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Improvement of canine *in vitro* fertilization system using frozen-thawed sperm

개 정자 동결법 개발 및 이를 통한 체외수정 시스템 구축

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Improvement of canine *in vitro* fertilization system using frozen-thawed sperm

by Erif Maha Nugraha Setyawan

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

in

Theriogenology and Biotechnology

Department of Veterinary Medicine, Graduate School

Seoul National University

We accept this thesis as confirming to the required standard

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Declaration

This thesis is submitted by the undersigned for examination for the degree of Doctor of Philosophy to the Seoul National University. This thesis has not been submitted for the purposes of obtaining any other degree or qualification from any other academic institution.

I hereby declare that the composition and experiment of this thesis and the work presented in it are entirely my own.

Erif Maha Nugraha Setyawan

Improvement of canine *in vitro* fertilization system using frozen-thawed sperm

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ABSTRACT

In vitro fertilization (IVF) in dogs has always been the main obstacle for researchers in opening up the possibility of preserving endangered species of dogs, understanding the inherited diseases between dogs and humans, and applying gene editing techniques. The first successful IVF with live puppies was reported towards the end of 2015, using fresh semen and *in vivo* matured oocytes combined with embryo freezing. A small number of studies have been performed in IVF using frozen-thawed sperm with *in vivo/vitro* matured oocytes, but low

penetration and cleavage rates, delayed cleavage and high degenerated embryos were the main problems faced in this field. The post-thawing quality of sperm became one of several key elements in overcoming these problems. The aim of this study was to improve canine IVF using enhanced frozen-thawed sperm through a modified freezing protocol, antioxidant supplementation during cryopreservation, and adding conditioned media during capacitation.

A multistep freezing protocol, comprising serial loading and dilution of cryoprotective agents by dividing the total volume of extender into 4 steps (14%, 19%, 27%, and 40%) every 30 sec, was compared to a single step method in sperm function, morphology and osmolytes content. A comparison of the effects of glycerol and ethylene glycol were also performed. A spermine treatment using 0, 0.1, 1, 5, or 10 mM was analyzed for its effect on sperm quality, reactive oxygen species (ROS) level, cryocapacitation rate and gene expression related oxidation. The conditioned media (CM) from human adipose-derived stem cells (ASCs) which was used as supplement in canine capacitating medium (CCM) during frozen-thawed sperm capacitation. The 0, 25 and 50% CM supplementation were compared and the viability and gene expression related fertility of those groups were determined. The optimum CM combination were used for IVF then cleavage rate and embryo transfer was evaluated.

Frozen–thawed spermatozoa in the multistep group showed superior quality compared to those in the single step group, with regards to progressive motility, intactness of membrane and bend in tail. The multistep protocol also succeeded in minimizing osmolytes loss, such as carnitine and glutamate, compared to the

single step group. Moreover, using glycerol with the multistep group was more advantageous in maintaining high sperm quality compared to using ethylene glycol. Although motility did not increase with spermine treatment, membrane integrity was significantly increased. Higher percentages of linearity and straightness with a lower amplitude of lateral head displacement (ALH) in the spermine treated group indicated that spermine inhibited hyperactivation. Concentrations of intracellular and extracellular ROS were decreased in the treatment groups. Higher expression of an anti-apoptotic gene (*BCL2*) and lower expression of a pro-apoptotic gene (*BAX*), together with decreased expression of mitochondrial ROS modulator 1 (*ROMO1*), DNA repair due to oxidative damage (*OGG1*), spermine synthase (*SMS*), NADPH oxidase associated with motility (*NOX5*) and spermine amino oxidase (*SMOX*), showed that 5 mM spermine treatment was beneficial for the spermatozoa. Furthermore, after thawing, the proportion of live spermatozoa with intact acrosomes in the treatment group was higher than in the control. After incubation of the spermatozoa in CCM, numbers of live capacitated spermatozoa with reacted acrosomes were higher in the spermine treated group than in the control. CCM supplemented with 25% CM resulted in a significantly higher percentage of motility, progressive motility, linearity and viability than control and 50% CM groups. The expression of gene related to DNA packaging, motility and fertility in the 25% CM group were significantly upregulated compared with the control group. The percentage of live sperm reacted acrosome in the treated group was also significantly greater than the control group. We collected the oocytes from 37 bitches and mature oocytes

were recovered from 30 (81.1%) dogs then could produce 70.5% cleavage rate after IVF. Immature oocytes recovered from 3 bitches showed 25.0% cleavage rate and aging oocytes from 4 bitches (10.8%) showed 51.4% cleavage rate. Optimum cleavage rate were produced by IVF using mature oocytes compared with other stages. Moreover, IVF using frozen-thawed sperm resulted in a cleavage rate of more than 60%, which is higher compared to those of other studies.

In conclusion, the multistep freezing method is superior at maintaining sperm function and osmolyte content. Spermine supplementation reduces ROS levels and decreases cryocapacitation, and adding 25% CM in CCM increases sperm motility, viability and fertility. Furthermore, an IVF system using enhanced frozen-thawed sperm can improve the canine embryo production.

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Keywords: canine, frozen-thawed sperm, capacitation, *in vitro* fertilization, cryopreservation

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AE	**Acrosome exocytosis**
ANOVA	**Analysis of variance**
ART	**Assisted reproductive technology**
ASCs	**Human adipose-derived stem cells**
BDNF	**Brain-derived neurotropic factor**
CASA	**Computer assisted sperm analysis**
CCM	**Canine capacitation media**
cDNA	**Complementary DNA**
CM	**Conditioned media from human adipose-derived stem cells**
CPAs	**Cryoprotectant agents**
DMSO	**Dimethyl sulfoxide**
DNA	**Deoxyribonucleic acid**
DSIA	**Dead spermatozoa with intact acrosomes**
DSRA	**Dead spermatozoa with reacted acrosomes**
EGF	**Epidermal growth factor**
ELISA	**Enzyme-linked immunosorbent assay**
FBS	**Fetal bovine serum**
FGF	**Fibroblast growth factor**
FGFR	**Fibroblast growth factor receptors**
IGF1	**Insulin-like growth factor 1**
IVC	***In vitro* culture**

IVF	***In vitro* fertilization**
KSOM	**Potassium simplex optimization medium**
LN_2	**Liquid nitrogen**
LSIA	**Live spermatozoa with intact acrosomes**
LSRA	**Live spermatozoa with reacted acrosomes**
MAPK	**Mitogen-activated protein kinase**
Mg	**Magnesium**
mSOF	**Modified synthetic oviduct fluid**
NBT	**Nitro blue tetrazolium**
NCBI	**National center for biotechnology information**
NGF	**Nerve growth factor**
P4	**Progesterone**
PBS	**Phosphate buffered saline**
PCR	**Polymerase chain reaction**
PI3K	**Phosphatidylinositol 3-kinases**
PRM1	**Protamine 1**
PRM2	**Protamine 2**
PVS	**Perivitelline space**
qPCR	**Quantitative real time polymerase chain reaction**
RNA	**Ribonucleic acid**
ROS	**Reactive oxygen species**
RVD	**Regulatory volume decrease**
RVI	**Regulatory volume increase**

SPACA3	**Sperm acrosome associated 3**
TGFβ	**Transforming growth factor beta**
TNFα	**Tumor necrosis factor alpha**

PUBLICATION LISTS

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PART I

GENERAL INTRODUCTION

1. Literature review

1.1. Physiology of canine spermatozoa

A normal mature spermatozoon has two main parts in its structure: the head and the tail. The head consists of a nucleus, an acrosome and a post-nuclear cap. Two-thirds of the anterior surface is covered by membrane-bound lysosome with hydrolytic enzymes, called an acrosome. The tail is a self-powered flagellum, composed of the capitulum, a middle piece, the principal piece and the terminal piece [1].

During ejaculation, spermatozoa are transported immediately from the lumen of the cauda epididymis to the orificium urethrae externa, located at the end of glans penis, and are mixed with seminal plasma secreted by accessory sex glands. In the dogs, the prostate gland is the major organ that supports most of the seminal plasma. The mixtures are complex secretions, originally provided by the testes, epididymis and accessory sex glands. The seminal plasma is also made from essential factors which can modulate the fertilizing ability of spermatozoa [2]. A dog ejaculates in three fractions: the first and third fractions consist of prostatic fluid and original fluid from the testes and epididymis. The second fraction is sperm rich suspension [3]. The proportion of prostatic fluid is more than 95% of the total volume of a dog's ejaculate. The remaining parts consist of sperm and other components from the epididymis and testes [2]. There are contradicting reports on the function of prostatic fluid on sperm quality. Some studies found that the presence of significant volumes of prostatic fluid in the sperm suspension had detrimental effects on the post-thawing

quality of the dog sperm [4-7]. In contrast, other researchers reported that an adequate amount of prostatic fluid could help maintain sperm function and enhance the fertility of frozen-thawed dog semen [8, 9].

The mechanical process is the main point during sperm penetration into perivitelline space (PVS) which combines the hyperactivated motility pattern, or the star-spin movement, with the softening of the matrix in the zona pellucida by acrosin from the acrosome enzyme. The sperm fertilizes the oocyte by entering the PVS from the inner face of zona pellucida after the acrosome reaction is performed. The acrosomal membrane reacts only in the anterior cap region of the sperm head, with the equatorial surface remaining intact with the mid-piece part. The sperm movement is stopped after attaching to the oolemma, and the fusion of gametes is initiated by the oocyte in order to complete sperm engulfment. After the sperm is inserted into the ooplasm, the sperm chromatin and nucleus undergo decondensation. The male pronucleus is assembled after a new nuclear envelope is formed from the nucleus components. During sperm and oocyte interaction, a cortical reaction of the oocyte is initiated to stimulate the hardening of the zona and to avoid polyspermy. After that, when the male and female pronuclei enclose each other, the nuclear envelope is broken down. Their chromosomes are released into the ooplasm, which initiates the first cell division. A new genetic descendant is created by the completion of syngamy from parental genetic material [10-13].

1.1.1. Osmotic pressure in canine spermatozoa

The sperm motility parameter was more susceptible to osmotic stress than the sperm plasma membrane integrity and mitochondrial function, regardless of the source of the spermatozoa. The motility already proved to be an important parameter in the assessment of the sperm's ability to fertilize the oocyte. It also gave accurate information about the effect of osmotic pressure in medium on the sperm quality [14]. Reduced motility caused by osmotic stress in dogs might be determined by the different compositions of membrane compartments in sperm, which would exhibit varying sensitivity levels to osmotic conditions. This phenomenon showed valuable information about the cryopreservability of dog semen and should be understood before performing sperm cryopreservation [15]. Higher bent tail abnormality of fresh semen indicates that membrane of dog sperm is more sensitive to osmotic pressure of the medium than other mammals [14, 16]. A bent tail might reflect a structural weakness [17] and has been found to be the most common abnormality associated with decreased function of canine spermatozoa [16].

Different components of the prostatic fluid might be the source of variation in the osmotolerance. These compositions support that the spermatozoa from whole ejaculates could tolerate a wider range of osmolality than those from the second fraction. In contrast, the rapid loss of sperm motility from the whole ejaculates in an isosmotic condition indicates that the sperm motility apparatus was not protected by prostatic fluids. It is difficult to predict the causes of this phenomenon because the

prostatic fluid in dog semen is supplying the sperm-coating substances, which support the maintenance of sperm function [7, 18].

1.1.2. Antioxidant in spermatozoa

Another characteristic of canine sperm is sensitivity to oxidative stress. Oxidative stress is correlated with high rates of oxidation of cellular components and excessive production of reactive oxygen species (ROS) which is created by centrifugation or changing of the medium [19]. Negative effects of ROS on sperm quality have been reported, such as lipid peroxidation of the sperm cell membrane followed by damage of membrane structure [20], influence on the mitochondrial function and loss of fertilization capacity [21]. Sperm motility could be reduced by chromosomal and deoxyribonucleic acid (DNA) fragmentation caused by high concentrations of ROS. This can lead to disruption of mitochondrial and plasma membranes [22].

This is consistent with the results of the physiological characteristics from computer-assisted sperm analysis (CASA). Similar results reported that the detrimental effects of ROS were reduced by the addition of antioxidants to the extenders in alpaca, boar and canine, respectively [23, 24]. The imbalance between ROS production and degradation caused oxidative injury to the sperm membrane and a consequent impairment of related functional properties [25]. ROS was believed to start the cascade of lipid peroxidation in the sperm membrane, while membrane lipid peroxidation was correlated with decreased sperm motility and membrane

damage [26]. These results indicate that sperm membrane alterations, promoted by lipid peroxidation, could be avoided by antioxidant supplementation [20].

1.1.3. Growth factors in spermatozoa

Growth factors play an essential role in controlling male reproductive development and function, but an excessive release of growth factors can interfere with male sexual behavior and fertility, especially in sperm [27]. The growth factors participate in various aspects of the physiological regulation of male fertility and stimulation of sperm viability and fertility

The physiological regulation of the male fertility are stimulated by growth factors which participated in various aspects of viability and fertility. Many studies have reported that the seminal plasma contains many growth factors including the transforming growth factor beta 1 (TGFβ1) [28, 29], the epidermal growth factor (EGF) [30], the insulin-like growth factor 1 (IGF1) [31] and other cytokines and interleukins [32]. However, the concentrations of these growth factors can not only show their proportions in the medium but also provide the various interactions between exogenous and endogenous growth factors with the spermatozoa [33]. Some exogenous growth factors using supra-physiological concentrations (*in vitro*) are affecting to the motility and promoting the acrosomal reaction such as EGF in ram [34], TGFβ1, EGF and IGF1 in human sperm [33]. Higher expression of TGFβ1 also upregulate sperm activity [35].

The fertilizing ability of sperm is determined primarily by sperm motility, which is also the most important parameter in sperm function. Sperm motility is

regulated by a number of growth factors such as EGF, fibroblast growth factor (FGF) [36] and nerve growth factor (NGF) [37]. The addition of NGF into a semen extender can significantly increase the post-thawing motility of semen, which may promote the clinical application of NGF in assisted reproductive technologies (ART) [37]. With the addition of exogenous NGF at 0.5 ng/ml [38] and 10 μM [39], motility of sperm was increased significantly in humans. Exogenous NGF has improved sperm viability and decreased apoptosis levels in human spermatozoa [38, 40]. Exogenous NGF also had significant effects on the secretion of leptin, cell viability, and reduction of sperm apoptosis on bovine [41].

Fibroblast growth factor 2 (FGF2) is the best-characterized member of FGFs family, constituting the family of 17–34 kDa proteins, and can be found in sperm and oocytes [42]. The FGFs have specific receptors (fibroblast growth factor receptors, FGFRs) which are found in the cell membrane. When bound, they will activate the tyrosine kinase and form: 3 extracellular immunoglobulin-like domains, a single transmembrane domain, and 2 highly conserved cytoplasmic domains. Both ligands and receptors have been reported to be found in several locations such as the central nervous system or in reproductive and gastrointestinal tissues [43]. Their functions have been determined as cell proliferation, differentiation, adhesion, viability, apoptosis, and motility. The FGF/FGFR pathway and their components have also been reported in the male reproductive tract tissues [44]. The variant FGFR is involved in sperm production and perform sperm capacitation in subfertile male transgenic mice [45]. This report suggests that FGFR1 or FGFR2 mediate FGF signal for modulating sperm capacitation by differentially influencing the

downstream of phosphatidylinositol 3-kinases (PI3K) and mitogen-activated protein kinase (MAPK) activity [45].

The effects of IGF1 on canine sperm function during cooled and freeze-thaw storage limited the reduction of progressive motility and led to an improvement in mitochondrial membrane potential [46]. In addition, the role of IGF1 in maintaining sperm motility is assumed to be through metabolism by enhanced mitochondrial membrane potential [47], reducing the free radicals by antioxidant effects [48] and providing high intracellular calcium level by increased ion transport [49]. In contrast, the high activity of fructose metabolism in buffalo sperm as a result of IGF1 supplementation is related to the generation of superoxide, which promote oxidative damage [50].

1.2. Cryobiology of canine spermatozoa

The principal variables of cryobiology derived from the study of animal gametes are cooling and warming rates, developmental stage and species, intracellular ice formation, cell volume excursions during cooling, osmotic responses, temperature, and chilling injury [14, 51]. Cryopreservation of canine semen as pellets exhibited better post-thawing progressive motility compared with freezing in 0.5 or 0.25 mL straws because of the extender used. Overall, the total motility and progressive motility are lower in frozen-thawed semen than with fresh or chilled semen [2]. Farstad [10] reported that post-thaw motility of 40% or greater is desirable and the highest rates of cell survival rate chosen for freezing is paired with an appropriate rate of thawing.

1.2.1. Development of cryopreservation in dogs

Many freezing methods for canines have been developed and the derivation of these protocols generally improved based on the applicative approach. Peña and Linde-Forsberg [52] reported the effects of a one-step dilution, in which both extenders were added before equilibration, compared with those of a two-step dilution, in which the second buffer was added after equilibration and immediately before freezing. Two freezing procedures were also performed to determine the effects of keeping the straws horizontally above the LN_2 surface in a Styrofoam box compared with gradually moving them vertically closer to LN_2 in a tank. They also compared the effects of thawing on post-thaw viability of a frozen dog by placing its sperm in a water bath at 70 °C for 8 sec, and by warming the frozen semen at 37 °C

for 15 sec. Then they proceeded to test the effects of freezing canine semen at different concentrations and diluting the sperm immediately after thawing with Tris buffer at different concentrations on the post-thaw sperm motility and membrane integrity. The results exhibited 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 compared to sperm frozen at lower or higher concentrations and/or not diluted after thawing. The best procedure was to dilute the sperm into two steps and to freeze them using a Styrofoam box method, and fast-thawing them in a water bath at 70 °C 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, as the pellet method can freeze small droplets of sperm on dry ice and store them in LN_2. Then, the sperm can be thawed rapidly, simultaneously reducing the cryoprotectant concentration by direct immersion into a solution. A high lactose solution including egg yolk and glycerol is widely used in several species for the pellet freezing method and it is successfully used in dogs.

1.2.2. Cryodamage in canine spermatozoa

Many considerable improvements have been achieved in canine sperm cryopreservation during the past decades of research, however, cryodamage still takes place and influences sperm quality during the process of sperm freezing and thawing. In recent years, some researchers suggested that excessive ROS production

may be a significant contributing factor to cryodamage [53]. Addition of antioxidants, such as trehalose, cysteamine, taurine, and hyaluronan in ram [54], trehalose, hypotaurine, taurine in bull [55] and also trehalose and raffinose mouse sperm [26] effectively reduced the cryodamage. The motility of frozen-thawed sperm of ram in 100 mM trehalose and 50 mM taurine groups was significantly greater than groups without taurine [54]. Similarly, the supplementation of taurine or trehalose could significantly improve frozen-thawed sperm quality in Karan fries [56] and murine [57].

It is most likely that ROS were responsible for the decline in sperm quality in some species during cryopreservation. Frozen-thawed sperm membrane integrity and mitochondrial function, measured by flow cytometrical analysis, were improved by the addition of trehalose and taurine [54, 55]. These results indicate that the protective effect of antioxidants on the frozen-thawed sperm was not caused by influencing the antioxidant enzymatic system but by antioxidants directly neutralizing the excessive ROS [25].

Some researchers suggest that mitochondrial DNA and membrane structure might be an important factor in explaining the impaired fertility and motility of cryopreserved sperm [58]. Cryodamage usually occurs during freezing and thawing due to ice crystal formation, osmotic stress, cryoprotectant toxicity and so on [59]. Fertilizing capability, velocity, and motility may significantly decrease because of cellular damage and because the cryodamage mechanism has not been completely determined.

With the rapid development of sperm cryopreservation, some researches have conducted antioxidant supplementation *in vitro* to improve techniques for sperm storage and cryopreservation [54, 60]. However, dilution in the extender before cryopreservation decreased the concentration of original antioxidant components in the seminal plasma, diminishing the antioxidant protection of sperm [25, 61]. The addition of antioxidant may be capable of neutralizing ROS and maintaining the balance of the production and scavenging of ROS generated during the cryopreservation process [62, 63]. Many varieties of antioxidant substances have been used in sperm cryopreservation, such as trehalose, cysteamine, taurine, and hyaluronan on ram semen [54] and Equex™ as free radical scavengers which composed of vitamin and enzyme [60]. However, the effect of each antioxidant was species-specific, dependent on the type of a molecule and concentration [56].

1.2.3. Cryoprotectant in canine sperm cryopreservation

Many different compounds from possible chemicals have been used for canine sperm freezing and based on their characters, the cryoprotectant agent can be assigned into two categories: permeate cells (glycerol, dimethyl sulfoxide and ethylene glycol) and non-permeate cells (proteins, sugars and synthetic macromolecules). Canine spermatozoa were frozen using many different extenders and methods but the successful sperm cryopreservation mostly used buffer/extender supplemented with glycerol as a cryoprotectant agent. Applying high concentrations of glycerol has a negative impact on fertility [64, 65]. Glycerol is the most used cryoprotectant in most species, but some experiments compared the use of glycerol

with dimethyl sulfoxide and its combinations [66]. 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 are reached between 4% to 11% (v/v) [67].

Egg yolk has been shown to protect cell membranes from cold shock and has mild cryoprotectant characteristics. They are regularly incorporated into diluents for spermatozoa [66]. The concentration of egg yolk used varies among species but is commonly used at concentrations between 3 to 25% (w/v) [67]. For the preservation of dog spermatozoa, a concentration of 20% egg yolk has been used in several studies [60, 68, 69]. A study about the influence of diluents, cryoprotectants, and sperm processing procedures on post-thaw motility of canine spermatozoa frozen in straws showed that post-thaw motility was highest when the diluent was egg yolk-Tris containing 2 to 4% glycerol. Frozen-thawed spermatozoa appeared to tolerate a range of glycerol concentrations, and the optimal glycerol concentration depended on the type of diluent used. Post-thaw motility increased as warming rate increased. Motility was highest when specimens were thawed in a 75 °C water bath for 12 sec [70].

1.3. Canine *in vitro* fertilization

The first IVF rabbits were born in the 1950s [71], the first human from IVF was born in 1978 [72] and domestic cattle produced many IVF offspring in the 1980s [73]. IVF has been successfully practiced for decades, and its combination with gene editing could give a brighter future for breeds that suffer from inherited diseases, allowing scientists to understand genetic defects in the bud and produce generations of disease free embryos. The success of IVF in domestic dogs also kindles hope for preserving the genetic diversity of endangered canids and opens up a chance to understand inherited diseases, which will then allow us to produce an animal model for humans [74].

1.3.1. The importance of canine IVF

The IVF was used for conserving the endangered Iberian lynx (*Lynx pardinus*) [75] and other wildlife animals such as pumas [76], tigers [77], cheetahs [78], Indian desert cats [79], gaurs [80], Armenian red sheep [81], llamas [82], African elephants [83], gorillas [84] and European mouflon [85]. There were also endangered species of Canidae families but their conservation using IVF or other ARTs was not as developed as other animals due to the limitations of oocyte availability and quality [86]. Ethiopian wolves (*Canis simensis*), the Mexican gray wolf (*Canis lupus baileyi*), the Red wolf (*Canis rufus*), Darwin's fox (*Lycalopex fulvipes*) and the Island fox (*Urocyon littoralis*) are the most endangered canidae in the recent decade [87]. Many studies have been conducted to preserve these species. However, there have

been no successful reports regarding the preservation of endangered canines using the IVF system.

The ability to produce, culture and manipulate domestic dog embryos *in vitro* also gives an opportunity to understand the inherited diseases between humans and dogs, which is important as canines and humans share 413 similar diseases [88]. Retinal dystrophy, muscular dystrophy, ocular dysplasia and lysosomal storage diseases have been extensively studied in dogs and found to have similar phenotypes with inherited diseases in humans [89]. The IVF system can be used to understand the mutations by evaluating the puppies and their sires after applying gene therapy. These gene therapies, currently under development, will also be valuable strategies for improving human health [90]. IVF is also important for improving the health of pet animals, because it opens up the possibility for scientists to identify certain disease-inducing genes and to fix them [91].

The canine IVF can be used to apply new gene editing technologies in dogs such as TALENs and CRISPR/Cas9 in order to perform gene repair [92]. The first dog models using CRISPR/Cas9 for knocking out Myostatin followed by embryo microinjection was published on 2015 [93]. This method would effectively allow the removal of deleterious genes in a specific locus without losing genetic diversity, solving the problem of the new deleterious traits potentially becoming fixed in a population. Genetically valuable domestic dogs that are unable to reproduce on their own could also benefit from this technique in the same way it has helped infertile human couples. Note that although CRISPR/Cas9 is now performed routinely and is highly efficient at producing germline modifications in mice, some hereditary

diseases will not be amenable for gene repair through this approach, due to varying/spontaneous mutations or lack of sequence specificity in the target region. Canine IVF provides an opportunity to repair some of the genetic defects and to ensure the production of genetically valuable individuals that result in the specific pathologies of breeds, endangered species or working dogs [74].

1.3.2. Canine IVF studies

Many studies have been conducted to produce canine embryos using the IVF method, and there started experiments to test aspects such as sperm fertility, quality of mature oocyte, culture medium and embryo transfer procedure [74]. Some studies provided oocytes from IVM [94-96] and others began from *in vivo* matured oocytes [91]. When IVF is performed after the IVM period, spermatozoa only penetrate 10–50% of oocytes and only around 4–10% of all oocytes subjected to maturation and fertilization procedures go through normal fertilization with two pronuclei formation [95-98]. Furthermore, 47% of 60 *in vitro* fertilized oocytes were penetrated with 2-12 spermatozoa per oocyte (an average of 3.3 spermatozoa per oocyte) [99, 100] and were recognized as polyspermy. Moreover, the *in vitro*-produced canine embryos remain exceptional, and blockage occurs around the four- to eight-cell stage [101, 102]. Only a few morula and blastocysts with approximately 0.5% development rates have been reported [94, 103]. In 2001, one pregnancy has been obtained with *in vitro*-produced embryos after the transfer of Day 2 embryos but were aborted at Day 36 [104].

A capacitation medium to support sperm fertility was previously developed for canine capacitation by Mahi *et al.* [97] without magnesium (Mg) to reduce the delayed incidence of spontaneous acrosome reaction. De Los Reyes *et al.* in 2009 reported a study which compared the time effect of frozen sperm, chilling sperm and fresh semen on the penetration of the zona pellucida. In this study they also compared the sperm penetration of immature and *in vitro* mature canine oocytes using different co-culture times between 1 to 10 h. Their results showed that fresh sperm exhibited the highest penetration rate but chilled and frozen-thawed sperm at the 1st hour showed higher penetration when compared with fresh semen at the same time. The use of chlortetracycline (CTC) assay with frozen dog semen demonstrated a significant increase in the number of capacitated sperm between 0 and 2 h of incubation in the capacitating medium [98]. In contrast to the previous study, Nagashima *et al.* [91] modified the canine capacitation medium by adding magnesium, which promoted acrosome exocytosis and physiological acrosome reaction stimulation by progesterone (P4) and/or protein from the zona pellucida.

1.3.3. Struggle in IVF with frozen-thawed sperm

The critical factors of successful IVF are capacitated sperm that have acquired the ability to fertilize [105], high quality oocytes [96], optimal fertilization/embryo culture conditions [94], and an embryo transfer recipient with an oviductal/uterine environment that will support implantation and successful pregnancy [106]. Lack of any one or several of these requirements will lead to failure [91].

Many studies have been reported some disadvantages of cryopreservation in sperm, such as: osmotic stress caused by glycerol movement through the cell membrane, followed by cell shrinking and swelling, ice crystal formation which causes disruption of the cellular membrane and organelles by extracellular and intracellular ice crystals, oxidative stress caused by removal of seminal plasma during centrifugation which makes limited intracellular antioxidant and stimulates peroxidative membrane damage, and the use of cryoprotectant which is toxic to the intracellular components [25, 64, 67, 107-109]. These detrimental effects of cryopreservation should be overcome before IVF is performed, especially in sperm motility, viability and fertility to acquire optimum results.

Canine IVF studies have been difficult to develop due to their specifications in reproductive physiology such as prolonged ovarian inactivity or anestrus [110], darkness due to high lipid content of oocytes [111], ovulation at an immature stage once or twice annually, and the required 48-72h in the oviduct for complete maturation [112]. Oocytes with very low MII rates were found around 10% to 30% after 72–96 h culture [113, 114].

Studies about canine IVF using frozen-thawed sperm have been conducted [95, 96, 98] but no blastocysts have been reported and sperm penetration rates only reached 26.4% to 34.2%. It has been hypothesized that cryopreservation reduced fertility, made sperm acquire capacitation-like changes [115, 116] and reduced longevity *in vivo* [107] and *in vitro* [117]. Sperm freezing process affects the time of sperm penetration and the final percentage of fertilized oocytes as reported previously in frozen-thawed sperm from sheep and cattle [118, 119]. In a study

conducted by freezing procedure generated an alteration of the acrosome and the plasma membrane, which led to a failure in the regulation of sperm intracellular calcium levels [116, 120].

2. General objective

The purpose of this study is to enhance the quality of canine frozen-thawed sperm using a modified the freezing protocol, antioxidant supplementation during cryopreservation, addition of conditioned media during capacitation and to establish canine IVF using the enhanced frozen-thawed sperm. This thesis is composed of 5 parts. In part I; the motivation behind this project was described as a general introduction. In part II; the general methodology used was described. In part III; the various methods used to enhance canine sperm were investigated, including: 1) the multistep freezing protocol and different cryoprotective agents used for maintaining canine sperm function and osmolyte content and 2) spermine reduced reactive oxygen species levels and decreased cryocapacitation in canine sperm cryopreservation. In part IV; the details of how frozen-thawed sperm supplemented with conditioned media in capacitation system were used in IVF and their results was described. In part V; a final conclusion of this study was described.

PART II

GENERAL METHODOLOGY

1. Chemicals and materials

All chemicals were obtained from Sigma-Aldrich Co. LLC. (Missouri, USA) unless otherwise stated.

2. Animal use

In this study, five sexually mature male beagles aged between 4 to 6 years and weighing 10 to 15 kg were used as semen sources, and mixed-breed female dogs (*Canis familiaris*) between 2 to 5 years were used as oocyte donors and embryo transfer recipients. All dogs were housed separately in indoor cages with animal care facilities and procedures following standards established by the Committee for Accreditation of Laboratory Animal Care at Seoul National University. The use of animals in this study was according to "The Guide for the Care and Use of Laboratory Animals" at Seoul National University.

3. Canine sperm preparation and evaluation

3.1. Semen collection

Semen were collected from the five male beagles twice a week using a digital manipulation method, and only normal ejaculates with sperm concentrations ≥ 100 × 10^6 /mL, motility ≥ 70%, viability ≥ 80% and normal morphology ≥ 80% were pooled in 15 mL tubes.

3.2. Sperm motility and kinematic parameters

In total, five different fields were analyzed for each sample and the computer-assisted sperm analysis (CASA) system was used for evaluating the kinematic parameters of at least 200 motile spermatozoa, which is shown in Fig. 1 using a sperm analysis imaging system (FSA2011 premium edition, Medical Supply Co., Ltd., Gangwon, Korea).

3.3. Membrane integrity and morphological defects

Sperm membrane integrity and morphology were evaluated by the eosin–nigrosin staining method. Sperm suspension smears, consisting of a sperm sample and stain (1:1), were spread onto a warm glass slide and dried. Afterwards, membrane integrity and morphological defects were assessed under a microscope with 100 X magnification.

3.4. Real time PCR

RNA samples were obtained in triplicates from five pairs of canine frozen-thawed spermatozoa from the control group and treatment group. Quantitative real-time PCR (qPCR) was conducted to assess transcript abundance using oligonucleotide primer sequences. The primers for canine genes were designed from sequences obtained from NCBI and all primers were tested using gel electrophoresis and standardized using a standard curve. The mRNA expression of apoptotic genes and others genes related to this study were analyzed by qPCR. Total RNA was extracted using TRIzol™ Reagent (Invitrogen, Carlsbad, CA, USA), according to

the manufacturer's protocol, and complementary DNA was produced using amfiRivert cDNA Synthesis Platinum Master Mix (GenDepot, Barker, TX, USA). The qPCR was performed using an ABI 7300 Real Time PCR System (Applied Biosystems, Forest City, CA, USA) and the expression of each target genes was quantified relative to that of the internal control gene (*β-actin*) using the equation $R = 2^{-[\Delta Ct\ sample - \Delta Ct\ control]}$ as previously described [115].

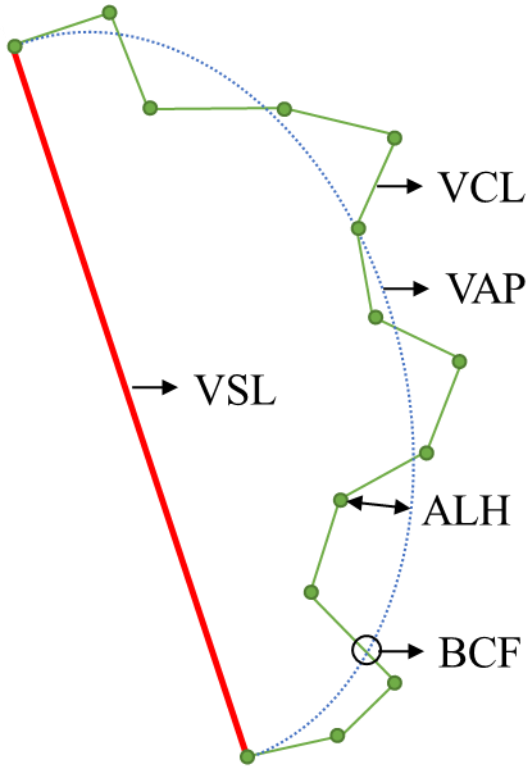

Figure 1. Schematic representation of motility and kinematic parameters of a spermatozoa evaluated by computer-assisted sperm analysis (CASA)

VCL, curvilinear velocity; VAP, average path velocity; VSL, straight-line velocity; ALH, amplitude of lateral head displacement; BCF, beat-cross frequency.

4. Sperm cryopreservation

4.1. Sperm freezing

The pooled ejaculate was washed by adding an equal volume of the first buffer (24 g/L Tris [hydroxymethyl] amino methane, 14 g/L citric acid, 8 g/L glucose, 0.6 g/L Na–benzyl penicillin, and 1 g/L streptomycin sulfate in distilled water [pH 6.60, 290 mOsm]) and centrifuged at 700*g* for 5 min. The pellet was resuspended by adding the first buffer to achieve a sperm concentration of 200 x 10^6 /mL. The second buffer was made by mixing 54% (v/v) first buffer, 40% (v/v) egg yolk and 6% (v/v) glycerol [115].

Each aliquot was mixed with the second buffer to obtain a sperm concentration of 100 × 10^6 /mL at room temperature. The required volume of second buffer was added all at once for the single step protocol or divided into 14%, 19%, 27% and 40% for the multistep protocol and loaded serially at 30 sec intervals [115, 121]. The extended sperm suspension was filled into 0.25 mL straws (Minitub, Tiefenbach, Germany) and incubated at 4 °C for 1 h. After equilibration, straws were placed horizontally, 2 cm above the surface of liquid nitrogen (LN_2) for 10 min then plunged into the LN_2. The straws were stored in the LN_2 container for one week before being thawed for the next step.

4.2. Sperm thawing

Thawing was performed in a water bath at 60 °C for 7 sec and then the sperm samples were diluted (1:5) with the first buffer at once for single step protocol or

divided into 14%, 19%, 27% and finally 40% of the total volume for the multistep protocol [121].

5. *In vitro* fertilization

5.1. Oocytes collection

In vivo matured oocytes were obtained by the oviducts flushing method with a Hepes-buffered tissue culture medium 199 (TCM 199, Invitrogen, Carlsbad, CA, USA) [86]. Blood was drawn alternately from cephalic and saphenous veins 3 to 7 days a week. Daily blood sampling was performed when proestrus was detected (the presence of serosanguinous discharge from the vulva and/or serum with P4 values higher than 1.0 μg/mL). Collected blood was first allowed to clot, then was centrifuged at 700*g* for 10 min to separate serum which was evaluated *via* Immulite 1000 (Siemens Medical Solutions Diagnostics, Los Angeles, CA). The days of the LH surge and ovulation were identified based on P4 values of 1.5–2.5 and 4.0–9.9 ng/ml, respectively [122, 123]. Approximately 70 to 76 h after ovulation, the dogs were subjected to the oocyte collection procedure.

The dogs were pre-anesthetized with 5 mg/kg ketamine HCl (Yuhan, Seoul, Korea) and 1 mg/kg xylazine (Bayer, Leverkusen, Germany), and anesthesia was maintained with 2% isoflurane (Hana Pharm., Seoul, Korea). After the abdominal region was prepared aseptically, a midline incision was made, and the ovary was pulled out. A 16 gauge flushing needle was inserted into the opening of the infundibulum and tied in with a ligature. An intravenous catheter was inserted into the caudal portion of the oviduct. The Hepes-buffered TCM-199 supplemented with

10% (v/v) fetal bovine serum (FBS) was introduced into the oviduct using a 5 mL syringe. The flushed medium containing oocytes was collected into a petri dish from the flushing needle. The quality of the recovered oocytes was determined with a micromanipulator based on the morphology and width of the PVS. Oocytes without PVS and first polar body were considered immature. Oocytes with PVS around 15 μm and more than 25 μm were respectively classified as mature and aging [124]. Only oocytes with first polar body and PVS less than 25 μm were used.

5.2. Sperm capacitation

One straw of cryopreserved spermatozoa was thawed in a water bath at 60 °C for 7 sec then divided into 2 aliquots and directly incubated in canine capacitating medium (CCM) supplemented with 1.0 mM $MgCl_2$ and 10 mM P4 for 2 h [91].

5.3. *In vitro* fertilization and embryo culture

Oocytes were washed in a potassium simplex optimization medium (KSOM; MTI-GlobalStem, Maryland, USA) before they were transferred to fresh, pre-equilibrated 90 μL droplets of the medium covered with mineral oil for IVF. *In vitro* capacitated sperm (incubated for 2 h under capacitating conditions) in 10 μL suspension was added to the oocytes in the IVF medium droplet at a final concentration of 1 x 10^6 sperm / mL. The gametes were co-incubated for 3 h at 38 °C with 5% CO_2 and 90% N_2 in an incubator. The 3 to 5 zygotes were transferred to pre-equilibrated 50 μL droplets covered with mineral oil. Embryo cleavage was evaluated 48 h post-IVF and from then on embryos development was observed at

every 12 h. All inseminated oocytes were transferred to the KSOM medium and cultured for 3 to 21 h in an incubator with 5% CO_2, 7% O_2 and 88% N_2 for identification of sperm head decondensation and the cell number of the embryo was examined at 168 h. Cleavage rates were recorded to assess the *in vitro* developmental capacity of embryos at 48 h. The cell number of surviving embryos was investigated at 168 h after IVF by bisbenzimide (Hoechst 33342) staining.

6. Statistical analysis

The Student's t-test for data with normal distribution was used to determine differences between two groups. The one-way analysis of variance (ANOVA) for data with normal distribution was used to determine differences among three or more groups. Tukey's Multiple Comparison Test was used to compare all pairs of columns. Linear regression and correlation analysis were used for determining the relationship between a scalar dependent variable and independent variables. All data was presented as the mean ± standard error of the mean (SEM) and a $P < 0.05$ was taken to indicate statistical significance. All experiments were replicated at least five times and GraphPad Prism version 5 (GraphPad Software, Inc., San Diego, CA, USA) was used for statistical analysis and graphical presentation.

PART III

ENHANCING THE CANINE SPERM CRYOPRESERVATION METHOD BY USING MULTISTEP PROTOCOL AND ANTIOXIDANT TREATMENT

Chapter I. Maintaining canine sperm function and osmolyte content with multistep freezing protocol and different cryoprotective agents

1. Introduction

Sperm cryopreservation is an essential aspect of ART in canids, because other ARTs such as intracytoplasmic sperm injection, IVF, *in vitro* maturation of oocytes and *in vitro* culture of embryos have not yet been established in this species [10]. Some ARTs need fertile spermatozoa as genetic resources, and sperm banking has benefits, especially for long-term storage of genetic materials, conservation of fertility of individuals possessing high genetic merit, or for preservation of breeds threatened with extinction due to disease or sudden death [125]. Cryopreservation also performs ice formation and high osmotic pressure that lead to cryoinjury and induces both loss of sperm function and viability post-thawing [126]. Although a variety of extenders [14] and cryopreservation protocols [127] have been developed since the first successful pregnancies using frozen-thawed dog semen in 1969, the whelping rates with frozen-thawed sperm (50.0–70.8%) have not been as satisfactory as those with fresh semen (81.8–83.7%) [69]. This might be due to the existence of many unknown factors such as sperm cryosensitivity to varying osmolalities, optimal cryoprotective agents (CPAs), freezing/thawing procedures [14] and CPAs loading/dilution [128, 129].

Osmolytes, such as carnitine, sorbitol, myo-inositol and glutamate, are small organic solutes that are loaded into spermatozoa when they are transported in the epididymis [130] and are used to maintain cell volume [131, 132]. During cryopreservation, spermatozoa are exposed to hypertonic conditions induced by adding CPAs and water efflux along with permeable solutes, which leads to cell shrinkage [133]. Cell shrinkage causes spermatozoa of several mammalian species (mouse [134], boar [135], human [136] and bull [137]) to exhibit regulatory volume increase (RVI) abilities for maintaining their cellular functionality [131]. In contrast, the prevailing hypotonic environment during thawing causes water influx and cell swelling [135]. In order to prevent spermatozoa from becoming excessively large, regulatory volume decrease (RVD) takes place to makes water with osmolytes flow out of the cells [135, 138]. If RVD fails, spermatozoa remain swollen, forming flagellar contortions and exhibiting flagellar angulation with the tail reflected backwards [131].

The conventional method for canine sperm cryopreservation performs a one-step protocol for adding and removing CPAs [14, 139, 140]. The rapid change of osmotic pressure leads to mechanical damage of the plasma membrane [141], altered cellular metabolism and oxidative injury [139]. Furthermore, a single step protocol caused rapid loss of sperm motility and membrane integrity in equine spermatozoa [129]. It also increased the frequency of several kinds of structural abnormalities in the flagellar region of cat [128], dog [140] and wolf [14] spermatozoa. However, the multiple steps protocol minimized the loss of sperm motility and membrane disruption in felids sperm cryopreservation [128]. Stepwise dilution (fixed molarity

and fixed volume-dilutions) for removal of glycerol in equine fresh semen reduced post-hyperosmotic stress, which improved the maintenance of motility, viability and membrane integrity compared to the one-step dilution method [129]. Similar results have also been reported with humans [142] and bovines [143]: that multistep addition and removal of CPAs could reduce sperm damage and maintain the osmolytes content and sperm function. Moreover, multistep protocol allows gradual changes in osmotic pressure and limits the extent of osmotic swelling at each step [142, 143], and could thereby control the degree of dehydration without exposing cells to lethal salt concentrations [14]. Therefore, the method of controlling osmotic changes and dehydration using a protocol for adding or removing CPAs needs to be addressed.

For canine sperm freezing, the degree of cell damage is also related to the characteristics of CPAs used, such as ethylene glycol [144] and glycerol [145]. Both CPAs have been widely used for canine sperm cryopreservation [65, 139] but ethylene glycol resulted in similar or better preservation in some studies [68, 144, 146] while giving inferior results in another [139]. Aside from these contradictory results, ethylene glycol and glycerol reduced osmolytes content in bovine, which is associated with sperm quality decrease [143]. Maintaining the number of osmolytes is also important in preserving mouse sperm volume [147]. There is no information about their effects on osmolytes content of canine spermatozoa and also regarding the relationship between osmolytes and sperm function. There are no studies using multistep protocol in dogs, and osmolytes loss could be reduced with multistep treatment [143]. Therefore, the objectives of the present study are to determine if the

multistep loading and dilution protocol can be used to overcome the osmotic sensitivity of canine spermatozoa, to confirm the type of CPAs that can maintain osmolyte content and to determine which types of osmolytes are lost during cryopreservation.

2. Materials and methods

2.1. Animal use

Procedures for animal use were described in general methodology.

2.2. Semen collection and preparation

Semen was collected twice a week from the five beagles and only normal ejaculates with sperm concentrations ≥ 100 × 10^6 /mL, motility ≥ 70%, viability ≥ 80% and normal morphology ≥ 80% were pooled in 15 mL tubes. The pooled ejaculate was washed by adding an equal volume of the first buffer (24 g/L Tris [hydroxymethyl] amino methane, 14 g/L citric acid, 8 g/L glucose, 0.6 g/L Na-benzyl penicillin, and 1 g/L streptomycin sulfate in distilled water [pH 6.60, 290 mOsm]) and centrifuged at 700*g* for 5 min. The pellet was resuspended by adding the first buffer to achieve a sperm concentration of 200 x 10^6 /mL. Washed semen was divided into 5 aliquots: 2 aliquots for the multistep method, 2 aliquots for the single step method, and 1 aliquot for the CPA-free group. The second buffer was made by mixing 50% (v/v) first buffer, 40% (v/v) egg yolk and 10% (v/v) cryoprotectant. Either glycerol or ethylene glycol was used as a cryoprotectant in the second buffer. All chemicals were purchased from Sigma-Aldrich, St. Louis, MO, USA.

2.3. Sperm freezing and thawing

The basic procedures for sperm freezing and thawing were described in general methodology. Each aliquot was mixed with the second buffer to obtain a sperm concentration of 100×10^6 /mL at room temperature (20-22 °C) for either the multistep or single step methods. For the multistep loading protocol, 14%, 19%, 27% and 40% of the second buffer volume was loaded serially at 30 sec intervals, while 100% of the second buffer was added all at once for the single step. These protocols were used for both the 5% glycerol and 5% ethylene glycol groups. The second buffer without cryoprotectant was used for the CPA-free group. The extended sperm suspension was filled into 0.25 mL straws and placed horizontally 2 cm above the surface of the LN_2 for 10 min then plunged into the LN_2. The straws were stored in the LN_2 container for one week before being thawed for evaluation.

The straws were thawed in a water bath at 37 °C for 30 sec and diluted (1:5) with the first buffer using the multistep or single step methods. For multistep dilution protocol, 14%, 19%, 27% and 40% of the first buffer volume was added serially at 30 sec intervals, while 100% of the first buffer was loaded all at once for the single step. The CPAs-free group was diluted by adding 100% of the first buffer all at once immediately after thawing. All thawed sperm suspensions were evaluated as follows.

2.4. Sperm evaluation

2.4.1. Sperm motility and kinematic parameters

In total, 3 different fields were analyzed for each sample and the kinematic parameters of at least 200 motile spermatozoa were tracked for 1 sec at 25 Hz [143,

148] with a sperm analysis imaging system (FSA2011 premium edition version 2011, Medical Supply Co., Ltd., Korea).

2.4.2. Membrane integrity and morphological defects.

The sperm membrane integrity and morphology were evaluated by the eosin–nigrosin staining method. Sperm suspension smears, consisting of a sperm sample and stain (1:1), were spread on a warm slide and dried [139, 148]. Afterwards, membrane integrity and morphological defects were assessed.

2.4.3. Mucus penetration test

Surrogate mucus was prepared freshly for each experiment by dissolving 6 mg/mL hyaluronic acid in the first buffer. Surrogate mucus was aspirated into marked flat capillary tubes (10 cm long, 3 mm deep; Camlab, Cambridge, UK). One end was sealed with polyvinyl alcohol and the tube was stood vertically with the sealed end on top for 15 min to remove bubbles and ensure the seal was intact. Then, the capillary tube was inserted into an Eppendorf tube containing 100 μL sperm suspension. The tubes were placed horizontally for 2 h at 20-22 ℃. The numbers of spermatozoa reaching 1 cm and 3 cm markers were counted [143].

2.4.4. Osmolyte measurements

Thawed sperm suspensions were centrifuged at 900*g* for 20 min and sperm pellets were suspended in 500 μL of 10 mmol/L Tris buffer at pH 7.0, mixed by pipetting and vortexing. Samples were sonicated three times for 10 sec on ice at 50

W and an amplitude setting of 30 in a UP50H Ultrasonic Processor (Hielscher Ultrasonics GmbH) fitted with a 1.5-mm tip. Centrifugation was performed at 16,000*g* for 15 min at 4 ºC and the supernatant was stored at −20 ºC before assay. The intracellular osmolytes content was determined by end-point fluorimetric assays performed in 96-well plates with top standards and sensitivities, respectively, of (μmol/L): glutamate 20, 0.09; carnitine 500, 14; *myo*-inositol 600, 11; sorbitol 600, 20. A fluorimetric kinetic assay for glucose-6-phosphate dehydrogenase (G6PDH) [143] was developed, and could be quantified by excitation at 560 nm and emission at 587 nm. Readings were taken every 40 sec for 10 min at 30 °C. Standard enzyme (up to 20 U/mL; sensitivity, 1.5 U/mL) and buffer blanks were included in each plate. The osmolyte content was expressed in nmol per U G6PDH activity to avoid data bias concerning the number of spermatozoa in the last treatment; those measurements were made in a GENios (Tecan, Switzerland, Austria).

2.5. Statistical analysis

Procedures for statistical analysis were described in general methodology.

3. Results

3.1. Sperm motility

Total sperm motility in fresh semen was 82.2 ± 1.5 % with 76.4 ± 0.9 % progressive motility and 62.7 ± 0.7 % linearity as shown in Table 1. After freezing, all motility parameters were significantly decreased but the multistep group retained the highest level of motility compared to the other groups. Total and progressive motility were significantly higher in the multistep group (51.5 ± 2.2 % and 23.3 ± 1.3 %, respectively) than in single step (37.0 ± 1.7 % and 12.5 ± 1.6 %, respectively) and CPAs-free (10.2 ± 1.4 % and 5.0 ± 1.2 %, respectively) groups. Linearity of motile spermatozoa in the multistep group (51.4 ± 1.5 %) was significantly higher than in single step (35.3 ± 1.9 %) and CPAs-free (19.4 ± 3.0 %) groups.

Total motility in the multistep protocol showed similar percentages between the 5% glycerol and 5% ethylene glycol supplemented groups (57.4 ± 2.4 % and 45.5 ± 2.4 %, respectively, $P > 0.05$) but progressive motility and linearity in the 5% glycerol group (27.1 ± 1.5 % and 54.4 ± 1.5 %, respectively) were significantly higher compared to those in the 5% ethylene glycol group (19.6 ± 0.9 % and 48.4 ± 2.4 %, respectively).

3.2. Mucus penetration test

Table 1 shows that more than 200 spermatozoa from fresh semen reached the 1 cm marker and 49.8 ± 2.2 cells gained the 3 cm marker after 2h incubation. There

were more spermatozoa at the 1 cm and 3 cm markers with the multistep protocol (67.9 ± 4.6 and 22.3 ± 2.9 cells, respectively) than with the single step (37.2 ± 1.8 and 10.7 ± 2.2 cells, respectively).

The numbers of spermatozoa in the 5% glycerol group reaching the 1 cm and 3 cm markers were significantly higher (84.7 ± 2.0 and 22.4 ± 2.4 cells, respectively) than in the 5% ethylene glycol group (51.0 ± 2.5 and 12.2 ± 0.9 cells, respectively) with the multistep protocol. However, the single step protocol gave similar values at the 1 cm marker in both CPAs but glycerol treatment supported higher sperm numbers at 3 cm than ethylene glycol (19.1 ± 0.7 *vs.* 2.4 ± 0.5 cells, respectively).

Table 1. The function of canine spermatozoa in fresh semen, frozen-thawed sperm with multistep and single step and cryoprotectant agent (CPA) free protocols

Group	Total motility (%)	Progressive motility (%)	Linearity (%)	Sperm count in 1 cm marker (N)	Sperm count in 3 cm marker (N)	Intact membranes (%)	Coiled tail (%)	Bent tail (%)
Fresh semen	82.2 ± 1.5^{De}	76.4 ± 0.9^{Dd}	62.7 ± 0.7^{Dd}	218.1 ± 5.9^{De}	49.8 ± 2.2^{Dd}	86.4 ± 1.6^{Dd}	1.8 ± 0.2^{Cc}	4.2 ± 1.1^{Cc}
Multistep	51.5 ± 2.2^{C}	23.3 ± 1.3^{C}	51.4 ± 1.6^{C}	67.9 ± 4.6^{C}	22.3 ± 2.9^{C}	66.5 ± 2.8^{C}	1.8 ± 0.3^{C}	29.2 ± 3.2^{B}
5% Glycerol	57.4 ± 2.4^{d}	27.1 ± 1.5^{c}	54.4 ± 1.5^{cd}	84.7 ± 2.0^{d}	22.4 ± 2.4^{c}	74.5 ± 3.3^{c}	1.4 ± 0.3^{c}	22.0 ± 0.3^{b}
5% E. Glycol	45.5 ± 2.4^{c}	19.6 ± 0.9^{b}	48.4 ± 2.4^{c}	51.0 ± 2.5^{c}	12.2 ± 0.9^{b}	58.5 ± 0.8^{b}	2.1 ± 0.4^{bc}	36.5 ± 5.2^{a}
Single step	37.0 ± 1.7^{B}	12.5 ± 1.6^{B}	35.3 ± 1.9^{B}	37.2 ± 1.8^{B}	10.7 ± 2.2^{B}	49.5 ± 2.6^{B}	3.3 ± 0.3^{B}	46.2 ± 1.9^{A}
5% Glycerol	41.7 ± 2.1^{c}	17.0 ± 2.0^{b}	39.3 ± 1.7^{bc}	39.3 ± 2.4^{bc}	19.1 ± 0.7^{c}	54.5 ± 3.6^{b}	3.2 ± 0.7^{bc}	45.3 ± 3.3^{a}
5% E. Glycol	32.2 ± 1.4^{b}	6.7 ± 0.4^{a}	31.4 ± 3.0^{b}	35.1 ± 2.5^{b}	2.4 ± 0.5^{a}	44.6 ± 2.6^{b}	3.4 ± 0.2^{b}	47.1 ± 2.3^{a}
CPA-free	10.2 ± 1.4^{Aa}	5.0 ± 1.2^{Aa}	19.4 ± 3.0^{Aa}	10.8 ± 2.1^{Aa}	0.0 ± 0.0^{Aa}	17.6 ± 2.6^{Aa}	10.8 ± 0.3^{Aa}	23.3 ± 1.4^{Bb}

$^{A-D}$ within a column, values with different superscripts differ significantly among four groups (fresh semen, multistep, single step and cryoprotectant agent (CPA) free groups, $P < 0.05$, $n = 8$).

$^{a-e}$ within a column, values with different superscripts differ significantly among six groups (fresh semen, 5% Glycerol and Ethylene Glycol of multistep, 5% Glycerol and Ethylene Glycol of single step and cryoprotectant agent (CPA) free groups, $P < 0.05$, $n = 8$).

3.3. Sperm morphology

The morphology of spermatozoa in fresh semen showed 86.4 ± 1.6% with intact membranes, 1.8 ± 0.2 % with coiled tails, and 4.2 ± 1.1 % with bent tails (Table 1). Photomicrographs of normal and abnormal sperm are presented in Fig. 2. Although the proportion of coiled tail spermatozoa in the multistep group (1.8 ± 0.3 %) was similar to that in fresh semen, the bent tail percentage in the multistep group (29.2 ± 3.2 %) was significantly higher than in fresh semen. However, the multistep group showed a higher percentage of intact membranes (66.5 ± 2.8 %) and a lower value of coiled and bent tails compared to the single step group (49.5 ± 2.6 %, 3.3 ± 0.3 % and 46.2 ± 1.9 %, respectively). The CPA-free group had the highest abnormality frequencies of coiled (10.8 ± 0.3 %) and bent tails (23.3 ± 1.4 %).

The 5% glycerol treatment with the multistep maintained a higher percentage of intact membranes, and had lower coiled tails and bent tails (74.5 ± 3.3 %, 1.4 ± 0.3 % and 22.0 ± 0.3 %, respectively) than 5% ethylene glycol treatment (58.5 ± 0.8 %, 2.1 ± 0.4 % and 36.5 ± 5.2 %, respectively). The bent tail percentage in 5% glycerol (22.0 ± 0.3 %) had a similar value to CPA-free (23.3 ± 1.4 %) while 5% ethylene glycol increased the bent frequency (36.5 ± 5.2 %).

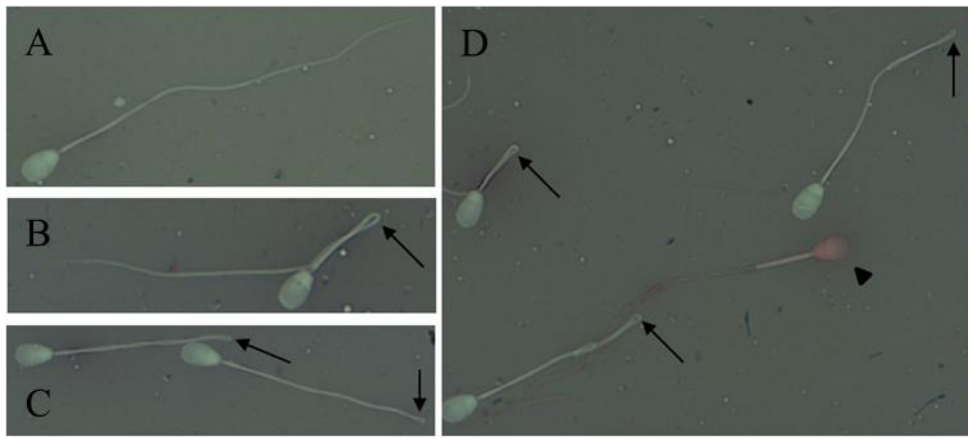

Figure 2. Morphology of canine spermatozoa with normal tail, bent tail and damaged membranes

This figure shows A) normal tail; B) bent tail in the middle; C) bent tail in the end; D) bent tail and damaged membranes (head arrow).

3.4. Changes in osmolytes content

Osmolyte contents in Table 2 were expressed in nmol/U G6PDH because glucose-6-phosphate dehydrogenase is known as a marker of cytoplasm and reflects the number of intact spermatozoa after treatment [143]. In fresh semen, osmolyte concentrations were: carnitine (36.0 ± 0.7 nmol/U G6PDH), glutamate (31.9 ± 0.7 nmol/U G6PDH), inositol (40.8 ± 2.3 nmol/U G6PDH) and sorbitol (40.6 ± 1.8 nmol/U G6PDH). Although carnitine and glutamate contents in all treatment groups were decreased, higher concentrations of carnitine were found in multistep (20.6 ± 2.0 nmol/U G6PDH) than in single step groups (10.8 ± 2.1 nmol/U G6PDH) and the amount of glutamate was not different between multistep and single step groups (18.4 ± 1.6 *vs.* 14.4 ± 0.8 nmol/U G6PDH). There were no significant differences in inositol and sorbitol content among multistep and single step groups.

All of the osmolytes were higher in the 5% glycerol group compared to those in the 5% ethylene glycol group in both multistep and single step groups. Within the 5% glycerol treatment, the multistep protocol gave higher carnitine content (28.0 ± 0.8 nmol/U G6PDH) than the single step (18.4 ± 0.9 nmol/U G6PDH) while other groups had similar values without significant differences.

Table 2. The osmolyte content of canine spermatozoa in fresh semen, frozen-thawed sperm with multistep and single step loading/dilution and cryoprotectant agent (CPA) free protocols

Group	Carnitine (nmol/U G6PDH)	Glutamate (nmol/U G6PDH)	Inositol (nmol/U G6PDH)	Sorbitol (nmol/U G6PDH)
Fresh semen	36.0 ± 0.7^{Ce}	31.9 ± 0.7^{Cd}	40.8 ± 2.3^{Bc}	40.6 ± 1.8^{Bc}
Multistep	20.6 ± 2.0^{B}	18.4 ± 1.6^{B}	35.7 ± 1.5^{B}	35.2 ± 1.4^{B}
5% Glycerol	28.0 ± 0.8^{d}	24.4 ± 0.7^{c}	38.7 ± 0.8^{c}	39.7 ± 1.3^{c}
5% E. Glycol	13.2 ± 0.8^{c}	12.5 ± 0.5^{b}	30.9 ± 1.0^{b}	30.7 ± 0.9^{b}
Single step	10.8 ± 2.1^{A}	14.4 ± 0.8^{B}	35.1 ± 1.1^{B}	35.6 ± 1.5^{B}
5% Glycerol	18.4 ± 0.9^{b}	16.5 ± 0.8^{c}	40.4 ± 1.2^{c}	40.6 ± 0.8^{c}
5% E. Glycol	3.2 ± 1.0^{a}	12.2 ± 0.9^{b}	31.5 ± 0.9^{b}	30.6 ± 1.3^{b}
CPA-free	3.0 ± 1.0^{Aa}	6.1 ± 1.0^{Aa}	23.5 ± 0.9^{Aa}	21.6 ± 1.3^{Aa}

$^{A-D}$ within a column, values with different superscripts differ significantly among four groups (fresh semen, multistep, single step and cryoprotectant agent (CPA) free groups, $P < 0.05$, $n = 8$).

$^{a-e}$ within a column, values with different superscripts differ significantly among six groups (fresh semen, 5% Glycerol and Ethylene Glycol of multistep, 5% Glycerol and Ethylene Glycol of single step and cryoprotectant agent (CPA) free groups, $P < 0.05$, $n = 8$).

3.5. Correlation of intracellular osmolytes with sperm function

A similar pattern was found between the carnitine and glutamate contents with progressive motility and sperm number at the 3 cm marker in all groups as seen in Fig. 3. The regression and correlation analysis showed that carnitine and glutamate had a positive correlation with progressive motility (R=0.8638 and R=0.8846, respectively) and sperm numbers at the 3 cm marker (R=0.9049 and R=0.8958, respectively). Meanwhile, inositol and sorbitol were not different in fresh semen, multistep and single step protocols.

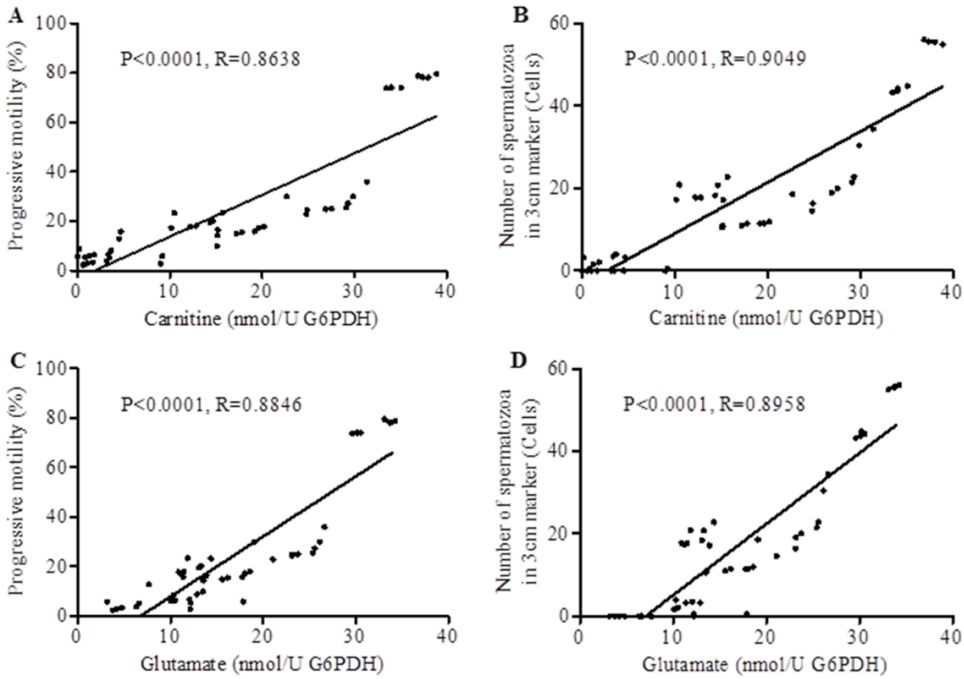

Figure 3. Correlation between carnitine and glutamate contents against progressive motility and number of spermatozoa at the 3 cm marker

A) carnitine *vs.* progressive motility; B) carnitine *vs.* number of spermatozoa at the 3 cm marker; C) glutamate *vs.* progressive motility; D) glutamate *vs.* number of spermatozoa at the 3 cm marker; osmolyte contents showed per U G6PDH activity of all groups (fresh semen, multistep, single step, and cryoprotectant agent (CPA) free).

4. Discussion

Several freezing methods using single step protocols have been developed for canine spermatozoa and showed decreases in the total motility of frozen-thawed sperm around 26.2% to 40.2% from initial motility [65, 139, 140]. Studies about cryoprotectant loading without freezing has been performed in several species, such as feline [128], equine [129], human [142] and bovine [143]. Base on those studies, a multistep protocol was designed using 4 steps in different sequence volumes (14%, 19%, 27% and 40%) that provide the same increase of osmolality in each step [143] so that the water and osmolytes transport can be controlled. These gradual osmotic challenges were reported to be more advantageous in maintaining osmolytes content and sperm function without freezing [142, 143]. This method was proposed to overcome the osmotic sensitivity of canine spermatozoa indicated by higher bent tail abnormality than other mammals [14, 16]. In this study, a bent tail was also found to be the most common abnormality associated with decreased function of canine frozen-thawed spermatozoa using single step protocols. It might reflect a structural weakness due to freezing-induced injury [14] and high sensitivity to osmotic pressure [17]. I hypothesized that gradual osmotic changes induced by dividing the total volume of extender into several steps could reduce these solution effects. Higher motility and number of spermatozoa with intact membranes in the multistep *vs.* single step groups in our study (Table 1) might be due to (1) a minimized osmolality gradient across the plasma membrane [143] and (2) volumetric shrinkage that relates to the water transport and ice formation [141]. These mechanisms most

likely induced gradual changes in cell volume and allow the cells to avoid swelling with enough RVD and prevent the cell membrane so that motility could be maintained. Interestingly, the multistep protocol used in our study showed a similar proportion of coiled tail to those in fresh semen, although there was a significantly higher bent tail percentage compared to the fresh group. Furthermore, bent tail percentage in the CPA-free group has a similar value to the multistep group and a smaller value smaller than single step group (Table 1). These phenomena might be generated by moderate osmotic stress in the multistep protocol compared with the single step, so that sperm tails showing a simple bending of the distal portion rather than a coiled shape [126].

CPAs in buffer are essential components for sperm cryopreservation, allowing modification of the membrane's water permeability [141, 146] and protecting cells from freezing-induced injury [14]. Glycerol and ethylene glycol have been widely used as CPAs for canine sperm cryopreservation due to their advantages such as rapid membrane penetration [149], tolerated osmotic effects [14] and moderate toxicity [68]. The uses of glycerol and ethylene glycol on canine semen cryopreservation have controversial results [68, 139, 144, 146]. Based on these facts, I used both CPAs to analyze their effects on the osmolyte content in canine frozen-thawed spermatozoa and also performed cryopreservation without CPAs to assure their effects. Our results showed superior results in progressive motility, mucus penetration test, tail morphology and intact membranes of the glycerol group (Table 1). Similar studies in canine have reported that ethylene glycol showed lower post-thaw motility, viability, plasma-membrane integrity, and acrosome-membrane

integrity compared to glycerol [109, 139]. Furthermore, other studies showed that the ethylene glycol induced acrosome reaction and capacitation after cryopreservation which resulted in membrane destabilization and eventually led to cell death [126, 139, 146]. These results indicated that 5% ethylene glycol have detrimental effects on canine sperm. and might be related to its characteristic on the potassium channels functionality then affected the flux of ions and organic osmolytes [150]. The channel dysfunctions caused osmolytes lost from canine spermatozoa during cryopreservation and from our study, it can be seen that carnitine, glutamate, inositol and sorbitol were significantly decreased compare to glycerol group (Table 2). Even though both CPAs have detrimental effects on the canine spermatozoa, 5% ethylene glycol showed excessive responses compared to 5% glycerol. The CPA-free freezing produced some motile spermatozoa and normal morphology with intact membrane in very low values. These results indicated that intracellular components of spermatozoa might act as natural CPAs [139] but not enough for maintaining frozen-thawed sperm quality.

Several organic osmolytes including L-carnitine, D-glutamate, *myo*-inositol and sorbitol are involved in volume regulation of spermatozoa [151] and it has been postulated that they would provide better sperm survival rates in the female reproductive tract [17, 152]. I hypothesized that canine spermatozoa would lose different types of osmolytes under osmotic stress during cryopreservation and this would affect sperm motility. Our results showed that canine spermatozoa in multistep and single step groups lose carnitine and glutamate content compared to fresh semen and there were no differences between inositol and sorbitol content

(Table 1). I also suggest that the higher carnitine content found in multistep compared to the single step group indicated that small sequential steps of osmotic challenge could minimize the loss of osmolytes compared with a single large osmotic insult in single step protocols. A low level of osmotic stress would reduce cell swelling and induce less RVD [17, 138], resulting in lower numbers of spermatozoa with coiled tails (Table 1). The similar glutamate content between the multistep and single step groups showed that there was a selective osmolyte loss in canine sperm cryopreservation. It seems that glutamate declines during cryopreservation but is not affected by the modified protocol. These osmolyte losses might be a consequence of a different volume regulation that helps to maintain sperm function [17] during freeze-thawing using both protocols. A positive correlation between carnitine or glutamate content and progressive motility or the sperm migration test (Fig. 3) also suggest that these zwitterion organic osmolytes can protect sperm function. In line with these results, bovine sperm treated with ethylene glycol without freezing had a positive correlation between the carnitine or glutamate content and the mucus penetration test [143]. The content of carnitine and glutamate in the glycerol group was higher than with ethylene glycol (Table 2), showing that the rapid penetration of ethylene glycol [139] allowed a larger efflux of water and osmolytes following a quick loss of carnitine and glutamate [153] compared to the glycerol group.

In conclusion, the multistep loading/dilution protocol used in this study could deliver a gradual osmotic challenge and help in maintaining sperm osmolytes content, morphology and motility. The advantages of the multistep protocol on canine semen cryopreservation can be enhanced using glycerol as a CPA. Further studies are

needed to maximize the frozen–thawed dog sperm quality, perhaps by adding carnitine and glutamate before the cryopreservation process.

Chapter II. Spermine reduces reactive oxygen species levels and decreases cryocapacitation in canine sperm cryopreservation

1. Introduction

Cryopreservation is an important tool in assisted reproduction, although the fertility of frozen/thawed spermatozoa is reduced [154] including in canines [155]. During sperm cryopreservation, ROS are generated endogenously and exogenously [25]. Freeze-thaw techniques contribute to ROS production and induce cryocapacitation [154] which result in acrosome damage due to freeze-thaw process [12]. Prolonged generation of ROS leads to oxidative stress which has a negative impact on sperm quality, especially membrane integrity [61], and stimulates apoptosis [156]. Oxidative stress occurs from an imbalance between systemic ROS production and the ability to detoxify ROS by mechanisms such as antioxidant defenses [156]. However, antioxidants should not completely eliminate ROS [157] because some ROS are needed for normal reproductive functions [158]. Therefore, maintaining physiological ROS levels and preventing oxidative stress are important criteria in selecting antioxidants for use with *in vitro* techniques such as cryopreservation [61].

Removing seminal plasma prior to cryopreservation is standard procedure to omit negative effects of prostatic fluid which reduces the motility and vitality of frozen-thawed spermatozoa [155, 157]. However, that procedure also leads to loss

of helpful seminal fluid components such as nutrients, buffers, antioxidants and other factors that support sperm quality [157]; thus, antioxidant treatment is particularly important because spermatozoa have little cytoplasmic fluid [25].

Several studies have been performed concerning oxidative stress on canine sperm chilling using numerous antioxidant agents such as vitamins E and C, dimethylsulphoxide, taurine, hypotaurine and N-acetylcysteine [24]. However, antioxidant treatment using polyamines to improve canine sperm cryopreservation has not yet been explored. Spermine is one of the polyamines that play important roles in protecting cells from oxidative damage and maintaining membrane structure/function [159]. Natural spermine is found in seminal plasma of humans and rats but not in dogs, rabbits or mice [160]. The protective effect of spermine on DNA integrity depends on its concentration which ranges from 1-10 mM in bacteria, yeast and mammalian cells [159, 161]. The spermine is present in all living cells and a wide variety of polyamines are formed in nature according to the species [159] which reported for first time as a component of seminal plasma by Leeuwenhoek in 1678 [162]. Mammals produce only spermine, spermidine, and the diamine putrescine (Fig. 4) which play important roles in many cellular processes including the regulation of transcription and translation, control of the activity of ion channels, modulation of kinase activities, effects on the cell cycle, protection from oxidative damage, the maintenance of membrane structure/function, and contribution to nucleic acid structure and stability [163-165]. Polyamines play an important role in the protection from ROS in bacteria, yeast, and mammalian cells. Polyamines have been shown to act as free radical scavengers, to quench singlet molecular oxygen

and shield phage and microbial DNA from oxidative damage. They also mediate defense from oxidative damage by stimulating the synthesis of protective gene products such as superoxide dismutase, heat shock proteins, and cell cycle regulators [163]. Spermidine is produced by spermidine synthase, which catalyzes the reaction of decarboxylated S-adenosylmethionine and putrescine to generate spermidine and 5-methylthioadenosine (Fig. 4). Although spermidine has some activity in this respect, spermine is considerably more effective [159].

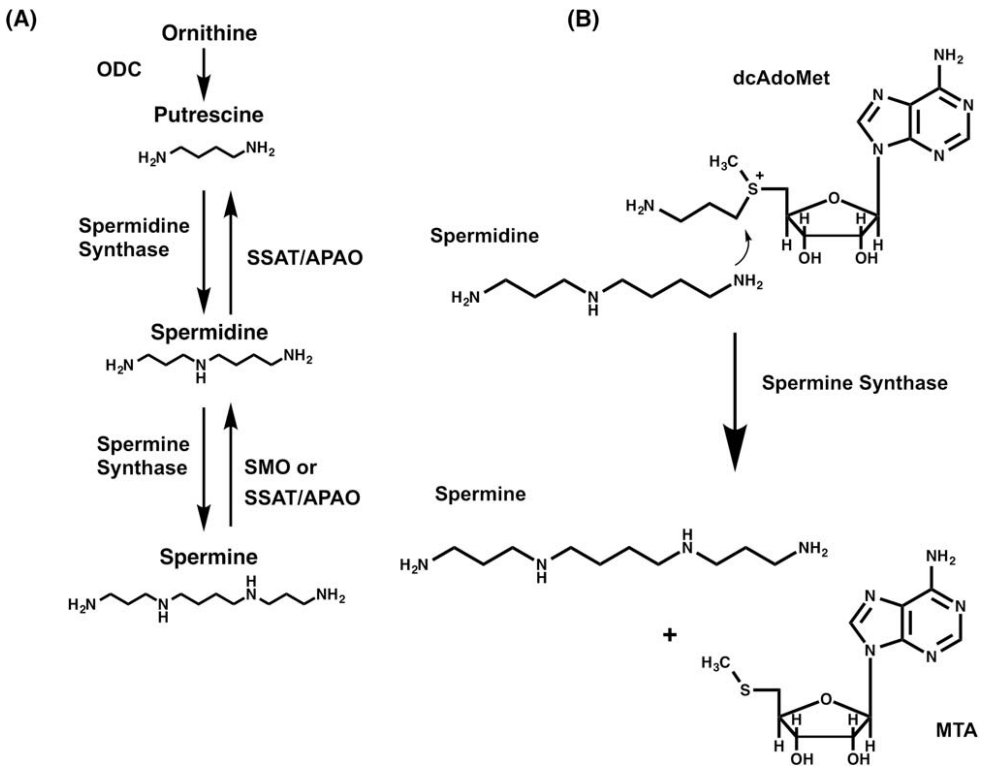

Figure 4. Spermine synthesis and metabolism

Panel (A) shows the biosynthetic aminopropyl transferase reactions leading to spermine which are effectively irreversible and the oxidative catabolic reactions converting spermine back to spermidine and putrescine. Panel (B) shows the reaction catalyzed by spermine synthase [159].

In spermatozoa, spermine was found to enhance the glycolytic rate, adenylate cyclase and ATPase and to inhibit phosphodiesterase. Furthermore, binding of spermatozoa to oocytes was affected by polyamines. The acrosome reaction was stimulated by the polyamines polyarginine. Spermine and other polyamines serve as natural acceptor amines for seminal transglutaminase action, thus attenuating protein cross-linking and premature clotting of the ejaculate [120, 166]. Polyamines bind reversibly to spermatozoa by electrostatic interactions whether the other cell was used metabolic energy to bind with spermine. Moreover, the rates of spermine-sperm binding and release were significantly faster compared with other cell types [167].

Spermine stimulate the progressive motility, straight linear velocity, curvilinear velocity and average path velocity *via* activation of the protein kinase G signaling pathway [168] through protein s-nitrosylation which sensitize the Ca^{2+} stores in the midpiece, causing a slow elevation of Ca^{2+}. This leads to a modulation of flagellar activity, particularly bending in the middle piece and contributing to the hyper activation that is vital for penetration of the oocyte [120, 166].

Spermine induces 85–100% escalation in human sperm capacitation [169] and increases acrosomal exocytosis directly in mouse spermatozoa *in vitro* [170]. Spermine is involved in capacitation through two mechanisms; 1) dependence on cAMP/PKA and 2) dependence on the ERK pathway by increases in the level of P–Thr–Glu–Tyr–P [171]. During the capacitation process, several alterations in the plasma membrane occur and spermine increases the mechanical stability of membranes [159]. In another study, the seminal plasma was reported to contain decapacitating factors which stabilize the sperm membrane and prevent premature

acrosome reaction. Several seminal proteins, including caltrin and acrosome-stabilizing factor, were identified as decapacitating factors and spermine is the major seminal decapacitating factor [166].

Based on the aforementioned studies, I aimed to determine the optimal concentration of spermine and analyze its effects on enhancing sperm motility, maintaining membrane function, and controlling ROS levels and cryocapacitation during canine sperm cryopreservation.

2. Materials and methods

2.1. Animal use

Procedures for animal use were described in general methodology.

2.2. Semen collection and preparation

Semen were collected twice a week from the beagles and only normal ejaculates with sperm concentrations ≥ 100 × 10^6 /mL, motility ≥ 70%, viability ≥ 80% and normal morphology ≥ 80% were pooled in a 15 mL tube. The pooled ejaculates were washed by adding an equal volume of the first buffer (24 g/L Tris [hydroxymethyl] amino methane, 14 g/L citric acid, 8 g/L glucose, 0.6 g/L Na–benzyl penicillin and 1 g/L streptomycin sulfate in distilled water [pH 6.6, 290 mOsm]) and centrifuged at 700*g* for 5 min. The pellets were resuspended by adding the first buffer to achieve a sperm concentration of 200 x 10^6 sperm/mL. This sperm suspension was divided into 5 aliquots for treatment with 0, 0.1, 1, 5 or 10 mM spermine in the second buffer which was made by mixing 54% (v/v) first buffer, 40% (v/v) egg yolk and 6% (v/v) glycerol [121]. All chemicals were purchased from Sigma–Aldrich, St. Louis, MO, USA.

2.3. Sperm freezing and thawing

Each aliquot was mixed with the second buffer to obtain a sperm concentration of 100 × 10^6 /mL at room temperature. The required volume of second buffer was divided into 14%, 19%, 27% and 40% and loaded serially at 30 sec intervals [121].

The extended sperm suspension was filled into 0.25 mL straws and incubated at 4 °C for 1 h. After equilibration, straws were placed horizontally at 2 cm above the surface of LN_2 for 10 min then plunged into the LN_2. The straws were stored in the LN_2 container for one week before being thawed for evaluation.

Thawing was performed in a water bath at 37 °C for 30 sec and then the sperm samples were diluted (1:5) with the first buffer to 14%, 19%, 27% and finally 40% of the total volume [121]. All thawed sperm suspensions were evaluated as follows.

2.4. Sperm motility and kinematic parameters

In total, 5 different fields were analyzed for each sample and the kinematic parameters of at least 200 motile spermatozoa were tracked with a sperm analysis imaging system (FSA2011 premium edition version 2011, Medical Supply Co., Ltd., Korea).

2.5. Membrane integrity and morphological defects

Sperm membrane integrity and morphology were evaluated by the eosin–nigrosin staining method. Sperm suspension smears, consisting of a sperm sample and stain (1:1), were spread onto a warm slide and dried. Afterwards, membrane integrity and morphological defects were assessed under a microscope.

2.6. Assessment of intracellular ROS levels

Thawed sperm cells from each group were washed twice by centrifuging 200

μL of sample in 1000 μL PBS (pH = 7.2, Gibco) at 300*g* for 5 min. The washed spermatozoa were resuspended in 200 μL of PBS, divided into 100 μL aliquots, and each aliquot was incubated with an equal volume of 0.1% nitro blue tetrazolium (NBT) at 37 °C for 45 min. Sperm cells containing the formazan product were washed twice in PBS for 10 min at 500*g* to remove all residual NBT, leaving only a sperm pellet. The intracellular formazan product was solubilized in 60 μL, each of 2 M KOH and dimethyl sulphoxide (DMSO). The reaction mixture after 5 min was dispensed into a microplate (96-well Immuno Plate, SPL Life Sciences Co., Ltd., Korea) and the resulting color was measured using an enzyme-linked immunosorbent assay (ELISA) reader (Sunrise, Tecan, Switzerland, Austria) at 655 nm [172]. ROS production was expressed as micrograms of formazan per 10^7 spermatozoa, derived from a standard curve plotted with the absorbance values *vs.* known concentrations of formazan substrate solubilized in DMSO [172].

2.7. Assessment of extracellular ROS levels

The supernatants were incubated with NBT (0.33 mg/mL, diluted in PBS) in a 96-well plate with 100 μL reaction mixture in each well. The plate was incubated at room temperature in the dark for 5 min, followed by addition of 50 μL DMSO into each well to dissolve the purple-colored formazan crystals. The absorbance was recorded at 655 nm using an ELISA reader with standard substrates.

2.8. Gene expression analysis using real-time polymerase chain reaction

RNA samples were obtained in triplicates from five pairs of canine frozen-thawed spermatozoa from the control group and the 5 mM spermine treatment group. Quantitative real-time PCR (qPCR) was conducted to assess transcript abundance using oligonucleotide primer sequences (Table 3). The mRNA expression of apoptotic genes (*BCL2* and *BAX*), a mitochondrial ROS modulator (*ROMO1*), a gene for repairing DNA damage caused by oxidation (*OGG1*), a spermine synthesis gene (*SMS*), the NADPH oxidase gene associated with motility (*NOX5*) and the spermine amino oxidase gene (*SMOX*) were analyzed by qPCR. Total RNA was extracted using Trizol reagent, according to the manufacturer's protocol, and complementary DNA was produced using amfiRivert cDNA Synthesis Platinum Master Mix (GenDepot, Barker, TX, USA). The qPCR were performed using an ABI 7300 Real Time PCR System (Applied Biosystems, Forest City, CA, USA) and the expression of each target genes were quantified relative to that of the internal control gene (*β-actin*) using the equation, $R = 2^{-[\Delta Ct\ sample\ -\ \Delta Ct\ control]}$ as previously described [173].

Table 3. Primer sequences used for gene expression analysis

Gene	Primer sequences (5'-3')	Product size (bp)	NCBI accession number
ACTB	F: GGCATCCTGACCCTGAAGTA	148	NM_001195845.1
	R: GGTGTGGTGCCAGATCTTCT		
BCL2	F: CTCCTGGCTGTCTCTGAAGG	145	NM_001002949.1
	R: GTGGCAGGCCTACTGACTTC		
BAX	F: GACGGCCTCCTCTCCTACTT	120	NM_001003011.1
	R: GGTGAGTGACGCAGTAAGCA		
ROMO1	F: TGTCTCAGGATCGGAATGCG	100	XM_534406.4
	R: TCCCGATGGCCATGAATGTG		
SMS	F: GTCGCCTGGTTGAGTATGACA	144	XM_005641195.1
	R: ATGCCAAATCACTCTCCGCC		
OGG1	F: CGCATCACTGGCATGGTAGA	133	XM_541781.3
	R: TCCTGAGCTGAGCCTCTACT		
SMOX	F: AGAAGTGTGATGACGAGGCG	128	XM_855324.3
	R: TCGGAAGTATGGGTTGCTGC		
NOX5	F: ACCTGAACATCCCCACCATC	101	NM_001103218.1
	R: TTCAGACCGGATGTGTAGCC		

2.9. Sperm capacitation with reacted acrosomes

One straw of cryopreserved spermatozoa was thawed in a water bath at 37 °C for 30 sec then divided into 2 aliquots. The first aliquot was directly incubated in canine capacitating medium (CCM) supplemented with 1.0 mM $MgCl_2$ and 10 mM P4 for 4 h [91]. The second aliquot was diluted 1:10 with 0.9% NaCl then stained with an equal volume of trypan blue 0.27% (v/v) in normal saline. Two smears were made then fixed in 37% formaldehyde solution for 2 min and then rinsed with distilled water. Slides were dipped in 7.5% (v/v) of Giemsa stock solution freshly made in distilled water and placed in an incubator at 37 °C for 2 h, then rinsed again in distilled water, air-dried and cover-slipped. At least 200 sperm cells were counted for each group with 5 independent replications. The following categories were assessed: 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). Dead spermatozoa stained dark blue while live spermatozoa appeared sky-blue. The anterior part of the sperm head with intact acrosomes was purple, those with damaged acrosomes were lavender, and those with no acrosome were pale gray [174] as shown in Fig. 5. The first aliquot was also stained for acrosomes status after incubation in CCM and compared with the second aliquot.

2.10. Statistical analysis

Procedures for statistical analysis were described in general methodology.

3. Results

3.1. Effect of spermine concentration on sperm motility and kinematic parameters

The average motility of fresh spermatozoa from 5 independent replications was 88.5 ± 0.5 % with linearity of 30.2 ± 0.2 % and straightness of 56.6 ± 0.3 %. The value for spermatozoa with intact membranes was 84.7 ± 2.4 %, while those for bent tail and coiled tail were 7.2 ± 0.4 % and 1.1 ± 0.6 %, respectively.

First, I evaluated the effect of different spermine concentrations (0, 0.1, 1, 5 and 10 mM) on post-thaw sperm motility and kinematic parameters. The percentages of post-thaw motility in all groups were not different but linearity in 5 and 10 mM spermine showed higher percentages (51.6 ± 3.0 % and 49.2 ± 1.8 %, respectively) than the control (38.0 ± 1.6 %, Table 4). Both concentrations increased the straightness percentage (71.9 ± 2.3 % and 70.6 ± 1.4 %, respectively) *vs.* the control (60.8 ± 1.5 %) and also significantly reduced the amplitude of lateral head displacement (ALH) (5.6 ± 0.2 % and 5.4 ± 0.2 %, respectively) *vs.* the control (7.3 ± 0.4 %, $P < 0.05$). There were no differences between 5 and 10 mM spermine on linearity, straightness and ALH.

Table 4. Sperm function and sperm morphology in frozen-thawed spermatozoa with spermine treatment

Spermine (mM)	Motility (%)	VCL (μm/sec)	VSL (μm/sec)	VAP (μm/sec)	Linearity (%)	Straightness (%)	ALH (μm)	Intact membranes (%)	Bent tail (%)	Coiled tail (%)
0	49.7 ± 3.4	138.4 ± 6.9^{a}	43.7 ± 1.7^{a}	86.1 ± 1.9^{a}	38.0 ± 1.6^{a}	60.8 ± 1.5^{a}	7.3 ± 0.4^{a}	60.7 ± 1.3^{a}	16.2 ± 1.2	3.4 ± 0.8^{ab}
0.1	50.7 ± 3.5	135.8 ± 7.5^{a}	46.7 ± 1.5^{ab}	86.8 ± 3.1^{a}	40.5 ± 2.1^{a}	63.0 ± 2.0^{ab}	7.1 ± 0.3^{a}	61.6 ± 2.2^{ab}	15.6 ± 1.2	2.1 ± 0.5^{ab}
1	50.9 ± 3.4	99.8 ± 6.8^{b}	52.2 ± 0.7^{bc}	65.1 ± 2.7^{b}	44.6 ± 3.6^{ab}	67.4 ± 3.2^{ab}	6.9 ± 0.3^{a}	73.1 ± 4.3^{bc}	13.8 ± 1.2	1.2 ± 0.3^{a}
5	51.1 ± 2.7	98.5 ± 4.7^{b}	54.5 ± 1.4^{c}	70.2 ± 2.0^{b}	51.6 ± 3.0^{c}	71.9 ± 2.3^{b}	5.6 ± 0.2^{b}	73.7 ± 2.9^{c}	13.1 ± 1.0	2.5 ± 0.6^{ab}
10	49.6 ± 2.8	95.6 ± 5.1^{b}	50.3 ± 1.1^{bc}	66.4 ± 2.8^{b}	49.2 ± 1.8^{bc}	70.6 ± 1.4^{b}	5.4 ± 0.2^{b}	72.9 ± 2.0^{bc}	15.8 ± 1.4	5.4 ± 1.6^{b}

$^{a-c}$ within a column, values with different superscripts differ significantly among five spermine concentrations (0, 0.1, 1, 5 and 10 mM, $P < 0.05$, $n = 5$). VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; ALH, amplitude of lateral head displacement.

3.2. Effect of spermine concentrations on membrane integrity and morphological defects

A total of 5,137 spermatozoa were analyzed in five independent replicates to evaluate the effect of spermine on membrane integrity and morphological defects after freezing (Table 4). Membrane integrity in the 1, 5 and 10 mM spermine groups showed similar percentages (73.1 ± 4.3%, 73.7 ± 2.9% and 72.9 ± 2.0%, respectively) but these values were significantly higher than the control and 0.1 mM spermine groups (60.7 ± 1.3% and 61.6 ± 2.2%, respectively). There were no significant differences for bent tail and coiled tail parameters between the 5 and 10 mM groups compared to the control.

3.3. Effect of spermine concentrations on intracellular and extracellular ROS levels

Concentrations of intracellular ROS in all treatment groups were significantly lower than in the control (71.5 ± 2.2 μg formazan/ 10^7 spermatozoa) and the lowest values were found with 5 and 10 mM spermine groups (13.0 ± 3.2 and 12.4 ± 2.9 μg formazan/ 10^7 spermatozoa, respectively). Both treatments also reduced extracellular ROS (6.6 ± 1.4 and 6.5 ± 1.4 μg formazan/ ml supernatant, respectively) compared with the control (11.7 ± 0.2 μg formazan/ ml supernatant, $P < 0.05$) which is shown in Table 5 and Fig. 7. However, ROS production with 10 mM spermine treatment did not differ from that of 5 mM spermine treatment.

Table 5. Intracellular and extracellular reactive oxygent species (ROS) levels in frozen-thawed spermatozoa with spermine treatment

Spermine (mM)	Intracellular ROS (μg formazan/ 10^7 sperm)	Extracellular ROS (μg formazan/ mL supernatant)
0	71.5 ± 2.2^{a}	11.7 ± 0.2^{a}
0.1	53.1 ± 6.7^{b}	9.6 ± 1.0^{ab}
1	33.7 ± 4.5^{c}	8.2 ± 1.5^{ab}
5	13.0 ± 3.2^{d}	6.6 ± 1.4^{b}
10	12.4 ± 2.9^{d}	6.5 ± 1.4^{b}

$^{a-c}$ within a column, values with different superscripts differ significantly among five spermine concentrations (0, 0.1, 1, 5 and 10 mM, $P < 0.05$, $n = 5$).

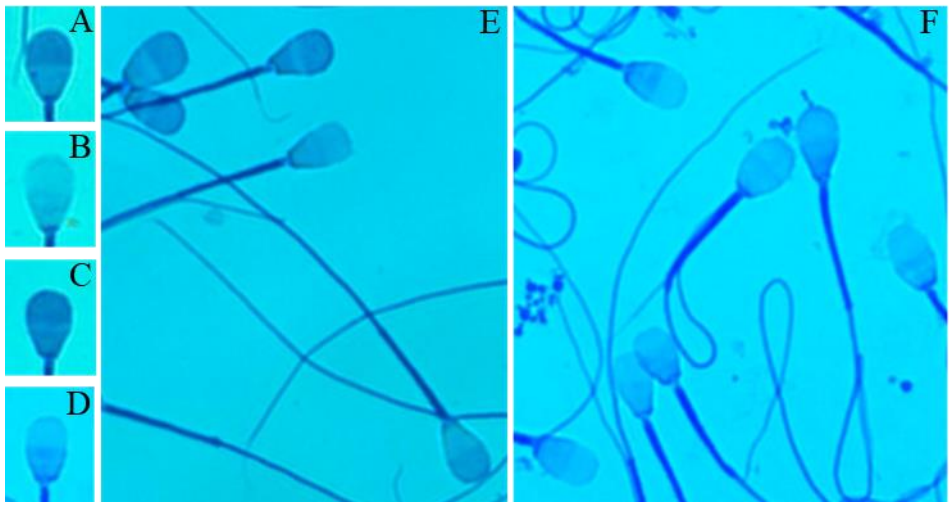

Figure 5. The acrosome staining of frozen-thawed sperm before and after capacitation

A) LSIA, live sperm intact acrosome; B) LSDA, live sperm damaged acrosome; C) DSIA, dead sperm intact acrosome; D) DSDA, dead sperm damaged acrosome; E) post thawing and F) after incubating in canine capacitating medium (CCM) supplemented with 1 mM MgCl2 and 10 mM P4 for 4 hours.

3.4. Effect of 5 mM spermine on gene expression

The effect of 10 mM spermine on sperm motility, membrane integrity and ROS concentration was not significantly improved from those with 5 mM spermine. Because high concentrations of spermine could have detrimental effects on sperm cells, 5 mM was chosen as the optimum spermine concentration for further experiments. Treatment with 5 mM spermine during canine sperm cryopreservation significantly increased *BCL2* transcript levels by 1.5-fold and decreased *BAX* to one-fourth compared with the control as shown in Fig. 6. The expression of *ROMO1* and *NOX5* was significantly reduced to around one-third in the treated group compared with the control. Furthermore, gene expression of *SMS*, *OGG1* and *SMOX* also decreased to about 25% of control levels ($P < 0.05$).

3.5. Effect of 5 mM spermine on capacitation of frozen-thawed spermatozoa

The percentage of LSIA in the treatment group (7.9 ± 0.3 %) was higher than in the control (4.4 ± 0.3 %). Treatment with spermine during cryopreservation also reduced the percentage of DSDA (26.8 ± 1.3 %) compared with the control (33.2 ± 1.8 %). However, there were similar percentages of LSRA and DSIA as shown in Table 6. Interestingly, after incubation in CCM for 4 h, the percentage of LSRA in the treatment group (64.8 ± 1.2 %) was significantly higher than in the control (59.8 ± 1.6 %, $P < 0.05$). The DSRA percentage in the treated group was also lower than in the control (29.6 ± 1.4 % and 35.1 ± 1.6 %, respectively).

Table 6. Acrosome staining results post thawing and after incubation in canine capacitating medium (CCM)

Spermine (mM)	Post thawing				After incubation in CCM			
	LSIA (%)	LSRA (%)	DSIA (%)	DSRA (%)	LSIA (%)	LSRA (%)	DSIA (%)	DSRA (%)
0	4.4 ± 0.3[a]	56.2 ± 1.4	5.1 ± 0.5	33.2 ± 1.8[a]	0.0 ± 0.0	59.8 ± 1.6[a]	6.2 ± 0.9	35.1 ± 1.6[a]
5	7.9 ± 0.3[b]	58.7 ± 1.4	5.6 ± 1.1	26.8 ± 1.3[b]	0.0 ± 0.0	64.8 ± 1.2[b]	6.6 ± 1.0	29.6 ± 1.4[b]

[a-b] within a column, values with different superscripts differ significantly among two spermine concentrations (0 mM and 5 mM, $P < 0.05$, $n = 5$). LSIA, live sperm intact acrosome; LSRA, live sperm reacted acrosome; DSIA, dead sperm intact acrosome; DSRA, dead sperm reacted acrosome

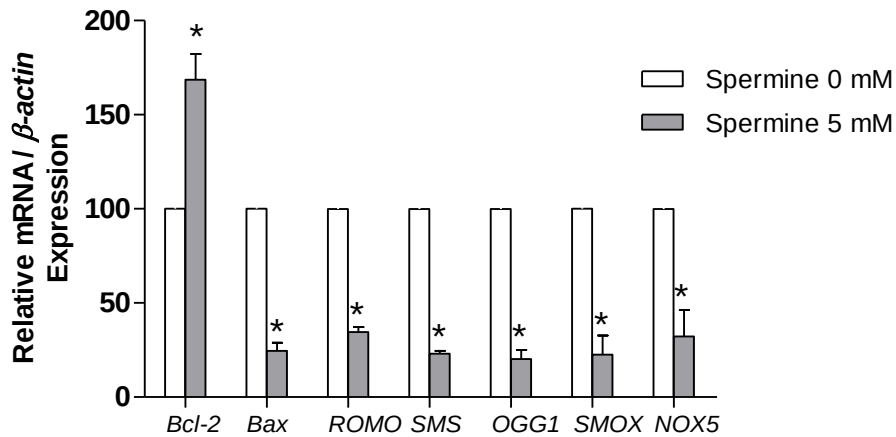

Figure 6. Gene expression related apoptosis and oxidative stress using 0 mM and 5 mM spermine

Bcl-2, B-cell lymphoma 2; *Bax*, Bcl-2–associated X protein; *ROMO1*, a mitochondrial ROS modulator; *OGG1*, a gene for repairing DNA damage caused by oxidation; *SMS*, a spermine synthesis gene; *NOX5*, the NADPH oxidase gene associated with motility and *SMOX*, the spermine amino oxidase gene (*$P < 0.05$).

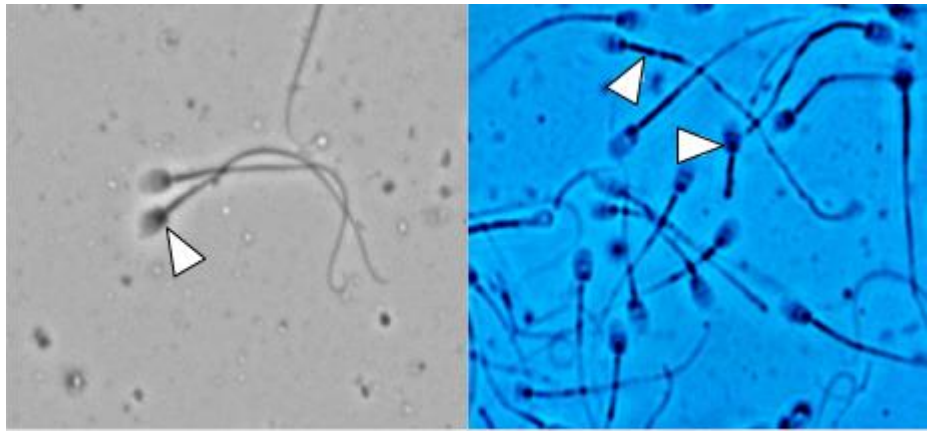

Figure 7. Intracellular ROS in head and midpiece of sperm

White arrows show the formazan complex in the head and midpiece of spermatozoa (1,000 X magnification).

4. Discussion

Accumulated ROS is responsible for damage to mammalian spermatozoa and dysfunction associated with the reduced sperm quality [156], especially during cryopreservation [157]. Spermatozoa are vulnerable to ROS damage due to the high polyunsaturated fatty acid content in their cell membranes, requiring antioxidants to reduce the negative impact of oxygen-induced damage and lipid peroxidation [161]. In order to reduce these adverse effects of ROS during cryopreservation, I treated the second buffer (cryopreservation extender/diluent) with spermine, which is a natural component of polyamines in the seminal fluid of humans and rats [160] and protects against oxidative stress in several ways including maintaining membrane structure and function, nucleic acid structure and stability [159]. However, toxicity of spermine on mammalian cells has been reported [159, 175] so that an appropriate concentration should be carefully determined; moreover, canine spermatozoa are also hyper-sensitive to osmotic pressure [121] and ROS [156].

In this study, the second buffer used to treat spermatozoa was supplemented with 0.1, 1, 5 and 10 mM spermine, followed by cryopreservation and sperm quality analysis. Our results showed that while post-thaw motility in 5 and 10 mM spermine was not improved, linearity and straightness values were higher with a lower percentage of ALH compared with controls (Table 4). Although standard values of linearity, straightness and ALH for dog spermatozoa have not been established, a previous study demonstrated that a significant decrease in linearity or straightness and an increase in ALH are generally regarded as indicative of hyperactivation [13].

Based on that report, our finding of higher percentages of linearity and straightness along with lower ALH indicated that both 5 mM and 10 mM spermine treatments could prevent hyperactivation.

Oxidative processes and ROS generation produce chemical and physical stress on sperm membranes [24, 158], which could reduce sperm fertility [61]. After treatment with 0.1, 1, 5 or 10 mM spermine, the percentages of spermatozoa with intact membranes were higher than in the control. The higher sperm membrane integrity in treatment groups indicated an ability of spermine to maintain cell structure [159]. Ambrosi *et al.* [176] also reported that addition of 0.1 mM spermine before ram sperm cryopreservation could act as an antioxidant and stabilize sperm membrane properties. This study only used a small amount of spermine, equivalent to its levels in ram seminal fluid and spermatozoa [160]. Assessment of ROS levels using NBT together with ELISA in canine spermatozoa is reported for the first time and appears to be effective and feasible to perform as a regular evaluation procedure, as it is in humans [172]. The formazan complex can be identified as purple particles in the head and midpiece of canine spermatozoa (Fig. 6) which reflects intracellular ROS production, while purple particles are also found in supernatant that exhibited extracellular ROS [25, 172]. The low concentration of intracellular and extracellular ROS (Table 5) exhibited after 5 and 10 mM spermine treatments indicates that spermine can suppress ROS production during cryopreservation.

All the assessments of canine sperm quality and ROS production suggested that 0.1 to 10 mM spermine treatment had positive effects on frozen-thawed sperm. Moreover, the motility, kinematic parameters, morphology and ROS levels all

showed that treatment with 10 mM spermine did not provide more advantage compared to 5 mM spermine. Because of deleterious effects of high concentrations of spermine [161, 173] and the risk of activating amine oxidase [159, 175], I consider that 5 mM is the optimum concentration for spermine supplementation in canine sperm cryopreservation.

To investigate the effect of 5 mM spermine on apoptosis, I analyzed the gene expression of *BAX* and *BCL2*. Expression of the proapoptotic gene, *BAX*, was reduced significantly while expression of the antiapoptotic gene, *BCL2*, was significantly higher in the spermine treated group. These results indicate that spermine treatment might reduce apoptosis during sperm cryopreservation. Low expression of *ROMO1*, an essential gene for inducing ROS production, in the treatment group indicated lower ROS production [177]. The beneficial effects of spermine as an antioxidant were also reflected in down-regulated gene expression of *OGG1*, *SMS*, *NOX5* and *SMOX* as shown in Fig. 5. Low expression of *OGG1* indicated that there was only a small amount of DNA damage [178] in spermatozoa during cryopreservation and low expression of *NOX5* showed lower levels of oxidized NADPH [179]. The *SMS* and *SMOX* in the spermine treated group also resulted low expression which means the spermatozoa exhibited a low oxidative stress response [159, 175].

The benefit of reducing ROS production by treatment with spermine could also be seen in the pattern of acrosome reactions [156]. The acrosome staining analysis in the spermine-treated group showed higher percentages of live spermatozoa with intact acrosomes than in the control group. Moreover, after

incubation in modified CCM [91], the proportion of live spermatozoa with reacted acrosomes was significantly higher than the control (Table 6). These results indicate that spermine protects sperm cells during cryopreservation, thus allowing more live cells to survive and then undergo acrosome reaction in CCM, while without spermine, fewer cells survive and so there are fewer live spermatozoa able to undergo acrosome reaction. Similar advantages of spermine treatment were also reported in a study on cryopreservation of ram spermatozoa which concluded that spermine played some roles in plasma membrane stabilization and prevention of premature capacitation [176].

In conclusion, supplementation of second buffer with 5 mM spermine can protect canine spermatozoa during cryopreservation sufficiently to maintain post-thaw motility, improve kinematic parameters and membrane integrity, reduce cryocapacitation during cryopreservation and increase the proportions of capacitated spermatozoa after incubation in CCM. Further studies are needed to determine the fertilizing capability of canine frozen-thawed spermatozoa *via* artificial insemination or *in vitro* fertilization.

PART IV

CANINE *IN VITRO* FERTILIZATION WITH FROZEN-THAWED SPERM

Chapter I. Effect of canine capacitation media supplemented with conditioned media on canine *in vitro* fertilization.

1. Introduction

Cryopreservation of semen results in reduced fertility compared with fresh semen, due to a combination of loss of sperm viability and reduced fertilizing ability of the surviving cells [10]. However, interaction of canine gametes offers a useful approach to evaluating presumptive fertilizing ability of spermatozoa, because this interaction is a complex process that requires several sperm functions involving initial recognition, attachment followed by binding, acrosome reaction and penetration of the zona matrix [114].

Many studies have been conducted to overcome the disadvantages of cryopreservation in sperm, such as: 1) osmotic stress caused by glycerol movement through the cell membrane followed by cell shrinkage and swelling, 2) intracellular and extracellular ice crystal formation that leads to disruption of cellular membrane and organelles, 3) oxidative stress caused by removal of seminal plasma during centrifugation which limits intracellular antioxidant and stimulates peroxidative membrane damage, 4) the use of cryoprotectant which is toxic to the intracellular component [25, 64, 67, 107-109]. Recently, some scientists have used exogenous growth factors for alleviating these detrimental effects of sperm freezing and many reports showed the superior results in sperm quality, viability and fertility. For brief periods of time, IGF1 maintain progressive motility and mitochondrial membrane

potential, FGF2 would increase sperm kinematics and progressive motility, HGF induce potent motogenic effects including motility, transforming growth factor beta (TGFβ) maintain homeostasis of sperm, brain-derived neurotropic factor (BDNF) associated with some types of male infertility, tumor necrosis factor alpha (TNFα) maintain homeostasis of sperm, and NGF improve sperm viability and motility [34, 40, 180-183].

Various studies on stem cell-derived secreted factors show that the secreted factor alone, without the stem cell itself, may cause tissue repair in various conditions that involve tissue/organ damage [184-187]. The secreted factors are referred to as secretome, microvesicles, or exosome and can be found in the medium where the stem cells are cultured; thus, the medium is called conditioned medium [188, 189]. Conditioned medium contains various growth factors and tissue regenerative agents, which are secreted by stem cells. The fact that stem cells secrete various growth factors was also shown by various proteomic studies, which revealed the presence of various growth factors and other cytokines in the CM [186, 188-190]. The growth factors which were secreted by ASCs included BDNF, NGF, basic fibroblast growth factor (bFGF), keratinocyte growth factor (KGF), TGFβ, HGF, vascular endothelial growth factor (VEGF) and IGF1 into CM, which might have mediated the physiological process in many organic systems [186, 189, 191].

Basic research and preclinical studies in the field of regenerative medicine have been conducted to overcome clinical shortcomings with the use of mesenchymal stem cells (MSCs). MSCs are present in adult tissues, including the

bone marrow and adipose tissues [192]. However, recent studies have shown that subcutaneous adipose tissue provides a clear advantage over other stem cell sources due to the ease with which adipose tissue can be accessed as well as the ease of isolating stem cells from harvested tissue [193]. ASCs are a kind of mesenchymal stem cell within the stromal-vascular fraction of subcutaneous adipose tissue that display a multilineage developmental potential and secrete various growth factors [186, 189, 191]. ASCs exhibit stable growth and proliferation kinetics and can differentiate toward osteogenic, chondrogenic, adipogenic, myogenic, or neurogenic lineages *in vitro* [194, 195].

There is no consensus when it comes to the nomenclature used to describe progenitor cells from adipose tissue-derived stroma, which can sometimes lead to confusion. The term ASCs refers to adipose-derived stem cells, and this will be used throughout this thesis.

ASCs are capable of self-renewal and are able to differentiate into various types of cell populations, including adipocytes, osteoblasts, chondrocytes, myocytes and neurons. Thus, ASCs are valuable sources of stem cells for use in regenerative medicine and cosmetic applications [196]. Similar with embryonic stem cells and induced pluripotent stem cells, ASCs contribute to tissues/cells rejuvenation and restoration, as well as reproductive organs, bone and cartilage formation. These therapeutic effects of ASCs are considered to result from the differentiation of ASCs into multiple cell types, as well as the paracrine action of the diverse cytokines and growth factors [187, 188].

There have been few reports regarding IVF and IVC of canine embryos [96, 114]. The first attempt to conduct IVM/IVF in dogs was reported by Mahi and Yanagimachi [97]; they achieved approximately 20–30% fertilization rates, as judged by the presence of swelling sperm nuclei within the oocyte. Subsequent studies yielded low proportions of embryos developing beyond the 8-cell stage [197, 198] and only one blastocyst [103]. A successful IVF was reported in the end of 2015 using fresh semen and an *in vivo* matured oocyte combined with embryo freezing [91]. The generation of a successful protocol for IVF in the dog lays the foundation for application of gene-editing technologies and also provides a means to perform gamete rescue in endangered canid species [199].

The objectives of the current study are to determine 1) the optimum concentration of conditioned media from human ASCs in supporting post thawing sperm quality and sperm capacitation which is essential for fertilization competence; 2) the optimal oocyte stage for IVF; 3) the *in vitro* developmental competence of canine IVF embryos using frozen-thawed sperm.

2. Materials and methods

2.1. Animals use

Procedures for animal use were described in general methodology.

2.2. Sperm collection and freezing

Semen were collected twice a week from the five beagles and only normal ejaculates with sperm concentrations ≥ 100 × 10^6 /mL, motility ≥ 70%, viability ≥ 80% and normal morphology ≥ 80% were pooled in 15 mL tubes. The pooled ejaculate was washed by adding an equal volume of the first buffer (24 g/L Tris [hydroxymethyl] amino methane, 14 g/L citric acid, 8 g/L glucose, 0.6 g/L Na–benzyl penicillin, and 1 g/L streptomycin sulfate in distilled water [pH 6.60, 290 mOsm]) and was centrifuged at 700*g* for 5 min. The pellet was resuspended by adding the first buffer to achieve a sperm concentration of 200 x 10^6 /mL. 5 mM spermine was added to the sperm suspension in the second buffer, which was made by mixing 54% (v/v) first buffer, 40% (v/v) egg yolk and 6% (v/v) glycerol [115].

The basic procedures for sperm freezing and thawing were described in general methodology. Each aliquot was mixed with the second buffer to obtain a sperm concentration of 100 × 10^6 /mL at room temperature. The required volume of the second buffer was divided into 14%, 19%, 27% and 40% and loaded serially at 30 sec intervals [115, 121]. The extended sperm suspension was filled into 0.25 mL straws and incubated at 4 °C for 1 h. After equilibration, straws were placed horizontally, 2 cm above the surface of LN_2 for 10 min then plunged into the LN_2.

The straws were stored in the LN_2 container for one week before being thawed for evaluation.

Thawing was performed in a water bath at 37 °C for 30 sec and then the sperm samples were diluted (1:5) with the first buffer to 14%, 19%, 27% and finally 40% of the total volume [121].

2.3. Oocyte recovery

In vivo matured oocytes were obtained by using the oviducts flushing method with Hepes-buffered TCM199 [86]. Briefly, blood was drawn alternately from cephalic and saphenous veins 3–7 days a week. Daily blood sampling was performed when proestrus was detected (the presence of serosanguinous discharge from the vulva and/or serum P4 values higher than 0.4 μg/mL). Collected blood was allowed to clot then centrifuged at 700*g* for 10 min to separate the serum, which was then evaluated *via* chemiluminescent immunoassay [123]. The days of the LH surge and ovulation were identified based on P4 values of 1.5–2.5 and 4.0–9.9 ng/mL, respectively. Approximately 70–76 h after ovulation, the dogs was subjected to the oocyte collection procedure.

The dogs were pre-anesthetized with 5 mg/kg ketamine HCl and 1 mg/kg xylazine, and anesthesia was maintained with 2% isoflurane. After the abdominal region was prepared aseptically, a midline incision was made, and the ovary was pulled out. A 16 gauge flushing needle was inserted into the opening of the infundibulum and tied in with a ligature. An intravenous catheter was inserted into the caudal portion of oviduct. The Hepes-buffered TCM-199 supplemented with 10%

(v/v) FBS was introduced into the oviduct using a 5 mL syringe. The flushed medium containing oocytes was collected from the flushing needle. The quality of the recovered oocytes was determined with a micromanipulator based on the morphology and width of the PVS. Oocytes without PVS and first polar body were regarded as immature. Oocytes with PVS around 15 μm and more than 25 μm were respectively classified as mature and aging [200]. Only oocytes with the first polar body were used.

2.4. Sperm capacitation and staining

One straw of cryopreserved spermatozoa was thawed in a water bath at 60 °C for 7 sec then divided into 2 aliquots. They were then directly incubated in CCM supplemented with 1.0 mM $MgCl_2$ and 10 mM P4 for 2 h [91]. 25% conditioned media was added to the first aliquot and the second aliquot was diluted with 25% CCM, then stained with an equal volume of trypan blue 0.27% (v/v) in normal saline. Two smears were made and then were fixed in 37% formaldehyde solution for 2 min before being rinsed with distilled water. Slides were dipped in 7.5% (v/v) of Giemsa stock solution, freshly made in distilled water, and placed in an incubator at 37 °C for 2 h. They were rinsed again in distilled water, air-dried and cover-slipped. At least 200 sperm cells were counted for each group with 5 independent replications. The following categories were assessed: 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). Dead spermatozoa stained dark blue while live spermatozoa appeared sky-blue. The

anterior part of the sperm head with intact acrosomes was purple, those with damaged acrosomes were lavender, and those with no acrosome were pale gray [174]. The first aliquot was also stained for acrosomes status after incubation in CCM and the results were compared with those of the second aliquot.

2.5. PCR and real-time PCR

RNA samples were obtained in triplicates from five pairs of canine frozen-thawed spermatozoa from the control group with 100% CCM and treatment groups with 25% CM and 50% CM supplementation. Quantitative real-time PCR (qPCR) was conducted to assess transcript abundance using oligonucleotide primer sequences (Table 9). The mRNA expression of apoptotic genes (*BCL2* and *BAX*), protamine 2 (*PRM2*), protamine 3 (*PRM3*), and sperm acrosome associated 3 (*SPACA3*) were analyzed by qPCR. Total RNA was extracted using Trizol reagent, according to the manufacturer's protocol, and complementary DNA was produced using amfiRivert cDNA Synthesis Platinum Master Mix (GenDepot, Barker, TX, USA). The qPCR were performed using an ABI 7300 Real Time PCR System (Applied Biosystems, Forest City, CA, USA) and the expression of each target genes were quantified relative to that of the internal control gene (*β-actin*) using the equation, $R = 2^{-[\Delta Ct\ sample - \Delta Ct\ control]}$ as previously described [115].

2.6. *In vitro* fertilization and embryo culture

Oocytes were washed in KSOM medium before transfer to fresh, pre-equilibrated 90 µl droplets of the medium covered with mineral oil for IVF. *In vitro*

capacitated sperm (incubated for 2 h under capacitating conditions) in 10 µl were added to the oocytes IVF droplet at a final concentration of 1 x 10^6 sperm / mL. The gametes were co-incubated for 3 h at 38 °C and 5% CO_2 and 90% N_2 in an incubator. The 3 to 5 zygotes were transferred to pre-equilibrated 50 µL droplets in mineral oil. Embryo cleavage was evaluated at 48 h post-IVF, then embryos were re-evaluating cleavage stage every 12 h.

All inseminated oocytes were transferred to KSOM medium and cultured 3-21 h in incubator at 5% CO_2, 7% O_2 and 88% N_2 for identification of sperm head decondensation (MPN) and examined cell number of expanded blastocyst at 168 h. Cleavage rates (Day 2 and 5) were recorded to assess the *in vitro* developmental capacity of embryos. The rate of survival and cleavage and the cell number of survived embryos were investigated at 168 h after IVF by bisbenzimide (Hoechst 33342) staining.

2.7. Embryo staining and gel electrophoresis

Half of the IVF embryos from each experimental group were washed in PBS, and the nuclei were stained with 25 µg/mL bisbenzamide (Hoechst) for 1 h at 37 °C. Stained blastocysts were mounted on a glass slide in a drop of glycerol, gently flattened with a cover glass, and examined for cell counting with a fluorescence microscope using a 346 nm excitation filter. Digital photographs were also taken for total cell counting using ImageJ 1.42q software.

The cDNA of IVF embryos were subjected to PCR using a Maxime PCR PreMix kit-i-StarTaq (Intron Biotech., Seoul, Republic of Korea). The PCR

amplification was carried out for one cycle of denaturation at 95 °C for 5 min and a subsequent 40 cycles with denaturation at 95 °C, annealing for 30 sec, extension at 72 °C for 45 sec, and a final extension at 72 °C for 5 min. Ten microliters of PCR products were fractionated on a 1% agarose gel (iNtRON Biotechnology, Inc., Korea) and stained with RedSafe ™ (iNtRON Biotechnology, Inc.). In all assays, cDNA template negative and reactions without RT resulted in negative amplification. The positive control was a cDNA of canine sperm, and the negative control was NWF. The base procedures for the IVF were described in general methodology. Gel electrophoresis was used for confirming canine embryos using *β-actin*.

2.8. Transfer embryo

Embryos were transferred into recipient using a surgical method. Recipients were prepared by predicting ovulation time based on serum P4 concentrations, and embryo transfer was held around 72 h after ovulation. Anesthesia was induced with 5 mg/kg ketamine HCl and 1 mg/kg xylazine, and general anesthesia was maintained with 2% isoflurane. Recipients in dorsal recumbence were aseptically prepared for surgery and a midline ventral incision was made to expose the reproductive tract. The zygotes or embryos were transferred using a 3.5-Fr Tom Cat Catheter that was connected to a 1 mL syringe, inserted into the ampullary portion of the oviducts. The reproductive tract was put back gently before closing the abdomen. A pregnancy diagnosis was done approximately 31 days after embryo transfer, using an ultrasonography imaging diagnosis system [200, 201].

Table 7. Primer sequences used for gene expression analysis in dogs

Gene	Primer sequences (5' → 3')	Product size (bp)	GenBank No.
B-actin	F- GATCTGGCACCACACCTTCT	148	NM_001195845.1
	R- GTACATGGCTGGGGTGTTGA		
BAX	F- CGAATGTCTCAAGCGCATCG	120	NM_001003011.1
	R- AACATCTCAGCTGCCACTCG		
BCL2	F- TCATGTGTGTGGAGAGCGTC	145	NM_001002949.1
	R- GGGCCGTACAGTTCCACAAA		
PRM2	F- AGGAGGAGATACAGGAGGTGC	148	NM_001287148
	R- CTTGCAAACTCAGGGCTTGG		
PRM3	F- GGCCACGAATCCTCCATGAA	128	XM_847270.4
	R- AGCTCCTCCTCTTCCTCCT		
SPACA3	F- GGATTTCGGCATGGAGGGAT	149	NM_001197087.1
	R- ACTTCCGGCTGTTGATCTGG		

2.9. Experimental design

2.9.1. The supplementation of conditioned media in sperm capacitation which essential for fertilization competence

The sperm motility parameter using a CASA system, the capacitation rate by double staining and gene expression related to fertility were examined after incubation in different capacitating media of control group (100% CCM) and CM treatment group (75% CCM + 25% CM). Differences between groups were evaluated using the $P < 0.05$ significance level.

2.9.2. The optimal oocyte stage for IVF

The recovered oocyte was determined as immature, mature and aging based on PVS [200] then used for IVF using frozen-thawed sperm and *in vitro* cultured with KSOM. The *in vitro* embryo development and cleavage rates were analyzed on cleavage 2-cell, 4-cell and > 8-cells.

2.9.3. The effect of conditioned media from human ASCs in supporting pregnancy rate after embryo transfer

The pregnancy rates from the control group compared with CM treatment groups were investigated after 15 h post-IVF followed by embryo transfer using a 3.5-Fr Tom Cat Catheter (Sherwood, St. Louis, MO, USA) into the ampullary portion of the oviducts of naturally synchronous recipients. Recipients were prepared by naturally synchronizing with oocyte donor dogs based on the serum P4 concentrations for predicting ovulation time. Pregnancy diagnoses were assessed

with SonoScape S8 (Sonoscape Medical Corp., Guangdong, China) approximately 31 days after embryo transfer.

2.10. Statistical analysis

Procedures for statistical analysis were described in general methodology.

3. Results

3.1. The supplementation of conditioned media in sperm capacitation which is essential for fertilization competence

The motility of frozen-thawed sperm after incubation in 75% CCM supplemented with 25% CM for 2 h (56.2 ± 1.6 %) was significantly higher than 50% CM treatment and only CCM groups (32.2 ± 1.6 % and 49.7 ± 1.4 %, respectively). The progressive motility and linearity also showed similar patterns in that they were significantly higher in 25% CM groups compared to 75% and 0% of CM supplementation groups as shown in Table 8. The other valuable parameter was viability. Supplementation of 25% CM in CCM resulted in a high percentage of post thawing sperm viability (74.5 ± 3.3 %) compared with 75% and without CM (44.6 ± 2.7 % and 58.5 ± 0.8 %).

Acrosome staining results after incubation on CCM supplemented with 25% CM for 2 h expressed higher percentage of LSRA compared with only CCM (64.6 ± 1.3 % *vs.* 55.8 ± 1.2 %). The DSRA in 25% CM group (25.4 ± 1.5 %) showed lower percentage than CCM group (31.8 ± 1.2 %, Table 9).

The gene expression related apoptosis (*BAX* and *BCL2*) were not different between CCM and 25% CM groups. The expression of genes related DNA packaging (*PRM2*), sperm motility (*PRM3*) and egg recognition-fertilization (*SPACA3*) were significantly up regulated after incubating in 25% CM supplementation (Fig. 8).

Table 8. The function of frozen-thawed sperm after incubation on canine capacitating medium (CCM) supplemented with conditioned medium (CM)

Groups	Motility (%)	Progressive Motility (%)	VCL (μm/sec)	VSL (μm/sec)	VAP (μm/sec)	Linearity (%)	Straightness (%)	Viability (%)
100% CCM	49.7± 1.4^{b}	23.9± 1.4^{b}	99.5 ± 2.0^{c}	52.9 ± 1.8^{b}	64.9 ± 2.1^{ab}	53.2± 1.4^{a}	82.2± 4.2^{a}	58.5± 0.8^{a}
75% CCM + 25% CM	56.2± 1.6^{c}	30.1± 1.7^{c}	88.0 ± 1.3^{b}	54.8 ± 1.9^{b}	71.3 ± 1.7^{b}	62.4± 2.6^{b}	77.1± 3.2^{a}	74.5± 3.3^{b}
50% CCM + 50% CM	32.2± 1.6^{a}	16.7± 0.5^{a}	76.3 ± 1.7^{a}	40.8 ± 1.8^{a}	60.5 ± 1.8^{a}	53.7± 2.9^{a}	67.6± 2.4^{b}	44.6± 2.7^{c}

$^{a-c}$ within a column, values with different superscripts differ significantly among groups ($P < 0.05$, $n = 5$).

Table 9. Acrosome staining results after incubation on canine capacitating medium (CCM) supplemented with conditioned medium (CM)

Treatment	After incubate in CCM for 2 h			
	LSIA (%)	LSRA (%)	DSIA (%)	DSRA (%)
100% CCM	5.8 ± 0.6	55.8 ± 1.2[a]	6.6 ±1.0	31.8 ± 1.2[a]
75% CCM + 25% CM	3.8 ± 0.7	64.6 ± 1.3[b]	6.2 ± 0.9	25.4 ± 1.5[b]

[a-b] within a column, values with different superscripts differ significantly among groups ($P < 0.05$, $n = 5$).

Conditioned medium (CM) was harvested from media of human adipose-derived stem cells. LSIA, live sperm intact acrosome; LSRA, live sperm reacted acrosome; DSIA, dead sperm intact acrosome; DSRA, dead sperm reacted acrosome

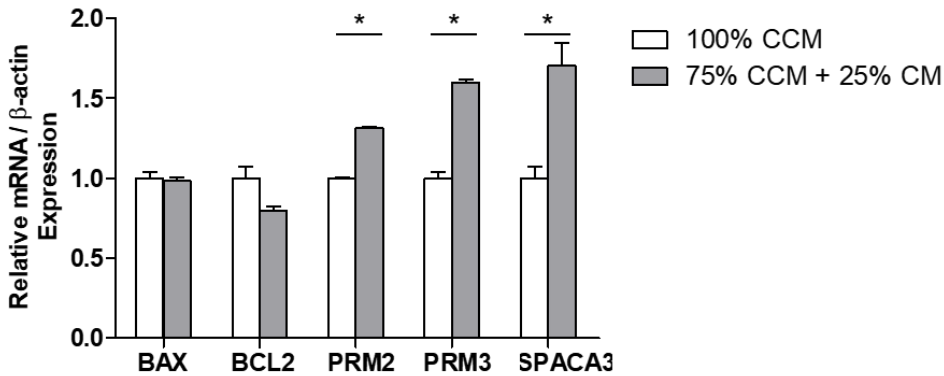

Figure 8. Gene expression from frozen-thawed sperm after capacitation

The gene expression from frozen-thawed sperm after incubation in canine capacitation media (100% CCM) and 75% CCM with 25% conditioned media (CM). *BAX*, Bcl-2–associated X protein; *BCL2*, B-cell lymphoma 2; *PRM2*, protamine 2 (control the sperm DNA packaging/DNA-binding proteins in the nucleus); *PRM3*, protamine 3 (manage sperm motility/flagellated sperm motility); *SPACA3*, sperm acrosome associated 3 (have roles in fertilization, sperm egg recognition and catabolic process). The CM was harvested from media of human adipose-derived stem cells.

3.2. The efficiency of ovulated oocyte prediction based on P4 level to obtain developmentally competent oocytes

The oocyte recovery by predicted ovulation time based on P4 level at 4.2 ± 0.3 ng/mL showed 81.1% (30/37) of female dogs producing mature oocytes, 8.1% (3/37) producing immature oocytes and 10.8% (4/37) producing aged oocytes as showed in Table 10 and Fig. 9A-C. All oocytes of these classifications were used for IVF and resulted in a cleavage rate of 25.0% from immature oocytes, 70.5% from mature oocytes and 51.4% from aged oocytes.

3.3 The effect of KSOM defined media during IVF and embryo culture

The use of KSOM during IVF and embryo culture in frozen-thawed sperm could result cleavage rate. The number of embryos developing to > 4 cells with fresh sperm also resulted in a higher percentage than frozen-thawed sperm, as shown in Table 11. The cleavage rate in IVF using frozen-thawed sperm and KSOM as culture media was 62.6 ± 3.8 % which is shown in Table 11.

The sperm penetration to fertilization stages shown in Fig. 10 reveal that some oocytes exhibited a pronucleus while other oocytes were not fertilized after incubating for 17 to 21 h in culture media. The randomly chosen penetrated oocytes and serial canine embryo developments from 4 cells up to > 8 cells are also shown in Fig. 10.

Table 10. The optimal recovered oocyte stage for canine IVF based on progesterone level

Recovered oocytes stage	Progesterone (ng/mL) on Day 0	Number of bitches	Cleavage rate
Immature	5.1 ± 0.9	3 (8.1%)	25.0%
Mature	4.2 ± 0.3	30 (81.1%)	70.5%
Aging	4.7 ± 0.8	4 (10.8%)	51.4%

Day 0 is predicted ovulation day; n = 37

Table 11. The cleavage rate after *in vitro* fertilization using fresh and frozen-thawed sperm

Sperm Type	Number of Oocytes	Number of embryos (%) developed to *				Cleavage rate
		1-cell	2-cell	4-cell	> 4-cell	
Frozen	50	18	9	12	11	62.6 ± 3.8 %

* Percentage of the number of oocytes cultured.

Canine capacitation media in both groups were supplemented with 25% conditioned media. Potassium simplex optimization medium (KSOM) was used as culture media in both groups.

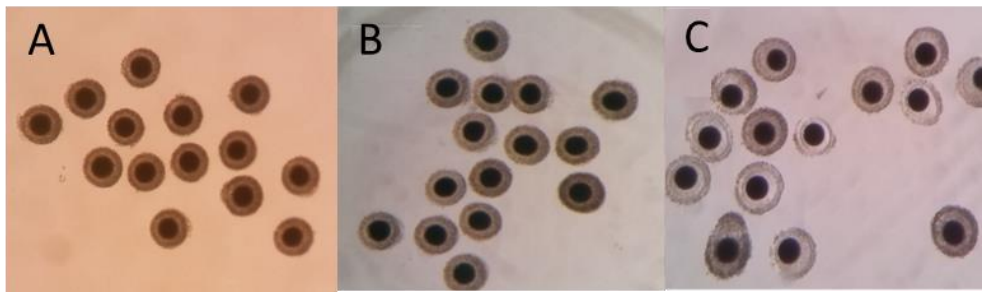

Figure 9. Recovered canine oocytes after 72 h post predicted ovulation

This picture shows A) immature stage, B) mature stage and C) aging stage of canine oocytes after 72 h post predicted ovulation.

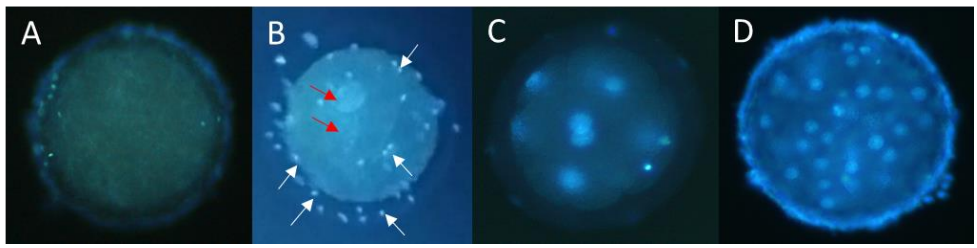

Figure 10. Canine *in vitro* embryo development after *in vitro* fertilization (IVF) using frozen-thawed sperm

A) Sperm penetration after 17 h post IVF; B) pronuclear formation after 17 h post-IVF with white arrows showing sperm heads and red arrows pointing to pronuclei; C) early development after 72 h post-IVF; D) late development after 96 h post-IVF; red arrows are pronuclei; white arrows are sperm. Canine capacitation media was supplemented with 25% conditioned media. Potassium simplex optimization medium (KSOM) was used as culture media.

3.4. The effect of conditioned media from human ASCs in improving pregnancy rates after embryo transfer

Seventy-eight embryos from treatment and control groups after 15-17 h post-IVF were transferred into 6 naturally synchronous recipient female dogs as shown in Table 12. Neither group resulted in a pregnancy.

Table 12. The result of embryo transfer at 15-17 h after *in vitro* fertilization (IVF)

Embryo Transfer	After 15-17 h post-IVF		
	Number of Embryos	Number of Recipients	Number of Sacs
100% CCM	45	3	0
75% CCM + 25% CM	33	3	0

Matured oocytes, frozen-thawed sperm and 25% conditioned media supplementation in canine capacitation media (CCM) were used. Potassium simplex optimization medium (KSOM) was used as culture media in both groups.

4. Discussion

IVF using frozen-thawed sperm in canine result in very low rates of embryo development [95, 96]. I initially hypothesized that this lack of success was due to prior use of frozen-sperm which lowers sperm motility and fertility. In this study, the exogenous growth factors derived from conditioned media of human ASCs were simultaneously added to capacitation media followed by IVC using KSOM, which would produce embryos consistently with high efficiency.

Motile, viable and fertile sperm are imperative in IVF as necessary genetic resources to activate the oocyte. These standards have to be met by frozen-thawed sperm, even though cryopreservation also implies ice formation and high osmotic pressure that can lead to cryoinjury and induce both a loss of sperm function and viability post-thawing [126]. Optimum frozen-thawed sperm conditions must be maintained, especially for the long-term storage of genetic material to conserve the fertility of individuals possessing high genetic merit, or preservation of breeds threatened with extinction due to disease or sudden death [125]. Another factor that must be considered is oocyte competence, primarily nuclear and cytoplasmic maturation. The difficulty in obtaining mature oocytes and a low maturation rate during canine IVM [96, 103] led researchers to test for the optimum protocol for *in vivo* to recover competent oocytes [91, 122]. When IVM is paired with IVF, previous studies reported low rates of embryo production (2.2%-33.6%), only 1 blastocyst [103] and 3 morulae [94] out of hundreds of oocytes, and no live births.

To maintain the motility, viability and fertility of frozen-thawed sperm, I performed capacitation using magnesium (Mg) and P4, then supplemented with CM from human ASCs. In the current study, the presence of P4 and Mg in CCM were critical to canine sperm capacitation. This combination resulted in more effective capacitation and cleavage rates (62.6 ± 3.8 %) than in other studies, such as heparin in frozen-thawed sperm (33.6 ± 1.2 %) [96], a combination of Mg and BSA in frozen-thawed sperm [95], and heparin and BSA in fresh semen [94] which only resulted in around 30% cleavage rates. Acrosome exocytosis (AE) and hypermotility were essential functions in fertilizing an egg. Physiological AE is stimulated by P4 and/or zona pellucida proteins [202], meanwhile magnesium is an important co-factor for glycolytic enzymes and promote AE *via* a Ca^{2+}-Mg^{2+}-ATPase in bull and ram spermatozoa [120]. The supplementation of CM, which is rich with growth factors [190], had positive effect in supporting post-thaw sperm quality (Table 8) and sperm capacitation (Table 9) which is essential for fertilization competence.

Adding growth factors into the traditional sperm culture medium to increase the efficiency of the medium has been studied for decades. Human ASCs secrete high levels of several growth factors which have roles on sperm quality such as IGF1, FGF2, BDNF, NGF, TNFα and TGFβ [185, 203, 204]. Adding CM in capacitation media is similar to adding all growth factors secreted by ASCs, and would give more benefits simultaneously. I found that addition of CM from ASCs increase the motility, progressive motility, linearity, viability and fertility parameters of canine frozen-thawed sperm (Table 8). This result is in agreement with supplementation of exogenous growth factors on fresh or frozen-thawed sperm shown in several studies.

Exogenous IGF1 could be helpful in maintaining progressive motility of canine spermatozoa during hypothermic storage *via* increased mitochondrial membrane potential [46]. Moreover, IGF1 maintain sperm functions following the cooling storage and elevate sperm fertilizing ability *in vitro* in ram [205] and yak [181]. The presence of an IGF1 receptor in canine spermatozoa *via* the IGF1 effect [46] stimulated motility, viability, capacitation and acrosome reaction through energy metabolism (glucose uptake, lactate production, pyruvate dehydrogenase activity etc.) [47], antioxidant effects [48] and high intracellular calcium level by increased ion transport [49]. In contrast, activation of cellular metabolism by IGF1 may also be related to the generation of free radicals [50].

The expression of FGFR1, 2, 3 and 4 mRNAs and proteins in ejaculated sperm were localized to the acrosomal region and flagellum [182]. Sperm exposure to FGF2 caused an increase in flagellar FGFR phosphorylation and the activation of extracellular signal-regulated kinase (ERK) and protein kinase B (PKB or Akt) signaling pathways [206]. Incubation with FGF2 led to a significant increase in the percentage of total and progressive sperm motility, as well as in sperm kinematics [182]. Sperm FGF2 levels showed a positive correlation with sperm concentration, motility, total sperm number and total motile cells per ejaculate [206].

The neurotrophins family, including NGF and BDNF, were potential markers for semen quality and NGF receptors were found in the tail and head of sperm [180]. Supplementation of NGF improved sperm motility and the number of A grade spermatozoa [39], increased intracellular nitric oxide concentration, decreased apoptosis content in normal human spermatozoa [38], and increased viability and

sperm apoptosis in bovine [180]. The role of NGF in supporting sperm viability might be involved in the phosphoinositide-3 kinase (PI3K) signaling pathway by increasing insulin and leptin in uncapacitated sperm [207] which activate PI3K to regulate cell survival and antiapoptotics signals [208]. TNFα and TGFβ play an important role in barrier regulation, associated with lower spermatozoa motility parameters and male infertility [183].

In order to determine the optimum stage of oocyte for IVF, oocyte collection was performed 72 h after the P4 level reached 4.0–9.9 ng/mL [122]. We collected the oocytes from 37 female dogs and mature oocytes recovered from 30 (81.1%) dogs could then produce a 70.5% cleavage rate after IVF. Immature oocytes recovered from 3 female dogs resulted in a 25.0% cleavage rate and aging oocytes from 4 female dogs (10.8%) resulted in a 51.4% cleavage rate (Table 10). Optimum cleavage rate was produced by IVF using mature oocytes compared with other stages. These results were in agreement with previous studies that reported that immature canine oocytes could be penetrated by sperm during IVF [97, 100] and the sperm penetration would induce meiosis resumption, which can lead to some of the penetrated immature oocytes developing to metaphase 1, 2 and forming two pronuclei [99, 112]. The aging oocytes also resulted in cleavage but at a lower percentage than the mature oocytes because the physiological states were significantly changed from metaphase toward degenerative interphase during cytoplasmic aging [209]. The cytoplasm sensitivity of metaphase 2 to the internal calcium perturbation was also reduced during aging and it led the oocytes to fail to activate [210].

Many defined culture media were developed and each of them have their own advantages. In canine IVF, cNCSU media was used by Nagashima *et al.* [91] and resulted in a 73.7% cleavage rate using fresh semen. J. Saikhun *et al.* [96] performed canine IVF with frozen-thawed sperm and used an SOF media supplemented with FBS. He reported a 33.6% cleavage rate. Our study using frozen-thawed sperm exhibited a higher cleavage rate (62.6 ± 3.8 %, Table 11) compared with Saikhun *et al*. [96] which reported a cleavage rate of 33.6 ± 1.2 %.

Serial staining from 3, 6, 17, and 21 h after IVF were performed and I found that less sperm reached zona pelucida after 17-21 h post-IVF and most oocytes were not fertilized. The cumulus thickness seems to have an effect on the time required for sperm penetration; oocytes with less cumulus exhibit cleavage faster than oocytes with more layers of cumulus as reported in other mammals [211]. This observation could also explain the delayed cleavage phenomenon in canine IVF that was reported by previous teams [91, 94]. The second reason is a stress on the embryo during embryo loading up to dispositioning, reported by Paternot *et al.* [212]. The changing media gives extra stress to the embryo, caused by differing microenvironments and osmolality. Moreover, Bouillon *et al.* [213] wrote that environmental stresses such as oxidative and ammonium stress could be provoke adverse effects from epigenetic changes up to early embryonic death.

Because there is no established culture system for the canine embryo, I designed the embryo transfer to be performed 15 h after IVF using a 3.5-Fr Tom Cat Catheter into the ampullary portion of the oviducts of naturally synchronous recipients [201]. Pregnancy diagnosis was performed on 31 day after ET and there

was no pregnancy detected (Table 12). This might be caused by 1) delayed fertilization between 14-20 h after IVF [94], 2) penetration time affected by the cumulus thickness in mammalian fertilization [211] which could explain the delayed fertilization in canine, 3) the negative effect of cryopreservation which could have affected the time of sperm penetration and the final percentage of fertilized oocytes as reported previously in other species [118, 119] and 4) a stress on embryos caused by media change [212] during embryo transfer. A transfer in 2cell stage, already performed by Nagashima *et al.* [91], seems to be the better option for further study with frozen-thawed embryos.

PART V

FINAL CONCLUSION

This thesis was conducted in order to establish a canine IVF protocol using frozen-thawed sperm with modifications in the freezing protocol, antioxidant supplementation during cryopreservation, addition of conditioned media during capacitation and the usage of different defined culture media.

First, the multistep loading/dilution protocol that used in this study express a gradual osmotic challenge and help maintain their osmolytes content, shape and motility. The advantages of the multistep protocol on canine semen cryopreservation can be enhanced using glycerol as a CPA. Further studies are needed to maximize the frozen–thawed dog sperm quality, perhaps by adding carnitine and glutamate before the cryopreservation process.

Second, supplementation of the second buffer with 5 mM spermine can protect canine spermatozoa during cryopreservation sufficiently enough to maintain post-thaw motility, improve kinematic parameters and membrane integrity, reduce cryocapacitation during cryopreservation and increase the proportions of capacitated spermatozoa after incubation in CCM. Further studies are needed to determine the fertilizing capability of canine frozen-thawed spermatozoa *via* artificial insemination or *in vitro* fertilization.

Finally, conditioned media from human ASCs could elevate post thawing quality, linearity and viability, up regulate the gene expression related fertility and increase the capacitation rate in canine frozen-thawed sperm. The combination of Mg and P4 were effective in stimulating acrosome reaction in canine sperm.

In conclusion, the multistep freezing method achieved superior results in maintaining sperm function and osmolyte content, spermine supplementation

reduced ROS levels and decreased cryocapacitation, and adding 25% CdM in CCM increased sperm motility, viability and fertility. Furthermore, enhanced frozen-thawed sperm can improve canine IVF as an alternative way for canine embryo production.

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국문초록

개 정자 동결법 개발 및 이를 통한 체외수정 시스템 구축

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수의학과 수의산과·생물공학 전공

개의 체외수정 (IVF)은 멸종 위기 종의 보존, 개와 인간 사이의 유전 질병의 이해, 유전 교정 기술의 적용을 연구하는 과학자들에게는 항상 주요한 장벽이었다. 2015 년말 신선 정액과 체내 성숙 난자의 IVF 동결배아를 이용해 개에서 최초의 IVF 산자 생산이 보고되었다. 비록 몇몇 연구들이 동결 정액과 체내/체외 성숙 난자의 IVF 를 수행하였으나, 낮은 정자침투율과 분할율 및 발달의 지연, 그리고 수정란의 퇴화와 같은 문제점들이 발생하였다. 동결-융해 정자의 질적 상태는 이러한 문제점들을 극복하기 위한 주요한 열쇠 중 하나로 알려져 있다. 따라서 본 학위 연구의 목적은 다단계 동결법,

항산화제가 첨가된 동결보존제, 조정배지 첨가된 수정능 획득 배지 개발로 동결-융해 정자의 질적 향상을 위한 정액동결법을 확립하여 개의 체외수정 시스템을 향상시키는 것이다.

희석제의 총 부피를 30 초마다 4 단계 (14 %, 19 %, 27 %, 40 %)로 나누어 연속적으로 첨가하면서 동결보호제를 희석시키는 다단계 정자 동결/해동법이 한 번에 희석제를 첨가하는 단일 동결/해동법에 비해 정자 기능, 형태학 및 삼투물질에 미치는 영향을 비교하였다. 글리세롤과 에틸렌 글리콜의 영향도 비교하였다. 여러 농도의 (0, 0.1, 1, 5, 10 mM) 스페르민 처리가 정자의 질, 활성산소, cryocapacitation 및 산화 관련 유전자 발현에 미치는 영향을 분석하였다. 사람 지방줄기세포 유래 조정배지로 동결-융해 정자의 수정능을 획득하였고, IVF 후 대리모에 이식하였다. 수정능 획득 정자의 생존율 및 수정능 관련 유전자 발현, 그리고 배아의 분할율을 분석하였다.

다단계 실험군의 동결-융해 정자는 단일 실험군에 비하여 전진운동능, 온전한 세포막, 구부러진 꼬리의 평가에서 질적으로 향상되었다. 다단계 실험군은 또한 단일 실험군에 비해 카르니틴과 글루타메이트와 같은 삼투물질의 손실을 최소화하는데 성공했다. 더욱이, 다단계 실험군에서 글리세롤이 에틸렌 글리콜보다 정자의 질을 유지하는데 더 유리했다. 스페르민 처리는 운동성을 향상시키지

못했으나 세포막은 유의적으로 보다 온전하게 유지시켰다. 스페르민 처리군의 높은 선형도 및 직진도, 낮은 측두이동거리는 스페르민이 과활성화를 억제했음을 의미한다. 스페르민 처리군에서는 세포 내 및 세포 외 활성산소의 농도가 감소했다. 미토콘드리아 활성산소 조절자 1 (*ROMO1*), 산화적 손상으로 인한 DNA 수선 (*OGG1*), 스페르민 합성효소 (*SMS*), NADPH 산화효소 관련 운동능 (*NOX5*), 스페르민 아미노 산화효소 (*SMOX*) 유전자 발현 감소와 항세포사멸 유전자 (*BCL2*)의 발현 증가 및 전세포사멸 유전자 (*Bax*)의 발현 감소는 5mM 스페르민 처리가 정자에 유익하다는 것을 보여 주었다. 뿐만 아니라, 처리군에서 융해 후 손상되지 않은 첨체를 가진 생존 정자의 비율이 대조군보다 높았다. 개 수정능 획득배지에서 정자를 배양한 후, 반응 첨체를 가진 생존 수정능 획득 정자의 수는 대조군보다 스페르민 처리군에서 더 높았다. 25% 조정배지가 첨가된 개 수정능 획득배지를 사용했을 때, 운동능, 전진운동능, 선형도, 생존률이 대조군이나 50% 조정배지 실험군보다 유의적으로 높았다. 25 % 조정배지 실험군에서 DNA 패키징, 운동능, 수정능과 관련된 유전자 발현이 대조군에 비해 유의적으로 증가하였다. 처리군에서 반응첨체를 가진 생존 정자 또한 대조군보다 유의하게 증가하였다. 배란 시 프로게스테론 농도를 4.2 ± 0.3 ng/mL 로 예상하였을 때 81.1 %의 암컷에서 성숙난자를 회수하였다. 게다가, 동결-융해

정자를 이용한 IVF 는 기존의 다른 연구들보다 높은 60% 이상이라는 높은 분할율을 나타내었다.

결론적으로, 개 정액동결 시 다단계 정액 동결/해동법 개발로 정자의 기능과 삼투물질을 유지하였고, 스페르민 첨가법 개발로 활성산소와 cryocapacitation 을 감소시켰으며, 25% 조정배지가 첨가된 개 수정능 획득 배지를 개발함으로써 정자의 운동능, 생존율, 수정능을 향상시켰다. 뿐만 아니라 향상된 동결-융해 정자를 이용하여 개 IVF 배아 생산을 향상시킬 수 있었다.

주요어: 개, 체외 수정, 동결 정자, 수정 능 획득, 분할률

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