FULL PAPER

Theriogenology

Resveratrol supplementation into extender protects against cryodamage in dog post-thaw sperm

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ABSTRACT. Antioxidants have multiple protective roles in a variety of cells and thus can be used to protect sperm against cryo-damage during freezing, which affects fertility. The antioxidant resveratrol (3,5,4-trihydroxytrans-stilbene; RSV) has been reported to protect the animal sperm during cryopreservation, including human sperm. In this study, we assessed the protective effects of RSV supplementation on dog sperm cryopreservation. Semen was collected from four dogs and the effect of different concentrations of RSV (0, 100, 200, and 400 μM) on post-thaw sperm quality was examined. After thawing, sperm motility was assessed using computer-aided sperm analysis, and the structural integrity of the plasma membrane, acrosome, and chromatin was examined. In addition, their mitochondrial activity and gene expression were also assessed. Dog sperm cryopreserved with 200 μM RSV showed significant improvement in post-thaw sperm motility and viability 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*), reactive oxygen species (ROS) modulator oxidative stress-related (*ROMO1*) and 8-oxoguanine DNA glycosylase 1 (*OGG1*) whereas higher expression levels of anti-apoptotic (*BCL2*), protamine-2 (*PRM2*), protamine-3 (*PRM3*) and sperm acrosome-associated 3 (*SPACA3*) genes than control. Our results suggest that RSV, at its optimum concentration, can be efficiently used as an antioxidant in the cryopreservation of dog sperm.

KEY WORDS: antioxidant, cryopreservation, dog, resveratrol, sperm

Preservation of the genetic materials enables the conservation of endangered animal species, artificial reproduction, and the selective breeding of the required pedigree [8, 20, 23]. The cryopreservation method of genetic conservation, commonly employed in livestock reproduction and conservation, has various limitations in terms of maintaining the fertility parameters and one of the common limitations is cryo-induced damage [7, 9, 33, 49]. Uncontrolled excessive production of free radicals from the mitochondria can overwhelm the natural antioxidant systems of the body causing oxidative damage to important cellular components such as lipids, proteins, and carbohydrates in cell membranes, and nucleic acids [29]. To alleviate this, the cryopreservation medium was supplemented with various antioxidants and proved to enhance the post-thaw sperm fertility. 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 [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) [40, 44]. 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 [31]. The mechanism behind cryodamage may be related to osmotic stress, cold shock, intracellular ice crystal formation, excessive production of ROS [12, 24, 51], alterations in antioxidant defense systems [51], and combinations of these processes. Antioxidants are the main defense factors against oxidative stress induced by ROS [26]. Many investigators have focused on the use of antioxidants in the freezing media to reduce the negative effects of ROS on sperm [2, 4, 10]. Our previous

findings showed that supplementation of dog cryopreservation medium with kinetin [42] and astaxanthin [41] 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 powerful antioxidant, which acts by scavenging ROS and chelating divalent cations [47]. 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 [15]. 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 [46]. In a recent study on post-thaw buck sperm, supplementation of a commercial extender with RSV enhanced their viability [32]. 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 [27]. In buffalo, the addition of RSV to a Tris-citric acid extender ameliorated post-thaw quality parameters, antioxidant enzyme levels, and sperm fertility [1].

By extension, we hypothesized that the antioxidant RSV could be used as a supplement in cryopreservation extender 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.

MATERIALS AND METHODS

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 according procedures reported in our previous study [41].

Preparation of buffers

The sperm washing and dilution system was prepared as previously described [41]. Buffer 1 used for sperm washing was composed of Tris (hydroxymethyl) aminomethane, 198.11 mM; 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).

Dogs and semen collection

Semen samples were collected from four Beagle dogs managed in the University premises, aged from 3–4 years and weighing 8–12 kg. The dog's management followed standard protocols for pets including protection from excessive noise, provision with standard diet and 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). Semen samples with $\geq$70% motility, $\geq$80% viability and a sperm concentration $\geq$100 × 10^6 cells/ml were pooled together and used for further processing. At total of 8 replications for treatment were performed, collecting sperm samples from each male twice a week for one month.

Semen cryopreservation and thawing

Pooled sperm samples from four dogs were adjusted to a concentration of 200 × 10^6 cells/ml with buffer 1. The sperm suspension was then divided and diluted with freezing extender (buffer 2) supplemented with 100, 200, or 400 µM RSV, or no RSV (control). The semen was extended following a multi-step dilution process to a final concentration of 100 × 10^6 cells/ml. The diluted semen was filled in 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 at a level of 2 cm above the surface of liquid nitrogen (LN$_2$) for 15 min. After storage for 1 week in LN$_2$, the frozen semen straws were thawed in a 37°C water bath for 30 sec.

Sperm motility, kinematic parameters, and viability

CASA software imaging system (Sperm Class Analyzer$^®$ CASA system, MICROPTIC, Nikon Ci-L, Tokyo, Japan) was employed to assess the kinematic parameters of post-thawed sperm. In brief, sperm samples (5 µl) were placed on a counting slide for assessment and for each sample, 5 different fields were randomly examined. For each sample at least 200 sperm were tracked for 1 sec 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 analysis of each sample was repeated 12 times for increasing precisions of measurements. Sperm survival was considered on the basis of plasma membrane integrity, assessed using the eosin-nigrosin staining procedure. Briefly, sperm were stained 1:1 with the staining reagent and then smeared on a warm slide and air-dried. At least 200 sperm per slide were examined at 1,000× magnification (Eclipse Ts 2, Nikon) 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.

Integrity of plasma and acrosomal membrane

Plasma membrane integrity was analyzed using the hypo-osmotic swelling (HOS) assay. One drop (~50 µl) of post-thaw semen from each group was mixed with 0.5 ml of HOS solution and incubated at 37°C for 30 min [22]. 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, Tokyo, Japan). The 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 (~50 µl) was smeared on a glass slide, air-dried, fixed with methanol at 20–22°C for 10 min, and stained. After staining, the smears were rinsed with PBS and air-dried. The sperm acrosome was observed using an epifluorescence microscope (1,000× magnification; Eclipse Ts2, 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.

Mitochondrial activity and chromatin 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 [43]. Briefly, 30 µl of R123 solution (5 mg/ml in dimethyl sulfoxide) was diluted with 120 µl of dimethyl sulfoxide and divided into 30 µl aliquots for storage. Post-thaw sperm samples were diluted with buffer 1 to achieve a concentration of 20×10^6 sperm/ml and 3 µl of R123 working solution was added. The semen-stain suspension was incubated for 15 min in the dark at 37°C. After incubation, 10 µl 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 500 g for 5 min, and the sperm pellet was re-suspended in 1 ml of PBS. One drop (~10 µl) of the sperm suspension was placed on a microscopic slide, mounted with a coverslip, and evaluated using an epifluorescence microscope (Eclipse Ts2, 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 [18, 21]. Post-thawed semen (5 µl) 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 (1,000×). Sperm with abnormal chromatin were indicated by blue-stained nuclei, whereas sperm with normal chromatin were indicated by unstained nuclei [6].

Mucus penetration test

Mucus penetration tests were performed using surrogate mucus (modified synthetic oviductal fluid) [34, 39]. The fluid was loaded into flat capillary tubes (80 ± 0.5 mm long, 1.25 ± 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 µl of semen suspension and placed vertically for 2 hr 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.

Gene expression

Only in post-thaw sperm samples of the control and 200 µM RSV, quantitative real-time polymerase chain reaction (RT-qPCR) was used to analyze the expression of genes related to apoptosis such as B-cell lymphoma 2 (*BCL2*) and BCL2-associated X (*BAX*); protamine 2 (*PRM2*) and protamine 3 (*PRM3*), as well as mitochondrial ROS modulator 1 (*ROMO1*), sperm acrosome-associated 3 (*SPACA3*) and oxidative induced DNA damage repair 8-oxoguanine DNA glycosylase 1 (*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 $R=2^{-[\Delta Ct\ sample\ -\Delta Ct\ control]}$ [28].

Statistical analysis

Statistical Package for Social Sciences version 21.0 software (IBM, Armonk, NY, USA) was employed to perform data analysis. The measurements of various parameters were expressed as the mean ± standard error of the mean (SEM), and $P<0.05$ was considered for statistical significance. For analysis of variance, one-way analysis of variance (ANOVA) to compare differences among treatments and control. Tukey's multiple comparison test were used to analyze data related to motion characteristics, membrane integrity, percentages of live sperm and mucus penetration.

The comparison of gene expression was performed using independent sample *t*-test.

RESULTS

Determination of optimum concentration of RSV

The optimum concentration of RSV was selected based on improvement in the post-thaw sperm quality and kinetic parameters of dog sperm supplemented with different concentrations of RSV (Table 2). Sperm samples supplemented with 200 µM RSV showed the highest post-thaw motility (50.4 ± 1.4%) compared to that of other RSV concentrations and the control ($P<0.05$). In addition, the progressive motility (19.8 ± 1.3%), straightness (76.3 ± 1.3%), and linearity (55.9 ± 1.3%) of sperm treated with 200 µM RSV were all significantly higher than that of the other groups ($P<0.05$).

Sperm survival rate

The eosin-nigrosin staining demonstrated that the post-thaw live sperm percentage was significantly increased in the samples supplemented with 200 µM RSV than in the control sample (54.8 ± 1.4% vs. 43.1 ± 2.9%) (Table 3).

Integrity of the plasma membrane and acrosome

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 µM RSV (60.1 ± 1.7% vs. 61.6 ± 2.6%, respectively) (Table 3). The results of FITC-PNA staining illustrated that RSV supplementation was effective in protecting the acrosomal integrity of post-thaw dog sperm. The number of sperm with an intact acrosome was significantly higher in samples supplemented with 200 µM RSV than in the control group ($P<0.05$; Table 3).

Mitochondrial activity and chromatin integrity

Sperm supplemented with 200 µM RSV showed a significant increase in mitochondrial activity compared to that of the control group ($P<0.05$; Table 3). Post-thaw sperm evaluation revealed an increase in the number of sperm with normal chromatin in the sample supplemented with RSV. Aniline blue staining revealed that the percentage of sperm with normal chromatin was significantly higher in sperm samples supplemented with 200 µM RSV than in the control samples (78.1 ± 1.4% vs. 63.1 ± 2.1%, respectively, $P<0.05$; Table 3).

Mucus penetration test

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 µM RSV-supplemented samples than in the control samples (Table 4; $P<0.05$).

Gene expression

We analyzed gene expression levels in post-thaw semen samples supplemented with RSV to reaffirm the post-thaw sperm quality. Sperm samples supplemented with 200 µM RSV showed a significantly enhanced expression level of *BCL2* and reduced expression of *BAX* compared with that of the control group ($P<0.05$; Fig. 1). The expression levels of *PRM2, PRM3*, and *SPACA3* in post-thaw sperm were significantly higher in 200 µM RSV-supplemented semen samples than in the control group ($P<0.05$; Fig. 1). Furthermore, the expression level of *ROMO1* and *OGG1* genes was significantly reduced in 200 µM RSV-supplemented sperm samples compared with that of the control ($P<0.05$; Fig. 1). These results showed an overall improvement in sperm characteristics and a reduction in apoptosis; therefore, one would expect post-thaw quality to be better than that of the control.

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 µM RSV to the Modena extender significantly improved motility and protected the boar sperm against oxidative stress [48]. The mechanism of RSV in reducing inflammation and associated pathologies involves decreasing interleukin-1β secretion and gene expression for cell apoptosis; this leads to a strong reduction in the activity of the inflammasome pathway subsequently leading to apoptosis [11]. In addition, the anti-inflammatory potential of RSV has been demonstrated by counteracting an inflammatory challenge in U-937 macrophages at representative plasma concentrations [50]. On these bases, reduction of inflammasome pathway by RSV might be the mechanism to improve the motility and survival rate of post-thaw dog sperm. We also anticipate RSV could be playing role in reducing the apoptosis process triggered by sperm cryopreservation.

The optimum concentration of RSV supplementation in dog sperm was 200 µM, higher than the optimum concentration of 50 µM in boar [48] and 40 µM in roosters [35]. The variation could be due to species differences, type of medium and buffer used, and emphasizes the need to optimize conditions when using RSV or other antioxidants in cryopreservation. Similar to our findings, the relatively high level of mitochondrial activity and integrity of the acrosome and chromatin in RSV observed in boar semen [19]. Moderate doses of RSV induced mitochondrial biogenesis and protected against a 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 [13]. Similarly, the effects of RSV supplementation were observed on low quality stallion semen, in which it can ameliorate mitochondrial activity and prevent DNA damage [36]. The maintenance of overall cellular integrity and higher mitochondrial activity of RSV-supplemented sperm supports better fertility potential as compared to control samples.

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

Gene	Primer sequence (5′–3′)	Product size (bp)	NCBI accession No.
β-actin	F: GAGGCATCCTGACTCTGA R: TCTGGCACCACACTTTCT	87	XM_544346.3
BAX	F: CCAAGAAGCTGAGCGAATG R: CTGCCACTCGGAAGAAGAC	123	NM_001003011.1
BCL2	F: GACAGAGAGGATCATGCTGT R: TGGCATGAGATGCAGGAAAT	141	NM_001002949.1
PRM2	F: CTCCAGAAGGGTCAGGAG R: GGCTCCTTGCAAACTCAG	169	NM_001287148.1
PRM3	F: TCTGGAGAGGCAGCCAGA R: AGGCCATGAGCTTCTTCA	101	XM_022420065.1
ROMO1	F: CTACGTGCTCCCGGAAGT R: TCGCTCAGTTCTACGTCTCAC	100	XM_534406.6
SPACA3	F: AACACAGCTGCTGTGGAC R: ACCACTTCCGGCTGTTGA	76	NM_001197087.1
OGG1	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*, 8-oxoguanine DNA glycosylase 1.

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

Groups	Motility (%)	Progress motility (%)	VCL (μm/sec)	VAP (μm/sec)	VSL (μm/sec)	Straightness (%)	Linearity (%)	ALH (μm)
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 μM 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 μM 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 μM 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]

Curvilinear velocity (VCL), average path velocity (VAP), straight-line velocity (VSL), amplitude of lateral head displacement (ALH). [a–c] Values with different lowercase superscripts letters in a column differ significantly ($P<0.05$, n=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 μM 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 μM 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 μM 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).

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 μM RSV	62.0 ± 1.5 [ab]	22.0 ± 1.3 [ab]
200 μM RSV	67.1 ± 1.2 [a]	31.5 ± 1.0 [a]
400 μM 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).
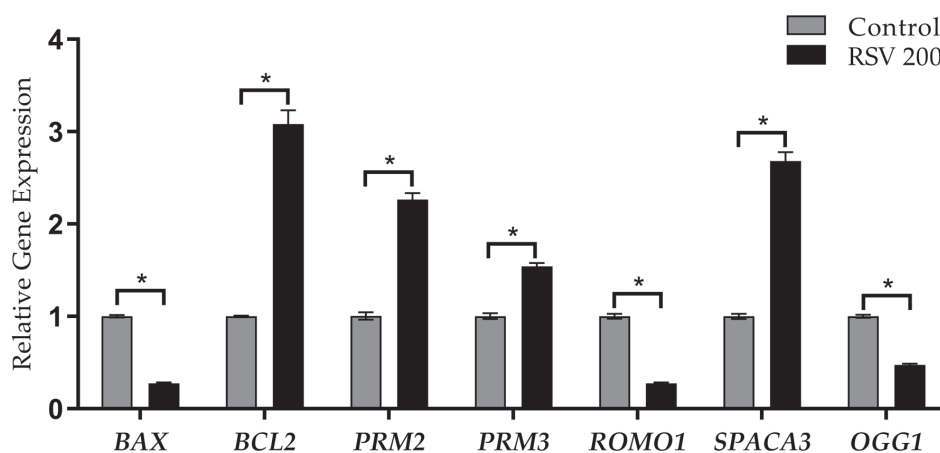
Fig. 1. Gene expression levels of pro-apoptotic BCL2-associated X (*BAX*), anti-apoptotic B-cell lymphoma (*BCL2*), protamine 2 (*PRM2*), protamine 3 (*PRM3*), the mitochondrial reactive oxygen species modulator 1 (*ROMO1*), sperm acrosome-associated *3* (*SPACA3*) and 8-oxoguanine DNA glycosylase 1 (*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 ± SEM. *Indicates significant differences ($P<0.05$).

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 after vitrification of mouse embryos [17]. In mouse oocyte cryopreservation, 25 µM RSV reduced the oxidative stress of vitrified oocytes by decreasing the ROS levels and increasing the glutathione levels. RSV also alleviated the abnormal mitochondrial distribution pattern in oocytes after vitrification [37], which is similar to the function of RSV in sperm cryopreservation. These findings suggest that RSV can be supplemented as cryoprotective extender in both oocytes/embryos and sperm to improve quality. In the current study, RSV has showed significant effect though lipid-solubility limiting its bioavailability. However, the development of bio-carriers for intracellular transportation of RSV like apolipoprotein E3 [25] is believed to make the effects of RSV even better and could be the best antioxidant. This mechanism of action of RSV is believed to limit to early hyperactivation of sperms and contribute positively in maintaining the fertility potential of sperm as it is needed later for the fusing stage with ovum [14].

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 [19] and rats [16]. This suggests that RSV improves the survival rate of sperm by reducing the stress-induced apoptotic pathways of cell death. The better survival rate of sperm in samples supplemented with 200 µM RSV in current study is a promising effect to enhance fertility in similar as to that observed in post-thaw sperm of buffalo where RSV prevented capacitation like changes and improve *in vitro* fertilizing capacity [30]. The chromatin integrity is essential for attaining the fertility of sperm and RSV supplementation has showed better chromatin integrity which is also observed in increased gene expression of *PRM2* and *PRM3*. This finding is in line with effect of RSV supplementation observed in human sperm [3]; this was likely due to the activation of AMP-activated protein kinase [45]. The reduced expression of *BAX* in RSV-supplemented dog sperm was also observed in RSV-supplemented rats exposed to *Cadmium bromide*. The reduced expression of *OGG1* in RSV supplemented sperm is an indication of lower DNA damage, which is also reported to be important to the subsequent success in embryo development and outcome of reproduction [52]. This indicates that RSV protects sperm by reducing the 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 *in vitro* fertility, and it can be considered as an alternative antioxidant.

RSV should be considered one of the best antioxidant supplements for dog sperm cryopreservation to preserve quality; we recommend an optimal concentration of 200 µM 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 though appropriate precautions should be taken as high doses could lead to toxic effects. Finally, we recommend that species- and cryopreservation condition-specific studies and verification be conducted before using an antioxidant supplement on a broader scale.

CONFLICT OF INTEREST. The authors declare no conflict of interest.

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