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Utilization of Oocytes Collected from Preserved Ovarian for *In Vitro* Production of Cat Embryos

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Abstract

Preservation of ovarian tissue from severely injured or dead valuable animals has the potential to preserve female germ cells of animals. The ability to maturize and fertilize the oocytes from preserved ovary of endangered species will allow us to sustain genetic and global biodiversities. The aims of this study were to investigate the viability of oocytes collected from the preserved ovary and its potential utilization for the production of cat embryos followed by *in vitro* maturation and fertilization. Ovary was preserved immediately in phosphate buffer saline (PBS) at 4°C for 24 or 48 hours. The quality and viability of oocytes after the maturation process were identified microscopically using aceto-orcein staining. Biological function of the oocytes was evaluated by using *in vitro* culture technique for the maturation and fertilization rate in CR1aa medium culture. The results showed that the percentage of oocytes collected from preserved ovary for 24 and 48 hours that remained at the stage of metaphase-II were 29.4% and 21.9% respectively. Fertilization rates produced in the IVF using oocytes collected from ovary preserved for 24 or 48 hours were significantly lower (30%) than that of unpreserved control (36.7%). In conclusion, female germ cells of cat ovary preserved at 4°C in PBS for 2 days were still viable for *in vitro* fertilization and thus can be utilized for *in vitro* production of cat embryos. Information obtained can be used as a basis of knowledge of using a combination of physiological reagent and cold-based preservation technique in modern reproductive technology for animals.

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INTRODUCTION

Reproduction is essential for the survival of a species because each individual in a species has a limited life span and only by doing reproduction it maintains its existence (Eriani *et al*., 2013). Unfortunately, reproduction in some species, especially wild animals, has been disrupted by some obstacles resulted from massive natural disasters or by human intervention. These obstacles have significantly diminished animal populations and even risked them in an endangered situation.

One species that has been considered as endangered animal is the family of Felidae covering both big felids (Panthera) and small felids (Felis). All felid species, with the exception of the domestic cat, are listed on the Red list of the International Union for Conservation of Nature (IUCN) for endangered species (IUCN Red List). Pope (2000) stated that more than 36 wild cats were classified as endangered species. Several wild cat species that considered to be in the risk of extinction in Indonesia are *Panthera tigris* sumatrensis, *Felis mormorata*, *Felis temminckii*, *Pardofelis nebulosa*, *Prionailurus planiceps*, *Felis bengalensis* (Leuser Management Unit, 2002). Sumatran tiger is the only endangered wild cats categorized into critically endangered (CR) species that being protected by the government..

Data from the Directorate General of Forestry and Natural Conservation, Department of Forestry of the Republic of Indonesia (2007) showed that population of Sumatran tiger had reduced from 1000 in 1970 to 660 in 1987 and to 127 in 2007. The tiger was listed in the Appendix I, the category of animals that are forbidden to trade under the CITES (Convention on International Trade in Endangered Species) in 1975.

The death of endangered animals in remote areas located far from conservation laboratory may cause the sources of genetic materials cannot be saved. A cryopreservation protocol has been developed for preserving cat ovarian tissues with minimum effect on the survival of the follicles (Giaretta *et al*., 2013). However, feline oocytes seem to express a higher sensitivity towards cryopreservation protocols (Fassbender *et al*., 2007). Shorter preservation procedures using physiological solutions such as PBS stored at 4-5°C can provide an alternative way to save the source of germ cells such as ovary and testes. Reduced viability and fertility of germinal cells collected from the preserved reproductive organs are important issues need to be addressed. The availability of mature oocytes in the preserved gonads is the limiting factor of assisted reproductive technology (Jewgenow & Paris, 2006).This study was performed to investigate the adaptive capability of ovary preserved in PBS at 4°C in maintaining oocytes' viability. It is expected that this treatment could minimize oocyte damages caused by constrain of distance and time. The information obtained can be used as a basis of knowledge of using a combination of physiological reagent and cold-based preservation technique in modern reproductive technology for animals, especially in cats.

METHODS

Ovary preservation and oocyte collection

Ovarian samples were collected from 12 female cats aged 2-3 years old and weighed 2.0-3.5 kg by ovariectomy, put in PBS, and stored in the refrigerator in 4°C for 24 and 48 hours. Collection and determination of oocytes quality were done according to protocols described by Eriani *et al*. (2008).

Histological examination

Ovaries were preserved for 0, 24 and 48 hours. Then, they were fixed in Bouin's solution for 24 hours and stored in 70% ethanol until the next process. After dehydration process in ethanol solution grades ascend from 80% to 100%, ovaries were cleared in xylol and embedded in paraffin. The paraffin-embedded ovaries were cut using a rotary microtome with a thickness of 5 μm, mounted on glass objects and stored in a 37°C incubator for 24 hours. In the end, tissues were stained using HE solution and subjected for microscopic evaluation.

Evaluation was carried out by calculating the percentage of primordial, primary, secondary, tertiary and Graafian follicles as well as the percentage of normal corpus luteum within five fields of view. The criteria of follicles were as the following: primordial follicles consisted of oocytes surrounded by one layer of flat cells; primary follicles comprised of oocytes surrounded by one layer of cube cells, secondary follicles comprised of oocytes surrounded by two or more layers of cube cells. At the late stage of secondary follicle development, a small anthrum starts to emerge, enlarges (tertiary follicles) and separate from the oocytes (Graafian follicles). The calculation of each follicle incision was only performed once, when the incision reached the nucleus. Six different incisions (with five multiplications) were calculated from each ovary. In order to avoid double counting, triplicate calculations of the secondary

and tertiary follicles were performed based on the occurrence of nucleoli. The percentage of normal follicles was determined by dividing the number of follicles that had a normal morphology with the total follicles observed.

Oocyte Maturation

The maturation of oocytes harvested from the preserved ovaries (24 and 48 h) was analyzed by using the modified protocol of Eriani *et al*. (2008). Oocytes were washed three times with CR1aa media supplemented with FBS 10% (v/v), BSA 0.03%, FSH 10 μg/ml, LH 20 μg/ml, estradiol 1 μg/ml, and antibiotics (10,000 IU/ml penicillin and 10 mg/ml streptomycin). Fifteen Grade A to B oocytes were put in a drop of 50 μl of CR1aa medium in a culture dish and covered with mineral oil. The oocytes were then cultured in an incubator containing CO_2 5% at 37°C for 24 hours.

The maturity of oocytes nucleus was determined microscopically by using aceto-orcein staining. Criteria used were GV (germinal vesicle) as immature oocytes, GVBD (germinal vesicle break down), metaphase-I (Mt-I) as oocytes underwent maturation, and Mt-II as mature oocytes. Oocyte maturation level was calculated from the number of oocytes that reached Mt-II per numbers of total oocytes.

Oocytes fertilization

Spermatozoa suspension harvested from the ductus deferens of male cats was inserted into a vial and centrifuged at 500 xg for 5 minutes. Spermatozoa cells were resuspended in CR1aa medium supplemented with 2.5 mM caffeine, 36 μl of heparin and appropriate antibiotics. Motility of spermatozoa used for IVF was selected by swim-up (Younglai *et al.*, 2001). Final concentration of spermatozoa used for IVF was $2x10^6$/ml. As much as 100 μl of the suspension was then prepared as drop medium and covered with mineral oil. Fifteen *in vitro* maturated oocytes were added in the medium and cultured for 18 hours in the incubator. Oocytes were then fixed and stained in aceto-orcein solution. Fertilization rate was determined by measuring the number of pronuclei. In order to monitor the developmental stages of embryos, oocytes were transferred into new drop of CR1aa medium and cultured for 4 days. The developmental stages of the embryos during this culture were observed microscopically.

Experimental Design and Data Analysis

The complete random design with three replications was used for this study. The data collected was analyzed by using the analysis of variance. When significant differences were identified in any treatment groups, further analysis were done by using Tukey-Kramer HSD test.

RESULTS AND DISCUSSION

The quality of oocytes

Morphological observation indicated that the percentages of good quality (grade A-B) oocytes per ovary collected after 0, 24 and 48 hours of preservation were 71.0%, 63.2%, and 60.6%, respectively (Table 1). Although the numbers of good quality oocytes declined according to preservation time, the decrease was statistically insignificant (p>0.05). These data indicating that preservation of ovary in PBS at 4°C for up to 48 hours were able to maintain the quality of oocytes to meet the standards for *in vitro* maturation.

According to Karja *et al*. (2002), preservation of ovary at 38°C for 1-6 hours might cause a spontaneous maturation of nuclei within the follicles (3.7%). This effect, however, was not observed in the ovary preserved at the low temperatures. Low temperatures inhibit to minimizing the metabolic processes in cells. According to Lehninger (2008) the decrease of temperature will reduce the movement of molecules and minimize metabolic interaction between enzymes and their substrates, resulting in suboptimum enzymatic reactions in the metabolism. As consequences, the preservation of ovary at refrigerator of 4°C would slow down the metabolisms in oocytes and thus delay the process of nuclei maturation.

The percentages of degenerated oocytes per total oocytes collected from each ovary preserved for 0, 24 and 48 hours were 3.9%, 5.7% and 9.1%, respectively (Table 1). A degenerated oocyte was characterized by inhomogeneous and pale cytoplasm with a smaller, dark nucleus. This degeneration occurred probably due to the effects of low temperatures on membrane stability. However, the numbers of degenerated oocytes in this study were relatively small as the ovary was preserved in the PBS, a solution had an ability to maintain pH under physiological ranges (7.2-7.6). Consequenly, good quality oocytes can still be found in up to 48 hours of preservation. According to Dave and Ghaly (2011), preservation of biological materials in physiological solutions prevents autolysis because these solutions have an isotonic pressure. Furthermore, Gencoglu *et al*. (2011) sugessted that the physiological solution could reduce cell damages caused by unbalanced intra- and extracellular osmotic pressures. Therefore, ovaries stored in physiological solutions are

Table 1. The size of follicle and the quality of oocytes after theovary preservation

Preservation time	Number of ovary	Follicle size (mm)				Σ oocyte	Oocyte quality (%)		
		<2mm	2-4mm	>4mm	CL		A – B	C – D	Deg
0	4	23[a]	11[a]	-	5[a]	76	54 (71.0)[a]	19 (25.0)[a]	3 (3.9)[a]
24	6	30[a]	22[a]	3[a]	2[b]	87	55 (63.2)[a]	27 (31.0)[a]	5 (5.7)[a]
48	6	38[a]	17[a]	-	-	165	100 (60.6)[a]	50 (30.3)[a]	15 (9.1)[a]

Numbers followed by the same letter in the same column are not significantly different at the 5% probability (Tukey-Kramer HSD). Deg=degeneration, CL=corpus luteum.

able to maintain the osmolality in both ovary and oocytes by preventing active movement of particles from preservation media into cells or vice versa.

Evaluation of follicle quality based on histological changes

Histological observations showed that viability of oocytes in the tertiary follicles after preservation for 0, 24 and 48 hours were 78.6±6.2%, 72.2±4.8% and 69.5±4.8%, respectively (Table 2). These indicating that preservation time did not significantly affect the viability of tertiary follicles compared with controls. On the other hand, viability of oocytes in the Graafian follicle in control (86.1±2.4%) was slightly different (p<0.05) from those in 24 and 48 hour groups (80.5±4.8% and 72.2±4.8, respectively). This phenomenon was also seen in the primary and secondary follicles (Table 2). In contrast, a significant difference (p<0.05) was found in the viability of oocytes in the primordial follicles of control (70.63±4.18%) and those of 24 hours group (46.9±8.0%) and 48 hours group (44.6±8.8%). This suggested that preservation time influenced the quality of small oocytes (range from 0.3 to 1 mm in diameter), but did not affect the quality of larger oocytes (more than 3 mm in diameter).

The quality of oocytes in the larger follicles is better probably because of the follicles with a diameter more than 3 mm have anthrum filled with follicular fluid that protects oocytes from the influence of external environment. This could minimize the damages caused by preservation process. According to Wood *et al*. (1997), level of oocytes degeneration occurred in follicles have larger diameter is higher than those in smaller follicles due to larger number granulose cells surround the oocytes. This phenomenon is still not fully understood yet. However, there are suggestions that granulose cells produce a compound that can be utilized by oocytes to maintain their viability even in a low temperature conditions. Storage process at low temperatures was assumed to cause damage to the pellucid zone and plasma membrane as well as cytoplasmic contents, resulting in oocyte degeneration. According to Palsdottir and Hunte (2004) changes in the lipid-protein of bilayer membrane might result in membrane destabilization. Similar to normal follicle, viable oocytes have intact granulose cell capable of procuring and transporting nutrients and metabolic precursors (Jewgenow & Paris, 2006). Intact granulose cells are also able to minimize cellular damage caused by the influence of low temperature stress (Tharasanit *et al*., 2005).

In the degenerated follicles, several damages were observed in the cuboidal or cylindrical cells at the basal line (Figure 2-B and C). Degeneration could also be detected from the visible appearance of the oocytes with in homogeneous cytoplasm (Figure 2-D). According to Wiedemann *et al*. (2013), degenerated follicles can be detected by the numbers of lipid vacuoles presence in the cytoplasm of oocytes, around the nuclei (Figure 2-F). Since lipid vacuoles normally located in the peripheral of oocytes, the occurrence of more vacuoles near the nuclei indicated a higher level of

Table 2. Percentage of normal follicles after the preservation

Ovary Preservation (hour)	Normal Oocytes (%)				
	Primordial	Primer	Secondary	Tertiary	Graafian
Control	70.6±4.2[a]	84.4±3.9[a]	84.7±2.4[a]	78.6±6.2[a]	86.1±2.4[a]
24	46.9±8.0[b]	77.8±4.8[b]	81.9±6.4[a]	72.2±4.8[a]	80.5±4.8[ab]
48	44.6±8.8[b]	57.9±7.5[b]	69.4±4.8[b]	69.5±4.8[a]	72.2±4.8[b]

Numbers followed by the same letter in the same column were not significantly differentat 5% probability (Tukey-Kramer HSD).

damages or degeneration of oocytes. However, histological observations in this study indicated that preservation in low temperature for 24 and 48 hours did not strongly affect the quality of oocytes found in the Graafian, secondary and tertiary follicles compared to that were found in control ovaries.

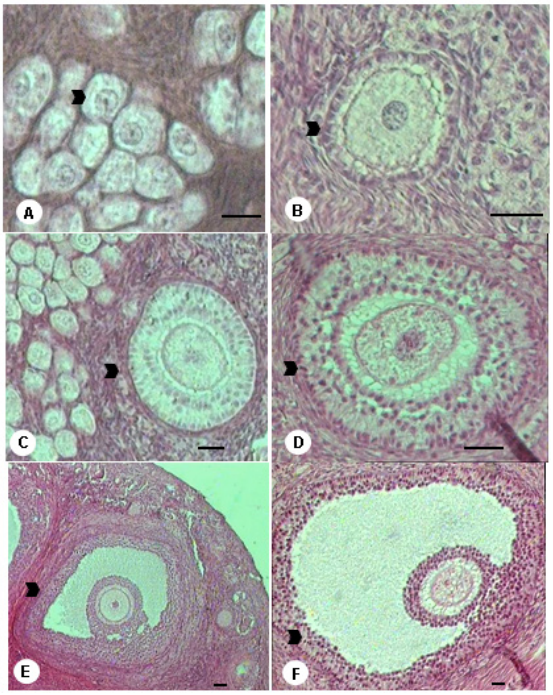

Figure 1, Histological appearance of normal ovarian follicles from cats. A) primordial follicles, B) primary follicles, C) secondary follicles, D) tertiary follicles, E) and F) Graafian follicle. HE staining. Bar=20 μm.

The high damage (degeneration) in the primordial follicles was probably caused by the unavailability of an intact granulose cell layer to protect basal membrane as found in other stages of the follicle development. As consequence, this follicle could not maintain its biological function when preserved at low temperatures. The damage was shown by the pale (inhomogeneous) cytoplasm, non-intact nuclear membrane, picnotic nucleus and a decreased in the cell cohesiveness. According to Wiedemann *et al*. (2013) characteristics of marked features in the next taphonomic changes were fragmentation of oocyte cytoplasm, loss of membrane integrity between cells and cells were eosinophillic-stained. In this study the changes were not visible on the ovaries stored in the PBS for 24 to 48 hours in cold temperatures.

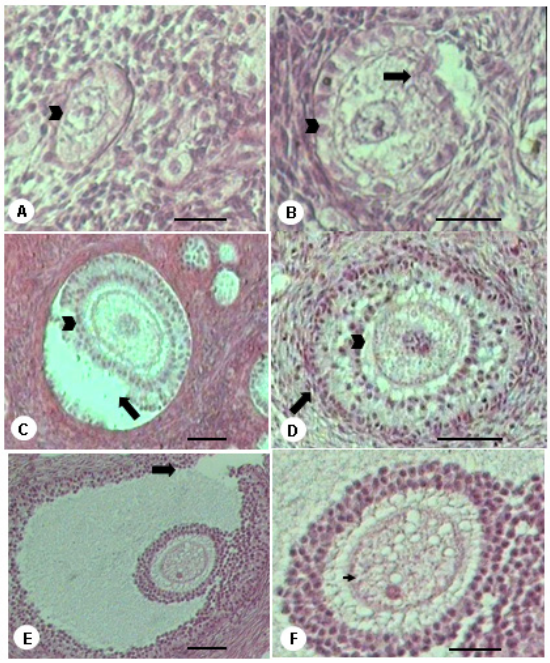

Figure 2. Histological appearance of degenerated cat ovary as characterized by lysis and picnotic (arrow heads) nuclei, picnotic (arrows) granulose cells. A) primordial follicles, B) primary follicles, C) secondary follicles, D) tertiary follicles, E) Graafian and F). Enlargement of the oocyte from the follicle Graafian with lipid vacuoles (pointer). HE staining. Bar=20 μm.

Level of oocyte maturation

In vitro maturation requires the same environmental conditions with *in vivo* environment where cytoplasm and the nucleus of oocytes underwent simultaneous maturation. Cumulus cells play a role in regulating oocyte activity. Some researchers stated that the expansion of cumulus cells is closely linked to the maturation of oocytes. According to Souza-Fabjan *et al*. (2013), a normal fertilization rate was achieved by simultaneous changes in the cumulus cells, pellucidal zone and oocyte vitellin membrane during the maturation of both nucleus and cytoplasm.

Data in Table 3 showed that preservation time influences the maturity level of nuclei after oocytes were *in vitro* maturated for 24 and 48 hours. Maturity level of oocytes reached the second metaphase stage after being preserved for 24 and 48 hours was 29.4% and 21.9%, respectively. Statistical analysis indicated that maturation levels in these groups were significantly different (p<0.05) from the one in control group (0.0%). This finding suggested that preservation time at

Table 3. Percentage of oocyte maturation rate after ovary preservation

Ovary Preservation (hours)	Σ Oocytes	Oocyte maturation(%)				
		Σ GV	Σ GVBD	Σ Mt-I	Σ Mt-II	Σ GV-block
Control	60	5 (8.3)[b]	8 (13.3)[a]	17 (28.3)[a]	30 (50.0)[a]	0 (0)[a]
24	102	16 (15.7)[a]	31 (30.4)[a]	18 (17.6)[b]	30 (29.4)[b]	7 (6.86)[a]
48	91	19 (20.9)[a]	21 (23.0)[a]	28 (30.7)[a]	20 (21.9)[b]	3 (3.30)[a]

Numbers followed by the same letter in the same column are not significantly different at 5% level test (Tukey-Kramer HSD). GV=germinal vesicle, GVBD=germinal vesicle breaks down, Mt=Metaphase

4°C could affect the environmental conditions in the ovary that ultimately affected the formation of meiotic spindles when oocytes matured, but did not affect chromosome condensation (Figure3A). According to Prentice and Anzar (2010), the exposure of oocytes to low temperatures could affect the formation of microtubules of chromosome. Although the formation of microtubules occurred, the shape of spindles was abnormal.

The percentages of declining rate of nucleus maturity for 24 and 48-hour periods of preservation were 20.6% and 28.1%, respectively. The decrease might be related to the interference in the release of PB. Tremoleda *et al*. (2001) previously described that the failure of oocytes to reach maturation stage of Mt-II was caused by the interruption in the process of meiotic spindle formation which in turn inhibit the release of PB. This suggested that meiotic spindles were extremely sensitive to low temperatures. There were indications that disturbance in this polar body formation could also lead to the formation of GV block during oocyte maturation. The number of GV block observed in oocytes collected from ovaries preserved for 24 and 48 hours were 6.86% and 3.30% compared to those from control (0%). According to Ackert *et al*. (2001), GV blocks are oocytes at the GV stage of meiosis that fail to proceed to the Mt-II stage. These GV blocks are characterized by bright ooplasma without a space between the plasma membrane and pellucidal zone, indicating the lysis of plasma membrane.

It has been known that only at the Mt-I and Mt-II oocyte has a perfect spindle microtubules arrangement. Cold temperatures can cause some effects on the microtubules arrangement during the Mt-II stage of oocyte maturation in cattle (Yang & Fortune, 2006). Cooling (low temperature) may cause depolymerization and the loss of central regulator of microtubule. Sun and Schatten (2006) stated that on the stage of GV, oocytes did not contain any spindle microtubules. When the oocyte was mature, the chromosomes begin to condense, and microtubules become well coordinated. Spindle formation is controlled by several factors including maturation promoting factor (MPF) and mitogen-activated protein kinase (MAPK). Based on their study, Sun and Schatten (2006) concluded that storage at 0 and 4°C do not directly involve in the microtubule spindle formation, but affect the formation and release of the next meiotic spindle pole body. This suggested that low temperatures may damage some key regulatory factors such as MPF, MAPK or others which in turn affect microtubule arrangement.

In this study, from the ovaries preserved at 4°C for 24 and 48 hours we still could obtain oocytes with nucleus reached the Mt-II stage. This probably because after oocytes were collected and subsequently matured *in vitro*, meiotic coil of microtubules reorganized so that the oocytes remained can resume meiosis (Do *et al*., 2014). Wiedemann *et al*. (2013) stated that cooling process at 20°C or 4°C could lead to microtubule depolymerization. Microtubule coil was capable of being reversible if bovine oocytes were incubated at 37°C for 60 minutes even though the potency for genetic anomalies due to errors in chromosome movement during the process is still a major concern need to investigate further.

The cat oocytes are unique in their ability to maintain viability after being collected from ovaries preserved at low temperature for 24 hours. Oocytes are still capable of reaching Mt-II (51%) after being matured *in vitro*. However, Wood *et al*. (1997) found that the ability of oocytes to mature and divide decrease at rate of 25% if ovary is stored at cold temperature for 48 hours. Further study is necessary to evaluate DNA integrity in oocytes collected from ovaries stored at cold temperatures.

We also should consider the data from the study of Otoi *et al*. (2001) which showed that there is a strong relationship between the diameter and meiotic capability of nuclei from cat oocytes stored at 4°C (77.7%) compared with oocytes that are stored at 38°C (89.2%) for 24 hours. Particu-

larly, in oocytes that were stored at 4°C metaphase-II achievement will decline along with the decrease in oocyte diameter. This phenomenon can be attributed to data obtained from histological observation of the preserved ovary. From these data we could conclude that there was a trend of decline in the quality of oocytes in the small-diameter follicles (primordial, primary and secondary).

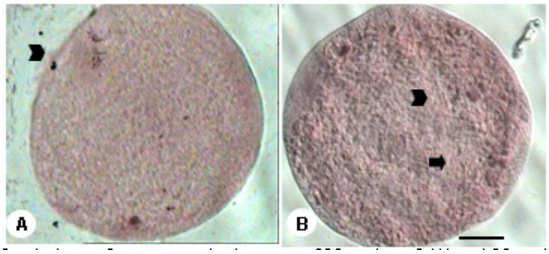

Figure 3. Morphology of cat oocytes in the stage of Metaphase-I (A) and Metaphase-II (B) as indicated by the presence of polar bodies (arrows). Arrow heads indicate the chromosomes in metaphase. Bar=20 μm.

According to Poschmann *et al*. (2008), although 4°C temperatures can inhibit ovarian taphonomic changes, DNA degradation started to occur in the granulose cells after 12 hours preservation. Metabolic relationship between the oocytes and granulose cells plays an important role in oocyte maturation. Here granulose cells transferred small molecules such as energetic substrates, nucleotides and amino acids in oocytes. Jewgenow and Paris (2006) stated that degradation of granulose cells initiates the loss of oocytes developmental competence before eventually die. Frequency of degeneration among oocytes preserved at 4°C declined with the decrease in oocyte diameter. The high frequency of oocyte degeneration after preservation treatment at 4°C might be caused by degeneration of granulose cells during preservation at cold temperatures and the high sensitivity of small diameter oocytes to low temperatures.

Fertilization rate

As shown in Table 4, normal fertilization rate (2 PN) for preservation time of 24 and 48 hours, that was 30%, showed no differences with those of 0 hours (36.7%). This suggested the possibility of doing IVF using fresh spermatozoa and the ovum collected from ovaries preserved for 24 and 48 hours. The low fertilization rate obtained in this study was possibly related to the spindle morphology of oocytes matured *in vitro*. Wiedemann *et al*. (2013) confirmed that normal fertilization and subsequent embryo development are highly dependent on the normal morphology of the spindle in the stage of Mt-II of matured oocytes. Karja *et al*. (2002) also stated that when oocytes were stored at 0-4°C, the percentage of oocytes having normal Mt-II spindles was very low. If these oocytes were used for fertilization the fertilization percentage obtained will also decrease.

We found a high levels of polyspermia (40%) in oocytes collected after 48 hours of preservation at 4°C compared with those in control (16.7%). This was probably caused by lower response of pellucidal zone of the oocytes to provide polysperm blocking so that other spermatozoa cells could go through the pellucidal zone. According to Liu (2011), an increase in calcium ion concentration will result in intracellular fusion of cortical granules with oolema so that their contents leak out into perivitelline space. Granule contains enzymes that acted on the ZP and would block the attachment and penetration of the next spermatozoa, natural mechanism prevents polyspermia. The breakdown of glycoproteins ZP2 (by protease) and those of polysaccharide parts of ZP3 (by β-hexosaminidase B) prevent the binding of the next spermatozoa to ZP3 since this protein no longer has a full ligand. Low temperature seems to reduce the quality of oocytes so the oocyte lost the capability to performed sequential chemical reactions required to prevent polyspermia. If we looked at the stages polysperm block, there was a possibility that low temperature might affect the enzymes that contribute to hardening of pellucidal zone. The longer preservation time

Table 4. Effects of ovary preservation on the fertilization rate in vitro

Ovary preservation (hour)	Σ fertilized Oocytes	Rate of nuclei development (%)			
		1 PN	2 PN	> 2 PN	PN + sperm
0	30	8 (26.7)[a]	11 (36.7)[a]	5 (16.7)[a]	6 (20.0)[a]
24	30	10 (33.3)[a]	9 (30.0)[a]	7 (23.3)[a]	4 (13.3)[a]
48	30	5 (16.7)[a]	9 (30.0)[a]	12 (40.0)[a]	4 (13.3)[a]

Numbers followed by same letter in the same column are not significantly different at 5% level test (Tukey-Kramer HSD).

would result in worse oocytes' viability.

According to Teperek and Miyamoto (2013), fertilization success was also greatly influenced by the maturity level of the nuclei and the changes in the oocyte cytoplasm that can trigger the formation of male pronucleus. Oocytes are very sensitive to the time of exposure to spermatozoa in the culture condition *in vitro*. Imperfect cytoplasmic maturation could inhibit the production of male pronucleus growth factor that is responsible for decondensation of spermatozoa chromatin. Yasmin *et al*. (2015) confirmed that the meiotic spindles in bovine oocytes are very sensitive to low temperatures that can affect the level of maturation and fertilization. Feline oocytes seem to express a higher sensitivity to chilling (Fassbender *et al*., 2007).

Overall, results of this study showed the potential of using preservation technique to maintain female germ cells obtained from mammals. In the future, results of this study can be used to cultivate germ cells from dead or injured endangered mammals and protect the population through *in vitro* maturation and fertilization.

CONCLUSION

The present study suggested that preservation of ovary in the PBS at 4°C for 48 hours could maintain biological functions of oocytes. This allows meiosis process in the oocytes proceed to the stage if metaphase-II when being cultured in *vitro* and required for IVF.

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REFERENCES

Ackert, C. L., Gittens, J. E., O'Brien, M. J., Eppig, J. J. & Kidder, G. M. (2001). Intercellular communication via connexin 43 gap junctions is required for ovarian folliculogenesis in the mouse. *Dev Biol*. 233, 258-270.

Dave, D. & Ghaly, A. E. (2011). Meat spoilage mechanisms and preservation techniques: A critical review. *Am J Agricult Biol Sci.* 6(4), 486-510

Do, L. T., Namula, Z., Luu, V. V., Sato, Y., Taniguchi, M., Isobe, T., Kikuchi, K. & Otoi, T. (2014). Effect of sericin supplementation during in vitro maturation on the maturation, fertilization and development of porcine oocytes. *Reprod Domest Anim*. 49, 17-20.

Eriani, K., Boediono, A., Djuwita, I., Sumarsono, S. H. & A. Azhar. (2008). Development of domestic cat embryo produced by preserved sperms. *Hayati J Biosci*. 15(4), 155-160

Eriani, K., Sukra, Y., Boediono, A., Djuwita, I. & Sumarsono, S. H. (2013). Produksi embrio kucing secara *in vitro* dari spermatozoa hasil preservasi melalui fertilisasi mikro [*In vitro* production of cat embryo from preserved sperms by micro fertilization] *J Kedokteran Hewan*. 7(1), 37-42.

Fassbender, M., Hildebrandt, T. B., Paris, M. C, Colenbrander, B. & Jewgenow, K. (2007). High-resolution ultrasonography of xenografted domestic cat ovarian cortex. *J Reprod Dev.* 53(5), 1023-1034.

Gencoglu, A., Camacho-Alanis, F., Nguyen, V. T., Nakano, A., Ros, A. & Minerick, A. R. (2011). Quantification of pH gradients and implications in insulator-based dielectrophoresis of biomolecules. *Electrophoresis*. 32, 2436-2447. doi:10.1002/elps.201100090

Giaretta, E., Spinaci, M., Bucci, D., Tamanini, C. & Galeati G. (2013). Effect of resveratrol on vitried porcine oocytes. *Oxid Med Cell Longev*. 2013, 1-7. doi: 10.1155/2013/920257

Jewgenow, K. & Paris MC. (2006). Preservation of female germ cells from ovaries of cat species. *Theriogenology* 66(1), 93-100.

Karja, N. W. K., Otoi, T., Murakami, M., Fahrudin, M. & Suzuki, T. (2002). *In vitro* maturation, fertilization and development of domestic cat oocytes recovered from ovaries collected at three stages of the reproductive cycle. *Theriogenology* 57, 2289-2298.

Lehninger, A. (2008). Principles of Biochemistry. 5th ed. Freeman, W. H. & Company, New York.

Liu, M. (2011). The biology and dynamics of mammalian cortical granules. *Reprod Biol Endocrinol*. 9, 149-166. doi: 10.1186/1477-7827-9-149

Otoi, T., Murakami, M., Ooaka, A., Karja, N. W. K & Suzuki, T. (2001). Effects of size and stroge temperature on meiotic competence of domestic cat oocytes. *Vet Rec*: 148, 160-8.

Palsdottir, H & Hunte, C. (2004). Lipids in membrane protein structures. *Biochimica et Biophysica Acta (BBA)-Biomembranes*. 1666(1-2), 2-18.

Pope, C. E. (2000). Embryo technology in conservation efforts for endangered felids. *Theriogenology.* 53, 163-174.

Poschmann, M., Fassbender, M., Lopes, C., Dorresteijn, A. & Jewgenow, K. (2008). Viability assessment of primordial follicles of domestic cats to develop cryopreservation protocols for ovarian tissue. *Reprod Domest Anim.* 43, 23-25.

Prentice, R. J. & Anzar, M. (2010). Cryopreservation of mammalian oocyte for conservation of animal genetics. *Vet Med Int.* 2011, 1-11

Souza-Fabjan, J. M., Pereira, A. F., Melo, C. H., Sanchez, D. J., Oba, E., Mermillod, P., Melo, L.

M., Teixeira, D. I. & Freitas, V. J. (2013). Assessment of the reproductive parameters, laparoscopic oocyte recovery and the first embryos produced in vitro from endangered Caninde goats (*Capra hircus*). *Reprod Biol*. 13, 325-332.

Sun, Q. & Schatten, H. (2006). Regulation of dynamic events by microfilaments during oocyte maturation and fertilization. *Reproduction*. 131, 193–205

Teperek, M. & Miyamoto, K. (2013). Nuclear reprogramming of sperm and somatic nuclei in eggs and oocytes. *Reprod Med Biol*. 12, 133-149. DOI 10.1007/s12522-013-0155-z

Tharasanit, T., Colenbrander, B. & Stout, T. A. E. (2005). Effect of cryopreservation on the cellular integrity of equine embryos. *Reproduction*. 129, 789-798. DOI: 10.1530/rep.1.00622

Tremoleda, J. L., Schoevers, E. J., Stout, T. A. E., Colenbrander, B. & Bevers, M. M. (2001). Organisation of the cytoskeleton during in vitro maturation of horse oocytes. *Mol Reprod Dev*. 60, 260-269.

Wiedemann, C., Zahmel, J., & Jewgenow, K. (2013). Short-term culture of ovarian cortex pieces to assess the cryopreservation outcome in wild felids for genome conservation. *Vet Res*. 9, 37.

Wood, T. C., Montali, R. J & Wildt, D. E. (1997). Follicle-oocytes atresia and temporal taphonomy in cold-stored domestic cat ovaries. *Mol Rep Dev*. 46, 190-200.

Yang, M. Y. & Fortune, J. E. (2006). Testosterone stimulates the primary to secondary follicle transition in bovine follicles *in vitro*. *Biol Reprod.* 75(6), 924-932.

Yasmin, C., Otoi, T., Setiadi, M. & Karja, N. W. K. (2015). Maturation and fertilisation of sheep oocytes cultured in serum-free medium containing silk protein sericin. *Acta Vet Hungarica*. 63(1), 110-117.

Younglai, E. V., Holt, D., Brown, P., Jurisicova, A. & Casper, R. F. (2001). Sperm swim-up techniques and DNA fragmentation. *Hum. Reprod*. 16(9), 1950-53.