A THESIS FOR THE DEGREE OF MASTER

수의학 석사 학위논문

Conditioned medium from canine amniotic membrane-derived stem cells improved dog sperm post-thaw quality-related parameters

개에서 양막유래 줄기세포 조정배지가 동결정액의 성상에 미치는 영향 연구

2021년 2월

서울대학교 대학원

수의과대학 임상수의학 전공

페리엘 야스민 마히딘

Conditioned medium from canine amniotic membrane-derived stem cells improved dog sperm post-thaw quality-related parameters

개에서 양막유래 줄기세포 조정배지가 동결정액의 성상에 미치는 영향 연구

지도교수 윤화영

이 논문을 수의학 석사 학위논문으로 제출함

2020년 11 월

서울대학교 대학원

수의과대학 임상수의학 전공

페리엘 야스민 마히딘

페리엘 야스민 마히딘의 석사학위논문을 인준함

2020년 12 월

위 원 장 박 세 창 (인)

부 위 원 윤 화 영 (인)

위 원 Junpei Kimura (인)

Conditioned medium from canine amniotic membrane-derived stem cells improved dog sperm post-thaw quality-related parameters

by Feriel Yasmine Mahiddine

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF SCIENCE

in

Veterinary Clinical Sciences

Department of Veterinary Medicine

Graduate school

Seoul National University

We accept this thesis as confirming to the required standard

PARK SE CHANG 朴世昌

Hwa Young Youn

Junpei Kimura 木村順平

Seoul National University

본 논문작성자는 한국정부초청장학금 (Global Korea Scholarship)을 지원받은 장학생임

Conditioned medium from canine amniotic membrane-derived stem cells improved dog sperm post-thaw quality-related parameters

Feriel Yasmine Mahiddine

(Supervised by Professor Yoon Hwa Yeong)

Department of Veterinary Clinical Science

College of Veterinary Medicine

Graduate School of Seoul National University

ABSTRACT

Mesenchymal stem cells and their derivatives are used in clinical studies for their anti-apoptotic, anti-oxidant, immunomodulatory, and regenerative properties. Their

use in reproductive medicine is increasing as they have been proved to be beneficial for infertility treatment. Mesenchymal stem cells can secrete factors that influence biological processes in target tissues or cells; these factors are either directly secreted by the cells or mediated through their derivatives. Although the amniotic membrane is easy to obtain and is a good source of stem cells, clinical trials using amniotic membrane-derived mesenchymal stem cells are still uncommon, especially in the field of reproduction and artificial reproductive technologies. This study investigated the effects of conditioned medium (CM) from canine amniotic membrane-derived MSCs (cAMSCs) on canine sperm cryopreservation. For this purpose, flow cytometry analysis was performed to characterize cAMSCs. The CM prepared from cAMSCs was subjected to proteomic analysis for the identification of proteins present in the medium. Sperm samples were treated with freezing medium supplemented with 0%, 5%, 10%, and 15% of the CM, and kinetic parameters were evaluated after 4–6 h of chilling at 4 ℃ to select the best concentration before proceeding to cryopreservation. Quality-related parameters of frozen–thawed sperm, including motility; kinetic parameters; viability; integrity of the plasma membrane, chromatin, and acrosome; and mitochondrial activity were investigated. The results showed that 10% of the CM significantly enhanced motility, viability, mitochondrial activity, and membrane integrity ($p<0.05$); the analysis of chromatin and acrosome integrity, however, showed no significant differences between the treatment and control groups. Therefore, I concluded that the addition of 10% CM derived from cAMSC in the freezing medium protected canine sperm during the cryopreservation

process.

This research was conducted to evaluate the cyto-protective potential of cAMSC-CM on canine sperm cryopreservation, with the hypothesis that the factors contained in the CM would enhance the post-thaw quality-related parameters. The results obtained from my study show that cAMSC-CM addition in the cryopreservation medium was beneficial to canine sperm cells during the freezing-thawing process. The innocuity and practical advantages of the CM makes it an interesting candidate for more advanced research in the field of reproduction. Moreover, its beneficial effects on sperm cryopreservation gives us more perspectives and hopes for better sperm cryopreservation protocols in endangered species, especially canine endangered species such as grey wolves, African wild dogs, Island foxes and dholes.

……

Keywords: biotechnology, conditioned medium, cryopreservation, sperm, stem cells,

Student number: 2019-29434

Contents

List of Tables

List of Figures

List of abbreviations

ALH	**Amplitude of lateral head**
ANOVA	**Analysis of variance**
cAMSCs	**Canine amniotic membrane-derived mesenchymal stem cells**
CM	**Conditioned medium**
CO_2	**Carbon dioxide**
DNA	**Deoxyribonucleic acid**
ECM	**Extracellular matrix**
FACS	**Fluorescence-activated cell sorting**
FITC	**Fluorescein isothiocyanate**
h	**Hour**
HOST	**Hypo-osmotic swelling test**
LIN	**Linearity**
LN_2	**Liquid nitrogen**
LPO	**Lipid peroxidation**
LC-MS/MS	**Liquid Chromatography with tandem mass spectrometry**
mL	**Milliliter**
MSCs	**Mesenchymal stem cells**

nm	**Nanometer**
PBS	**Phosphate buffered saline**
PE	**Phyceorythrin**
PI	**Propidium iodide**
PNA	**Peanut agglutinin**
qPCR	**quantitative Polymerase chain reaction**
R123	**Rhodamine 123**
ROS	**Reactive oxygen species**
RNA	**Ribonucleic acid**
SEM	**Standard error of the mean**
STR	**Straightness**
VAP	**Average path velocity**
VCL	**Average curvilinear velocity**
VSL	**Average straight-line velocity**
xg	**Gravity**
µm	**Micrometer**
µL	**Microliter**

Literature Review

1. Canine sperm physiology and function

Understanding the characteristics of canine sperm physiology and function are essential for the development of assisted reproductive technologies and research in this species and their wild and endangered relatives. Sperm cells are produced in the testes section and their transport, maturation, storage and de-capacitation process occur in the epididymis section [1].

In general, mammalian spermatozoa structure is the same, and sperm cells have a distinct morphology in comparison with somatic cells as they are formed by a head with a nucleus and an acrosome, a tail, and a sperm plasma membrane surrounding the entire cell. The sperm plasma membrane contains phospholipids, glycoproteins, and cholesterol. The latter component is responsible for sperm permeability and along with double bonds, gives the membrane its stiffness. However, these compounds are targeted during chemical reactions, such as the ones triggered during cryopreservation, and it can lead to deleterious effects on sperm function and membrane morphology [2].

1.1. Sperm head

The sperm head is formed by the nucleus and acrosome. The nucleus is considered as the most important structure in the sperm cells as it contains the genetic information. It is formed by condensed chromatin that takes almost all the space in the head. The chromatin organization in the sperm head is typical and starts during the spermatogenesis; in the DNA, histones are replaced by protamines through a process referred to as protamination. Protamines are twice as small as histones and after the protamination process, the chromatin area becomes smaller in the sperm head. Protamines 1 and 2 are found in sperm and are correlated with the DNA status after the freezing-thawing process. In mammalian sperm, a lack of protamine 2 is positively correlated with cryo-resistance. In comparison with other

mammalian sperms, canine sperm is more resistant to cryo-damages on the nucleus, and this is probably due to the fact that canine sperm cells are characterized by a lack of protamine 2. This is particularly important in fertility and assisted reproductive technologies since sperm cells with damaged chromatin can fertilize an oocyte but a successful embryo development is related to the severity of the damages. In this case, cryopreserved canine spermatozoa might have higher fertility abilities than other mammalian cryopreserved spermatozoa [3].

1.2. Acrosome

The acrosome is a typical structure of sperm cells. It is located at the top of the head and contains hydrolytic enzymes that will be released during the acrosome reaction, which makes the acrosome an essential element during fertilization. In canine species, the proportion of sperm cells displaying acrosome damages is relatively low. Cryo-injuries induce acrosome damages, especially in old animals, but with no significant importance [3].

1.3. Sperm tail

The sperm tail is another specific part of the sperm cell. It is mainly formed by a mid-piece containing mitochondria, and an axoneme as the basic element of the tail. The most important structure in the tail is the mitochondria since it is the main source of energy in the sperm cell. ATP is produced in the inner mitochondrial membrane. Moreover, mitochondria are also involved in apoptosis and calcium homeostasis in sperm. Therefore, dysfunction of sperm mitochondria can lead to deleterious effects on sperm function and survival [3, 4].

2. Cryopreservation and cryo-damages on sperm

Cryopreservation is widely used in clinics and research centers to preserve valuable sperm samples. However, this process induces various changes in the spermatozoa ultra-structure and function, which ultimately compromise sperm frozen-thawed-related quality parameters. These changes are induced by cryo-damages during the freezing and thawing steps [5].

Cryo-damages, or cryo-injuries, are induced when intra- and extra-cellular water phases change. The change in temperature, especially between -15℃ and -60℃, results in deleterious osmotic changes in the milieu. At first, if the cooling rate from -5℃ to -15℃ is slow, ice crystals are formed in the extra-cellular phase only, which creates a higher chemical potential that leads to hypertonic stress, water leakage from the cells, dehydration, shrinking of organelles and later cell lysis during the thawing process. On the other side, if the cooling rate is fast, ice crystals are also formed in the intra-cellular water, more precisely in the cytoplasm, and ultimately leads to cryo-injuries [6].

To cope with the cryo-damages induced during the cooling phase, different temperatures have been experimented to find optimal cooling rates adapted to different cell types. However, it should be noted that post-thaw survival and function are dependent on both cooling and thawing rates together. An adequate cooling rate alone cannot ensure good post-thaw parameters [6]. In dog sperm, except for some individual variabilities, a cooling rate of 10℃/min associated with a fast thawing give the highest survival rate of spermatozoa cells [7].

In general, each type of cell has a different cryo-tolerance level depending on their hydraulic conductivity and its activation energy, and the cell's surface area/volume ratio. Incidentally, different cryo-tolerance thresholds have been noticed in individuals, including dogs. For sperm cells, their sensitivity to freezing-thawing process is higher than other cells. It has been shown in numerous studies that spermatozoa cells are more sensitive to the osmotic changes occurring during cryopreservation. Moreover, it has been demonstrated that canine sperm reaction to hypertonic stress during cryopreservation is different than other cells since they are sensitive to dehydration but not to shrinking and swelling [8]. However, post-thaw quality-related parameters are still low and new techniques, medium or cryo-protectants need to be developed [9].

3. Mesenchymal stem cells

The first scientist to emit the theory that bone marrow contained mesenchymal tissues was A.J. Friedenstein, back in the 1960s [10]. In 1966, his work on bone marrow grafts and osteogenesis of the grafted tissues confirmed his theory. Based on their differentiation abilities during embryonic development and in bone marrow grafts, scientists first hypothesized that mesenchymal progenitors were formed by heterogeneous cell populations [11]. This hypothesis gained more popularity in the late 1970s when a second type of stem cell involved in hematopoiesis was suspected to be present in the bone marrow. It was only in 1991 that Caplan identified these mesenchymal progenitors as one kind of stem cell that has the ability to differentiate into different and distinct cell types [12]. He named them mesenchymal stem cells (MSCs), as an alternative to stromal or osteogenic stem cells. Later, MSCs have been successfully characterized, and their function and differentiation abilities have been studied more deeply. Nowadays, they are used in numerous in vitro and in vivo research studies and proved to be efficient in regenerative medicine [11].

The potentials harbored by MSCs make them an ideal candidate for clinical trials. Their functions, characteristics, and differentiation abilities can be beneficial to various conditions and treatments [13]. MSCs can differentiate into stromal cells, osteocytes, chondrocytes, fibroblasts, myotubes and adipocytes, which supports their use in transplantation trials for bone, cartilage, musculoskeletal, and myocardial tissues, nervous system, skin, and liver regeneration [14]. Furthermore, their pluripotency, immunomodulatory, immunosuppressive, and anti-inflammatory abilities encourage the use of MSCs in preclinical and clinical regenerative medicine [13].

However, despite the hype around MSCs and their healing potentials, safety issues have been reported in numerous studies [13]. The use of MSCs is associated with fibrosis, MSCs migration, pro-inflammation, and high risks of tumorigenicity, as some cases of MSCs grafts evolving into tumors were previously reported. Additionally, MSCs have the ability to secrete growth factors, pro-angiogenic factors, and chemokines, which creates an ideal micro-environment for tumors development. The association of these factors, and the immunosuppressive effects of MSCs, tumor growth, and propagation in other organs become easier, making the use of stem cell-based therapies controversial [13].

4. Stem cell-free therapy

The current advances in medical and biological sciences have made it possible for researchers to study deeply about MSCs and the characterization of their secretome. Cells can communicate with their surrounding environment through the secretion of molecules in the extracellular space. This communication system is used by cells or tissues to convey a signal/information and the contents of the secretome change depending on the environment, and the cells' physiological or pathological state. This secretome consists mostly of soluble proteins, nucleic acids, lipids, and extra-cellular vesicles, including apoptotic bodies, exosomes, and micro-vesicles. Recently, the use of this secretome in pre-clinical and clinical trials have become more common as CM obtained from MSCs cultures or extra-cellular vesicles extracted from CM convey the same effects as MSCs [15].

The main advantage of this secretome is that it is an alternative to MSCs-based therapies since it can exert MSCs' effects without the risks associated with their use. In comparison with cell-based therapies or extra-cellular vesicles-based therapies, the use of CM derived from MSCs presents other key advantages in regenerative medicine such an easy conservation, storage and manipulation, a more economical and practical use, a possible mass production at low cost, an easier administration, and a faster and easier production and collection. Various treatments using MSCs-derived CM were tried and proved CM's efficiency and harmlessness in regenerative medicine [16].

With such a rich secretome, MSCs-derived CM can be beneficial in the treatment of various conditions. CM can convey MSCs' anti-apoptotic activity to cells by decreasing the expression of pro-apoptotic factors such as BAX, and increasing the expression of anti-apoptotic factors. Besides, CM contributes to tissue regeneration by enhancing the proliferation ability, epithelial regeneration, and it can also prevent scar formation through its anti-fibrotic and angiogenic effects [16]. Surprisingly, CMs contain both angiogenic inhibitors and stimulators, which creates a perfect equilibrium during angiogenesis [16, 17]. The use of MSCs and their secretome in grafts studies is mostly supported by their anti-inflammatory and immunomodulation activity. CM contains both anti and pro-inflammatory cytokines and the balance between them will determine the final effect on the targeted tissue. Studies also demonstrated that CM can inhibit immune cells recognition and differentiation. Furthermore, in addition to the chemokines and growth factors in CM, anti-oxidants, anti-bacterial agents, extra-cellular matrix components (ECM) and enzymes and proteins associated with cell metabolism can also be found [15, 16].

The MSCs-derived CMs general actions on tissues and organisms are similar regardless of the MSCs origin as they all carry the same regenerative potential; however, there are still variations in the CMs compositions depending on the MSCs origin, and more proteomic analyses are needed to compare and evaluate these differences [18].

Introduction

Sperm cryopreservation is used to store sperm samples from cancer patients or endangered species, and for other activities such as breeding, shipping, and research; the post-thaw quality obtained is, however, low when compared with the fresh samples [19]. During cryopreservation, cold shock and crystal formation from intra- and extra-cellular water induce cryo-injuries that disturb the sperm plasma membrane integrity and lipid composition, resulting in the leakage of intracellular contents. Consequently, sperm metabolism is reduced [20] and apoptotic-like changes occur in the cells [21-23]. Sperm cells, in particular, are more sensitive to environmental changes because of their limited protein and lipid biosynthetic abilities [24], and the absence of DNA repair mechanism [25]. These events ultimately result in a weakened oxidative stress defence that exposes the sperm cells to reactive oxygen species (ROS). An increase in the ROS production during cryopreservation destroys sperm lipid matrix structures, and subsequently, causes the loss of membrane integrity, an increase in lipid peroxidation and excessive DNA fragmentation [25, 26].

To counteract the consequences of cryo-injuries, chemicals with protective properties can be added to the cells prior to or during cryopreservation. Cyto-protective agents such as cryo-protectants, anti-oxidants, or anti-apoptotic factors, act on different levels to protect cells from cryo-injuries. Anti-apoptotic factors such as the anti-cell death FNK protein or curcumin prevent cell death [27, 28]; cryo-protectants such as glycerol protect cells from intracellular ice formation and reduce osmotic damage [29], while anti-oxidants such as vitamin E protect sperm ultra-structure, function, and mitochondrial DNA from oxidative stress [30, 31]. Therefore, efficient and effective cryopreservation requires the addition of protective chemicals in the freezing media to increase cellular defences and reduce ROS generation [28, 32, 33]. However, the commercial and homemade available freezing medium

does not fully satisfy the requirements needed for the complete protection of sperm during the cryopreservation process [34]. Although Tris-egg yolk buffer is the most commonly used diluent for mammalian sperm, many studies show that supplementation of anti-apoptotic factors, anti-oxidants, post-thaw enhancing chemicals, or novel cryo-protective agents is required to get good post-thaw results [19, 23, 35, 36]. Thus, various molecules such as metformin [37], cholesterol [38], or α-tocopherol [39] have been successfully used in canine sperm cryopreservation to reduce oxidative stress, DNA damage [37, 38], improve motility [39], and protect plasma membrane and acrosome integrity [38].

Mesenchymal stem cells (MSCs) and their derivatives are commonly used in regenerative medicine and have proved their clinical efficacy in the treatment of infertility [40-42]. The MSCs enhance anti-oxidant defences in several tissues, including testis [42], through the secretion of proteins that reduce ROS production by scavenging free radicals [42, 43], and enhance mitochondrial function through the Akt1 pathway [44]. They also secrete anti-inflammatory molecules and growth factors that protect cells from apoptosis when exposed to injuries [45, 46]. Amniotic membrane-derived MSCs (AMSCs) have been isolated in dogs and humans [47, 48]; although human AMSCs already proved to be useful in regenerative medicine [49], the use of canine AMSCs in this field have only been suggested but never applied [50, 51]. In comparison with other stem cells, AMSCs are easier to obtain and isolate, which make them an ideal candidate for clinical trials [49, 52].

The effects of MSCs on live tissues are mostly due to their paracrine signaling [53] since their secretome is rich in anti-oxidants and anti-apoptotic factors, which makes them a good alternative to cell therapy [54, 55]. The derivatives of MSCs confer the same effects as the cells from which they originate [55], and have regenerative and protective properties [56] that could positively affect sperm cells. Conditioned medium (CM), in particular, has been studied and used in several clinical trials since it is easy to get, safer than cell-based therapies [57], and has a low immunogenicity [57], anti-oxidant and anti-apoptotic properties [58-60]. Moreover, CM can be used in many types of research studies since it can be manipulated more easily in comparison with cells [61] and it can also be added in solutions [62].

It is known that MSCs-derived CM obtained from starved cells consists of paracrine factors that enhance cell defence and trigger anti-apoptotic and anti-oxidative mechanisms [63]. The use of these factors may protect sperm from the detrimental effects of cryopreservation such as oxidative stress, apoptosis, DNA damage, and loss of mitochondrial activity. Therefore, I hypothesized that CM prepared from canine amniotic membrane-derived MSCs (cAMSC-CM) would have cyto-protective effects on canine sperm during the freeze–thaw process.

The purpose of my study is to find a novel cyto-protective agent for sperm cryopreservation. My hypothesis is that cAMSC secretome contains factors able to protect sperm function during the freezing-thawing process. The experiments I conducted were aimed to confirm the phenotype of cAMSC, unveil the components of its CM, determine the right concentration of cAMSC-CM for canine sperm cryopreservation and its effects on quality-related parameters after freezing-thawing process.

Materials and methods

1. Experimental design

Experiment 1 focused on the characterization of cAMSCs by flow cytometric analysis and pluripotency genes confirmation, preparation of cAMSC-CM and analysis of its components. Experiment 2 was conducted using cAMSC-CM. First, high and low ranges of cAMSC-CM concentrations were added to a freezing medium and used on dog sperm during chilling and cryopreservation process; however, high concentrations of cAMSC-CM had deleterious effects on sperm cells and therefore the experiments were conducted using a lower range of concentrations from 0 to 15% of cAMSC-CM. The optimal concentration of cAMSC-CM was selected by evaluating sperm kinetic parameters and viability after 4 to 6 h of chilling in the freezing medium supplemented with cAMSC-CM. Afterwards, in experiment 3, the optimal concentration of cAMSC-CM determined in Experiment 2 was used for dog sperm cryopreservation and post-thaw quality-related parameters were evaluated and compared with the control group. All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2. Cell culture

Canine amniotic membrane-derived mesenchymal stem cells (cAMSC) and their culture medium were provided by Naturecell Co., Ltd (Seoul, Republic of Korea). In brief, cAMSCs were seeded and cultured in tissue culture dishes with RCMEP media (Stem Cell Research Center, Biostar, Seoul, Republic of Korea), supplemented with serum and antibiotics. Cells were incubated in a humidified environment containing 5% CO2 at 37 °C. The cells used for characterization of cAMSC were cultured until passage two at 90% confluency, and the cells used to make the (CM) were cultured until passage three).

3. Flow cytometric analysis

Fluorescence-activated cell sorting (FACS) was used to determine cAMSC immunophenotype. Cells were washed two times with phosphate-buffered saline (PBS; Thermo Fisher Scientific, MA, USA) TrypLE™ Express (Gibco, Grand Island, NY, USA) was used to detach the cells. Cells were washed with PBS (Thermo Fisher Scientific) two times, counted and aliquoted in a 96 well plate (1×10^5 cells/100 µL per well). In each well, 5µl of fluorochrome-conjugated antibodies or isotype control antibodies with fluorescein isothiocyanate (FITC) or phycoerythrin (PE)—CD29 Monoclonal Antibody-PE (Invitrogen, CA, USA), CD44 Monoclonal Antibody-FITC, CD90 (Thy-1) Monoclonal Antibody-PE, CD34 Monoclonal Antibody-PE, CD45 Monoclonal Antibody-FITC, Rat IgG2a kappa Isotype Control-FITC, Rat IgG2b kappa Isotype Control-PE, Mouse IgG1 kappa Isotype Control-PE, Rat IgG2b kappa Isotype Control-FITC (eBioscience, CA, USA)—were added to the aliquoted cell suspensions. After 30 min of incubation at 4 ℃, the cells were centrifuged (1500rpm/3 min) and washed with PBS two times. Cells were transferred in round tubes with 5mL of PBS, analyzed using FACSCalibur™ and Cell Quest software (BD Biosciences, USA) was used to calculate CD marker percentages. For each antibody, 10000 cells were used.

4. Quantitative polymerase chain reaction

Pluripotent genes expression was confirmed by quantitative polymerase chain reaction (qPCR). To summarize, RNA was extracted from cAMSC cultures at passage two using the RNeasy Mini kit (Qiagen, Hilden, Germany). Next, cDNA was synthesized by RNA reverse transcription using DiaStar 2X RT Pre-Mix (Solgent, Daejeon, Republic of Korea) and Random Hexamers (Invitrogen). Synthesized cDNA was amplified by PCR and real-time PCR was performed using Agilent ariaMX Real-Time PCR (Agilent, Santa Clara, USA). Primers used for qPCR are displayed in Table 1 and beta-actin was used as an endogenous control. For gel electrophoresis, 10 µL of each real-time PCR product were loaded in wells and subjected to 1% agarose gel electrophoresis for 20 min.

5. Conditioned medium preparation

The cAMSCs were maintained in their culture media until they reached 80% confluency at passage three. The cells were starved by changing the media to serum-free Dulbecco's Modified Eagle Medium. After 48 h [64], CM was retrieved, centrifuged (2000 × *g*/30 min), and filtered with a 0.22 µm filter to remove cell debris. The CM aliquots were stored at −80 °C.

6. Proteomic analysis and CM composition

To identify and quantify cAMSC-CM protein composition, a one-dimensional electrophoresis-liquid chromatography tandem mass spectrometry (1-DE-LC-MS/MS) system coupled with a Q Exactive Plus mass spectrometer (Thermo Scientific) was used. In brief, cAMSC-CM was collected and submitted to precipitation (ppt) for protein purification using ammonium sulfate (AS) saturated at 80%. The CM was then centrifuged at 18000rpm/1

h for precipitation, dissolved using 20mM tris-HCl pH 8.0 and AS was removed using Viva spin (50kD). Protein lysates were separated using 12% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (12% SDS-PAGE) followed by in-gel digestion with trypsin. MASCOT software (Matrix Science Inc., Boston, USA) was used to indentify the proteins, generate the exponentially modified protein abundance index (emPAI) and then mole percentage was calculated according to emPAI values. Obtained data were analyzed using UniProtKB (UniProt Knowledgebase) database (*Canis lupus familiaris*). Identified proteins were classified by groups based on their functions, and the mole percentages for each group of proteins present in the cAMSC-CM was calculated.

7. Animal use for semen collection

All dogs used for the study were kept in individual cages. They were fed with commercial adult dry food, and water was provided *ad libitum*. All the experiments and studies were conducted in accordance with the recommendations described in "The Guide for the Care and Use of Laboratory Animals" published by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University (approval numbers; SNU-180731-2-1 and SNU-200409-3). Semen was obtained from beagle dogs by using digital manipulation twice a week, and only the second fraction of the ejaculate was collected and processed. The samples with an appropriate concentration (> 300×10^6 sperm/mL), motility higher than 80%, and mass motility of 4/5 at least (on a scale of 0–5) were selected and pooled to avoid individual variations. One ejaculate per male was obtained from five males, and four independent replicates were performed using the pooled semen.

8. Determination of CM optimal concentration

The pooled semen was diluted with Tris-extender (1:1, v/v)—distilled water, tris (hydroxymethyl) aminomethane 24 g/L, citric acid 14 g/L, fructose 8 g/L, kanamycin sulfate 0.15 g/L; pH 6.6, 290 mOsm—and centrifuged at 700 × *g* for 1 min. The supernatant was collected and centrifuged (500 × *g*/5 min) and only the pellet was re-suspended in Tris-extender to achieve a concentration of 200×10^6 sperm cells/mL. Different concentrations of CM (0%, 5%, 10%, and 15%) were added to the freezing media—54% (v/v) Tris-extender, 40% (v/v) egg yolk, and 6% (v/v) glycerol —and the samples were chilled in it for 4–6 h at 4 ℃. The sperm analysis imaging system (FSA2011 premium edition version 2011; Medical Supply, Republic of Korea) was used to evaluate sperm motility, curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), linearity (LIN), straightness (or VSL/VAP) (STR), amplitude of lateral head (ALH) and viability parameters. The treated group with the best results was selected for the rest of the experiments as previously described [65, 66].

9. Semen cryopreservation and thawing

The pooled semen was diluted with Tris-extender, washed, and centrifuged. The pellet was resuspended in Tris-extender to achieve a concentration of 200×10^6 sperm cells/mL. The semen was divided into aliquots to be used for the control and the treatment groups. The freezing medium, with or without CM, was added by a multistep loading protocol [67], and 14%, 19%, 27%, and 40% of the freezing medium was added every 30 s. The samples were loaded in 0.5 mL straws (Minitube, Tiefenbach, Germany) and were kept at 4 ℃ for 1 h to reach equilibration. They were then placed horizontally to freeze at 5 cm above liquid nitrogen (LN_2) for 15 min, before being transferred to LN_2 tanks (-196 ℃). The semen straws

were then thawed in a water bath at 37 °C for 30 s. The samples were diluted with Tris-extender (1:5, v/v) stepwise using 14%, 19%, 27%, and 40% of the total volume at intervals of 30 s. The samples were then washed, and afterward, we proceeded with the analysis.

10. Sperm kinetic parameters analysis

Sperm kinetic parameters were analyzed using a computer-assisted sperm analysis (CASA; Sperm Class Analyzer® System version 6.4.0.93, Microptic, Barcelona, Spain). The system included a Nikon Eclipse ci-L microscope (Nikon, Tokyo, Japan) with a 10× phase-contrast objective and a heating stage at 37 ℃. Leja 20 µm chamber slides (Leja, Gynotec Malden, Nieuw Vennep, Netherlands) were used for the analysis, and the frame rate was set at 25 frames/s. Various parameters such as sperm motility, progressive motility,VCL, VSL, VAP, LIN, STR, ALH, and the percentage of rapid and immotile spermatozoa were analyzed.

11. Eosin-nigrosin staining

Eosin-nigrosin staining was used to determine the percentage of sperm cells alive and tail morphology defects in each group. In brief, the frozen–thawed samples were washed, and a drop of 10 µL from the sperm pellet with an equal amount of eosin and nigrosin was mixed, and smeared onto warm glass slides. The slides were then air-dried, and the sperm was evaluated afterward. For each stained smear, 200 sperms were examined with a light microscope (Eclipse Ts 2, Nikon, Tokyo, Japan) with oil immersion objective lens (1000× magnification). The unstained sperms were counted as alive, and the stained ones were counted as dead cells. The results are expressed as the percentage of live sperm cells [65]. Sperm with a coiling of the mid piece were counted as cells with a coiled tail, and sperm with a bending of the mid piece or the entire tail were counted as cells with a bent tail [68].

12. Aniline blue staining

The frozen–thawed samples were washed and 20 µL of sperm pellet was smeared on a glass slide, air-dried and fixed with a solution of 3% buffered glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 30 min. The slides were then stained with 5% aqueous aniline blue solution mixed with 4% acetic acid (pH 3.5) for 5 min. In each group, 200 sperm cells were evaluated with a light microscope (Eclipse Ts 2, Nikon) in oil immersion objective lens (1000× magnification). The cells with unstained nuclei were considered normal (mature chromatin), and those with blue-stained nuclei were considered abnormal (immature chromatin). The results are expressed as the percentages of aniline blue-positive sperm (abnormal) [69].

13. Hypo-osmotic swelling test

The hypo-osmotic swelling test (HOST) was performed to evaluate the percentage of sperm cells with an intact plasma membrane. In brief, 100 µL of sperm was added to 900 µL of a hypo-osmotic solution (150–155 mOsm) and incubated at 37 ℃ for 30 min [70]. A drop of HOST solution with sperm was then placed on a warm slide and covered, and at least 100 spermatozoa were counted using a phase-contrast microscope (Eclipse Ts 2, Nikon). The cells with a coiled tail were counted as HOST-positive sperm.

14. Acrosome assessment test

The sperm acrosome membrane was analyzed using fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) as described previously. In brief, semen was smeared on glass slides, air-dried, fixed in absolute methanol, stained, and mounted with anti-fade mounting medium (VECTASHIELD® , Vector Laboratories, CA, USA). The integrity

of sperm acrosome membrane was analyzed using an epifluorescence phase-contrast microscope (Eclipse Ts 2, Nikon) and classified as intact acrosome (strong green fluorescence) or non-intact acrosome (partial or no fluorescence) [71].

15. Mitochondria activity assessment

The percentage of live sperm cells with functional mitochondria was assessed using a combination of fluorescent stains Rhodamine 123 (R123) (Molecular Probes, OR, USA) and propidium iodide (PI) as described previously. In each slide, 200 spermatozoa were examined under an epifluorescence phase-contrast microscope (Eclipse Ts 2, Nikon) at 600× magnification, equipped with an excitation/barrier filter of 490/515 nm for R123 (blue excitation), excitation/barrier filter of 545/590 nm for PI (green excitation), and a digital camera (Olympus DP 11, Tokyo, Japan). The sperm cells displaying green fluorescence in the mid-piece region and no red fluorescence in the head were considered viable with functional mitochondria, whereas cells exhibiting red fluorescence in the head were counted as dead [72].

16. Statistical analysis

Prior to analysis, D'Agostino and Pearson omnibus test was performed. Optimal concentration data were analyzed using one-way analysis of variance (ANOVA) following by a Tukey's multiple comparison test. For the control and 10% CM-treated groups, the independent sample *t*-test was used. For each experiment, four replicates were performed and the statistical analysis was performed using GraphPad Prism 5.0 (GraphPad, CA, USA). The values are expressed as mean ± standard error of the mean (SEM), and the values less than $p<0.05$ were considered statistically significant.

Table 1. List of primers used for quantitative polymerase chain reaction

Gene	Primer Sequence (5′-3′)	Product size (bp)	Temperature (°C)	NCBI Accession No.
β-ACT[1]	F - GCTACGTCGCCCTGGACTTC R - GCCCGTCGGGTAGTTCGTAG	86	60	AF021873.2
Oct3/4[2]	F - CGAGTGAGAGGCAACCTGGAGA R - CCACACTCGGACCACATCCTTC	114	60	XM_538830
Sox2[3]	F - CAGACCTACATGAACGGCTCGC R - CCTGGAGTGGGAGGAGGAGGTA	147	60	XM_005639752
Nanog[4]	F - GAATAACCCGAATTGGAGCA R - AGTTGTGGAGCGGATTGTTC	125	60	XM_022411387

[1] β-*ACT*, beta-actin; [2] *Oct3/4*, octamer binding transcription factor 3/4, [3] *Sox2*, SRY (sex determining region Y)-box 2, [4]*Nanog*, homeobox protein

Results

1. cAMSC and cAMSC-CM characterization

1.1. Confirmation of the surface markers

Flow cytometry analysis of cAMSC showed that the expression of CD29, CD44, and CD90 markers was high (93.73, 94.28, and 90.10%, respectively), whereas the expression of CD34 and CD45 markers was low (0.39 and 0.35%, respectively) (Figure 1). Therefore, based on the presence of these surface markers, we could infer that these cells were MSCs.

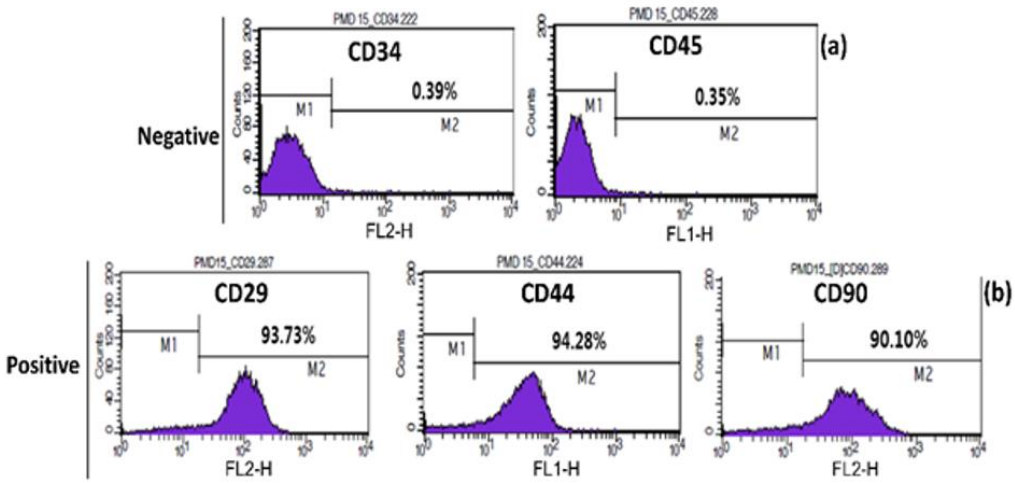

Figure 1. Confirmation of surface markers of canine amniotic membrane-derived mesenchymal stem cells using fluorescence-activated cell sorting (FACS); (**a**) negative surface markers CD34, CD45; (**b**) positive surface markers CD29, CD44 and CD90 surface makers analyzed by FACS.

1.2. Confirmation of pluripotency genes expression

The qPCR analysis was performed to analyze the expression of pluripotency genes. qPCR results confirmed the expression of pluripotency genes Oct3/4, Sox2, and Nanog in cAMSC (Figure 2), which proves that cAMSCs exhibit pluripotency potential.

1.3. cAMSC-CM proteome

The proteomic analysis showed the presence of 86 proteins (Table 2) and was expressed in mole percentage. Intermediate filaments (26%), other types of proteins involved in cell metabolism (21%), growth factors (18%), extracellular matrix components (15%), anti-oxidants (13%), and enzymes (7%) were found in the cAMSC-CM (Table 3).

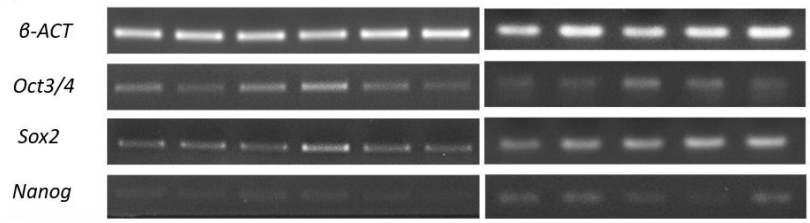

Figure 2. Confirmation of pluripotent genes expressions in all canine amniotic membrane-derived mesenchymal stem cell lines (n=11)

Table 2. Proteins found in canine amniotic membrane-derived mesenchymal stem cells-derived conditioned medium

Category / Function	Protein name	Gene	Molecular weight (Da)	Accessions
Extracellular matrix component, growth factor, anti-apoptosis, calcium ion binding	Secreted protein acidic and cysteine rich/Osteonectin	SPARC	35390	E2RMA3
Growth factor, anti-oxidant, metal binding	Serum albumin	ALB	70558	F2Z4Q6
Intermediate filament	Keratin, type I cytoskeletal 10	KRT10	57821	F1PYU9
Extra-cellular matrix component, cell proliferation	Lumican	LUM	38665	E2R416
Intermediate filament	Keratin, type II cytoskeletal 1	KRT1	63846	F1PTY1
Growth factor, glycoprotein, steroidogenesis stimulation	Metalloproteinase inhibitor 1	TIMP1	29250	F1PQS2
Growth factor	Insulin like growth factor binding protein 7	IGFBP7	29907	E2RNL2

Apolipoprotein, cholesterol metabolism, anti-oxidant	Apolipoprotein E	APOE	37258	F1PJ74
Intermediate filament	Keratin 14	KRT14	52621	F1Q0R0
Cell metabolism	Actin, cytoplasmic 1	ACTB	O18840	42052
Intermediate filament	Vimentin	VIM	53622	F1PLS4
Intermediate filament	Keratin 75	KRT75	59713	E2R917
Extra-cellular matrix component	Follistatin like 1	FSTL1	36073	F1PY69
Intermediate filament	Uncharacterized protein	KRT5	62919	E2R8Z5
Extra-cellular matrix component	Collagen type III alpha 1 chain	COL3A1	139948	F1PG69
Intermediate filament	Uncharacterized protein	KRT6A	60975	F1PTS8
Anti-oxidant	Thioredoxin	TXN	11665	J9NWJ5
Intermediate filament	Uncharacterized protein	KRT42	50182	E2R7W6
Enzyme	Malate dehydrogenase	MDH1	38564	E2QV08
Growth factor	Alpha 2-HS glycoprotein	AHSG	40021	E2QUV3

Cell metabolism	GC, vitamin D binding protein	GC	55997	F1P841
Cell metabolism	Plasminogen activator, urokinase receptor	PLAUR	27149	E2RGN0
Cell metabolism	Alpha-fetoprotein	AFP	70491	F1PXN2
Cell metabolism	Uncharacterized protein	YWHAZ	27899	F1PBL1
Apolipoprotein, sperm motility, cholesterol metabolism	Apolipoprotein A-I	APOA1	30178	P02648
Intermediate filament	Uncharacterized protein	KRT4	64360	E2QUU4
Intermediate filament	Keratin, type I cytoskeletal 9	KRT9	65274	F1Q0N7
Cell metabolism	Uncharacterized protein	LOC477072	112612	F6V1W9
Enzyme	Triosephosphate isomerase	TPI1	32085	A0A0A0MPD0
Enzyme	Purine nucleoside phosphorylase	PNP	32566	F1PQM1
Cell metabolism	Hemoglobin subunit beta	HBB	16100	P60524
Growth factor	Fibronectin	FN1	270608	F1P6H7

Extra-cellular matrix component	Collagen alpha-1(I) chain	COL1A1	140042	F1Q3I5
Intermediate filament	Keratin 24	KRT24	56819	E2R150
Enzyme	ATP synthase subunit d, mitochondrial	ATP5PD	18679	J9P8U0
Anti-oxidant	Ferritin	LOC102154317	19901	E2RRH1
Anti-oxidant	Decorin	DCN	40297	Q29393
Extra-cellular matrix component	Uncharacterized protein	LGALS3BP	63197	E2RKQ6
Intermediate filament	Keratin, type II cytoskeletal 2 epidermal	KRT2	65016	F1PTX4
Cell metabolism	SAP domain containing ribonucleoprotein	SARNP	23759	F1PVE3
Extra-cellular matrix component	Dermatopontin	DPT	24578	E2RPQ6
Cell metabolism	Proteasome subunit beta	PSMB6	25686	E2R0B6
Intermediate filament	Keratin 78	KRT78	56429	E2R4B0
Cell metabolism	Uncharacterized protein	LOC475521	26945	F1PCE8
Intermediate filament	Keratin 72	KRT72	57646	E2QUT7

Enzyme	Nicotinate-nucleotide pyrophosphorylase [carboxylating]	QPRT	31301	J9NZ55
Cell metabolism	Growth arrest specific 2	GAS2	35495	F1PBV2
Cell metabolism	Olfactory receptor	OR6K6	38505	E2R0T9
Cell metabolism	Olfactory receptor	LOC100687010	35594	F1PU69
Cell metabolism	Lysosomal protein transmembrane 4 beta	LAPTM4B	37360	F1PGH9
Growth factor	Fetuin B	FETUB	43162	E2R9B6
Enzyme	L-lactate dehydrogenase	LDHA	40058	F1PVW0
Enzyme	L-lactate dehydrogenase	LDHB	36931	J9NT18
Cell metabolism	Tropomyosin 1	TPM1	37436	F1P912
Cell metabolism	Annexin A2	ANXA2	38915	Q6TEQ7
Extra-cellular matrix component	Collagen alpha-2(I) chain	COL1A2	129835	O46392
Growth factor	Cellular communication network factor 2	CCN2	39909	J9NTX8
Extra-cellular matrix component	Thrombospondin 1	THBS1	133521	F1PBI6

Extra-cellular matrix component	Biglycan	BGN	42268	G1K2D8
Cell metabolism	Serpin family A member 7	SERPINA7	46789	F1PB85
Cell metabolism	Serpin family F member 1	SERPINF1	46586	F2Z4Q7
Cell metabolism	Nerve growth factor receptor	NGFR	47024	F1PAR7
Cell metabolism	Mitogen-activated protein kinase associated protein 1	MAPKAP1	59657	E2RAY0
Cell metabolism	Serpin family C member 1	SERPINC1	53003	E2RES2
Cell metabolism	Uncharacterized protein	ZNF385C	53432	F1PZ48
Extra-cellular matrix component	Collagen type V alpha 2 chain	COL5A2	145807	F1PG08
Enzyme	Dihydropyrimidinase like 3	DPYSL3	62236	F1Q3Y2
Cell metabolism	Fragile X mental retardation 1	FMR1	69446	F1Q2U8
Enzyme	TBK1 binding protein 1	TBKBP1	67443	F1P609
Enzyme	Protein phosphatase 1 regulatory subunit	PPP1R12C	85052	E2QUK2
Anti-oxidant	Uncharacterized protein	A2M	166540	F6UME0

Cell metabolism	Potassium calcium-activated channel subfamily N member 2	KCNN2	93690	F1PA33
Cell metabolism	Otoancorin	OTOA	127728	E2RIN3
Cell metabolism	KIAA0319	KIAA0319L	106022	F6XGU5
Other, binding protein	Latent transforming growth factor beta binding protein 1	LTBP1	178675	F1PLA1
Cell metabolism	Rho guanine nucleotide exchange factor 40	ARHGEF40	175931	F1PME6
Enzyme, energy metabolism	Adenylate cyclase 10	ADCY10	189494	E2RDZ3
Extra-cellular matrix component	Collagen type V alpha 1 chain	COL5A1	180707	F1PHX8
Cell metabolism	Fibronectin type III domain containing 3B	FNDC3B	134875	E2REV4
Cell metabolism	Uveal autoantigen with coiled-coil domains and ankyrin repeats	UACA	163865	Q9GL21
Growth factor	Fms related tyrosine kinase 1	FLT1	152451	F1PKV5
Cell metabolism	Zinc finger protein 142	ZNF142	211301	J9P6V2
Cell metabolism	Ryanodine receptor 1	RYR1	572146	F1PIS0

Enzyme	Citron rho-interacting serine/threonine kinase	CIT	236845	E2RPN3
Extra-cellular matrix component	Laminin subunit alpha 3	LAMA3	376682	E2RPP1
Enzyme	DNA-dependent protein kinase catalytic subunit	PRKDC	476572	F1Q3H1

Table 3. Mole percentages of canine amniotic membrane-derived mesenchymal stem cells CM components from proteomics analysis

Type of proteins	Total mole percentage by type of proteins (%)
Intermediate filaments	26
Cell metabolism	21
Growth factors	18
Extra-cellular matrix components	15
Anti-oxidants	13
Enzymes	7

2. Determination of cAMSC-CM optimal concentration

Sperm viability and VCL were higher in the treated groups, 5%, 10%, and 15% CM (5% CM, 75.4 ± 6.7% and 83.9 ± 2.8%; 10% CM, 87.2 ± 8.1% and 90.1 ± 2.8%; 15% CM, 75.4 ± 6.7% and 86.8 ± 3.5%, respectively) and the group treated with 10% CM was significantly higher ($p<0.05$) in comparison with the control group (74.2 ± 4.4% and 80.8 ± 2.0%, respectively). The 10% CM-treated group showed significantly higher motility and ALH (79.2 ± 2.6% and 4.8 ± 0.3 µm) than the other groups (control, 67.3 ± 2.5% and 4.1 ± 0.1 µm; 5% CM, 72.4 ± 2.5% and 4.1 ± 0.1 µm; 15% CM, 72.1 ± 3.9% and 4.1 ± 0.3 µm) ($p<0.05$), (Table 4). Therefore, the 10% CM-treated group was selected for the rest of the experiment.

3. cAMSC-CM effects on sperm cryopreservation

3.1. Motility and velocity parameters

The CASA system results showed that 10% CM treatment significantly enhanced ($p<0.05$) motility and LIN (54.3 ± 1.9% and 50.3 ± 3.1%, respectively) of sperm compared to that of the control group (42.1 ± 2.1% and 47.0 ± 3.4%, respectively). The percentage of immotile spermatozoa was significantly reduced in the treatment group (45.7 ± 1.9%), when compared with the control group (57.9 ± 2.1%) ($p<0.05$), (Figure 3). The rest of the parameters showed no significant differences between the groups ($p<0.05$) *(*Table 5).

Table 4. Motility and velocity parameters of 4h chilled sperm using different concentrations of canine amniotic membrane-derived mesenchymal stem cells conditioned media

Concentration of CM (%)	Motility (%)	Viability (%)	VCL (µm/sec)	VSL (µm/sec)	VAP (µm/sec)	LIN (%)	STR (%)	ALH (µm)
0	67.3 ± 2.5^{b}	80.8 ± 2.0^{b}	74.2 ± 4.4^{b}	21.4 ± 1.3	45.8 ± 2.0	29.0 ± 1.3	47.4 ± 1.7	4.1 ± 0.1^{b}
5	72.4 ± 2.5^{b}	83.9 ± 2.8^{ab}	75.4 ± 6.7^{ab}	20.4 ± 1.0	46.8 ± 3.1	29.0 ± 1.5	44.1 ± 1.4	4.2 ± 0.3^{b}
10	79.2 ± 2.6^{a}	90.1 ± 2.8^{a}	87.2 ± 8.1^{a}	23.8 ± 1.8	54.0 ± 4.0	31.2 ± 1.6	44.5 ± 1.2	4.8 ± 0.3^{a}
15	72.1 ± 3.9^{b}	86.8 ± 3.5^{ab}	75.4 ± 6.7^{ab}	24.6 ± 3.7	46.7 ± 4.0	31.6 ± 2.1	46.4 ± 2.3	4.1 ± 0.3^{b}

VCL, average curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity (average ratio of VSL/VCL); STR, straightness (average value of the ratio VSL/VAP); ALH, amplitude of lateral head. All results show means ± SEM. Values within marked with the letters “a” or “b” are significantly different ($p<0.05$, n= 4)

Table 5. Motility and velocity parameters of frozen-thawed sperm in control and 10 % of conditioned media treatment groups

Concentration of CM (%)	Motility (%)	Progressive motility (%)	VCL (µm/sec)	VSL (µm/sec)	VAP (µm/sec)	LIN (%)	STR (%)	ALH (µm)
0	42.1 ± 2.1[b]	22.8 ± 3.4	81.5 ± 6.4	49.4 ± 5.6	57.2 ± 5.6	47.0 ± 3.4[b]	68.1 ± 2.4	3.1 ± 0.3
10	54.3 ± 1.9[a]	26.2 ± 4.2	74.5 ± 7.8	46.3 ± 7.1	53.3 ± 7.2	50.3 ± 3.1[a]	70.0 ± 2.2	2.8 ± 0.2

VCL, average curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity (average ratio of VSL/VCL); STR, straightness (average value of the ratio VSL/VAP); ALH, amplitude of lateral head. Values are presented as means ± standard error of the mean (SEM). Values within columns marked with the letters "a" or "b" are significantly different ($p<0.05$, n= 4)

(**a**) (**b**)

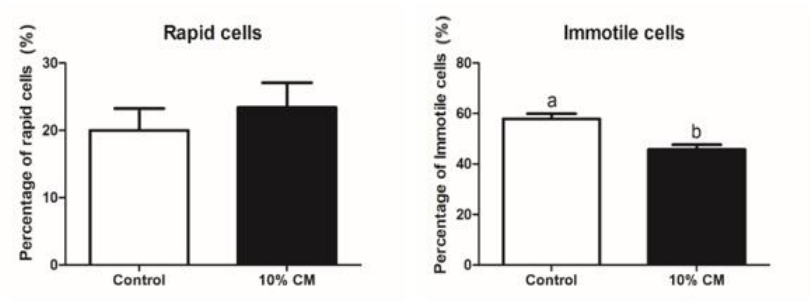

Figure 3. Percentages of rapid and immotile in frozen-thawed sperm. (**a**) Percentage of rapid sperm cells; (**b**) Percentage of immotile sperm cells. Bars with the letters "a" or "b" are values with a statistically significant difference ($p<0.05$, n= 4)

3.2. Live/ dead count and morphology assessment

The percentage of live sperm cells was higher in the 10% CM-treated group (55.2 ± 3.0%) than that of the control group (43.9 ± 4.3%), and the percentage of spermatozoa with a bent tail was lower in the treatment group (1.8 ± 0.6%) than the control group (3.1 ± 0.7%) ($p<0.05$), (Table 6). However, the percentage of cells with a coiled tail was not significantly different between the control and the 10% CM-treated groups (3.0 ± 1.2% and 3.0 ± 1.8%, respectively). These results suggest that cAMSC-CM probably has an anti-apoptotic effect during sperm cryopreservation.

3.3. Chromatin integrity

The percentage of spermatozoa with abnormal chromatin condensation-stained nuclei was not significantly different in both the groups (control, 34.0 ± 2.9%; 10% CM, 31.0 ± 3.1%) ($p<0.05$), (Table 7). These results suggest that cAMSC-CM has no protective effect on DNA integrity.

Table 6. Live sperm percentage and morphological defects of frozen-thawed sperm in control and 10 % of conditioned media treatment groups

Concentration of CM (%)	Live sperm (%)	Coiled tail (%)	Bent tail (%)
0	43.9 ± 4.3^{a}	3.0 ± 1.2	3.1 ± 0.7^{a}
10	55.2 ± 3.0^{b}	3.0 ± 1.8	1.8 ± 0.6^{b}

Values are presented as means ± standard error of the mean (SEM). Values within columns marked with the letters "a" or "b" are significantly different ($p<0.05$, n= 4)

Table 7. Percentage of frozen-thawed sperm with an abnormal chromatin condensation in control and 10 % of conditioned media (CM) treatment groups

Concentration of CM (%)	Aniline blue positive sperm (%)
0	34.0 ±2.9
10	31.0 ± 3.1

All results show means ± SEM (n= 4)

3.4. Acrosome and membrane integrity assessment

The percentage of sperm cells with intact plasma membrane was significantly higher ($p<0.05$) in the treatment group (66.5 ± 2.3%) than the control group (54.5 ± 2.9%). The FITC-PNA test revealed no significant difference ($p<0.05$) in the percentage of spermatozoa with an intact acrosome between the two groups (control, 74.0 ± 4.3%; 10% CM, 76.6 ± 4.0%) (Table 8).

3.5. Mitochondria activity assessment

The R123 dye was used to assess mitochondrial activity, and PI was used to stain dead sperm cells. Both the data were used to calculate the percentage of live sperm with active mitochondria in each group. The 10% CM-treated group showed significantly enhanced ($p<0.05$) mitochondrial activity (49.6 ± 0.7%) compared with the control group (36.4 ± 2.5%) (Figure 4).

Table 8. Percentages of intact acrosome and membrane in frozen-thawed sperm in control and 10 % of conditioned media treatment groups

Concentration of CM (%)	Intact acrosome (%)	Intact membrane (%)
0	74.0 ± 4.3	54.5 ± 2.9[b]
10	76.6 ± 4.0	66.5 ± 2.3[a]

Values are presented as means ± standard error of the mean (SEM). Values within columns marked with the letters "a" or "b" are significantly different ($p<0.05$, n= 4)

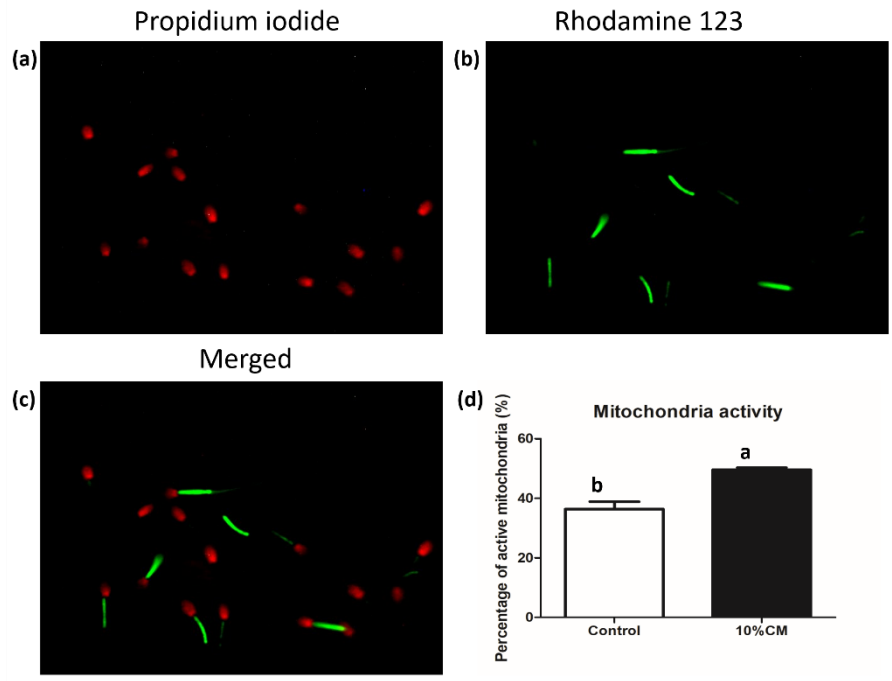

Figure 4. Mitochondria activity in frozen-thawed sperm using Rhodamine 123 (R123) and Propidium iodide (PI) dual staining; (**a**) dead sperm stained with PI; (**b**) live sperm with R123-stained mitochondria; (**c**) Merged channels showing both PI and R123 stained sperm; (**d**) percentage of sperm with active mitochondria. Bars with the letters "a" or "b" are values with a statistically significant difference ($p<0.05$, $n= 4$)

Discussion

Since the isolation of MSCs from amniotic tissues, researchers have displayed a growing interest in the study of their characteristics, properties and possible applications [49, 52, 73]. Thus, they have become an interesting alternative to embryonic stem cells as they can be obtained by non-invasive methods [49], and the ethical issues associated with the use of amniotic tissues-derived stem cells are minor [52], since they are obtained from the placenta that is usually discarded after caesarian sections [47]. They are non-teratogenic [73], pluripotent, and also have regenerative properties, and low immunogenicity [49, 52, 73]. Canine MSCs derived from the amniotic membrane have recently been isolated and proved to have the same characteristics, biosafety and properties as other MSCs, which makes them a good candidate for clinical trials [50, 74]. The composition and potential use of cAMSC-CM have not been studied yet, but since CM contains cell paracrine factors [55], the application of cAMSC-CM in clinical studies seems to be promising. In my study, cAMSC phenotype was confirmed through FACS analysis and showed a high expression of CD29, CD44, and CD90 surface markers, whereas CD34 and CD45 expression, which are associated with hematopoietic stem cells [75, 76], was low (Figure 1). This phenotype has also been found in other studies using canine MSCs [77-79], and CD29, CD44, CD90 surface antigens are found on adipose, bone marrow and umbilical cord blood-derived mesenchymal stem cells [80, 81]. Pluripotency is an important factor in MSCs since it is involved in regenerative pathways [82]. The analysis of pluripotency gene expression (*Oct3/4*, *Nanog*, and *Sox2*) also confirmed the MSC phenotype (Figure 2). The analysed genes are essential for self-renewal of MSCs, determination of pluripotency and maintenance of cells' undifferentiated state [83, 84]. Their expression by cAMSCs is an indicator that cAMSCs could be used in regenerative medicine.

Stem cells-derived CM contains growth factors and anti-apoptotic factors, and has been

used in cell-free therapies to mediate stem cells paracrine effects on tissues [57, 59, 60]. Each CM has a different composition depending on the cells of origin, cell state, and the microenvironment surrounding them [53]. Previously, canine MSC-derived CM was used for different clinical applications and showed good results in xenogeneic tissues wound healing [85], laryngotracheal stenosis healing [86], and stem cells survival and differentiation [87]. However, no study has unveiled the effects of cAMSC-CM and its proteomic analysis. In this study, the composition of cAMSC-CM was investigated and the proteomic analysis revealed the presence of intermediate filaments, extra-cellular matrix components (ECM), anti-oxidants, growth factors, enzymes, and other proteins involved in the cell metabolism (Tables 2 and 3). My results corroborate those of previous studies showing that one of the main components of CMs were growth factors and anti-oxidants [57]. Furthermore, amniotic membrane stem cells proteome from another study [88] showed the presence of ECM components involved in cellular processes through the focal adhesion kinase signaling pathway such as lumican, collagen, and fibronectin, that were also found in cAMSC-CM in my study (Table 2 and 3). Apolipoproteins, especially Apolipoprotein A-1 which is involved in sperm capacitation and motility [89]; and Apolipoprotein E which has an anti-oxidant activity [90], were also found in cAMSC-CM (Table 2). Some of the growth factors found in my study include Thioredoxin, which has an anti-oxidant activity and plays a role in fertility [91-93]; and serum albumin that might also play an important role in fertility improvement [94] and sperm cryopreservation, by enhancing post-thaw motility and protecting sperm morphology, and the integrity of the plasma membrane, acrosome, and DNA [95, 96].

Although sperm cells have the ability to adapt to osmotic changes [97], freezing medium does not fully protect them from the osmotic stress happening during freezing and thawing that leads to increase in ROS production, apoptosis-like changes, DNA damage and

an increase in tail defects [98-100]. Therefore, I hypothesized that the addition of cAMSC-CM to the freezing medium would increase sperm tolerance to osmotic changes, oxidative defence, and help protect their ultra-structure, because it contains proteins with anti-oxidant, regenerative, and anti-apoptotic effects (Table 2). To date, no study has depicted the addition of CM in sperm cryopreservation, and the range of CM concentrations added to the freezing medium remains unknown. Here, I evaluated the optimal concentration of CM starting from a low range of concentrations, from 0 to 15% of the CM, among which 10% of the CM revealed to be the best concentration (Table 4). I assumed that a concentration higher than 15% would influence sperm homeostasis, as it would probably change the osmolarity of the milieu. In fact, during my preliminary study we observed that the use of a higher range of concentrations (from 25 to 75%) negatively impacted the quality-related parameters of frozen–thawed sperms (data not shown). Neverthless, from the low range of concentrations, the supplementation of 10% CM in the freezing medium was found to be the optimal one and was further used for cryopreservation.

My results showed the percentage of live sperm was significantly increased when cAMSC-CM was added to the freezing medium (Table 6) which could be explained by the regenerative and anti-apoptotic effects of cAMSC-CM proteins. When CM is prepared, the components released from the starvation phase resulting from the MSC survival state activation, causes the release of protective factors that protects cells from apoptosis and oxidative stress [63]. These factors probably improved the anti-oxidant defence and anti-apoptotic mechanisms in sperm. However, they were not sufficient to protect chromatin integrity as aniline blue test results showed no significant differences between the control and cAMSC-CM treated groups (Table 7). Aniline blue dye binds to nucleoproteins and allows researchers to evaluate chromatin integrity, however, DNA denaturation and

fragmentation induced by freezing and thawing are not always immediately apparent. A study showed that canine sperm further incubated after thawing showed an increase in the DNA fragmentation index [101].

The cryopreservation process leads to an increase in sperm tail abnormalities [98, 102], altered acrosome and plasma membrane integrity [103], and a decrease in the mitochondrial activity [22, 100] and motility [98, 100]. In my study, I found that the percentage of coiled tail sperm was the same in both groups, but the percentage of sperm with a bent tail was significantly reduced in the 10% CM-treated group in comparison with the control group (Table 6). The proteins present in seminal plasma can interact with sperm at the surface, and repair plasma membranes and mitochondrial DNA [104]. In my study, some of the proteins found in cAMSC-CM, including collagen, olfactory receptors, zinc finger protein, vitamin D binding protein, fibronectin, and serum albumin (Table 2), have also been found in the seminal plasma collected from fertile men [105], boar [106, 107], alpaca and camels [107]. These proteins, along with the protective nature of other cAMSC-CM factors, might explain the positive effects on sperm ultra-structural characteristics (Table 6). Moreover, the positive correlation between some of these proteins and fertility [94, 105] and their role in mitochondrial DNA repair [104] might explain post-thaw improved sperm motility, LIN (Table 5), and the enhanced mitochondrial activity (Figure 4). Mitochondrial activity is essential for sperm motility, and a decrease in its activity results in increased apoptosis [108]. Furthermore, the addition of 10% cAMSC-CM reduced the percentage of immotile sperm (45.7 ± 1.9%) (Figure 3). I hypothesized that proteins in cAMSC-CM might have reduced ROS production and have had a positive role in mitochondrial DNA repair. However, the percentage of sperm with an intact acrosome was high in both the groups (74.0 ± 4.3% and 76.6 ± 4.0% respectively), but there was no significant difference between them (Table 8). A

study showed that acrosome integrity is above 60% in canine sperm at 0 h after thawing, when 6–8% glycerol is used [109]. In my freezing medium, 6% glycerol was used and the acrosome integrity test was performed within few minutes after thawing. This might explain the high percentage of intact acrosomes observed in both groups.

Preservation of sperm membrane integrity and mitochondrial function during the freeze–thaw process is important for successful fertilization, but the commercial freezing media do not protect sperm from the loss of these functions [34]. The addition of cyto-protective factors in the freezing medium can prevent the deleterious effects of cryopreservation [110] because they can protect cells from the negative effects of ROS on sperm motility, mitochondrial activity, and DNA integrity [111]. In my study, the preservation of membrane integrity was more effective in the 10% CM-treated group (66.5 ± 2.3%) in comparison with the control group (54.5 ± 2.9%). This might be due to the proteins present in cAMSC-CM, especially apolipoproteins (Table 2). ROS targets poly-unsaturated fatty acids and cholesterol [112], which disturbs the integrity of plasma membrane that is essential to sperm homeostasis [113]. Cholesterol, in particular, is important for membrane stability and can determine sperm freezability, as disturbed cholesterol to phospholipid ratio can negatively impact the outcome of cryopreservation [114]. Apolipoproteins play a crucial role in cholesterol homeostasis in the epididymis [115], and are involved in lipid exchange, capacitation, and membrane remodeling [89, 116]. In addition, fibronectin, an ECM component that acts as a growth factor [117], was found in the cAMSC-CM, which is also involved in protecting membrane integrity (Table 2 and 3). Qamar et al. [118] showed that fibronectin was expressed in adipose-derived MSCs, and that plasma membrane integrity was protected when adipose-derived MSCs were added to the freezing media. This suggests that cAMSC-CM protected plasma membrane integrity in the present study (Table 8).

Conclusion

In conclusion, cAMSC-CM has been successfully characterized and used for canine sperm cryopreservation. To summarize briefly, my results showed that cAMSC-CM contained proteins, including growth factors, anti-oxidants, enzymes and ECM components, which protect sperm functions and ultra-structure characteristics during cryopreservation. The addition of cAMSC-CM in the freezing medium enhanced sperm motility and viability, membrane integrity, and mitochondrial activity.

These results gave me more insights on the wide benefits of stem cells and their derivatives, but also more perspectives of the potential fields they might be useful in. Moreover, this was the first study to reveal the composition of cAMSC-CM and its effect on sperm cryopreservation. In the future, more studies characterizing cAMSC and cAMSC-CM will be needed to unveil their components and spectrum of activity.

Further *in vitro* studies to evaluate cAMSC- CM effects on sperm capacitation and fertilizing ability should be conducted. *In vivo* studies should also be conducted to reveal the effects of cAMSC-CM proteins on fertility, along with a much deeper study on the proteins implicated in canine sperm fertility.

References

[1] Amann RP, Hammerstedt RH, Veeramachaneni DN. The epididymis and sperm maturation: a perspective. Reprod Fertil Dev. 1993;5:361-81.

[2] Silva AR, Fontenele-Neto JD, Cardoso R, Silva L, Chirinéa V, Lopes M. Description of ultrastructural damages in frozen-thawed canine spermatozoa. Ciência Animal Brasileira. 2009;10:595-601.

[3] Chłopik A, Wysokińska A. Canine spermatozoa—What do we know about their morphology and physiology? An overview. Reprod Domest Anim. 2020;55:113-26.

[4] Rodriguez-Gil JE, Montserrat A, Rigau T. Effects of hypoosmotic incubation on acrosome and tail structure on canine spermatozoa. Theriogenology. 1994;42:815-29.

[5] Baran A, Ozdas OB, Sandal AI, Ak K. Effects of Skim Milk and Tris Extender on Frozen-Thawed Canine Sperm Morphology. J Anim Vet Adv. 2012;11:3242-6.

[6] Yeste M. Sperm cryopreservation update: Cryodamage, markers, and factors affecting the sperm freezability in pigs. Theriogenology. 2016;85:47-64.

[7] Thirumala S, Ferrer MS, Al-Jarrah A, Eilts BE, Paccamonti DL, Devireddy RV. Cryopreservation of canine spermatozoa: theoretical prediction of optimal cooling rates in the presence and absence of cryoprotective agents. Cryobiology. 2003;47:109-24.

[8] Songsasen N, Yu I, Murton S, Paccamonti DL, Eilts BE, Godke RA, et al. Osmotic sensitivity of canine spermatozoa. Cryobiology. 2002;44:79-90.

[9] O'Connell M, McClure N, Lewis SEM. The effects of cryopreservation on sperm morphology, motility and mitochondrial function. Hum Reprod. 2002;17:704-9.

[10] Friedenstein AJ, Piatetzky S, II, Petrakova KV. Osteogenesis in transplants of bone marrow cells. J Embryol Exp Morphol. 1966;16:381-90.

[11] Caplan AI, Correa D. The MSC: an injury drugstore. Cell Stem Cell. 2011;9:11-5.

[12] Caplan AI. Mesenchymal stem cells. J Orthop Res. 1991;9:641-50.

[13] Han Y, Li X, Zhang Y, Han Y, Chang F, Ding J. Mesenchymal Stem Cells for Regenerative Medicine. Cells. 2019;8:886.

[14] Li Z, Hu X, Zhong JF. Mesenchymal stem cells: characteristics, function, and application. Hindawi; 2019.

[15] Konala VB, Mamidi MK, Bhonde R, Das AK, Pochampally R, Pal R. The current landscape of the mesenchymal stromal cell secretome: A new paradigm for cell-free regeneration. Cytotherapy. 2016;18:13-24.

[16] Vizoso FJ, Eiro N, Cid S, Schneider J, Perez-Fernandez R. Mesenchymal Stem Cell Secretome: Toward Cell-Free Therapeutic Strategies in Regenerative Medicine. Int J Mol Sci. 2017;18:1852.

[17] Zanotti L, Angioni R, Cali B, Soldani C, Ploia C, Moalli F, Gargesha M, D'Amico G, Elliman S, Tedeschi G, Maffioli E, Negri A, Zacchigna S, Sarukhan A, Stein JV, Viola A. Mouse mesenchymal stem cells inhibit high endothelial cell activation and lymphocyte homing to lymph nodes by releasing TIMP-1. Leukemia. 2016;30:1143-54.

[18] Pires AO, Mendes-Pinheiro B, Teixeira FG, Anjo SI, Ribeiro-Samy S, Gomes ED, et al. Unveiling the Differences of Secretome of Human Bone Marrow Mesenchymal Stem Cells, Adipose Tissue-Derived Stem Cells, and Human Umbilical Cord Perivascular Cells: A Proteomic Analysis. Stem Cells Dev. 2016;25:1073-83.

[19] Bencharif D, Dordas-Perpinya M. Canine semen cryoconservation: Emerging data over the last 20 years. Reprod Domest Anim. 2020;55 Suppl 2:61-5.

[20] Desrosiers P, Legare C, Leclerc P, Sullivan R. Membranous and structural damage that occur during cryopreservation of human sperm may be time-related events. Fertil Steril. 2006;85:1744-52.

[21] Pena FJ, Nunez-Martinez I, Moran JM. Semen technologies in dog breeding: An update.

Reprod Domest Anim. 2006;41:21-9.

[22] Martin G, Sabido O, Durand P, Levy R. Cryopreservation induces an apoptosis-like mechanism in bull sperm. Biol Reprod. 2004;71:28-37.

[23] Ezzati M, Shanehbandi D, Hamdi K, Rahbar S, Pashaiasl M. Influence of cryopreservation on structure and function of mammalian spermatozoa: an overview. Cell Tissue Bank. 2020;21:1-15.

[24] Hammerstedt RH, Parks JE. Changes in sperm surfaces associated with epididymal transit. J Reprod Fertil Suppl. 1987;34:133-49.

[25] Gonzalez-Marin C, Gosalvez J, Roy R. Types, Causes, Detection and Repair of DNA Fragmentation in Animal and Human Sperm Cells. Int J Mol Sci. 2012;13:14026-52.

[26] Sicherle CC, de Souza FF, Freitas-Dell'Aqua CP, Mothe GB, Padovani CR, Papa FO, et al. Effects of the cryopreservation process on dog sperm integrity. Anim Reprod. 2020;17:e20190081.

[27] Sudo K, Asoh S, Ohsawa I, Ozaki D, Yamagata K, Ito H, et al. The anti-cell death FNK protein protects cells from death induced by freezing and thawing. Biochem Biophys Res Commun. 2005;330:850-6.

[28] Kanitkar M, Bhonde RR. Curcumin treatment enhances islet recovery by induction of heat shock response proteins, Hsp70 and heme oxygenase-1, during cryopreservation. Life Sci. 2008;82:182-9.

[29] Storey BT, Noiles EE, Thompson KA. Comparison of glycerol, other polyols, trehalose, and raffinose to provide a defined cryoprotectant medium for mouse sperm cryopreservation. Cryobiology. 1998;37:46-58.

[30] Cummins JM, Jequier AM, Kan R. Molecular biology of human male infertility: links with aging, mitochondrial genetics, and oxidative stress? Mol Reprod Dev. 1994;37:345-62.

[31] Silva SV, Soares AT, Batista AM, Almeida FC, Nunes JF, Peixoto CA, et al. Vitamin E (Trolox) addition to Tris-egg yolk extender preserves ram spermatozoon structure and kinematics after cryopreservation. Anim Reprod Sci. 2013;137:37-44.

[32] Wang AW, Zhang H, Ikemoto I, Anderson DJ, Loughlin KR. Reactive oxygen species generation by seminal cells during cryopreservation. Urology. 1997;49:921-5.

[33] Sieme H, Oldenhof H, Wolkers WF. Mode of action of cryoprotectants for sperm preservation. Anim Reprod Sci. 2016;169:2-5.

[34] Miguel-Jimenez S, del Alamo MMR, Alvarez-Rodriguez M, Hidalgo CO, Pena AI, Muino R, et al. In vitro assessment of egg yolk-, soya bean lecithin- and liposome-based extenders for cryopreservation of dairy bull semen. Anim Reprod Sci. 2020;215:106315.

[35] Ugur MR, Saber Abdelrahman A, Evans HC, Gilmore AA, Hitit M, Arifiantini RI, et al. Advances in Cryopreservation of Bull Sperm. Front Vet Sci. 2019;6:268.

[36] Kumar A, Prasad J, Srivastava N, Ghosh SJB, Biobanking. Strategies to minimize various stress-related freeze–thaw damages during conventional cryopreservation of mammalian spermatozoa. Biopreserv Biobank. 2019;17:603-12.

[37] Grandhaye J, Partyka A, Ligocka Z, Dudek A, Nizanski W, Jeanpierre E, et al. Metformin Improves Quality of Post-Thaw Canine Semen. Animals. 2020;10:287.

[38] Khan J, Tahir MZ, Khalid A, Sattar A, Ahmad N. Effect of cholesterol-loaded cyclodextrins on cryosurvival of dog spermatozoa. Reprod Domest Anim. 2017;52 Suppl 2:265-8.

[39] Belala R, Fatmi S, Kaidi R, Iguer-Ouada M. Benefits of cholesterol and α-tocopherol loaded cyclodextrins in dog semen cryopreservation. Revue Méd Vét. 2016;167:22-7.

[40] Cakici C, Buyrukcu B, Duruksu G, Haliloglu AH, Aksoy A, Isik A, et al. Recovery of fertility in azoospermia rats after injection of adipose-tissue-derived mesenchymal stem cells:

the sperm generation. Biomed Res Int. 2013;2013:529589.

[41] Mehrabani D, Hassanshahi MA, Tamadon A, Zare S, Keshavarz S, Rahmanifar F, et al. Adipose tissue-derived mesenchymal stem cells repair germinal cells of seminiferous tubules of busulfan-induced azoospermic rats. J Hum Reprod Sci. 2015;8:103-10.

[42] Hassan AI, Alam SS. Evaluation of mesenchymal stem cells in treatment of infertility in male rats. Stem Cell Res Ther. 2014;5:131.

[43] Stavely R, Nurgali K. The emerging antioxidant paradigm of mesenchymal stem cell therapy. Stem Cells Transl Med. 2020.

[44] DeSantiago J, Bare DJ, Banach K. Ischemia/Reperfusion injury protection by mesenchymal stem cell derived antioxidant capacity. Stem Cells Dev. 2013;22:2497-507.

[45] Song YS, Joo HW, Park IH, Shen GY, Lee Y, Shin JH, et al. Bone marrow mesenchymal stem cell-derived vascular endothelial growth factor attenuates cardiac apoptosis via regulation of cardiac miRNA-23a and miRNA-92a in a rat model of myocardial infarction. PLoS One. 2017;12:e0179972.

[46] Richardson SM, Kalamegam G, Pushparaj PN, Matta C, Memic A, Khademhosseini A, et al. Mesenchymal stem cells in regenerative medicine: Focus on articular cartilage and intervertebral disc regeneration. Methods. 2016;99:69-80.

[47] Park SB, Seo MS, Kim HS, Kang KS. Isolation and characterization of canine amniotic membrane-derived multipotent stem cells. PLoS One. 2012;7:e44693.

[48] Zhao P, Ise H, Hongo M, Ota M, Konishi I, Nikaido T. Human amniotic mesenchymal cells have some characteristics of cardiomyocytes. Transplantation. 2005;79:528-35.

[49] Kim EY, Lee KB, Kim MK. The potential of mesenchymal stem cells derived from amniotic membrane and amniotic fluid for neuronal regenerative therapy. BMB Rep. 2014;47:135-40.

[50] Borghesi J, Ferreira Lima M, Mario LC, de Almeida da Anunciacao AR, Silveira Rabelo AC, Giancoli Kato Cano da Silva M, et al. Canine amniotic membrane mesenchymal stromal/stem cells: Isolation, characterization and differentiation. Tissue Cell. 2019;58:99-106.

[51] Pall E, Pop RA, Ciupe S, Cenariu M, Groza IS. Canine Amniotic Membrane Derived Mesenchymal Stem Cells-Potential Sources for Regenerative Medicine. "Agriculture for Life, Life for Agriculture" Conference Proceedings: Sciendo; 2018. p. 461-4.

[52] Fauza D. Amniotic fluid and placental stem cells. Best Pract Res Clin Obstet Gynaecol. 2004;18:877-91.

[53] Kusuma GD, Carthew J, Lim R, Frith JE. Effect of the Microenvironment on Mesenchymal Stem Cell Paracrine Signaling: Opportunities to Engineer the Therapeutic Effect. Stem Cells Dev. 2017;26:617-31.

[54] Kim WS, Park BS, Kim HK, Park JS, Kim KJ, Choi JS, et al. Evidence supporting antioxidant action of adipose-derived stem cells: protection of human dermal fibroblasts from oxidative stress. J Dermatol Sci. 2008;49:133-42.

[55] Yuan Ql, Zhang Yg, Chen QJTAR. Mesenchymal Stem Cell (MSC)-Derived Extracellular Vesicles: Potential Therapeutics as MSC Trophic Mediators in Regenerative Medicine. Anat Rec. 2020;303:1735-42.

[56] Green EM, Lee RT. Proteins and small molecules for cellular regenerative medicine. Physiol Rev. 2013;93:311-25.

[57] Gunawardena TNA, Rahman MT, Abdullah BJJ, Abu Kasim NH. Conditioned media derived from mesenchymal stem cell cultures: The next generation for regenerative medicine. J Tissue Eng Regen Med. 2019;13:569-86.

[58] Ra K, Oh HJ, Kim GA, Kang SK, Ra JC, Lee BC. High Frequency of Intravenous

Injection of Human Adipose Stem Cell Conditioned Medium Improved Embryo Development of Mice in Advanced Maternal Age through Antioxidant Effects. Animals. 2020;10:978.

[59] Chen YX, Zeng ZC, Sun J, Zeng HY, Huang Y, Zhang ZY. Mesenchymal stem cell-conditioned medium prevents radiation-induced liver injury by inhibiting inflammation and protecting sinusoidal endothelial cells. J Radiat Res. 2015;56:700-8.

[60] Yamaguchi S, Shibata R, Yamamoto N, Nishikawa M, Hibi H, Tanigawa T, et al. Dental pulp-derived stem cell conditioned medium reduces cardiac injury following ischemia-reperfusion. Sci Rep. 2015;5:16295.

[61] Di Santo S, Yang Z, von Ballmoos MW, Voelzmann J, Diehm N, Baumgartner I, et al. Novel cell-free strategy for therapeutic angiogenesis: in vitro generated conditioned medium can replace progenitor cell transplantation. PLoS One. 2009;4:e5643.

[62] Korkmaz-Icöz S, Li K, Loganathan S, Ding Q, Ruppert M, Radovits T, et al. Brain-dead donor heart conservation with a preservation solution supplemented by a conditioned medium from mesenchymal stem cells improves graft contractility after transplantation. American Journal of Transplantation. 2020.

[63] Ferro F, Spelat R, Shaw G, Duffy N, Islam MN, O'Shea PM, et al. Survival/Adaptation of Bone Marrow-Derived Mesenchymal Stem Cells After Long-Term Starvation Through Selective Processes. Stem Cells. 2019;37:813-27.

[64] Ando Y, Matsubara K, Ishikawa J, Fujio M, Shohara R, Hibi H, et al. Stem cell-conditioned medium accelerates distraction osteogenesis through multiple regenerative mechanisms. Bone. 2014;61:82-90.

[65] Qamar AY, Fang X, Kim MJ, Cho J. Improved Post-Thaw Quality of Canine Semen after Treatment with Exosomes from Conditioned Medium of Adipose-Derived

Mesenchymal Stem Cells. Animals. 2019;9:865.

[66] Abdillah DA, Setyawan EMN, Oh HJ, Ra K, Lee SH, Kim MJ, et al. Iodixanol supplementation during sperm cryopreservation improves protamine level and reduces reactive oxygen species of canine sperm. J Vet Sci. 2019;20:79-86.

[67] Setyawan EM, Kim MJ, Oh HJ, Kim GA, Jo YK, Lee SH, et al. Maintaining canine sperm function and osmolyte content with multistep freezing protocol and different cryoprotective agents. Cryobiology. 2015;71:344-9.

[68] Brito LF. Evaluation of stallion sperm morphology. Clinical Techniques in Equine Practice. 2007;6:249-64.

[69] Sati L, Huszar G. Methodology of aniline blue staining of chromatin and the assessment of the associated nuclear and cytoplasmic attributes in human sperm. Spermatogenesis: Springer; 2013. p. 425-36.

[70] Pinto CR, Kozink DM. Simplified hypoosmotic swelling testing (HOST) of fresh and frozen-thawed canine spermatozoa. Anim Reprod Sci. 2008;104:450-5.

[71] Ren F, Fang Q, Feng T, Li Y, Wang Y, Zhu H, et al. Lycium barbarum and Laminaria japonica polysaccharides improve Cashmere goat sperm quality and fertility rate after cryopreservation. Theriogenology. 2019;129:29-36.

[72] Fraser L, Dziekonska A, Strzezek R, Strzezek J. Dialysis of boar semen prior to freezing-thawing: its effects on post-thaw sperm characteristics. Theriogenology. 2007;67:994-1003.

[73] Petsche Connell J, Camci-Unal G, Khademhosseini A, Jacot JG. Amniotic fluid-derived stem cells for cardiovascular tissue engineering applications. Tissue Eng Part B Rev. 2013;19:368-79.

[74] de Oliveira Pinheiro A, Lara VM, Souza AF, Casals JB, Bressan FF, Fantinato Neto P, et al. Characterization and Immunomodulation of Canine Amniotic Membrane Stem Cells.

Stem Cells Cloning. 2020;13:43-55.

[75] Wang J, Zhao HP, Lin G, Xie CQ, Nie DS, Wang QR, et al. In vitro hematopoietic differentiation of human embryonic stem cells induced by co-culture with human bone marrow stromal cells and low dose cytokines. Cell Biol Int. 2005;29:654-61.

[76] Park KS, Pang B, Park SJ, Lee YG, Bae JY, Park S, et al. Identification and functional characterization of ion channels in CD34(+) hematopoietic stem cells from human peripheral blood. Mol Cells. 2011;32:181-8.

[77] Takemitsu H, Zhao D, Yamamoto I, Harada Y, Michishita M, Arai T. Comparison of bone marrow and adipose tissue-derived canine mesenchymal stem cells. BMC Vet Res. 2012;8:150.

[78] Vieira NM, Brandalise V, Zucconi E, Secco M, Strauss BE, Zatz M. Isolation, characterization, and differentiation potential of canine adipose-derived stem cells. Cell Transplant. 2010;19:279-89.

[79] Trindade Hill A, Therrien J, Garcia J, Smith LJCP. Mesenchymal-like stem cells in canine ovary show high differentiation potential. Cell Proliferat. 2017;50:e12391.

[80] Kern S, Eichler H, Stoeve J, Kluter H, Bieback K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells. 2006;24:1294-301.

[81] Zeng G, Wang G, Guan F, Chang K, Jiao H, Gao W, et al. Human amniotic membrane-derived mesenchymal stem cells labeled with superparamagnetic iron oxide nanoparticles: the effect on neuron-like differentiation in vitro. Mol Cell Biochem. 2011;357:331-41.

[82] Greenow K, Clarke AR. Controlling the stem cell compartment and regeneration in vivo: the role of pluripotency pathways. Physiol Rev. 2012;92:75-99.

[83] Han SM, Han SH, Coh YR, Jang G, Ra JC, Kang SK, et al. Enhanced proliferation and

differentiation of Oct4-and Sox2-overexpressing human adipose tissue mesenchymal stem cells. Exp Mol Med. 2014;46:e101-e.

[84] Liu TM, Wu YN, Guo XM, Hui JH, Lee EH, Lim B. Effects of ectopic Nanog and Oct4 overexpression on mesenchymal stem cells. Stem Cells Dev. 2009;18:1013-22.

[85] Ansari MM, Sreekumar T, Chandra V, Dubey P, Kumar G, Amarpal S. Therapeutic potential of canine bone marrow derived mesenchymal stem cells and its conditioned media in diabetic rat wound healing. J Stem Cell Res Ther. 2013;3:2.

[86] Iravani K, Sobhanmanesh A, Ashraf MJ, Hashemi SB, Mehrabani D, Zare S. The Healing Effect of Conditioned Media and Bone Marrow-Derived Stem Cells in Laryngotracheal Stenosis: A Comparison in Experimental Dog Model. World J Plast Surg. 2017;6:190-7.

[87] Nakamura M, Nishida H, Yoshizaki K, Akiyoshi H, Hatoya S, Sugiura K, et al. Canine mesenchymal stromal cell-conditioned medium promotes survival and neurite outgrowth of neural stem cells. J Vet Med Sci. 2020;82:668-72.

[88] Baharvand H, Heidari M, Ebrahimi M, Valadbeigi T, Salekdeh GH. Proteomic analysis of epithelium-denuded human amniotic membrane as a limbal stem cell niche. Mol Vis. 2007;13:1711-21.

[89] Jha KN, Shumilin IA, Digilio LC, Chertihin O, Zheng H, Schmitz G, et al. Biochemical and structural characterization of apolipoprotein AI binding protein, a novel phosphoprotein with a potential role in sperm capacitation. Endocrinology. 2008;149:2108-20.

[90] Pham T, Kodvawala A, Hui DY. The receptor binding domain of apolipoprotein E is responsible for its antioxidant activity. Biochemistry-Us. 2005;44:7577-82.

[91] Arner ESJ, Holmgren A. Physiological functions of thioredoxin and thioredoxin reductase. Eur J Biochem. 2000;267:6102-9.

[92] Gasdaska JR, Berggren M, Powis G. Cell growth stimulation by the redox protein thioredoxin occurs by a novel helper mechanism. Cell Growth Differ. 1995;6:1643-50.

[93] Kuribayashi Y, Gagnon C. Effect of catalase and thioredoxin addition to sperm incubation medium before in vitro fertilization on sperm capacity to support embryo development. Fertil Steril. 1996;66:1012-7.

[94] Chen Y, Foote RH, Brockett CC. Effect of sucrose, trehalose, hypotaurine, taurine, and blood serum on survival of frozen bull sperm. Cryobiology. 1993;30:423-31.

[95] Uysal O, Bucak MN. Effects of oxidized glutathione, bovine serum albumin, cysteine and lycopene on the quality of frozen-thawed ram semen. Acta Vet Brno. 2007;76:383-90.

[96] Sarıözkan S, Türk G, Cantürk F, Yay A, Eken A, Akçay A. The effect of bovine serum albumin and fetal calf serum on sperm quality, DNA fragmentation and lipid peroxidation of the liquid stored rabbit semen. Cryobiology. 2013;67:1-6.

[97] Yeung CH, Anapolski M, Setiawan I, Lang F, Cooper TG. Effects of putative epididymal osmolytes on sperm volume regulation of fertile and infertile c-ros transgenic Mice. J Androl. 2004;25:216-23.

[98] Raad G, Lteif L, Lahoud R, Azoury J, Azoury J, Tanios J, et al. Cryopreservation media differentially affect sperm motility, morphology and DNA integrity. Andrology. 2018;6:836-45.

[99] Ball BA. Oxidative stress, osmotic stress and apoptosis: impacts on sperm function and preservation in the horse. Anim Reprod Sci. 2008;107:257-67.

[100] Martins AD, Agarwal A, Henkel R. Sperm cryopreservation. In vitro fertilization: Springer; 2019. p. 625-42.

[101] Koderle M, Aurich C, Schafer-Somi S. The influence of cryopreservation and seminal plasma on the chromatin structure of dog spermatozoa. Theriogenology. 2009;72:1215-20.

[102] Woolley DM, Richardson DW. Ultrastructural injury to human spermatozoa after freezing and thawing. J Reprod Fertil. 1978;53:389-94.

[103] Ozkavukcu S, Erdemli E, Isik A, Oztuna D, Karahuseyinoglu S. Effects of cryopreservation on sperm parameters and ultrastructural morphology of human spermatozoa. J Assist Reprod Genet. 2008;25:403-11.

[104] Bernardini A, Hozbor F, Sanchez E, Fornes MW, Alberio RH, Cesari A. Conserved ram seminal plasma proteins bind to the sperm membrane and repair cryopreservation damage. Theriogenology. 2011;76:436-47.

[105] Milardi D, Grande G, Vincenzoni F, Messana I, Pontecorvi A, De Marinis L, et al. Proteomic approach in the identification of fertility pattern in seminal plasma of fertile men. Fertil Steril. 2012;97:67-73 e1.

[106] Gonzalez-Cadavid V, Martins JAM, Moreno FB, Andrade TS, Santos ACL, Monteiro-Moreira ACO, et al. Seminal plasma proteins of adult boars and correlations with sperm parameters. Theriogenology. 2014;82:697-707.

[107] Druart X, Rickard JP, Mactier S, Kohnke PL, Kershaw-Young CM, Bathgate R, et al. Proteomic characterization and cross species comparison of mammalian seminal plasma. J Proteomics. 2013;91:13-22.

[108] Durairajanayagam D, Singh D, Agarwal A, Henkel R. Causes and consequences of sperm mitochondrial dysfunction. Andrologia. 2020:e13666.

[109] Pena AI, Barrio F, Quintela LA, Herradon PG. Effect of different glycerol treatments on frozen-thawed dog sperm longevity and acrosomal integrity. Theriogenology. 1998;50:163-74.

[110] Gungor S, Ata A, InanC ME, Kastelic JP. Effect of various antioxidants and their combinations on bull semen cryopreservation. Turk J Vet Anim Sci. 2019;43:590-5.

[111] Chai RR, Chen GW, Shi HJ, Wai-Sum O, Martin-DeLeon PA, Chen H. Prohibitin involvement in the generation of mitochondrial superoxide at complex I in human sperm. J Cell Mol Med. 2017;21:121-9.

[112] Lenzi A, Picardo M, Gandini L, Dondero F. Lipids of the sperm plasma membrane: From polyunsaturated fatty acids considered as markers of sperm function to possible scavenger therapy. Hum Reprod Update. 1996;2:246-56.

[113] Hossain MS, Johannisson A, Wallgren M, Nagy S, Siqueira AP, Rodriguez-Martinez H. Flow cytometry for the assessment of animal sperm integrity and functionality: state of the art. Asian J Androl. 2011;13:406-19.

[114] Leahy T, Gadella BM. New insights into the regulation of cholesterol efflux from the sperm membrane. Asian J Androl. 2015;17:561-7.

[115] Saez F, Ouvrier A, Drevet JR. Epididymis cholesterol homeostasis and sperm fertilizing ability. Asian J Androl. 2011;13:11-7.

[116] Argraves WS, Morales CR. Immunolocalization of cubilin, megalin, apolipoprotein J, and apolipoprotein A-I in the uterus and oviduct. Mol Reprod Dev. 2004;69:419-27.

[117] Bitterman PB, Rennard SI, Adelberg S, Crystal RG. Role of Fibronectin as a Growth-Factor for Fibroblasts. J Cell Biol. 1983;97:1925-32.

[118] Qamar AY, Fang X, Kim MJ, Cho J. Improved viability and fertility of frozen-thawed dog sperm using adipose-derived mesenchymal stem cells. Sci Rep-Uk. 2020;10:1-10.

국문초록

개에서 양막유래 줄기세포 조정배지가 동결정액의 성상에 미치는 영향 연구

페리엘 야스민 마히딘

(지도교수: 윤화영)

서울대학교 대학원

수의학과 임상수의학 전공

중간엽 줄기 세포(MSC)와 그 유도체는 항 세포사멸, 항산화, 면역 조절 및 재생 특성에 대한 임상 연구에 사용된다. 또한, 불임 치료에 유익한 것이 증명되면서 생식 의학에서의 사용이 증가하고 있다. MSC는 표적 조직이나 세포에서 생물학적 과정에 영향을 미치는 요소들을 분비할 수 있는데, 이 요소들은 세포에 의해 직접 분비되거나 유도체를 통해 매개된다

. 양막은 줄기 세포의 좋은 공급원이지만 임상 실험 특히, 생식 의학이나 인공 생식 기술에서 양막 유래 MSC를 사용하는 경우는 드물다.

본 연구는 개 정액 동결보존에서 개 양막 유래 중간엽 줄기 세포(cAMSCs) 유래 조정배지(CM)의 영향을 분석하였다. 먼저cAMSCs 확인을 위해 유세포 분석이 수행되었다. 그 후, 단백질 분석을 통해cAMSC로부터 제조된 CM에 존재하는 단백질을 확인하였다. 마지막으로 동결보존 전, CM의 최적 농도 설정을 위해 각각 0 %, 5 %, 10 %, 및 15 %의 CM이 함유된 동결 배지가 정자 샘플에 처리되었다. 처리된 정자 샘플들은 4 ° C에서 4 ~ 6 시간 보관된 후 동결융해 정자 품질관련 지표(운동성, 운동학적 지표, 생존력, 원형질막과 염색질 및 첨체의 완전성 및 미토콘드리아 활성) 분석에 사용되었다.

그 결과, 염색질 및 첨체의 완전성은 모든 농도의 CM처리군에서 유의미한 차이를 보이지 않았다. 그러나, 10%의 농도로 CM처리 시 정자의 운동성, 생존력, 미토콘드리아 활성 및 멤브레인 완전성이 유의미하게 향상된 것을 확인하였다 ($p < 0.05$). 따라서, cAMSCs 유래 CM이 10% 첨가된 동결배지는 개의 정액 동결 과정에서 품질보존에 도움을 줄 수 있다.

……

주요어: 정액, 동결, 줄기세포, 조정배지, 개

학번: 2019-29434