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Viability and Growth of Preantral Follicles Derived from Cryopreserved Ovarian Tissues of a Cheetah (*Acinonyx jubatus*) Post-mortem

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Abstract

This study aimed to investigate freezing effects of ovarian tissues on survival of preantral follicles and observing *in vitro* growing of preantral follicles retrieved from cryopreserved ovarian cortical tissues of a cheetah post-mortem. After 29-hour cold storage, ovarian cortices were cut into small pieces (2.0 x 2.0 x 1.0 mm³) and allocated to be frozen using a passive cooling container (n = 3 pieces) or vitrification (n=3 pieces). After one year of storage, 43 (10/23) and 21% (12/58) follicles isolated from ovarian tissues cryopreserved using a passive cooling device (slow freezing rate) and vitrification, respectively, were viable (positively stained with neutral red). Thereafter, the viable follicles were *in vitro* grown in a culture medium containing M199 supplemented with growth hormone (GH), follicular-stimulating hormone (FSH), insulin-like growth factor I (IGF-I) and activin A for 7 days. Diameters and diameter gains were examined on Days 0, 3 and 7. Follicle viability was assessed on Days 0 and 7. Diameters of follicles frozen by the slow freezing decreased gradually from 53.5±14.2 µm on Day 0 to 50.9±17.1µm with 2 out of 10 viables, whereas those frozen using vitrification maintained their diameters between 50.7±15.6 µm and 50.5±17.9 µm on Days 0 and 7, respectively, with 2 of 12 viable. In conclusion, the passive cooling container is suggested to perform a slow freezing rate for ovarian tissue cryopreservation. Although the cheetah ovarian follicles obtained from cryopreserved tissues can be grown *in vitro* for 7 days, optimization of culture medium is required to improve the viability and growing rate.

Keywords: feline, freezing, gamete rescue

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บทคัดย่อ

การมีชีวิตและการเจริญของฟริแอนทรัลฟอลลิเคิลที่ได้จากเนื้อเยื่อรังไข่แช่แข็งของเสือชีตาร์หลังตาย

กฤษณรงค์ วงศ์บ้านตุ๋¹ เนติ์ ต้นประดิษฐ์¹ ดารกา ทองไทยนนท์¹ ปวีณา ฐะระนุติ¹ เกวลี ฉัตรตรงค์^{1*}

การทดลองนี้มีวัตถุประสงค์เพื่อศึกษาผลกระทบของการแช่แข็งเนื้อเยื่อรังไข่ต่อการรอดชีวิตของฟริแอนทรัลฟอลลิเคิลและศึกษาการเจริญภายนอกร่างกายของฟอลลิเคิลที่แยกได้จากเนื้อเยื่อรังไข่ส่วนคอร์เท็กซ์แช่แข็งของเสือชีตาร์หลังตาย รังไข่ของเสือชีตาร์ถูกเก็บแช่เย็นและขนส่งไปยังห้องปฏิบัติการภายในเวลา 29 ชั่วโมง ตัดเนื้อเยื่อรังไข่ส่วนคอร์เท็กซ์เป็นชิ้นเล็ก ๆ (ขนาด 2.0 x 2.0 x 1.0 มม.) นำชิ้นเนื้อผ่านกระบวนการแช่แข็งด้วยวิธีลดอุณหภูมิแบบช้าในภาชนะสำหรับแช่แข็ง และแช่แข็งด้วยวิธีวิทริฟิเคชัน หลังการเก็บชิ้นเนื้อไว้ 1 ปี นำชิ้นเนื้อที่ผ่านกระบวนการแช่แข็งทั้งสองวิธี ๆ ละ 3 ชิ้นเนื้อ มาละลายและแยกฟริแอนทรัลฟอลลิเคิลออกจากชิ้นเนื้อ ประเมินการมีชีวิต (โดยใช้การย้อมสีนิวทรัลเรด) และคุณภาพฟอลลิเคิลจากลักษณะภายนอกภายใต้กล้องสเตอริโอ เลือกฟอลลิเคิลที่มีคุณภาพดีและมีชีวิตไปเลี้ยงเป็นเวลา 7 วัน ในน้ำยาเอ็ม 199 ที่มี โกรทฮอร์โมน ฟอลลิคูล่าสตีมูลेटติ้งฮอร์โมน อินซูลินไลโคโทรทแพคเตอร์วัน และแอกทิวิตินเอ วัดขนาดเส้นผ่านศูนย์กลางและเส้นผ่านศูนย์กลางที่เพิ่มขึ้นในวันที่ 0 3 และ 7 ของการเลี้ยง ฟอลลิเคิลที่แยกได้จากเนื้อเยื่อรังไข่ที่ผ่านการแช่แข็งด้วยวิธีลดอุณหภูมิแบบช้ามีจำนวน 2๓ ฟอลลิเคิล และจากเนื้อเยื่อรังไข่ที่ผ่านการแช่แข็งด้วยวิธีวิทริฟิเคชันมีจำนวน 58 ฟอลลิเคิล ฟอลลิเคิลที่รอดชีวิตและนำไปเลี้ยงในน้ำยามีจำนวน 10 และ 12 ฟอลลิเคิลจากเนื้อเยื่อที่ผ่านการแช่แข็งสองวิธีดังกล่าวตามลำดับ ขนาดเส้นผ่านศูนย์กลางของฟอลลิเคิลที่ได้จากเนื้อเยื่อที่แช่แข็งด้วยวิธีลดอุณหภูมิแบบช้าค่อย ๆ ลดลงจาก 53.5±14.2 ไมโครเมตรในวันที่ 0 เป็น 50.9±17.1 ไมโครเมตรในวันที่ 7 และมีฟอลลิเคิลที่มีชีวิต 2 จาก 10 ฟอลลิเคิล ขนาดเส้นผ่านศูนย์กลางของฟอลลิเคิลที่ได้จากเนื้อเยื่อที่แช่แข็งด้วยวิธีวิทริฟิเคชันรักษาขนาดเส้นผ่านศูนย์กลางไว้ได้คงเดิมคือ 50.7±15.6 และ 50.5±17.9 ไมโครเมตรในวันที่ 0 และ 7 ตามลำดับ โดยมีฟอลลิเคิลมีชีวิต 2 จาก 12 ฟอลลิเคิล กล่าวโดยสรุป ควรใช้การแช่แข็งรังไข่ด้วยวิธีลดอุณหภูมิแบบช้าโดยใช้ภาชนะสำหรับแช่แข็ง และถึงแม้ว่าฟริแอนทรัลฟอลลิเคิลที่เก็บได้จากเนื้อเยื่อรังไข่ซึ่งผ่านการแช่แข็งสามารถเจริญภายนอกร่างกายได้นาน 7 วัน ควรมีการพัฒนาวิธีการแช่แข็งและศึกษาหาส่วนประกอบของน้ำยาที่เหมาะสมในการเลี้ยงฟริแอนทรัลฟอลลิเคิลเพื่อเพิ่มอัตราการรอดชีวิตและอัตราการเจริญเติบโต

คำสำคัญ: สัตว์ตระกูลแมว การแช่แข็ง การกักเซลล์สืบพันธุ์

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Introduction

Preantral follicle culture is a promising approach to restore fertility in human and endangered animals (Smitz et al., 2010). This tool provides great opportunity to preserve child-bearing potential in cancer patients, particularly pre-pubertal women prior to receiving a radiation or chemotherapy for treatment of cancers (Rodriguez-Wallberg and Oktay, 2012). Restoration of fertilizable follicles from cryopreserved ovarian tissues is feasible through a tissue re-implantation or follicle *in vitro* culture. Although ovarian implantation has exhibited achievements with birth of 18 human babies (Andersen et al., 2012; Wiedemann et al., 2012), risk of reintroduction of malignant cells back to recipients

has been concerned (Kim, 2010). In rare animal species, female gametes may be recovered when animals die accidentally or underwent spaying for medical reasons (Jewgenow and Paris, 2006). Rescue of gamete and ovarian cryopreservation followed by *in vitro* follicle culture offers promising approach for fertility preservation in these valuable animals. Live birth has been reported from *in vitro* culture of cryopreserved ovarian follicles in mice (Wang et al., 2011).

Decrease in populations mainly by habitat destruction and poaching, cheetah (*Acinonyx jubatus*) is classified as vulnerable by International Union for Conservation of Nature (IUCN, 2012). Although practical efforts with assisted reproductive techniques

(ART) including induction of folliculogenesis using hormones, *in vitro* oocyte maturation (IVM), *in vitro* fertilization (IVF), artificial insemination (AI) and embryo transfer (ET) have been utilized to improve reproductive capacity in captive population, success in giving live offspring of this vulnerable species is low (Pelican et al., 2006). Therefore, alternative approaches by conserving immature follicles in ovarian tissues to enhance propagation of the cheetah are essential to ensure existence of the species in the future. Current protocols for ovarian tissue cryopreservation have been categorized into 2 methods; conventional slow freezing and rapid freezing or vitrification (Isachenko et al., 2007). While the slow freezing has been employed as a standard protocol for preserving oocytes and various somatic tissues, vitrification is an alternative technique requiring lesser steps of freezing and freezing equipment, consequently simplifying cryopreservation process. Comparative outcomes of slow freezing and vitrification promoting ovarian graft survival after transplantation have been exhibited in monkeys (Yeoman et al., 2005) and mice (Kim et al., 2011). In felids, successful ovarian cryopreservation has been demonstrated in domestic cats (Lima et al., 2006) and lions (Wiedemann et al., 2012) using programmable slow freezing. Recently, a passive freezing container has replaced a programmable freezer to convey the slow freezing rate. However, application of the device has not been investigated in cats.

In vitro follicle culture in felids has been reported in the domestic cats indicating beneficial effects of culture medium, proteins, gonadotropins and ovarian growth factors on promoting preantral follicle growth and viability (Jewgenow and Pitra, 1993; Jewgenow, 1996; Wongbandue et al., 2013). In addition, secondary follicles were shown capable of developing to antral stage *in vitro* (Jewgenow and Pitra, 1993). However, these investigations limited only in preantral follicles collected from fresh ovarian tissues of the domestic cats, and have not been studied in other wild species. This study aimed to: 1) compare two freezing methods for ovarian tissues retrieved from a cheetah post-mortem, and 2) perform *in vitro* growing of preantral follicle extracted from cryopreserved ovarian tissues.

Materials and Methods

All chemicals and reagents used in this study were purchased from Sigma-Aldrich Company (St. Louis, MO, USA), unless otherwise stated.

Animal and collection of ovaries: A pair of ovary was obtained from a cheetah which died in captivity at Khaokeow Open Zoo (Chonburi, Thailand). Ovaries were removed after 3 hours of necropsy, stored in 0.9% (v/w) normal saline solution (NSS) at 4°C, and shipped to the laboratory in 29 hours. Upon arrival, connective tissues and blood vessels were trimmed off and ovaries were washed 3 times in NSS supplemented with 100 IU/ml penicillin and 0.1 mg/ml streptomycin. Each ovary was cut into small

pieces of 2.0x2.0x1.0 mm³. Pieces of cortical tissues were then allocated to be frozen using a passive cooling container (n = 3) or vitrification (n = 3).

Cryopreservation

Slow freezing and thawing: Ovarian cortices were incubated in cryopreservation medium containing 1.5 M dimethyl sulphoxide (DMSO) and 0.1 M sucrose in phosphate buffered solution (PBS) at 4°C for 15 min. Thereafter, the tissues were placed into cryovials and incubated for 15 min at 4°C. Next, vials containing ovarian cortices were transferred into pre-cooled passive cooling device (Coolcell, Biocision, Lakspur, CA, USA) and placed in -80°C freezer for 24 hours to achieve cooling rate of -1°C/min. Afterward, frozen vials were stored in liquid nitrogen (-196°C) until analyzed. Thawing of cryopreserved tissues was performed according to Cleary et al. (2001). In brief, cryovials were removed from liquid nitrogen and placed in a water bath at 37°C for 3 min. The tissues were then incubated in a thawing medium (PBS added with 0.75 mol/l DMSO and 0.2 mol/l sucrose) for 10 min at room temperature before being transferred to a dissection medium (M199 supplemented with 25 mM HEPES, 0.23 mM sodium pyruvate, 2 mM L-glutamine, 0.3% (w/v) bovine serum albumin (BSA), 100 IU/ml penicillin, and 0.1 mg/ml streptomycin).

Vitrification and warming: Vitrification of ovarian tissues was modified from a previous report using this method for freezing testicular tissue (two-step freezing technique) (Thuwanut and Chatdarong, 2012). The tissues were incubated in an equilibration medium containing HEPES M199 supplemented with 7.5% (w/v) DMSO, 7.5% (w/v) ethylene glycol (EG) and 20% (v/v) fetal calf serum (FCS) at room temperature for 15 min, followed by transferring into a vitrification medium (HEPES M199 supplemented with 15% (w/v) DMSO, 15% (w/v) EG and 0.5 M sucrose) at 4°C for 15 min. Subsequently, the ovarian tissues were immersed into liquid nitrogen. For thawing, cryovials were immersed in a waterbath at 37°C for 3 min. Thawed tissues were then placed into a warming medium containing 1 M sucrose and 20% FCS in HEPES M199 at 37°C for 10 min.

Preantral follicle isolation and selection: Preantral follicles were isolated from ovarian tissues by mechanical technique. In brief, frozen-thawed ovarian tissues were placed on a petridish containing the dissection medium. Thereafter, they were held with surgical forceps and finely sliced with surgical blades and needles. Isolated preantral follicles of normal morphology, characterized by an intact basement membrane, round or oval in shape, and absence of pigmented granulosa cells were selected under a stereomicroscope (SMZ645, Nikon, Japan) at x 50 magnification. Follicle viability was determined by staining with 50 µg/ml neutral red (38°C, 20 min) (Wongbandue et al., 2013). Only morphologically normal and viable follicles stained red were chosen for *in vitro* culture.

Culture of preantral follicles: Selected preantral follicles were washed in the dissection medium before

transferring to a culture medium that composed of M199 supplemented with 12.5 mM HEPES, 0.23 mM sodium pyruvate, 2 mM L-glutamine, 0.3% (w/v) BSA, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, 1% (v/v) insulin-transferrin-selenite solution (ITS), 2.13 µg/ml follicle stimulating hormone (FSH), 10 ng/ml insulin-like growth factor I (IGF-I), 1.0 mIU/ml growth hormone (GH) and 10 ng/ml activin A (rhAct A; R & D Systems, Abingdon, UK) (Wongbandue et al., 2013). The follicles were individually placed into 20-µl drops of medium, which were prepared in tissue culture dishes and pre-equilibrated for 2 hours at 38.5°C, 5% CO₂ in air. Thereafter, the droplets containing follicles were overlaid with mineral oil and incubated at 38.5°C, 5% CO₂ in air for 7 days. Every two days, half of the medium was replaced with freshly prepared medium.

Assessment of preantral follicle growth: To allow assessment of follicle growth, photographs of each follicle were taken on Days 0, 3 and 7 using an inverted microscope (CKX41, Olympus, Japan) installed with a digital microscopic camera (DP20, Olympus, Japan). Mean diameters of follicles were determined with assistance of the program DP2-BSW (Olympus, Japan) by measuring 2 maximum diameters perpendicularly through the center of each follicle. In addition, diameter gains were calculated by subtractions of the follicle diameters between Days 0 and 3, and Days 3 and 7.

Viability assessment: On Day 7, follicles were examined for viability by staining with 50 µg/ml neutral red for 20 min and immediately assessed under an inverted microscope (CKX41, Olympus, Japan) at x 400 magnification. Follicles with >50% granulosa cells stained red in cytoplasmic lysosome were classified as viable.

Results and Discussion

A total of 23 and 58 preantral follicles were isolated from cryopreserved ovarian tissues underwent slow freezing and vitrification, respectively. The pattern of preantral follicle growing is represented in Fig 1. The preantral follicles retrieved from slow freezing exhibited viability of 43% (10 of 23) compared to 21% (12 of 58) of those vitrified (Table 1). On Day 0, the initial diameters of the selected follicles were 53.4±4.5 µm and 50.7±4.5 µm in slow freezing (n = 10) and vitrification group (n = 12), respectively (Fig 2A). The follicles in the slow freezing group showed decreased diameter during Days 3 to 7 compared to the vitrification group (Fig 2B). After frozen-thawed, follicles recovered from ovarian tissues cryopreserved using the slow freezing method presented higher percentages of viability than the vitrification group (Table 1). However, after 7-day culture, the percentages of viability were similar between the two groups.

The present study is the first to demonstrate the survival of cheetah preantral follicles after ovarian tissue was cryopreserved and cultured for 7 days. Ovarian tissue cryopreservation has been performed in wildlife post-mortem, including elephants (Gunasena et al., 1998), wombats (Cleary et al., 2004)

and lions (Wiedemann et al., 2012). The constraints of this technique in wild animals usually involve health of the animals prior to ovarian recovery (Johnston et al., 1991), duration of organ transportation and storage temperature. Moreover, time delay between animal's death and ovarian recovery resulted in dramatic decrease in survived follicles (Cleary et al., 2001). Miao et al. (2007) revealed an increase in granulosa cell apoptosis leading to follicle and oocyte degeneration when excision of ovaries was delayed up to 30 minutes in mice carcasses. Duration and temperature during transportation affecting viability and morphology of preantral follicles was demonstrated in dogs (Lopes et al., 2009). In line with the above reports, health status, duration of time from animal's death to ovarian recovery and transportation of the cheetah in this study contributed to initial poor condition of preantral follicle which might account for small numbers of follicles recovered from cryopreserved ovarian tissues.

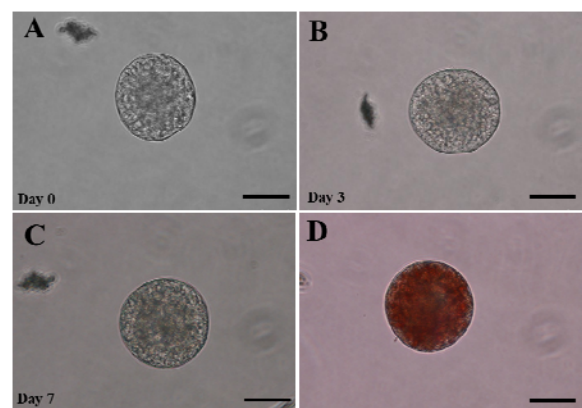


Figure 1 Vitrified cheetah preantral follicle after frozen-thawed (A), after 3 days (B) and 7 days of *in vitro* (C) and stained red with neutral red on Day 7 of culture (D). Bar = 50 µm.

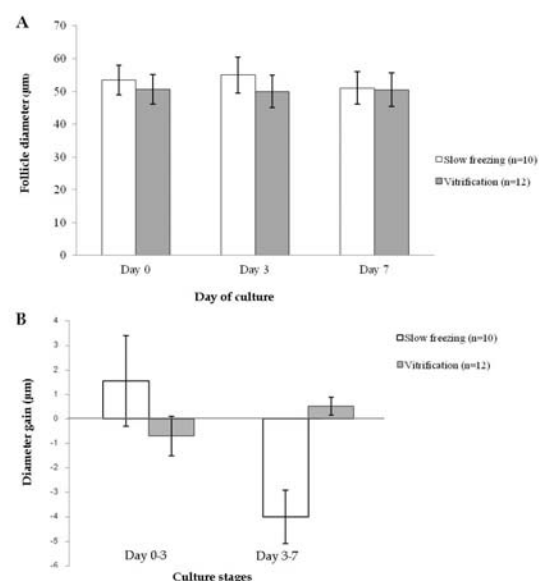


Figure 2 Diameter (A) and diameter gain (B) of cryopreserved cheetah preantral follicles during 7 days of *in vitro* culture.

Table 1 Percentages of viable preantral follicles extracted from ovarian tissues cryopreserved using slow freezing and vitrification before and after cultured for 7 days.

Freezing techniques	Isolated follicles (N)	% Viable follicles (N)	
		Day 0	Day 7
Slow freezing	23	43 (10)	20 (2)
Vitrification	58	21 (12)	17 (2)

In the present study, the proportion of viable cheetah follicles in the slow freezing group (43%) was comparable to that in the lions (37-59%) (Wiedemann et al., 2012) and the domestic cats (39%) (Lima et al., 2006). In addition, the survival rates of preantral follicles isolated from vitrified-warmed ovarian tissues were slightly higher than the previous study in the cats (21% vs 18%, respectively) (Galiguis et al., 2012). However, assessment of follicle viability in those studies was based on histological morphology and sizes of preantral follicles within ovarian tissues, whereas the follicle viability in our study indicated physiological function by cell uptake of non-toxic dye neutral red. The ovarian tissues frozen using the slow freezing resulted in a higher post-thawed survival rate of preantral follicles compared to the vitrified-warmed ovarian tissues in this study. Although a recent histological study in monkey indicated an advantage of vitrification preserving ovarian stromal integrity and intact follicle morphology above slow-freezing (Ting et al., 2011), high cryoprotectant concentration was used. The decrease in follicle viability and estradiol production has been reported in the vitrified-warmed ovarian tissues compared to the slow freezing group (Milenkovic et al., 2012). Taken together, the lower number of viable follicles in this study was likely contributed to cryoprotectant toxicity in the vitrification medium.

Advances in *in vitro* preantral follicle culture have been reported in various species including human (Telfer et al., 2008), primate (Xu et al., 2009b), ovine (Arunakumari et al., 2010) and murine (Wang et al., 2011) whereas developments in felids are limited. In felid species, achievements of *in vitro* follicle culture were demonstrated only in the domestic cats (Jewgenow and Pitra, 1993; Wongbandue et al., 2013). In addition, this study revealed the use of FSH and growth factors (IGF-I and activin A) supplements in the culture medium according to Wongbandue et al. (2013) in the domestic cats. However, the beneficial effects of these supplements were not obviously presented in *in vitro* culture of cheetah follicles during 7-day culture period. The survival rate of these cryopreserved ovarian follicles (17% and 20%) after 7 days of culture were lower than that of the fresh follicles of domestic cats (46.9%) (Wongbandue et al., 2013). The results were likely contributed to the damage caused by cryopreservation process.

Proliferation and differentiation of granulosa cells are controlled by oocytes, while development and meiotic transcription of oocytes are regulated by granulosa cells (Matzuk et al., 2002). These regulatory

processes require communication of oocytes and granulosa cells via gap junctions (Navarro-Costa et al., 2005). The present study demonstrated that the slow freezing was more likely to preserve structural integrity of granulosa cells and oocytes. However, investigation of expression levels of gap junction protein genes; connexin 37 (Gja4) and connexin 43 (Gja1) which facilitated interaction of granulosa-granulosa cells and oocyte-granulosa cells, is required to confirm the viability and function (Xu et al., 2009a). Cryopreservation has been shown to induce mitochondrial deformities and formation of vacuoles in the zona pellucida (Kim et al., 2011). The freezing also impaired cellular organelles and caused failure of meiotic spindle stabilization in the oocytes (Camboni et al., 2008). These effects influence physiological function of preantral follicle compartments and could account for the low survival and growth rate of cryopreserved ovarian follicles culture *in vitro* in this study.

In conclusion, the preantral follicles retrieved from ovarian tissues of the cheetah 29 hours post-mortem survived after cryopreservation using the slow freezing and vitrification. In addition, the technique of slow freezing with passive cooling container supported viability of the follicles post-thawing better than vitrification. This study also presents the ability of cheetah preantral follicles to grow *in vitro* up to 7 days. The report represents possibility of female gamete rescue in the felid species post-mortem.

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