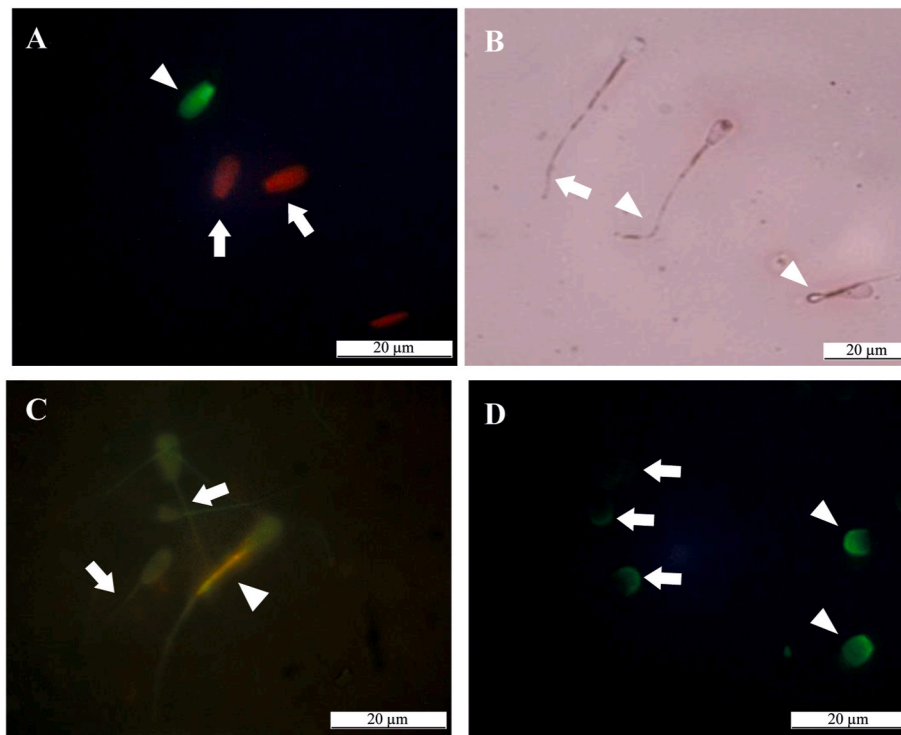


Fig. 2. Assessment of post-thawed bull sperm viability, plasma membrane integrity, mitochondrial membrane potential (MMP), and acrosome integrity. (A) Fluorescence micrograph of spermatozoa stained with SYBR-14 and propidium iodide (PI). Viable spermatozoa exhibit bright green fluorescence (SYBR-14, indicated by arrowheads), while non-viable spermatozoa fluoresce red (PI, indicated by arrows). (B) Hypo-osmotic swelling test (HOST) for evaluating plasma membrane integrity under light microscopy. Spermatozoa displaying coiled tails (intact membranes; arrowheads) are distinguished from those with straight tails (compromised membranes; arrows). (C) JC-1 staining for analysis of mitochondrial membrane potential. Spermatozoa with high MMP emit orange/red fluorescence in the midpiece (arrowheads), whereas those with low MMP fluoresce green (arrows). (D) Evaluation of acrosome integrity using FITC-conjugated Pisum sativum agglutinin (FITC-PSA) under fluorescence microscopy. Spermatozoa showing bright green fluorescence localized to the acrosomal region are considered intact, while those lacking fluorescence in the acrosomal cap are classified as acrosome-reacted or damaged. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

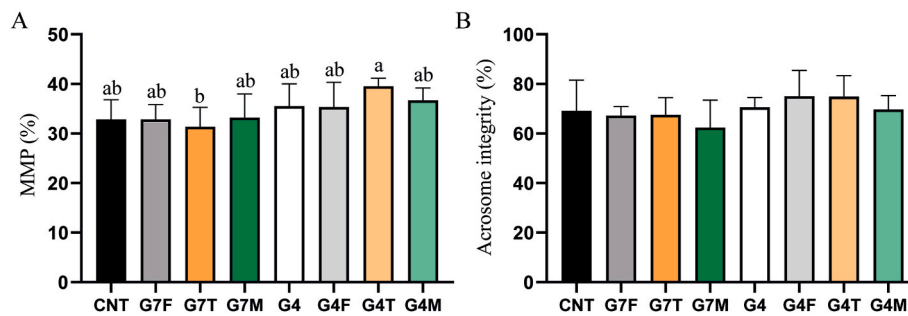


Fig. 3. Statistical analysis of mitochondrial membrane potential (MMP) and acrosome integrity in post-thawed bull spermatozoa. (A) MMP levels measured using JC-1 staining. (B) Acrosome integrity assessed via FITC-PSA staining. Data are presented as mean \pm SEM. Bars with different superscript letters (a, b) represent statistically significant differences between groups ($P < 0.05$).

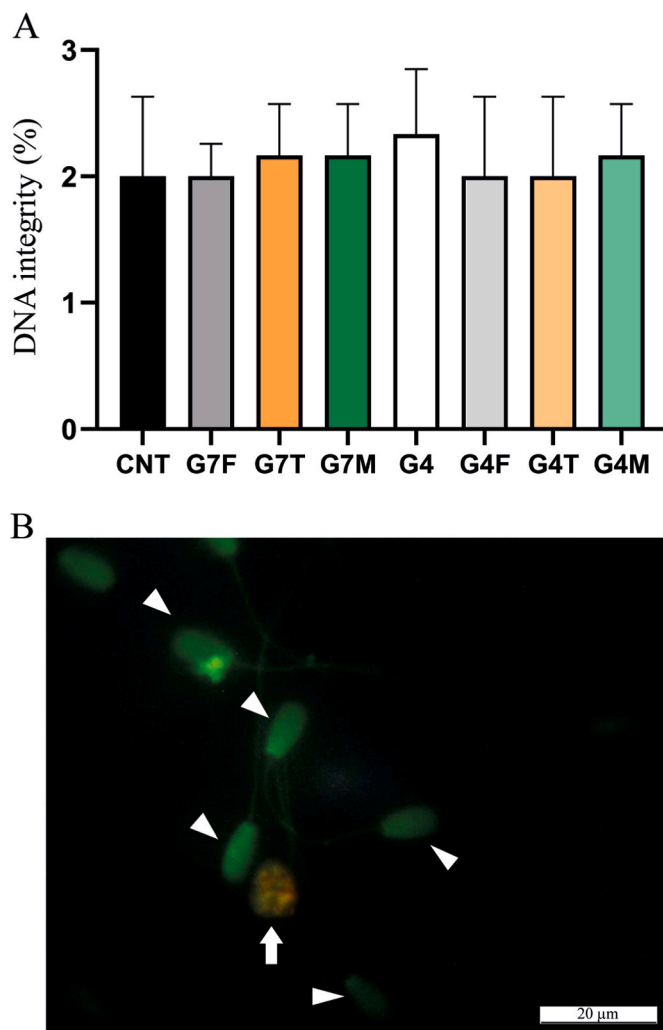


Fig. 4. Assessment of DNA fragmentation in post-thawed bull spermatozoa. (A) Statistical analysis of DNA fragmentation across treatment groups. (B) Representative micrograph of spermatozoa stained with acridine orange. Sperm cells emitting green fluorescence (indicated by arrowheads) represent intact double-stranded DNA, whereas cells showing red or yellow fluorescence indicate fragmented or denatured DNA, classified as damaged or abnormal. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

leads to reduced membrane fluidity, impaired membrane permeability, and decreased osmotic resistance, ultimately compromising sperm viability and fertilizing capacity [27]. Successful cryopreservation requires the preservation of sperm viability, motility, and metabolic

function throughout the process [28,29]. Glycerol is the most widely used intracellular cryoprotectant in sperm cryopreservation protocols [30]. It protects the sperm membrane by forming hydrogen bonds between its hydroxyl groups and the phosphate groups of membrane phospholipids, thereby stabilizing the membrane structure during freezing [9]. Although glycerol is commonly added to semen extenders at concentrations between 3% and 10% [12], higher concentrations can exert cytotoxic effects, potentially compromising sperm function and structural integrity [31].

In this study, various cryoprotectants and antioxidants, including fetuin, taxifolin, and trehalose, were incorporated into bull sperm extenders containing either 4% or 7% glycerol. The primary objective was to evaluate the potential of these compounds to reduce glycerol concentration from 7% to 4% while maintaining or improving sperm quality. Fetuin, a commercially available glycoprotein, has been reported to enhance sperm motility [32]. However, previous research demonstrated that fetuin did not significantly affect motility at 3 mg/mL but promoted forward motility at a higher concentration of 80 mg/mL [6]. Consistent with these findings, our results showed that fetuin alone did not improve sperm motility or other functional parameters. Moreover, when combined with trehalose, fetuin exhibited a detrimental effect on sperm quality.

Another compound used in this study was trehalose, a disaccharide known for its cryoprotective properties. Trehalose stabilizes cell membranes during cooling by forming hydrogen bonds with the polar head groups of membrane phospholipids, thereby preventing molecular rearrangements and reducing osmotic stress during the freeze-thaw process [33]. It exhibits two primary mechanisms of cryoprotection: (1) stabilization of membrane phospholipids against cryoinjury, and (2) interaction with extracellular ice crystals, similar to glycerol. Under hypertonic extracellular conditions, water efflux is promoted, thereby minimizing intracellular ice crystal formation. Trehalose has been widely used as a cryoprotectant to reduce the cytotoxic effects of glycerol in bull sperm extenders [34]. This combination enhances cellular resistance to thermal, osmotic, and oxidative stress [34]. The beneficial effects of trehalose have been demonstrated in cryopreservation of various tissues and cells, including pancreatic islets, adipose tissue, fetal skin, trachea, red blood cells, plant cells, embryos, oocytes, hematopoietic stem cells, and spermatogonial stem cells [35]. In the context of sperm cryopreservation, numerous studies across species—including human [36], ram [37], and bull [38]—have reported that the addition of trehalose to the freezing medium improves post-thaw sperm quality. Furthermore, several studies have shown that trehalose can partially compensate for reduced glycerol concentrations, maintaining cryoprotective efficiency while minimizing glycerol-related cytotoxicity [32, 39]. In our study, 50 mM trehalose was added to the extender containing 4% glycerol, aiming to mitigate the toxic effects of glycerol and reduce its concentration from the typical 7%–4%, which has been associated with contraceptive effects. However, the results indicated that the G4T50 group (4% glycerol + 50 mM trehalose) exhibited the lowest

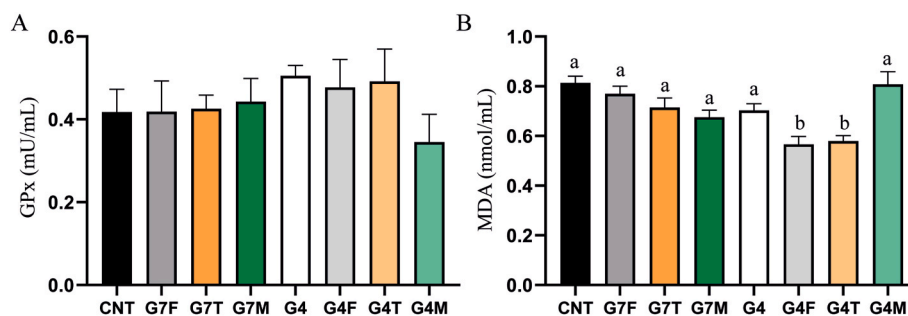


Fig. 5. Statistical analysis of antioxidant and oxidative stress biomarkers in post-thawed bull spermatozoa. (A) Glutathione peroxidase (GPx) activity levels across treatment groups. (B) Malondialdehyde (MDA) concentrations as an indicator of lipid peroxidation in treatment groups. Data are presented as means \pm SEM. Different letters (a, b) denote statistically significant differences among the groups ($P < 0.05$).

percentages of total and progressive motility compared to the control and other treatment groups.

Previous studies have demonstrated the protective effects of trehalose on sperm function during cryopreservation. For instance, the addition of 5 mM trehalose to rat sperm freezing media effectively preserved functional parameters against chilling injury [40]. Similarly, in Merino rams, supplementation of a Tris-based extender with 10 or 25 mM trehalose significantly preserved acrosome integrity, mitochondrial activity, motility, and viability during liquid storage [41]. In contrast, our findings showed that trehalose alone, when added to the semen extender, did not enhance total or progressive motility or improve mitochondrial membrane potential (MMP) in bull sperm. However, the combination of trehalose with taxifolin led to improved motility indices and enhanced some functional parameters compared to the control group.

Taxifolin is a potent hydroxyl radical ($\bullet\text{OH}$) scavenger known to protect bone marrow-derived mesenchymal stem cells from oxidative damage. It exerts both direct and indirect antioxidant effects [42]. Histopathological and biochemical analyses have shown that taxifolin mitigates pulmonary oxidative stress by reducing oxidant levels and preserving endogenous antioxidant defenses [43]. Based on our findings, supplementation of the freezing extender with 50 μM taxifolin hydrate, along with 4 % glycerol and 50 mM trehalose, provided effective cryoprotection—particularly improving sperm motility, viability, and acrosome integrity. However, the concentration of taxifolin is a critical factor; higher levels may adversely affect these sperm quality parameters. Thus, it can be suggested that taxifolin, at an optimal concentration, serves as a beneficial antioxidant that mitigates the detrimental effects of cryopreservation on acrosome integrity, mitochondrial function, sperm motility, and viability in bull spermatozoa.

DNA damage resulting from sperm cryopreservation can significantly impair embryo development, fetal viability, and overall reproductive success, thereby affecting the outcomes of both natural mating and assisted reproductive techniques [44]. The degree of DNA fragmentation is widely recognized as a critical marker of sperm quality and fertilizing potential [44], underscoring the importance of reliable assessment methods. In the present study, no statistically significant differences in DNA fragmentation were observed among the treatment groups. This finding suggests that, under the applied freeze-thaw conditions, sperm DNA remained relatively stable. In contrast, previous studies have demonstrated the protective effects of taxifolin against oxidative DNA damage. For instance, taxifolin has been shown to reduce hydrogen peroxide (H_2O_2)-induced DNA fragmentation in human whole blood cells at concentrations of 100, 250, and 500 $\mu\text{g}/\text{mL}$ [45]. Similarly, taxifolin concentrations ranging from 0 to 100 μM have been reported to inhibit DNA damage in human keratinocytes [46].

The plasma membrane of spermatozoa is highly susceptible to oxidative damage during the freeze-thaw cycle due to its rich content of polyunsaturated fatty acids [25]. Compared to fresh spermatozoa,

frozen-thawed sperm are significantly more vulnerable to lipid peroxidation [47]. Elevated levels of malondialdehyde, a byproduct of lipid peroxidation, are indicative of increased oxidative stress and are associated with reduced semen quality post-thaw [48]. This study investigated the effects of different concentrations of fetuin and taxifolin on MDA levels in frozen-thawed spermatozoa. Previous studies have shown that oral administration of taxifolin significantly reduced MDA levels in various rat tissues, including mammary gland, kidney, liver, and serum, following dimethylbenzanthracene (DMBA)-induced carcinogenesis, in a dose-dependent manner. Moreover, taxifolin was shown to restore superoxide dismutase (SOD) activity diminished by DMBA exposure [49]. In another study, taxifolin treatment reversed rotenone-induced oxidative stress in rats, improving glutathione levels and reducing lipid peroxidation by enhancing glutathione S-transferase and SOD activity [50]. In our study, all treatment groups supplemented with taxifolin and/or fetuin combined with 4 % glycerol exhibited reduced MDA levels compared to the 7 % glycerol control group. This suggests that reducing glycerol concentration while incorporating taxifolin and fetuin may enhance intracellular ROS management and mitigate glycerol-induced toxicity. However, no significant differences were observed in glutathione peroxidase activity between treatment and control groups. Based on these findings, the use of a tris-based cryopreservation extender containing 4 % glycerol, 50 mM trehalose, 50 μM taxifolin, and 5 mg/mL fetuin is recommended to optimize post-thaw quality of bull spermatozoa.

5. Conclusion

Our findings demonstrated that supplementing the semen extender with 4 % glycerol, in combination with trehalose, taxifolin, and fetuin, improved post-thaw bull sperm quality specifically in terms of motility, viability, mitochondrial membrane potential (MMP), and regulation of malondialdehyde (MDA) compared to the conventional 7 % glycerol extender. Based on these results, it is recommended to formulate a tris-based cryopreservation extender containing 4 % glycerol supplemented with 50 mM trehalose, 50 μM taxifolin, and 5 mg/mL fetuin to enhance the post-thaw quality of bull spermatozoa. Further *in vitro* and *in vivo* studies are warranted to assess the fertilization potential and reproductive performance of frozen-thawed bull spermatozoa supplemented with various concentrations of taxifolin, fetuin and trehalose.

CRedit authorship contribution statement

Mustafa Numan Bucak: Investigation, Conceptualization. **Morteza Taravat:** Writing – original draft, Methodology. **Oya Korkmaz:** Writing – review & editing, Methodology. **Ali Soliemanzadeh:** Writing – review & editing, Methodology. **Mojtaba Karimpour:** Methodology. **Mohammad Saber Maroufi:** Methodology. **Neda Abedpour:** Methodology. **Sadik Küçükgünay:** Methodology, Formal analysis. **Tohid Rezaei Topraggaleh:** Supervision.

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Declaration of competing interest

None.

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Data availability

All relevant data are included in the paper.

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