Quercetin improves the apoptotic index and oxidative stress in post-thaw dog sperm

Seonggyu Bang1 · Ahmad Yar Qamar1,2 · Bereket Molla Tanga1,3 · Xun Fang1 · Gyeonghwan Seong1 · Abdelbagi Hamad Talha Nabeel4,5 · Il-Jeoung Yu4 · Islam M. Saadeldin1,6 · Jongki Cho1

Received: 1 September 2021 / Accepted: 4 November 2021 / Published online: 13 November 2021 © The Author(s), under exclusive licence to Springer-Verlag GmbH Germany, part of Springer Nature 2021

Abstract
Freeze storage of ejaculated sperms is a crucial technique for the semen preservation of valuable pet animals such as dogs. The current 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 μM) in the freezing of dog semen. Reactive oxygen species levels were significantly affected (p < 0.05) between the various concentrations of QRN and the control (17.56 ± 1.02, 7.54 ± 0.48, 5.66 ± 0.80, and 10.41 ± 0.69), respectively. The apoptosis index was 9.1 ± 1.34, 6.66 ± 0.58, 6.77 ± 0.66, and 5.38 ± 0.86 in the control, and 25, 50, and 100 μM 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 μM QRN showed significantly higher motility (51.8 ± 2.1% vs. 43.1 ± 1.4%, P < 0.05), survival rates (46.9 ± 0.7% vs. 43.9 ± 0.4%, P < 0.05), and mucus penetration than control group, respectively. Results also indicated that higher concentrations of QRN (100 μM) were not effective on sperm quality and parameters when compared with the medium levels (50 μM). In conclusion, supplementation of freezing buffer with 50 μM QRN reduced oxidative damage and improved the quality of post-thaw dog sperm.

Keywords Dog · Oxidative stress · Post-thaw · Quercetin · Sperm

1 College of Veterinary Medicine, Chungnam National University, 34134 Daejeon, Republic of Korea
2 Department of Clinical Sciences, College of Veterinary and Animal Sciences, Jhang, Sub-Campus University of Veterinary and Animal Sciences, Lahore, Pakistan
3 Faculty of Veterinary Medicine, Hawassa University, 05, Hawassa, Ethiopia
4 Laboratory of Theriogenology and Reproductive Biotechnology, College of Veterinary Medicine and Bio-Safety Research Institute, Jeonbuk National University, Iksan, Republic of Korea
5 Department of Veterinary Medicine and Surgery, College of Veterinary Medicine, Sudan University of Science and Technology, Khartoum, Sudan
6 Research Institute of Veterinary Medicine, Chungnam National University, 34134 Daejeon, Republic of Korea
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) or free radicals are resulted from physiological cellular metabolic processes that lead to cellular early aging and apoptosis (Redza-Dutordoir and Averill-Bates, 2016). Living 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 and Chandel, 2014).

In semen cryopreservation, seminal plasma is usually discarded and therefore sperms lack extracellular antioxidants that defeat ROS (Alahmar 2019, Fraser et al. 2011, Iwasaaki and Gagnon 1992, Kashou et al. 2013, Papas et al. 2019, Sabeti et al. 2016). Consequently, cell cryopreservation increases intracellular ROS, promotes the oxidation of phospholipids in the cell membrane, and leads to DNA fragmentation and cell membrane damage (Bansal and Bilaspuri 2010, El-Said et al. 2014; Su et al. 2019). This effect is propagated in case of gametes cryopreservation and compromises the fertility quality of post-thaw sperm (Baumber et al. 2000; Sariözkan et al. 2009). Therefore, supplementation with antioxidants has been applied to scavenge ROS, reduce its effect on cell components, and retain the fertility quality of sperm, such as motility and viability (Barciszewski et al. 2000; Liu et al. 2021; Snezhkina et al. 2019).

Interestingly, externalization and translocation of phosphatidylserine (PS) from the inner leaflet of sperm membranes to the external leaflet is considered 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 and Linner 2020, Ricci et al. 2002).

Antioxidant-supplemented sperm showed reduced lipid peroxidation, enabling the plasma membrane to maintain normal physiological and metabolic activity, ultimately resulting in enhanced viability (Alvarez and Storey 1989, Bansal and 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 maintain the functional integrity of spermatozoa during the freezing process (Bansal and Bilaspuri 2010). For instance, α-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).

Quercetin is a flavonol from the flavonoid group of polyphenols found in many fruits, vegetables, and seeds (Formica and Regelson 1995). Quercetin prevents peroxidation in several organisms; displays anti-cancer, antibacterial, and anti-inflammatory effects; reduces nanoparticles toxicity; and improves oocyte in vitro maturation (Bungau et al. 2019; Ezzati et al. 2020; Grewal et al. 2021; Han et al. 2021; Hussein et al. 2016; Kang et al. 2016; Kim et al. 2020; Moodi et al. 2021; Russo et al. 2012). Quercetin 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 H2O2 production and protecting mitochondrial function and integrity (Carrasco-Pozo et al. 2012; Tanga et al. 2021). Quercetin supplementation in human and bovine sperm caused significant improvements in frozen/thawed spermatozoa motility, viability, and DNA integrity, and prevents apoptosis (Azadi et al. 2017, Diao et al. 2019, Moon and Morris 2007, Tvrda et al. 2016, Zribi et al. 2012).

Conversely, 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 and Ashida 2008). Therefore, determining the optimum concentration and conditions for the use of quercetin is vital for its application.

Therefore, 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. We examined the effects of supplementing various concentrations of quercetin on ROS, oxidative stress, and sperm apoptosis as well as some structural and functional analysis of post-thaw dog sperms as indicators of fertility quality.

Materials and methods

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.

**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 100×10^6 sperms/mL, 70% motility, and 80% viability were selected, pooled, and processed. Cell debris was discarded after centrifugation at 100×g for 1 min (at 25 °C); then, the supernatant was used. The supernatant was mixed with buffer 1 (1v/1v, buffer 1: Tris (hydroxymethyl) aminomethane (198.11 mM), citric acid (72.87 mM), fructose (44.39 mM), and kanamycin sulfate (0.25 mM)) and centrifuged at 700×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 200×10^6 sperm/mL and was then adjusted to 100×10^6 sperms/mL by adding a freezing extender (freezing extender) which was prepared using 54% buffer 1 (v:v), 40% egg yolk (v:v), and 6% glycerol (v:v)) that contained 0 (control), 25, 50, or 100 μM 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 (LN2) for 10–15 min. The frozen straws were stored in an LN2 container (−196 °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.

**Computer-aided sperm analysis (CASA) of thawed sperms**

Five microliters of post-thaw sperm samples was 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).

**Flow cytometric analysis of ROS and PS translocation index**

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), annexin V-FITC (apoptosis detection kit I (BD Biosciences), and propidium iodide (Mallo et al. 2010) 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 μL/min, 16-μm core). Sperm populations were divided into regions and quadrants. The data were analyzed using BD Accuri™ C6 Plus Flow cytometer software. Intracellular ROS levels were detected using H2DCFDA according to the experimental protocol of Guthrie and Welch (2010, Mahfouz et al. 2009). The level of ROS was evaluated by calculating the percentage of sperm stained with H2DCFDA from the total percentage of live sperm (stained negatively with PI). The PS translocation status was assessed through the annexin V-FITC detection kit. Briefly, spermatozoa were pelleted twice using PBS at 300×g for 5 min and then diluted in 1 mL of 1×annexin buffer (5×10^6 sperm/mL). From this suspension, 100 μL was collected in new 1.5 mL tubes and mixed with 5 μL annexin-FITC stain and 5 μL propidium iodide (Mallo et al. 2010). The mixture was maintained in the dark at room temperature (25 °C) for 15 min. Thereafter, 400 μL of 1×annexin buffer was mixed into the tubes and analyzed by flow cytometry.

**Sperm plasma membrane and acrosome 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 μL of FITC-PNA (100 μL/mL in PBS) was added to the fixed smear and incubated in a humid dark place for 30 min. Stained spermatozoa (n > 200) were examined through an epifluorescence microscope (1000×magnification; Eclipse Ts2, 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).

Measuring sperm mitochondrial activity and chromatin integrity

Mitochondrial activity was measured according to our previous method (Bang et al. 2021). In brief, 1 mg rhodamine 123 (R123) fluorescent stain was mixed with post-thaw diluted sperms (20×10⁶ sperm/mL) and incubated at 37 °C for 15 min in a dark chamber. Ten microliters of PI was mixed with the mixture and was kept at 37 °C for 10 min. Sperms were retrieved by centrifugation (500×g for 5 min at room temperature) and the pellets were resuspended in phosphate-buffered saline (PBS). One drop of sperm mixture was placed on a glass slide, covered with a coverslip, and examined through an epifluorescence microscope (Eclipse Ts2, Nikon, Japan) to detect the functional mitochondria-containing sperms with green fluorescence color. Sperm chromatin integrity was estimated by the acidic aniline blue staining method (Kazerooni et al. 2009). In brief, semen samples were smeared and buffered glutaraldehyde (3:100 w/v) was added for fixing the smears for 30 min. Acidic aniline blue was added for 7 min to stain the smears, rinsed with purified water, and air-dried. Sperms were examined under the oil immersion lens and the blue-stained heads were considered as abnormal chromatin, while the non-stained heads were considered as normal ones.

Sperm mucus penetration test

With the aid of modified synthetic oviductal fluid on flat capillary tubes (80 ± 0.5 mm long, 1.25 ± 0.05 mm wide; Hilgenberg GmBH, Stutzerbach, Germany), the sperm penetration test was evaluated. After sealing one periphery of the flat capillary tube, it was filled with mucus and left horizontally at 25 °C for 2 h. The numbers of sperms that reached the marks of 1 and 3 cm were counted.

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 ± standard error of the means (SEM). Data of motion characteristics and percentages of live sperms were analyzed with a one-way analysis of variance (ANOVA) and Tukey’s multiple comparison test. P < 0.05 was considered a statistical significance.

Results

Effect of quercetin on ROS level

The Control group showed increased ROS levels (P < 0.05) when compared with the quercetin-supplemented groups (Fig. 1). ROS proportions were 17.56 ± 1.02, 7.54 ± 0.48, 5.66 ± 0.80, and 10.41 ± 0.69 in the control, and the 25, 50, and 100 μM quercetin treatment groups, respectively. ROS levels were statistically significantly different (p < 0.05) among the various concentrations of quercetin and the control (Fig. 1). Of the treatment groups, the 50 μM quercetin group was found to have the most significantly lower ROS level, which indicates that the optimal concentration for antioxidant activity is 50 μM.

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 ± 1.34, 6.66 ± 0.58, 6.77 ± 0.66, and 5.38 ± 0.86 in the control, and the 25, 50, and 100 μM quercetin treatment groups, respectively; the control group showed significantly higher values than the quercetin-supplemented groups. However, no differences were observed between the quercetin-supplemented groups (Fig. 2).

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 μM quercetin (51.8 ± 2.1%) showed higher motility than control samples (43.1 ± 1.4%) (P < 0.05). Motion characteristics showed no difference between the control and 25 μM quercetin groups, while the values, except for ALH, tended to be reduced as quercetin concentration increased. In addition, the percentage of live sperms was increased in the samples frozen with 50 μM quercetin (46.9 ± 0.7%) when compared with the control samples (43.9 ± 0.4%) (P < 0.05). However, there was no difference between the control, 25 μM (45.7 ± 1.6) and 100 μM (43.6 ± 0.9) quercetin groups (Table 2). The motility and viability of post-thaw sperm were significantly increased in sperms supplemented with 50 μM quercetin when compared with the other groups.
Fig. 1 Effects of quercetin on ROS level in post-thaw dog spermatozoa. a Control, b quercetin (QRN) 25, c QRN 50, d QRN 100. Panels 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. Superscript letters (a–d) indicate that the differences between the groups are different at $P < 0.05$.

Fig. 2 Impacts of quercetin on the apoptosis of post-thaw dog sperm. a Control, b quercetin (QRN) 25, c QRN 50, d QRN 100. Panels a–d are the results of Annexin V-FITC/PI staining and analysis by flow cytometry. Superscript letters (a–b) indicate that the differences between the groups are significantly different at $P < 0.05$. 

Effect of quercetin on sperm plasma membrane integrity, mitochondrial activity, and chromatin integrity

The HOS test showed a significant increase in plasma membrane integrity of the 50 μM QRN groups compared to the control group (54.0 ± 0.6% vs. 46.6 ± 0.7%, respectively). However, there was a significant decrease in the membrane integrity of sperm samples supplemented with 100 μM quercetin (45.2 ± 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. Similar patterns were observed on mitochondrial activity and chromatin integrity and results showed the effectiveness of QRN at 50 μM when compared with the control and 100 μM groups (Table 2).

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 the 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 (Fig. 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;

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

| Groups   | Motility (%) | Progressive motility (%) | VCL (μm/s) | VAP (μm/s) | VSL (μm/s) | Straight (%) | Linearity (%) | ALH (μm) |
|----------|--------------|--------------------------|------------|------------|------------|--------------|--------------|----------|
| Control  | 43.1 ± 1.4b  | 22.4 ± 1.4b              | 70.7 ± 2.3a | 58.6 ± 2.3a | 54.2 ± 2.4a | 77.3 ± 0.8a  | 61.5 ± 0.8a  | 2.0 ± 0.0b |
| 25 μM QRN| 49.6 ± 2.7a  | 27.4 ± 2.5a              | 72.5 ± 2.9a | 58.9 ± 2.5a | 54.4 ± 2.5a | 75.6 ± 1.6ab | 59.6 ± 1.6a  | 2.1 ± 0.0b |
| 50 μM QRN| 51.8 ± 2.1a  | 22.5 ± 3.4b              | 65.3 ± 4.5ab| 52.3 ± 4.4b | 47.2 ± 4.5ab| 70.3 ± 1.9b  | 52.9 ± 2.7b  | 2.0 ± 0.1b |
| 100 μM QRN| 40.7 ± 4.4b | 19.8 ± 2.6b              | 63.1 ± 6.8b | 50.0 ± 2.9b | 45.2 ± 3.2b | 72.3 ± 2.4b  | 51.3 ± 2.7b  | 2.3 ± 0.1a |

Table 2 Effects of quercetin (QRN) supplementation on live sperms, plasma membrane integrity, acrosome, mitochondrial activity, and chromatin integrity of post-thaw dog spermatozoa

| Groups   | Live sperms (%) | Plasma membrane integrity (%) | Acrosome integrity (%) | Mitochondrial activity (%) | Chromatin integrity (%) |
|----------|-----------------|-------------------------------|------------------------|---------------------------|-------------------------|
| Control  | 43.9 ± 0.4b     | 46.6 ± 0.7b                   | 61.6 ± 3.2             | 44.4 ± 0.5b               | 58.9 ± 0.9b             |
| 25 μM QRN| 45.7 ± 1.6a     | 49.2 ± 0.6ab                  | 53.7 ± 2.7             | 47.1 ± 0.7ab              | 61.6 ± 0.8ab            |
| 50 μM QRN| 46.9 ± 0.7a     | 54.0 ± 0.6a                   | 60.7 ± 4.4             | 49.5 ± 1.8a               | 64.3 ± 0.7a             |
| 100 μM QRN| 43.6 ± 0.9ab   | 45.2 ± 0.6b                   | 54.9 ± 1.7             | 45.5 ± 1.6b               | 57.1 ± 0.5b             |

---

Fig. 3 Effects of quercetin (QRN) supplementation on the mucus penetration ability of post-thaw dog spermatozoa. a–c Within the same parameter, values with different superscripts differ significantly (p<0.05, n=7)
however, the effect of 100 μM was relatively higher than that of 25 μM quercetin at 1 cm (Fig. 3).

**Discussion**

Cryopreservation is a crucial tool for preserving 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 the 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 and 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 conserving 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 cryopreservation. 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).

ROS generation during cryopreservation triggers the apoptosis of spermatozoa, eventually leading to sperm loss of function or death (Said et al. 2004, 2010). Moreover, under oxidative stress conditions, the mitochondrial membrane potential decreases and apoptosis is enhanced by ROS generation (Redza-Dutordoir and Averill-Bates, 2016). Quercetin displayed potent antioxidative properties, improved membrane integrity, improved mitochondrial activity, and chromatin integrity owing to its ROS scavenging activity (Mazzi et al. 2012). Quercetin 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-Jedrzejowska 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 α (TNF-α) and interleukin (IL-1α) (Bureau et al. 2008). This effect of quercetin could be an additional mechanism for the reduced apoptosis and enhanced integrity of the sperm; however, further studies are needed to confirm this. Our results coincide with the recent results of Kawasaki et al. (2020) who observed that the skim milk-based extender supplemented with 5 μg/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).

The current results revealed that supplementation of a freezing extender with 50 μM quercetin improved motility and survival as well as the proportion of total and progressively motile 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). 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. 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. 4.

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 and 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.

Acknowledgements

The authors would like to express their gratitude to Prof. Chul Park at Jeonbuk National University and Kyu-Pil Lee & Je-Won Ko at Chungnam National University for permitting the use of their laboratory facilities.

Author contribution

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.

Funding

This work was supported by the Ministry of Science and ICT through the National Research Foundation of Korea (NRF) (grant no. 2021R1A2C2009294) and the Brain Pool program (grant no.: 2021H1D3A2A02040098).

Data availability

The data that support the findings of this study will be available from the corresponding author upon a reasonable request.

Declarations

Ethical approval

All experimental procedures and animal care were approved by Chungnam National University (approval no. 202006A-CNU-103).

Consent to participate

Not applicable.

Consent to publish

Not applicable.

Competing interests

The authors declare no competing interests.

References

Ahmed W, Lingner J (2020) PRDX1 counteracts catastrophic telomeric cleavage events that are triggered by DNA repair activities post oxidative damage. Cell Rep 33:108347

Alahmar AT (2019) Role of oxidative stress in male infertility: an updated review. J Hum Reprod Sci 12:4–18

Almubarak AM, Kim W, Abdelbagi NH, Balla SE, Yu I-J, Jeon Y (2021) Washing solution and centrifugation affect kinematics of cryopreserved boar semen. J Anim Reprod Biotechnol 36:65–75

Alvarez JG, Storey BT (1989) Role of glutathione peroxidase in protecting mammalian spermatozoa from loss of motility caused by spontaneous lipid peroxidation. Gamete Res 23:77–90

Ashida H, Fukuda I, Yamashita T, Kanazawa K (2000) Flavones and flavonols at dietary levels inhibit a transformation of aryl hydrocarbon receptor induced by dioxin. FEBS Lett 476:213–217

Avdatek F, Yeni D, İnanç ME, Çil B, Tuncer BP, Türkmen R, Taşdemir U (2018) Supplementation of quercetin for advanced DNA integrity in bull semen cryopreservation. Andrologia 50:e12975

Azadi L, Tavalaeec M, Deemeh MR, Arabian M, Nasr-Esfahani MH (2017) Effects of tempol and quercetin on human sperm function after cryopreservation. Cryo Letters 38:29–36

Ball EB, Ergin V, Rackova L, Bayraktar O, Kütükboyaci N, Karasu Ç (2014) Olive leaf extracts protect cardiomyocytes against 4-hydroxynonenal-induced toxicity in vitro: comparison with oleuropein, hydroxytyrosol, and quercetin. Planta Med 80:984–992

Bang S, Qamar AY, Tanga BM, Fang X, Cho J (2021) Resveratrol supplementation into extender protects against cryodamage in dog post-thaw sperm. J Vet Med Sci 83:973–980

Bansal AK, Bilaspuri GS (2010) Impacts of oxidative stress and antioxidants on semen functions. Vet Med Int 2010:686137

Barciszewski J, Siboska G, Clark BF, Rattan SI (2000) Cytokinin formation by oxidative metabolism. J Plant Physiol 157:587–588

Baumber J, Ball BA, Gravance CG, Medina V, Davies-Morel MC (2000) The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential, and membrane lipid peroxidation. J Androl 21:895–902

Bungau S, Abdel-Daim MM, Tit DM, Ghanem E, Maruyama-Inoue M, Yamane S, Kadonosono K (2019) Health benefits of polyphenols and carotenoids in age-related eye diseases. Oxid Med Cell Longev 2019:1–22

Bureau G, Longpré F, Martinoli MG (2008) Resveratrol and quercetin, two natural polyphenols, reduce apoptotic neuronal cell death induced by neuroinflammation. J Neurosci Res 86:403–410

Carrasco-Pozo C, Mizzign ML, Speisky H, Gotteland M (2012) Differential protective effects of quercetin, resveratrol, rutin and epigallocatechin gallate against mitochondrial dysfunction induced by indomethacin in Caco-2 cells. Chem Biol Interact 195:199–205
Kashou AH, Sharma R, Agarwal A (2013) Assessment of oxidative stress in sperm and semen. Spermatogenesis. Springer, pp. 351–361

Kawasaki Y, Sakurai D, Yoshihara T, Tsuchida M, Harakawa S, Suzuki H (2020) Effect of quercetin on the motility of cryopreserved canine spermatozoa. Cryobiology 96:50–54

Kazerooni T, Asadi N, Javid L, Kazerooni M, Ghanadi A, GhaifarPasand F, Kazerooni Y, Zolghadr J (2009) Evaluation of sperm’s chromatin quality with acridine orange test, chromomycin A3 and aniline blue staining in couples with unexplained recurrent abortion. J Assist Reprod Genet 26:591–596

Kim TY, Leem E, Lee JM, Kim SR (2020) Control of reactive oxygen species for the prevention of Parkinson’s disease: the possible application of flavonoids. Antioxidants (base) 9:583

Liu X, Xu Y, Liu F, Pan Y, Miao L, Zhu Q, Tan S (2021) The feasibility of antioxidants avoiding oxidative damages from reactive oxygen species in cryopreservation. Front Chem 9:648684

Mahlouz R, Sharma R, Lackner J, Aziz N, Agarwal A (2009) Evaluation of chemiluminescence and flow cytometry as tools in assessing production of hydrogen peroxide and superoxide anion in human spermatozoa. Fertil Steril 92:819–827

Maizoub A, Agarwal A (2020) Antioxidants in sperm cryopreservation. In: Parekkattil SJ, Esteves SC, Agarwal A (eds) Male infertility: contemporary clinical approaches, andrology, ART and antioxidants. Springer International Publishing, Cham, pp 671–678

Malo C, Gil L, Gonzalez N, Martinez F, Cano R, de Blas I, Espinosa E (2010) Anti-oxidant supplementation improves boar sperm characteristics and fertility after cryopreservation: comparison between cysteine and rosemary (Rosmarinus officinalis). Cryobiology 61:142–147

Martin G, Sabido O, Durand P, Levy R (2005) Phosphatidylycerine externalization in human sperm induced by calcium ionophore A23187: relationship with apoptosis, membrane scrambling and the acrosome reaction. Hum Reprod 20:3459–3468

Mazzi L, Geminiani M, Coltello G, Iacoponi F, Martini S, Bonechi C, Rossi C, Moretti E (2012) Quercetin and rutin: effects of two flavonoids on induced oxidative stress in human ejaculated sperm. J Siena Acad Sci 3:22–26

Moodi Z, Bagherzade G, Peters J (2021) Quercetin as a precursor for biochanin A disposition in rats. Mol Pharm 4:865–872

Najafi A, Kia HD, Mehdipour M, Hamishehkar H, Alvarez-Rodriguez M (2020) Effect of quercetin loaded liposomes or nanostructured lipid carrier (NLC) on post-thawed sperm quality and fertility of rooster sperm. Theriogenology 152:122–128

Pasap S, Sindhu B, Arroyo L, Bassols A, Miró J, Yeste M (2019) Specific activity of superoxide dismutase in stallion seminal plasma is related to sperm cryotolerance. Antioxidants (base) 8:539

Qamar AY, Fahim S, Kim MJ, Cho J (2020) Effects of kinetin supplementation on the post-thaw motility, viability, and structural integrity of dog sperm. Cryobiology 95:90–96

Rakha BA, Qurratul A, Ansari MS, Akhter S, Akhter A, Awan MA, Santiago-Moreno J (2020) Effect of quercetin on oxidative stress, mitochondrial activity, and quality of Indian red jungle fowl (Gallus gallus muskari) sperm. Biopreserv Biobank 18:311–320

Redza-Dutordoir M, Averill-Bates DA (2016) Activation of apoptosis signalling pathways by reactive oxygen species. Biochim Biophys Acta 1863:2977–2992

Ricci G, Peticarari S, Fragonas E, Giolo E, Canova S, Pozzobon C, Guaschino S, Presani G (2002) Apoptosis in human sperm: its...
correlation with semen quality and the presence of leukocytes. Hum Reprod 17:2665–2672
Russo M, Spagnuolo C, Tedesco I, Bilotto S, Russo GL (2012) The flavonoid quercetin in disease prevention and therapy: facts and fancies. Biochem Pharmacol 83:6–15
Sabeti P, Pourmasumi S, Rahiminia T, Akyash F, Talebi AR (2016) Etiologies of sperm oxidative stress. Int J Reprod Biomed 14:231–240
Said TM, Agarwal A, Sharma RK, Mascha E, Sikka SC, Thomas AJ Jr (2004) Human sperm superoxide anion generation and correlation with semen quality in patients with male infertility. Fertil Steril 82:871–877
Said TM, Gagliani A, Agarwal A (2010) Implication of apoptosis in sperm cryoinjury. Reprod Biomed Online 21:456–462
Sariözkan S, Bucak MN, Tuncer PB, Ulutas PA, Bilgen A (2009) The influence of cysteine and taurine on microscopic-oxidative stress parameters and fertilizing ability of bull semen following cryopreservation. Cryobiology 58:134–138
Schieber M, Chandel NS (2014) ROS function in redox signaling and oxidative stress. Curr Biol 24:R453–R462
Seifi-Jamadi A, Kohram H, ZareShahneh A, Ansari M, Maclas-García B (2016) Quercetin ameliorate motility in frozen-thawed Turkmen stallions sperm. J Equine Vet 45:73–77
Seytawan EMN, Kim MJ, Oh HJ, Kim YK, Lee SH, Choi YB, Lee BC (2016) Spermine reduces reactive oxygen species levels and decreases cryocapacitation in canine sperm cryopreservation. Biochem Biophys Res Commun 479:927–932
Shiratsuchi A, Umeda M, Ohba Y, Nakanishi Y (1997) Recognition of phosphatidylserine on the surface of apoptotic spermatogenic cells and subsequent phagocytosis by Sertoli cells of the rat. J Biol Chem 272:2354–2358
Snezhkina AV, Kudryavtseva AV, Kardymon OL, Savvateeva MV, Melnikova NV, Krasnov GS, Dmitriev AA (2019) ROS generation and antioxidant defense systems in normal and malignant cells. Oxid Med Cell Longev 2019:6175804
Su L-J, Zhang J-H, Gomez H, Murugan R, Hong X, Xu D, Jiang F, Peng Z-Y (2019) Reactive oxygen species-induced lipid peroxidation in apoptosis, autophagy, and ferroptosis. Oxid Med Cell Longev 2019:5080843
Tanga BM, Qamar AY, Raza S, Bang S, Fang X, Yoon K, Cho J (2021) Sperm evaluation: methodological advancements in sperm quality-specific fertility assessment - a review. Anim Biosci 34:1253–1270
Tvrdá E, Kováčik A, Tušimová E, Paál D, Mackovich A, Alimov J, Lukáč N (2016) Antioxidant efficiency of lycopene on oxidative stress - induced damage in bovine spermatozoa. J Anim Sci Biotechnol 7:50
Vieira NMG, Losano JDA, Angrimani DSR, Kawai GKV, Bicudo LC, Rui BR, da Silva B, Assumpção M, Nichi M (2018) Induced sperm oxidative stress in dogs: Susceptibility against different reactive oxygen species and protective role of seminal plasma. Theriogenology 108:39–45
Walczak-Jedrzejowska R, Wolski JK, Slowikowska-Hilczer J (2013) The role of oxidative stress and antioxidants in male fertility. Cent European J Urol 66:60–67
Yang D, Wang T, Long M, Li P (2020) Quercetin: its main pharmacological activity and potential application in clinical medicine. Oxid Med Cell Longev 2020:8825387
Yelumalai S, Giribabu N, Karim K, Omar SZ, Salleh NB (2019) In vivo administration of quercetin ameliorates sperm oxidative stress, inflammation, preserves sperm morphology and functions in streptozotocin-nicotinamide induced adult male diabetic rats. Arch Med Sci AMS 15:240–249
Yoshimoto T, Nakamura S, Yamauchi S, Muto N, Nakada T, Ashizawa K, Tatamoto H (2008) Improvement of the post-thaw qualities of Okinawan native pig spermatozoa frozen in an extender supplemented with ascorbic acid 2-O-alpha-glucoside. Cryobiology 57:30–36
Zribi N, Chakroun NF, Ben Abdallah F, Elleuch H, Sellami A, Gargouri J, Rebai T, Fakhfakh F, Keskes LA (2012) Effect of freezing-thawing process and quercetin on human sperm survival and DNA integrity. Cryobiology 65:326–331
Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.