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PROSPECTS OF CULTURE AND CRYOPRESERVATION OF CANINE PREANTRAL FOLLICLES

UNIVERSITY OF VETERINARY MEDICINE, BUDAPEST

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1. Abstract

Abstract in English

Ovary of the mammals contains high numbers of preantral follicles. These follicles are suitable for in vitro culture, can be cryopreserved, and provides a large repository of oocytes. Following in vitro maturation, these oocytes can be fertilized. The procedure makes the opportunity to preserve fertility in animals with unique genetics or in women before anti-cancer treatment. However, in vitro culture protocols are not standardized, and the basal media and supplements, and preservation methods, differ among laboratories and species as well. Our study aimed to analyze the characteristics of in vitro growth of canine follicles. Furthermore, we aimed to investigate whether the open-pulled straw vitrification or the cryotube cryopreservation method is more suitable for the long-term storage of the samples.

Canine preantral follicles were isolated from ovaries after ovariohysterectomy and treated with collagenase. After morphological selection, follicles were divided into three groups: fresh, OPS, and CT. Fresh follicles were put into a culture medium and cultured for 14 days in 20 ul droplets of modified culture medium (Advanced-MEM + 5% FBS + 100 mIU/ml eCG), covered by mineral oil in a CO2 incubator. Half of the media was exchanged to fresh on every other day. Morphological and growth assessments were carried out on the same days. OPS follicles were vitrified with the open-pulled straw method, and CT follicles were cryopreserved with the cryotube method. Samples were stored in liquid nitrogen for one week. Thawing was carried out with a decreasing concentration of sucrose. The frozen-thawed follicles were put into a culture medium and cultured with the same protocol as that of fresh ones. Besides the assessment of growth

assessment, the rate of live/dead cells was measured in each group with the calcein-AM assay.

The growth of the follicles was continuous (perimeters were 2-fold greater on day5 than that on day0) in each group. The rate of live/dead cells in the follicles was not significant between fresh and OPS-vitrified samples. However, we found differences between fresh vs. CT ($p<0.001$), and OPS vs.CT ($p<0.001$) follicles (Kruskal-Wallis-test).

Our data shows that our culture medium is suitable for culturing canine preantral follicles in vitro. Furthermore, the follicles can be vitrified with the OPS method, which has no detrimental effect on the live cell rate of follicles.

Abstract in Hungarian

Az emlősök petefészke nagyszámú preantrális tüszőt tartalmaz. Ezek a tüszők alkalmasak in vitro tenyésztésre, fagyasztva tárolhatók, és nagy mennyiségű petesejtet biztosítanak. Az in vitro maturációt követően pedig lehetőség van az in vitro fertilizásióra. Az eljárás lehetőséget ad a termékenység megőrzésére egyedi genetikájú állatokban vagy nőknél rákellenes kezelés előtt. Az in vitro tenyésztési protokollok azonban jelenleg nem egységesek, és a mediumok, valamint a krioprezerváció módja laboratóriumonként és fajonként is eltérőek. Vizsgálatunk célja egy kutya preantrális tüszők számára alkalmas tenyésztési protokoll kialakítása volt. Továbbá megvizsgáltuk, hogy az OPS vitrifikáció és a kriocsöves fagyasztás milyen hatással van a minták olvasztás utáni minőségére.

A preantrális tüszőket ovariohiszterectomiát követően nyertük ki a petefészkekből, és kollagenázzal izoláltuk őket. Morfológiai szelekció után a tüszőket három csoportba osztottuk: friss, OPS (vitrifikált) és CT (kriocsöves fagyasztás). A friss tüszőket hormonal kiegészített tápfolyadékba (Advanced-MEM + 5% FBS + 100 mIU/ml eCG) helyeztük és 14 napig in vitro tenyésztettük, 20 µl-es cseppekben olajjal lefedve, CO2 inkubátorban. A táptalajok felét minden második napon frissre cseréltük. A morfológiai és növekedési paraméterek rögzítését ugyanazokon a napokon végeztük el. Az OPS csoport tüszőit vitrifikáltuk, a CT tüszőket pedig a cryotube módszerrel fagyasztottuk. A mintákat egy hétig folyékony nitrogénben tároltuk. A felolvasztást mkövetően a tüszőket táptalajba helyeztük, és a friss tüszőkkel azonos protokoll szerint tenyésztettük. A növekedés értékelésén kívül az élő/elhalt sejtek arányát minden csoportban calcein-AM festési módszerrel mértük.

A tüszők növekedése minden csoportban folyamatos volt (az 5. napon a

tüszők kerülete átlagosan 2-szer nagyobb voltak, mint a 0. napon). A tüszőkben az élő/elhaló sejtek aránya nem különbözött a friss és az OPS-vitrifikált minták között. Azonban szignifikánsan alacsonyabb volt ez az érték a CT tüszőkben, mind a friss ($p<0.001$), mind az OPS ($p<0.001$) tüszőkhöz képest (Kruskal-Wallis-teszt). Adataink azt mutatják, hogy az általunk kialakított rendszer alkalmas a kutyák preantralis tüszőinek in vitro tenyésztésére. Továbbá a tüszők vitrifikálhatók az OPS módszerrel, ami nincs káros hatással a tüszők élő sejtek arányára.

2. **List of abbreviations**

ART: assisted reproductive technologies

FSH: Follicle-stimulating hormone

r-hCG: Recombinant Human Chronic Gonadotropin

rLH: Recombinant Luteinizing Hormone

FBS: Fetal Bovine Serum

PBS: phosphate buffered saline

MEM: Minimum Essential Medium

HM: Holding Medium

SM: Sucrose Medium

DMSO: Dimethyl sulfoxide

OPS: Open Pulled Straw

3. Introduction/ Literature Review

The primary function of the female reproductive organ is to release the mature oocytes for fertilization which leads to the successful survival of the species. The number of follicles is already set at birth, and once the animal hits puberty, folliculogenesis takes place in the ovaries. A small cohort of primordial follicles is recruited and undergoes a process of maturation and development. They become primary, secondary (pre-antral), and antral follicles until they reach the preovulatory stage (McGee& Hsueh, 2000). It is widely accepted that the mammalian ovaries usually reserve thousands to millions of follicles, with preantral follicles making up around 90 % of them. However, the great majority of the follicles end up experiencing follicular death called atresia during the process, while only one or two are successfully ovulated at the end, depending on the species (Figueiredo et al., 2018).

Utilizing the preantral follicles could multiply the number of oocytes, thus enhancing the chance of producing more offspring. This potentially plays a significant role in managing the endangered species whose individual contribution to the genetic pool is invaluable. After the animal's death, oocytes can be saved from the ovaries and cryopreserved, which is a useful technique to maximize an individual's genetic contribution to the species even in later times. Before applying the techniques to endangered animals, it is essential to establish a model by using relatives (Pukzhenthi et al., 2006). For instance, Crosier et al. produced the first cheetah cabs with thawed semen by using the method developed with domestic cat models. Establishing models for other species is essential for wildlife conservation. Therefore, in our research, domestic dogs were selected to be examined.

Another advance in using domestic dogs is that there is an increasing interest in

them as experimental animals. Because there are numerous distinct benefits over other often-employed laboratory animals, dogs are being recognized more and more as suitable models for a variety of biomedical research areas. For example, dogs live with their owners and typically receive quality care, including proper healthcare. The similar condition of life to that of humans, as well as their genetic diversity, let them have the common clinical signs of comparable diseases, especially for hereditary diseases (Tsai et al., 2007; Switonski, 2014). Moreover, they are highly sought-after models for cancer research because they tend to develop the same varieties of cancers as humans (Rowell et al., 2011; Davis et al., 2014). Moreover, domestic dogs can be a good translational model to expand the field of drug uses, find the genetic target of the drug including the causative genes for certain diseases, and new drug targets, pharmacokinetics, and dynamics in the human biomedical field (Fleischer et al., 2008; Momozawa 2019).

However, there are some difficulties in using bitches' follicles. The efficiency of in vitro maturation for bitch oocytes produced from antral follicles is very poor, so the use of reproductive techniques is not frequent in dogs compared to other mammals. This could be explained by the fact that the female dog's developmental biology and reproductive physiology are different from other animals. For instance, oocytes complete their meiotic maturation in the oviduct after being released at immature diploid stage. As a result, the follicles spend a lot of time in the reproductive tract before implantation. Because of these unique characteristics, canine species are less well understood than many other domestic mammalian species in terms of the actual situation and mechanisms of early development, such as oocyte maturation, fertilization, and subsequent embryogenesis. (Abe et al., 2008) In spite of the differences, preantral follicles

would serve as an alternative follicular source because they are more numerous and present throughout the animal's reproductive life (Reynaud et al., 2006; Lunardon et al., 2015).

Also, it is challenging to create an in vitro culture environment that promotes the development of the preantral follicles in general and a culture medium loaded with an energy source and amino acids with other materials are required to stimulate the growth. Among these, bovine serum albumin, insulin-transferrin-selenium (ITS), and growth hormones, such as FSH, are the notable compounds (Serafim et al., 2010). Furthermore, one of the unique characteristics of the canine is its dark-colored cytoplasm caused by a significant amount of lipid content, which implies that it is difficult to freeze. (Amstislavsky et al., 2019) Thus, it is crucial to investigate the different vitrification methods to avoid as many cryo injuries as possible.

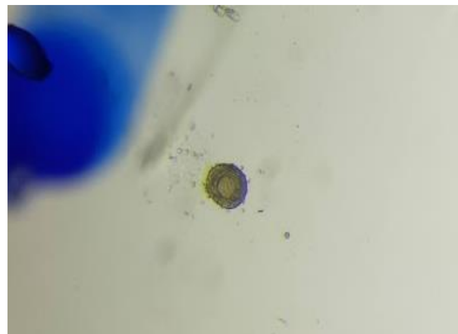

Fig. 3-1: A mouse follicle

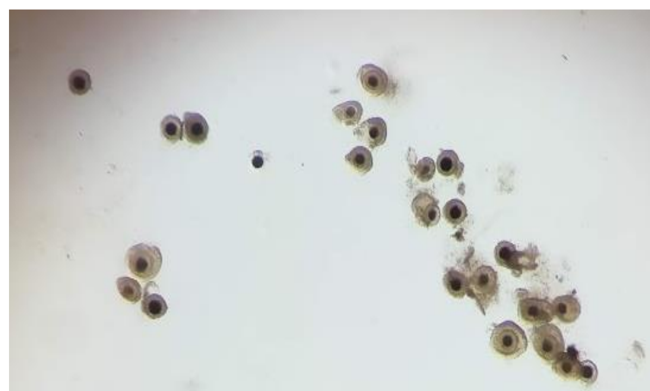

Fig. 3-2: Dog follicles appearing black due to high amount of lipids

Therefore, this study aims to reveal the characteristics of in vitro growth of the canine preantral follicles. Additionally, the research sought to determine which method of long-term storage – open-pulled straw vitrification or cryotube preservation – was more effective and less invasive.

Specific questions are the following:

1. Is our media suitable for in vitro follicle growth?
2. Which is the best method to cryopreserve and how does it affect the survival rate of the follicles after thawing?
3. Are the growth rate differences between the fresh follicles and thawed ones?

4. Materials and Methods

Animals

Ovariohysterectomies on female dogs were performed at the small animal clinic of the university of veterinary medicine Budapest. After the ovaries are collected, they are immediately placed in a sterile tube that is filled with phosphate buffered saline (PBS) at room temperature. They then are delivered to the lab on the same property in order to isolate the preantral follicles from them on the same day.

Isolation of Preantral follicles

The ovaries were cleaned and trimmed off of excess fat in a solution consisting of PBS + 10% FBS (Sigma-Aldrich, Canada). Using a surgical blade, they were sliced into 3mm thin pieces and moved into collagenase bath at 37℃ for 90 minutes. The preantral follicles were mechanically isolated from the enzymatically digested pieces with a 27G needle under a microscope.

Fig. 4-1

Vitrification and thawing

Cryotube and OPS, two distinct vitrification protocols, have been used to cryopreserve the morphologically healthy, undamaged preantral follicles.

Cryotube vitrification was performed as follows (Gupta et al., 2021):

1. Follicles were incubated in vitrification solution (VS1) comprising of a base medium (Medium 199 (Sigma-Aldrich, Canada) +10% FBS) + 10% Ethylene glycol + 10% DMSO + 0.3 M sucrose.
2. Samples were then transferred into VS2 (base medium+ 25% EG + 25% DMSO and 0.3 M sucrose) and allowed to settle for 45 seconds.
3. After settling in VS2, follicles were deposited into cryotubes (18 follicles per container) which carry 200μL VS2. The containers were submerged in liquid nitrogen and stored for later use.
4. To thaw, they were initially placed in a water bath at 37℃ for one minute.
5. The follicles were placed and thawed in three solutions of successively diluted sucrose concentration (base medium + 0.3M, 0.15M, or 0.075M sucrose, respectively). Each step lasted for 5 minutes

OPS cryopreservation was carried out as follows (Vajta et al., 1998) :

1. Preantral follicles were rinsed in a holding medium (HM) (Medium 199+ 20% FBS) before being incubated in an equilibration solution (ES) (HM+ 7.5% Ethylene glycol + 7.5% DMSO) for 3 minutes.
2. Subsequently, follicles were incubated and placed in a vitrification solution (VS) (Medium 199 + 20% FBS + 1M sucrose + 16.5% EG + 16.5% DMSO) for 30 seconds, then hoovered up into open pulled straws (10 follicles/ straw).

3. Each straw was dipped in liquid nitrogen for 30 seconds and stored in a holding (0.5mL) straw that was closed with an ultrasonic seal and kept in liquid nitrogen storage.
4. In order to thaw, OPS were taken out of the holding straw that was in liquid nitrogen, and follicles inside were immediately released and immersed in HM+50% SM (Medium 199 + 1M sucrose + 20% FBS) for 5 minutes.
5. The process was repeated 3 more times with decreased concentration of SM medium. (50% SM, 25%,0%, respectively)

Viability inspection

To detect follicles' viability, they were treated in a 75μl droplet obtained from a staining solution (PBS + 2 μM ethidium homodimer 1 + 4 μM calcein AM) for 30 minutes (LIVE / DEAD Viability/cytotoxicity Kit, Invitrogen). The follicles were then fixed in a 2% PFA solution and analyzed under a fluorescent microscope (Olympus SZX7).

Staining was performed on fresh samples shortly after collection or thawed ones (OPS and cryotubes). Viability was measured by assessing the proportion of living to dead cells (live cell rate). Image J software was used to analyze the images (NIH, Bethesda MD, USA).

Post-thawing in vitro growth

The ability of both fresh and thawed preantral follicles to grow was evaluated by in vitro cultures. Each follicle was put in a 20 μL droplet of culture medium, which was fully covered with mineral oil. The culture medium consists of Advanced-MEM (Gibco, Grand Island, NY) + 5% FBS+ 1% ITS (Gibco, Grand

Island, NY) + 0.5% penicillin-streptomycin (Lonza, Belgium) + 1% L-glutamine (Gibco, Grand Island, NY) and 0.2% of FSH (R&D Systems, MN). Half of the medium was exchanged every other day with the newly prepared medium, while morphological and growth checks of the follicles were carried out. After day 7 in the incubator, either r-hCG or r-LH was added at 1.4% concentration.

Statistical analysis

R (R Core Team) version 3.6.2 was used for data analysis (2019). *R: The Language and Environment for Statistical Computing*. R Foundation for Statistical Computing. https://www.R-project.org/)

Follicle viability in each treatment was analyzed with Kruskal-Wallis rank sum test, with post hoc pairwise comparison using Wilcoxon rank sum test (p-adjustment method: Benjammini&Hochberg). Data on oocyte viability and ovulation rate were analyzed with Fisher’s exact test.

5. Results

Post-thawing growth and cryopreserved follicles

Measurement of the diameter of the follicles were done on day 0, day 2 and day5 and the mean of the diameter was calculated.

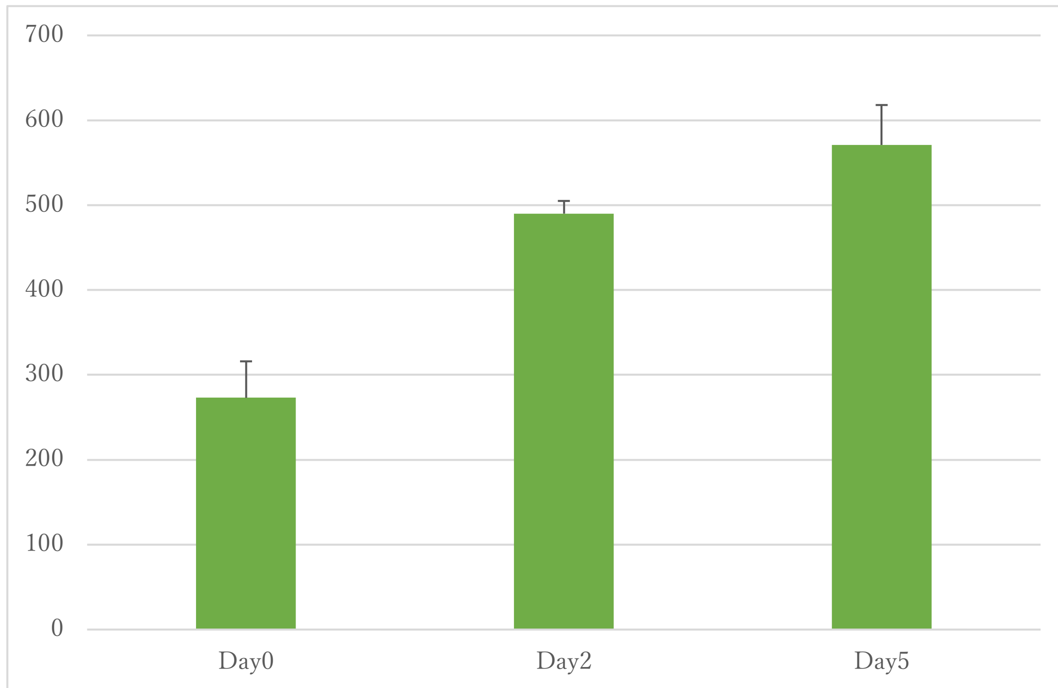

Fig. 5-1: growth of the diameter (µm) of the follicles

The mean diameter of the day 0 was 273 (±43)µm, Day 2 was 49 (±15) µm and Day 5 turned out 571 (±47) µm. They showed the continuous growth and reached about 2-fold of the original size in diameter by Day 5.

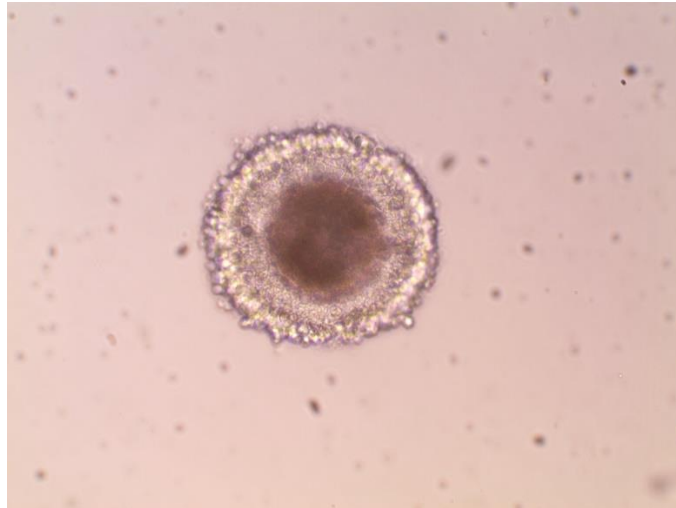

Fig. 5-2: Day 0 of a follicl (magnification 20X)

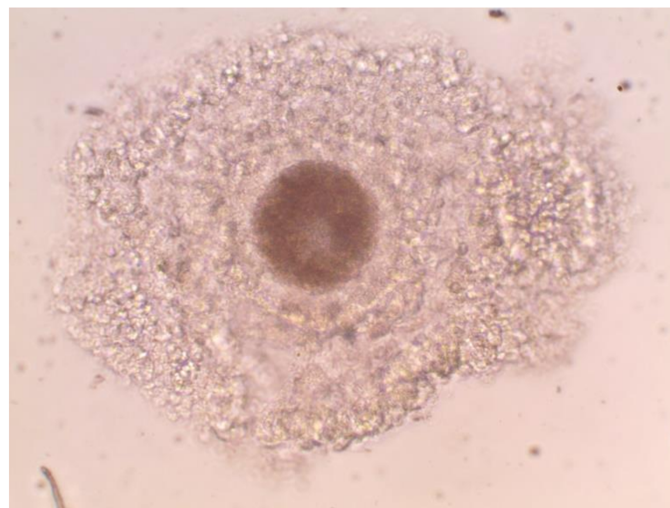

Fig. 5-3: Day 2 (magnification 20X)

Live cell rate

Firstly, the viability of the cryopreserved follicles was compared to the fresh control group. The intact part of calcein-AM-ethidium homodimer-treated follicles appears green under a fluorescent microscope, whereas it turns red if dead. The area of both colors is measured and calculated to show in percentage. The number of samples for the control (Fresh), OPS, and cryotube was 59, 50, and 87, respectively.

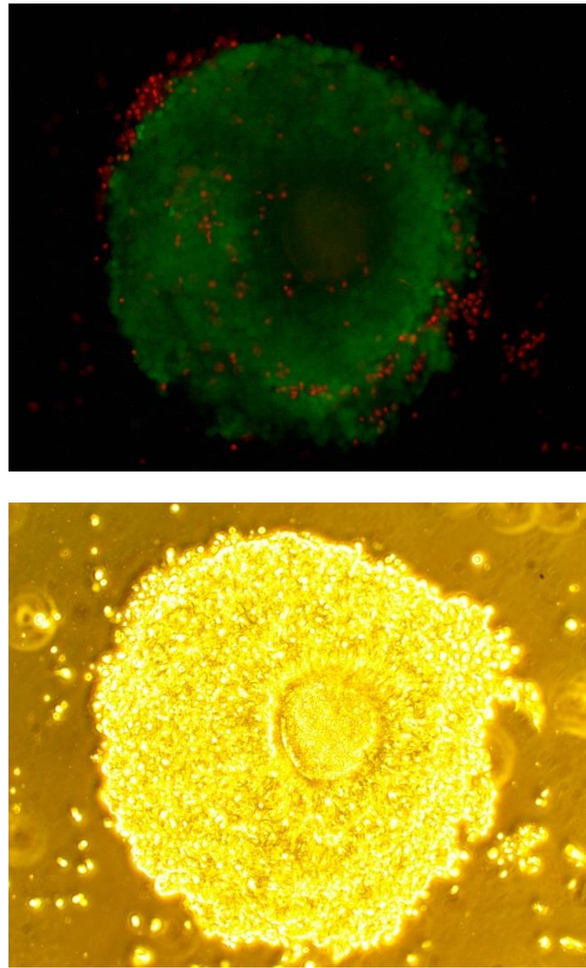

Fig. 5-4: a follicle after calcein AM and ethidium homodimer staining.
Upper image: under a fluorescent microscope
Lower: under dark background

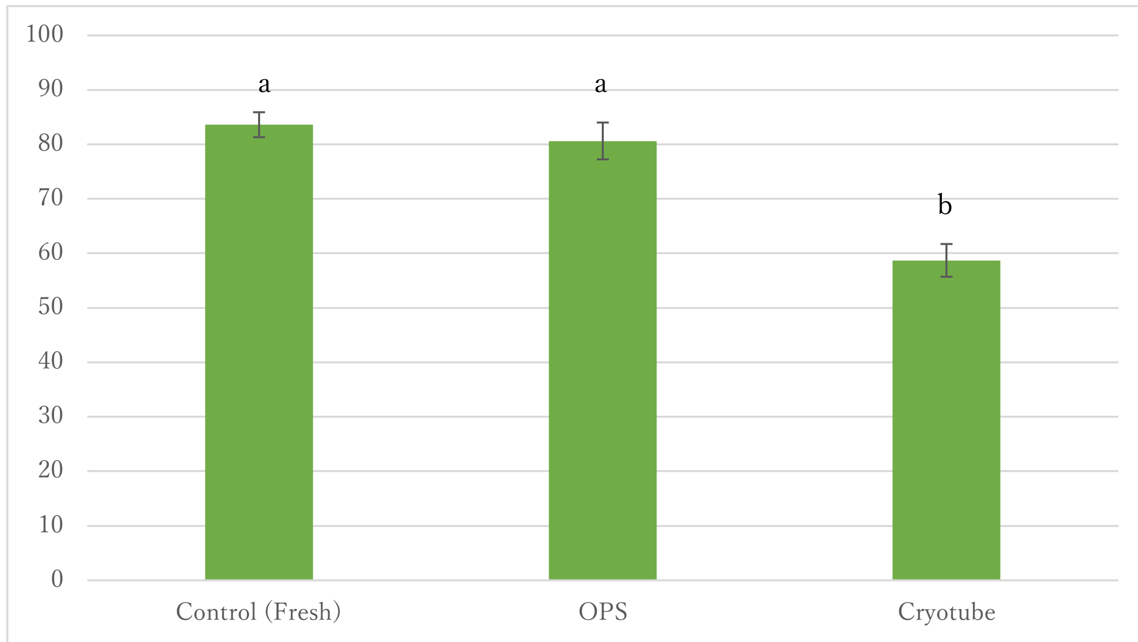

Fig. 5-5: The survival rate of mean (±SD) of each sample; a,b ($p<0.001$), Krsuskal-Wallis test

As shown in Fig.5-2, the mean value for the survival rate of the control group was 83.61 (±17.47) % and the 95% confidence interval (CI) was 72.46- 94.76. There was no difference between the fresh group and OPS whose average was 80.62 (±23.65)% (NS). However, the result of the cryotube groups marked the lowest mean survival rate with 58.71(±27.84) %. This was significantly lower compared to other 2 groups. The CI for the OPS group was 65.45-95.80 and the cryotube 41.11-76.31, respectively.

(p value of the fresh and OPS: non significant; fresh and cryotube: $p<0.0001$; OPS and cyotube: $p<0.0001$)

Oocytes viability after cryopreservation

Treatment	Dead	Live	The total number of samples (n)	Live %
Control (Fresh)	32	57	89	64
OPS	12	46	58	79.30
Cryotube	60	38	98	38.80

Fig. 5-6: the viability of fresh an frozen-thawed oocyes; Control/OPS, Cryotube ($p<0.01$), Fisher's exact test

The oocyte viability test has shown the result above with a significant viability rate difference between the fresh and cryotube samples ($p<0.001$), while it was non-significant between the fresh and control.

The ovulation rate of each group

Treatment	ovulated	non ovulated	%
Control (Fresh)	21	278	7%
OPS	2	118	1.70%
Cryotube	25	255	8.90%

Fig. 5-7: Effect of different cryopreservation on ovulation rate; Control/ Cryotube, OPS ($p<0.01$) Fisher's exact test

While the cryotube group and control did not have a significant difference in ovulation rate, the OPS group showed a significantly lower rate compared to both fresh ($p<0.05$) and cryotube ($p<0.001$) by using Fisher's exact test.

6. Discussions

In this study, the effect of different cryopreservation methods on the live cells was tested by the viability investigation with calcein AM and ethidium homodimer staining. The test indicated that there is no significant difference prior to and after the OPS method. OPS were significantly more effective in saving the viability of the follicles than the cryotubes ($p<0.001$). The difference between the two methods is explained by the fact that fast cooling and warming are crucial to prevent crystallization in the cells and avoid cryoinjuries. In research from Gao and Crsiter (2000), they found out that the cells are most vulnerable between -15℃ to -60 ℃, unlike the common theory that the difficulty with cryopreserving cells is their ability to withstand storage at low temperatures. Cryotubes, which are much bigger and contained a greater amount of liquid (200μL) compared to Open pulled straws, took more time to freeze and the temperature didn't drop to -196℃ as fast as the OPS. The cryotube samples also took a longer time to warm up and put the follicles at risk of re-crystallizing during the temperature rise. Crystallization during warming is particularly a big problem when cryoprotectants did not reacha high enough concentration and it could be avoided by very rapid warming up speed according to Pegg and Diaper (1990). Furthermore, an osmotic imbalance typically occurs while adding or removing the cryoprotectants. The cells swell if the concentration the of outer environment is too low, or they shrink if the environment is too high. Therefore, a long time in vitrification solution before being placed in liquid nitrogen could hurt the cellular integrity. In our case, the cryotube method required the follicles to be settled in the vitrification solution for a longer time, namely 45 seconds, than the OPS method's 30 seconds.

In the scientific literature, there are only a few results on canine oocyte

vitrification. Abdel-Ghani and Suzuki (2014) vitrified canine cumulus-oocyte-complexes (COC), partly denuded, and completely denudes oocytes, using the Cryotop method. After thawing, they analyzed the viability rate which was 52.8%, 43.9%, and 33.9%, respectively. Boutelle et al. (2011) vitrified Mexican grey wolf and canine oocyte, while establishing a gene bank. The post-warming viability of the oocytes was 57.1% and 61%, respectively. Although we vitrified preantral follicles and not COCs, our viability rate was higher in the OPS group (79.3%). Cryotop and OPS are very similar techniques to the point that both are open systems, require the same vitrification media and apply minimum volume cryopreservation. In the above-mentioned studies, authors analyzed the viability of oocytes based on membrane integrity (propidium-iodide staining). In our study, we classify the oocytes based on a functional test (calcein-AM), which shows if a cell has active metabolism. Considering this fact, our viability check system seems more reliable than that of previous studies in the field. Moreover, to our knowledge, this is the first result comparing different cryopreservation protocols on canine isolated preantral follicles.

As for the oocyte viability rate, the result was aligned with the lice cell proportion. Cryotubes were significantly lower in comparison to both control and OPS ($p<0.001$). In fact, the OPS showed even higher (79.30%) viability rate than the fresh group (64%), which was statistically not significant.

In addition to the survival rate and viability oocyte rate, our study found out that the medium used in this experiment was suitable for canine preantral follicles to grow. Research on in vitro culture of dog preantral follicles has increased during the past decade. However, most of the data are from the research team of Songsasen and collagues (Nagashima et al., 2010; Sangsaen et al., 2011;Thongkittidilok et al.,

2020). Since the ability to culture and grow preantral follicles, and obtain oocytes is still a challenge, data on these topics are still limited. The average of the diameter of the follicles became at least twice bigger than Day 0 average. The growth rate was very similar to that of mice (Bordás, 2021). In our culture system, we could reach in vitro ovulation both fresh and cryopreserved follicles, after adding LH to the culture medium. However, the ovulation rate showed differences from one another. While the control and cryotube had no significant difference, the OPS paired with cryotubes, or control had significant differences. Since our study focused on the prospects of in vitro culture and cryopreservation of canine PAFs, we did not mature the oocytes. Oocyte maturation of the dog is quite special and highly different from other domestic mammals which makes in vitro maturation quite a challenge (Van Soom et al., 2014) The bitch ovulates an immature oocyte [germinal vesicle (GV)] that corresponds to the prophase of the first meiotic division. The germinal vesicle breakdown (GVBD) occurs in the oviduct under high progesterone concentrations at 48–96 h after ovulation. To date, the best results lie within 20 and 36.5% MII rate – however, these data are obtained from in vivo ovulated oocytes. (Rodriguez, 2021)

In the future, the plan of our research team is to improve our culture system to make it suitable for matured oocytes.

7. Summary/ conclusion

In conclusion, the research was successful in comparing the two different methods of vitrification of canine preantral follicles as well as the suitability of the present cultural medium for them to grow in vitro. Examining the possibility of maturation of the oocyte and subsequent fertilization in vitro requires further research in the future. In our case, we will focus on improving our culture system and making it suitable for matured oocytes.

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