수의학 석사 학위논문

Maintaining DNA integrity of canine frozen-thawed sperm using iodixanol supplementation

개 동결융해 정자의 DNA 온전성 유지를 위한 Iodixanol 첨가

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수의학과 임상수의학(수의산과학) 전공

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이 논문을 수의학 석사 학위논문으로 제출함

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Maintaining DNA integrity of canine frozen-thawed sperm using iodixanol supplementation

by Dimas Arya Abdillah

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in

Veterinary Clinical Sciences(Theriogenology)
Department of Veterinary Medicine, Graduate School
Seoul National University

We accept this thesis as confirming to the required standard

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Seoul National University

Maintaining DNA Integrity of Canine Frozen-Thawed sperm using iodixanol supplementation

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ABSTRACT

Sperm cryopreservation has been considered as an essential step for artificial insemination (AI). The cryopreservation sperm was found first time in the late 1930s and early 1940s. However, the number of fertile spermatozoa after post-

thawing has always been a challenge for researchers to preserve high genetic value organism and endangered species of dogs. In addition, successful sperm cryopreservation can bring several benefits such as preventing sexually transmitted disease, reducing animal stress during transportation, and etc. Because of those reasons above, the quality of sperm post-thawing has become the main concern to overcome successful artificial insemination. Thus, the purpose of this study was to improve canine sperm cryopreservation quality using cryoprotectant agent supplementation.

Cryoprotectant is one of the basic parts in the freezing extender to develop post-thawing motility quality of sperm. However, adding cryoprotectant into extender sometimes gives deleterious effect to sperm quality. Therefore, it is needed to find out the optimum concentration of the cryoprotectant agent in canine sperm. In this study, we treat sperm with Optiprep™ (iodixanol 60% in water) as a cryoprotectant supplementation. It has been widely used as supplementation in several species. Nonetheless, there is no study on Optiprep™ supplementation in canine sperm cryopreservation.

Determining Optiprep™ concentration was performed at room temperature within 1 hour. We compared sperm viability and kinematic parameter of fresh spermatozoa in control and treatment groups. As a results, 2.5% concentration of Optiprep™ was the optimum concentration compared to the control group. Based on experiment results in fresh spermatozoa, we decide to analyze 2.5% concentration of Optiprep™ in cryopreservation process.

Frozen–thawed spermatozoa in 2.5% Optiprep™ treatment group showed better quality in motility compared to the control. In addition, the comparison between control and treatment group mentioned that cryopreservation using 2.5% Optiprep™ was more useful in enhancing high sperm quality. Moreover, improved expression level of protamine-2 (*PRM2)*, protamine-3 (*PRM3)*, anti-apoptotic gene (*BCL2*), and sperm acrosome associated-3 (*SPACA3*), together with lower expression ROS modulator (*ROMO1*) and pro-apoptotic gene (*BAX*) than control, showed that 2.5% Optiprep™ treatment was advantageous for the spermatozoa.

Furthermore, after thawing process, the quantity of live spermatozoa in Optiprep™ group showed significantly higher than control. The number of live capacitated spermatozoa which undergoes acrosomes reaction post-incubation in CCM was greater in the Optiprep™ treated group than in the control. In mucus penetration test, the number of sperm traveled three cm marker was also higher than the control.

In conclusion, the Iodixanol supplementation in canine sperm cryopreservation significantly enhanced post-thawing sperm quality. Iodixanol supplementation can reduce ROS levels, decrease cryocapacitation, and increase viability, motility, and fertility

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Keywords: canine, frozen-thawed sperm, cryopreservation, protamine

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LIST OF ABBREVIATIONS

AE	**Acrosome exocytosis**
AI	**Artificial insemination**
ANOVA	**Analysis of variance**
ART	**Assisted reproductive technology**
CASA	**Computer assisted sperm analysis**
CCM	**Canine capacitation media**
cDNA	**Complementary DNA**
CPAs	**Cryoprotectant agents**
DNA	**Deoxyribonucleic acid**
DSIA	**Dead spermatozoa with intact acrosomes**
DSRA	**Dead spermatozoa with reacted acrosomes**
LN_2	**Liquid nitrogen**
LSIA	**Live spermatozoa with intact acrosomes**
LSRA	**Live spermatozoa with reacted acrosomes**
Mg	**Magnesium**
mSOF	**Modified synthetic oviduct fluid**
NCBI	**National center for biotechnology information**
P4	**Progesterone**
PRM1	**Protamine 1**
PRM2	**Protamine 2**

qPCR	**Quantitative real time polymerase chain reaction**
RNA	**Ribonucleic acid**
ROS	**Reactive oxygen species**
SPACA3	**Sperm acrosome associated 3**

PUBLICATION LISTS

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1. **Dimas Arya Abdillah**, MinJung Kim, HyunJu Oh, Kihae Ra, Seok Hee Lee, ByeongChun Lee. Optiprep™ effect on protamine level of canine sperm during cryopreservation. Australasian extracellular vesicle conference. 2018.
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3. **Dimas Arya Abdillah**, MinJung Kim, HyunJu Oh, Kihae Ra, SeokHee Lee, ByeongChun Lee. Sulforaphane supplementation effect on canine sperm cryopreservation. The korean society of veterinary science (KSVS). 2018.
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LITERATURE REVIEW

1. Physiology of canine spermatozoa

The Golgi apparatus from immature spermatid create lysozyme which is full of proteolytic enzyme to form acrosome during differentiation. Furthermore, centriole will initiate the growth of the flagellum. The middle piece or neck spermatozoa are formed by the mitochondria. The nucleus passes important structural change. The nucleus becomes compacts when histones are replaced by protamines [1]. Non-motile sperm gain their fertility in epididymis. They will get some modification to make it functional [2].

In the short time, spermatozoa are mixed with seminal plasma before ejaculation. Spermatozoa are moved from cauda epididymis to the orificium urethra externa at the end of glans penis. Prostate gland in dog is the gland that produce major substance for constructing seminal plasma. Even though some seminal plasma components nourish the spermatozoa, it also has a critical role to support spermatozoa fertility [3]. There are three kind of fractions in dog's ejaculate: the main fraction is the second fraction which contains sperm rich suspension. The testes and epididymis sc prostatic fluid which is used for the first and the third fraction in dog ejaculate [4]. In one dog's ejaculate, above 95% composition of the total volume is consisting of prostatic fluid [3]. The function of prostatic fluid is still in debate. Some researchers mentioned that the existence of large amount of prostatic fluid in the sperm suspension had negative effects on the post-thawing quality of the dog sperm [5-8]. However, other researchers reported that an sufficienst amount of

prostatic fluid improved fertility and maintain frozen thawed dog sperm function [9, 10].

Fertilization process needs fusion between one spermatozoa and one egg. During capacitation spermatozoa obtains hyperactivation and the competence to experience the acrosome reaction that are important for fertilization [11]. Sperm surface lose cholesterol and release other sterols and glycoproteins obtained in epididymis. These mechanisms create sperm membrane more fluid for fertilization [12]. Hyperactivation is also needed to penetrate glycoprotein matrix called the zona pellucida (ZP) [13]. Eggs are covered by ZP. It assembled inside a hyaluronic acid-rich jelly consisting of somatic cells. Normally, it is called as cumulus oocyte complex (COC). After sperm penetrate zona pelucida, they continue to move until the oolema is found. Inside the perivitelline space, the sperm movement stop immediately after attaching oolema and sperm membrane can fuse with it. The sperm binds the membrane-anchoring glycosylphosphatidylinositol (GPI), an egg essential protein for fertilization [14]. After that, pronucleus is made by sperm chromatin decondensation and nuclear envelope formation. The pronucleus of male is assembled after a envelope of a new nuclear is made from the nucleus parts [15]. Cortical reaction of the oocyte is started to prevent polyspermy by solidifying ZP during sperm and oocyte fusion. The centrosome organizes microtubules that pull pronuclei from sperm and egg to the center of the ovum cytoplasm. The two pronuclei membrane rupture and mixing nuclear material are necessary to form zygote. The result is creating new genetic from parental genetic material [16]

2. Cryopreservation in canine spermatozoa

The sperm motility has been proved to be an important parameter for sperm's capability to fertilize oocyte assessment. Comparing to activity of mitochondrial and integrity of sperm membrane, motility is vulnerable to osmotic stress. The accurate information about the osmotic pressure in medium effect on the sperm quality is also given [17]. Osmotic stress that can reduce motility in dogs might be figured by the different sperm membrane compartments composition, showing different levels of sensitivity to osmotic conditions. This event showed important information about the dog semen cryopreservability and must be known before carrying out sperm cryopreservation [18]. Higher bent tail abnormality of fresh semen can be indicator that dog sperm membrane was more susceptible to osmotic pressure of the medium compared to other mammals [17, 19]. A structural weakness might be reflected by bent tail existence [20] and had been found to be the most popular abnormality linked with decreased canine spermatozoa function [19].

The source of osmotolerance variation might come from different prostatic fluid components. These compositions accommodated the spermatozoa from whole ejaculates to be capable of tolerating a wider osmolality range than those from the second fraction. In contrast, the sperm motility loss from the whole ejaculates in an isosmotic condition indicated that prostatic fluid did not protect the sperm motility. Predicting the causes of this event was hard because the prostatic fluid in dog semen was supplying the sperm-coating substances, supporting the sperm activity maintenance [8, 21]. The cryobiology principal variables derived from animal

gametes studies were cooling and warming rates, ice formation inside cell, excursions volume of cell in cooling process, chilling injury, temperature, and response of osmotic pressure [17, 22].

3. Development of canine sperm cryopreservation

There are three kind of cryopreservation method that still develop, which are slow freezing (conventional freezing), rapid-freezing, and ultra-rapid freezing (vitrification) [23]. Approximately, two until four hours are needed to perform manual slow freezing. The sample must reach 5 ℃ gradually at rate around 0.5-1 ℃ /min. Then the rate of temperature declined to 1-10 ℃/minute until -80 ℃. In the end, LN_2 (-196 ℃) was kept the sample [24]. In the rapid freezing protocol, after adding cryoprotectant to the sample, sample is loaded into straw in 15 until 20 cm above from LN_2 for 15 minutes. Then kept in the LN_2 (-196 ℃). There are several approaches in vitrification method. One approach is making ice from pure water slowly, until temperature point of vitrification reached, and usually called as 'Tg' (glass transition temperature). Another approach is either by frozen portion of ice sublimation at sub-zero temperatures to remove excess water, or direct vacuum/air drying to evaporate liquid phase, or softening glassy material left from lyophilization result [25].

The way of making dilution on freezing sperm preparation can be completed in one or two steps. One step and two step dilution effect was explained well by Peña

and Linde-Forsberg [26], in which the different thing was the extender administration time. In one step dilution, the extenders were administered to sperm before equilibration, on the other hand, in two-step dilution, after equilibration and before freezing the second buffer was administered. Those procedures were also performed to investigate the effects of placing the straws horizontally abouve LN_2 and gradually moving close to LN_2. In addition, the researchers compared the sperm viability after thawing at 70 ℃ for 8 sec, and by warming the frozen semen at 37 ℃ for 15 sec. Then they evaluated the effects of extender using different concentration when freezing canine semen and diluting the sperm with Tris buffer after thawing at different concentrations on the post-thaw sperm motility and membrane integrity. The results mentioned that canine sperm frozen in an extender at a concentration of 200×10^6 cells/ml and diluted 1:4 or 1:2 in a plain Tris buffer immediately post-thaw saw increased viability. The combination of two steps and fast-thawingThe best procedure was using the sperm into two steps and fast-thawing them in a water bath at 70 ℃ for 8 sec.

Most canine sperm freezing methods use 0.25 mL (mini straws) and 0.5 mL (midi straws) for containing the frozen specimen before storing them in liquid nitrogen (LN_2). However, canine sperm frozen by the pellet technique is still used in some clinics. Dry ice is utilized for cooling small drops of sperm in pellet technique and preserved them inside LN_2. Then, the sperm can be thawed rapidly, simultaneously reducing the cryoprotectant concentration by direct immersion into

a solution. Glycerol, egg yolk, and a high lactose contents are extensively applied in several species for the pellet freezing protocol and it is successfully used in dogs.

4. Cryodamage in canine spermatozoa

The consequences of sperm cryoinjury are caused by osmotic and oxidative stress that dramatically alter the acrosome status, composition of membrane lipid, sperm motility, and viability. Sperm plasma membrane is the primary site for modification due to cryopreservation process. Likewise, cryopreserved sperm are in a incompletely capacitated condition [27]. This process is usually called cryocapacitation. Many reports mentioned, that mostly cholesterol efflux leads to the increasing of membrane bilayer permeability and fluidity that make sperm cell capacitated more. Cholesterol loss is probably result of peroxidative damage to membrane lipids that occur rapidly due to lipid peroxidation [28]. The molecular action of tyrosine phosphorylation throughout freezing step is induced by cholesterol efflux in membrane lipid components. The protein tyrosine phosphoryilation pattern and deficiency of sperm superficial protein in freezing procedure were reported to be similar with *in vitro* capacitated spermatozoa [29].

A numerous studies mention that reactive oxygen species (ROS) has several vital roles in fertility/infertility. In natural sperm activity, such as capacitation, acrosome reaction, and hyperactivation, ROS has significant physiologic roles. However, when ROS is produced excessively, and antioxidants is disrupted, ROS will transform into oxidative stress. ROS can decrease probability of oocyte

diffusion, block sperm-egg fusion, arrest motility of sperm, and sperm DNA damage, leading to serious problem in embryo development [30].

Mitochondrial DNA and membrane are believed to be the cause of impaired fertility and motility due to cryopreservation process. The cryodamage mechanism in cryopereservation that decreases the motility and sperm fertilizing ability has not been determined clearly. In ram semen, 4-hydroxynonenal as a lipid peroxidation derivatives, have been reported to be negate sperm motility [31]. Moreover, important cell structure such as mitochondria and DNA integrity will be affected. Sperm mitochondrial has a critical function to maintain cell's energy, motility, and further for fertility [32]. Recently, overproduction of ROS in sperm mitochondria affected motility and induced DNA damage. Many researchers are involved to solve this important issue. The addition of chemical such as antioxidant and cryoprotectant agent may reduce cryodamage effects.

5. Cryoprotectant in canine sperm cryopreservation

It is broadly understood that irreversible injury is caused by intracellular ice formation as a cellular stress. It has long been suspected that CPA alter liquid water transitions become ice. In early history of cryopreservation, Nash mention that hydrogen bonding between water and CPA was linked to transform water properties [33]. The cryoprotectant agent can be divided into two categories permeate cells (ethylene glycol, glycerol, and dimethyl sulfoxide) and non-permeate cells (sugar, proteins, sugars and synthetic macromolecules) based on their capability to enter the

cell. Generally, adding cryoprotectants to cryopreservation in cell is necessary but there may be potential for toxic effects of these substance. Canine spermatozoa were frozen using many different extenders and methods but most of successful sperm cryopreservation used buffer/extender supplemented with glycerol which normally act as cryoprotectant agent in freezing protocol.

Applying high glycerol contents has a adverse impact on fertility [34, 35]. Glycerol is the most used cryoprotectant in most species, but some experiments compare the use of glycerol with dimethyl sulfoxide and its combinations [36]. The diluent, cooling method, and species determine the optimal concentration of glycerol. The cooling rate also assigns the concentration; for example: faster cooling rates require a lower concentration. Thus, the range of glycerol concentrations for optimal results is reached between 4% to 11% (v/v) [37].

Besides cell membrane protection from cold shock, egg yolk has mild cryoprotectant characteristics. They are normally assimilated into diluents with spermatozoa [36]. The egg yolk concentration around 3 to 25 % (w/v) is usually applied among species [37]. In several studies, for dog spermatozoa preservation has used approximately 20% concentration of egg yolk [38-40]. A study explained about the effect of processing protocols, cryoprotectants, and diluents of sperm on post-thaw canine spermatozoa motility in straws. It is showed that tris-egg yolk based extender with 2 to 4% glycerol had highest motility after thawing. Glycerol concentration seemed to be useful for frozen-thawed spermatozoa in certain concentration. The diluent type used was determined the optimal glycerol

concentration. Thawing specimens in water bath with 75 ℃ temperature water bath for 12 sec give the highest motility [41].

INTRODUCTION

Sperm cryopreservation is an essential step for artificial insemination (AI), which is the most widely utilized 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 [42]. In addition, sperm cryopreservation for AI can prevent sexually transmitted diseases such as brucellosis and herpes virus infections [43]. In wild animals, AI with cryopreserved spermatozoa has had a considerable effect in conservation management of African wild dogs [44]. 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 [45].

During the cryopreservation process, osmotic pressure and ice formation can cause cryoinjury and loss of viability and sperm function post-thawing [46]. 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) [47] [48]. An disproportion between ROS output during cryopreservation and antioxidant defenses of spermatozoa result in oxidative stress [49] and lipid peroxidation of plasma membranes that composed huge number of polyunsaturated fatty acids [50]. Consequently, important cell components such as DNA are damaged by cryopreservation [51]. On the other hand, complete elimination of ROS is also detrimental because several ROS are required for natural reproductive events [52].

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 [53] and results in the construction of toroids, which are condensed DNA strands that protect sperm chromatin from oxidative damage [54]. After a freeze-thaw cycle, bonding of disulfide bridges in protamine is disturbed, and DNA damage is increased [55]. In addition, less compaction of DNA and more susceptibility to damage has been observed in frozen-thawed canine spermatozoa [56], and a higher quantity of sperm DNA loss reduces the quality of the embryo [57]. Insufficient sperm chromatin content is also correlated with an elevated early pregnancy loss risk throughout *in vitro* fertilization (IVF) and intracytoplasmic sperm injection process [58]. 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 [59, 60]. Adding Optiprep™ (60% iodixanol in water) could change ice crystal formation into a intracellular dendritic form, which results in a more sperm-friendly environment [61]. However, the mechanism associated with iodixanol protecting canine sperm from DNA damage during freezing step has not yet been determined. We assumed that iodixanol treatment conserves sperm protamines to help maintain DNA integrity throughout freezing process.

MATERIALS AND METHODS

1. Chemical use

All chemicals used in this experiment were purchased from Sigma-Aldrich Co., LLC. (St. Louis, MO, USA) unless otherwise stated.

2. Semen collection and preparation

The subjects used in this experiment were 4 healthy and mature male beagles, 4–6 years old, and weighing 8–10 kg. The dogs were maintained individually inside indoor cages. Their housing, facilities and procedures of animal care followed criterias installed 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 number; SNU-160602-6-4). Semen samples from the dog were acquired twice weekly and we used only ejaculates having sperm concentrations $\geq 100 \times 10^6$/mL, motility $\geq$ 70%, viability $\geq$ 80%, and normal morphology $\geq$ 80%.

3. Determination of Optiprep^{TM} concentration

Optiprep^{TM} (60% iodixanol in water) was mixed into the first buffer. The first buffer is consisting of 24 g/L Tris [hydroxymethyl] aminomethane, 14 g/L citric acid, 8 g/L fructose, and 0.15 g/L kanamycin sulfate. All ingredients of first buffer were mixed in distilled water until pH 6.6 and 290 mOsm. The ejaculates were collected from 4 male beagles and pooled into 15 mL tubes. The combined ejaculates were washed by centrifuging at 100*g* for 1 min at room temperature to get rid of

debris [62]. 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%, 2.5%, and 5% treatment groups.

Afterward, motility and kinematic parameters were evaluated. We dropped 10 μL of sperm on a slide and mounted using a coverslip. Spermatozoa in 5 different fields were assessed and tracked at least 200 motile spermatozoa for the kinematic parameters in each experiment by using a sperm examination imaging system (FSA2011 premium edition version 2011, Medical Supply Co., Ltd., Korea).

Additionally, sperm viability was examined by using an eosin-nigrosin staining technique. Briefly, smears from sperm suspension were made after adding the stain (1:1) to the first buffer with Optiprep™. 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.

4. Sperm freezing and thawing

The second experiment was executed to inspect the effects of iodixanol on cryopreservation. The first buffer with equal volume was put to pooled ejaculates for washing. Then, it was centrifuged at 700*g* for 5 min at room temperature. The pellet was refilled by mixing the first buffer to obtain 200×10^6 sperm/mL concentration. Every aliquot was combined with the second buffer (40% [v/v] egg yolk, 54% [v/v] first buffer, and 6% [v/v] glycerol) and iodixanol. The second buffer volume was

added carefully to produce 14%, 19%, 27%, and 40% of the total volume and filled sequentially around 30 sec intervals as described in a previous study [63]. Straws (0.25 mL; Minitub, Germany) were used to store the extended sperm suspension. In equilibration phase, within 1 h, straws were kept at 4℃. Subsequently, all straws were put horizontally 2 cm above liquid nitrogen (LN_2) in 15 min. After that, they were dropped into the LN_2. After one week, the sperm was thawed by using 60℃ water bath for 7 sec. To obtain 14%, 19%, 27%, and 40% of the total volume, all samples had to be diluted (1:5) by adding the first buffer. Thawed sperm motility and kinematic parameters were determined with a sperm examination imaging system as mentioned in the Determination of Optiprep™ concentration section. Then, all thawed spermatozoa were evaluated as described in the following sections.

5. Gene expression analysis and chromomycin A3 staining

Briefly, frozen-thawed sperm samples from all treatment groups were used for RNA measurement. To check transcript abundances using oligonucleotide primer sequences, we utilized Quantitative real-time PCR (qPCR). All of primer sequences are listed in Table 1. The gene 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. The RNA was isolated using Trizol reagent (Invitrogen, USA), referring to the manufacturer's protocol. We utilized Maxime RT PreMix (Intronbio, Korea) to produce complementary DNA. The qPCR evaluations were executed using a Step

One Plus Real-Time PCR System (Applied Biosystems, USA). Every target gene's expression was measured relative to that of the internal gene (*ACTB*) using the equation, $R = 2^{-[\Delta Ct\ sample - \Delta Ct\ control]}$ [64].

Frozen-thawed canine sperm smears were fixed in methanol/glacial acetic acid (3:1) at 4℃ for 5 min. Control and treatment group slides were treated for 20 min with 100 µL CMA_3 solution. The CMA_3 solution contained 0.25 mg/mL CMA_3 in McIlvane's buffer (pH 7.0) supplemented with 10 mM $MgCl_2$. 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 1000× magnification. A total of 200 spermatozoa were randomly evaluated on each slide. Evaluation of CMA_3 was completed by identifying two categories of staining forms: bright green fluorescence of the sperm head (CMA_3 positive/abnormal chromatin packaging) and dull green staining (CMA3 negative/normal chromatin packaging) of the sperm head.

6. Sperm capacitation test

The contents of one cryopreserved straw were divided into 2 aliquots after thawing in 37℃ water bath 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. Next, a same trypan blue volume 0.27% (v/v) was added and the combination was

mixed on a slide. Sperm smears were then fixed in a 37% formaldehyde solution was used to fix sperm smears. It took about 2 min. Then, sperm smears were washed with distilled water. The Giemsa stock solution was newly prepared by combining to distilled water. Slides were plunged in 7.5% (v/v) of Giemsa stain, air-dried in a vertical position, then mounted with cover slip. 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 reacted acrosomes (LSRA), dead spermatozoa with intact acrosomes (DSIA), and dead spermatozoa with reacted acrosomes (DSRA). 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), reacted acrosomes (lavender), and those without acrosomes (pale gray). A canine capacitation medium (CCM) mixed with 1.0 mM $MgCl_2$ 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 [65].

7. Mucus penetration test

Modified synthetic oviduct fluid (mSOF), as a surrogate mucus, was loaded into marked flat capillary tubes (10 cm long, 3 mm deep; Camlab, UK) that were closed 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,

Eppendorf tube with 100 µL sperm suspension inside was inserted by the capillary tube. Samples were laid horizontally for 2 h at room temperature. The spermatozoa accomplishing the 1 cm and 3 cm markers in the capillary tube were then counted.

8. Statistical analysis

All values in this experiment are mentioned as mean ± standard error of the 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 version 5 (GraphPad Software, USA) was used for the statistical analyses.

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

Gene	Primer Sequence (5'-3')	Product size (bp)	NCBI accession number
ACTB	F: GGCATCCTGACCCTGAAGTA	148	NM_001195845.1
	R: GGTGTGGTGCCAGATCTTCT		
BCL2	F: CTCCTGGCTGTCTCTGAAGG	120	NM_001002949.1
	R: GTGGCAGGCCTACTGACTTC		
BAX	F: GACGGCCTCCTCTCCTACTT	145	NM_001003011.1
	R: GGTGAGTGACGCAGTAAGCA		
ROMO1	F: TGTCTCAGGATCGGAATGCG	100	XM_534406.4
	R: TCCCGATGGCCATGAATGTG		
PRM2	F: AGGAGGAGATACAGGAGGTGC	148	NM_001287148
	R: CTTGCAAACTCAGGGCTTGG		
PRM3	F: GGCCACGAATCCTCCATGGA	128	XM_847270.4
	R: AGCTCCTCCTCTTCCTCCT		
SPACA3	F: GGATTTCGGCATGGAGGGAT	149	NM_001197087.1
	R: ACTTCCGGCTGTTGATCTGG		

RESULTS

1. Determination of optimum Optiprep™ concentration

The percentage of fresh spermatozoa exhibiting motility in the 5% Optiprep™ group was significantly decreased (48.0% ± 6.3%) compared to the percentages in the 0%, 1%, and 2.5% Optiprep™ groups (81.5% ± 1.9%, 75.2% ± 7.9%, and 76.8% ± 7.0%, respectively) (Table 2).

Four independent replicates were examined to assess iodixanol effect (as Optiprep™) on fresh spermatozoa viability. The dead spermatozoa percentage in the 2.5% Optiprep™ group was significantly reduce (4.1% ± 0.4%) than in the control (9.9% ± 1.2%) and 5% Optiprep™ (11.1% ± 1.4%) groups (Fig. 1). We did not acquire any significant difference among the 1% Optiprep™ and control groups.

Table 2. Motility, linearity, straightness, ALH, and dead canine spermatozoa 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
1% Optiprep™	75.2 ± 7.9[a]	29.7 ± 1.9	8.6 ± 1.4[a]	4.0 ± 0.9	44.1 ± 1.4
2.5% Optiprep™	76.8 ± 7.0[a]	29.2 ± 2.2	4.1 ± 0.4[b]	4.4 ± 0.6	44.3 ± 1.7
5% Optiprep™	48.0 ± 6.3[b]	25.6 ± 3.7	11.1 ± 1.4[a]	2.6 ± 0.3	41.7 ± 1.9

[a,b] Values within a column with different superscripts differ significantly among control, 1%, 2.5%, and 5% Optiprep™ ($p < 0.05$, 4 replicates). Straightness, the leaving of the cell track from straight line; Linearity, the straightness of the track; ALH, amplitude of lateral displacement

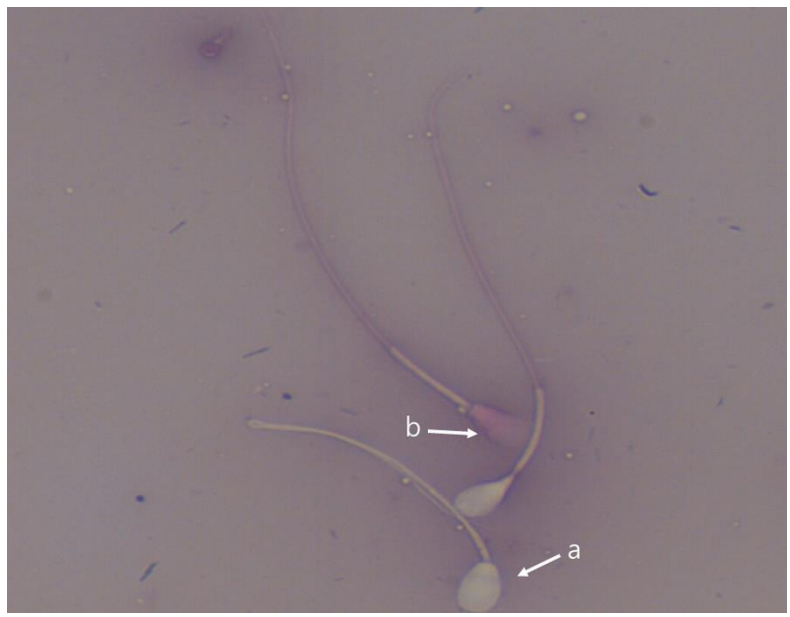

Fig 1. Image of eosin nigrosin canine sperm staining, live spermatozoa (a), dead spermatozoa (b).

2. Effect of Optiprep™ on frozen-thawed sperm kinematic parameters and mucus penetration test

The frozen-thawed sperm percentage exhibiting motility after treatment with 2.5% Optiprep™ was significantly higher (54.0% ± 0.9%) than that in the control group (46.6% ± 2.2%, Table 3). In our result, we had no significant differences in linearity, ALH, and straightness percentages between the treatment and control groups (30.6% ± 2.4% *vs*. 28.7% ± 2.2%; 3.9% ± 0.4% *vs*. 3.4% ± 0.4%; 56.1% ± 2.6% *vs*. 57.0% ± 1.9%, respectively). The mucus penetration test results showed that sperm counts in the 2.5% Optiprep™ treatment group were significantly higher at both the 1 cm and 3 cm markers (147.9 ± 3.1 and 56.4 ± 5.3 cells, respectively) compared to those in the control group (138.6 ± 2.9 and 40.7 ± 4.7 cells, respectively, Table 3).

Table 3. Motility, linearity, straightness, ALH, and canine sperm mucus penetration test using frozen-thawed spermatozoa treated with iodixanol

Group	Motility (%)	Linearity (%)	ALH (μm)	Straightness (%)	Sperm count in 1 cm marker	Sperm count in 3 cm marker
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]
2.5% Optiprep™	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]

[a-b] Within a column, values with different superscripts differ significantly among control and 2.5% Optiprep™ ($p < 0.05$, 6 replicates). Straightness, the leaving of the cell track from straight line; Linearity, the straightness of the track; ALH, amplitude of lateral head displacement.

3. Effect of Optiprep™ on gene expression and CMA_3 staining

Treatment with 2.5% Optiprep™ during sperm cryopreservation significantly increased the *BCL2* transcript level by 1.6-fold and decreased the *BAX* level by 0.5-fold related with the control group (Fig. 2). Transcript levels of *PRM2* and *PRM3* significantly improved by 2.1-fold and 1.8-fold, respectively, related with the control. Gene expression of *ROMO1* was significantly reduced by 0.8-fold compared with the control. Next, *SPACA3* gene expression significantly increased to about 1.4-fold of the control level.

CMA_3 staining was performed for detection of protamine deficiency. Frozen-thawed spermatozoa treated with 2.5% Optiprep™ showed significantly higher protamine levels than that in the control (25.5 ± 2.5 % and 34.8 ± 1.3 %, respectively) (Fig. 3).

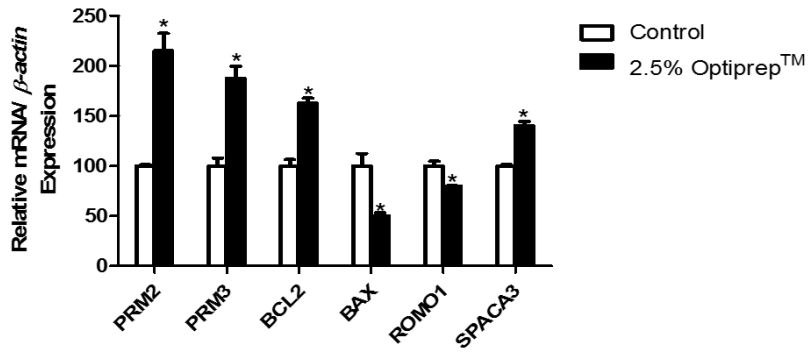

Fig 2. Expression of gene associated to apoptosis, protamines, and oxidative stress with sperm cryopreservation in control and 2.5% OptiprepTM (* $p < 0.05$).

PRM2, Protamine 2 (control DNA packaging in the nucleus); *PRM3*, Protamine 3 (control sperm motility); *BCL2*, B-cell lymphoma 2; *BAX*, X protein associate with *BCL2*; *ROMO1,* a mitochondrial ROS modulator; *SPACA3*, sperm associated 3 (have roles in sperm egg recognition and fertilization).

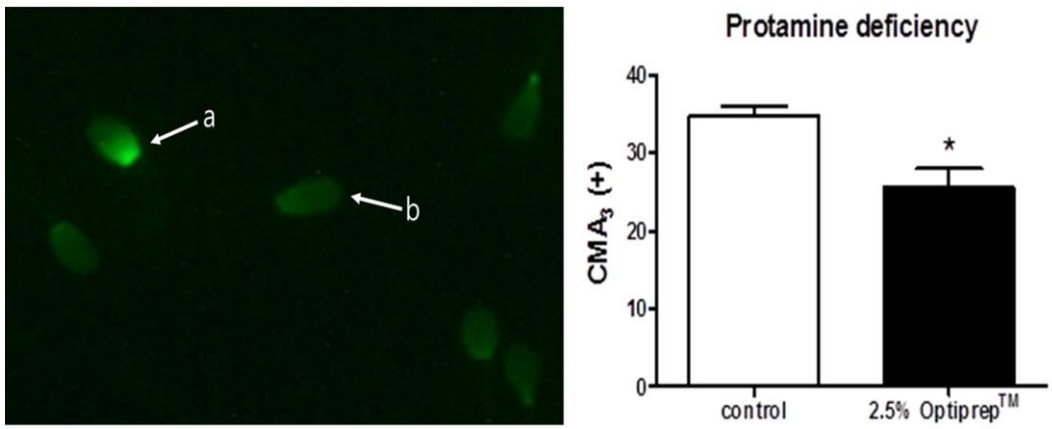

Fig 3. CMA_3 staining of sperm chromatin, spermatozoa with protamine deficiency (a), normal spermatozoa (b). Results of CMA_3 staining in control and 2.5% Optiprep™ (* $p < 0.05$, 5 replicates)

4. Effect of 2.5% Optiprep™ on the sperm capacitation test

The live sperm percentage post-thawing in the 2.5% Optiprep™ treatment group (71.9% ± 0.9%) was significantly better than in the control group (59.1% ± 0.3%). In addition, adding Optiprep™ 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). After incubation for 4h in CCM, all sperm acrosomes in both the treatment and control groups were lost. The percentage of LSRA 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 DSRA percentage was significantly poorer than that in in the control (21.4% ± 0.9% *vs.* 26.5% ± 1.2%, respectively) as shown in Table 4.

Table 4. The staining results of acrosome after thawing and incubation in canine capacitation medium (CCM)

Group	Post thawing		After incubation in CCM			
	Live sperm (%)	**Dead sperm (%)**	**LSIA (%)**	**LSRA (%)**	**DSIA (%)**	**DSRA (%)**
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]
2.5% 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]

a-b Values within a column with different superscripts differ significantly between control and 2.5% Optiprep™ ($p < 0.05$, 5 replicates). LSIA, live spermatozoa with intact acrosome; LSRA, live spermatozoa with reacted acrosome; DSIA, dead spermatozoa with intact acrosome; DSRA, dead spermatozoa with reacted acrosome.

DISCUSSION

Single step protocols have been reported reduces 26.2% to 40.2% of motility after post thawing step [35, 66, 67]. The critical parameter of sperm is motility that can be used for predicting capability of sperm to fertilize oocyte and it also more sensitive to osmotic stress. Based on that report, I used multistep protocol that give better result rather than single step protocol. Multistep protocol is believed can bring gradual osmotic challenge and support sperm shape and motility [68]. That reducing motility event is well known as the key of cryopreservation which is also related to cryoprotectant agent. The key problem above is possibly because of ROS, which can reduce viability of mammalian sperm during freezing process [69], it may associate with chromatin damage [51].

There are evidences that paternal factor is also a key in embryo quality. Abnormalities in the paternal DNA give negative effect to embryo quality. Late pronuclear construction and slow embryonic development are connected with poor sperm quality [70]. The deleterious effects of sperm DNA damage also cause delayed embryo cleavage, poor morphology, lower blastocyst formation and lower implantation rates [71, 72]. Poor blastocyst quality is the result of postponement further DNA repair pathways forces activation in cell division [73, 74]. Further, elevation of sperm DNA fragmentation is related to failure of assisted reproduction technology (ART) result [75].

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 Optiprep™ using fresh spermatozoa and a method described in a previous study [76]. The reduced motility and increased proportions of dead spermatozoa in 5% Optiprep™ (Table 2) might be due to a toxic effect of iodixanol on spermatozoa. The optimal Optiprep™ concentration found in our study was 2.5% (Table 3), which is similar to those for bovine [61] and buffalo [77] and comparable to ram [78], rats [79], 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 [61]. It seems that a loose net of dendritic ice, as created by 2.5% iodixanol, was suitable in dogs and cattle due to canine and bovine spermatozoa similarities in retinoic acid receptors, which support sperm function and structure [80]. Based on our results, 2.5% Optiprep™ was chosen for iodixanol treatment of canine spermatozoa in the subsequent experiments.

To explore Iodixanol effect on freezing sperm gene expression level, I analyzed several genes such as *BCL2*, *BAX*, *ROMO1*, *SPACA3*, *PRM3*, and *PRM2*. 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. Overexpressed *BAX* in cells counters the effect of *BCL2* gene on cellular survival that can accelerate apoptotic death [81]. In our result we confirmed that iodixanol treatment could reduce apoptosis. *ROMO1* is a key gene for generating production of mitochondrial ROS [82], and a high level of endogenous ROS created in the mitochondrial respiratory chain can cause random genetic

mutations and lead to programmed cell death [83]. After treatment with iodixanol, the gene expression level of *ROMO1* was significantly reduce compared to the control. Our results suggest that iodixanol could protect mitochondria during the freezing and thawing process [84] and result in a ROS reduction. The advantage of a lower ROS production could be seen in the acrosome reaction [85]. Indeed, the viable spermatozoa number 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 [86]. 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 [87]. Low expression of the *PRM2* gene may also be related to abnormal morphology and entry to the apoptotic pathway [88]. The *PRM3* gene is expressed in the cytoplasm and is important for sperm motility [89].

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.

CMA_3 staining is a simple and cheaper method to assess sperm protamination degree. In accordance with this result, any damage from freezing and thawing process inflicted on sperm DNA and also the previous studies have explained that a significant negative correlation was proved between CMA_3 (+) and *in vitro* fertilization (IVF) efficiency [90]. CMA_3 staining result mentioned to be of prognostic value for early pregnancy injury in IVF and the other researcher have reported DNA protamination connected with decreased fertilization and embryo development [91].

The spermatozoa penetration ability, IVF outcomes, and pregnancy *in vivo* was associated with mucus penetration result [92]. The principal of sperm mucus penetration test is to test the capability of sperm to move and survive in female tract fluid [93]. As mentioned by previous researchers, oviduct involve in important process such as capacitation, sperm selection, and embryo development [94]. 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, 2.5% Optiprep™ supplementation of the second buffer protected canine spermatozoa during cryopreservation and maintained chromatin packing, effects that resulted in improved post-thaw motility and lowered cryocapacitation in frozen-thawed spermatozoa. Further studies, including examination of *in vitro* fertilization and AI results, are necessary to fully describe

the fertilizing capability of cryopreserved canine spermatozoa protected by the Optiprep™ supplementation.

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국문초록

개 동결융해 정자의 DNA 온전성 유지를 위한 Iodixanol 첨가

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수의학과 수의산과·생물공학 전공

정액 동결은 인공 수정(AI)을 위한 필수 단계로 간주되어왔고, 1930년대 후반과 1940 년대 초반에 정액 동결법이 최초로 개발되었다. 그러나 높은 유전적 가치를 지닌 생물체와 개의 멸종위기종을 보존하기 위해 융해 후에도 생식능을 가진 정자의 수를 유지시키는 것은 항상 어려운 연구로 여겨졌다. 성공적인 정액 동결은 성매개질환의 예방, 운송 중 동물의 스트레스 감소 등과 같은 여러 이점을 가져올 수 있다. 융해 후 정자의 품질은 성공적인 인공 수정을 위한 중요한 요소이다. 본 연구의 목적은 동결 보호제의 첨가를 이용하여 개의 정액 동결을 향상시키는

것이다.

동결 보호제는 정자의 융해 후 운동성을 증진시키기 위한 동결 희석액의 기본 요소 중 하나이다. 그러나 동결 보호제와 같은 희석액에 일부 화학 물질을 첨가하면 정자의 품질에 해로운 영향을 미치는 경우도 존재한다. 따라서 개의 정액에서 동결 보존제 첨가의 최적 농도를 결정하는 것이 요구되었다. Optiprep™ (iodixanol 60 % in water)은 여러 종에서 희석액으로 널리 사용되고 있다. 그러나 개의 정액동결에서의 Optiprep™ 첨가에 대한 연구는 이루어지지 않았다.

Optiprep™ 농도 결정은 상온에서 1 시간 이내에 수행되었다. 해당 연구는 대조군과 처리군 사이의 신선 정자의 정자 생존 능력과 운동학적 지표를 비교했다. 결과적으로, 2.5% 농도의 Optiprep™ 이 대조군 및 다른 군에 비교하여 최적의 농도였다. 이 결과를 바탕으로 정액동결 과정에서 2.5% 농도의 Optiprep™를 분석하였다.

2.5 % Optiprep™ 처리군에서의 동결-융해 정자는 대조군에 비해 더 우수한 품질을 보였다. 처리군의 운동능은 대조군보다 유의적으로 향상되었다. 또한, 2.5 % Optiprep™을 이용한 정액동결은 정자의 품질을 향상시키는데 대조군보다 더욱 효과적이었다. 또한 프로타민 -2 (*PRM2*), 프로타민 -3 (*PRM3*), 항 - 세포 사멸 유전자 (*BCL2*) 및 정자 첨체 관련 유전자 -3 (*SPACA3*)의 발현의 높은 발현과 ROS 조

절자 (*ROMO1*) 및 세포 자멸사 유전자 (*BAX*)의 낮은 발현은 2.5 % Optiprep™ 처리가 정자에 유익하다는 것을 보여 주었다.

또한 해동 과정 후에 Optiprep™ 처리군에서 살아있는 정자의 비율이 대조군보다 유의적으로 높게 나타났다. CCM에서 배양 후 반응 첨체를 가진 살아있는 정자의 수는 Optiprep™ 처리군에서 대조군보다 더 높았다. 점액 침투 검사에서 3cm 마커를 이동한 정자의 수 또한 대조군보다 높았다.

결론적으로, 개의 정액 동결시 Iodixanol 처리는 해동 후 정자의 품질을 유의적으로 향상시켰다. Iodixanol의 처리는 ROS 수준을 감소시키고 수정능 획득을 줄이며 생존력, 운동성 및 번식능을 증가시킬 수 있다.

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주요어: 개, 체외 수정, 분할률, 수정 능 획득, 분할률, 프로타민

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