Original Article

Restoration of tiger (*Panthera tigris*) follicles from frozen-thawed ovarian tissues

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Abstract

Ovarian tissue cryopreservation has been proposed as a tool to conserve the valuable genetics of endangered species. However, sustaining the viability of follicles and oocytes enclosed within the frozen-thawed tissues is important for the application of this technology. This study evaluated the impact of eCG supplementation on the viability and growth of tiger follicles and oocytes within frozen-thawed ovarian tissues during *in vitro* culture. Six frozen-thawed ovarian fragments from a tiger obtained post-ovariohysterectomy were included. Twelve antral follicles were mechanically isolated from the ovarian fragments and randomly allocated to two culture conditions (control and 0.05 IU/mL eCG supplementation) and cultured in an alginate hydrogel for 3 days. Follicle growth was evaluated on Day 0 and 3 using Image J software. At the end of the culture, follicles were removed from the hydrogel and stained with neutral red to assess follicle viability. The oocytes were thereafter recovered and evaluated for nuclear status using Hoechst 33258 dye. Follicle diameter in the control group significantly decreased ($P < 0.05$), whereas that of the eCG supplementation group increased their relative growth ($P < 0.05$). The oocyte recovery rate from cultured follicles was 33% and 67% in the control and eCG groups respectively ($P > 0.05$). All recovered oocytes remained in the germinal vesicle phase. The present study showed that tiger frozen-thawed antral follicles could maintain their viability and structure when eCG was supplemented in the culture medium. In conclusion, viable oocytes within follicles can be rescued from the tiger ovarian tissues post-mortem.

Keywords: antral follicle, eCG, Endangered, Gamete rescue

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Introduction

During the past decades, many wild felids have gradually decreased in numbers. Of the 38 wild felids worldwide, five species including the tiger (*Panthera tigris*) have been classified as endangered by the International Union for the Conservation of Nature (IUCN 2019. The IUCN Red List of Threatened Species. Version 2019-1. <https://www.iucnredlist.org>). The tiger population has rapidly decreased because of the loss of habitat, declining prey population and direct persecution (Goodrich, 2010) contributing to the need for a captive breeding program establishment to secure populations, as well as for education and research purposes.

To date, various assisted reproductive technologies (ARTs) developed for the domestic cat have been applied to aid in the genetic management of wild felid populations. Ovarian stimulation protocols followed by either *in vitro* fertilization or artificial insemination with fresh semen have resulted in the production of tiger cubs (Donoghue *et al*., 1990; Donoghue *et al*., 1993). Tiger embryos obtained from *in vitro* fertilization with frozen-thawed sperm have also been reported (Crichton *et al*., 2003).

For animal post-mortem, cryopreservation of female gametes enclosed in the ovary is an option for preserving female gametes to expand the gene pool. Moreover, ovarian cryopreservation could solve the delay needs for oocyte maturation and embryo production. There are three options to cryopreserve oocytes within the follicles: whole ovary, ovarian cortex or isolated follicles, using slow freezing or vitrification methods. After cryopreservation, frozen-thawed ovarian tissues can be cultured or transplanted to the renal capsules of SCID mice (Bosch *et al*., 2004; Dos Santos *et al*., 2016) to produce mature fertilizable oocytes. However, the cryopreservation of ovarian cortex provides more success because it allows more cryoprotectant perfusion than the whole ovary. Therefore, the *in vitro* culture of ovarian cortical tissue, which contain abundant of primordial follicles, has been performed in many mammalian species including humans (Amorim *et al*., 2011; Herraiz *et al*., 2014), mice (Wang *et al*., 2011; Youm *et al*., 2014), cats (Luvoni *et al*., 2012; Mouttham and Comizzoli, 2016; Mouttham and Comizzoli, 2017; Brito *et al*., 2018; Martins *et al*., 2018), cows (Herraiz *et al*., 2014), sheep (Fathi *et al*., 2011) and goats (Carvalho *et al*., 2013). Furthermore, the advantage of ovarian tissue vitrification in human is to preserve fertility in cancer patients before chemotherapy or radiotherapy (Paulini *et al*., 2016; Dolmans, 2018). This approach also provides options for rescuing gametes of valuable species that die abruptly or are ovariohysterectomized for medical reasons (Jewgenow and Paris, 2006). Although gamete resource banking (GRB) is considered as an important approach to conserve endangered species, the limitation is the availability of mature oocytes. Previous studies of frozen-thawed ovarian tissues in the domestic cats (Luvoni *et al*., 2012; Mouttham and Comizzoli, 2017; Brito *et al*., 2018; Martins *et al*., 2018) and wild felids (Wiedemann *et al*., 2012; 2013) have been conducted with the focus on: 1) cumulus oocyte complexes culture (Luvoni *et al*., 2012); 2) preantral follicle morphology/viability evaluation (Tanpradit *et al*., 2015; Mouttham and Comizzoli, 2016; Martins *et al*., 2018); and 3) transplantation of ovarian tissue (Demirel *et al*., 2018) but the method of isolated follicle culture is lacking.

To date, the information on isolated follicle culture from the cryopreserved tissues especially in the tigers is limited. The aims of the present study were to 1) evaluate tiger follicle growth in an alginate hydrogel three-dimensional culture system supplemented with or without eCG and 2) investigate the feasibility of female gamete restoration from frozen-thawed tiger ovaries.

Materials and Methods

Chemicals: All chemical used in this study were purchased from Sigma Aldrich, St Louis, MO, USA, unless otherwise indicated.

Samples: Ovaries of an adult tiger (9 years old), housed at the Zoological Park Organization under the Royal Patronage of His Majesty the King, were obtained after ovariohysterectomy in 2013. The ovaries were transported at 4°C in sterile normal saline (NSS) supplemented with 1% penicillin–streptomycin to our laboratory within 24 h. Upon arrival, blood surrounding the tissue was removed and the ovaries were then washed in NSS supplemented with 1% penicillin–streptomycin. The ovaries were cut into small piece before cryopreservation.

Ovarian tissue cryopreservation: The ovarian tissue dimension of 5 x 5 x 3 mm3 (75 mm3) was previously frozen (Thuwanut and Chatdarong, 2012). Briefly, ovarian tissues were incubated in the equilibration solution, composed of medium 199 with Earle's salts (M 199), 20.0% (v/v) fetal calf serum (FCS), 7.5% dimethyl sulfoxide (DMSO) and 7.5% ethylene glycol (EG), for 10 min and placed in vitrification medium, which consisted of 0.5 M sucrose, 20.0% (v/v) FCS, 15.0% (v/v) DMSO and 15.0% (v/v) EG in M 199, at 4°C for 30 mins. Tissues were vitrified by plunging on to aluminum foil containing liquid nitrogen for 10 mins, transfer to frozen cryogenic tubes and kept in a liquid nitrogen tank. The vitrified tissues were donated to the Research unit of Obstetrics and Reproduction in Animals, Department of Obstetrics, Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand.

Ovarian tissue thawing: The ovarian tissues were taken out of the cryotubes and transferred into a thawing solution, composed of M 199, 20.0% (v/v) FCS and 1 M sucrose at 25°C for 10 min and then transferred to the holding medium composed of M 199 containing 25 mM HEPES and 10.0% (v/v) FCS prior to follicle culture.

Follicle isolation, encapsulation and culture: Antral follicles (>500 µm; n = 12) were isolated from ovarian cortical strips using 25G gauge needles. Individual normal follicles with intact basement membrane without vacuoles or dark cytoplasm (Antonino *et al*., 2019), were then encapsulated into a bead of alginate

solution with the first step being transferred into a 5 to 10 µL droplet (dependent on follicular size) of 0.5% alginate using a 20 µL pipette. Single droplets were immersed into a solution containing 50 mM CaCl2 and 140 mM NaCl and alginate beads were allowed to cross-link for 2 min, and then washed three times in a culture medium: MEM (supplemented with 3 mg/mL BSA, 2 mM L-glutamine, 10 ng/mL activin, 10 µg/mL insulin, 1.9 µg/mL transferrin, 5 µg/mL selenium, 10 IU/mL penicillin G sodium and 10 mg/mL streptomycin sulfate). The entire procedure was performed on a warm plate set at 38°C. For each experimental replication, two follicles were cultured in 500 µL of culture medium supplemented with 0 (control) or 0.05 IU/mL eCG at 38.5 °C in 5.0% CO_2 in humidified air for 3 days. There are three replications in the study, totaling six follicles for each treatment.

***Assessment of follicle growth*:** Follicle diameter was assessed under an inverted microscope at x 1,000 magnification. Screen shots were taken with digital camera at Olympus BX51 microscope (Olympus, Germany). Each follicle image was sized from the outer layer of somatic cells, with the measurements including the widest diameter and perpendicular width in the initial assessment by Image J software, and then, the actual follicle diameter calculated by comparing to the scale bar. The mean of these two metrics was calculated and reported in terms of diameter. The mean diameters of each follicle on Day 0 and Day 3 were recorded. The relative growth of the follicles (compared to day 0) was calculated.

***Follicle survival and oocyte nuclear configuration*:** Viability of isolated follicles was primarily evaluated by staining with 50 ng/mL neutral red at 38 °C in 5.0% CO_2 in atmospheric air for 30 min. Follicles with no red coloration were considered dead follicle. Follicles incubated with neutral red were classified as surviving follicles when the oocyte and more than 75.0% of the granulosa cells stained positive for neutral red (Bulgarelli *et al*., 2018). The oocytes within positively stained follicles were collected and transferred into a mixture of 2 µM Hoechst 33258 in 500 µL holding medium for 15 mins in the dark. The nuclear configuration of stained oocytes was examined under a fluorescent microscope (BX51; Olympus) at X 2,000 magnification.

Statistical analysis: Data was analyzed using IBM SPSS Statistics for Windows, Version 23.0 (Armonk, NY: IBM Corp.). Normal distribution and equal variance were tested by the Shapiro test and Bartlett's test, respectively. Differences in mean follicle diameter between Days 0 and 3 and the relative growth of the follicle at the end of the culture between control and 0.05 IU/mL eCG supplementation were determined by paired *t* test. Pearson Chi-Square test was used to evaluate the differences in the numbers of oocytes recovery between the control and eCG treated group at the end of culture. For all statistical analysis, differences were regarded as significant if $P < 0.05$.

Results

The results of frozen-thawed tiger ovarian tissue are presented in Table 1. At the end of the culture, the follicle diameter significantly decreased in the control group ($P < 0.05$), whereas the follicle diameter of eCG treated group sustained their initial size ($P > 0.05$). The relative growth of the follicles was significantly different between the control and eCG treated groups ($P < 0.05$). In 100% follicle survival (Fig. 1B), four (66.7%) and two (33.3%) oocytes were viable (Table 1). All oocyte size measured without zona pellucida was ~ 140 µm (141.8 ± 0.9 µm). Hoechst 33258 staining revealed that the nuclear configuration of all recovered, viable oocytes remained in the germinal vesicle stage (Fig. 2).

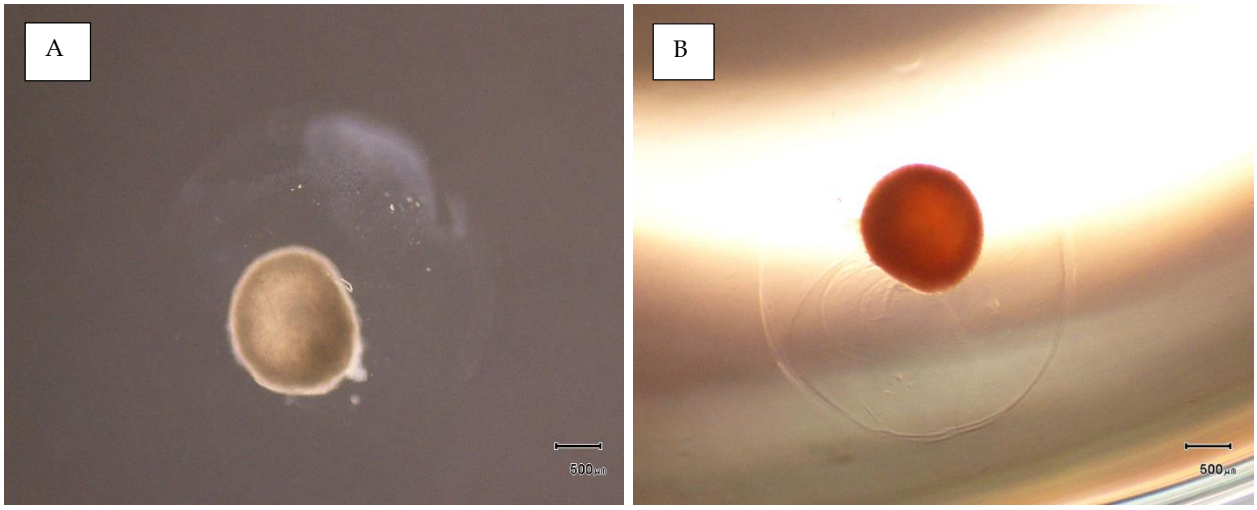

Figure 1 Micrographs of (A) Tiger antral follicle encapsulated in an alginate hydrogel on Day 0. and (B) neutral-red positive tiger antral follicle at the end of the culture. Scale bar, 500 µm.

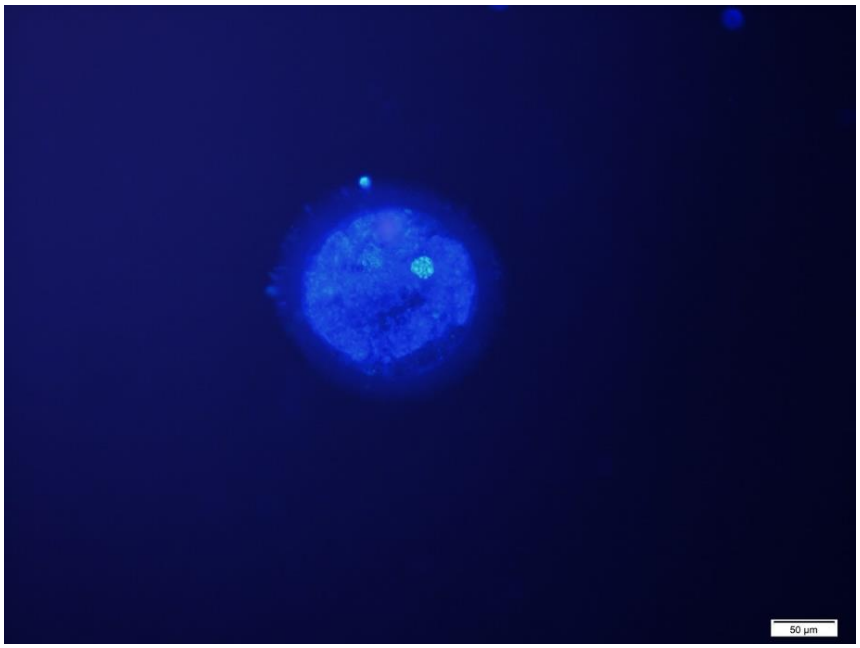

Figure 2 The germinal vesicle stage oocyte stained with Hoechst 33258 at the end of the culture. Scale bar, 50 µm.

Table 1 Mean ± SEM of follicle diameter on Day 0 and 3 of culture and the percentage of oocyte recovery at the end of culture.

Group	n	Follicle diameter (µm)		Relative growth of the follicle	Oocyte recovery (%)
		Day 0	Day 3		
Control	6	1,553.10 ± 269.50[a]	1,514.58 ± 270.85[b]	0.97±0.01[B]	33.33
eCG	6	1,541.45 ± 210.54[a]	1,644.73 ± 230.90[a]	1.07±0.03[A]	66.67

Value with different superscripts (a, b) differ significantly (P<0.05) between culture days.
Value with different superscripts (A, B) differ significantly (P<0.05) between groups.

Discussion

In this study, the tiger ovarian tissues were vitrified 5 years ago. Although the successful vitrification protocols for feline ovarian tissue have been established (Mouttham and Comizzoli, 2017; Martins *et al*., 2018), the tissues in our experiment showed signs of degeneration (dark and decomposed appearance) post-thawing. In fact, the essential part of vitrification is to permeate the tissue with cryoprotectant to minimize ice formation. Apart from the selected type of cryoprotectants, an optimal equilibration duration is important and can affect post-thaw cellular survival (Newton *et al*., 1998). Using the same protocol, the sperm plasma membrane and DNA integrity have been protected in the cat testicular tissues (tissue dimension of 4 x 4 x 4 mm^3) (Thuwanut and Chatdarong, 2012). The protocol likely provided different results for the cat ovarian tissues in this study. In addition, the post-thawed degeneration appearance might have occurred because of the initial large tissue size. In domestic cats, ovarian cortical tissues sliced into 1 x 1 x 0.2 mm^3 obtained 47% of normal follicles after thawing (Mouttham and Comizzoli, 2017) which was higher than our study. In baboons, the percentage of normal follicles in frozen-thawed ovarian tissues sliced into 0.5 x 1.5 x 1 mm^3 was higher than those sliced into 2 x 1 x 1 mm3 (Lu *et al*., 2014). Similarly, the previous investigation of the ovarian tissue size confirmed that tissue dimension of 3 x 3 x 3 mm^3 fragments was adequate for cryoprotectant perfusion than the greater dimensions (3 x 3 x 5 mm^3 and 3 x 3 x 7 mm^3) (Gorricho *et al*., 2018). Altogether, the optimal CPA incubation duration and tissue dimension appeared as the important factors contributing to the degeneration of frozen-thawed ovarian tissues in the present study. However, in this study, the antral follicles were obtained from tissue edges that were probably perfused well by the CPA, resulting in some surviving.

To the best of our knowledge, there have been no studies of isolated follicle culture in tigers. Our study is the first report of survival of antral follicles cultured from frozen-thawed tiger ovarian tissue after long-term storage. The germinal vesicle stage of the oocytes revealed by that the tiger ovarian tissues is capable of restoring their viability after long-time storage in liquid nitrogen. Moreover, this study showed that the tiger antral follicles responded to eCG similar to those in the domestic cats (Chansaenroj *et al*., 2019). Granulosa cell apoptosis and follicular atresia of antral follicles seemed to be prevented by eCG as described previously (Li *et al*., 1998). In this study, the initial oocyte size could not be measured because of the large volume of antral cavity. However, the oocytes were evaluated at the end of the culture. The different culture conditions had no significant effect on the oocyte recovery rate. The recovery oocyte diameters were approximately 140 µm which is larger than the normal size for cat oocytes obtained from antral follicles (120 µm) (Izumi *et al*., 2012). Our finding suggested that the fully-grown oocytes of the tigers are slightly larger than domestic cats (Izumi *et al*., 2012). The relation between oocyte diameter and meiotic competence has been reported previously in cold storage domestic cat ovaries. The small diameter oocyte is sensitive to low temperatures, granulosa cell degeneration precedes the loss of developmental

competence of oocytes (Otoi *et al*., 2001). However, the information on this relation in tigers is limited. Future research to assess the ability of tiger oocytes recovered from cryopreserved ovarian tissue to develop to complete nuclear maturation are warranted.

In conclusion, the rapid transport of tissue at 4°C is crucial for rescuing the female gametes of valuable species. The ovarian tissue size for vitrification is recommended as being less than 3 x 3 x 3 mm^3 in order to preserve the survival of follicle post-thaw. In this study, we demonstrated the feasibility of female gamete restoration from frozen-thawed tiger ovaries. The recovered antral follicles can grow and sustain the viability of immature tiger oocytes in a three-dimensional alginate hydrogel culture system supplemented with eCG. However, appropriate conditions to complete meiotic maturation and in vitro fertilization are worth investigating.

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