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RESEARCH ARTICLE

Effect of Quercetin Supplementation in Tris-based Extender on the Post-thaw Sperm Quality, Oxidative stress, DNA Integrity and Fertility of Kundhi Buffalo Bulls

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ABSTRACT

Cryopreservation-induced oxidative stress significantly impairs sperm quality and fertility in buffalo semen. Quercetin is a natural antioxidant flavonoid known for its potent ability to scavenge reactive oxygen species and reduce oxidative stress. The present study investigated the oxidative stress biomarkers and DNA integrity in cryopreserved sperm following supplementation of various concentrations (0, 25, 50 and 100µM) of quercetin to a Tris-based extender. In this study, 32 semen ejaculates were collected from four sexually active buffalo bulls of Kundhi breed, twice weekly for four weeks. Semen ejaculates with ≥80% sperm motility were selected for processing. Ejaculates collected from four bulls on each collection day were pooled, diluted in Tris-based extender, cryopreserved and stored for 24h in liquid nitrogen. Following cryopreservation, the quercetin 100µM group showed the highest sperm viability (69.12±1.24%), plasma membrane integrity (62.25±1.08%), total motility (57.75±1.15%) and progressive motility (49.25±0.96%). Similarly, the activities of antioxidant enzymes-catalase (2.50±0.05U/mL), superoxide dismutase (50.625±1.02U/mL) and total glutathione (49.875±0.95µM) were significantly elevated, whereas malondialdehyde (MDA) levels (38.37±0.84nmol ml^{-1}) and DNA damage (8.625±0.42%) were significantly reduced, in the 100µM group compared with the control group ($P<0.05$). *In vivo* fertility trials demonstrated a higher conception rate in the quercetin-treated group (65%) compared with 40% in the control group ($P<0.05$). In conclusion, quercetin supplementation at 100µM to the Tris-based extender effectively alleviates oxidative stress during cryopreservation and enhances post-thaw sperm quality and improves fertility of semen of Kundhi buffalo bulls.

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INTRODUCTION

The water buffalo, *Bubalus bubalis*, plays an important role in the socio-economic development of masses in the South and Southeast Asia, as it produces milk, meat, and provides draught power for various farm operations. Pakistan possesses two internationally acclaimed buffalo breeds, i.e. Nili-Ravi and Kundhi. The population of Kundhi buffaloes in the country is approximately 5.5 million heads, which contribute around 2000-2400 liters of milk per lactation (Abid *et al.,* 2024). Genetic improvement of these breeds is essential for sustainable livestock production (Khan, 2009; Andrabi, 2009). The central feature of buffalo genetic improvement program is artificial insemination (AI), using deep-frozen semen, but efficiency of this procedure is still poor with the conception rate as low as 33-45% under field conditions (Anzar *et al.*, 2003). The major reason of this low fertility is deterioration of the quality of semen following cryopreservation, which affects the biological integrity and fertilizing ability of spermatozoa.

Cryopreservation causes various types of physiological stresses on spermatozoa such as thermal shock, osmotic imbalance and intracellular ice crystal formation. Oxidative stress induced by the excess production of ROS has been acknowledged as one of the main mechanisms of the frozen-thawed sperm dysfunction (Agarwal *et al.*, 2008). Although physiological levels of

ROS are required to stimulate the capacitation of sperm, acrosome reaction and oocyte fusion, excessive production of ROS during a cryopreservation process leads to the onset of an oxidative cascade that includes lipid peroxidation (LPO) of the plasma membranes, protein carbonylation, and DNA strand fragmentation (Kadirvel *et al.*, 2021; Aitken and Gibb, 2022). Buffalo spermatozoa are especially vulnerable to oxidative damage, as the plasma membrane contains an extremely high amount of polyunsaturated fatty acids (PUFAs), which are known to be the predictable targets of lipid peroxidation (Andrabi, 2009).

Sperm DNA fragmentation (SDF) is a relatively new well-known predictor of male fertility. High levels of SDF correlate with low fertilization, impaired embryonic development, high rates of spontaneous abortion, and low success in assisted reproductive technologies (Leushuis *et al.*, 2019; Agarwal *et al.*, 2020). Incomplete protamination and apoptotic nucleases activation during cryopreservation also play a role in DNA strand damage via oxidative attack on sperm chromatin by ROS as well (Zribi *et al.*, 2012; Priyanto *et al.*, 2019). High levels of structural genomic damages persist after thawing and can potentially impair fertility despite apparently normal semen conventional quality parameters. This underscores the clinical significance of DNA integrity analysis in frozen buffalo semen (Castro *et al.,* 2025).

The spermatozoa possess an endogenous enzymatic system of antioxidant defense that is not capable to counter the high burden of ROS produced during cold storage of semen (Bilodeau *et al.*, 2000; Nair *et al.*, 2006). The indirect effect of this enzymatic deficiency is added to by the fact that seminal plasma antioxidants are lost following dilution with extenders, making the sperm especially susceptible to the effects of cryostress (Garg *et al.*, 2009). Thus, the use of exogenous antioxidants in supplementing semen extenders has become one of the viable and effective approaches to alleviate oxidative damage, maintain structural integrity of sperm, and improve post-thaw fertility (Arora *et al.*, 2021; Yánez-Ortiz *et al.*, 2022).

One of the most common and bioactive dietary flavonoids is quercetin 7-pentahydroxyflavone ($C_{15}H_{10}O_7$; MW 302.24g/mol). It is commonly and abundantly found in different types of vegetables, fruits and grains. Rich dietary sources of quercetin include berries, oranges, onions, tea, and red wine (Sampson *et al.*, 2002). It has a strong antioxidant ability because of five hydroxyl groups, especially the catechol moiety on the B-ring, which allows it to effectively donate hydrogen atoms and chelate transition metal ions, thus halting radical chain reactions (Stojanovic *et al.*, 2001; Boots *et al.*, 2008;). Quercetin also has a better ROS-scavenging activity than the conventional antioxidants (including vitamins C and E) and exhibits other pharmacological activities such as anti-inflammatory, anti-apoptotic, and membrane-stabilizing (Moretti *et al.*, 2012; Seifi-Jamadi *et al.*, 2017).

The use of quercetin as a supplement in semen extenders has been investigated in various species, such as stallions (Seifi-Jamadi *et al.*, 2016)), goats (Batool *et al.*, 2024), boars (Fraser *et al.*, 2010), poultry (Rakha *et al.*, 2020) and men (Cheraghi *et al.*, 2021), and in each case has shown improvement in sperm progressive motility, sperm viability, membrane remnancy, and antioxidant status of frozen-thawed semen. It is worth noting that its effectiveness has been established earlier in buffalo semen, where El-Khawagah *et al.* (2020) have observed that the supplementation with quercetin leads to better sperm motility and a ratio of reduced antioxidative stress indicators in Egyptian buffalo bulls. Nevertheless, there has not been a thorough analysis that includes sperm DNA integrity test, and *in vivo* reproductive success, especially in Kundhi buffalo bulls which has been a poorly represented breed in the reproductive research.

Thus, the current study was planned to systematically evaluate dose-dependent effects of quercetin supplementation (0, 25, 50, and 100μM) in a Tris-citric acid extender on post-thaw semen quality parameters, oxidative stress markers, antioxidant enzyme activities, sperm DNA fragmentation, and *in vivo* fertility of Kundhi buffalo bulls. The findings of this study are expected to provide evidence-based recommendations for optimizing quercetin supplementation in buffalo bull semen cryopreservation protocols and to contribute to the broader field of livestock reproductive biotechnology.

MATERIALS AND METHODS

Animals and management: The experiment was conducted in Semen Production Unit, Department of Animal Reproduction, Sindh Agriculture University, Tandojam, Pakistan, for a period of four months from November 2025 to February 2026. Four clinically healthy and sexually active Kundhi buffalo bulls, aged 3-4 years with average body weight of 500 to 600kg, were housed in individual bull sheds. They were fed 5kg green fodder, 1.5kg wheat straw, 0.5kg concentrate and 50g mineral mixture per 100kg body weight per day, with *ad libitum* supply of water.

Semen collection and initial evaluation: Semen from four experimental bulls was collected twice weekly over a period of four weeks. Resultantly, 32 ejaculates from four bulls were collected through an artificial vagina that was pre-warmed to 42°C. Following collection, all collected ejaculates were taken to the laboratory, where they were kept at 37°C in a water bath. The ejaculates with a minimum concentration of 500×10^6 sperm/ml and progressive motility of 80% were included in the study. Ejaculates collected from four bulls on each collection day were pooled to avoid variations in the results due to individual bulls. Thus, eight samples were available for further processing.

Semen extension and cryopreservation: Pooled semen samples were extended using a Tris-based extender containing Tris (199.8mM), citric acid (69.75mM), fructose (55.56mM), glycerol (7%), egg yolk (20%), benzyl penicillin and streptomycin, and supplemented with different concentrations of quercetin including 0, 25, 50 and 100uM (Sigma Aldrich, USA). The semen was diluted at 5°C to achieve final concentration of $50x10^6$ sperm/mL. Then diluted semen was placed in cold cabinet for 4h for equilibration. After equilibration, 0.5mL straws of different colors corresponding to each treatment group (white for 0uM, red for 25uM, blue for 50uM and yellow

for 100uM) were filled using filling machine (Repulsion motor, MULTI-FLEX Betriebsart: IP21). Then open ends of straws were manually sealed with polyvinyl chloride (PVC) powder. Vapor freezing of the chilled straws was performed for 7 minutes at 4cm above in liquid nitrogen vapors, after which straws were dipped into liquid nitrogen (−196°C) for freezing (Ali *et al.*, 2024).

Post-thaw semen evaluation: After at least 24h of storage in liquid nitrogen, straws were placed at 37°C in the water bath for 30sec for thawing. Post-thaw semen evaluation comprising sperm motility, sperm viability, sperm plasma membrane integrity, DNA fragmentation, antioxidant enzyme activity and lipid peroxidation was carried out, as per methods described below:

Sperm viability: On a pre-warmed (37^0C) glass slide, a 10µL of post-thawed semen sample was placed and mixed with two to three drops of eosin-nigrosin stain. After 3 minutes, a thin smear was made, the extra stain was rinsed with water and slides were dehydrated with ethanol. Under a phase contrast microscope, dried smear was examined for live and dead sperm at 400X. Sperm that did not absorb eosin stain and showed white head were regarded as live (Ahmad *et al.*, 2011), however pinkish head sperm were those that absorbed the eosin stain and were considered as dead. The eosin-nigrosine stain contained eosin 0.67g and nigrosine 5.0g, dissolved in 100mL distilled water.

Plasma membrane integrity: Hypo-osmotic swelling test (HOST) was applied for assessing sperm plasma membrane integrity, as described earlier (Ijaz *et al.*, 2009). Briefly, 1.0mL of the hypo-osmotic solution was mixed with 100µL of semen and incubated at 37°C for one hour. Then, a small drop of semen was placed on pre-warmed glass slide and examined using a phase-contrast microscope at 400X. A total of 100 spermatozoa were examined and sperm showing swollen or curled tail in response to HOST were considered as having intact plasma membrane.

Antioxidant enzyme analysis: The levels of antioxidant enzymes, including catalase, superoxide dismutase (SOD) and total glutathione were determined in post-thaw sperm. To separate seminal plasma, semen samples (120µL) were centrifuged at 1600×g for 5min at 25°C and supernatant was discarded. Then, 360µL of 1% Triton X-100 was added to the precipitate and incubated for 20min at 25°C. After incubation, the suspension was centrifuged at 4000g for 30min at 25°C and the supernatant containing crude enzyme extract was collected (Hu *et al.*, 2010). The supernatant was utilized in the analysis of the enzymes. ELISA kits were used to determine enzyme activities based on the protocols provided by the manufacturer. The activity of catalase was determined with the help of OxiSelect 339STA-339 (Cell Biolabs, USA), SOD with the help of OxiSelect 19160 kit (Sigma-Aldrich, USA) and total glutathione with the help of OxiSelect 312STA-312 (Tuncer *et al.*, 2013; El-Khawagah *et al.*, 2020).

Oxidative stress (malondialdehyde level) analysis: The lipid peroxidation was determined in terms of malondialdehyde (MDA) level measured by the thiobarbituric acid reactive substances (TBARS) assay (Mughal *et al.* 2013). For this purpose, 800µL semen sample containing $40x10^6$ spermatozoa were mixed with 200µL 8% (w/v) sodium dodecyl sulfate solution, followed by addition of 20% (v/v) acetic acid (pH 3.5) in graduated tube. Subsequently, 1.5mL of 0.8% (w/v) thiobarbituric acid solution and distilled water were added to bring the total volume to 4mL. The solution was heated at 95°C for 60min and allowed to cool down to room temperature. The final volume was adjusted to 5mL by adding distilled water. Thereafter, 5mL of butanol:pyridine solution (15:1, v/v) was added, and the mixture was subjected to centrifugation at 4000g for 10min. Finally, 50µL of supernatant was collected, and absorbance was measured at 492nm using an ELISA reader (HER-480, HT Company Ltd., Essex, UK) (Khosrowbeygi and Zarghami, 2007). The concentration of MDA of each sample (nmol mL^{-1}) was calculated from standard curve prepared using known concentrations of tetramethoxypropane (TMP): 0.00, 0.78, 1.50, 3.10, 6.20, 12.50, 25.00, 50.00, and 100.00µM.

Evaluation of sperm DNA damage: DNA fragmentation in post-thaw semen was assessed by using Halomax kit (Halomax Kit, Halotech DNA, Spain). Semen samples were diluted to achieve sperm concentration of 15-20 million cells/mL by using phosphate-buffered saline (PBS). Agarose was melted in water bath at 90-100°C for 5min and cooled to 37°C. Thereafter, 25µL sample was mixed with agarose solution in micro tube. A 25µL aliquot of this mixture was spread on slides, covered with cover-slip and incubated for 5min at 4°C. Coverslip was then gently removed and lysis solution was added to completely cover the agarose. Slides were incubated at room temperature for 5min, followed by immersion in distilled water for 5min. Dehydration was carried out sequentially using ethanol 70, 90 and 100% (4min each), after which the slides were air dried. The sample was incubated in distilled water for 5min, dried, and stained with eosin for 5min, followed by rinsing for 2min in distilled water. Then, slides were stained with methylene blue for 5min and again rinsed with distilled water for 2min. Finally, the specimen was observed under a microscope at 400x magnification using a green filter. At least 500 spermatozoa per sample were assessed regarding the DNA fragmentation. Sperm with fragmented DNA exhibited nucleoid surrounded by a large dispersed and irregular (spotty) halo chromatin, while sperm without fragmented DNA were exhibiting nucleoids with small, compact and well-defined halo chromatin (Prabowo *et al.,* 2023).

Sperm motility, velocity distribution and kinematics analysis: Post-thaw sperm motility parameters, velocity distribution and kinematic characteristics were evaluated using computer assisted sperm analysis system (CASA; CEROS, Version 12.3, Hamilton-Thorne Biosciences, USA). The analysis was performed under standardized settings as follow: 60Hz frame rate, 56 minimum contrast and 5 pixels minimum cell size; average path velocity (VAP) cutoff of 20.0µm/s; progressive minimum VAP 80.0µm/s; straight line velocity (VSL) cutoff, 0.0µm/s.

For evaluation, post-thawed semen (7µL) was applied onto a pre-warmed slide (37°C) and examined under a 10x objective lens. Measurements which were made included total sperm motility, progressive sperm motility, velocity distribution (rapid, medium, slow), average path velocity (VAP), curvilinear velocity (VCL), straight-line velocity (VSL), beat cross frequency (BCF) and amplitude of lateral head displacement (ALH), as has been described earlier (El-Khawagah *et al.*, 2020). At least 200 sperm cells were examined per sample.

In vivo fertility: According to the *in vitro* findings, 100µM quercetin supplemented semen exhibited the best post-thaw semen quality results, as well as minimized the presence of oxidative stress and DNA damages. Therefore, frozen-thawed semen supplemented with 100µM of quercetin was used for *in vivo* fertility trials. These trials were carried out through 80 artificial inseminations, using 40 control and 40 treatment doses.

Estrus synchronization: For *in vivo* fertility trial, 80 adult Kundhi buffaloes of 4.5 to 6.0 years of age, 2nd to 3rd lactation, with 3 to 4 BCS and clinically normal reproductive tract, were selected and divided into two groups (n=40/group). Estrus synchronization was done with the Ovsynch protocol. At day 0, 50µg Lecirelin acetate, a synthetic analogue of GnRH (2mL Dalmarelin, FATRO) was administered intramuscularly to each animal. On the 7th day, the 150µg Cloprostenol, a synthetic analogue of PGF2α (2mL Dalmazin, FATRO) was injected. On day 9, the second injection of 50µg Lecirelin acetate (2mL Dalmarelin, FATRO) was administered.

Artificial insemination and pregnancy diagnosis: Frozen-thawed semen was used for fixed time artificial insemination of buffaloes 16h following the second injection of GnRH. The trans- rectal ultrasonography was used to confirm pregnancy 45 days after the insemination, using a portable veterinary ultrasound system (HS-102V, Japan), fitted with a 5.0MHz linear-array rectal transducer.

Statistical analysis: The data collected during the study were analyzed using SPSS software (version 24.0, IBM, USA). The results have been presented as mean±standard error (SE). Analysis for differences within groups of treatments was based on one-way analysis of variance (ANOVA). Tukey's post hoc test was used for multiple means comparison, where necessary. Chi-square test was applied for analysis of data on pregnancy rates between animals of two groups. Probability level of P<0.05 was followed for description of statistical significance.

RESULTS

Post-thaw sperm viability and plasma membrane integrity: The effects of quercetin supplementation on post-thaw sperm viability and sperm plasma membrane integrity have been summarized in Fig. 1. Sperm viability and plasma membrane integrity were significantly (P<0.05) influenced by quercetin concentration. The highest sperm viability and plasma membrane integrity were observed in the 100µM quercetin group, which differed significantly from the control, 25 and 50µM quercetin groups. Similarly, 50µM quercetin group showed higher sperm viability and plasma membrane integrity than the control and 25µM groups (P<0.05). Control and 25µM groups showed the lowest values for both parameters.

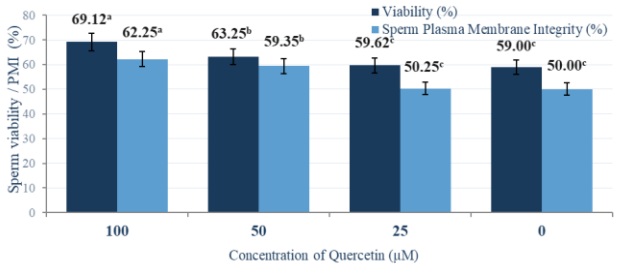

Fig. 1: Effect of quercetin supplementation in extender on sperm viability and plasma membrane integrity of Kundhi bull post-thaw. Values with different letters for each parameter indicate significant differences among groups (P<0.05).

Post-thaw CASA motility and kinematic parameters: Sperm total motility, progressive motility, progressive fast motility and progressive slow motility increased significantly (P<0.05) with increasing quercetin concentration (Fig. 2). The 100µM quercetin group exhibited the highest total motility, progressive motility, progressive fast motility and progressive slow motility, while the control group showed the lowest values for these parameters.

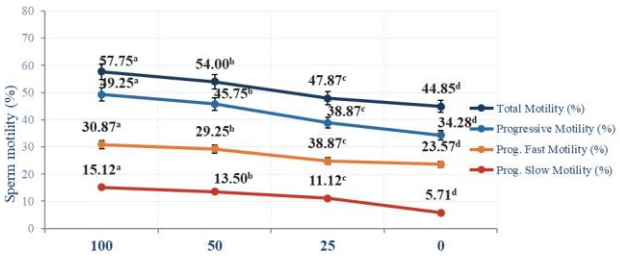

Fig. 2: Effect of quercetin supplementation in extender on sperm total motility, progressive motility, progressive fast motility and progressive slow motility of post-thaw Kundhi bull semen. Values with different letters for each parameter differ significantly (P<0.05).

As shown in Figure 3, sperm velocity parameters including curvilinear velocity (VCL) and straight-line velocity (VSL) were significantly (P<0.05) improved as the quercetin concentration was increased, with 100µM group showed the highest values, while the control had the lowest values. A similar trend was seen for average path velocity (VAP), except that the difference between 50 and 100µM quercetin-treated groups was non-significant.

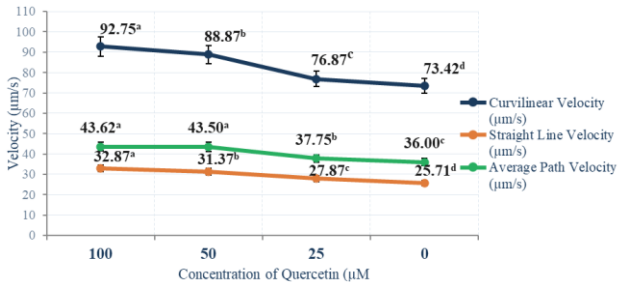

Fig. 3: Effect of quercetin supplementation to extender on VCL, VSL and VAP of post-thaw Kundhi bull sperm. Values with different letters for each parameter differ significantly (P<0.05).

Among distance parameters (Fig. 4), curvilinear distance and straight-line distance increased significantly (P<0.05) with increase in quercetin supplementation; except that a non-significant difference was recorded between 25 and 50µM quercetin groups. For average path distance, values for 50 and 100µM were significantly higher than those for 25µM and control groups (P<0.05); differences in average path distance between the former two groups, as well as the latter two groups, were non-significant.

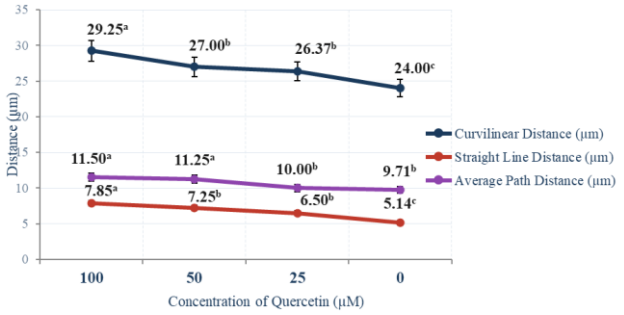

Fig. 4: Effect of quercetin supplementation to extender on curvilinear distance, straight-line distance and average path distance in post-thaw Kundhi dull sperm. Values with different letters for each parameter differ significantly (P<0.05).

The values for amplitude of lateral head displacement were significantly higher (P<0.05) in the 25, 50 and 100µM groups compared to the control (P<0.05); differences among 25, 50 and 100µM groups were non-significant (Table 1). Beat cross frequency values were significantly higher (P<0.05) in the 50 and 100µM groups compared to the 25µM and controls. Differences between control and 25µM groups, as well as between 50 and 100µM groups, were non-significant. Linearity showed a significant improvement at 100µM than 50µM and control (P<0.05), whereas straightness remained statistically similar among all experimental groups, including the control.

Table 1: Effect of quercetin supplementation in extender on Amplitude of lateral head displacement, Beat cross frequency, Linearity and Straightness of Kundhi bull sperm post-thaw

Semen quality parameters	Concentration of Quercetin (µM)			
	0.00	25	50	100
Amplitude of lateral head displacement (µm)	0.7714±0.021^{b}	0.9362±0.028^{a}	0.957±0.024^{a}	0.970±0.026^{a}
Beat cross frequency	4.000±0.150^{b}	4.375±0.172^{b}	5.500±0.210^{a}	5.875±0.225^{a}
Linearity (VSL/VCL)	0.3486±009^{b}	0.3550±0.010^{ab}	0.3488±0.008^{b}	0.3650±0.011^{a}
Straightness (VSL/VAP)	0.7186±0.012^{a}	0.7200±0.010^{b}	0.7200±0.011^{b}	0.7200±009^{a}

Values with different superscripts in the same row indicate significant differences among groups (P<0.05).

Antioxidant enzymes profile: Activities of catalase, super oxide dismutase and glutathione peroxidase enzymes were higher in 50 and 100µM quercetin supplemented groups than those of the control and 25µM supplemented groups (P<0.05; Table 2). There was no difference in these enzyme activities between control and 25µM groups. Superoxide dismutase and glutathione peroxidase activities for 100µM were significantly higher than for 50µM group, but for catalase there was non-significant difference between 50 and 100µM supplemented groups.

Table 2: Effect of quercetin supplementation in extender on antioxidant enzyme activities of Kundhi bull semen post thaw

Antioxidant enzymes in semen	Concentration of Quercetin (µM)			
	0.00	25	50	100
Catalase (U/mL)	1.6200±0.05^{b}	1.6712±0.04^{b}	2.335±0.06^{a}	2.5000±0.05^{a}
Super oxide dismutase (U/mL)	42.143±0.082^{c}	43.375±0.75^{c}	46.250±0.91^{b}	50.625±1.02^{a}
Glutathione peroxidase(µM)	39.571±0.68^{c}	40.000±0.71^{c}	44.000±0.80^{b}	49.875±0.95^{a}

Values with different superscripts in the same row indicate significant differences among groups (P<0.05).

Lipid peroxidation: Lipid peroxidation was assessed in terms of MDA concentrations. Semen MDA concentration decreased significantly (P<0.05) in 50 and 100µM groups compared to control and 25µM groups (Fig. 5). The lowest MDA level was observed in the 100µM group, whereas the difference in MDA between control and 25µM group was non-significant.

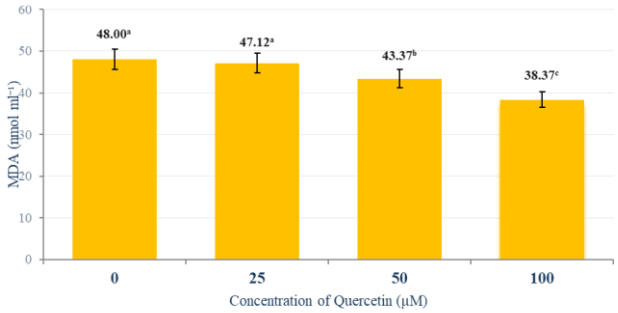

Fig. 5: Effect of quercetin supplementation in extender on MDA levels in Kundhi bull semen post-thaw. Values with different letters indicate significant differences among groups (P<0.05).

Sperm DNA damage: Sperm DNA damage was significantly reduced (P<0.05) in 50 and 100µM quercetin supplemented groups than 25µM quercetin supplemented and control groups (Fig. 6). Sperm DNA was significantly lower in 100µM than 50µM group. The minimum DNA damage was recorded in the 100µM quercetin group, while the control and 25µM groups showed the highest DNA fragmentation values, with non-significant difference between these two groups.

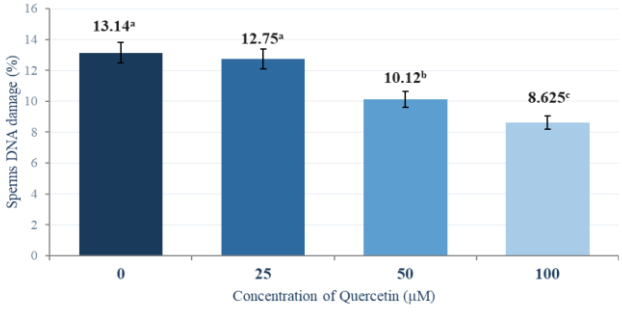

Fig. 6: Effect of quercetin supplementation in extender on sperm DNA damage in post-thaw Kundhi bull semen. Values with different letters indicate significant differences among groups (P<0.05).

In vivo fertility: In vivo fertility results demonstrated a significant improvement in pregnancy rate with 100µM quercetin supplementation (Table 3). The pregnancy rate increased from 40% in the control group to 65% in 100µM group, with chi-square analysis indicated a significant difference (P<0.05) in pregnancy rates between groups.

Table 3: Effect of quercetin supplementation in extender on *in vivo* fertility of Kundhi bull semen

Concentration of Quercetin (µM)	No. of animals inseminated	Pregnancy rate (%)	Chi-square value	P-value
0.00	40	40.00	5.02	0.025*
100.00	40	65.00		

*Significant (P<0.05).

DISCUSSION

The present study provides comprehensive evidence that quercetin supplementation in Tris-based extender exerts concentration-dependent cytoprotective effects on Kundhi buffalo bull spermatozoa during cryopreservation. Significant improvements were recorded in post-thaw sperm viability, plasma membrane integrity, CASA motility and kinematic parameters, antioxidant enzyme activities, and sperm DNA integrity, alongside a marked reduction in lipid peroxidation. Supplementation of 100µM quercetin to semen extender consistently yielded the most favorable results across all parameters, including a clinically meaningful improvement of *in vivo* pregnancy rates. These results align with and extend the existing literature on quercetin antioxidative role in sperm cryobiology.

Sperm viability and plasma membrane integrity were improved in 100µM and 50µM quercetin groups than the control and 25µM groups. Phospholipids present in the plasma membrane are attacked by ROS induced by cryopreservation and trigger the lipid peroxidation cascades, which impairs the membrane fluidity and selective permeability; eventually plasma membrane ruptures, causing death of sperm (Agarwal *et al.*, 2008). By its high membrane affinity and capacity to intercalate into the phospholipid bilayers due to its five hydroxyl groups, quercetin stabilizes sperm membranes by scavenging ROS and preventing lipid peroxidation during cryostress (Tammela *et al.*, 2004; Moretti *et al.*, 2012).

Similar improvement in the integrity of sperm plasma membranes after quercetin supplementation was previously been observed in different mammalian and avian species. El-Khawagah *et al.* (2020) found sperm plasma membrane integrity improvement in Egyptian buffalo bulls fed on quercetin at 10µM. In crossbred Kamori goats, Batool *et al.* (2024) reported that quercetin at concentrations of physiological relevance enhanced post-thaw sperm membrane functionality and viability due to its direct free radical scavenging, as well as membrane lipid stabilization activity. In contrast, Rakha *et al.* (2020) stated that quercetin did not affect sperm viability in Indian red jungle fowl.

Following supplementation to semen extender, quercetin showed significant dose-effective results on sperm total motility, progressive motility and various kinematic variables such as VCL, VSL, VAP, ALH, and BCF. The 100µM quercetin supplemented group showed higher total motility and progressive motility compared to control and other quercetin supplemented groups. Sperm motility plays a key role in fertilizing capacity of sperm and is especially vulnerable to the membrane lipid peroxidation and mitochondrial dysfunction caused by oxidative stress (Kadirvel *et al.*, 2021).

In previous studies, Arora *et al.* (2021) and Yanez-Ortiz *et al.* (2022) reported that the improvement of sperm velocity parameters is presumably an indication of maintenance of mitochondrial membrane potential and axonemal integrity by quercetin. Semen cryopreservation is one of the important contributors to impaired sperm energy metabolism through the mitochondrial ROS generation, which is directly associated with a reduction in flagellar beating frequency and progressive motility. Quercetin supplementation appears to suppress mitochondrial superoxide production through upregulation of SOD and inhibition of electron chain uncoupling. The increase in ALH and BCF at 50 and 100µM levels suggests the increase in sperm hyperactivation capability to be a requirement in zona pellucida penetration during fertilization (Seifi-Jamadi *et al.*, 2017). Similar dose-dependent kinematic effects of quercetin have been reported in Türkmen stallions (Seifi-Jamadi *et al.*, 2016) and in human sperm (Cheraghi *et al.*, 2021), supporting the idea that quercetin exhibits its motility enhancing effect across different species.

Results of the current study showed that SOD, CAT and GPx enzyme activities in post-thaw semen were higher for 50 and 100µM quercetin-treated groups compared to the control and 25µM groups, with the highest activities were observed for 100µM. These results imply that quercetin not only scavenges the ROS but also enhances the endogenous antioxidant enzyme systems of the sperm, which improves the inherent protective system of the cell against oxidative damage.

The quercetin-induced antioxidant enzyme activities have been shown to be correlated with the fact that it activates the Nrf2 (Nuclear factor erythroid 2-related factor 2)/ARE (Antioxidant Response Element) pathway, which transcriptionally up-regulates the expression of phase II cytoprotective enzymes, including SOD, CAT and GPx (Pintus and Ros-Santaella, 2021; Tanaka *et al.*, 2022). This nuclear signaling pathway offers a molecular explanation for the durability of the antioxidant effect of quercetin supplemented semen despite the immediate effect on free radical scavenging. The generalizability of these findings to buffalo reproductive biology can be suggested because similarly increased antioxidant enzyme profiles have been reported across species, such as goats (Batool *et al.*, 2024), poultry (Rakha *et al.*, 2020) and human sperm (Cheraghi *et al.*, 2021).

The concentration of MDA, which is a commonly used biomarker of lipid peroxidation, was decreased significantly by quercetin supplementation in 50 and 100µM compared to control and 25µM groups, with the lowest value was observed for 100µM. This indicates that quercetin is effective in inhibiting the peroxidative degradation of sperm membrane phospholipids during the freeze-thaw cycle, a process which is mechanistically associated with decreased sperm motility, viability and fertilizing potential (Bilodeau *et al.*, 2000; Nair *et al.*, 2006).

The decrease in MDA levels by quercetin supplementation of extender recorded in this study is supported by El-Khawagah *et al.* (2020), who recorded quercetin-mediated inhibition of lipid peroxidation alongside enhanced sperm kinematic parameters in Egyptian buffalo bulls. The reduction of MDA levels in 50 and 100µM supplemented groups indicates that antioxidant effects of quercetin on the sperm plasma membrane take a concentration-bound limit in the physiological range of concentration tested, and the 100µM level is the optimal concentration of quercetin to maximize the protection of lipid peroxidation in this species. Such protective effect is of particular importance because Kundhi buffalo sperm membranes are characterized by a high concentration of PUFA, and this molecular vulnerability of this species to oxidative stress following cryopreservation is the main factor for poor post-thaw sperm quality (Andrabi, 2009).

Quercetin supplementation at 50 and 100µM concentrations significantly reduced post-thaw sperm DNA fragmentation compared to control and 25µM groups. Whereas in fresh semen, DNA damage was minimal (3.13%; unpublished data), the freeze-thaw cycle led to high DNA damage across all groups, which validates the claim that the freeze-thaw cycle is an important genotoxic stressor for buffalo spermatozoa. The fact that SDF is significantly attenuated in quercetin-treated groups reflects the value of antioxidant cytotoxic protection against genomic integrity during cryostress.

Molecular pathways involved in quercetin to protect DNA are multi-complementary. The cryopreservation-induced SDF is mainly due to oxidative attack on sperm chromatin, mediated by ROS, and mostly appears as 8-oxo-7,8-dihydro-deoxyguanosine (8-OHdG) lesions and strand breaks, while free radical scavenging activity of quercetin directly interrupts this oxidative cascade (Agarwal *et al.*, 2008; Zribi *et al.*, 2012). Moreover, the anti-apoptotic effects, such as the inhibition of caspase-3 activation and maintenance of the mitochondrial membrane potential, can help to decrease the apoptosis-related endonuclease-mediated DNA degradation during cryopreservation (Leushuis *et al.*, 2019; Yan *et al.*, 2022). According to Zribi *et al.* (2012), quercetin can maintain the integrity of the human sperm DNA during freeze-thaw process because it suppresses the oxidative stress markers. Batool *et al.* (2024) also reported a decrease in SDF in goat semen after quercetin supplementation. Considering the already established association between high levels of SDF and poor fertility rates, the fact that quercetin considerably lowers the occurrence of DNA damage is a rather convincing argument in the context of integrating quercetin into the process of buffalo bull semen cryopreservation.

The *in vivo* fertility experiment showed that the pregnancy rate in 100µM quercetin-supplemented group (65%) was higher compared to 40% in the control group. The buffalo breeding programs have a direct practical value with this 25-percentage-point increase in conception rate. The increase in pregnancy rate is determined by the compounding effect of better post-thaw sperm quality under a variety of parameters including motility, viability, membrane integrity, and DNA integrity, each of which contributes to the fertilizing ability of spermatozoa *in vivo*.

According to Batool *et al.* (2024), pregnancy rates are higher in Kamori goats inseminated with frozen-thawed semen supplemented with quercetin, which supports findings of this study, where pregnancy rates were higher in buffaloes inseminated with frozen-thawed semen supplemented with 100µM quercetin compared to the control group. Notably, pregnancy rates are an integrative indicator of the quality of semen, not only the motility of sperm and the integrity of their membrane but also the preservation of DNA, the loss of which is detrimental during the early embryonic development (Priyanto *et al.*, 2019; Agarwal *et al.*, 2020). The resultant decrease in SDF, which has been recorded in the current study, is probably a factor in the increased pregnancy rate due to a decrease in early embryonic death caused by paternally the source of DNA damage. These results are indicative of the translational application of quercetin supplementation using laboratory measures of semen quality into field performance of fertility in Kundhi buffalo bulls.

Conclusions: The present study demonstrated that supplementation of quercetin to a tris-based extender significantly improved post-thaw semen quality of Kundhi buffalo bulls. Quercetin effectively reduced oxidative stress, enhanced sperm motility and membrane integrity, and preserved DNA integrity during the cryopreservation process. These improvements ultimately contributed to better fertility results. It is concluded that quercetin can be considered as a beneficial antioxidant supplement for semen extenders to enhance the efficiency of frozen-thawed buffalo bull semen and improve fertility rates in females.

Ethical approval: All the experiment procedures in this study were duly approved by the relevant ethics committee of Sindh Agriculture University, Tandojam, Pakistan.

Data availability: The data supporting the findings of this study can be obtained from the corresponding author upon reasonable request.

Competing interests: The authors declare no competing interests.

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Authors contribution: KAM and NAK conceived the idea and planned the study. The experiment and data collection were done by KAM, HST and AK. The laboratory tests and data analysis were carried out and the manuscript was prepared by KAM. The study was supervised by NAK, who also critically revised the manuscript. The final version of the manuscript was critically reviewed and approved by all authors.

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