

***Corresponding author:**

Jongki Cho
College of Veterinary Medicine, Chungnam
National University, 99 Daehak-ro, Yuseong-
gu, Daejeon 34134, Korea
Tel: +82-42-821-6788
E-mail: cjki@cnu.ac.kr

ORCID:
<https://orcid.org/0000-0002-8431-0457>

Conflict of interest:
The authors declare no conflict of interest.

Received: July 8, 2021
Revised: August 23, 2021
Accepted: August 31, 2021

Supplementation of cryoprotective extender with resveratrol decreases apoptosis index and reactive oxygen species levels in post-thaw dog sperm

Seonggyu Bang¹, Bereket Molla Tanga^{1,2}, Ahmad Yar Qamar^{1,3},
Xun Fang¹, Gyeonghwan Seong¹, Abdelbagi Hamad Talha Nabeel^{4,5},
Iljeoung Yu⁴, Jongki Cho^{1,*}

¹College of Veterinary Medicine, Chungnam National University, Daejeon 34134, Korea

²Faculty of Veterinary Medicine, Hawassa University, 05 Hawassa, Ethiopia

³Department of Clinical Sciences, College of Veterinary and Animal Sciences, Jhang, Sub-campus University of Veterinary and Animal Sciences, Lahore, Pakistan

⁴Laboratory of Theriogenology and Reproductive Biotechnology, College of Veterinary Medicine and Bio-safety Research Institute, Jeonbuk National University, Iksan 54596, Korea

⁵Department of Veterinary Medicine and Surgery, College of Veterinary Medicine, Sudan University of Science and Technology, Khartoum, Sudan

Resveratrol (RSV, 3,5,4'-trihydroxytrans-stilbene) protects sperm from cryo-induced damage in various animal and human species. In this study, we aimed to assess the effect of dog sperm cryoprotective extender containing RSV on the quality of post-thaw dog sperm. Sperm were collected from 4 Beagles and supplemented with different concentrations of RSV (0, 100, 200, and 400 μ M). After thawing, apoptosis index, and reactive oxygen species (ROS) levels were assessed to determine post-thaw sperm quality. Dog sperm cryopreserved with 400 μ M RSV showed significant improvement in post-thaw sperm quality with lower apoptosis index and ROS levels ($p < 0.05$). Our results showed that the supplementation of dog sperm cryoprotective extender with RSV at a concentration of 400 μ M improved the post-thaw dog sperm quality in the term of sperm ROS production and apoptosis. In addition, we emphasize the necessity of testing the ROS levels and apoptosis index using flow cytometry to determine the quality of post-thaw semen.

Keywords: apoptosis; cryopreservation; dog; resveratrol; reactive oxygen species

Introduction

The use of artificial insemination (AI) has expanded widely in livestock and canine reproduction. This technique reduces the use of breeding dogs, which requires transportation of animals, is relatively costly, is time-consuming, and poses risks of disease transmission [1-3]. AI also facilitates breeding of the required, usually genetically superior dogs, both within and among countries [4,5]. Cryopreservation of genetic materials in livestock has various limitations that negatively affect fertility quality [6-9]. Cryo-induced damage causes alteration of sperm cell homeostasis and leads to failure of the antioxidant system, which plays a major role in controlling the level of oxidative stress (OS) and is responsible for controlling excessive free radicals [10].

Various reports have shown that supplementation of antioxidants in cryopreser-

vation medium improves post-thaw sperm quality [4,11]. Cryopreservation efforts involving freezing and thawing inevitably affect sperm homeostasis, leading to significant physiological and chemical changes in the sperm, affecting OS homeostasis and causing excessive production of reactive oxygen species (ROS) [12,13]. This is mainly due to the removal of seminal plasma during the cryopreservation process, which reduces antioxidant defenses in sperm and makes sperm vulnerable to OS [14]. Other effects of cryopreservation include osmotic stress, cold shock, and intracellular ice crystal formation [15-17]. Various antioxidants have been shown to improve post-thaw sperm quality in different animal species by suppressing ROS production and its associated effects [18,19]. Previous studies showed that supplementation of cryoprotectant extender with kinetin and astaxanthin improved post-thaw motility and structural integrity of dog sperm [20,21]. Resveratrol (RSV, 3,5,4'-trihydroxytrans-stilbene) exhibits its antioxidant effects by scavenging free radicals, chelating divalent cations, and inhibiting ROS formation [22]. Supplementation of extender with RSV also decreased mitochondrial membrane potential and reduced ROS levels [23]. RSV supplementation in the cryopreservation of human sperm has been reported to improve the quality and function of post-thaw sperm [24].

Accordingly, we expedite the possible application of flow cytometry, for the detection of the survival rate depend on apoptosis index, which is vital for the sperm quality [25]. Besides, apoptosis elicited from cryopreservation and post-thaw effects, sperm are experiencing from relatively higher ROS [26]. During freezing, an increase in ROS level in sperm leads to fatty oxidation. This action induces DNA fragmentation in sperm, leading to apoptosis by relying on damage to the genetic mechanism [27]. Thus, we aimed to use the flow cytometry as independent test for assessing the quality of post-thaw sperm in dog sperm. We applied the apoptosis index and ROS level for the detection of the quality of sperms analysis of the control and RSV treated. In fact, even there are reports of application of flow cytometry, there are few and most of reports used as an additional method of assessment to conventional techniques. Therefore, this study aims to evaluate the effect of RSV on ROS levels and apoptosis in dog sperm, as measured by flow cytometry, independently analyze post-thaw quality.

Materials and Methods

All chemicals used in the experiment were purchased from Sigma-Aldrich (USA), unless otherwise stated. Sperm cryopreservation and post-thaw quality assessments were performed

following previously reported protocols [13]. All experimental procedures were conducted according to the guidelines for the care and use of laboratory animals at Chungnam National University (approval number: 202006A-CNU-103).

Preparation of buffers

The sperm washing and dilution system was prepared as described previously [20]. 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 (volume per volume [v:v]), 40% egg yolk (v:v), and 6% glycerol (v:v).

Study animals and semen collection

Four healthy male Beagles, 3- to 4-year-old and weighing 8 to 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 with sufficient area for exercise and rest. Semen collection was performed twice a week for a total of 8 times using digital manipulation. Semen samples were initially assessed using a CASA software imaging system (MICROPTIC CASA Systems, SCA class analyzer; Josep Tarradellas, Spain). Samples with $\geq 70\%$ motility, $\geq 80\%$ viability, and a sperm concentration $\geq 100 \times 10^6$ cells/mL were pooled and used for further processing.

Semen cryopreservation and thawing

Sperm cryopreservation and post-thaw quality assessment were performed following previously reported protocols [20] and are described in detail in this paper. 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, 400 μM RSV, or no RSV (control). Semen was extended in a multi-step dilution process to a final concentration of 100×10^6 cells/mL. Diluted semen was used to fill 0.5 mL semen straws (Ref. 13408/0010: Minitüb GmbH, Germany), which were then equilibrated at 4°C for 45 to 60 minutes. Freezing of the semen straws was performed by horizontally placing the straws 2 cm above the surface of liquid nitrogen for 15 minutes. After storage in liquid nitrogen for a week, the frozen semen straws were thawed in a 37°C water bath for 30 seconds for evaluation.

ROS level assessment

Sperm ROS levels were assessed according to the protocol de-

scribed by Guthrie and Welch [28]. 2,7-dichlorodihydrofluorescein diacetate (H₂DCFDA; Molecular Probes Inc., USA) was used to detect H₂O₂. The mean fluorescence intensity [29] of DCF was measured to evaluate the intracellular mean H₂O₂ per viable sperm population. All fluorescence signals of labeled sperm were analyzed using a BD Accuri C6 Plus flow cytometer (BD Biosciences, USA). The data are expressed as the percentage of viable sperm with H₂O₂ (high DCF fluorescence).

Apoptosis status assessment

The sperm apoptosis status was evaluated according to Meh-dipour et al. [30]. Sperm were washed in Dulbecco's Phosphate Buffered Saline and readjusted to 100 mL at a concentration of 1×10^6 sperm/mL in $1 \times$ binding buffer (10 mM HEPES/NaOH, pH 7.5, containing 0.14 M NaCl and 2.5 mM CaCl₂), and 5 μ L annexin V fluorescein isothiocyanate (FITC) (50 μ g/mL stock) and 5 μ L propidium iodide (PI) solutions were added. The cells were incubated for 15 minutes at room temperature in the dark. Sperm were categorized into 4 different groups: viable non-apoptotic, early apoptotic, late apoptotic, and necrotic cells. The last group contained late apoptotic and necrotic cells, which were categorized as dead cells. The apoptosis index was calculated as the ratio of annexin V detected among live sperm.

Statistical analysis

The IBM SPSS Statistics ver. 24.0 (IBM Corp., USA) was used

to analyze the data. Apoptosis status and ROS levels were analyzed using bivariate correlation coefficients. The measurements of different parameters were expressed as the mean \pm standard error of the mean, and $p < 0.05$ indicating statistical significance.

Results

ROS level

ROS level is calculated on the bases of the proportion of ROS detected sperm from total number of live sperm. In the quadrant, the upper and lower parts indicate the presence or absence of PI staining, and depending on this result, the sperm is categorized as live or dead. In addition, the left and right sides of the quadrant indicate is on the bases of DCFDA staining, which indicates the higher level of ROS {lower right quadrants / (lower left quadrants + lower right quadrants)}. The results of showed that the ROS level gradually decreased in the group treated with RSV at increasing concentrations ($p < 0.05$) (Fig. 1). In particular, there was a significant difference between the control group and the RSV 400 μ M treatment group (measured as 4.31 ± 0.51 and 1.43 ± 0.18 in control and 400 μ M RSV, respectively).

Phosphatidylserine translocation status (apoptosis)

The apoptosis index was calculated as the probability that an-

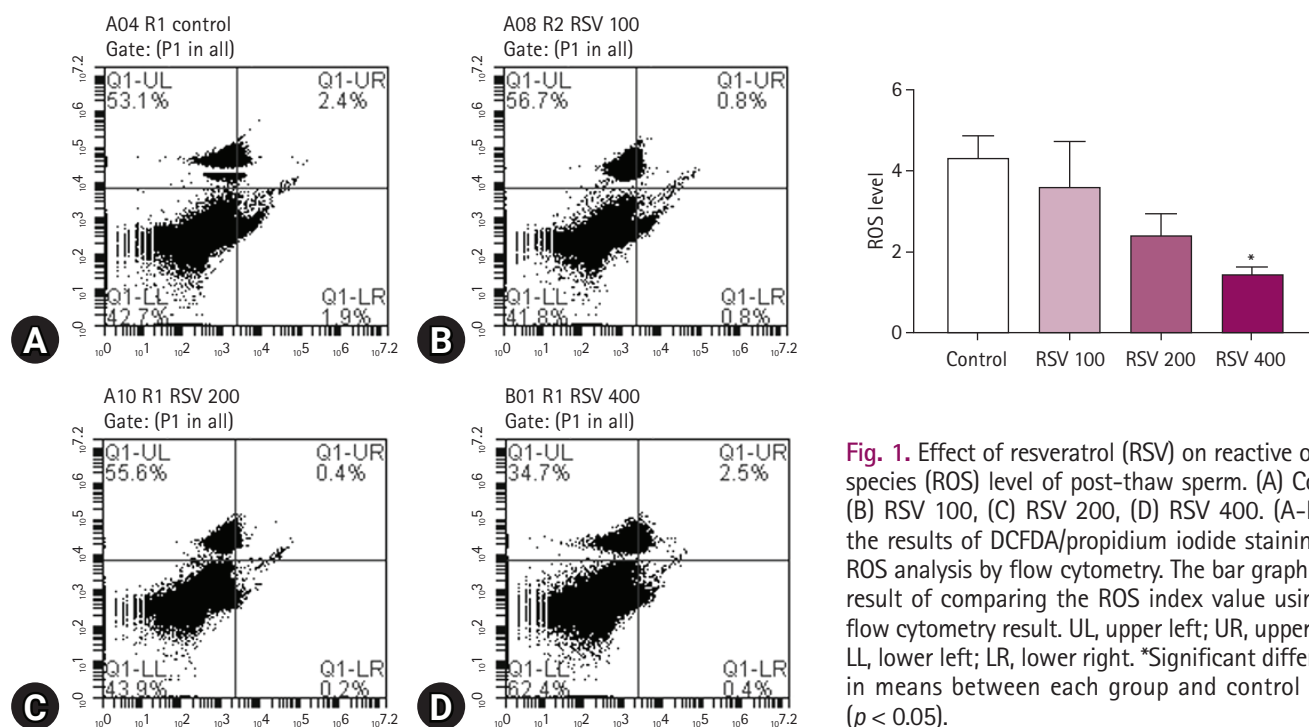


Fig. 1. Effect of resveratrol (RSV) on reactive oxygen species (ROS) level of post-thaw sperm. (A) Control, (B) RSV 100, (C) RSV 200, (D) RSV 400. (A-D) are the results of DCFDA/propidium iodide staining and ROS analysis by flow cytometry. The bar graph is the result of comparing the ROS index value using the flow cytometry result. UL, upper left; UR, upper right; LL, lower left; LR, lower right. *Significant differences in means between each group and control group ($p < 0.05$).

nexin V were detected depending on the number of live sperm. In the quadrant, the upper and lower parts indicate the presence or absence of PI staining, and depending on this result, the sperm is categorized as live or dead. In addition, the left and right sides of the quadrant indicate the presence or absence of annexin V FITC staining, which indicates the expression of apoptosis {lower right quadrants / (lower left quadrants + lower right quadrants)}. The apoptosis index showed a marked difference between the RSV-treated groups and the control group ($p < 0.05$) (Fig. 2). RSV supplementation at 400 μM in the cryoprotectant extender resulted in decreased apoptosis index (measured as 5.65 ± 0.58 and 3.29 ± 0.21 in control and 400 μM RSV, respectively).

Discussion

RSV is an activating factor of SIRT1, which is responsible to reduce and improve ROS levels and thereby reduces apoptosis in cells [31]. The flow cytometry method of apoptosis index and ROS determination of post-thaw sperm quality revealed relatively lower apoptosis index and ROS level at 400 μM RSV supplemented group is statistically significantly different than control ($p < 0.05$). The finding of current study, optimum concentration of RSV, as 400 μM in dog post-thaw sperm is higher than, 40 μM RSV reported to be efficient in roosters [32], which could be due to species-specific differences and usage of differ-

ent media types. RSV has been shown to affect autophagy factor 1, which is an outer mitochondrial membrane protein, responsible for calcium metabolism, anti-apoptosis, and anti-autophagy [33,34]. Therefore, supplementation with RSV will up-regulate the development of mitochondrial organisms, allowing sperm to obtain the energy source required for activity after thawing [35].

The conventional sperm quality evaluation methods analyze mainly cell membrane, mitochondria, DNA and acrosome and so far used in combination as a useful approach to determine the sperm quality [36]. Whereas advances in molecular cytometry enable to analyze sperm quality objectively and in relatively faster rate. These findings are similar to previous reports where apoptotic biomarkers were used to determine the male fertility potential [37]. Therefore, the excessive ROS levels in the control group could be responsible for physical and chemical stress on the sperm and affect fertility quality. This finding is in line with a previous report demonstrating that antioxidant supplementation using kinetin maintains the motility and survival rate of cryopreserved dog sperm [20].

The lower apoptosis index in sperm groups treated with RSV is an indication of better survival of the sperm in the treated groups as compared to that of the control group. In general, apoptosis is gene-activated and occurs as a normal consequence of cell aging or as a result of cellular stress [38]. The reduced apoptotic index in the treated groups is a mechanism to reduce

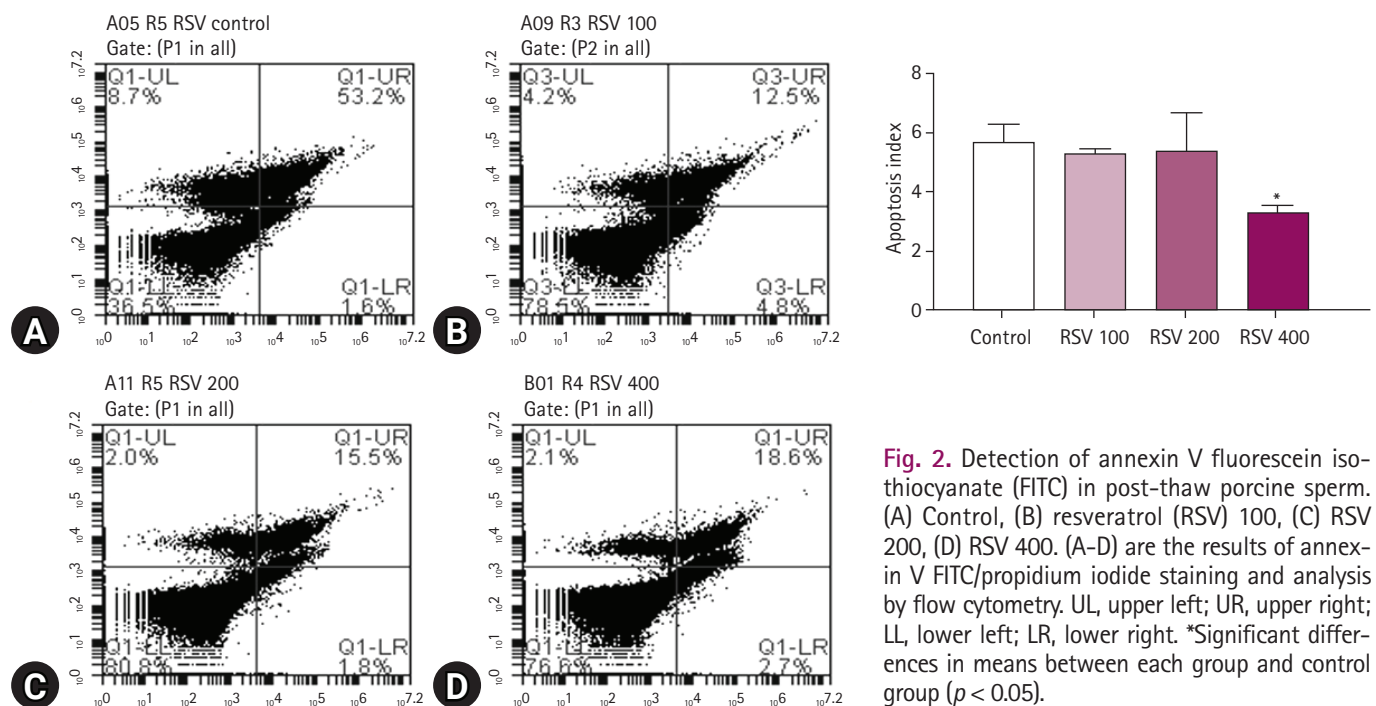


Fig. 2. Detection of annexin V fluorescein isothiocyanate (FITC) in post-thaw porcine sperm. (A) Control, (B) resveratrol (RSV) 100, (C) RSV 200, (D) RSV 400. (A-D) are the results of annexin V FITC/propidium iodide staining and analysis by flow cytometry. UL, upper left; UR, upper right; LL, lower left; LR, lower right. *Significant differences in means between each group and control group ($p < 0.05$).

the cryo-induced damage of the apoptotic process that occurs during freezing and thawing. This, in turn, is believed to contribute to maintaining the fertility quality of post-thaw sperm. On the other hand, high levels of ROS are a major cause of DNA damage that may result in genetic mutations and consequently different pathological conditions including aging, leading to apoptosis and cancer [39]. Damage to sperm DNA affects various fertility parameters [40].

RSV lowers the apoptotic index and ROS levels and should be considered as an effective antioxidant for post-thaw dog sperm at 400 μ M. Furthermore, flow cytometry methods are effective for post-thaw sperm quality analysis, revealing similar results as those of conventional sperm motility and survival tests. We encourage further studies in this regard, particularly on the effects of higher concentrations of RSV and a wide-scale validation of the results of this study. Finally, we note that the study species and the types of cryopreservation media used are important factors to consider while determining the optimum concentration of RSV.

Acknowledgments

This work was supported by research fund of Chungnam National University.

ORCID

Seonggyu Bang, <https://orcid.org/0000-0002-2513-4887>

Bereket Molla Tanga, <https://orcid.org/0000-0001-9901-5875>

Ahmad Yar Qamar, <https://orcid.org/0000-0002-0366-3913>

Xun Fang, <https://orcid.org/0000-0001-8714-0679>

Gyeonghwan Seong, <https://orcid.org/0000-0002-4007-5379>

Abdelbagi Hamad Talha Nabeel, <https://orcid.org/0000-0002-0939-6705>

Iljeoung Yu, <https://orcid.org/0000-0002-5530-5974>

Jongki Cho, <https://orcid.org/0000-0002-8431-0457>

References

1. Waberski D, Riesenbeck A, Schulze M, Weitze KE, Johnson L. Application of preserved boar semen for artificial insemination: past, present and future challenges. *Theriogenology* 2019;137:2–7.
2. Jha PK, Sapkota S, Gorkhali NA, Pokharel BR, Jha AK, Bhandari S, Shrestha BS. Seminal attributes and semen cryo-banking of Nepalese Indigenous Achhami (*Bos indicus*) bull under ex-situ conservation. *J Anim Reprod Biotechnol* 2019;34:272–

- 279.
3. Kang SS, Kim UH, Lee MS, Lee SD, Cho SR. Spermatozoa motility, viability, acrosome integrity, mitochondrial membrane potential and plasma membrane integrity in 0.25 mL and 0.5 mL straw after frozen-thawing in Hanwoo bull. *J Anim Reprod Biotechnol* 2020;35:307–314.
4. Thomassen R, Farstad W. Artificial insemination in canids: a useful tool in breeding and conservation. *Theriogenology* 2009;71:190–199.
5. Mahiddine FY, Qamar AY, Kim MJ. Canine amniotic membrane derived mesenchymal stem cells exosomes addition in canine sperm freezing medium. *J Anim Reprod Biotechnol* 2020;35:268–272.
6. Bailey JL, Lessard C, Jacques J, Brèque C, Dobrinski I, Zeng W, Galantino-Homer HL. Cryopreservation of boar semen and its future importance to the industry. *Theriogenology* 2008;70:1251–1259.
7. Ugur MR, Saber Abdelrahman A, Evans HC, Gilmore AA, Hitit M, Arifiantini RI, Purwantara B, Kaya A, Memili E. Advances in cryopreservation of bull sperm. *Front Vet Sci* 2019;6:268.
8. Baumber J, Ball BA, Linfor JJ, Meyers SA. Reactive oxygen species and cryopreservation promote DNA fragmentation in equine spermatozoa. *J Androl* 2003;24:621–628.
9. Kim DS, Hwangbo Y, Cheong HT, Park CK. Effects of discontinuous Percoll gradient containing alpha-linolenic acid on characteristics of frozen-thawed boar spermatozoa. *J Anim Reprod Biotechnol* 2020;35:58–64.
10. Aprioku JS. Pharmacology of free radicals and the impact of reactive oxygen species on the testis. *J Reprod Infertil* 2013;14:158–172.
11. Banday MN, Lone FA, Rasool F, Rashid M, Shikari A. Use of antioxidants reduce lipid peroxidation and improve quality of crossbred ram sperm during its cryopreservation. *Cryobiology* 2017;74:25–30.
12. Said TM, Gaglani A, Agarwal A. Implication of apoptosis in sperm cryoinjury. *Reprod Biomed Online* 2010;21:456–462.
13. Qamar AY, Fang X, Kim MJ, Cho J. Improved viability and fertility of frozen-thawed dog sperm using adipose-derived mesenchymal stem cells. *Sci Rep* 2020;10:7034.
14. Lucio CF, Regazzi FM, Silva LC, Angrimani DS, Nichi M, Vannucchi CI. Oxidative stress at different stages of two-step semen cryopreservation procedures in dogs. *Theriogenology* 2016;85:1568–1575.
15. Chatterjee S, Gagnon C. Production of reactive oxygen species by spermatozoa undergoing cooling, freezing, and thawing. *Mol Reprod Dev* 2001;59:451–458.

16. Wang AW, Zhang H, Ikemoto I, Anderson DJ, Loughlin KR. Reactive oxygen species generation by seminal cells during cryopreservation. *Urology* 1997;49:921–925.
17. Kim EJ, Talha NA, Jeon YB, Yu IJ. Effect of κ -carrageenan on sperm quality in cryopreservation of canine semen. *J Anim Reprod Biotechnol* 2019;34:57–63.
18. Aitken RJ, Buckingham DW, Brindle J, Gomez E, Baker HW, Irvine DS. Analysis of sperm movement in relation to the oxidative stress created by leukocytes in washed sperm preparations and seminal plasma. *Hum Reprod* 1995;10:2061–2071.
19. Bilodeau JF, Chatterjee S, Sirard MA, Gagnon C. Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. *Mol Reprod Dev* 2000;55:282–288.
20. Qamar AY, Fang X, Bang S, Kim MJ, Cho J. Effects of kinetin supplementation on the post-thaw motility, viability, and structural integrity of dog sperm. *Cryobiology* 2020;95:90–96.
21. Qamar AY, Fang X, Bang S, Shin ST, Cho J. The effect of astaxanthin supplementation on the post-thaw quality of dog semen. *Reprod Domest Anim* 2020;55:1163–1171.
22. Stojanović S, Sprinz H, Brede O. Efficiency and mechanism of the antioxidant action of trans-resveratrol and its analogues in the radical liposome oxidation. *Arch Biochem Biophys* 2001;391:79–89.
23. Silva EC, Cajueiro JF, Silva SV, Soares PC, Guerra MM. Effect of antioxidants resveratrol and quercetin on in vitro evaluation of frozen ram sperm. *Theriogenology* 2012;77:1722–1726.
24. Collodel G, Federico MG, Geminiani M, Martini S, Bonechi C, Rossi C, Figura N, Moretti E. Effect of trans-resveratrol on induced oxidative stress in human sperm and in rat germinal cells. *Reprod Toxicol* 2011;31:239–246.
25. Hossain MS, Johannisson A, Wallgren M, Nagy S, Siqueira AP, Rodriguez-Martinez H. Flow cytometry for the assessment of animal sperm integrity and functionality: state of the art. *Asian J Androl* 2011;13:406–419.
26. Tanga BM, Qamar AY, Raza S, Bang S, Fang X, Yoon K, Cho J. Semen evaluation: methodological advancements in sperm quality-specific fertility assessment: a review. *Anim Biosci* 2021;34:1253–1270.
27. Moustafa MH, Sharma RK, Thornton J, Mascha E, Abdel-Hafez MA, Thomas AJ Jr, Agarwal A. Relationship between ROS production, apoptosis and DNA denaturation in spermatozoa from patients examined for infertility. *Hum Reprod* 2004;19:129–138.
28. Guthrie HD, Welch GR. Use of fluorescence-activated flow cytometry to determine membrane lipid peroxidation during hypothermic liquid storage and freeze-thawing of viable boar sperm loaded with 4, 4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid. *J Anim Sci* 2007;85:1402–1411.
29. Bomfim MM, Andrade GM, Del Collado M, Sangalli JR, Fontes PK, Nogueira MF, Meirelles FV, da Silveira JC, Perecin F. Antioxidant responses and deregulation of epigenetic writers and erasers link oxidative stress and DNA methylation in bovine blastocysts. *Mol Reprod Dev* 2017;84:1296–1305.
30. Mehdipour M, Daghighi Kia H, Nazari M, Najafi A. Effect of lecithin nanoliposome or soybean lecithin supplemented by pomegranate extract on post-thaw flow cytometric, microscopic and oxidative parameters in ram semen. *Cryobiology* 2017;78:34–40.
31. Zhang T, Chi Y, Ren Y, Du C, Shi Y, Li Y. Resveratrol reduces oxidative stress and apoptosis in podocytes via Sir2-related enzymes, sirtuins1 (SIRT1)/peroxisome proliferator-activated receptor γ co-activator 1 α (PGC-1 α) axis. *Med Sci Monit* 2019;25:1220–1231.
32. Najafi A, Daghighi Kia H, Hamishehkar H, Moghaddam G, Alijani S. Effect of resveratrol-loaded nanostructured lipid carriers supplementation in cryopreservation medium on post-thawed sperm quality and fertility of roosters. *Anim Reprod Sci* 2019;201:32–40.
33. Cheng L, Yan B, Chen K, Jiang Z, Zhou C, Cao J, Qian W, Li J, Sun L, Ma J, Ma Q, Sha H. Resveratrol-induced downregulation of NAF-1 enhances the sensitivity of pancreatic cancer cells to gemcitabine via the ROS/Nrf2 signaling pathways. *Oxid Med Cell Longev* 2018;2018:9482018.
34. Cheng CK, Luo JY, Lau CW, Chen ZY, Tian XY, Huang Y. Pharmacological basis and new insights of resveratrol action in the cardiovascular system. *Br J Pharmacol* 2020;177:1258–1277.
35. Chen TT, Peng S, Wang Y, Hu Y, Shen Y, Xu Y, Yin J, Liu C, Cao J. Improvement of mitochondrial activity and fibrosis by resveratrol treatment in mice with *Schistosoma japonicum* infection. *Biomolecules* 2019;9:658.
36. Guzik DS, Overstreet JW, Factor-Litvak P, Brazil CK, Nakajima ST, Coutifaris C, Carson SA, Cisneros P, Steinkampf MP, Hill JA, Xu D, Vogel DL; National Cooperative Reproductive Medicine Network. Sperm morphology, motility, and concentration in fertile and infertile men. *N Engl J Med* 2001;345:1388–1393.
37. Zorn B, Golob B, Ihan A, Kopitar A, Kolbezen M. Apoptotic sperm biomarkers and their correlation with conventional

- sperm parameters and male fertility potential. *J Assist Reprod Genet* 2012;29:357–364.
38. Elmore S. Apoptosis: a review of programmed cell death. *Toxicol Pathol* 2007;35:495–516.
39. Waris G, Ahsan H. Reactive oxygen species: role in the development of cancer and various chronic conditions. *J Carcinog* 2006;5:14.
40. Henkel R, Hajimohammad M, Stalf T, Hoogendijk C, Mehnert C, Menkveld R, Gips H, Schill WB, Kruger TF. Influence of deoxyribonucleic acid damage on fertilization and pregnancy. *Fertil Steril* 2004;81:965–972.