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# Impact of Quercetin implementation in skimmed milk extender on the quality of cooled-stored Arabian stallion's semen

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**ABSTRACT**

Cold storage of stallion semen alters semen characteristics and exposes spermatozoa to oxidative stress. The current study aimed to evaluate the roles of quercetin supplementation in skim milk extender in improving semen quality and preventing lipid peroxidation. Semen was collected from three proven fertile Arabian stallions over 8 weeks using a Missouri artificial vagina. Collected ejaculates were extended with skim milk extender (Kenney extender) supplemented with quercetin at 0 (control), 25 µM, 50 µM, and 100 µM concentrations and cooled to 4 ºC. Cold-stored extended semen was evaluated at 12-hours intervals for up to 72 hours for sperm progressive motility, livability, and total abnormalities. Sperm-free extender samples were assessed for acid phosphatase (ACP), alkaline phosphatase, aspartate aminotransferase (AST), lactate dehydrogenase, Catalase (CAT), superoxide dismutase and glutathione peroxidase, Malondialdehyde (MDA) and acrosin activity. The results showed that quercetin had a significant effect on stallion sperm progressive motility and livability (improved at 60 and 72 hours, respectively), ACP and MDA (reduced at 12 hours), AST (reduced at 24 hours), acrosin (reduced at 1-3 days) and CAT (increased at 1-3 days) during cold storage. The effect of quercetin was prominent at a concentration of 50 µM. In conclusion, quercetin supplementation to the cooled stallion semen extender is beneficial in maintaining progressive motility and livability, lower extra-cellular escape of enzymes and decreasing lipid peroxidation perhaps accused to enhancement of catalase enzyme activity during short term-storage.

## 1. INTRODUCTION

The practice of cooled stallion semen is constrained by its short-term fertilizing capacity in association with peroxidation of sperm membrane lipids (Aurich et al., 1997). Exposure of stallion spermatozoa to low temperature during storage induces stressful conditions and reduces cell viability. Successful preservation of stallion spermatozoa is difficult due to lipid peroxidation-induced damage of sperm during cold storage. The highly unsaturated fatty acids (e.g., arachidonic acid, docosapentaenoic acid and docosahexaenoic acid) in stallion plasma membranes contribute to membrane fluidity and proper sperm functions (Macías García et al., 2011) although they predispose to high liability to oxidative stress (Wathes et al., 2007).

Stallion spermatozoa exhibit a powerful metabolic activity, where oxidative phosphorylation in the mitochondria is the primary ATP generator for motility, and maintaining physically and physiologically intact spermatozoa (Davila et al., 2016). Oxidative phosphorylation produces reactive oxygen species (ROS) e.g., superoxide and hydrogen peroxide (Wallace, 1992), which can induce intracellular damage (Bayir, 2005). This oxidative stress contributes to decreased sperm motility, viability, and mitochondrial function (Peña and Gibb, 2022). Therefore, there is a great need to circumvent oxidative stress to maintain spermatozoa longevity outside the body without affecting their fertilizing capacity and/or the consequent pregnancy rate.

Stallion sperm cells have a relatively low cytoplasm and low intracellular antioxidant activity (Ball, 2009). In the meantime, seminal plasma contains diverse antioxidants that can defend against excess ROS accumulation and subsequently protect against oxidative stress (Baumber et al., 2005). There is a high correlation between the enzymatic and non-enzymatic antioxidants of seminal plasma and the tolerance of stallion spermatozoa to cryopreservation (Catalán et al., 2022). Nevertheless, semen dilution for preservation reduces the enzymatic antioxidant (catalase, superoxide dismutase and glutathione peroxidase) contents of seminal plasma compared to raw semen (Bustamante Filho et al., 2009).

Spermatozoa damage due to bacteria, leukocytes and excessive oxidants can be prevented by antioxidants (Bansal and Bilaspuri, 2010). Former studies reviewed the addition of either enzymatic or non-enzymatic antioxidants to guard against oxidative stress during *in vitro* manipulation of spermatozoa. Baumber et al. (2005) showed that the addition of antioxidants (CAT, SOD, GPx, α-tocopherol) to the extender did not improve the quality of equine spermatozoa after thawing. Also, Saad et al. (2021) showed that cysteine and ascorbic acid did not seem to be efficient to counteract oxidative stress during

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cryopreservation of stallion semen. On the other hand, Ghallab et al. (2017) showed that trehalose or zinc sulphate improved cryopreserved Arabian stallion spermatozoa. The use of antioxidant hydroxytyrosol increased the viability and DNA intactness of stallion frozen-thawed semen (Alharbi et al., 2024).

Quercetin is a natural flavanol capable of scavenging hydroxyl radicals and reactive species (Boots et al., 2008) due to the existence and location of the hydroxyl (OH) substitutions and the catechol-type B ring (Stojanović et al., 2001; Moretti et al., 2012). Quercetin antioxidant power is similar to trans-Resveratrol (nonflavonoid polyphenol) and stronger than vitamin E or C (Stojanović et al., 2001) in protection against the sperm damage of lipid peroxidation (Moretti et al., 2012). Quercetin, compared to catalase or cysteine, enhances frozen-thawed motility and zona binding ability of non-sorted stallion spermatozoa, and DNA fragmentation in sex-sorted stallion sperm (Gibb et al., 2013).

To our knowledge, few studies regarding the protective effect of quercetin against the damage of stallion semen during cold storage. Therefore, the present study aims to investigate the effect of quercetin supplemented in skimmed milk-glucose (Kenney) extender on stallion spermatozoa characteristics and biochemistry of sperm-free extender during cold storage.

## 2. MATERIAL AND METHODS

All procedures applied here were approved by the Animal Welfare and Ethics Committees, Fac. Vet. Med., Benha University, Egypt (BUFVTM 02-02–23).

*2.1. Animals and semen collection*

Semen donors were three proven fertile Arabian stallions, 4 - 8 years old, and 3-4 body condition score, reared at The Egyptian Army Equestrian Club, Cairo, Egypt during August-September 2021. Animals were kept solely in a paddock with a distance of 5 meters in length and 3 meters in width. Stallions were fed hay (1 kg/head/day) or clover (4 kg/head/day), and a local pelleted ration 9% protein (6 kg/head/day) with free access to water and mineral blocks.

Semen was collected from stallions twice weekly for two successive months (n= 48 ejaculates) using Missouri artificial vagina (Nasco, Ft. Atkinson, WI, USA) on mares in estrus according to Arifiantini et al. (2013). The inner temperature of the artificial vagina was adjusted to 45–50 °C with hot water, and a gauze filter was added on the top of the collection receptacle to separate the gel part of the semen post-ejaculation.

*2.2. Extenders preparation and semen processing*

Skimmed milk extender (Kenney extender) was prepared according to Kenney et al. (1975) from Non-fat dry skimmed milk (2.40 g), Glucose (4.90 g) and $NaHCO_3$ (8.4%, 2.00 ml) in bi-glass distilled water (100 ml). The extender was heated at 92-95 °C for 10 min, then cooled and filtered. An antibiotic mixture form streptomycin (1 mg/ml) and penicillin (1000 IU/ml) was added to the extender before use. The prepared extender was divided into four portions supplemented with quercetin (Code 117-39-5, Sigma-Aldrich®, USA) at 0 (control), 25 µM, 50 µM, and 100 µM concentration according to a pilot study done before the experiment.

Collected semen samples were evaluated for motility, viability, morphology, and concentration just after collection. Semen from each stallion was diluted to a final concentration of $25\times10^6$ motile sperm/ml using the prepared warmed extender containing quercetin, cooled gradually to 5 ºC within two hours and stored at 5 ºC in the refrigerator for three days (Ghallab et al., 2017).

*2.3. Assessment of chilled semen*

Aliquot part (2 ml) from each treatment was collected at 12-hours intervals during the storage period (up to 72 hours) for evaluation blindly by the same experts. Half ml of extended semen samples from each supplement was used for semen quality assessment, while the rest of the semen aliquots were centrifuged at 1800 ×g (Hermal Compact Centrifuge Z206-A, Germany) for 5 min (Hoogewijs et al., 2010), and the supernatant was harvested and kept at -20 ºC until analysis for biochemical parameters.

*2.4. Evaluation of extended semen quality*

2.4.1. Evaluation of sperm characteristics in the extended semen

Before evaluating semen quality, extended semen from each supplement was warmed at 35 ºC for 10 minutes (Vokrouhlíková et al., 2021).

The sperm progressive motility was assessed by computer-assisted sperm analysis (CEROS II sperm analysis, Code 024905, France) equipped with a pre-warmed phase contrast microscope (Nikon Eclipse E200, phase contrast). The extended semen (3-µl) was loaded on a Leja slide (20 µm depth, Leja® Amsterdam, Netherlands) warmed at 37 °C, and ten microscopic fields were blindly evaluated (Vokrouhlíková et al., 2021).

Sperm livability (n= 3 slides/treatment/sample/time point) was assessed using an eosin-nigrosine stain (Łącka et al., 2016). Extended semen, 5% eosin and 10% nigrosine were mixed at 1:2:4 ratios on a warm slide, smeared, and air dried on a heating stage (37 °C). About 100-200 sperm per slide were counted using a light microscope (×40). The live sperm has clear/white cell head.

The total sperm abnormalities (n= 3 slides/ treatment/sample /time point) in eosin/nigrosine-stained slides according to Dowsett et al. (1984). At least two hundred sperm in each slide were examined.

2.4.2. Assessment of semen biochemical parameters

Sperm-free extender from each treatment was spectrophotometrically (Jenway 6300, USA) assessed for acid phosphatase (ACP, Catalog # AC1011, Randox, Spain), alkaline phosphatase (ALP, Catalog # AP3802, Randox, Spain), aspartate aminotransferase (AST, Catalog # AS3804, Randox, Spain), and lactate dehydrogenase (LDH, Catalog # LD3842, Randox, Spain) were assessed using the appropriate commercial kits.

Catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPX) activities were determined using commercial kits (Catalog #K773-100, #K335-100, and #K762-100, BioVision, USA, respectively) according to Goth (1991), Marklund et al. (1982), and Paglia and Valentine (1967), respectively. Malondialdehyde (MDA) values were measured using colorimetric assay kit (Catalog # K739, BioVision, USA) according to Tavilani et al. (2005). Acrosin activity (µIU/ml) was assessed spectrophotometrically (Jenway 6300, USA) based on BNPNA (N-α-benzoyl-DL-arginine-p-nitroanilide) method according to Cui et al. (2000).

*2.5. Statistical analysis*

Data were presented as Mean ±SEM and statistically analyzed for normality by the Shapiro–Wilk test with SPSS (Ver. 25). The differences between treatment groups were

analyzed with One-Way Analysis of Variance (ANOVA) and Tukey HSD test. P< 0.05 defends statistical significance.

## 3. RESULTS

3.1. Changes in semen picture and biochemical characters of stallion chilled semen with Quercetin addition to extender at the 1st day post-cooling

There was numerical improvement in sperm progressive motility, livability and normality, but did not reach a statistical level at the 1st day post-cooling. ACP and MDA were reduced by 12 hrs, AST was reduced by 24 hrs, and acrosin was reduced, and CAT was increased by 12 and 24 hrs compared to control (Table 1).

3.2. Changes in semen picture and biochemical characters of stallion chilled semen with Quercetin addition to extender at the 2nd day post-cooling

There were little but non-significant differences in sperm progressive motility, livability and normality between Quercetin groups and control at the 2nd day post-cooling. Acrosin enzyme (P< 0.001) decreased, and CAT increased (P< 0.001) in quercetin groups compared to the control at the 2nd day post-cooling (Table 2).

3.3. Changes in semen picture and biochemical characters of stallion chilled semen with Quercetin addition to extender at the 3rd day post-cooling

Sperm progressive motility at 60 hrs and livability at 72 hrs substantially (P< 0.05) improved with 50 µM quercetin compared to control at the 3rd day post-cooling. There was a notable (P< 0.001) decrease in acrosin enzyme, and an increase in CAT in all quercetin groups compared to control at the 3rd day post-cooling (Table 3).

Table 1 Effect of Quercetin incorporation in Kenney extender on the quality of stallion chilled semen at the 1st day post-storage

| Time | Item | Abbrev. | Unit | Control | Q 25µg | Q 50µg | Q 100µg | P value |
|---|---|---|---|---|---|---|---|---|
| 12 hrs post-cooling | Sperm progressive motility | | % | 60.73±2.33 | 61.03±2.26 | 62.83±2.24 | 57.43±2.29 | 0.45 |
| | Sperm livability | | % | 66.67±3.53 | 69.33±4.26 | 73.33±4.26 | 65.33±2.33 | 0.47 |
| | Sperm total abnormalities | | % | 19.33±3.53 | 16.33±2.33 | 14.33±2.19 | 19.00±2.08 | 0.52 |
| | Acid phosphatase | ACP | U/L | 26.22±0.77a | 23.27±0.55b | 23.10±0.52b | 23.80±0.61ab | 0.025 |
| | Alkaline phosphatase | ALP | U/L | 14.51±1.55 | 14.62±1.57 | 14.62±1.57 | 16.07±1.67 | 0.88 |
| | Aspartate aminotransferase | AST | U/L | 195.9±4.3 | 189.8±0.3 | 189.3±0.6 | 190.9±0.8 | 0.22 |
| | Acrosin | Acr. | µIU/ml | 301.00±6.08a | 281.00±6.11ab | 263.33±9.53b | 270.33±9.21b | 0.05 |
| | Lactate dehydrogenase | LDH | U/L | 230.33±3.84 | 233.00±8.19 | 231.00±9.45 | 238.00±8.89 | 0.89 |
| | Catalase | Cat | U/L | 95.37±4.32b | 189.83±0.33a | 189.30±0.61a | 190.93±0.76a | 0.001 |
| | Superoxide dismutase | SOD | U/L | 103.73±2.83 | 102.73±2.72 | 105.37±2.85 | 102.57±2.73 | 0.88 |
| | Glutathione peroxidase | GPx | U/L | 327.33±9.94 | 329.33±3.84 | 327.00±3.61 | 340.33±6.17 | 0.45 |
| | Malondialdehyde level | MDA | nmol/mg | 36.17±0.77a | 33.27±0.55b | 33.10±0.52b | 33.80±0.61b | 0.05 |
| 24 hrs post-cooling | Sperm progressive motility | | % | 49.47±1.72 | 49.63±1.84 | 51.83±1.59 | 43.63±4.21 | 0.22 |
| | Sperm livability | | % | 59.33±2.91 | 65.33±4.81 | 70.33±4.81 | 57.00±2.08 | 0.14 |
| | Sperm total abnormalities | | % | 21.33±3.76 | 17.67±3.18 | 15.67±2.96 | 20.00±3.21 | 0.64 |
| | Acid phosphatase | ACP | U/L | 27.28±0.67 | 25.37±1.00 | 25.17±1.00 | 25.80±1.00 | 0.42 |
| | Alkaline phosphatase | ALP | U/L | 14.82±1.67 | 14.74±1.59 | 14.71±1.56 | 16.20±1.70 | 0.89 |
| | Aspartate aminotransferase | AST | U/L | 206.8±4.5a | 193.2±0.6b | 192.8±0.6b | 193.8±0.5b | 0.01 |
| | Acrosin | Acr. | µIU/ml | 265.67±2.60a | 260.67±1.45ab | 249.00±1.53c | 258.00±1.53b | 0.001 |
| | Lactate dehydrogenase | LDH | U/L | 236.33±1.76 | 240.67±10.59 | 234.67±9.49 | 244.33±10.73 | 0.86 |
| | Catalase | Cat | U/L | 106.30±4.51b | 193.23±0.58a | 192.77±0.61a | 193.80±0.47a | 0.001 |
| | Superoxide dismutase | SOD | U/L | 104.27±2.72 | 103.53±2.68 | 104.77±2.06 | 103.40±2.79 | 0.98 |
| | Glutathione peroxidase | GPx | U/L | 332.00±9.61 | 331.33±5.70 | 331.67±2.96 | 347.67±6.44 | 0.29 |
| | Malondialdehyde level | MDA | nmol/mg | 37.23±0.67 | 35.37±1.00 | 35.17±1.00 | 35.80±1.00 | 0.52 |

Values with different letters at the same row were significantly different

Table 2 Effect of Quercetin incorporation in Kenney extender on the quality of stallion chilled semen at the 2nd day post-storage

| Time | Item | Abbrev. | Unit | Control | Q 25µg | Q 50µg | Q 100µg | P value |
|---|---|---|---|---|---|---|---|---|
| 36 hrs post-storage | Sperm progressive motility | | % | 38.67±4.32 | 38.30±4.39 | 41.17±4.76 | 34.67±4.62 | 0.79 |
| | Sperm livability | | % | 48.67±3.53 | 57.00±5.57 | 61.33±5.24 | 39.33±7.45 | 0.10 |
| | Sperm total abnormalities | | % | 24.00±4.36 | 19.33±3.48 | 17.00±3.21 | 22.67±3.84 | 0.57 |
| | Acid phosphatase | ACP | U/L | 28.42±0.38 | 27.10±1.63 | 26.93±1.64 | 27.63±1.64 | 0.881 |
| | Alkaline phosphatase | ALP | U/L | 14.80±1.68 | 14.73±1.59 | 14.70±1.57 | 16.20±1.70 | 0.89 |
| | Aspartate aminotransferase | AST | U/L | 200.4±6.0 | 192.9±0.6 | 192.5±0.6 | 193.2±0.4 | 0.27 |
| | Acrosin | Acr. | µIU/ml | 263.67±2.60a | 259.00±1.15ab | 246.33±1.86c | 255.67±1.86b | 0.001 |
| | Lactate dehydrogenase | LDH | U/L | 242.00±4.16 | 245.67±10.04 | 241.00±10.26 | 250.67±9.84 | 0.86 |
| | Catalase | Cat | U/L | 99.93±6.01b | 192.90±0.61a | 192.50±0.61a | 193.17±0.37a | 0.001 |
| | Superoxide dismutase | SOD | U/L | 105.73±1.74 | 104.83±1.68 | 106.47±1.78 | 104.30±1.72 | 0.83 |
| | Glutathione peroxidase | GPx | U/L | 338.00±9.85 | 341.00±4.16 | 339.33±3.84 | 351.67±6.89 | 0.49 |
| | Malondialdehyde level | MDA | nmol/mg | 38.37±0.38 | 37.10±1.63 | 36.93±1.64 | 37.63±1.64 | 0.92 |
| 48 hrs post-storage | Sperm progressive motility | | % | 32.67±1.54ab | 32.33±1.55ab | 34.33±1.51a | 27.93±1.49b | 0.08 |
| | Sperm livability | | % | 44.67±3.53 | 52.67±4.37 | 56.33±3.71 | 43.67±2.85 | 0.11 |
| | Sperm total abnormalities | | % | 24.67±1.86 | 21.33±4.06 | 17.67±3.53 | 25.67±4.41 | 0.44 |
| | Acid phosphatase | ACP | U/L | 28.85±0.78 | 28.23±1.58 | 28.07±1.55 | 29.77±2.22 | 0.867 |
| | Alkaline phosphatase | ALP | U/L | 14.78±1.69 | 14.73±1.59 | 14.70±1.57 | 16.19±1.71 | 0.89 |
| | Aspartate aminotransferase | AST | U/L | 197.3±7.3 | 192.6±0.7 | 192.2±0.5 | 192.9±0.5 | 0.73 |
| | Acrosin | Acr. | µIU/ml | 261.67±2.60a | 256.67±0.88ab | 244.00±2.08c | 253.67±1.86b | 0.001 |
| | Lactate dehydrogenase | LDH | U/L | 244.33±4.41 | 242.33±11.35 | 236.33±11.46 | 248.67±10.35 | 0.84 |
| | Catalase | Cat | U/L | 96.83±7.32b | 192.57±0.71a | 192.17±0.52a | 192.87±0.52a | 0.001 |
| | Superoxide dismutase | SOD | U/L | 105.47±1.74 | 104.50±1.71 | 106.20±1.48 | 104.23±1.65 | 0.83 |
| | Glutathione peroxidase | GPx | U/L | 336.33±9.53 | 343.33±4.41 | 341.67±4.10 | 350.00±7.09 | 0.56 |
| | Malondialdehyde level | MDA | nmol/mg | 38.80±0.78 | 34.90±2.66 | 34.73±2.66 | 33.10±1.47 | 0.34 |

Values with different letters at the same row were significantly different

Table 3 Effect of Quercetin incorporation in Kenney extender on the quality of stallion chilled semen at the 3rd day post-storage

| Time | Item | Abbrev. | Unit | Control | Q 25µg | Q 50µg | Q 100µg | P value |
|---|---|---|---|---|---|---|---|---|
| 60 hrs post-storage | Sperm progressive motility | | % | 25.27±1.37ab | 24.90±1.31ab | 26.83±1.26a | 20.33±1.18b | 0.03 |
| | Sperm livability | | % | 43.33±3.71 | 51.00±4.51 | 54.00±3.61 | 42.33±3.18 | 0.15 |
| | Sperm total abnormalities | | % | 29.33±5.24 | 23.33±4.33 | 20.33±3.84 | 27.67±5.36 | 0.55 |
| | Acid phosphatase | ACP | U/L | 28.68±0.81 | 28.10±1.44 | 27.87±1.42 | 29.40±1.70 | 0.863 |
| | Alkaline phosphatase | ALP | U/L | 14.75±1.71 | 14.73±1.59 | 14.68±1.55 | 16.15±1.70 | 0.89 |
| | Aspartate aminotransferase | AST | U/L | 195.2±6.9 | 192.1±0.7 | 191.5±0.7 | 192.2±0.4 | 0.87 |
| | Acrosin | Acr. | µIU/ml | 259.00±2.65a | 253.00±1.00ab | 241.00±2.08c | 251.67±1.45b | 0.001 |
| | Lactate dehydrogenase | LDH | U/L | 243.00±4.36 | 239.67±11.39 | 233.67±11.70 | 245.33±11.68 | 0.86 |
| | Catalase | Cat | U/L | 94.73±6.87b | 192.07±0.69a | 191.53±0.74a | 192.20±0.38a | 0.001 |
| | Superoxide dismutase | SOD | U/L | 105.27±1.71 | 104.37±1.74 | 107.00±1.66 | 104.07±1.79 | 0.64 |
| | Glutathione peroxidase | GPx | U/L | 333.67±9.77 | 342.00±4.36 | 338.67±3.53 | 348.33±6.77 | 0.49 |
| | Malondialdehyde level | MDA | nmol/mg | 38.63±0.81 | 34.77±2.55 | 37.87±1.42 | 36.07±2.09 | 0.51 |
| 72 hrs post-storage | Sperm progressive motility | | % | 18.83±1.74 | 18.50±1.80 | 20.70±1.76 | 14.37±1.42 | 0.13 |
| | Sperm livability | | % | 39.00±2.65b | 48.33±4.67ab | 52.33±3.71a | 38.67±1.86b | 0.05 |
| | Sperm total abnormalities | | % | 31.00±5.51 | 24.67±4.06 | 21.33±3.84 | 29.00±5.86 | 0.53 |
| | Acid phosphatase | ACP | U/L | 28.32±0.84 | 27.37±1.36 | 27.17±1.36 | 28.33±1.39 | 0.866 |
| | Alkaline phosphatase | ALP | U/L | 14.75±1.70 | 14.72±1.59 | 14.67±1.55 | 16.13±1.69 | 0.90 |
| | Aspartate aminotransferase | AST | U/L | 194.9±6.8 | 191.8±0.6 | 191.0±0.8 | 191.9±0.3 | 0.85 |
| | Acrosin | Acr. | µIU/ml | 255.33±2.91a | 250.33±0.88a | 238.00±2.08b | 249.33±1.20a | 0.001 |
| | Lactate dehydrogenase | LDH | U/L | 241.00±4.36 | 237.00±12.01 | 231.00±11.79 | 243.33±11.68 | 0.84 |
| | Catalase | Cat | U/L | 94.40±6.79b | 191.77±0.65a | 191.00±0.75a | 191.87±0.28a | 0.001 |
| | Superoxide dismutase | SOD | U/L | 105.03±1.80 | 103.80±2.08 | 107.43±1.79 | 103.90±2.25 | 0.56 |
| | Glutathione peroxidase | GPx | U/L | 330.67±10.17 | 340.00±4.36 | 336.33±3.28 | 345.00±6.03 | 0.49 |
| | Malondialdehyde level | MDA | nmol/mg | 38.27±0.84 | 37.37±1.36 | 37.17±1.36 | 35.00±2.67 | 0.62 |

Values with different letters at the same row were significantly different

## 4. DISCUSSION

The cooled-shipped semen is routinely used in horse breeding (Heckenbichler et al., 2011), but with unpredictable results due to poor fertilizing capacity associated with oxidative stress. The applicability of an antioxidant to relief the oxidative stress during cold storage of stallion spermatozoa is extremely mandatory. Quercetin has the structural components of an antioxidant through its hydroxylation pattern of 3, 5, 7, 30, and 40 and a catechol B-ring (Silva et al., 2002; Rietjens et al., 2005). The strength of quercetin to counteract ROS and nitric oxide accumulation is higher than many antioxidants e.g., vitamin E or C (Stojanović et al., 2001). The current study revealed a significant positive effect to quercetin, natural antioxidant flavonoid, on stallion sperm progressive motility and livability (improved at 60 hrs and 72 hrs, respectively), ACP and MDA (reduced at 12 hrs), AST (reduced at 24 hrs), acrosin (reduced at 1-3 days) and CAT (increased at 1-3 days) during cold storage. There is a significant correlation between semen progressive motility, morphological normality and CASA, and pregnancy rate/cycle in equines (Jasko et al., 1990). Therefore, sperm quality traits could be used to mark stallion fertility. The stallion sperm motility, viability, and fertility are adversely affected during chilling and freezing storage (Aurich, 2005). This is attributed to the high concentration of sperm plasma membrane polyunsaturated fatty acids that make sperm more vulnerable to oxidative stress and lipid peroxidation, which damages DNA and reduces sperm motility and viability (LeFrapper et al., 2010; Rečková et al., 2022). Prevention/reduction of oxidation/reduction potential may be a promising clue to enhance sperm quality by competing with oxidative stress during cold storage (Seifi-Jamadi et al., 2016). In this study, slight numerical improvement in sperm progressive motility and livability was observed with 50 µM quercetin, and less with 25 µM, but with deteriorating effects with 100 µM during the first two days of storage. This semen traits reinforcement reached a statistically significant level at 60 hrs (motility) and 72 hrs (livability) post-storage. Transmission electron microscopy analysis revealed protective action of quercetin (30 µM) on damage induced by lipid peroxidation (Moretti et al., 2012). Nearby, McNiven and Richardson (2003) results showed that quercetin at 0.1 mM in standard skim milk extender maintained high sperm motility and membrane intactness at 48, 96 and 144 hrs post-cold storage, though its ATP-stimulating effect was evident at 96 and 144 hrs only. In frozen-thawed stallion semen, quercetin at 0.1 mM supplemented in egg yolk-based extender improved total and progressive motility (Seifi-Jamadi et al., 2016). The impact of quercetin on sperm traits e.g., motility and livability could be attributed to its function in increasing the efficiency of bioenergetic cost of motility in a manner comparable to its action on the Na+/K+ pump (Hammerstedt et al., 1988). Besides, quercetin interacts with Ca2+-ATPase which is an important enzyme that controls sperm motility (Williams and Ford, 2003). Furthermore, the reduction of the intracellular calcium concentrations mediates the synthesis of intracellular cyclic adenosine monophosphate (cAMP) which is essential for maintaining sperm motility (Si and Okuno,1999).

Reactive oxygen species built extracellularly can enter sperm cells, accumulate inside mitochondria, and prevent its function (Carrasco-Pozo et al., 2012; Avdatek et al., 2018). A negative relation has been reported between the level of ROS and the sperm mitochondrial membrane potential (Chatterjee and Gagnon, 2001). Semen extracellular-leaked enzyme activity is an indicative of sperm membrane stability and semen quality (Corteel, 1980; El-Khawagah et al., 2020). Cold shock alters the normal configuration of sperm membrane and causes membrane damage due to ROS production (Arifiantinia et al., 2013; Loureiro et al., 2020). In this study, quercetin supplementation reduced extracellular ACP and AST (12 and 24 hrs post-cooling, respectively). Quercetin also reduced lipid peroxidation (MDA) and acrosin enzyme (at 12 hrs and 1-3 days post-cooling, respectively) concomitant with increased CAT activity along the cold storage period. Dilution of equine centrifuged semen with skim milk-egg yolk-based extender equilibrates the non-enzymatic antioxidant protection, but not the enzymatic antioxidant defense, lost with seminal plasma separation (Bustamante Filho et al., 2009). So, the increase in CAT enzymatic activity in our study is assumed to be due to the quercetin effect. Low extra-cellular ACP and AST recorded here indicated plasma membrane intactness of cooled stallion

semen and matches to the study of McNiven and Richardson (2003), which showed high intact plasma membrane with quercetin compared to control at 48 hrs (55.6 vs. 46.1%), 96 hrs (44.4 vs. 24.7 %) and 144 hrs (26.2 vs.9.6%). The catalase enzyme is an indicator of male fertility. It splits $H_2O_2$ (ROS member involved in oxidative stress) into $H_2O$ and $O_2$. Low CAT and total antioxidant capacity of seminal plasma are also correlated with sperm abnormalities (Khosrowbeygi et al., 2004). Flavonoids e.g., Quercetin have diverse functions e.g., ROS scavenging, oxidase inhibition, metal chelation, and DNA protection (Afanas'ev et al., 1989).

## 5. CONCLUSIONS

Collectively, quercetin at a concentration of 50 µM, in cooled skim-milk extender is beneficial in maintaining high stallion sperm progressive motility and livability and decreasing the leaked cellular enzymes and lipid peroxidation in association with catalase enzyme activation during cold storage. Further studies are mandatory to verify the effect of improved semen quality with quercetin on its fertilizing capacity.

## CONFLICT OF INTEREST

No conflict of interest.

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