

# Quercetin Improves the Apoptotic Index and Oxidative Stress in Post-Thaw Dog Sperm

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## Research Article

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# Abstract

This study was conducted to investigate if quercetin (QRN) may ameliorate apoptosis and oxidative stress in post-thaw dog sperm. Herein, we evaluated the post-thaw apoptosis and oxidative stress after treatment with QRN (control, 25, 50, and 100  $\mu$ M) in freezing of dog semen. The oxidative stress index was significantly affected ( $p < 0.05$ ) between the various concentrations of QRN and the control ( $17.56 \pm 1.02$ ,  $7.54 \pm 0.48$ ,  $5.66 \pm 0.80$ , and  $10.41 \pm 0.69$ ), respectively. The apoptosis index was  $9.1 \pm 1.34$ ,  $6.66 \pm 0.58$ ,  $6.77 \pm 0.66$ , and  $5.38 \pm 0.86$  in the control, and 25, 50, and 100  $\mu$ M QRN treatment groups, respectively ( $p < 0.05$ ). The effects of ameliorated cryo-induced damage by QRN on post-thaw sperm quality were also observed through improved structural and functional tests. Sperm treated with 50  $\mu$ M QRN showed significantly higher motility ( $51.8 \pm 2.1\%$  vs.  $43.1 \pm 1.4\%$ ,  $P < 0.05$ ), survival rates ( $46.9 \pm 0.7\%$  vs.  $43.9 \pm 0.4\%$ ,  $P < 0.05$ ), and mucus penetration than control group, respectively. Results demonstrate that supplementing freezing buffer with 50  $\mu$ M QRN reduced oxidative damage and improved the quality of post-thaw dog sperm.

## 1. Introduction

Artificial insemination using frozen dog semen is associated with a sluggish progress compared with that of other animal species (England 1993). Identifying the best cryoprotectant for preventing cryodamage-induced molecular toxicity in dog sperm post-thaw is a focus for several research groups, including our group (Farstad 2009, Qamar et al. 2020). Reactive oxygen species (ROS) are resulted from physiological cellular metabolic processes, which pushes the cells to early aging and apoptosis (Redza-Dutordoir & Averill-Bates 2016). Organisms have diverse and complex systems to balance and maintain harmless intracellular ROS levels to protect phospholipids, proteins, and DNA from the adverse effects of ROS (Schieber & Chandel 2014).

In sperm cryopreservation, due to discarding the seminal plasma, sperms lack essential antioxidants that defeat ROS (Kashou et al. 2013), and further oxidative stress develops, which potentiates cryo-induced stress, damage, and subsequent apoptosis (Fraser et al. 2011, Iwasaki & Gagnon 1992, Papas et al. 2019). The cryopreservation of cells promotes the oxidation of phospholipids in the cell membrane, increases intracellular ROS, and thus leads to DNA fragmentation and cell membrane damage (El-Said et al. 2014, Su et al. 2019). This, in turn, compromises the fertility quality of post-thaw sperm. Therefore, supplementation with antioxidants has been applied to prevent ROS, reduce its effect on cell components, and retain the fertility quality of sperm, such as motility and viability (Snezhkina et al. 2019). These antioxidants reduce ROS generation and/or eliminate the generated ROS in the cells of an organism (Liu et al. 2021). Antioxidants play important roles as oxidation inhibitors by scavenging free radicals (Barciszewski et al. 2000). Antioxidant-supplemented sperm showed reduced lipid peroxidation, enabling the plasma membrane to maintain normal physiological and metabolic activity, ultimately resulting in enhanced viability (Alvarez & Storey 1989). Several studies reported the useful effects of antioxidants during semen freezing to minimize the adverse impacts of ROS on spermatozoa has been reported, thereby improving post-thaw quality (Bansal & Bilaspuri 2010, Malo et al. 2010, Qamar et al. 2020, Setyawan et al. 2016, Yoshimoto et al. 2008). However, finding an appropriate species-specific antioxidant is the target of several research groups to alleviate the cryodamage and maintaining functional integrity of spermatozoa during freezing process (Bansal & Bilaspuri 2010). For instance,  $\alpha$ -Linoleic acid was shown to suppress ROS generation by stabilizing the plasma membrane during the cryopreservation of boar sperm (Qamar et al. 2020). In canine species, several antioxidants and ROS

scavenger supplements were used for sperm cryopreservation, including rosemary and spermine (Setyawan et al. 2016, Vieira et al. 2018).

Interestingly, externalization and translocation of phosphatidylserine (PS) from the inner leaflet of sperm membranes to the external leaflet considers as an early marker of apoptosis in spermatozoa (Martin et al. 2005, Shiratsuchi et al. 1997). ROS production is significantly related to the activity of anti-apoptotic Bcl2 and pro-apoptotic BAX proteins (Setyawan et al. 2016). DNA integrity is also a concern, as cryopreservation alters the properties of the mitochondrial membrane and increases the generation of free radicals that affect DNA oxidation and lead to single- and double-strand DNA breaks (Ahmed & Lingner 2020, Ricci et al. 2002).

Quercetin is a flavonol from the flavonoid group of polyphenols found in many fruits, vegetables, and seeds (Formica & Regelson 1995). Quercetin prevents peroxidation in organisms, displays anti-cancer, antibacterial, and anti-inflammatory effects, reduces nanoparticles toxicity, and improves oocyte in vitro maturation (Ezzati et al. 2020, Han et al. 2021, Hussein et al. 2016, Kang et al. 2016, Moodi et al. 2021). Quercetin can scavenge ROS and hydroxyl radicals (Kim et al. 2020), and can modulate the mitochondrial membrane potential by restoring ATP levels, blocking caspase-3, and minimizing DNA unpacking (Bali et al. 2014). Electron transport chain and cytochrome *c* were reported to be the molecular targets of quercetin, preventing H<sub>2</sub>O<sub>2</sub> production and protecting mitochondrial function and integrity (Carrasco-Pozo et al. 2012, Tanga et al. 2021).

Quercetin supplementation in human sperm caused significant improvements in frozen/thawed spermatozoa motility, viability, and DNA integrity (Zribi et al. 2012). In human and bovine, quercetin was demonstrated to be a ROS scavenging, reduce H<sub>2</sub>O<sub>2</sub> level, and metal-chelating agent that prevented the alterations caused by ROS such as DNA fragmentation and preserved sperm function (Azadi et al. 2017, Diao et al. 2019, Tvrdá et al. 2016). At the molecular level, quercetin acts by reducing ROS levels inside the cell by scavenging free radical species (Russo et al. 2012). In other studies, quercetin was found to inhibit phosphorylation and prevent apoptosis (Moon & Morris 2007). Quercetin can be a pro-oxidative in the long-term uses (Ashida et al. 2000) and its action is dose-dependent, particularly in the cell culture conditions (Fukuda & Ashida 2008). Therefore, determining the optimum concentration and conditions for the use of quercetin is vital for its application.

We hypothesized that supplementing the freezing extender with quercetin could preserve sperm fertility in dogs by reducing free radical production, oxidative stress, and subsequent apoptosis. Therefore, we examined the impacts of supplementing various concentrations of quercetin on ROS, OS, and sperm apoptosis. Furthermore, we carried out some structural and functional tests on sperm to assess the indicators of fertility quality.

## **2. Materials And Methods**

### **2.1 Animals and ethics**

Four healthy beagles (age, 2–4 years old; weight, 8–12 kg) were used in the current study. Formulated food and water were available *ad libitum* to the beagles and were maintained in a comfortable facility and isolated from external stresses. All experimental procedures and animal care were approved by Chungnam National University (approval no. 202006A-CNU-103).

### **2.2 Semen collection and freezing**

Semen was collected two times per week using digital manipulation for a total of eight times. The pooled semen was washed, diluted, and analyzed using a computer-aided sperm analysis software (MICROTOPIC CASA System; SCA class analyzer, Josep Tarradellas, Barcelona, Spain). Samples of more than  $100 \times 10^6$  sperms/mL, 70% motility, and 80% viability were selected, pooled and processed. Cell debris was discarded after centrifugation at  $100 \times g$  for 1 min (at 25°C), then the supernatant was used. The supernatant was mixed buffer 1 (1v/1v) and centrifuged at  $700 \times g$  for 2 min at 25°C to collect the pellets (Bang et al. 2021). Sperm pellet was resuspended in buffer 1 to attain a  $200 \times 10^6$  sperm/mL, and was then adjusted to  $100 \times 10^6$  sperms/mL by adding a freezing extender that contained 0 (control), 25, 50, or 100  $\mu\text{M}$  quercetin. After dilution, the samples were loaded into 0.5 mL straws (Minitube, Tiefenbach, Germany) and cooled for 45–60 min at 4°C. Sperm freezing was performed through keeping the straws about 2–4 cm above the level of liquid nitrogen ( $\text{LN}_2$ ) for 10–15 min. The frozen straws were stored in an  $\text{LN}_2$  container ( $-196^\circ\text{C}$ ). For thawing, straws were maintained at 37°C for 30 s and semen was sequentially diluted with buffer 1 for further evaluation within 5–10 min after thawing.

## 2.3 Computer-aided sperm analysis (CASA) of thawed sperms

Five  $\mu\text{L}$  of post-thaw sperm samples were transferred onto a clean glass slide to assess the motility and motion characteristics using a CASA imaging system. For the analysis, five fields of more than 200 sperms for each semen sample were monitored for 1 s at 25 Hz. The proportions of total and progressive motility were analyzed. The recorded motion characteristics were curvilinear velocity (VCL), average path velocity (VAP), straight-line velocity (VSL), straightness, linearity, and amplitude of lateral head displacement (ALH).

## 2.4 Flow cytometric analysis

Flow cytometry analysis was performed through BD Accuri™ C6 plus (Becton Dickinson, BD Biosciences, Franklin Lakes, NJ, USA). The flow cytometer was fitted with blue (488-nm, solid state, 20 mW) and diode red (640 nm, 14.7 mW) excitation lasers. The fluorescent probes used in this experiment were 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA) (Molecular Probes, Inc., OR, USA), and annexin V-FITC (Apoptosis detection kit I (BD Biosciences), and propidium iodide (Malo et al.) were excited using a 488 nm blue-solid state laser. Live spermatozoa stained with H2DCFDA and annexin V were detected using a filter detector 533/30 BP (wavelength range 511–543 nm). The signal from dead sperm stained with propidium iodide was detected using a filter detector 586/42 BP (wavelength range 565–607 nm). The flow rate of the flow cytometer was medium (35  $\mu\text{L}/\text{min}$ , 16  $\mu\text{m}$  core). Sperm populations were divided into regions and quadrants. The data were analyzed using BD Accuri™ CFlow software.

## 2.5 Level of ROS and PS translocation index

ROS levels were assessed according to the experimental protocol of Guthrie and Welch (Guthrie & Welch 2010). Briefly, ROS in the sperm was detected using H2DCFDA. The level of ROS was evaluated by measuring the fluorescence intensity of H2DCFDA in the live sperm population. All samples were analyzed to derive their fluorescence signals using a BD Accuri™ C6 Plus flow cytometer. The data are expressed as the percentage of ROS molecules measured in live sperm. The PS translocation status was assessed through the annexin V-FITC detection Kit. Briefly, spermatozoa were pelleted twice using PBS at  $300 \times g$  for 5 min, and then diluted in 1 mL of  $1 \times$  annexin buffer ( $5 \times 10^6$  sperm/mL). From this suspension, 100  $\mu\text{L}$  was collected in new 1.5 mL tubes,

and mixed with 5  $\mu\text{L}$  annexin-FITC stain and 5  $\mu\text{L}$  propidium iodide (Malo et al.). The mixture was maintained in the dark at room temperature (25°C) for 15 min. Thereafter, 400  $\mu\text{L}$  of 1  $\times$  annexin buffer was mixed to the tubes and analyzed by flow cytometry.

## 2.6 Sperm plasma membrane integrity

Hypo-osmotic swelling (HOS) assay was used to assess the sperm plasma membrane integrity. In brief, approximately 200 sperms were incubated for 30 min in the HOS solution and then examined under a phase-contrast microscope (Eclipse Ts2; Nikon, Minato-Ku, Tokyo, Japan) within 5–10 min. Coiled sperm tails showing swelling indicate an intact sperm plasma membrane. Acrosome integrity was examined through using fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) stain (Almubarak et al. 2021, Kang et al. 2020). Briefly, smears were prepared and fixed with methanol. After washing with PBS, 30  $\mu\text{L}$  of FITC-PNA (100  $\mu\text{L}/\text{ml}$  in PBS) was added to the fixed smear and incubated in humid dark place for 30 min. Stained spermatozoa ( $n > 200$ ) were examined through an epifluorescence microscope (1000  $\times$  magnification; Eclipse Ts 2, Nikon), and categorized according to the presence or absence of fluorescence staining (i.e. acrosome-intact spermatozoa emitted intense green fluorescence on the anterior sperm).

## 2.7 Mucus penetration test

By the aid of modified synthetic oviductal fluid on flat capillary tubes (80  $\pm$  0.5 mm long, 1.25  $\pm$  0.05 mm wide; Hilgenberg GmbH, Stutzerbach, Germany), sperm penetration test was evaluated. After sealing one periphery of the flat capillary tube, it was filled with mucus and left to stand for 15 min. Thereafter, the open periphery of the flat capillary tubes was immersed in a 100  $\mu\text{L}$  of semen samples and left horizontally at 25°C for 2 h. The numbers of spermatozoa reached the marks of 1 and 3 cm were counted.

## 2.8 Statistical Analysis

Data were analyzed by the Statistical Package for Social Sciences (SPSS, version 24.0 software, IBM, Armonk, NY, USA). Data were expressed as the mean  $\pm$  standard error of the means (SEM). Data of motion characteristics and percentages of live sperms were analyzed with one-way analysis of variance (ANOVA) and Tukey's multiple comparison test.  $P < 0.05$  was considered a statistical significance.

## 3. Results

### 3.1 Effect of quercetin on ROS level

ROS levels were measured by calculating the percentage of sperm stained with H2DCFDA from the total percentage of live sperm (stained negatively with PI). Control group showed increased ROS levels ( $P < 0.05$ ) when compared with the quercetin-treated groups (Fig. 1). The oxidative stress index was 17.56  $\pm$  1.02, 7.54  $\pm$  0.48, 5.66  $\pm$  0.80, and 10.41  $\pm$  0.69 in the control, and the 25, 50 and 100  $\mu\text{M}$  quercetin treatment groups, respectively. The oxidative stress index was statistically significantly different ( $p < 0.05$ ) among the various concentrations of quercetin and the control (Fig. 1). Of the treatment groups, the 50  $\mu\text{M}$  quercetin group was found to have the most significantly lower ROS level, which indicates that the optimal concentration for antioxidant activity is 50  $\mu\text{M}$ .

## 3.2 Effect of quercetin on PS translocation index (Apoptosis status)

The apoptosis index was measured by evaluating the PS translocation. The percentage of apoptotic spermatozoa (annexin V+/PI-) was calculated from the total live spermatozoa (PI-), and the data were considered as the PS translocation index. The apoptosis index values were  $9.1 \pm 1.34$ ,  $6.66 \pm 0.58$ ,  $6.77 \pm 0.66$ , and  $5.38 \pm 0.86$  in the control, and the 25, 50, and 100  $\mu\text{M}$  quercetin treatment groups, respectively; the control group showed significantly higher values than the quercetin-treated groups. However, no differences were observed between the quercetin-treated groups (Fig. 2).

## 3.3 Effect of quercetin on sperm motility and percentage of live sperm

The motility, motion characteristics, and viability results are shown in Table 1. Post-thaw semen in samples frozen with 50  $\mu\text{M}$  quercetin ( $51.8 \pm 2.1\%$ ) showed higher motility than control samples ( $43.1 \pm 1.4\%$ ) ( $P < 0.05$ ). Motion characteristics showed no difference between the control and 25  $\mu\text{M}$  quercetin groups, while the values, except for ALH, tended to be reduced with as quercetin concentration increased. In addition, the percentage of live sperms was increased in the samples frozen with 50  $\mu\text{M}$  quercetin ( $46.9 \pm 0.7\%$ ) when compared with the control samples ( $43.9 \pm 0.4\%$ ) ( $P < 0.05$ ). However, there was no difference between the control, 25  $\mu\text{M}$  ( $49.6 \pm 2.7$ ) and 100  $\mu\text{M}$  ( $40.7 \pm 4.4$ ) quercetin groups (Table 1). The motility and viability of post-thaw sperm were significantly increased in sperms supplemented with 50  $\mu\text{M}$  quercetin when compared with the other groups.

Table 1  
Effects of quercetin (QRN) on the motility and motion variables of post-thaw dog spermatozoa.

Groups	Motility (%)	Progressive motility(%)	VCL ( $\mu\text{m/s}$ )	VAP ( $\mu\text{m/s}$ )	VSL ( $\mu\text{m/s}$ )	Straight (%)	Linearity (%)	ALH ( $\mu\text{m}$ )	Live sperms (%)
Control	43.1 $\pm$ 1.4 <sup>b</sup>	22.4 $\pm$ 1.4 <sup>b</sup>	70.7 $\pm$ 2.3 <sup>a</sup>	58.6 $\pm$ 2.3 <sup>a</sup>	54.2 $\pm$ 2.4 <sup>a</sup>	77.3 $\pm$ 0.8 <sup>a</sup>	61.5 $\pm$ 0.8 <sup>a</sup>	2.0 $\pm$ 0.0 <sup>b</sup>	43.9 $\pm$ 0.4 <sup>b</sup>
25 $\mu\text{M}$ QRN	49.6 $\pm$ 2.7 <sup>a</sup>	27.4 $\pm$ 2.5 <sup>a</sup>	72.5 $\pm$ 2.9 <sup>a</sup>	58.9 $\pm$ 2.5 <sup>a</sup>	54.4 $\pm$ 2.5 <sup>a</sup>	75.6 $\pm$ 1.6 <sup>ab</sup>	59.6 $\pm$ 1.6 <sup>a</sup>	2.1 $\pm$ 0.0 <sup>b</sup>	45.7 $\pm$ 1.6 <sup>a</sup>
50 $\mu\text{M}$ QRN	51.8 $\pm$ 2.1 <sup>a</sup>	22.5 $\pm$ 3.4 <sup>b</sup>	65.3 $\pm$ 4.5 <sup>ab</sup>	52.3 $\pm$ 4.4 <sup>b</sup>	47.2 $\pm$ 4.5 <sup>ab</sup>	70.3 $\pm$ 1.9 <sup>b</sup>	52.9 $\pm$ 2.7 <sup>b</sup>	2.0 $\pm$ 0.1 <sup>b</sup>	46.9 $\pm$ 0.7 <sup>a</sup>
100 $\mu\text{M}$ QRN	40.7 $\pm$ 4.4 <sup>b</sup>	19.8 $\pm$ 2.6 <sup>b</sup>	63.1 $\pm$ 6.8 <sup>b</sup>	50.0 $\pm$ 2.9 <sup>b</sup>	45.2 $\pm$ 3.2 <sup>b</sup>	72.3 $\pm$ 2.4 <sup>b</sup>	51.3 $\pm$ 2.7 <sup>b</sup>	2.3 $\pm$ 0.1 <sup>a</sup>	43.6 $\pm$ 0.9 <sup>ab</sup>
a-b Within the same column, values with different lowercase superscripts differ significantly ( $p < 0.05$ , $n = 7$ ).									
ALH, amplitude of lateral head displacement; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity.									

### 3.4 Effect of quercetin on sperm plasma membrane integrity

HOS test showed a significant increase in plasma membrane integrity of the quercetin-supplemented groups compared to the control group (54.0  $\pm$  0.6% vs. 46.6  $\pm$  0.7%, respectively). However, there was a significant decrease in the membrane integrity of sperm samples supplemented with 100  $\mu\text{M}$  quercetin (45.2  $\pm$  0.6%) (Table 2). FITC-PNA staining showed that quercetin supplementation did not effectively protect the acrosomal integrity of the cryopreserved dog sperm, as no statistically significant differences were found between the quercetin-supplemented sperm samples and the control samples.

Table 2

Effects of quercetin (QRN) supplementation on plasma membrane integrity, acrosome, and mitochondria of post-thaw dog spermatozoa.

Groups	HOS (%)	Mitochondrial activity (%)	Acrosome integrity (%)	Chromatin integrity (%)
Control	46.6 ± 0.7 <sup>b</sup>	44.4 ± 0.5 <sup>b</sup>	61.6 ± 3.2	58.9 ± 0.9 <sup>b</sup>
25 µM QRN	49.2 ± 0.6 <sup>ab</sup>	47.1 ± 0.7 <sup>ab</sup>	53.7 ± 2.7	61.6 ± 0.8 <sup>ab</sup>
50 µM QRN	54.0 ± 0.6 <sup>a</sup>	49.5 ± 1.8 <sup>a</sup>	60.7 ± 4.4	64.3 ± 0.7 <sup>a</sup>
100 µM QRN	45.2 ± 0.6 <sup>b</sup>	45.5 ± 1.6 <sup>b</sup>	54.9 ± 1.7	57.1 ± 0.5 <sup>b</sup>

<sup>a-b</sup>Values with different superscript lowercase letters in a column vary significantly ( $p < 0.05$ ,  $n = 7$ ).

### 3.6 Effect of quercetin on mucus penetration

Results showed that 50 µM quercetin-supplemented post-thaw sperm penetrated the modified synthetic oviductal fluid more effectively than control group (69.2 ± 1.3 vs. 51.5 ± 1.9 at 1 cm and 15.1 ± 1.6 vs. 27.0 ± 1.1 at 3 cm), respectively. The sperm counts for quercetin-supplemented sperm samples were significantly higher at both the 1- and 3-cm marks than those for the control sperm (Table 3). The motility-promoting effects of 25 and 100 µM quercetin were less than those of 50 µM quercetin at both 1 and 3 cm; however, the effect of 100 µM was relatively higher than that of 25 µM quercetin at 1 cm (Table 3).

Table 3

Effects of quercetin (QRN) supplementation on the mucus penetration ability of post-thaw dog spermatozoa.

Group	Numbers of spermatozoa penetrating the mucus	
	1 cm	3 cm
Control	51.5 ± 1.9 <sup>b</sup>	15.1 ± 1.6 <sup>b</sup>
25 µM QRN	59.0 ± 0.8 <sup>ab</sup>	20.2 ± 1.3 <sup>ab</sup>
50 µM QRN	69.2 ± 1.3 <sup>a</sup>	27.0 ± 1.1 <sup>a</sup>
100 µM QRN	61.7 ± 1.8 <sup>ab</sup>	19.2 ± 0.7 <sup>ab</sup>

<sup>a-b</sup> Within the same column, values with different superscripts differ significantly ( $p < 0.05$ ,  $n = 7$ ).

## 4. Discussion

Cryopreservation is a crucial tool for preserving of spermatozoa and the genetic merits of valuable species for a long time. However, cryodamage due to freezing and thawing compromises the fertility of sperm post-thaw. Cryodamage at the molecular level due to freezing and thawing is also a concern during long-term preservation of dog sperm (England 1993, Farstad 2009, Vieira et al. 2018). Cryodamage can be caused



through the generation of ROS in sperm. However, supplementing antioxidants during sperm freezing to reverse the effect of ROS has been used to ameliorate these adverse effects (Majzoub & Agarwal 2020).

Herein, the observed reduction in oxidative stress by the application of quercetin in dog sperm coincides with the results found in the rats, Red Jungle fowl and the roosters where supplementation with quercetin reduced oxidative stress (Najafi et al. 2020, Rakha et al. 2020, Yelumalai et al. 2019). The mechanism employed by quercetin to ameliorate oxidative stress in post-thaw sperm is thought to occur via an increase in the sperm total antioxidant capacity and the amelioration of lipid peroxidation (Papas et al. 2019). Quercetin has also caused improvements in advanced DNA integrity, owing to reduced ROS levels, as reported in bulls (Avdatek et al. 2018), which could have contributed to fertility. The lipid peroxidation reduction ability of quercetin (Yang et al. 2020) is believed to contribute to oxidative stress in post-thawed sperm. Accordingly, we anticipate that further sperm quality criteria and the in vitro fertility test could be conducted to further validate the effectiveness of sperm. Evaluation of post-thaw sperm revealed significantly improved viability in the quercetin-supplemented sperm samples compared to control samples. Moreover, higher percentages of sperm with intact membranes were observed in the quercetin-supplemented sperm samples than in the control samples. The greater membrane integrity of quercetin-supplemented sperm is an indicator of preserved sperm structure and ultimately the enhanced survival rate of sperm (Ismail et al. 2020).

Sperm are very sensitive to oxidative stress owing to their limited oxidative defense mechanisms (Alahmar 2019, Sabeti et al. 2016). The sensitivity to oxidative stress is further amplified by the dilution of semen antioxidative enzymes during semen dilution process and excessive ROS generation during post-thaw (Bansal & Bilaspuri 2010). The ROS produced during cryopreservation lead to a decrease in sperm motility, viability, acrosome membrane integrity, and fertilization capacity (Baumber et al. 2000, Sariözkan et al. 2009). Furthermore, ROS generation during cryopreservation triggers the apoptosis of spermatozoa, eventually leading to sperm loss of function or death (Said et al. 2004, Said et al. 2010). Moreover, under these stress conditions, the mitochondrial membrane potential decreases and apoptosis is enhanced by ROS generation (Redza-Dutordoir & Averill-Bates 2016). Quercetin displayed potent antioxidative properties owing to its preventative effects on ROS formation. It antagonizes the enzymatic (nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and nicotinamide adenine dinucleotide (NADH)-dependent oxidoreductase) and non-enzymatic systems involved the ROS formation (Walczak-Jedrzejska et al. 2013).

The reduced apoptotic index due to the application of quercetin is attributed to its ability to inhibit lipopolysaccharide-induced mRNA expression of tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and interleukin (IL-1 $\alpha$ ) (Bureau et al. 2008). This effect of quercetin could be an additional mechanism for the reduced apoptosis and integrity of the sperm; however, further studies are needed to confirm this. Our results coincide with the recent results of Kawasaki et al. (Kawasaki et al. 2020) who observed that the skim milk-based extender supplemented with 5  $\mu$ g/ml quercetin and 0.1% dimethyl sulfoxide improved the motility and fertility of cryopreserved dog spermatozoa. Similarly, the application of quercetin in stallion sperm cryopreservation caused improved post-thaw sperm quality, such as protecting DNA fragmentation, motility, and zona binding ability (Gibb et al. 2013, Seifi-Jamadi et al. 2016).

This study revealed that supplementation of a freezing extender with 50  $\mu$ M quercetin improved the motility and survival of frozen-thawed dog sperm. This finding aligns with that of previous studies on the effect of

quercetin on reducing oxidative stress in humans (15 µg/ml) (Zribi et al. 2012) and stallions (45 µg/ml) (Zribi et al. 2012). This effect of quercetin might be due to its free radical scavenging capacity and its effect on lipid peroxidation (Mazzi et al. 2012) which could reduce the level of ROS and subsequently decrease the number of apoptotic spermatozoa. However, quercetin is a versatile flavonoid; after displaying its antioxidant effect, it changes to a toxic product that might affect sperm quality (Mazzi et al. 2012) as observed in the 100 µM quercetin group in this study. Therefore, determining the optimum concentration and cryopreservation conditions should be performed to enable the effective application of antioxidants in cryopreservation. In this study, quercetin supplementation improved post-thaw motility as well as the proportion of total and progressively motile dog sperm, which reflects improved fertility. The mucus penetration test also confirmed the improved motility of the quercetin-supplemented sperm sample. The effects of quercetin supplementation on canine sperm cryopreservation are summarized in Fig. 3.

## 5. Conclusions

The quercetin-supplemented freezing extender had a significant effect in ameliorating cryo-induced oxidative stress and apoptosis, and protected the key fertility parameters of post-thaw dog sperm, such as mucus penetration, membrane integrity, and livability. Therefore, we recommended the addition of quercetin as a vital cryoprotectant supplement to maintain the survival and motility of post-thaw sperm (Collins & Ryan 2011, Henkel et al. 2004). Based on our analysis, the optimum concentration of quercetin for protecting post-thaw dog sperm against oxidative stress-induced damage is 50 µM.

## Declarations

**Author Contributions:** Conceptualization, S.B., A.Y.Q., and J.C.; methodology, S.B., A.Y.Q., B.M.T., X.F., G.S., A.H.T.N., I.J.Y., and J.C.; investigation, S.B., B.M.T., and A.Y.Q.; data curation, S.B., B.M.T., and A.Y.Q.; writing—original draft preparation, S.B., B.M.T. and A.H.T.N.; writing—review and editing, S.B., B.M.T., A.Y.Q., I.J.Y., I.M.S., and J.C.; supervision, J.C.; project administration, J.C.; funding acquisition, J.C.

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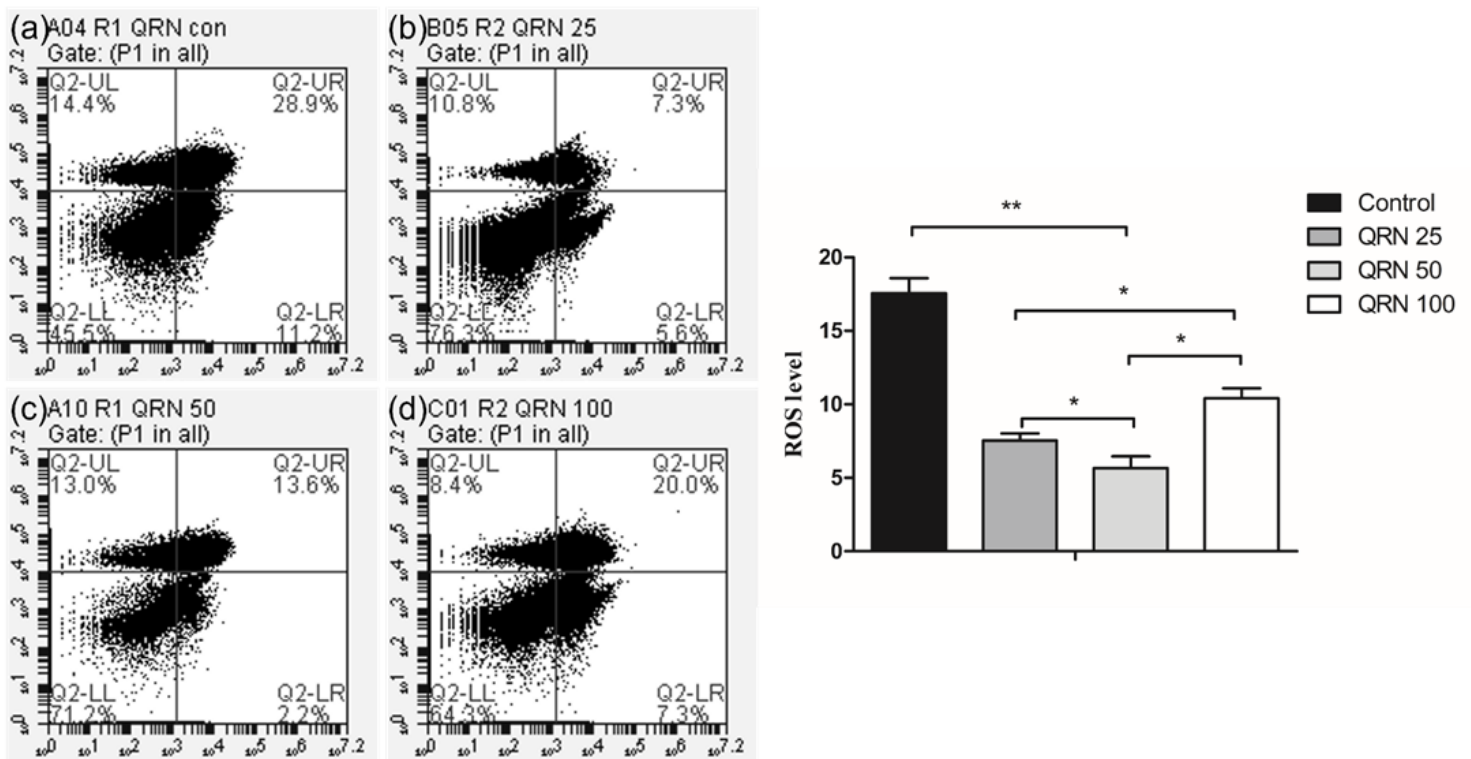
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## Figures



**Figure 1**

Effects of quercetin on ROS level in post-thaw dog spermatozoa. (a) control, (b) quercetin (QRN) 25, (c) QRN 50, (d) QRN 100. (a)-(d) are the results of DCFDA/PI staining and ROS analysis by flow cytometry. The bar graph is the result of comparing the ROS index value using the flow cytometry result. \* and \*\* indicate that the differences between the groups are different at  $P < 0.05$  and  $P < 0.01$ , respectively.

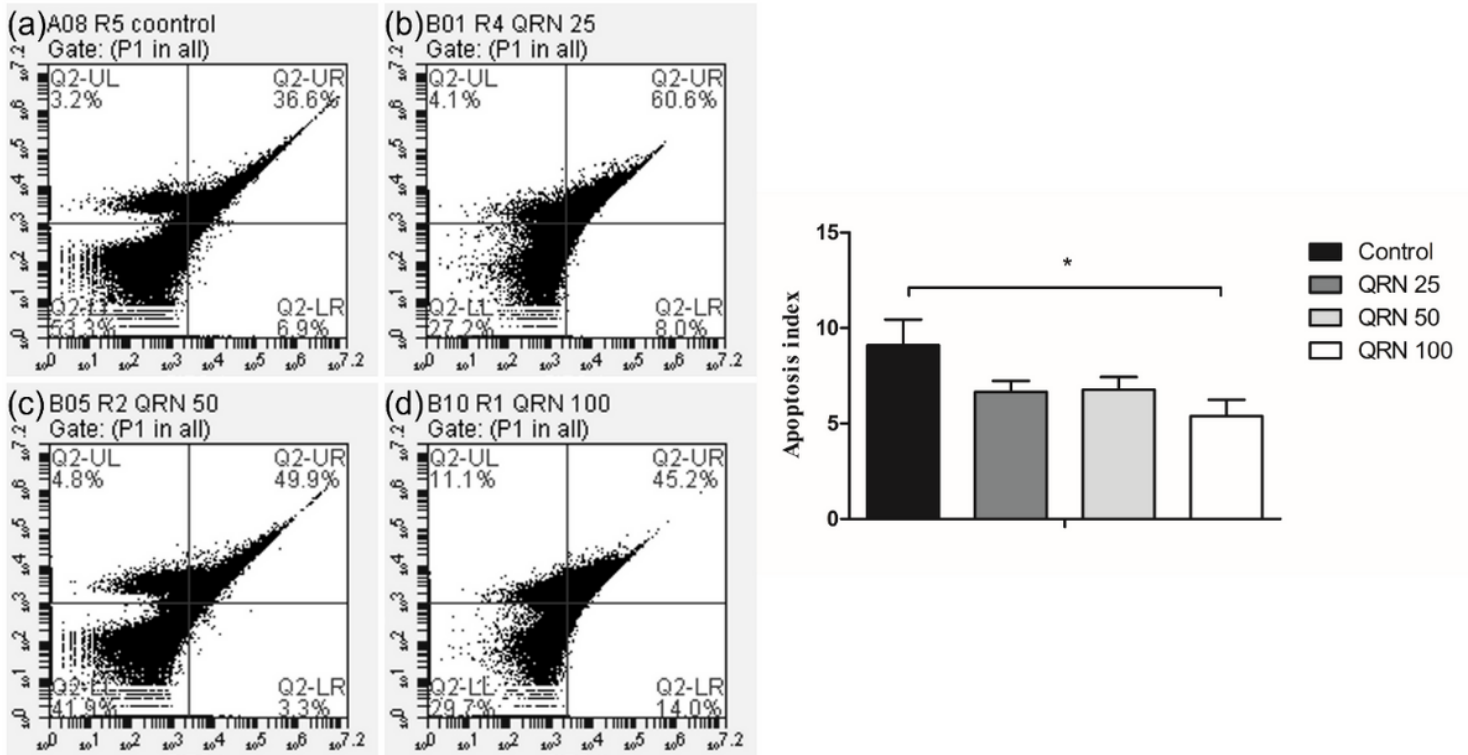


Figure 2

Impacts of quercetin on the apoptosis of post-thaw dog sperm. (a) control, (b) quercetin (QRN) 25, (c) QRN 50, (d) QRN 100. (a)-(d) are the results of Annexin V-FITC/PI staining and analysis by flow cy-tometry. \* that the differences between the groups are significantly different at  $P < 0.05$ .

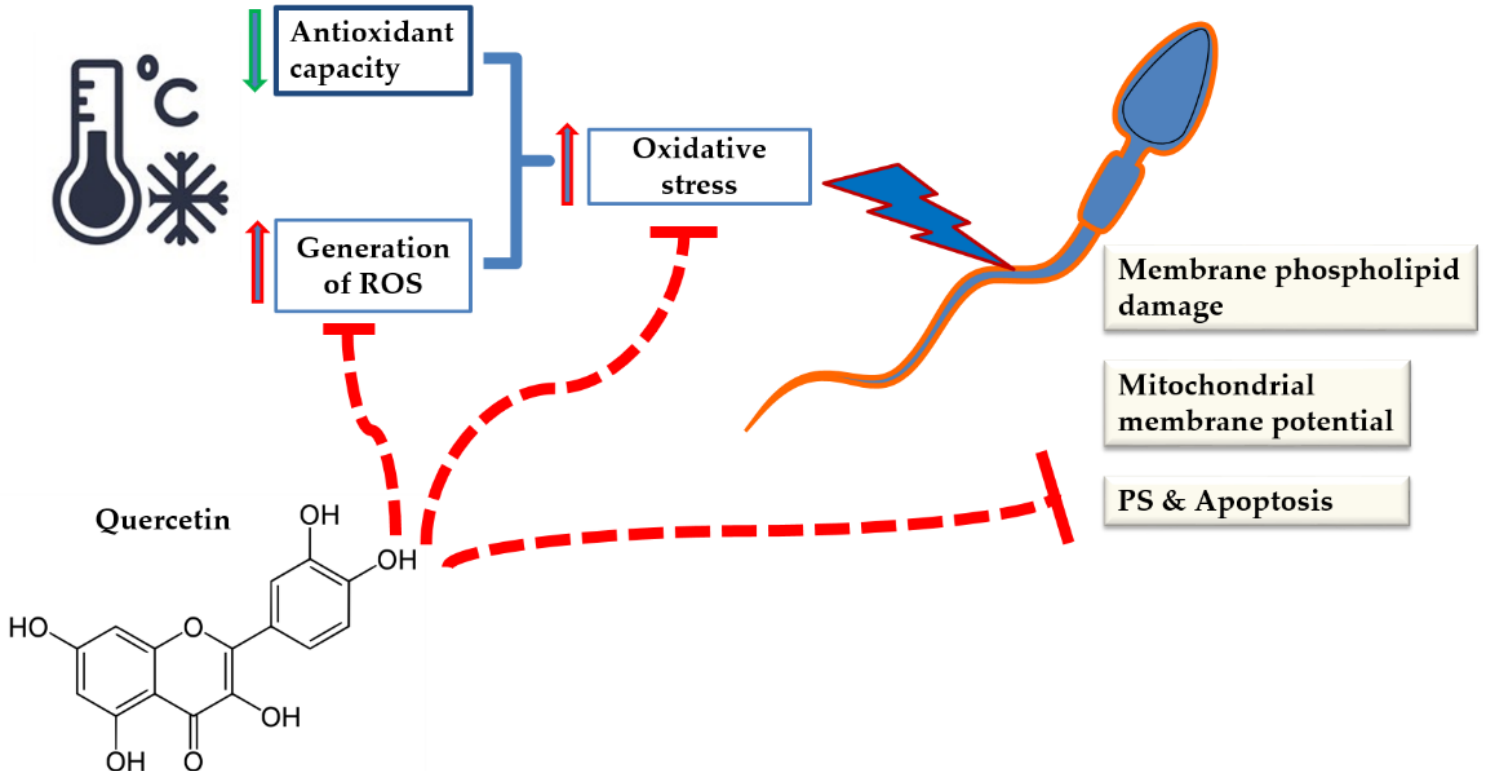


Figure 3



The proposed beneficial effects of supplementing quercetin during the dog sperm freezing-thawing process. Quercetin ameliorates reactive oxygen species (ROS) generation and increases the antioxidant capacity, thereby reducing the oxidative stress impacts on the sperms, such as phospholipid damage, mitochondrial membrane potential, and phosphatidylserine (PS) translocation, which are associated with apoptosis. Dashed line indicates suppression of the effects.