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Iodixanol supplementation during sperm cryopreservation improves protamine level and reduces reactive oxygen species of canine sperm

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The objective of this study was to analyze the protective effects of iodixanol on dog spermatozoa during cryopreservation. The optimal concentration of iodixanol, 1.5%, was determined using fresh spermatozoa and was applied in the following experiments. The 1.5% iodixanol group showed significantly increased sperm motility from that in the control ($p < 0.05$). Lower mitochondrial reactive oxygen species (ROS) modulator (*ROMO1*) and pro-apoptotic gene (*BAX*) expressions, together with higher expressions of protamine-2 (*PRM2*), protamine-3 (*PRM3*), anti-apoptotic B-cell lymphoma-2 (*BCL2*), and sperm acrosome associated-3 (*SPACA3*) genes were detected in the iodixanol-treated group. In addition, decreased protamine deficiency and cryocapacitation were observed in the treatment group. Our results show that supplementation with 1.5% iodixanol is ideal for reducing production of ROS and preventing detrimental effects during the canine sperm cryopreservation process, effects manifested as increased motility and reduced cryocapacitation in frozen-thawed spermatozoa.

Keywords: Dogs; Sperm; Cryopreservation; Reactive oxygen species; Protamine

Introduction

Sperm cryopreservation is an essential step for artificial insemination (AI), which is the most widely used assisted reproductive technology in Canidae. The main goal of sperm cryopreservation is to conserve the fertility of high genetic value organisms or to preserve endangered species [7]. In addition, sperm cryopreservation for AI can prevent sexually transmitted diseases such as brucellosis and herpes virus infections [10]. In wild animals, AI with cryopreserved spermatozoa has had a considerable effect in conservation management of African wild dogs [5]. Moreover, AI using cryopreserved spermatozoa provides a number of potential advantages including reduction of stress associated with transportation of animals, avoiding resistance to copulation due to behavioral issues (female aggressiveness), and overcoming quarantine restrictions placed on live animals [8].

During the cryopreservation process, osmotic pressure and ice formation can cause cryoinjury and loss of viability and sperm function post-thawing [25]. Osmotic stress following cell dehydration induces destabilization of sperm membranes by reducing their fluidity, and this is exacerbated by excessive production of endogenous and exogenous reactive oxygen species (ROS) [1,13]. An imbalance between ROS production during cryopreservation and antioxidant defenses of spermatozoa result in oxidative stress [3] and lipid peroxidation of plasma membranes that contain large amounts of polyunsaturated fatty acids [35]. Consequently, important cell components such as DNA are damaged by cryopreservation [16]. On the other hand, complete elimination of ROS is also detrimental because some ROS are required for normal reproductive events [15]. Thus, maintaining appropriate oxidative stress and ROS levels is necessary during sperm cryopreservation and thawing.

Protamine binding of large segments of DNA is stronger than histone binding [6] and results in the construction of toroids, which are condensed DNA strands that protect sperm chromatin from oxidative damage [28]. After a freeze-thaw cycle, bonding of disulfide bridges in protamine is disturbed, and DNA damage is increased [12]. In addition, less compaction of DNA and more susceptibility to damage has been observed in frozen-thawed canine spermatozoa [4], and a higher level of sperm DNA damage reduces the quality of the embryo [33]. Insufficient sperm chromatin content is also

correlated with an elevated early pregnancy loss risk after *in vitro* fertilization (IVF) and intracytoplasmic sperm injection [39]. Accordingly, conserving protamines during sperm cryopreservation and thawing is important to protect sperm chromatin and support a pregnancy.

Iodixanol is normally used as an x-ray contrast medium, because it is nontoxic, non-ionic, dimeric, and hexaiodinated [11,21]. Adding iodixanol could change ice crystal formation into a intracellular dendritic form, which results in a more sperm-friendly environment [26]. However, the mechanism associated with iodixanol protecting canine sperm from DNA damage during cryopreservation has not yet been determined. We hypothesized that iodixanol treatment conserves sperm protamines to help maintain DNA integrity during cryopreservation.

Materials and Methods

All chemicals used in this experiment were purchased from Sigma-Aldrich (USA) unless otherwise stated.

Animal use and semen preparation

The subjects used in this experiment were 4 healthy and mature male beagles, 4 to 6 years old, and weighing 8 to 10 kg. The dogs were maintained separately in indoor cages. Their housing, animal care facilities and procedures followed standards established by the Committee for Accreditation of Laboratory Animal Care at Seoul National University. The Guide for the Care and Use of Laboratory Animals at Seoul National University was the standard for animal use in this study (approval No. SNU-160602-6-4). Semen samples from the dog were collected twice a week and only ejaculates having sperm concentrations $\geq 100 \times 10^6$/mL, motility $\geq 70\%$, viability $\geq 80\%$, and normal morphology $\geq 80\%$ were used.

Determination of iodixanol concentration

Optiprep (60% iodixanol in water) was mixed into the first buffer (24 g/L Tris [hydroxymethyl] aminomethane, 14 g/L citric acid, 8 g/L fructose, and 0.15 g/L kanamycin sulfate in distilled water [pH 6.6, 290 mOsm]). The ejaculates were collected from 4 male beagles and pooled into 15 mL tubes. The pooled ejaculates were washed by centrifuging at $100 \times g$ for 1 min at room temperature to remove debris [24]. The debris was the pellet in the tube bottom and sperm was the supernatant. The supernatant was divided into 4 aliquots with different concentrations of Optiprep; 0% (control), 1% (0.6% iodixanol), 2.5% (1.5% iodixanol), and 5% (3% iodixanol) treatment groups.

Afterward, motility and kinematic parameters were evaluated. Approximately 10 μL of sperm was dropped onto a slide and mounted with a coverslip. Spermatozoa in 5 different fields were assessed and the kinematic parameters of at least 200 motile spermatozoa were tracked in each experiment by using a sperm analysis imaging system (FSA2011 premium edition version 2011; Medical Supply, Korea).

Additionally, sperm viability was examined by using an eosin-nigrosin staining method. Briefly, sperm suspension smears were made after adding the stain (1:1) to the first buffer with iodixanol. The sperm sample was then spread onto a slide and dried in air. Sperm viability was analyzed for at least 200 cells. Thereafter, the optimal concentration was chosen and used for the following experiment to investigate the effects of iodixanol on sperm cryopreservation.

Sperm freezing and thawing

The second experiment was performed to investigate the effects of iodixanol on cryopreservation. An equal volume of the first buffer was added to pooled ejaculates for washing and centrifuged at $700 \times g$ for 5 min at room temperature. The pellet was resuspended by adding the first buffer to achieve a sperm concentration of 200×10^6 sperm/mL. Each aliquot was mixed with the second buffer (40% [v/v] egg yolk, 54% [v/v] first buffer, and 6% [v/v] glycerol) and iodixanol. The suitable volume of the second buffer was added to produce 14%, 19%, 27%, and 40% of the total volume and loaded sequentially at 30 sec intervals as described in a previous study [29]. Straws (0.25 mL; Minitub, Germany) were used to store the extended sperm suspension and were incubated at 4°C for 1 h for equilibration. Subsequently, the straws were placed horizontally 2 cm above the surface of liquid nitrogen (LN_2) for 15 min then plunged into the LN_2. After one week, the sperm was thawed in a water bath at 60°C for 7 sec. The sperm samples were then diluted (1:5) by adding the first buffer to obtain 14%, 19%, 27%, and finally 40% of the total volume. Thawed sperm motility and kinematic parameters were determined with a sperm analysis imaging system as mentioned in the 'Determination of iodixanol concentration' section. Then, all thawed spermatozoa were evaluated as described in the following sections.

Gene expression analysis and chromomycin A3 staining

Briefly, triplicates from five pairs of canine frozen-thawed sperm samples from the control and from treatment groups were used for RNA measurement. Real-time quantitative polymerase chain reaction (RT-qPCR) was employed to assess transcript abundances using oligonucleotide primer sequences, all of which are listed in Table 1. The mRNA expressions of apoptotic genes (*BCL2* and *BAX*), a mitochondrial ROS modulator (*ROMO1*) gene, genes for measuring protamine levels (*PRM2* and *PRM3*), and a sperm acrosome associated-3 (*SPACA3*) gene were analyzed. Total RNA was extracted using Trizol reagent (Invitrogen, USA), according to the manufacturer's protocol, and complementary DNA was produced using Maxime RT PreMix (Intronbio, Korea). The RT-qPCR assays were performed using a Step One Plus

Table 1. Primer sequences used for gene expression analysis in spermatozoa treated with iodixanol during cryopreservation

Gene	Primer sequence (5′–3′)	Product size (bp)	GenBank accession No.
ACTB	F: GGCATCCTGACCCTGAAGTA R: GGTGTGGTGCCAGATCTTCT	148	NM_001195845.1
BCL2	F: CTCCTGGCTGTCTCTGAAGG R: GTGGCAGGCCTACTGACTTC	120	NM_001002949.1
BAX	F: GACGGCCTCCTCTCCTACTT R: GGTGAGTGACGCAGTAAGCA	145	NM_001003011.1
ROMO1	F: TGTCTCAGGATCGGAATGCG R: TCCCGATGGCCATGAATGTG	100	XM_534406.4
PRM2	F: AGGAGGAGATACAGGAGGTGC R: CTTGCAAACTCAGGGCTTGG	148	NM_001287148
PRM3	F: GGCCACGAATCCTCCATGGA R: AGCTCCTCCTCTTCCTCCT	128	XM_847270.4
SPACA3	F: GGATTTCGGCATGGAGGGAT R: ACTTCCGGCTGTTGATCTGG	149	NM_001197087.1

F, forward; R, reverse.

Real-Time PCR System (Applied Biosystems, USA) and each target gene's expression was quantified relative to that of the internal gene (*ACTB*) using the equation, $R = 2^{-[\Delta Ct\ sample\ -\ \Delta Ct\ control]}$ [17].

Frozen-thawed canine sperm smears were fixed in methanol/glacial acetic acid (3:1) at 4°C for 5 min. Control and treatment group slides were treated for 20 min with 100 μL CMA₃ solution. The CMA₃ solution contained 0.25 mg/mL CMA₃ in McIlvane's buffer (pH 7.0) supplemented with 10 mM MgCl₂. Slides were then rinsed in McIlvain's buffer and air dried. Microscopic analysis of slides was performed by measuring fluorescence with a Zeiss microscope at 1,000× magnification. A total of 200 spermatozoa were randomly evaluated on each slide. Evaluation of CMA₃ was completed by identifying two types of staining patterns: bright green fluorescence of the sperm head (CMA₃ positive/abnormal chromatin packaging) and dull green staining (CMA₃ negative/normal chromatin packaging) of the sperm head.

Sperm capacitation test

The contents of one cryopreserved straw were divided into 2 aliquots after thawing in a water bath at 37°C for 30 sec. Cryocapacitation was analyzed using the first aliquot, while capacitation ability after thawing was analyzed using the second aliquot.

The first aliquot was diluted by adding 1 part semen to 9 parts 0.9% NaCl then an equal volume of trypan blue 0.27% (v/v) was added and the combination was mixed on a slide. Sperm smears were then fixed in a 37% formaldehyde solution for 2 min, then rinsed with distilled water. The Giemsa stock solution was freshly made by adding the stain to distilled water. Slides were dipped in 7.5% (v/v) of Giemsa stain, air-dried in a vertical position, then cover-slipped. Slides were examined by examining 200 sperm cells with 5 independent replications. Assessment included counting the following: live spermatozoa with intact acrosomes (LSIA), live spermatozoa with damaged acrosomes (LSDA), dead spermatozoa with intact acrosomes (DSIA), and dead spermatozoa with damaged acrosomes (DSDA). The posterior part of the sperm head was dark blue in dead spermatozoa and sky-blue in live spermatozoa. Based on the color of anterior part sperm head, spermatozoa were divided into three groups, spermatozoa with intact acrosomes (purple), damage acrosomes (lavender), and those without acrosomes (pale gray). A canine capacitation medium (CCM) supplemented with 1.0 mM MgCl₂ and 10 mM progesterone was used for incubating the second aliquot for 4 h, following incubation the second aliquot was stained to determine acrosomal status as described for the first aliquot [30].

Mucus penetration test

Modified synthetic oviduct fluid, as a surrogate mucus, was loaded into marked flat capillary tubes (10 cm long, 3 mm deep; Camlab, UK) that were sealed on one end. The filled capillary tubes were left standing vertically with the sealed end on top to allow removal of bubbles and check seal tightness. Then, the capillary tube was inserted into an Eppendorf tube containing a 100 μL sperm suspension and laid horizontally for 2 h at room temperature. The spermatozoa reaching the 1 cm and 3 cm markers in the capillary tube were then counted.

Statistical analysis

All values are presented as mean ± SEM values, and a *p* value < 0.05 was used to indicate statistical significance. For multiple comparisons among treatments and control groups, one-way

analysis of variance and Tukey's multiple comparison tests were used. An unpaired *t*-test was used to compare results from the control and optimal iodixanol concentration groups. GraphPad Prism 5 (GraphPad Software, USA) was used for the statistical analyses.

Results

### Determination of optimum iodixanol concentration

The percentage of fresh spermatozoa exhibiting motility in the 3% iodixanol group was significantly decreased (48.0 ± 6.3%) compared to the percentages in the 0%, 0.6%, and 1.5% iodixanol groups (81.5 ± 1.9%, 75.2 ± 7.9%, and 76.8 ± 7.0%, respectively; Table 2).

Four independent replicates were examined to evaluate the effect of iodixanol on the viability of fresh spermatozoa. The percentage of dead spermatozoa in the 1.5% iodixanol group was significantly lower (4.1 ± 0.4%) than in the control (9.9 ± 1.2%) and 3% iodixanol (11.1 ± 1.4%) groups (Fig. 1). There was no significant difference between the 0.6% iodixanol and control groups.

Effect of iodixanol on frozen-thawed sperm motility, kinematic parameters, and mucus penetration test

The frozen-thawed sperm percentage exhibiting motility after treatment with 1.5% iodixanol was significantly higher (54.0 ± 0.9%) than that in the control group (46.6 ± 2.2%; Table 3). There were no significant differences in linearity, amplitude of lateral head displacement, and straightness percentages between the treatment and control groups (30.6 ± 2.4% *vs.* 28.7 ± 2.2%; 3.9 ± 0.4 μm *vs.* 3.4 ± 0.4 μm; 56.1 ± 2.6% *vs.* 57.0 ± 1.9%, respectively). The mucus penetration test results showed that sperm counts in the 1.5% iodixanol treatment group were
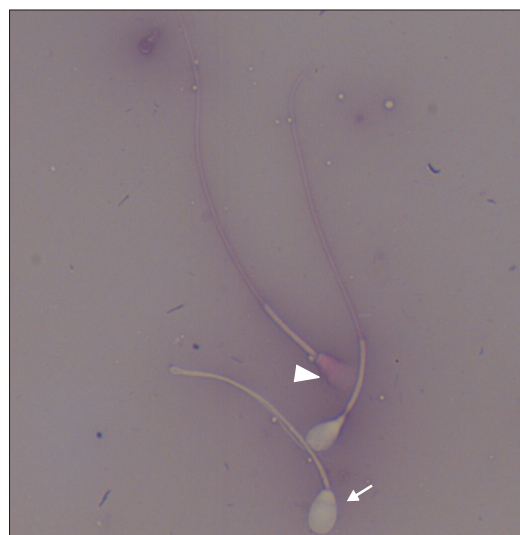
Fig. 1. Image of eosin-nigrosin stained sperm showing live (arrow) and dead (arrowhead) spermatozoa.

Table 2. Motility, linearity, dead canine spermatozoa, amplitude of lateral head displacement (ALH), and straightness with iodixanol treatment

Group	Motility (%)	Linearity (%)	Dead sperm (%)	ALH (μm)	Straightness (%)
Control	81.5 ± 1.9[a]	27.5 ± 1.9	9.9 ± 1.2[a]	4.7 ± 0.1	43.5 ± 1.6
0.6% iodixanol	75.2 ± 7.9[a]	29.7 ± 1.9	8.6 ± 1.4[ab]	4.0 ± 0.9	44.1 ± 1.4
1.5% iodixanol	76.8 ± 7.0[a]	29.2 ± 2.2	4.1 ± 0.4[b]	4.4 ± 0.6	44.3 ± 1.7
3% iodixanol	48.0 ± 6.3[b]	25.6 ± 3.7	11.1 ± 1.4[c]	2.6 ± 0.3	41.7 ± 1.9

Linearity, straightness of the path; Straightness, departure of the cell path from a straight line. [a–c]Within a column, values with different superscripts differ significantly ($p < 0.05$, 4 replicates).

Table 3. Motility, linearity, amplitude of lateral head displacement (ALH), straightness, and sperm mucus penetration distance (cell) in frozen-thawed spermatozoa treated with/without iodixanol

Group	Motility (%)	Linearity (%)	ALH (μm)	Straightness (%)	Sperm number in mucus penetration distance	
					1 cm	3 cm
Control	46.6 ± 2.2[a]	28.7 ± 2.2	3.4 ± 0.4	57.0 ± 1.9	138.6 ± 2.9[a]	40.7 ± 4.7[a]
1.5% iodixanol	54.0 ± 0.9[b]	30.6 ± 2.4	3.9 ± 0.4	56.1 ± 2.6	147.9 ± 3.1[b]	56.4 ± 5.3[b]

Linearity, straightness of the path; Straightness, departure of the cell path from straight line. [a,b]Within a column, values with different superscripts differ significantly ($p < 0.05$, 6 replicates).

significantly higher at both the 1 cm and 3 cm markers (147.9 ± 3.1 cells and 56.4 ± 5.3 cells, respectively) compared to those in the control group (138.6 ± 2.9 cells and 40.7 ± 4.7 cells, respectively; Table 3).

Effect of iodixanol on gene expression and CMA₃ staining

Treatment with 1.5% iodixanol during sperm cryopreservation significantly increased the *BCL2* transcript level by 1.6-fold and decreased the *BAX* level by 0.5-fold compared with the control group (Fig. 2). Transcript levels of *PRM2* and *PRM3* significantly increased by 2.1-fold and 1.8-fold, respectively, compared with the control. Gene expression of *ROMO1* was significantly reduced by 0.8-fold compared with the control. Furthermore, *SPACA3* gene expression significantly increased to about 1.4-fold of the control level.

CMA₃ staining was performed for detection of protamine deficiency. Frozen-thawed spermatozoa treated with 1.5% iodixanol showed significantly higher protamine levels than that in the control (25.5 ± 2.5% and 34.8 ± 1.3%, respectively; Fig. 3).

Effect of 1.5% iodixanol on sperm capacitation

The live sperm percentage post-thawing in the 1.5% iodixanol treatment group (71.9 ± 0.9%) was significantly greater than that in the control group (59.1 ± 0.3%). In addition, adding iodixanol to the buffer during cryopreservation also significantly reduced the dead sperm percentage compared to that in the control (28.1 ± 0.9% *vs.* 40.3 ± 2.3%, respectively).
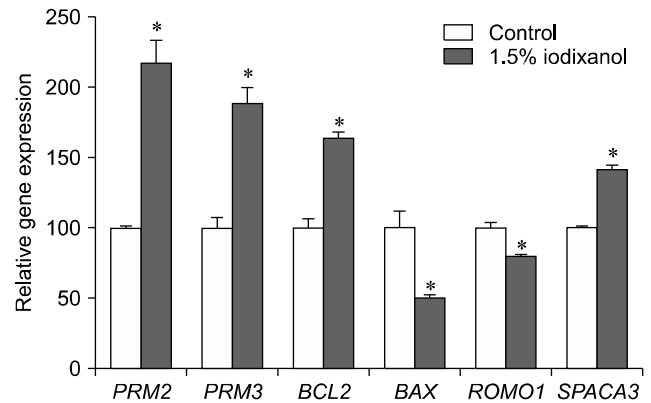
Fig. 2. Gene expression of apoptosis-, oxidative stress-, and protamine-related genes in control and 1.5% iodixanol-treated cryopreserved spermatozoa (*$p < 0.05$).
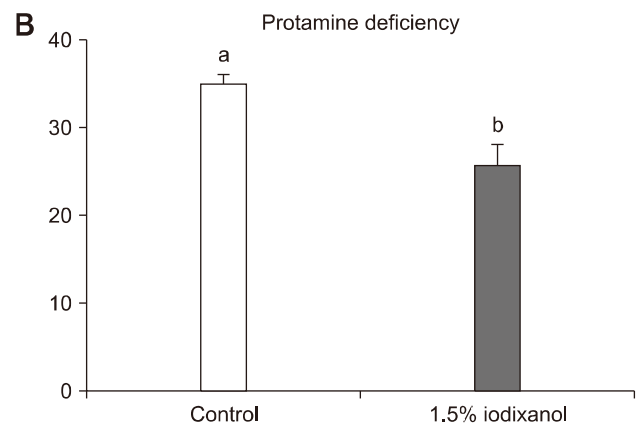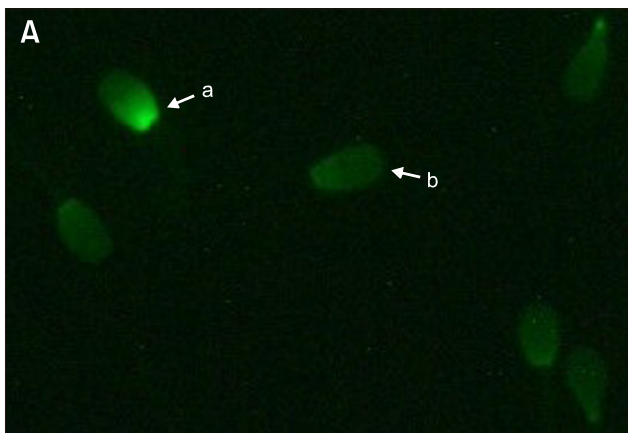
Fig. 3. (A) Results of CMA₃ staining of sperm chromatin for protamine-deficient spermatozoa (a) and normal spermatozoa (b). (B) Quantification of CMA₃ staining results for control- and 1.5% iodixanol-treated spermatozoa ([a,b]$p < 0.05$, 5 replicates).

Table 4. Acrosome staining results post-thawing and after incubation in canine capacitation medium (CCM)

Group	Post-thawing		After incubation in CCM			
	Live sperm (%)	Dead sperm (%)	LSIA (%)	LSDA (%)	DSIA (%)	DSDA (%)
Control	59.1 ± 0.3[a]	40.3 ± 2.3[a]	0.0 ± 0.0	59.1 ± 0.7[a]	13.6 ± 1.0	26.5 ± 1.2[a]
1.5% iodixanol	71.9 ± 0.9[b]	28.1 ± 0.9[b]	0.0 ± 0.0	65.8 ± 1.1[b]	12.8 ± 1.0	21.4 ± 0.9[b]

LSIA, live spermatozoa with intact acrosome; LSDA, live spermatozoa with damaged acrosome; DSIA, dead spermatozoa with intact acrosome; DSDA, dead spermatozoa with damaged acrosome. [a,b]Within a column, values with different superscripts differ significantly ($p < 0.05$, 5 replicates).

After incubation for 4 h in CCM, all sperm acrosomes in both the treatment and control groups were lost. The percentage of LSDA in the treatment group was significantly higher than that in the control (65.8 ± 1.1% and 59.1 ± 0.7%, respectively). In the treatment group, the DSDA percentage was significantly lower than that in in the control (21.4 ± 0.9% *vs*. 26.5 ± 1.2%, respectively) as shown in Table 4.

Discussion

Generation of ROS is a source of problems for mammalian sperm viability, especially during cryopreservation [23] in which chromatin damage can result [16]. In this study, iodixanol was used as a cryoprotectant to alleviate such problems. To minimize the direct effects of iodixanol on sperm cell metabolism, we determined the optimal concentration of iodixanol using fresh spermatozoa and a method described in a previous study [22]. The reduced motility and increased proportions of dead spermatozoa in 3% iodixanol (Table 2) might be due to a toxic effect of iodixanol on spermatozoa. The optimal iodixanol concentration found in our study was 1.5% (Table 3), which is similar to those for bovine [26] and buffalo [34] and comparable to ram [5], rats [19], spermatozoa. Ice crystal formation alteration in a non-colligative manner and elevation of glass transition temperature are possible mechanisms for obtaining an optimal environment for freezing [26]. It seems that a loose net of dendritic ice, as created by 1.5% iodixanol, was suitable in dogs and cattle due to canine and bovine spermatozoa similarities in retinoic acid receptors, which support sperm function and structure [18]. Based on our results, 1.5% iodixanol was chosen for iodixanol treatment of canine spermatozoa in the subsequent experiments.

Increased levels of the antiapoptotic gene *BCL2* and reduced levels of the proapoptotic gene *BAX* in our results indicate that iodixanol reduced apoptosis during cryopreservation. *ROMO1* is a key gene for generating production of mitochondrial ROS [31], and a high level of endogenous ROS produced in the mitochondrial respiratory chain can cause random genetic mutations and lead to programmed cell death [36]. Our results suggest that iodixanol could protect mitochondria during the freezing and thawing process [32] and result in a ROS reduction. The advantage of a lower ROS production could be seen in the acrosome reaction [3]. Indeed, the number of viable spermatozoa and the percentage of viable spermatozoa with reacted acrosomes significantly increased in the treatment group (Table 4). The *SPACA3* gene conserves subtract-binding sites for oligosaccharides of *N*-acetylglucosamine existing in the extracellular matrix around the plasma membrane [20]. Increased *SPACA3* expression in our study suggests that treating spermatozoa with iodixanol maintains their mechanical support, which could be the reason for the decreased apoptosis and increased mitochondrial protection observed in the iodixanol group.

Mechanical support in spermatozoa can also be important in conserving protamines in the sperm head. Protamine genes *PRM2* and *PRM3* have roles in sperm motility. For example, impaired histones, disturbed DNA-hypercondensation, and immotility, consequences of serious membrane defects, are the results of *PRM2* deficiency in spermatozoa [27]. Low expression of the *PRM2* gene may also be related to abnormal morphology and entry to the apoptotic pathway [38]. The *PRM3* gene is expressed in the cytoplasm and is important for sperm motility [14]. We confirmed the increased transcript expression of these protamine genes in the iodixanol treatment group (Fig. 2) by applying CMA$_3$ stain (Fig. 3), a detector of nicked DNA and protamine-deficient, loosely packaged chromatin in spermatozoa. A significant negative correlation has been detected between CMA$_3$ positivity and IVF efficiency [9].

The mucus penetration test shows the capability of spermatozoa to penetrate cervical mucus surrogates and it has been used to assess IVF and pregnancy results *in vivo* [2]. In our study, the increased motility and numbers of spermatozoa at distance results obtained during the mucus penetration test in the iodixanol group suggest that, compared to untreated spermatozoa, iodixanol-treated spermatozoa have more potential for producing fertilization and pregnancy success in the canine species.

In conclusion, 1.5% iodixanol supplementation of the second buffer protected canine spermatozoa during cryopreservation and maintained chromatin packing, effects that resulted in improved post-thaw motility and reduced cryocapacitation in frozen-thawed spermatozoa. Further studies, including examination of IVF and AI results, are necessary to fully describe the fertilizing capability of cryopreserved canine spermatozoa protected by the iodixanol supplementation.

Acknowledgments

Conflict of Interest

The authors declare no conflicts of interest.

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