

## Short Communication

# Canine amniotic membrane derived mesenchymal stem cells exosomes addition in canine sperm freezing medium

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**ABSTRACT** Amniotic membrane stem cells are considered as a good alternative to embryonic stem cells, but their use in clinical studies is still not common. Here, exosomes from canine amniotic membrane mesenchymal stem cells (cAmMSC-exo) were used for dog sperm cryopreservation. Upon cryopreserved straws using cryoprotectant containing 0, 0.5, 1, or 2 µg/mL of cAmMSC-exo were thawed, motility and membrane integrity were analyzed. However, results showed no significant differences between the groups. We concluded that cAmMSC-exo with lower than 2 µg/mL have no effects on sperm cryopreservation, and further studies to get higher concentrations of cAmMSC-exo should be conducted for clinical application.

**Keywords:** amniotic membrane, dog sperm, exosomes, mesenchymal stem cells, sperm cryopreservation

## INTRODUCTION

Exosomes have been discovered in 1983 (Pan and Johnstone, 1983) and are defined as 40-100 nm extracellular vesicles originating from the fusion of multi-vesicular bodies, also referred to as late endosomes, with the plasma membrane. They are known to mediate cell-to-cell communication as they can carry proteins, nucleic acids, and lipids, providing through that cargo a paracrine effect to the recipient tissues or cells (Mitchell et al., 2019). Thus, stem cell-derived exosomes have been used as a shuttle in cell-free therapies for the treatment of different conditions such as kidney diseases (Dorronsoro and Robbins, 2013) or osteoarthritis (Zhang et al., 2019). Among

stem cell types, perinatal tissue-derived stem cells are a good alternative to embryonic stem cells as they are obtained without major ethical issues since they come from discarded fetal annexa, but the use of their secretome is still not common as there are over 30 clinical trials using perinatal tissue-derived stem cells but less than 10 trials using their derived exosomes (<https://clinicaltrials.gov/>). Among the perinatal tissues, the amniotic membrane is a good source of mesenchymal stem cells (Alviano et al., 2007) but the use of these stem cells or their derived exosomes is not as spread out as the other stem cells types, although they have the same properties and their secretome is as rich in growth and anti-apoptotic factors (An et al., 2017).

Canine sperm cryopreservation is regularly performed by veterinarians in practice and research laboratories, but due to cryoinjuries and the deleterious effects on sperm post-thaw parameters, it still faces some hurdles during this process. Cryoinjuries affect spermatozoa cells membranes due to osmotic changes and it increases sperm DNA fragmentation which leads to low post-thaw parameters and a high percentage of dead sperm. To prevent that, penetrating and non-penetrating cryo-protectants have been added in the freezing media with antioxidants such as vitamin E to protect DNA integrity and anti-apoptotic factors.

Exosomes derived from mesenchymal stem cells carry anti-oxidants, anti-apoptotic, and growth factors (Zhou et al., 2013), which makes them ideal candidates for a new cryopreservation protocol to improve post-thaw sperm parameters. Thus, the aim of this study was to evaluate the effects of canine amniotic membrane mesenchymal stem cells derived exosomes (cAmMSCs-exo) on canine sperm cryopreservation.

## MATERIEL AND METHODS

### Cell culture and exosomes extraction

Canine amniotic mesenchymal stem cells (cAmMSC) and its culture media (RPME-5) were provided from Nature-cell Co., Ltd (Seoul, Republic of Korea). cAmMSCs were cultured with the culture media up to 80% confluency at passage 3 when serum-free Dulbecco's Modified Eagle Medium was added to the cells. After 48 h, the conditioned media was retrieved and centrifuged at 2.000 g. for 30 min. Exosomes were extracted using total isolation reagent (Invitrogen™, Vilnius, Lithuania) according to the manufacturer's instructions. Exosomes were identified under transmission electron microscopy after staining with 2% uranyl acetate, and Bradford assay was conducted for protein quantification.

### Animal use and semen processing

All dogs used for the study were housed under the same conditions, in individual cages and were fed with commercial adult dry food and water daily. All experiments were conducted in accordance with 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 (ap-

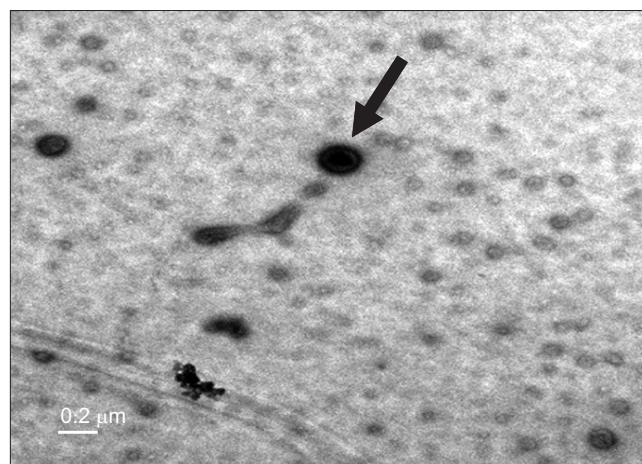
proval number: SNU-180731-2-1). Semen was obtained by digital manipulation, twice a week from five beagle dogs. Only the second fraction of the ejaculate was collected, then the samples were pooled to avoid individual variations and washed.

### Cryopreservation and thawing process

Semen was diluted with Tris-extender (1:1, v/v) and centrifuged at 700 g for 1 min. The supernatant was collected and centrifuged at 400 g for 5 min and only the pellet was re-suspended with TRIS buffer to adjust the concentration of  $100 \times 10^6$  sperm cells/mL when Tris-egg yolk-glycerol extender with or without exosome proteins (0.5, 1 or 2  $\mu\text{g}/\text{mL}$ ) will be added. The samples were loaded in 0.5 mL straws (Minitube, Tiefenbach, Germany) by multistep protocol (Setyawan et al., 2015), sealed, kept at 4°C during 1 h for equilibration, and were frozen horizontally 10 cm above liquid nitrogen (LN2) for 15 min before being transferred to LN2 tanks (-196°C). A week after, straws were thawed in a 37°C water bath for 30 s, then 14%, 19%, 27%, and 40% TRIS extender was added every 30 s.

### Sperm parameters evaluation

Post-thaw parameters were evaluated with a computer-assisted sperm analysis system (CASA) (FSA2011 premium edition version 2011, Medical Supply Co., Ltd., Korea) and a hypo-osmotic swelling test (HOST). Briefly, 100  $\mu\text{L}$  of sperm were added to 1 mL of a hypo-osmotic solution (150-155 mOsm) and incubated at 37°C for 30 min. A drop



**Fig. 1.** Transmission electron microscopy image of exosomes (arrow) extracted from canine amniotic membrane stem cells conditioned media.

of HOST solution with sperm was then put on a warm slide, covered and at least 100 sperm cells were counted under a microscope. All data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using GraphPad Prism 5.0 (Graphpad, San Diego, CA, USA). Values are presented as means ± standard error of the mean, and *p* values less than 0.05 were considered to be statistically significant.

## RESULTS AND DISCUSSION

Exosomes were extracted from 40 mL of conditioned media; 10 mL were used for protein quantification, to obtain a yield of  $35.7 \pm 6.5$  µg of exosome proteins, and the rest (30 mL) was used for sperm cryopreservation or electron microscopy. Exosomes were identified under transmission electron microscopy with a size ranging from 50 to 200 nm (Fig. 1). Upon sperm straws were thawed in 37°C for 1 min, CASA or HOST were used right away for motility and velocity parameters or membrane integrity check respectively. There were no significant differences among the groups (0, 0.5, 1 or 2 µg/mL) in post-thaw sperm parameters including motility, vitality, average curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), linearity (LIN, average ratio of VSL/VCL), straightness (STR, average value of the ratio VSL/VAP), amplitude of the lateral movement of the head (ALH) (Table 1), and they were on the reference ranges (Park et al., 2018). As for HOST results, although the statistical analysis showed no significant differences, the group treated with 2 µg/mL of exosome proteins showed the highest percentage of intact membrane  $61.3 \pm 7.4\%$  compared with the control group, 0, 0.5, and 1 µg/mL of exosome proteins treatment groups with  $51.3 \pm 4.3$ ,  $56.5 \pm 5.7$  and  $55.4 \pm 2.9\%$  respectively (Table 2).

The use of exosomes in sperm cryopreservation is still

new and up to date, only two studies have been reported (Mokarizadeh et al., 2013; Qamar et al., 2019). Paracrine secretions of mesenchymal stem cells are responsible for their therapeutical effects and stem cell-derived exosomes are known to carry anti-apoptotic and growth factors, chemokines, cytokines, mRNA, and miRNA (Zhou et al., 2013). We hypothesized that these anti-apoptotic and growth factors carried in exosomes could have beneficial effects on canine post-thaw sperm. Exosomal concentrations used in studies vary a lot as some experiments are successfully carried with low concentrations and others with high concentrations. Taking into consideration that exosomes can be obtained from different types of cells, each type of cell probably has different yields of exosomes with different contents. As for high concentrations of exosomes, experimental dosages vary from 50 µg/mL to 500 µg/mouse (Mokarizadeh et al., 2013; An et al., 2017; Qamar et al., 2019), and for cryopreservation, exosomes have been used at concentrations ranging from 25 to 100 µg of exosome proteins with beneficial obtained at the concentrations of 50 and 100 µg (Mokarizadeh et al., 2013; Qamar et al., 2019) with the concentration of 50 µg/mL having protective and regenerative effects. For low concentrations, 30 µg/mL of amniotic fluid stem cells derived exosomes have anti-apoptotic effects on granulosa cells exposed to chemotherapy, and the same effects were

**Table 2.** Hypo-osmotic swelling test results of post-thaw dog sperm using different concentrations of exosomes extracted from canine amniotic membrane stem cells conditioned media

Concentration (µg/mL)	Intact membrane (mean ± SEM)
0	51.3 ± 4.3
0.5	56.5 ± 5.7
1	55.4 ± 2.9
2	61.3 ± 7.4

All results show means ± SEM.

**Table 1.** Post-thaw sperm parameters using different concentrations of exosomes extracted from canine amniotic membrane mesenchymal stem cells conditioned media

Concentration (µg/mL)	Motility (%)	Vitality (%)	VCL (µm/sec)	VSL (µm/sec)	VAP (µm/sec)	LIN (%)	STR (%)	ALH (µm)
0	43.4 ± 2.1	59.8 ± 1.7	50.8 ± 4.1	14.3 ± 1.4	26.7 ± 2.0	24.3 ± 1.2	53.9 ± 2.2	2.5 ± 0.2
0.5	43.4 ± 1.1	61.1 ± 3.2	46.0 ± 5.0	12.0 ± 1.2	23.9 ± 1.6	23.9 ± 2.5	50.2 ± 1.9	2.4 ± 0.2
1	42.1 ± 2.5	61.6 ± 3.4	41.4 ± 5.5	13.0 ± 1.3	22.5 ± 2.3	26.4 ± 1.0	58.0 ± 1.9	2.1 ± 0.2
2	40.7 ± 2.3	62.4 ± 5.2	43.3 ± 4.0	12.4 ± 0.6	22.4 ± 1.6	27.4 ± 4.7	56.1 ± 2.0	2.2 ± 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 the lateral movement of the head. All results show means ± SEM.

obtained with 5 µg/mL (Xiao et al., 2016). Significant results were also obtained with low concentrations varying from 0.05 µg/mL to 0.4 µg/mouse of exosomes (Tan et al., 2014; Venugopal et al., 2017). Thus, we aimed to test the effects of low concentrations of cAmMSC-exo in our study, to give more insights about the effects of exosomes at low dosages. However, our results show that cAmMSC-exo have no effect on canine post-thaw sperm parameters.

The absence of beneficial effects of cAmMSC-exo could be explained by the cell type and thus the factors they secreted, the possible lack of exosome uptake from spermatozoa cells or by eventual cryo-damages induced on the exosomes, as they can also be affected by the cryopreservation process, resulting in a reduced biological activity (Bosch et al., 2016). Interestingly, exosomes can act on the same tissue differently depending on their concentration and the cell passaging (Venugopal et al., 2017). Furthermore, during the preliminary study, cAmMSC and adipose stem cells were cultured in the same conditions, with the same media but their average exosome concentrations of conditioned media were different (173 µg/mL for cAmMSC but 615.1 µg/mL for adipose stem cells exosomes). Thus, stem cells origin, cell passage, and environmental factors could influence the exosomal content, yield, and bioactivity (Salomon et al., 2013; Ban et al., 2015; Patel et al., 2016). Clinical studies using amniotic membrane stem cell-derived exosomes are still limited by the exosomal yield and quantification methods, but also by the determination of the right therapeutic dosage (Mehdiani et al., 2015).

## CONCLUSION

In conclusion, this is the first study to evaluate the effects of cAmMSC-exo on sperm cryopreservation. Our results showed that concentrations lower than 2 µg/mL of exosomes have neither harmful nor beneficial effects on canine cryopreservation. These findings give us more insights into the ranges of concentrations at which cAmMSC-exo might be effective. Further studies about cAmMSC-exo extraction methods are needed prior to its effects on sperm cryopreservation for clinical application.

## CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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