Accepted: 20 November 2017

DOI: 10.1111/and.12975

ORIGINAL ARTICLE

WILEY ANDROLOGIA

# Supplementation of quercetin for advanced DNA integrity in bull semen cryopreservation

F. Avdatek[1] | D. Yeni[1] | M. E. İnanç[2] | B. Çil[3] | B. P. Tuncer[4] | R. Türkmen[5] | U. Taşdemir[6]

[1]Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Afyon Kocatepe University, Afyonkarahisar, Turkey

[2]Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Mehmet Akif University, Burdur, Turkey

[3]Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Ankara University, Ankara, Turkey

[4]Technical Sciences Vocational School, Mersin University, Mersin, Turkey

[5]Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Afyon Kocatepe University, Afyon, Turkey

[6]Technical Sciences Vocational School, Aksaray University, Aksaray, Turkey

**Correspondence**
Umut Taşdemir, Technical Sciences Vocational School, Aksaray University, Aksaray, Turkey.
Email: tasdemiru@gmail.com

**Funding information**
This study was supported by General Directorate of Agribusiness, Ankara, Turkey.

## Summary

The aim of this study was to identify the effects of adding quercetin (Q) to Tris extender in order to identify levels of oxidative stress in bull sperm after freeze thawing. Ejaculates were collected via artificial vagina from Holstein bulls. Semen was divided into five tools and diluted to a final concentration of $15 \times 10^6$ spermatozoa/ml with the Tris extender containing Q (25, 50, 100 and 200 μg/ml) and no additive (control; C). All examples were equilibrated at 4°C during 4 hr then were loaded into 0.25-ml straws and frozen using a controlled rate. Sperm motility and motility characteristics were determined using the computer-assisted semen analyser. Sperm membrane integrity was assessed using the hypoosmotic swelling test. Sperm chromatin integrity was investigated using the single cell gel electrophoresis. Total antioxidant capacities were performed colorimetrically. Q supplementation used as an antioxidant did not produce better results in the proportion of sperm progressive and total motility, plasma membrane integrity and sperm abnormalities. Q supplementation exhibited the favourable tail length, tail DNA and tail moment. In conclusion, when whole parameters are considered, Q25 can be added to the Tris extender due to its positive effect on sperm DNA integrity and no adverse effect on the progressive and total motilities of sperm.

**KEYWORDS**
antioxidant activity, cryopreservation, DNA integrity, oxidative stress, quercetin

## 1 | INTRODUCTION

Favourable artificial insemination and pregnancy outcomes depend on the capability of cryopreserved spermatozoa to fertilise the oocyte in cattle (El-Harairy, Eid, Zeidan, Abd El-Salaam, & El-Kishk, 2011). Treatment of sperm cells at room temperature during the initial semen evaluations as well as cooling and freezing procedures can generate reactive oxygen species (ROS) and toxic oxidants in semen extender. This causes reduction in energy sources that are necessary for sperm cells, resulting in lipid peroxidation, DNA damage and apoptosis (Guthrie, Welch, & Long, 2008; Taşdemir et al., 2013). Free radicals are produced by ROS when semen is preserved for a long period of time at environmental or subfreezing temperatures (Aitken, 1994). Sperm structures are intensely vulnerable to oxidative stress, which leads to fertility loss and cell death (Aitken, Irvine, & Wu, 1991).

To prevent oxidative stress, decrease cryodamage to sperm or block premature sperm maturation, adding antioxidant supplementation to the semen extender may provide increased protection from potential ROS aggregation (Takahashi et al., 2000). Many natural plants are rich in flavonoids, and their antioxidant properties have been shown to be superior to synthetic antioxidants due to lower cytotoxicity (Gupta & Sharma, 2006). Quercetin (Q) is a flavonoid antioxidant and is able to scavenge reactive species and hydroxyl radicals (Boots, Haenen, & Bast, 2008) as well as providing beneficial health effects: for example, anticarcinogenic (Pereira et al., 1996), anti-inflammatory (Guardia, Rotelli, Juarez, & Pelzer, 2001) and antimicrobial properties (Nitiema, Savadogo, Simpore, Dianou,

  

& Traore, 2012). These flavonoid's positive effects have been related to various characteristics such as oxidative inhibition (Ben Abdallah, Fetoui, Zribi, Fakhfakh, & Keskes, 2013), metal chelating capability (Ferrali et al., 1997) and protection of DNA integrity (Seifi-Jamadi, Kohram, Shahneh, Ansari, & Macías-García, 2016). Supplementation of semen extender with Q has been reported to have beneficial antioxidant properties on post-thaw characteristics in sperm cells of bulls (Tvrda et al., 2016), horses (Seifi-Jamadi et al., 2016), boars (Kim et al., 2014), rams (Silva, Cajueiro, Silva, Soares, & Guerra, 2012) and bucks (Silva, Arruda, Silva, Souza, & Guerra, 2016). In other respects, more research is needed to optimise the use of Q in ROS associated with oxidative stress.

Thus, the aim of this study was to identify the effects of adding Q to Tris extender in order to identify levels of oxidative stress in bull sperm after freeze thawing.

## 2 | MATERIALS AND METHODS

### 2.1 | Animals and study plan

Semen samples were collected from Holstein breed bulls. The bulls are free from any genital or venereal diseases and were breed at Sultansuyu Agribusiness (Sultansuyu, Malatya, Turkey). The study was replicated nine times for each trial. All samples that had concentrations up to $1.0 \times 10^9$ spermatozoa/ml and >80% progressive motility were used as the semen source. Ejaculates were collected via an artificial vagina once a week. Semen samples were plunged into a 37°C water bath as far as they could be assessed for sperm concentration, progressive motility and total motility. The total volume and concentration were determined via graded collection tube and photometer (Minitube GmbH, Tiefenbach, Germany) respectively, soon after semen collection. A Tris-based extender (30.7 g Tris, 16.4 g citric acid, 12.6 g fructose, 20% (v/v) egg yolk, 6% (v/v) glycerol and 1,000 ml distilled water at pH 6.8) was used as the base extender for the trials. Extracted Q (10 mg) was diluted with 1 ml ethanol (Merck, 99%) to create the Q stock solution. Ejaculates were allocated to five fractions and diluted to a final concentration of $15 \times 10^6$ spermatozoa/ml with the base extender containing Q (25, 50, 100 and 200 μg/ml) and no additive (control: C). All dilutions were placed into 0.25-ml French straws. The samples were cooled slowly to 4°C and equilibrated for 4 hr and then were frozen using a controlled-rate freezer (SY LAB Gerate GmbH, Neupurkersdorf, Austria) at five programmed rates (0°C/min from +4°C to +4°C 3 min, −3°C/min from +4°C to −7°C 3.67 min, 0°C/min from −7°C to −7°C 1 min, −37.67°C/min from −7°C to −120°C 3 min, 0°C/min from −120°C to −120°C 5 min). Afterwards, the straws were submerged into liquid nitrogen at −196°C. The study's experimental design was approved by the Animal Care Committee of Afyon Kocatepe University Veterinary Medicine Faculty in terms of ethics, with the authorisation number 49533702/29.

### 2.2 | Assessment of spermatozoa motility and motility characteristics

Sperm motility and motility characteristics were evaluated using the sperm class analyser system (CASA; Microptic S.L., Barcelona, Spain) with a 10 × objective at 37°C. A 5 μl diluted semen specimen was put onto a pre-warmed slide covered with a coverslip and evaluated. Immediately after evaluation, motility values were recorded in percentages: nonprogressive motility, progressive motility and total motility. Average path velocity μm/s (VAP), straight linear velocity μm/s (VSL), curvilinear velocity μm/s (VCL), amplitude of lateral head displacement, μm (ALH), [VSL/VCL] × 100 (LIN), [VAP/VCL] × 100 (WOB), [VSL/VAP] × 100 (STR) and beat cross frequency were determined by measuring the frequency with which the sperm track crossed the cell path in either direction and changeable tracks. Spermatozoa with high velocities, namely hyperactivated spermatozoa, were also identified. The sperm motilities were calculated with speed standards set as fast >80 μm/s, medium >60 μm/s, slow >20 μm/s and static. For each evaluation, between 220 and 370 spermatozoa were analysed in six microscopic fields.

### 2.3 | Assessment of plasma membrane integrity

Sperm membrane integrity was assessed using the hypoosmotic swelling test (HOST), based on swollen and curled tails. Hypoosmotic swelling test was performed by incubating 30 μl of semen added to 300 μl of a 100 mOsm hypoosmotic solution (9 g fructose plus 4.9 g trisodium citrate in a litre of distilled water) at 37°C for 60 min. After incubation, 0.2 ml of the mix was spread on a warm slide with a coverslip. Two hundred sperm cells were evaluated using a phase-contrast microscope (400×, Olympus CX41, Tokyo, Japan). Spermatozoa with swollen or coiled tails showed that the plasma membrane integrity was intact (Uysal & Bucak, 2009).

### 2.4 | Assessment of abnormal spermatozoa

Sperm abnormalities were evaluated following protocols described previously (Schafer & Holzmann, 2000). Hancock solution—62.5 ml formalin (37%), 150 ml buffer solution, 150 ml saline solution and 500 ml double-distilled water—was used for evaluation. Three drops of each specimen were added to Eppendorf tubes containing 1 ml Hancock solution to assess sperm abnormalities. One drop of this mix was put on a slide and covered with a coverslip. The sperm abnormality proportions (head, acrosome, tail and total abnormalities) were recorded, under phase-contrast microscopy (1,000×, Olympus CX41, Tokyo, Japan) by counting a total of 200 oil-immersed sperm cells.

### 2.5 | Assessment of chromatin integrity

Sperm chromatin integrity was investigated using the single cell gel electrophoresis (comet) assay using a comet assay kit (Trevigen, Gaithersburg, MD, USA). Semen samples were thawed at 37°C for 30 s in a water bath and then centrifuged at 300 × g for 10 min at 4°C. To ensure that the final concentration of semen samples had $1 \times 10^5$ sperm cell/ml, the upper part of the supernatant was removed, and the remaining sperm cells were washed with phosphate buffer solution (free of Mg2 + and Ca2 + ). Each microscope slide was pre-coated with a layer of 1% high melting point agarose in PBS, After the slides were thoroughly dried at room temperature, 100 μl of 0.7% low melting point agarose was mixed

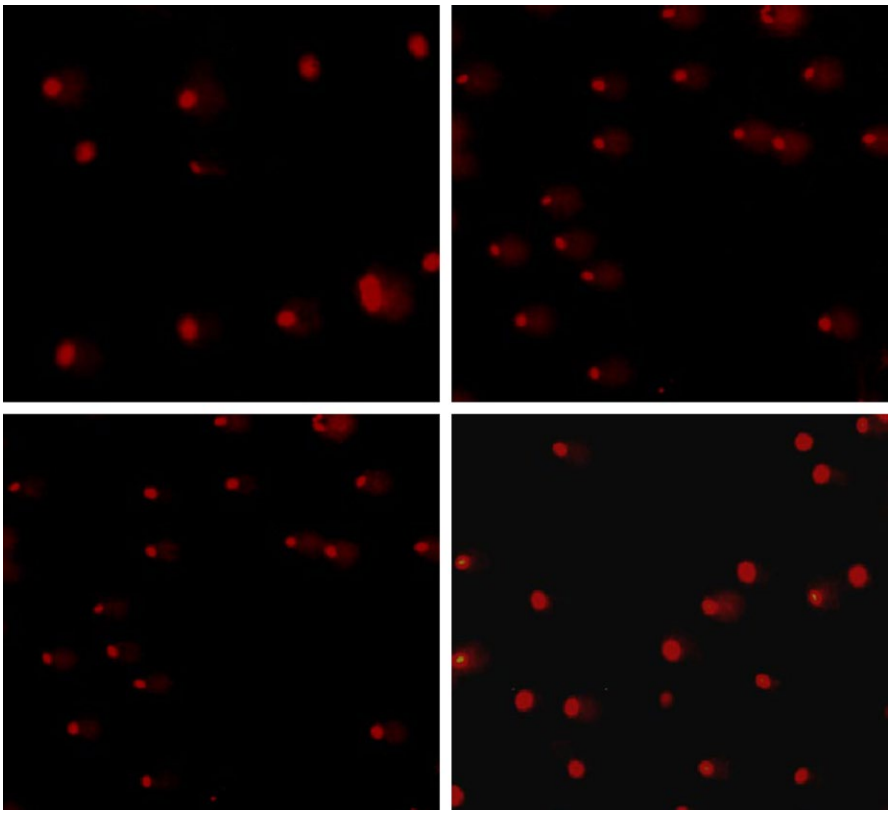

**FIGURE 1** DNA fragmentation visualised by the comet assay

with 10 μl of the cell suspension at 37°C and dropped on top of the first layer. Slides were allowed to solidify in a moist chamber at 4°C for 5 min. The coverslips were removed, and the slides were immersed in a freshly prepared cold lysis buffer containing 2.5 M NaCl, 100 mM Na2-EDTA, 10 mM Tris, 1% Triton X-100 and 40 mM dithiothreitol (pH: 10) at 4°C for 1 hr. The slides were further incubated overnight in 100 μg/ml proteinase K (Sigma) and added to the lysis buffer at 37°C. After cell lysis, all slides were washed with deionised water three times at 10-min intervals to remove salt and detergent from the microgels. The slides were then placed in a horizontal electrophoresis unit filled with fresh alkaline electrophoresis solution containing 300 mM NaOH and 1 mM EDTA (pH: 13) for 20 min to allow the DNA to unwind. Electrophoresis was performed at 25V for 20 min at room temperature and was adjusted to 300 mA. To suspend alkali and detergents, the slides were washed with a neutralising solution of 0.4 M Tris (pH: 7.5). Following neutralisation, the slides were stained with 50 μl of 2.5 μl/ml ethidium bromide (Sigma) and covered with a coverslip. Slides were observed under epifluorescent microscopy (Olympus CX31, Tokyo, Japan), and digital images were captured for subsequent analyses and scoring with comet assay TriTek Comet Score software (V. 1.5: see Figure 1). On each slide, a total of 100 sperm cells from five fields were counted for analysis. All stages were performed under dark room conditions to guard further chromatin damage (Gundogan, Yeni, Avdatek, & Fidas, 2010).

## 2.6 | Assessment of oxidative stress

Total antioxidant (TA) capacities were performed colorimetrically using a commercial kit (RelAssay®, Gaziantep, Turkey). Antioxidants in the specimen reduced dark blue-greencolored 2,2′-azinobis (3-ethyl benzothiazoline-6-sulphonic acid) ammonium salt (ABTS) radical to a colourless reduced ABTS form. The change of absorbance at 660 nm is related to the TA level of the sample. The results were indicated in mmol Trolox equivalents/l. Glutathione peroxidase (GPx) activity was measured using a GPx assay (Bioxytech® GPx-340™, OxisResearch™, Portland, Oregon, USA). The assay is based on an NADPH (nicotinamide adenine dinucleotide phosphate) coupled reaction where oxidised glutathione, produced by reduction of organic peroxide (tert-butylhydroperoxide) by GPx, is recycled to its reduced state by utilising the enzyme glutathione reductase and NADPH. The oxidation of NADPH to NADP+ is associated with a decrease in absorbance at 340 nm, shown using spectrophotometry to follow GPx activity. The rate of decline in absorbance at 340 nm is directly proportional to the GPx activity. The GPx activity is determined from the correlation of the rate of change of absorbance in the sample to the rate of change in absorbance of known concentrations of GPx. Lipid peroxide, derived from polyunsaturated fatty acids, is unstable and decomposes to form a complex series of compounds, of which the most abundant is malondialdehyde (MDA). Levels of lipid peroxidation, which depends on MDA, were identified using an MDA commercial kit (MDA-586; OxisResearch, Portland, OR, USA). One molecule of MDA reacts with two molecules of N-methyl-2-phenylindole (NMPI) to yield a stable substance with maximum absorption at 586 nm. The MDA was determined from the sample's absorbance at 586 nm in the sample, and the standard curve was prepared using the MDA standard provided in the kit. The results were indicated in μmol/ml (Kasimanickam, Pelzer, Kasimanickam, Swecker, & Thatcher, 2006).

**TABLE 1** Mean (±*SE*) sperm motility values in frozen thawed bull semen

| Analysis | Control | Q25 | Q50 | Q100 | Q200 | *p* |
|---|---|---|---|---|---|---|
| Nonprogressive motility (%) | 42.80 ± 2.56$^{ab}$ | 47.11 ± 3.06$^{a}$ | 36.55 ± 2.99$^{b}$ | 45.31 ± 2.52$^{ab}$ | 21.98 ± 3.36$^{c}$ | * |
| Progressive motility (%) | 37.36 ± 2.43$^{a}$ | 36.13 ± 3.36$^{a}$ | 22.45 ± 3.26$^{b}$ | 24.12 ± 2.31$^{b}$ | 3.55 ± 1.05$^{c}$ | * |
| Total motility (%) | 80.17 ± 3.31$^{a}$ | 83.26 ± 1.10$^{a}$ | 59.02 ± 4.94$^{b}$ | 69.42 ± 3.72$^{b}$ | 25.56 ± 4.30$^{c}$ | * |
| VAP (μm/s) | 57.16 ± 1.49$^{a}$ | 55.42 ± 2.72$^{a}$ | 49.11 ± 2.52$^{b}$ | 46.15 ± 2.06$^{b}$ | 26.25 ± 1.70$^{c}$ | * |
| VSL (μm/s) | 43.53 ± 1.60$^{a}$ | 41.45 ± 2.85$^{ab}$ | 36.85 ± 2.45$^{bc}$ | 33.05 ± 1.94$^{c}$ | 15.86 ± 1.39$^{d}$ | * |
| VCL (μm/s) | 90.57 ± 1.75$^{a}$ | 88.43 ± 2.99$^{a}$ | 78.93 ± 2.50$^{b}$ | 76.90 ± 2.62$^{b}$ | 49.61 ± 2.47$^{c}$ | * |
| ALH (μm/s) | 3.95 ± 0.05$^{a}$ | 4.02 ± 0.06$^{a}$ | 3.98 ± 0.12$^{a}$ | 3.95 ± 0.05$^{a}$ | 3.43 ± 0.15$^{b}$ | * |
| BCF (Hz) | 12.46 ± 0.32$^{a}$ | 11.82 ± 0.30$^{a}$ | 12.41 ± 0.30$^{a}$ | 11.75 ± 0.28$^{a}$ | 10.00 ± 0.54$^{b}$ | * |
| LIN (%) | 47.98 ± 1.13$^{a}$ | 46.50 ± 1.68$^{ab}$ | 46.41 ± 1.66$^{ab}$ | 42.73 ± 1.28$^{b}$ | 31.66 ± 1.51$^{c}$ | * |
| STR (%) | 76.01 ± 1.09$^{a}$ | 74.30 ± 1.51$^{ab}$ | 74.68 ± 1.12$^{ab}$ | 71.25 ± 1.21$^{b}$ | 59.90 ± 1.78$^{c}$ | * |
| WOB μm/s | 63.06 ± 0.69$^{a}$ | 62.43 ± 1.02$^{ab}$ | 61.98 ± 1.30$^{ab}$ | 59.86 ± 0.79$^{b}$ | 52.70 ± 1.16$^{c}$ | * |
| Hyperactivity μm/s | 36.67 ± 1.60$^{a}$ | 36.33 ± 2.45$^{a}$ | 22.11 ± 2.29$^{b}$ | 24.13 ± 1.99$^{b}$ | 3.60 ± 0.97$^{c}$ | * |

Different superscripts within the same row demonstrate significant differences (*$p$ < .05).

**TABLE 2** Mean (±*SE*) sperm plasma membrane integrity and abnormality values in frozen-thawed bull semen

| Analysis | Control | Q25 | Q50 | Q100 | Q200 | *p* |
|---|---|---|---|---|---|---|
| HOS T (%) | 34.87 ± 1.21$^{a}$ | 34.37 ± 1.71$^{a}$ | 32.62 ± 1.01$^{ab}$ | 29.87 ± 1.28$^{bc}$ | 27.00 ± 1.70$^{c}$ | * |
| Head abnormalities (%) | 2.60 ± 0.69$^{ab}$ | 3.69 ± 0.45$^{a}$ | 2.73 ± 0.55$^{ab}$ | 3.12 ± 0.50$^{ab}$ | 1.98 ± 0.42$^{b}$ | * |
| Mid-piece abnormalities (%) | 2.74 ± 0.64$^{ab}$ | 3.07 ± 0.39$^{ab}$ | 4.79 ± 0.77$^{a}$ | 3.58 ± 1.41$^{ab}$ | 2.02 ± 0.74$^{b}$ | * |
| Tail abnormalities (%) | 2.94 ± 0.69$^{ab}$ | 3.68 ± 0.73$^{a}$ | 1.17 ± 0.62$^{b}$ | 2.71 ± 0.69$^{ab}$ | 1.40 ± 0.32$^{b}$ | * |
| Total abnormalities (%) | 8.29 ± 1.28$^{ab}$ | 10.45 ± 1.01$^{a}$ | 8.70 ± 1.20$^{ab}$ | 9.41 ± 1.74$^{a}$ | 5.41 ± 0.97$^{b}$ | * |

Different superscripts within the same row demonstrate significant differences (*$p$ < .05).

### 2.7 | Statistical analysis

Parameters were normally distributed using the Shapiro–Wilk normality test. Homogeneities of variance with Levene's test groups were compared using the SPSS/PC computer programme (Version 13.0; SPSS/PC, Chicago, IL). The test ascertained that the variance was homogeneous. Afterwards, comparisons between the groups were made using analysis of variance with the Duncan's post hoc test. The differences were considered significant at $p$ < .05. The results were expressed as means or proportions (±standard error mean).

## 3 | RESULTS

As shown in Table 1, Q supplementation used as an antioxidant did not produce better results in the proportion of sperm progressive and total motility assessed by CASA after thawing. In particular, samples with more than 25 μg/ml Q concentrations exhibited adverse effects when compared to the C group ($p$ < 0.05). When sperm motility characteristics, plasma membrane integrity and sperm abnormalities were considered, the results indicated that current treatment did not improve any of the fields, as shown in Tables 1 and 2. In other findings, DNA integrity was significantly different among treatment groups ($p$ < .05). As shown in Table 3, Q supplementation exhibited favourable tail length, tail DNA and tail moment when compared to C ($p$ < .05). Although GPx activity was greater in Q50, Q100 and C than other groups, it did not enhance the plasma membrane integrity proportion in all groups except C. In addition, MDA and TA activities were not affected positively by the treatment as shown in table 4.

## 4 | DISCUSSION

Oxidative stress was proven to be imbalanced between the generation of ROS and antioxidant clearing activities (Sikka, 2001). Reactive oxygen species formation and membrane lipid peroxidation have negative effects on sperm parameters which are closely related to fertility (Guthrie & Welch, 2012). Motility and motility characteristics provide the most reliable information for predicting bull fertility and the fertilisation capacity of sperm (Verstegen, Iguer-Ouada, & Onclin, 2002). In the current study, Q supplementation did not produce better results in the proportion of both progressive and total sperm motility after

**TABLE 3** Mean (±*SE*) chromatin integrity values in frozen-thawed bull semen

| Analysis | Control | Q25 | Q50 | Q100 | Q200 | *p* |
|---|---|---|---|---|---|---|
| Tail length (μm/s) | 43.40 ± 2.20$^{a}$ | 33.18 ± 3.00$^{b}$ | 10.83 ± 2.07$^{c}$ | 14.44 ± 2.05$^{c}$ | 37.58 ± 2.90$^{ab}$ | * |
| Tail DNA (%) | 33.28 ± 2.19$^{a}$ | 18.54 ± 2.87$^{bc}$ | 15.20 ± 1.72$^{c}$ | 31.04 ± 7.77$^{ab}$ | 31.38 ± 4.18$^{ab}$ | * |
| Tail moment (μm/s) | 18.52 ± 1.45$^{b}$ | 7.31 ± 0.98$^{c}$ | 8.51 ± 1.58$^{c}$ | 44.48 ± 3.55$^{a}$ | 38.40 ± 3.12$^{a}$ | * |

Different superscripts within the same row demonstrate significant differences (*$p$ < .05).

**TABLE 4** Mean (±*SE*) glutathione peroxidase (GPx), malondialdehyde (MDA) and total antioxidant (TA) activities in frozen-thawed bull semen

| Analysis | Control | Q25 | Q50 | Q100 | Q200 | *p* |
|---|---|---|---|---|---|---|
| GPx (mU/ml) | 17.28 ± 1.61$^{a}$ | 11.07 ± 1.55$^{bc}$ | 14.19 ± 1.18$^{ab}$ | 16.78 ± 1.57$^{a}$ | 9.21 ± 0.70$^{c}$ | * |
| MDA (μmol/ml) | 4.23 ± 0.93$^{bc}$ | 2.92 ± 0.38$^{c}$ | 5.30 ± 0.64$^{b}$ | 8.21 ± 0.40$^{a}$ | 10.03 ± 0.93$^{a}$ | * |
| Total antioxidant activities (mmol/trolox/ml-$10^{9}$ cell/ml) | 0.32 ± 0.01$^{b}$ | 0.33 ± 0.02$^{b}$ | 0.43 ± 0.03$^{ab}$ | 0.46 ± 0.05$^{a}$ | 0.38 ± 0.03$^{ab}$ | * |

Different superscripts within the same row demonstrate significant differences (*$p$ < .05).

thawing. Samples with more than 25 μg/ml Q concentrations exhibited adverse effects.

Several previous studies in different species generated results similar to those found in our study. Supplementation of various concentrations of Q did not enhance progressive or total motility of sperm cells in rams (Silva et al., 2012) and stallions (Seifi-Jamadi et al., 2016) before cryopreservation. Khanduja, Verma, and Bhardwaj (2001) stated that Q neither protects sperm from ROS-mediated damages nor positively affects sperm motility (5–200 mM) in humans. In these studies, when higher concentrations of Q were used in semen extender, sperm motility was impaired. Even though the concentrations of Q used were different in the present study, the motility results were similar to those of the studies noted above. Tvrda et al. (2016) reported that Q densities ranging between 50 and 100 μmol/l were particularly effective in protecting sperm motility and mitochondrial activity against injury caused by lipid peroxidation of surplus ROS, which does not agree with our findings. Sapanidou et al. (2015) concluded that adding crocin (at the level of 1 mM) had a beneficial effect on total motility and progressive motility. In a boar sperm study, collectively Q and genistein have favourable effects on sperm motility and viability at between 1 and 50 μM for three- and 6-hr chilling periods. Moreover, Q (at levels of 1, 5 or 10 μM) seems to have a beneficial effect on porcine IVF embryo development (Kim et al., 2014).

Based on our results, we can infer that dissimilarity in the results may be due to the generation of Q's protective effect, which occurs only under extreme stress and does not necessarily reflect the physiological conditions with which the sperm cell is affected. When sperm motility characteristics, plasma membrane integrity and sperm abnormalities were considered, treatment did not improve any fields tested. The results of the present study indicate that neither resveratrol nor Q improved sperm morphology, plasma membrane, acrosomal integrity (Silva et al., 2016) and sperm motility characteristics (Mortimer, 2000). The addition of either methionine or Q did not ensure any effect on the sperm motility characteristics VAP and VCL in a study of chilled rabbit sperm (Johinke, de Graaf, & Bathgate, 2014). Taşdemir et al. (2014) used antioxidant supplement, fetuin, amino acid and cysteine as antioxidants; however, they did not identify positive results in sperm motility characteristics after thawing.

It has been stated in the literature that despite the fact that DNA integrity varies widely among mammalian species during external and internal environmental stress (Mukhopadhyay et al., 2011), dilution and cryopreservation of bull sperm—especially in egg yolk-based extenders—cause damage to both the intracellular and extracellular antioxidant defence systems (Bilodeau, Chatterjee, Sirard, & Gagnon, 2000). During cryopreservation, ice crystals significantly damage sperm DNA integrity, which adversely affects the motility parameters of bull sperm cells (Gilmore, Liu, Woods, Peter, & Crytser, 2000). Similar to our findings, Gibb, Butler, Morris, Maxwell, and Grupen (2013) found that Q significantly reduced DNA fragmentation in sex-sorted, cryopreserved stallion spermatozoa. Another study suggested that Q minimised post-thawing DNA damage and should be considered in the human cryopreservation process (Zribi et al., 2012). Adversely, the addition of Q did not have a beneficial effect on the percentage of spermatozoa with DNA integrity values (Seifi-Jamadi et al., 2016). Our results suggest that Q supplementation has a significant effect on the stability of sperm DNA integrity, although freezing and thawing trigger DNA fragmentation.

GPx activity is one of the demonstrative indicators to antioxidant capacity (Kumar, Kumar, Sikka, & Singhbal, 2015). Malondialdehyde is useful to estimate the degree of osmotic stress, which may assist clinicians to both elucidate the role of MDA in fertility and evaluate the effects of the antioxidant treatment (Lanzafame, 2009). In the present study, although GPx activity was greater in the Q50 and Q100 samples, they did not enhance progressive motility, plasma membrane or DNA integrity. Malondialdehyde and TA activity were not affected positively by the treatments. Present results contrast

with earlier reports, such as the study by Kumar et al. (2015) who indicated the GPx activity was found at higher levels and MDA was determined to be lower in the extender supplemented with sericin at the concentrations of 0.25% and 0.5%. Ben Abdallah, Zribi, and Ammar-Keskes (2011) postulated in their study that Q successfully decreased MDA level when supplemented in rat semen extender. In consensus, Khumran et al. (2015) found lesser concentrations (0.5–1.5 mM/ml) of butylated hydroxytoluene supplementation decreased MDA concentrations that resulted in protection against DNA damage by reducing oxidative stress. These reports and the findings of the present study indicate that antioxidant level is one of the major factors in improving sperm survival following the freeze-thawing process. This means that treatment groups may not improve the plasma membrane structure and motility functions, but Q25 can reduce chromatin damage.

## 5 | CONCLUSIONS

In conclusion, current findings showed that, although Q supplementation did not produce advanced results in the proportion of progressive and total sperm motility, treatments exhibited favourable tail length, tail DNA and tail moment. When whole parameters are considered, Q25 can be added to the Tris extender due to its positive effect on sperm DNA integrity and no adverse effect on the progressive and total motilities of sperm.


## ACKNOWLEDGEMENTS

This study was supported by General Directorate of Agribusiness, Ankara, Turkey.


## CONFLICT OF INTEREST

The authors confirm that they have no conflict of interest to declare.


## ORCID

*F. Avdatek* http://orcid.org/0000-0003-2345-8826

*M. E. İnanç* http://orcid.org/0000-0001-6954-6309

*U. Taşdemir* http://orcid.org/0000-0003-2827-1286


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**How to cite this article:** Avdatek F, Yeni D, İnanç ME, et al. Supplementation of quercetin for advanced DNA integrity in bull semen cryopreservation. *Andrologia*. 2018;e12975. https://doi.org/10.1111/and.12975