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Improving the quality of cryopreserved goat semen with a commercial bull extender supplemented with resveratrol*

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ABSTRACT

The purpose of the current study was to evaluate the effects of resveratrol (RSV) on the quality of frozen-thawed goat sperm. Semen samples from four bucks were divided into five aliquots and diluted with a commercial bull semen extender containing: no antioxidant (RSV-0, control), 10 μM RSV (RSV-10), 50 μM RSV (RSV-50), 100 μM RSV (RSV-100) and 250 μM RSV (RSV-250). After thawing, sperm motility, abnormal morphology, membrane and acrosome integrity, mitochondrial activity, phosphatidylserine (PS) distribution, and oxidative stress were evaluated. The results indicated that in comparison with the control, when the concentration of RSV was 10 or 50 μM, the total motility, progressive motility, membrane and acrosome integrity, and mitochondrial activity of post-thaw spermatozoa was greater ($P < 0.05$). Additionally, the use of extenders containing RSV-10 or RSV-50 resulted in a greater percentage of viable spermatozoa as compared to the other groups ($P < 0.05$). Importantly, there were more viable spermatozoa (49.61 ± 0.61%) and less non-viable spermatozoa (49.16 ± 1.01%) in the RSV-50 group compared to the other extenders ($P < 0.05$). Furthermore, the use of the extenders containing RSV-10 and -50 resulted in a reduction in ROS production in frozen-thawed spermatozoa as compared to the control ($P < 0.05$). There, however, was no difference among extenders for abnormal morphology and PS distribution. In conclusion, supplementation with RSV, at a concentration of 10 or 50 μM in the semen extender, can improve the post-thaw goat sperm quality, which may occur as a consequence of inhibition of ROS generation.

1. Introduction

The cryopreservation process can induce oxidative stress in mammalian spermatozoa. Oxidative stress decreases sperm motility and induces DNA damages which are underlying causes of the subsequent reduction of sperm fertilizing capacity (Alvarez and Storey, 1989; Bell et al., 1993; Aitken et al., 1998). Furthermore, sperm membranes have a relatively greater concentration of polyunsaturated fatty acids than most animal cells which make spermatozoa susceptible to oxidative stress caused by the freeze-thawing procedure (White, 1993; Aitken and Fisher, 1994; Sikka, 1996; Gandini et al., 2000; Moussa et al., 2002; Bucak et al., 2010). Furthermore, the process of semen cryopreservation results in production of large amounts of reactive oxygen species (ROS) (Chatterjee and Gagnon, 2001; Kim et al., 2010). The concentration of ROS in semen is determined by a balance between the cellular

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production of these molecules and the catabolism of antioxidative compounds, because seminal plasma contains different substances including the antioxidant molecules, membrane stabilizers and sugars (Maxwell and Johnson, 1999; Chen et al., 2002). During the dilution and cooling process, however, the antioxidant capacity of semen is markedly reduced. Thus, mammalian spermatozoa are deficient of adequate reserves of natural antioxidants that function to reduce the detrimental effects of ROS and prevent lipid peroxidation (LPO) during the cryopreservation process (Aurich et al., 1997; Storey, 1997). The addition of some external antioxidants to freezing extenders, therefore, may be an alternative practice to maintain values for spermatozoa variables that are involved in inhibition of oxidation after the freeze/thaw process (Naijian et al., 2013). Addition of antioxidants to the extenders leads to an enhancement in values for the post-thaw sperm quality variables in bull (Sariözkan et al., 2009), stallion (Nouri et al., 2018), red deer stag (Sánchez-Rubio et al., 2018), dog (Andersen et al., 2018), ram (Aisen et al., 2002), buck goat (Atessahin et al., 2008; Reddy et al., 2018) and boar (Gadea et al., 2004; Estrada et al., 2014) semen.

Among these compounds, resveratrol (3, 5, 4′-trihydroxystilbene) (RSV), a nonflavonoid polyphenol found mainly in grapes (Latruffe and Rifler, 2013), has an important function as an antioxidant. In some studies, RSV functions as an effective scavenger of free radicals including superoxide anion, hydroxyl radical, and metal-induced radicals (Leonard et al., 2003; Willcox et al., 2004). The RSV compound has many biological activities, such as anti-inflammation, cardioprotection, chemoprevention, and anti-apoptotic properties (Saiko et al., 2008; Longobardi et al., 2017). The RSV compound also has an important protective effect against lipid peroxidation (LPO) and DNA damage caused by free radicals in spermatozoa (Branco et al., 2010; Collodel et al., 2011). Previously, *in vivo* as well as *in vitro* studies have been conducted with results indicating RSV may improve sperm quality during cryopreservation (Garcez et al., 2010; Najafi et al., 2018). At the same time, results of pivotal studies have emphasized the potential effect of RSV as a suitable antioxidant supplement to semen extenders for ram, human, mouse, bull and boar semen (Sarlós et al., 2002; Collodel et al., 2011; Mojica-Villegas et al., 2014; Bucak et al., 2015; Bucci et al., 2018). There, however, is a lack of information regarding the effect of RSV supplementation in the extenders on goat semen during cryopreservation.

The purpose of the present study, therefore, was to evaluate the effects of adding RSV to the extender before freezing on the motility characteristics, morphology, membrane integrity, acrosome integrity, mitochondrial activity, PS distribution, and ROS production of cryopreserved goat spermatozoa, to explore whether RSV supplementation of semen extenders can improve the quality of frozen-thawed goat semen.

2. Materials and methods

2.1. Ethics statement

All experiments in the present study were approved by the ethical committee of Yunnan Animal Science and Veterinary Institute (Kunming city, Yunnan province, China).

2.2. Chemicals and reagents

All chemicals, reagents, or kits were purchased from Sigma Chemical Co. (St. Louis, Mo, USA) unless otherwise stated. All fluorochrome solutions were kept in the darkness at −20 °C.

2.3. Animals and management

Four goats (2–3 years old) were used for collection of semen. Anthelmintic treatments and vaccination against rabies and tetanus were performed routinely. The goats were managed with there being uniform feeding, housing, and light conditions. The feed was composed of corn (29.5%), soybean meal (23%), calcium monophosphate (1.5%), premix (1%), NaCL (0.5%), sodium bicarbonate (0.5%), broad bean bran (19%), alfalfa Grass (10%), and corn silage (15%). The goats had free access to water and salt.

2.4. Semen collection, dilution, freezing and thawing

Semen was collected twice per week per goat using an artificial vagina. A total of 50 ejaculates were collected from four bucks during the study. The volume of semen was quantified and color was observed. Mass motility was estimated by assessment of wave motion of fresh undiluted semen at 10× magnification (0–5 scale) (Domínguez et al., 2008). Spermatozoa concentration was determined using the NucleoCounter® SP-100™ (ChemoMetic AS, Allerød, Denmark). After initial evaluation, the ejaculates were used for the experiments if the following standards were satisfied: mass motility: ≥3.5; progressive motility: ≥80%; spermatozoa concentration: ≥2500 × 10^6 spermatozoa/mL; normal spermatozoa morphology: ≥80%. The pooled semen was divided into five equal aliquots and diluted in Optidyl® (Biovet, France) containing 0 μM RSV (RSV-0), 10 μM RSV (RSV-10), 50 μM RSV (RSV-50 μM), 100 μM RSV (RSV-100), and 250 μM RSV (RSV-250) to a final concentration of 0.2 × 10^9 spermatozoa/mL. Semen was cooled to 5 °C in a refrigerated room. After 4 h of equilibration, all straws were placed horizontally on a wire plate 4 cm above the nitrogen surface and frozen for 10 min, subsequently plunged into liquid nitrogen until thawing. After 7 days of storage in liquid nitrogen, samples were thawed in water at 37 °C for 30 s.

2.5. Semen evaluation

2.5.1. Sperm motility

The motility of post-thawed spermatozoa was analyzed using a computer-assisted semen analysis (CASA) system installed with the Sperm Class Analyzer (SCA) software (Version 5.1; Microptic, Barcelona, Spain), with a setting of 25 frames acquired to avoid sperm track overlapping, minimum contrast 10, minimum velocity of average path 30 μm/s, progressive motility greater than 80% straightness. This system has a specific calibration for goat sperm evaluation. In particular, calibrations were as follows: phase contrast; frame rate, 60 Hz; minimum contrast, 70; low and high static size gates, 0.6–4.32; low and high intensity gates, 0.20–1.92; low and high elongation gates, 7–91; default cell size, 10 pixels; default cell intensity, 80.

All spermatozoa samples were diluted using PBS to a concentration 20×10^6 spermatozoa/mL and 10 μl drop of spermatozoa suspension was placed on a slide and covered with a cover-slip (18 × 18 mm). A previously heated (37 °C) Makler chamber was placed on the phase-contrast microscope (Nikon, ECLIPSE E200, Japan) with magnification of 100X and the following motility values were recorded: total motility (TM, %) and progressive motility (PM, %). For each sample, more than three fields per drop were analyzed and a minimum of 500 spermatozoa were evaluated.

2.5.2. Sperm abnormal morphology

For the assessment of spermatozoa with abnormal morphology, at least 15 μL of each semen sample was added to tubes containing 1000 μL of Hancock solution (HS) prepared by mixing formalin (62.5 mL), sodium saline solution (150 mL), buffer solution (150 mL), and double-distilled water (500 mL) (Schäfer and Holzmann, 2000). These solutions were prepared as follows: sodium saline solution: 9.01 g NaCl in 500 mL of double-distilled water, buffer solution: (1) 21.7 g Na_2HPO_4 H_2O in 500 mL of double-distilled water, (2) 22.254 g KH_2PO_4 in 500 mL of double-distilled water, 100 mL of (1) and 50 mL of (2) were mixed to obtain 150 mL of buffer solution. To detect abnormalities of the sperm acrosome, head and tail, 10 μl of processed sperm was placed on a slide.

The percentages of spermatozoa with abnormal morphology were recorded by assessing a total of 200 spermatozoa using a phase-contrast microscope at 1000X (Nikon, ECLIPSE E200, Japan).

2.5.3. Sperm membrane integrity assessed by hypo-osmotic swelling test (HOST)

The hypo-osmotic swelling test (HOST) was conducted to assess the functional integrity of sperm plasma membrane using the procedure described previously (Revell and Mrode, 1994). For this, 20 μl of semen sample was diluted in 200 μl of the HOST medium (0.367 g of sodium citrate and 0.675 g of fructose were dissolved separately in 50 ml of distilled water. The final HOST medium was prepared by mixing equal volumes of sodium citrate and fructose solution), followed by its incubation for 60 min at 37 °C. The osmolality of the final HOST medium (100 mOsm) was measured by the FISKE ONE-TEN Osmometer (FISKE Associates). After incubation, 20 μl of incubated sample was placed on a clean grease free slide and a cover slip was applied and there was immediate examination at 400x magnification for calculation of HOST reacted spermatozoa. Spermatozoa with visible coiling of tail were considered as HOST reacted spermatozoa. A total of 200 spermatozoa were counted to determine percentage of HOST reacted spermatozoa

2.5.4. Flow cytometry

Flow cytometry analyses were conducted using a FacStar-plus flow cytometer (FAC- SCalibur, Becton Dickinson and Co., Franklin Lakes, NJ, USA). The excitation wavelength was 488 nm. The emission wavelength was 530 nm.

The FITC-PSA, Annexin-V-FITC, Rhodamine – 123, and 2,7- dichlorodihydrofluorescein diacetate (H_2DCFDA) fluorescence were detected using the detector FL1, and PI fluorescence was detected using the detector FL2. The examples of the density plots are included in Fig. 1. Data were collected from 10,000 events for further analysis using the Cell-Quest software (Becton Dickinson).

2.5.4.1. Acrosome integrity assessment. The acrosome status of goat spermatozoa was assessed using fluorescein isothiocyanate—labeled lectin from Pisum sativum (FITC-PSA) (Freitas et al., 2012). Briefly, 200 μL semen sample diluted in TALP to a final concentration of 10×10^6 spermatozoa/mL was mixed with 50 μL propidium iodide (PI) (50 μg/mL) and 0.5 μL FITC-PSA (2 mg/mL). The mixed suspensions were incubated in a darkened area and in a moist chamber for 15 min at 37 °C. The percentage of spermatozoa stained with FITC-PSA and PI was analyzed using flow cytometry. The percentage of viable spermatozoa with intact acrosome was detected in the FITC-PSA^-PI^- quadrant.

2.5.4.2. Mitochondrial activity. The assessment of mitochondrial activity was performed using the Rhodamine-123 kit (R123; KeyGEN Biology, Nanjing, Jiangsu province, China) and PI as described by Najafi et al. (2013). Briefly, 10 μL of R123 solution (0.01 mg/mL distilled water) was added to 500 μL of diluted semen samples (50×10^6 spermatozoa/mL) and incubated for 30 min at the room temperature (RT, 25 °C) in a darkened area. Samples were subsequently centrifuged at $500 \times g$ for 3 min at room temperature to remove the supernatant. The spermatozoa pellets were then re-suspended in 500 μL the assays buffer included in the kit. There was subsequent addition of 10 μL PI to the samples before the flow cytometric analysis was conducted. The percentages of viable spermatozoa with active and functional mitochondria were detected in the $R123^+$ PI^- quadrant.

2.5.4.3. Phosphatidylserine (PS) translocation. The PS translocation detection Commercial Kit (KeyGEN Biology, Nanjing, Jiangsu province, China) was used for determination of viable, apoptotic, and non-viable spermatozoa according to the manufacturer's instructions. Normally, PS is located at the inner side of plasma membrane. However, PS can transfer to the outside of plasma

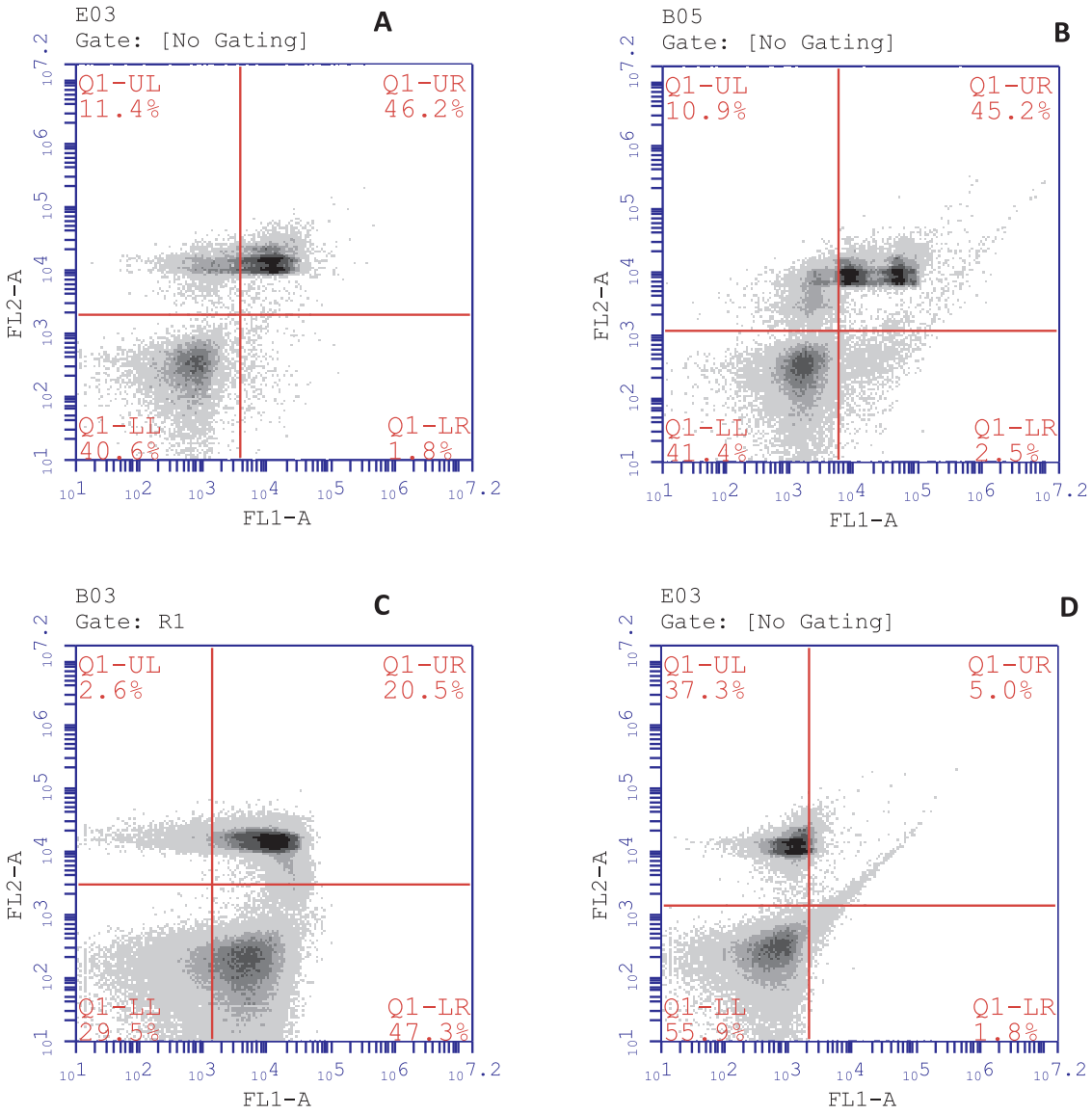

Fig. 1. Flow cytometry density plots of post-thaw spermatozoa stained with Annexin-V-TITC, FITC-PSA, Rhodamine-123, or H_2DCFDA and PI, as analyzed with flow cytometry; (A) viable spermatozoa were located in the Q1-LL field and not stained with Annexin-V and PI; (B) viable spermatozoa were located in the Q1-LL field and not stained with PSA and PI; (C) viable spermatozoa were located in Q1-LR and stained with Rhodamine-123 but not PI; (D) viable spermatozoa were located in the Q1-LL field and not stained with PI and H_2DCFDA.

membrane in some stressful environments. In general, PS exposure is thought as one of typical apoptotic features. Briefly, spermatozoa were washed twice in PBS and adjusted the concentration to 1.0×10^6 spermatozoa/mL in the binding buffer. Then, 10 µL of Annexin V-FITC was added to 100 µL spermatozoa suspension and incubated for 20 min at the room temperature. After that, 5 µL of PI were added and then incubated for 15 min at room temperature in a darkened area. The samples were subsequently analyzed by flow cytometry. The spermatozoa were classified into three subpopulations as follows: (1) viable non-apoptotic cells, negative for Annexin-V and excluding PI (A^-/PI^-); (2) cells presenting signs of early apoptosis, binding Annexin-V but still excluding PI (A^+/PI^-); (3) and non-viable spermatozoa, stained with PI (PI^+).

2.5.4.4. Evaluation of oxidative stress by the production of reactive oxygen species. The concentrations of reactive oxygen species (ROS) were measured by incubating spermatozoa in the presence of 2′, 7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) using procedures that have been previously described (Guthrie and Welch, 2006). Briefly, 500 µL semen sample diluted in the TALP buffer to a final concentration of 1×10^6 spermatozoa/mL was mixed with 0.5 µL H_2DCFDA (final concentration of 20 µM) and 50 µL PI (50 µg/mL). Then, the samples were incubated for 60 min at room temperature in darkness.

The spermatozoa suspension was analyzed using flow cytometry. Percentage of viable spermatozoa with low ROS was identified

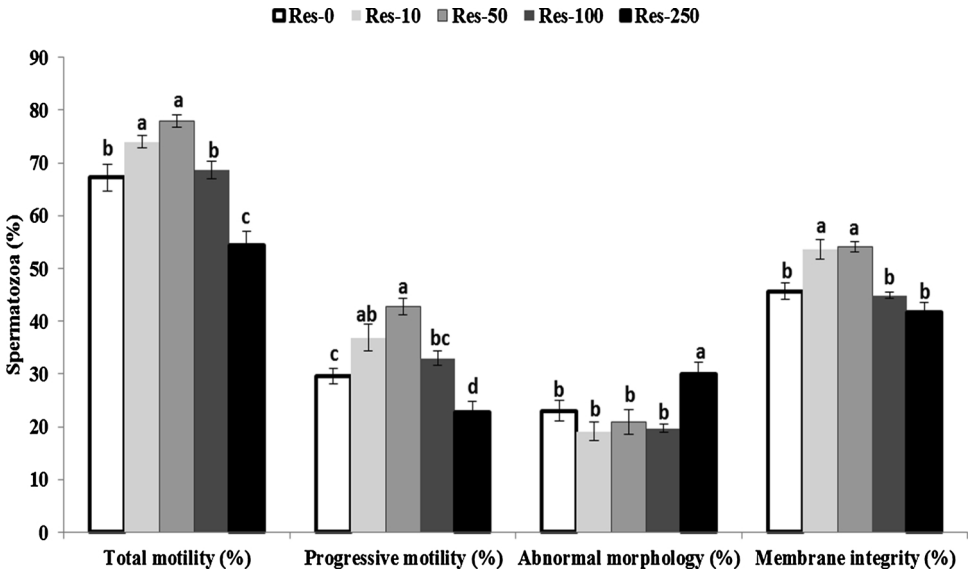

Fig. 2. Effects of resveratrol on total, progressive motility, abnormal morphology, and membrane integrity of goat semen cryopreserved in the Optidyl® extender; Data are presented as mean ± SEM; Different letters indicate differences ($P < 0.05$) in values for sperm variables among the extenders assessed in this study.

by H_2DCFDA negative and no PI staining.

2.6. Statistical analysis

Data were analyzed using the JMP10.0 software (SAS Institute Inc., Cory, NC, USA). Data normality and homogeneity of variances were verified using the Shapiro–Wilk normality tests and Levene's tests, respectively. Dependent variables with normal distribution were evaluated using analysis of variance with comparison of means using Tukey's test and t-student. Results were expressed as mean ± SEM. Differences with a value of $P < 0.05$ considered to be statistically significant.

3. Results

3.1. Effects of resveratrol on spermatozoa motility variables, morphology and membrane integrity

The data for results of total and progressive motility after the sperm cryopreservation in different concentrations of RSV are depicted in Fig. 2. The total and progressive motility in the RSV-10 μM and 50 μM groups were greater ($P < 0.05$) than that in the control, RSV-100, and RSV-250 groups. There was no difference in the percentage of TM and PM between the control and the RSV-100 groups ($P > 0.05$). There were the least rates for TM and PM in the RSV-250 when compared with the control and other groups ($P < 0.05$).

Additionally, as indicated by the depictions in Fig. 2, in terms of abnormal morphology, there was no difference in values for this variable with use of all extenders except with use of RSV at 250 μM there was a greater percentage of spermatozoa with abnormal morphology.

In terms of plasma membrane integrity, when the concentration of RSV in the extender was 10 or 50 μM, the post-thaw percentage of goat spermatozoa with an intact membrane was greater as compared to the control group (Fig.2). There, however, was no difference in values for this variable among the control, RSV-100, and RSV-250 groups ($P > 0.05$).

Table 1
Effects of resveratrol on acrosome integrity and mitochondrial activity of frozen goat spermatozoa.

Variable (unit)	Extender				
	Res-0	Res-10	Res-50	Res-100	Res-250
Acrosome integrity (%)	41.97 ± 2.73^{b}	51.35 ± 0.90^{a}	48.37 ± 2.15^{a}	46.87 ± 2.76^{ab}	41.35 ± 1.31^{b}
Mitochondria activity (%)	47.37 ± 0.80^{bc}	52.05 ± 1.97^{a}	52.27 ± 0.78^{a}	50.25 ± 0.30^{ab}	45.40 ± 1.41^{c}

Note: Acrosome integrity (FITC-PSA) and mitochondrial activity (%, $R123^{+}/PI^{-}$) variables were analyzed; Data are presented as mean ± SEM; Different superscripts within the same row indicate differences among groups ($P < 0.05$).

Table 2
Effects of resveratrol on Phosphatidylserine (PS) translocation distribution of goat spermatozoa during the cryopreservation process.

Sperm subpopulation	Extender				
	Res-0	Res-10	Res-50	Res-100	Res-250
Viable (%)	45.13 ± 0.87^{c}	46.36 ± 0.79^{b}	49.67 ± 0.61^{a}	41.9 ± 1.09^{c}	37.86 ± 1.58^{d}
Early Apoptotic (%)	3.20 ± 0.55^{a}	2.26 ± 0.17^{a}	2.43 ± 0.37^{a}	2.60 ± 0.38^{a}	2.20 ± 0.47^{a}
Non-viable (%)	54.66 ± 1.41^{ab}	51.36 ± 0.97^{bc}	49.16 ± 1.01^{c}	55.50 ± 1.00^{ab}	58.63 ± 2.85^{a}

Note: Viable (%, AnnexinV^{-}/PI^{-}), early apoptotic (%, AnnexinV^{+}/PI^{-}), and non-viable (%, PI^{+}) variables were analyzed; Data are presented as mean ± SEM; Different superscripts within the same row indicate differences among groups ($P < 0.05$).

3.2. Effect of resveratrol on acrosome integrity, mitochondrial activity, PS distribution, and ROS production

As per data included in Tables 1 and 2 and as depicted in Fig. 3, there were differences in values for variables with use of all extenders in terms of acrosome integrity, mitochondrial activity, apoptotic and oxidative stress. Results indicate that the percent acrosome integrity was considerably greater in the groups treated with RSV-10 (51.35 ± 0.90%) and RSV-50 (48.37 ± 2.15%) than the control group (41.97 ± 2.73%) ($P < 0.05$). Additionally, there was no difference in acrosome integrity between the RSV-250 and control group (Table 1).

The data for results related to mitochondrial activity, as evaluated using Rhodamine, are presented in the Table 1. The data indicate the percentage of viable spermatozoa with relatively greater mitochondrial activity was greater in the RSV-10 (52.05 ± 1.97%) or RSV-50 (52.27 ± 0.78%) group than the control group (47.37 ± 0.80%) ($P < 0.05$). There was no difference between the control (47.37 ± 0.80%) and RSV-100 group (50.25 ± 0.30%) ($P > 0.05$).

Regarding the percentage of viable, apoptotic and non-viable spermatozoa based on the phosphatidylserine translocation assay (Table 2), the results indicated the supplementation of RSV at a concentration of 50 μM resulted in the greatest percentage of viable spermatozoa among all tested groups. Furthermore, data indicated that the RSV-50 treated group had a lesser value for non-viable spermatozoa (49.16 ± 1.01%) as compared to when there was use of the other extenders ($P < 0.05$) (Table 2). There was a lesser value for viable spermatozoa when there was supplementation with RSV-250. In terms of early apoptotic spermatozoa with exposed PS molecules, there was no difference in values for this sperm variable with use of different extenders.

The data for evaluation oxidative stress using H_2DCFDA are depicted in the Fig. 3. The results indicate that when the concentration of RSV was 10 or 50 μM, the percentage of viable spermatozoa with less ROS production was greater as compared to the control group ($P < 0.05$). Additionally, there was no difference in values that are indicative of oxidative stress among the RSV-100, RSV-250, and control groups.

4. Discussion

During cryopreservation, there imposing of various types of stress on spermatozoa such as cold shock, osmotic, and ice crystal formation (Hammerstedt et al., 1990; Olaciregui et al., 2014). All these factors induce physical and chemical damage to spermatozoa, resulting in changes in values for various sperm variables (Hsieh et al., 2006; Agarwal et al., 2013; Gadea et al., 2013). Additionally, spermatozoa also undergo marked oxidative stress caused by the cryopreservation, due to the fact that the current commercially utilized freeze-thaw process is performed in a nearly open environment. As a result of these problems, using extracellular antioxidants for protection of mammalian spermatozoa against oxidative damage during the cryopreservation process became necessary (Jeong et al., 2009).

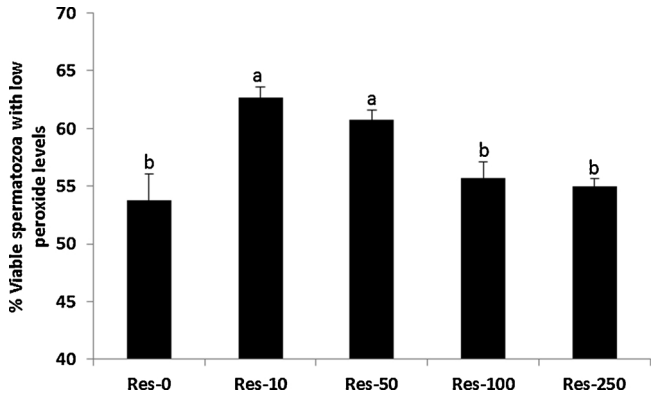

Fig. 3. Effect of different concentrations of RSV on oxidative stress using H_2DCFDA of post-thawed goat semen Data are presented as mean ± SEM; Different letters indicate differences among groups ($P < 0.05$).

One feature of the present study is that the effects of supplementation of a semen extender with RSV on the quality of frozen-thawed goat sperm are assessed by using a conventional freezing procedure. It is well known that the primary advantage for use of programmable freezers for semen cryopreservation is that the freezing curve can be customized, which may result in enhanced post-thaw sperm survival as compared to the conventional method where there is use of liquid nitrogen vapor to pre-freeze sperm. With use of this method, however, there is need for availability of a piece of equipment for which there needs to be considerable expertise to operate. Furthermore, the use of liquid nitrogen vapor to pre-freeze spermatozoa before storage in liquid nitrogen has been adopted in many studies (Purdy, 2006). Another feature is the addition of RSV to the commercial Optidyl® extender as an antioxidant for cryopreservation of frozen goat spermatozoa. Results of the present study clearly indicate cryopreservation in the Optidyl® extender supplemented with RSV-10 or RSV-50 results in greater protection of spermatozoa quality as compared to the control. The exact cryoprotective mechanism of RSV, however, is not completely elucidated. Additionally, the results of the present study indicate the greater amounts of RSV supplementation used does not inhibit the production of ROS and furthermore does not mitigate the cryodamage to goat sperm. To the best of our knowledge, there is only one published preliminary study for which there has been reporting of the evaluation of the effects of RSV on frozen goat spermatozoa (Silva et al., 2016). In the previous study, the relatively greater supplementations of RSV resulted in a reduction in the values for oscillation variables of frozen-thawed spermatozoa (Silva et al., 2016), indicating that greater than optimal concentrations of RSV in semen extenders for goats may negatively affect the pattern of motility of frozen-thawed spermatozoa.

Sperm motility is closely related to reproductive efficiency. Furthermore, motility of frozen-thawed spermatozoa may be a reliable indicator of successful cryopreservation and fertilizing capacity of spermatozoa (Mortimer, 2000; Del Olmo et al., 2013). Furthermore, the plasma membrane and acrosome integrity are important variables that are associated with the fertilizing capacity of spermatozoa (Silva and Gadella, 2006). Antioxidants in extenders have the capacity to protect spermatozoa and contribute to maintenance of sperm motility, membrane integrity, acrosome integrity, and fertilization capacity (Sariözkan et al., 2009; Bucak et al., 2010; Seifi-Jamadi et al., 2016). Results of another study indicated that the beneficial effects of RSV on spermatozoa motility may be explained by the effect of this molecule in decreasing ROS generation (Bucak et al., 2015). In this previous study, addition of RSV led to improvement in values for spermatozoa motility variables. Results of the present study were inconsistent with those of previous studies with spermatozoa of men (Garcez et al., 2010), boars (Martín-Hidalgo et al., 2013; Bucci et al., 2018; Gadani et al., 2017) and rams (Silva et al., 2012). Use of relatively greater concentrations of antioxidants may lead to a decrease in the functional integrity of the spermatozoa acrosome and membrane (Atessahin et al., 2008; Zhang et al., 2015). In the present study, the optimum functional RSV concentration was 10 or 50 μM which was different from the results of previous studies with stallion (Nouri et al., 2018) and bull (Bucak et al., 2015; Tvrdá et al., 2015) semen and the same as that when there was assessments with buffalo spermatozoa (Longobardi et al., 2017). This difference in results among these studies may have resulted from the nature of extender, animal breed, and cryopreservation protocols. In addition, it can be hypothesized that antioxidants function to maintain post-thawed spermatozoa membrane stability when there is oxidative stress. These observations together with the current findings raise the possibility that the inclusion of RSV in extenders may enhance goat spermatozoa motility, membrane integrity and acrosome integrity after thawing.

Mitochondria are the main organelle of ROS production in spermatozoa. It is well known that mitochondria are responsible for the synthesis of ATP. So, damage to this organelle leads to a disruption of ATP synthesis (Ravagnan et al., 2002). Mitochondria have a pivotal function in maintaining normal sperm function and energy homeostasis as a result of oxidative phosphorylation and ATP synthase activation, which is essential for spermatozoa motility acquisition (Ruiz-Pesini et al., 2007; Amaral et al., 2013). Loss of energy sources of spermatozoa results in an ATP deficiency, leading to lesser spermatozoa motility following the freezing-thawing processes (Medeiros et al., 2002). In the present study, there were a relatively greater percentage of viable spermatozoa with optimal mitochondrial activity with use of extenders supplemented with RSV-10 or RSV-50. At the same time, the spermatozoa of these two groups also had a superior TM and PM as compared to spermatozoa in the other groups. There is a potential relationship between spermatozoa motility and mitochondrial activity (Guthrie et al., 2008; Emamverdi et al., 2013). Similarly, Shabani Nashtaei et al. (2017) and Najafi et al. (2018) reported that supplementation with RSV led to improvements in mitochondrial activity of frozen-thawed spermatozoa. In the present study, results further confirmed this previous finding that RSV has cryoprotective effects on mitochondria activity, leading to a greater spermatozoa motility after cryopreservation.

Apoptotic-like changes represent a potential mechanism involved in the induction of spermatozoa DNA damage during cryopreservation (Said et al., 2010). Phosphatidylserines are translocated to the outside of spermatozoa plasma membrane during apoptosis. In previous studies, there was use of the Annexin-V assay to determine cell apoptosis (Martínez-Pastor et al., 2009). Results of the present study indicated that RSV supplementation at 10 μM and at the optimal concentration of 50 μM could be used to increase the percentage of viable spermatozoa and decrease the number of non-viable spermatozoa. Similar to the results of the present study, there are previous reports of detrimental effects of antioxidants when these were used at greater than optimal doses for spermatozoa preservation, whereas the spermatozoa quality can be improved when there are optimal concentrations of antioxidants included in semen extenders (Fernández-Santos et al., 2007). Furthermore, oxidative stress leads to phosphatidylserine translocations in human spermatozoa (Moustafa et al., 2004; Kotwicka et al., 2011), which can explain the improvement in number of viable spermatozoa with use of 10 and 50 μM to the semen extender in the present study as a result of the supplementation with these doses leading to there being lesser ROS production.

Regarding oxidative stress, the results of the present study indicate that the RSV-10 and RSV-50 treatments protected spermatozoa against oxidative stress by increasing the numbers of viable spermatozoa probably because of lesser peroxide concentrations. This result likely occurred because of the antioxidant property of RSV as a ROS scavenger (Juan et al., 2005). Furthermore, RSV has important functions in ROS absorption and neutralization due to the oxidation-reduction properties of this compound (de la Lastra

and Villegas, 2007).

In conclusion, the results of the present study provide evidence that RSV has the capacity to maintain goat sperm quality during the cryopreservation process. Additionally, RSV concentrations at 10 and 50 μM were particularly effective in protecting spermatozoa against the oxidative damage caused by cryopreservation through inhibiting ROS generation. When there are greater than optimal concentrations of RSV supplemented, however, the efficacious effects of RSV as an antioxidant are not present. Effects of RSV, therefore, may be dose-dependent. Further studies, such as *in vitro* fertilization or artificial insemination should be conducted to confirm the effects of RSV on the fertilizing capacity of goat spermatozoa.

Declaration of Competing Interest

None.

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