

Article

Effects of resveratrol supplementation on the motility, structural integrity, and mitochondrial function of freeze-thawed dog sperm

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Abstract: Antioxidants have multiple protective roles in cells and can be used as a supplement to protect cells against cryopreservation-induced detrimental effects, including protecting sperm fertility quality. The antioxidant resveratrol (3,5,4-trihydroxy-*trans*-stilbene; RSV) has been shown to be a protective supplement for the cryopreservation of animal sperm, including human sperm. In this study, we assessed the effect of RSV supplementation on canine sperm cryopreservation. Semen was collected from four dogs and the effect of different concentrations of RSV (0, 100, 200, and 400 μ M) on post-thaw quality of sperm was examined. After thawing, sperm motility was assessed using computer aided sperm analysis, and the structural integrity of the plasma membrane, acrosome, and chromatin were examined, as well as mitochondrial activity and gene expression were assessed. Dog sperm cryopreserved with 200 μ M RSV showed significant improvement in motility and viability following thawing compared with that of the control group ($p < 0.05$). Moreover, RSV-supplemented samples showed significantly higher numbers of sperm with an intact plasma membrane, active mitochondria, and structural integrity of acrosomes and chromatin than that of control samples ($p < 0.05$). Furthermore, gene expression showed that RSV supplemented samples showed lower expression of pro-apoptotic (*BAX*) oxidative stress-related (*ROMO1*) and oxidative induced DNA damage repair (*OGG1*) whereas higher expression levels of anti-apoptotic (*BCL2*) protamine-2 (*PRM2*), protamine-3 (*PRM3*) and sperm acrosome-associated (*SPACA3*) genes than control. Our results suggest that RSV, at its optimum concentration, can be efficiently used as an alternative antioxidant in the cryopreservation of dog sperm.

Keywords: antioxidant; cryopreservation; dog; resveratrol; sperm

1. Introduction

Antioxidants are substances, mainly of plant origin, that can prevent or reduce the magnitude of oxidative damage to cells caused by free radicals. There are two types of antioxidants: enzymatic and non-enzymatic [1]. Enzymatic antioxidants are also known as natural antioxidants and include glutathione peroxidase, glutathione reductase, superoxide dismutase, and catalase [2]. Non-enzymatic antioxidants, whether synthetic or dietary supplements, include reduced glutathione, urate, ascorbic acid, vitamin E (alpha-tocopherol), carotenoids (β -carotene), ubiquinones, taurine and hypotaurine, selenium, and zinc [2,3]. Antioxidants in the body of animals play a major role in controlling oxidative stress and maintaining cellular homeostasis by reducing excessive free radicals resulting from cell function or external effects [4]. Uncontrolled excessive production of free radicals from the

mitochondria can overwhelm the protective biochemical systems of the body causing oxidative damage to important cellular components such as lipids, proteins, and carbohydrates in cell membranes, and nucleic acids [5].

The sperm cryopreservation process involves freezing and thawing, leading to significant physiological and chemical changes in the sperm, which in turn causes the production of reactive oxygen species (ROS) [6,7]. This is mainly due to the removal of seminal plasma during the cryopreservation process, which reduces antioxidant defenses in sperm; thus, sperm become vulnerable to oxidative stress [8]. The mechanism behind cryodamage may be related to osmotic stress, cold shock, intracellular ice crystal formation, excessive production of ROS [9-11], alterations in antioxidant defense systems [10], and combinations of these processes. Antioxidants are the main defense factors against oxidative stress induced by ROS [12]. Many investigators have focused on the use of antioxidants in the freezing media to reduce the negative effects of ROS on sperm [13-15]. Our previous findings showed that supplementation of canine freezing extenders with kinetin [16] and astaxanthin [17] improved the post-thaw motility, viability, and structural integrity of dog sperm, and we aimed to assess the effect of resveratrol (RSV) as an alternative antioxidant.

RSV is a non-flavonoid with powerful antioxidant activity, which acts by scavenging free radicals and chelating divalent cations [18]. RSV has been found to be beneficial in the prevention of vascular diseases and involved in cellular signaling, enzymatic pathways, and apoptosis, mainly by inhibiting ROS formation [19]. RSV also reduces calcium release into the cell in rat ventricular myocytes, which can explain the beneficial effects of RSV on cardiovascular disease [20]. In rams, the addition of RSV (5–20 ng/mL) to a Tris-egg yolk–glycerol extender was found to decrease mitochondrial membrane potential, which protected sperm by reducing ROS levels [21]. In a recent study on post-thaw buck sperm, supplementation of a commercial extender with RSV enhanced their viability [22]. RSV-supplemented freezing medium has been reported to improve the quality and function of post-thaw human sperm by reducing the magnitude of cryoinjury and the level of ROS [23]. In buffalo, the addition of RSV to a Tris-citric acid extender ameliorated post-thaw quality parameters, antioxidant enzyme levels, and the fertility of the sperm [24].

By extension, we hypothesized that the antioxidant RSV could be used as a cryopreservation supplement to improve the post-thaw quality of dog sperm. Here, we determined the optimal concentration of RSV and assessed its effect on post-thaw dog sperm quality.

2. Materials and Methods

2.1 Materials and protocols

All chemical used in the experiment were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Sperm cryopreservation and post-thaw quality assessment were performed following previously reported protocols in our study [17].

2.2 Preparation of buffers

The sperm washing and dilution system was prepared as previously described [17]. Buffer 1 used for sperm washing was composed of Tris (hydroxymethyl) aminomethane, 198.11mM; citric acid, 72.87 mM; fructose, 44.39 mM; and kanamycin sulfate, 0.25 mM dissolved in distilled water. (pH 6.6, 290 mOsm). Buffer 2 (semen extender) was prepared using 54% buffer 1 (v/v), 40% egg yolk (v/v), and 6% glycerol (v/v).

2.3 Dogs and semen collection

Four healthy male beagle dogs, 3–4 years old, weighing 8–12 kg, were used for semen collection. Each dog was kept under the same conditions according to standard protocols, including protection from excessive noise and provided a standard diet in a space with sufficient area for exercise and rest. All experimental procedures were conducted following the guidelines for the care and use of laboratory animals at Chungnam National University (approval no. 202006A-CNU-103). Semen collection was performed using digital manipulation twice a week. Semen samples were initially assessed using a CASA software imaging system (MICROPTIC CASA Systems; SCA class analyzer, Josep Tarradellas, Barcelona, Spain). Samples with $\geq 70\%$ motility, $\geq 80\%$ viability, and a sperm concentration $\geq 100 \times 10^6$ cells/mL were pooled together and used for further processing.

2.4 Semen cryopreservation and thawing

Sperm cryopreservation and post-thaw quality assessment were performed following previously reported protocols [17] and as detailed here. Pooled sperm samples were adjusted to a concentration of 200×10^6 cells/mL with buffer 1. The sperm suspension was then divided and diluted with extender (buffer 2) supplemented with 100, 200, or 400 μM RSV, or no RSV (control). The semen was extended in a multi-step dilution process to a final concentration of 100×10^6 cells/mL. The diluted semen was used to fill 0.5 mL semen straws (Minitub GmbH, Ref. 13408/0010, Germany), which were then equilibrated at 4°C for 45–60 min. Freezing of the semen straws was performed by horizontally placing the straws 2 cm above the surface of liquid nitrogen for 15 min. After storage for 1 week in liquid nitrogen, the frozen semen straws were thawed in a 37°C water bath for 30 seconds.

2.5 Assessment of motility, kinematic parameters, and sperm viability

Post-thawed sperm samples (5 μL) were placed on a slide for assessment of motility using the CASA software imaging system. For each semen sample, 5 different fields were randomly selected and at least 200 sperm were tracked for 1 s at 25 Hz. The kinematic parameters analyzed included the percentage of motile sperm, progressive motility, curvilinear velocity (VCL), average path velocity (VAP), straight-line velocity (VSL), straightness, linearity, and amplitude of lateral head displacement (ALH). The CASA analysis was repeated 12 times for increasing precisions of measurements.

Sperm survival was assessed using the eosin-nigrosin staining procedure. Briefly, sperm were stained 1:1 with the staining reagent and then the smear was applied to a warm slide and air-dried. At least 200 sperm per slide were examined at $1000\times$ magnification to assess sperm survival. Sperm stained pink were considered non-viable, and unstained sperm were considered viable. The optimal RSV concentration required for cryopreservation of dog sperm was determined based on the results of parameters related to motion characteristics and percentage of live sperm.

2.6 Analysis of plasma membrane integrity

Plasma membrane integrity was analyzed using the hypo-osmotic swelling (HOS) assay. One drop ($\sim 50 \mu\text{L}$) of post-thaw semen from each group was mixed with 0.5 mL of HOS solution and incubated at 37°C for 30 min [25]. The incubated mixture was placed on a pre-warmed glass slide, and 200 sperm per sample were assessed within 5–10 min for their ability to expand using a phase-contrast microscope (Eclipse Ts2, Nikon, Minato-Ku, Tokyo, Japan). Swelling was indicated by the coiling of the sperm tail, and such sperm were considered to possess an intact plasma membrane. The integrity of the acrosome in post-thaw sperm samples was assessed using fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) staining. A sperm-drop ($\sim 50 \mu\text{L}$) was smeared on a glass slide, air-dried, fixed with methanol at $20\text{--}22^\circ\text{C}$ for 10 min, and stained. After staining, the smears were rinsed with PBS, air-dried, and glycerol was added. The sperm acrosome was observed using an epifluorescence microscope ($1000\times$ magnification; Eclipse Ts 2, Nikon). At least 200 sperm per smear were examined and classified according to the presence (strong green fluorescence) or absence (no fluorescence) of an intact acrosome.

2.7 Analysis of sperm mitochondrial activity and chromatic integrity

The assessment of post-thaw sperm for functional mitochondria was performed using a combination of fluorescent stains, including rhodamine 123 (R123) and propidium iodide (PI), as previously described [26]. Briefly, 30 μ L of R123 solution (5 mg/mL in dimethyl sulfoxide) was diluted with 120 μ L of dimethyl sulfoxide and divided into 30 μ L aliquots for storage. Post-thaw sperm samples were diluted with buffer 1 to achieve a concentration of 20×10^6 sperm/mL and 3 μ L of R123 working solution was added. The semen-stain suspension was incubated for 15 min in the dark at 37°C. After incubation, 10 μ L of PI solution (0.5 mg/mL in PBS) was added and the samples were incubated again for 10 min at 37.8°C. Incubation was followed by centrifugation at 500g for 5 min, and the sperm pellet was re-suspended in 1 mL of PBS. One drop (~10 μ L) of the sperm suspension was placed on a microscopic slide, mounted with a coverslip, and evaluated using an epifluorescence microscope (Eclipse Ts 2, Nikon). At least 200 sperm per sample were examined, and viable sperm with functionally active mitochondria were identified by the presence of green fluorescence at the midpiece of the sperm tail.

The chromatin status of post-thaw sperm samples treated with RSV was evaluated using acidic aniline blue staining [27,28]. Post-thawed semen (5 μ L) was smeared on a glass slide and air-dried. Buffered glutaraldehyde (3%) was used to fix the smears for 30 min before staining with 5% aqueous aniline blue mixed with 4% acetic acid (pH 3.5) for 7 min. Stained smears were rinsed with distilled water and air-dried. At least 200 sperm per sample were evaluated using a light microscope at oil immersion magnification (1000 \times). Sperm with abnormal chromatin were indicated by blue-stained nuclei, whereas sperm with normal chromatin were indicated by unstained nuclei [29].

2.8 Mucus penetration test

Mucus penetration tests were performed using surrogate mucus (modified synthetic oviductal fluid) [30,31]. The fluid was loaded into flat capillary tubes (80 \pm 0.5 mm long, 1.25 \pm 0.05 mm wide, Hilgenberg GMBH, Stutzerbach, Germany) sealed at one end. The capillary tubes were placed in the vertical position for 15 min to check the tightness of the seal and to remove bubbles, following which the open end of the capillary tube was inserted into an Eppendorf tube containing 100 μ L of semen suspension and placed vertically for 2 h at room temperature (25–28°C). Thereafter, the number of sperm that penetrated to the 1- and 3-cm marks in the capillary tube was counted.

2.9 Gene expression

Gene expression was assessed by quantitative real-time polymerase chain reaction (RT-qPCR) to analyze the expression of genes related to apoptosis such as B-cell lymphoma (*BCL2*) and *BCL2*-associated X (*BAX*); protamine 2 (*PRM2*) and protamine 3 (*PRM3*), as well as mitochondrial ROS modulator 1 (*ROMO1*), sperm acrosome-associated (*SPACA3*) and oxidative induced DNA damage repair 8-oxoG-DNA glycosylase (*OGG1*). Briefly, RNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) from post-thawed sperm cryopreserved with different concentrations of RSV, or no RSV (control). Complementary DNA synthesis was performed using the Compact cDNA synthesis kit (SJ Bioscience, Daejeon, Korea), according to the manufacturer's instructions. Transcript expression levels were analyzed by RT-qPCR using the SYBR Green Q-PCR Master Mix (SJ Bioscience) and the primers listed in Table 1. The expression of each target gene was quantified relative to that of the internal gene *β -actin* using the equation $R = 2^{-[\Delta Ct \text{ sample} - \Delta Ct \text{ control}]}$ [32].

2.10 Statistical analysis

The Statistical Package for Social Sciences (SPSS) version 24.0 software for analysis (SPSS Inc., Chicago, IL, USA) was used to analyze the data. The measurements of different parameters were

expressed as the mean \pm standard error of the mean (SEM), and $p < 0.05$ indicated statistical significance. For analysis of variance, one-way analysis of variance (ANOVA) and Tukey's multiple comparison test were used to analyze data related to motion characteristics and percentages of live sperm.

Table 1. Primer sequences used for analysis of gene expression in post-thaw canine sperm.

| Gene | Primer Sequence (5'–3') | Product Size (bp) | NCBI Accession No. |
|---------------------------------|---|-------------------|--------------------|
| <i>β-actin</i> | F: GAG GCA TCC TGA CTC TGA R: TCTGGCACCACACTTTCT | 87 | XM_544346.3 |
| <i>BAX</i> | F: CCAAGAAGCTGAGCGAATG R: CTGCCACTCGGAAGAAGAC | 123 | NM_001003011.1 |
| <i>BCL2</i> | F: GACAGAGAGGATCATGCTGT R: TGGCATGAGATGCAGGAAAT | 141 | NM_001002949.1 |
| <i>PRM2</i> | F: CTCCAGAAGGGTCAGGAG R: GGCTCCTTGCAAATCAG | 169 | NM_001287148.1 |
| <i>PRM3</i> | F: TCTGGAGAGGCAGCCAGA R: AGGCCATGAGCTTCTTCA | 101 | XM_022420065.1 |
| <i>ROMO1</i> | F: CTACGTGCTCCCGGAAGT R: TCGTCTAGTTCTACGTCTCAC | 100 | XM_534406.6 |
| <i>SPACA3</i> | F: AACACAGCTGCTGTGGAC R: ACCACTTCCGGCTGTTGA | 76 | NM_001197087.1 |
| <i>OGG1</i> | F: AACAACAACATTGCTCGCA R: GGAAGCCATGGTAGGTGAC | 100 | XM_022406407.1 |

F, forward; R, reverse; *BAX*, *BCL2*-associated X; *BCL2*, B-cell lymphoma; *PRM2*, protamine 2; *PRM3*, protamine 3; *ROMO1*, ROS modulator 1; *SPACA3*, sperm acrosome associated-3; *OGG1*, oxidation-induced DNA damage repair.

3. Results

3.1 Determination of optimum concentration of RSV

The optimum concentration of RSV was selected based on improvement in fertility quality and kinetic parameters of dog sperm compared to the control (no RSV) (Table 2). Sperm samples supplemented with 200 μ M RSV showed the highest post-thaw motility ($50.4 \pm 1.4\%$) compared to that of other RSV concentrations and the control ($p < 0.05$). In addition, the progressive motility ($19.8 \pm 1.3\%$), straightness ($76.3 \pm 1.3\%$), and linearity ($55.9 \pm 1.3\%$) of sperm treated with 200 μ M RSV were all significantly higher than that of the other groups ($p < 0.05$). On this basis, the optimum concentration of RSV for dog sperm cryopreservation was determined to be 200 μ M.

Table 2. Determination of optimal concentration of resveratrol (RSV) for semen cryopreservation.

| Groups | Mot. (%) | Pro. Mot. (%) | VCL (μ m/s) | VAP (μ m/s) | VSL (μ m/s) | Straight (%) | Linearity (%) | ALH (μ m) |
|-----------------|------------------------------|-----------------------------|------------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Control | 38.7 ± 3.1 ^{bc} | 15.7 ± 1.8 ^b | 61.4 ± 3.9 ^{ab} | 43.8 ± 4.0 ^a | 39.6 ± 3.9 ^b | 74.3 ± 2.4 ^b | 51.2 ± 3.3 ^b | 2.3 ± 0.1 ^a |
| 100 μ M RSV | 41.6 ± 2.1 ^b | 14.1 ± 1.6 ^b | 54.1 ± 3.1 ^{bc} | 36.2 ± 3.3 ^c | 31.1 ± 3.4 ^c | 70.0 ± 2.5 ^c | 46.9 ± 2.8 ^c | 2.2 ± 0.0 ^b |
| 200 μ M RSV | 50.4 ± 1.4 ^a | 19.8 ± 1.3 ^a | 58.5 ± 2.7 ^b | 43.5 ± 2.6 ^b | 41.5 ± 2.9 ^a | 76.3 ± 1.3 ^a | 55.9 ± 1.3 ^a | 2.1 ± 0.0 ^{bc} |
| 400 μ M RSV | 37.4 ± 3.4 ^c | 10.7 ± 1.7 ^c | 51.7 ± 3.0 ^c | 35.0 ± 3.0 ^c | 29.7 ± 3.1 ^c | 67.9 ± 3.3 ^c | 46.0 ± 3.4 ^c | 2.0 ± 0.0 ^c |

^{a-c} Values with different lowercase superscripts letters in a column differ significantly ($p < 0.05$, $n=3$)

3.2. Sperm survival rate

The eosin-nigrosin staining results of sperm post-thaw showed that the percentage of live sperm was significantly increased when supplemented with 200 μ M RSV ($p < 0.05$). The sperm survival rate was statistically higher in the 200 μ M RSV-treated case than in the control sample ($54.8 \pm 1.4\%$ vs. $43.1 \pm 2.9\%$) (Table 3).

Table 3. Effects of resveratrol (RSV) supplementation on the post-thaw integrity of the plasma membrane (HOS), acrosome, and chromatin, and mitochondrial activity of dog sperm.

| Groups | Live sperm (%) | HOS (%) | Mitochondrial activity (%) | Acrosome integrity (%) | Chromatin integrity (%) |
|-----------------|---------------------|------------------|----------------------------|------------------------|-------------------------|
| Control | 43.1 ± 2.9^b | 60.1 ± 1.7^a | 47.4 ± 1.2^b | 54.6 ± 3.2^b | 63.1 ± 2.1^c |
| 100 μ M RSV | 45.5 ± 1.9^b | 54.1 ± 2.3^b | 46.5 ± 2.0^b | 55.3 ± 2.2^b | 68.1 ± 2.0^b |
| 200 μ M RSV | 54.8 ± 3.8^a | 61.6 ± 2.6^a | 58.1 ± 1.4^a | 58.2 ± 3.6^a | 78.1 ± 1.4^a |
| 400 μ M RSV | 39.6 ± 0.7^{bc} | 48.8 ± 1.8^c | 40.5 ± 1.2^c | 58.7 ± 2.2^a | 69.3 ± 2.5^b |

^{a-c} Values with different superscript lowercase letters in a column differ significantly ($p < 0.05$, $n=3$).

3.3 Integrity of the plasma membrane and acrosome

The results of FITC-PNA staining illustrated that RSV supplementation was effective in protecting the acrosomal integrity of post-thaw dog sperm (Figure 1a). The number of sperm with an intact acrosome was significantly higher in samples treated with 200 μ M RSV than in the control group ($p < 0.05$; Table 3). Plasma membrane integrity was also evaluated using an HOS assay and showed that there was no significant difference between control samples and samples supplemented with 200 μ M RSV ($60.1 \pm 1.7\%$ vs. $61.6 \pm 2.6\%$, respectively) (Table 3).

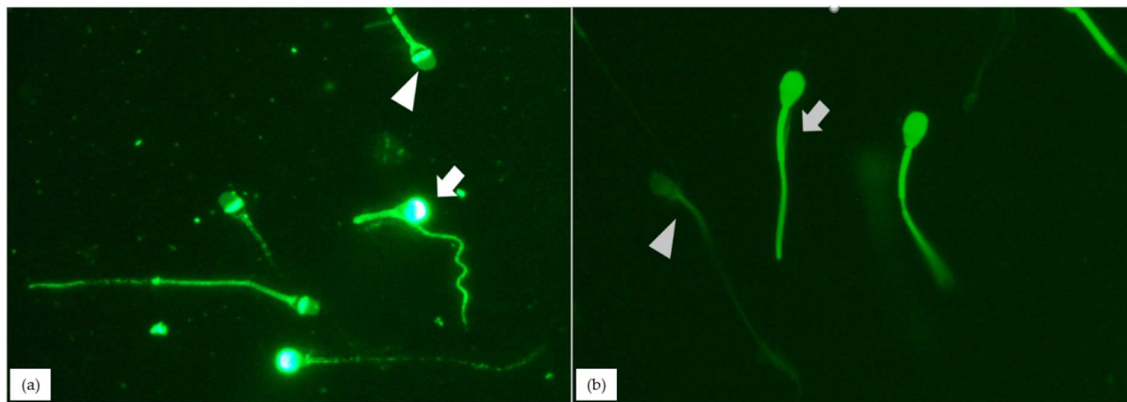


Figure 1. Sperm integrity. (a) Sperm stained with fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA). Intact acrosome (arrow) and damaged acrosome (arrowhead); (b) Rhodamine 123 and propidium iodide (R123/PI) staining of post-thaw sperm midpiece with normal mitochondrial core (arrow) and abnormal mitochondrial core (arrowhead).

3.4 Mitochondrial activity and chromatin integrity

Post-thaw evaluation of sperm mitochondrial integrity was carried out using R123/PI staining (Figure 1b). Sperm treated with 200 μ M RSV showed a significant increase in mitochondrial activity compared to that of the control group ($p < 0.05$; Table 3). After thawing, semen evaluation revealed an increase in the number of sperm with normal chromatin in the group supplemented with RSV. Aniline

blue staining revealed that the number of normal chromatin was significantly higher in sperm supplemented with 200 μ M RSV than in the control sperm ($78.1 \pm 1.4\%$ vs. $63.1 \pm 2.1\%$, respectively, $p < 0.05$; Table 3).

3.5 Mucus penetration ability

Results of the mucus penetration test showed that the number of sperm capable of penetrating mucus to the 1- or 3-cm mark was significantly higher in 200 μ M RSV-treated sperm than in the control group ($p < 0.05$). However, samples treated with 400 μ M RSV did not show a significant difference compared with that of the control at either the 1- or 3-cm mark (Table 4).

Table 4. Effects of resveratrol (RSV) supplementation on mucus penetrability of post-thaw dog sperm.

| Groups | Number of Sperm Penetrating the Mucus | |
|-----------------|---------------------------------------|---------------------|
| | 1 cm penetration | 3 cm penetration |
| Control | 51.4 ± 3.0^b | 16.7 ± 1.3^b |
| 100 μ M RSV | 62.0 ± 1.5^{ab} | 22.0 ± 1.3^{ab} |
| 200 μ M RSV | 67.1 ± 1.2^a | 31.5 ± 1.0^a |
| 400 μ M RSV | 52.0 ± 1.8^b | 14.7 ± 0.9^b |

^{a,b} Values with different superscript lowercase letters in a column differ significantly ($p < 0.05$, $n=3$).

3.6 Gene expression

The analysis of gene expression levels in post-thaw semen samples supplemented with 200 μ M RSV showed a significantly enhanced expression level of *BCL2* and reduced expression of *BAX* compared with that of the control group ($p < 0.05$ for *BCL2*; Figure 2). The expression levels of genes *PRM2* and *PRM3* and *SPACA3* in post-thaw semen samples were significantly higher in 200 μ M RSV-supplemented semen samples than in the control group ($p < 0.05$ in all except *PRM2*; Figure 2). Furthermore, the expression level of *ROMO1* and *OGG1* was significantly reduced in 200 μ M RSV-supplemented sperm samples compared with that of the control ($p < 0.05$; Figure 2). These results show an overall improvement in sperm characteristics and a reduction in apoptosis with RSV supplementation; therefore, one would expect fertility quality to be better than that of the control.

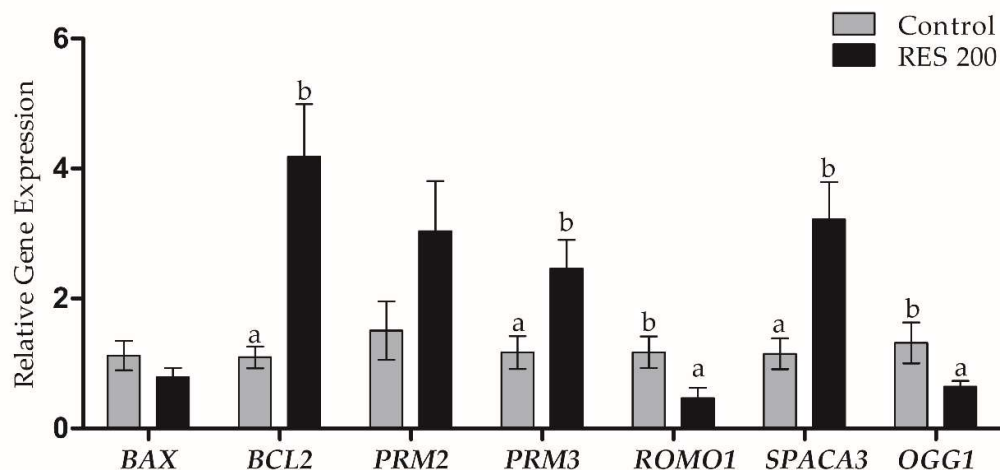


Figure 2. Gene expression levels of pro-apoptotic BCL2-associated X (*BAX*), anti-apoptotic B-cell lymphoma (*BCL2*), protamine 2 (*PRM2*), protamine 3 (*PRM3*), the mitochondrial reactive oxygen species modulator 1

(*ROMO1*), sperm acrosome-associated (*SPACA3*) and oxidative induced DNA damage repair (*OGG1*) using real-time quantitative polymerase chain reaction (RT-qPCR) in resveratrol (RSV)-supplemented sperm samples (dark bars) and control samples (light bars). Values are presented as the mean \pm SEM. Different lowercase letters (a or b) indicate significant differences ($p < 0.05$).

4. Discussion

The findings in the current study of improved motility and survival rate of RSV-supplemented cryopreserved dog sperm are in line with previous findings in boar sperm; the addition of 50 μ M RSV to the Modena extender significantly improved motility and protected the boar sperm against oxidative stress [33]. The mechanism of action of RSV in reducing inflammation and associated pathologies involves decreasing interleukin-1 β secretion and gene expression for cell apoptosis; this leads to a strong reduction in activity of the inflammasome pathway ending in apoptosis [34]. In addition, the anti-inflammatory potential of RSV has been demonstrated by counteracting an inflammatory challenge in U-937 macrophages at representative plasma concentrations [35]. On this basis, we hypothesized that the main mechanism of RSV in improving the motility and survival rate of post-thaw dog sperm is through reduced inflammasome pathway activity in addition to maintaining mitochondrial activity.

The optimum concentration of RSV supplementation in dog sperm was 200 μ M, higher than the optimum concentration of 50 μ M in boar [33] and 40 μ M in roosters [36]. The variation could be due to differences in species and the type of media and buffer used and emphasizes the need to optimize conditions when using RSV or other antioxidants in cryopreservation. The relatively high level of mitochondrial activity and integrity of the acrosome and chromatin in RSV-supplemented dog sperm was also observed in boar semen, in which enhanced mitochondrial function and decreased apoptosis, induced by both the death receptor- and mitochondria-mediated apoptotic pathways, were observed [37]. Moderate doses of RSV induced mitochondrial biogenesis and protected against metabolic decline in mice [38]. RSV has been shown to affect nutrient-deprivation autophagy factor-1, which is an outer mitochondrial membrane protein, known to play an important role in calcium metabolism, anti-apoptosis, and anti-autophagy [39]. The upregulation of mitochondrial biogenesis by RSV [40] was also supported by supplemented mice cells showing better mitochondrial function than untreated cells. The effect of RSV on dog sperm in our study is also in line with its effect on low-quality stallion semen, in which it can ameliorate mitochondrial activity and prevent DNA damage when added prior to cryopreservation [41]. The maintenance of overall cellular integrity and higher mitochondrial activity of RSV-supplemented sperm supports better fertility potential as compared to controls without RSV supplementation.

The positive effect of RSV supplementation is not only limited to sperm, but also has been reported to improve embryo growth and survivability after cryopreservation. RSV was found to help repair abnormal mitochondrial distribution and mitochondrial dysfunction in bovine embryos after vitrification of mouse embryos [42]. In mouse oocyte cryopreservation, 25 μ M RSV reduced the oxidative stress of vitrified oocytes by decreasing the levels of ROS and increasing the levels of glutathione. RSV also alleviated the abnormal mitochondrial distribution pattern in oocytes after vitrification [43], which is similar to the function of RSV in sperm cryopreservation. These findings suggest that RSV can be used as a cryoprotective extender supplement in both oocytes/embryos and sperm to improve quality. In the current study, RSV has showed significant effect though lipid-solubility limiting its bioavailability. But, the development of bio-carriers for intracellular transportation of RSV like apolipoprotein E3 [44] believed to make the effects of RSV even better and could be the best antioxidant. RSV also showed to act as a ligand for trans-membrane proteins [45] including voltage-gated calcium channels and plasma membrane calcium ATPase [46,47]. It also increases endoplasmic reticulum (ER) calcium concentrations and decrease release of calcium from intracellular stores [47-50] results in overall calcium homeostasis during states of cellular dysfunction

[51]. This mechanism of action of RSV is believed to limit to early hyperactivation of sperms and contribute positively in maintain the fertility potential of sperm as it is needed later for fusing stage with ovum[52].

The significant increase in the expression of *BCL2*, a mitochondria-mediated apoptotic pathway gene, by RSV-supplementation in post-thaw dog sperm agreed with the findings in RSV-supplemented boar sperm [53] and rats [54]. This suggests that RSV improves the survival rate of sperm by reducing the stress-induced apoptotic pathways of cell death. Similarly, increased expression of *PRM1* and *PRM2* in dog sperm with RSV supplementation was also observed in human sperm; this was likely due to the activation of AMP-activated protein kinase [55]. The reduced expression of *BAX* in RSV-supplemented dog sperm was also observed in RSV-supplemented rats exposed to Cd bromide. The reduced expression of *OGG1* in RSV supplemented sperm is an indication of lower DNA damage, is also reported which is important to the subsequent success in embryo development and outcome of reproduction [56]. This indicates that RSV protects sperm by reducing activity of the apoptotic pathway and in activating AMP-activated protein kinase. Almost all assessments have showed RSV has better effect in maintaining the quality of post-thawed sperm in terms of fertility, and it can be considered as alternative antioxidant.

5. Conclusions

RSV should be considered one of the best antioxidant supplements for dog sperm cryopreservation to preserve quality; we recommend an optimal concentration of 200 μ M RSV. Our findings indicate that there is still room for improvement in antioxidant use in reproductive technologies, cell biology, and pathological studies. In addition to its use in sperm cryopreservation, RSV is also a promising buffer supplement for embryo cryopreservation. Finally, we recommend that species- and cryopreservation condition-specific studies and verification be conducted before using an antioxidant supplement on a broader scale.

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