Original article

Mouse Ovarian Tissue Vitrification: effects of exposure time to cryoprotective agent

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Objective The objective of this study was to compare the survival rate and growth rate of isolated preantral follicles from vitrified/warmed mouse ovarian tissues with different exposure times to Vitrification Solution 2 (V2).

Methods Mouse ovaries were divided into a control and four experimental groups. All four experimental groups were vitrified and warmed with exposure times to V2 solution for 3, 5, 10 and 15-minutes. Preantral follicles were mechanically isolated from the vitrified/warmed ovarian tissue and individually cultured in vitro in 10-µL drops of culture medium under paraffin oil. Follicle diameter was measured every two days for 12 days. Primary outcome measurements were the survival rate and growth rate of the isolated preantral follicles.

Results Preantral follicles from vitrified/warmed ovarian tissues that were exposed to V2 solution for five and ten-minutes had the highest survival rates (73.20% and 72.17%, respectively). The three and fifteen-minute exposure groups had survival rates of only 65.37% and 56.55%, respectively. There was no difference in the mean diameter of the follicles during the first eight days. On day ten, the mean follicular diameter of the fifteen-minute group was lower than the control group (p<0.001). On day 12, diameters of the three and fifteen-minute groups were lower than the control group (p=0.005 and p<0.001, respectively). The preantral follicles from the five and ten-minute groups had growth rates comparable to the control group

conclusion Exposure of mouse ovarian tissues to V2 solution for five and ten-minutes yields the highest survival rate and growth rate of preantral follicles, while both longer and shorter exposures adversely affects the survival and subsequent development of preantral follicles. **Chiang Mai Medical Journal 2016;55(4):143-51.**

keywords: ovarian tissue, exposure time, vitrification solution

Introduction

Advances in the treatment of childhood and adult cancers have resulted in increased life expectancy, especially in young and adolescent female patients[1]. However, radiotherapy and chemotherapy treatment for ovarian cancer can damage the ovaries and may result in ovarian failure, infertility and sex steroid hormone depletion in later life[2-4]. For that reason, the preservation of ovarian function in female cancer patients who undergo radiotherapy or

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chemotherapy is an important issue[5-8].

Several methods of assisted reproductive technology have been developed to preserve the fertility of these patients. Long-term experience and good outcomes with oocyte and embryo cryopreservation prior to gonadotoxic treatment has made this a standard treatment option[6,9]. However, neither method is suitable in the following cases: (1) pre-pubertal girls whose ovaries are still immature and not responsive to ovarian stimulation; (2) cancer patients in need of immediate treatment who cannot spare two weeks or more for ovarian stimulation and oocyte harvest; and (3) women with estrogen-sensitive or estrogen-receptor-positive cancer, as the high estrogen level during ovarian stimulation may promote progression, metastasis or recurrence of the cancer[10]. In those cases, ovarian tissue cryopreservation is an a potentially attractive alternative.

Ovarian tissue cryopreservation can be performed at any time during the menstrual cycle. It is a promising investigational method that holds potential to become a standard treatment in the near future[9]. The tissue can be warmed and transplanted into the patients after the disease is cured and fertility treatment is desired. The transplanted tissues can produce both ovarian hormones and oocytes. As a result, patients can have their own biological children after surviving cancer[10-14]. Alternatively, ovarian follicles can be isolated from cryopreserved tissue and cultured in vitro[15].

Ovarian tissue cryopreservation is more difficult and challenging than oocyte/embryo cryopreservation due to the presence of heterogenous cellular components (oocytes, granulosa and stromal cells) with different cryobiological properties. The success of ovarian tissue cryopreservation depends on many factors including the types and concentrations of cryoprotective agents (CPAs), exposure time to the CPAs, the size of the ovarian tissue fragments and the speed of cooling and warming. Cryoprotectant is one of the most important factors for vitrification, in terms of its cryobiological properties and toxic effect to cells. Ethylene glycol (EG) and dimethyl sulfoxide (DMSO) are a very effective combination and have been used as the primary cryoprotectants in many studies[16-18]. However, although all the studies used the same type of tissue (mouse ovary) in their studies, exposure times to the cryoprotectant varied[16-18]. This leaves open the question whether exposure time to cryoprotectants may be a critical factor in the success of vitrification. As vitrification employs a high concentration of cryoprotectants, it is generally considered best to limit exposure time as much as possible[19,20]. However, exposure time must be long enough to allow the cryoprotectant to become distributed evenly throughout the tissue.

We are interested in ovarian tissue cryopreservation. In this study, our primary focus was on the effect of exposure time to the vitrification solution on the survival rate of preantral follicles and their subsequent development competency. A mouse model was employed as mouse ovarian tissues have a similar thickness to human ovarian cortex. The results of this study are intended to form the basis for future studies on human ovarian tissue vitrification.

Materials and methods

Outbred ICR mice were purchased from the National Animal Institute, Mahidol University, Bangkok, Thailand. They were kept at the Animal Husbandry Unit, Faculty of Medicine, Chiang Mai University, in a well-ventilated room at 25±2 °C, under 60-70% humidity with controlled 12 hour light/dark cycles. Before the experiment, they were left undisturbed for five days to avoid possible effect from the stress of transportation. The local Animal Ethics Committee of the Faculty of Medicine, Chiang Mai University, approved this study.

The five- to seven-week old ICR female mice were used in this study were sacrificed by cervical vertebrae dislocation following standard guidelines. Both whole ovaries from each animal were collected, and the experiment was carried out immediately.

1. Vitrification and Thawing Solutions

Vitrification and thawing solutions were modified from Youm *et al.*[16] as follows:

Vitrification Solution 1 (V1) was composed of phosphate buffered solution (PBS, GIBCO, NY, USA) containing 20% fetal bovine serum (FBS, Sigma, St Louis, MO), 7.5% ethylene glycol (EG) and 7.5% dimethyl sulfoxide (DMSO).Vitrification Solution 2 (V2)

was composed of PBS containing 20% FBS, 20% EG, 20% DMSO and 0.5M sucrose.

Thawing Solution 1 (T1) was composed of PBS, 20% FBS and 1M sucrose.

Thawing Solution 2 (T2) was composed of PBS, 20% FBS and 0.5M sucrose.

2. Ovarian tissue vitrification

A total of 80 ovaries from 40 female mice were collected and kept in our in-house culture medium before vitrification. In each of eight experiments, an ovary (size ~ 2x2x2 mm^3) was divided, using a surgical blade, into one control group and four experimental groups as follows:

1. Group 1: exposed to V2 solution for 3 minutes.
2. Group 2: exposed to V2 solution for 5 minutes.
3. Group 3: exposed to V2 solution for 10 minutes.
4. Group 4: exposed to V2 solution for 15 minutes.
5. Group 5: non-vitrified ovarian tissue (control).

In each of the experimental groups, ovarian tissues were equilibrated in V1 solution for 25 minutes at room temperature. They were then transferred into V2 solution at room temperature, with four different exposure times as previously described. Ovarian tissues with a thin-film of vitrification solution were placed on an aluminum foil spoon (Figure 1), and immediately plunged into liquid nitrogen. The ovarian tissue pieces were kept in cryovials and stored in a liquid nitrogen tank for a minimum of two weeks.

In the warming process, the aluminum foil spoon containing a piece of vitrified ovarian tissue was removed from the liquid nitrogen and immediately immersed in T1 solution at 37 °C for one minute. The ovarian tissue was then moved into T2 solution at room temperature for five minutes. All post-thaw ovarian tissues were equilibrated in culture media for at least ten minutes to clear all remaining cryoprotectants before follicle isolation.

3. Follicle isolation and in vitro culture

Vitrified/warmed ovarian tissue pieces were individually dissected under a dissecting microscope. Preantral follicles were mechanically isolated using a 27-G needle attached to a tuberculin syringe. All morphologically normal preantral follicles larger than 70 µm were collected for in vitro culture.

Each isolated preantral follicle was rinsed three times in culture medium then transferred into a 10-µL drop of culture medium under paraffin oil (Figure 1). The follicles were cultured individually for 12 days at 37 °C, 100% humidity, under an atmosphere of 5% O_2, 6% CO_2, and 89% N2. The culture medium was adapted from Fatehi *et al*[17] and Hasegawa *et al*[21], and consisted of alpha-minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum albumin, 100 mIU/mL recombinant FSH (Puregon, MSD), 5 mg/mL insulin, 5 mg/mL transferrin, 5 ng/mL sodium selenite (Gibco), 10 U of penicillin, 10 µg of streptomycin and 0.23 mM sodium pyruvate.

Every two days 5 µL of the culture medium was removed and replaced with fresh medium. Follicle diameter, including the granulosa-theca cell layer, was measured every other day under an inverted microscope (Nikon, Germany), using a program that accompanied the LYKOS clinical laser system. Follicles were considered viable when oocytes were clear and intact, and the granulosa-theca cell layer formed more than 50% of the follicle diameter.

4. Statistical analysis

The numbers of surviving preantral follicles from vitrified/warmed ovarian tissues from the four experimental groups and the control group were compared by Chi-square or Fisher exact test, as appropriate. The mean diameters of the follicles were compared by a general linear model repeated measures ANOVA, with Scheffe post-hoc tests as appropriate. Statistical anal-

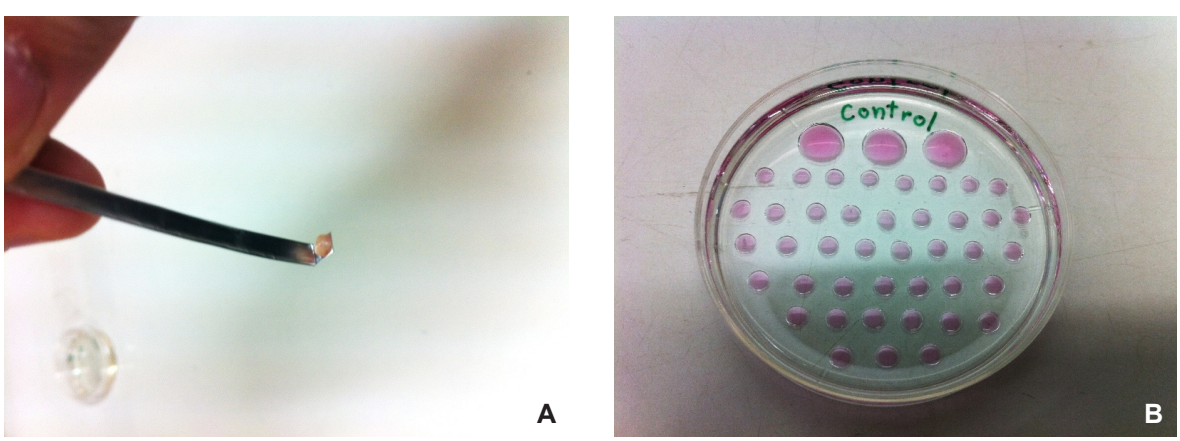

Figure 1. (A) Ovarian tissue placed on an aluminum foil spoon. (B) In vitro culture plate.

Table 1. Survival rates of preantral follicles from vitrified/warmed mouse ovarian tissues exposed to vitrification solution (V2) for different time periods and from the non-vitrified control group after 12 days of in vitro culture.

Group	Number of preantral follicles			Survival ratea
	Total	Death	Survive	
Control	353	32	321	90.94%
3-minute (group 1)	387	134	253	65.37%
5-minute (group 2)	362	97	265	73.20%
10-minute (group 3)	345	96	249	72.17%
15-minute (group 4)	336	146	190	56.55%

[a]Chi-square test, $p<0.001$

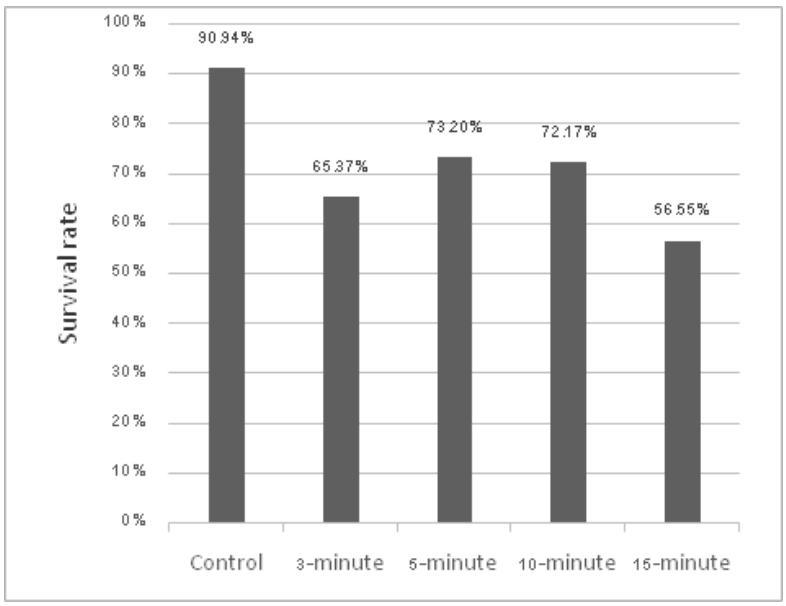

Figure 2. Survival rate of preantral follicles from vitrified/warmed mouse ovarian tissues that were exposed to vitrification solution (V2) for different time periods and the non-vitrified control group after 12 days of in vitro culture.

yses were performed using STATA program, version 8.2 (College Station, Texas). A two-tailed *p*-value < 0.05 was considered to indicate statistical significance.

Results

Eighty ovaries from 40 mice were included in the study. A total of 1,783 preantral follicles larger than 70 µm were mechanically isolated and divided into the four experimental groups and one control group (Table 1).

In the non-vitrified (control) group, 321 out of 353 (90.94%) of the preantral follicles survived after 12 days of in vitro culture. In the experimental groups, preantral follicles from vitrified/warmed ovarian tissues that were exposed to vitrification solution (V2) for five and ten minutes had the highest survival rates (73.20% and 72.17%, respectively), with no statistically significant difference between the two groups (p=0.759). Preantral follicles from vitrified/warmed ovarian tissues that were exposed to V2 for three and fifteen minutes had survival rates of only 65.37% and 56.55%, respectively (Table 1 and Figure 2).

To assess capacity for follicular growth, surviving preantral follicles were measured every other day for up to 12 days (Figure 3).

Baseline follicle size at day zero was not significantly different between groups (p=0.069) (Table 2). In all groups, the follicular diameter

Table 2. Growth of preantral follicles [mean diameter (µm) ± SD] from non-vitrified control group and vitrified/warmed mouse ovarian tissues that were exposed to vitrification solution (V2) for different time periods.

Group \ Day	0[a]	2	4	6	8	10[b]	12[c]
Control	95.60 ± 18.01	109.23 ± 25.26	137.80 ± 49.39	180.70 ± 75.51	235.46 ± 96.90	284.02 ± 107.13	318.13 ± 115.13
3 min (gr. 1)	93.68 ± 16.37	104.37 ± 25.34	139.91 ± 56.93	187.01 ± 81.65	231.57 ± 92.56	268.17 ± 93.88	296.19 ± 97.08
5 min (gr. 2)	97.51 ± 17.93	107.66 ± 23.55	142.43 ± 50.80	189.05 ± 79.91	249.09 ± 97.64	293.24 ± 106.12	317.09 ± 107.76
10 min (gr. 3)	97.91 ± 20.77	112.17 ± 29.24	147.22 ± 57.80	198.40 ± 85.79	251.40 ± 104.61	292.87 ± 106.99	327.50 ± 100.03
15 min (gr. 4)	95.14 ± 19.11	107.42 ± 23.90	130.80 ± 42.11	171.48 ± 70.65	211.37± 88.90	240.58 ± 98.58	271.34 ± 108.81

[a]ANOVA test, p=0.069
[b]ANOVA test, p<0.001; post-hoc test indicated a difference between control group and group 4 (15-minute exposure)
[c]ANOVA test, p<0.001; post-hoc test indicated a difference between control group and group 4 [15-minute exposure (p<0.001)], and between control group and group 1 [3-minute exposure p=0.005)]
min = minutes; gr. = group

increased continuously until day 12 (Figure 4).

There was no significant difference in the mean diameter of follicles from vitrified/warmed ovarian tissues compared to the non-vitrified control group from day zero to day eight (Table 2). On day ten, the mean follicular diameter of group 4 (15-minute exposure) was significantly lower than that in the non-vitrified control group (ANOVA test; p<0.001). On day 12, the mean follicular diameters of group 1 (3-minute exposure) and group 4 (15-minute exposure) were significantly lower than those in the non-vitrified control group (ANOVA test; p=0.005 and p<0.001, respectively). In contrast, the preantral follicles from group 2 (5-minute exposure) and group 3 (10-minute exposure) had follicular diameters comparable to the non-vitrified control group from baseline (day zero) up to day 12 of in-vitro culture.

Discussion

Ovarian tissue cryopreservation provides an alternative to fertility preservation for patients who are not suitable candidates for controlled ovarian stimulation and oocyte/embryo cryopreservation. Although this method is still experimental, there have been reports from many centers of live births from vitrified/warmed ovarian tissue autotransplantation[12].

Ovarian tissue cryopreservation can be performed using either standard slow programmable freezing or vitrification. Many studies found that vitrification had equal or better results than slow freezing in terms of follicular morphological integrity[22], estradiol production, follicular proliferation and apoptotic rates[23]. As vitrification is simple, takes less time to perform, and does not require an expensive computerized controlled-rate freezer, it is emerging as the method of choice for ovarian tissue cryopreservation.

The presence of various cellular components (oocytes, granulosa and stromal cells) in ovarian tissue makes cryopreservation challenging, especially with vitrification which requires exposure to high concentrations of cryoprotective agents.

Our study evaluated effects of exposure

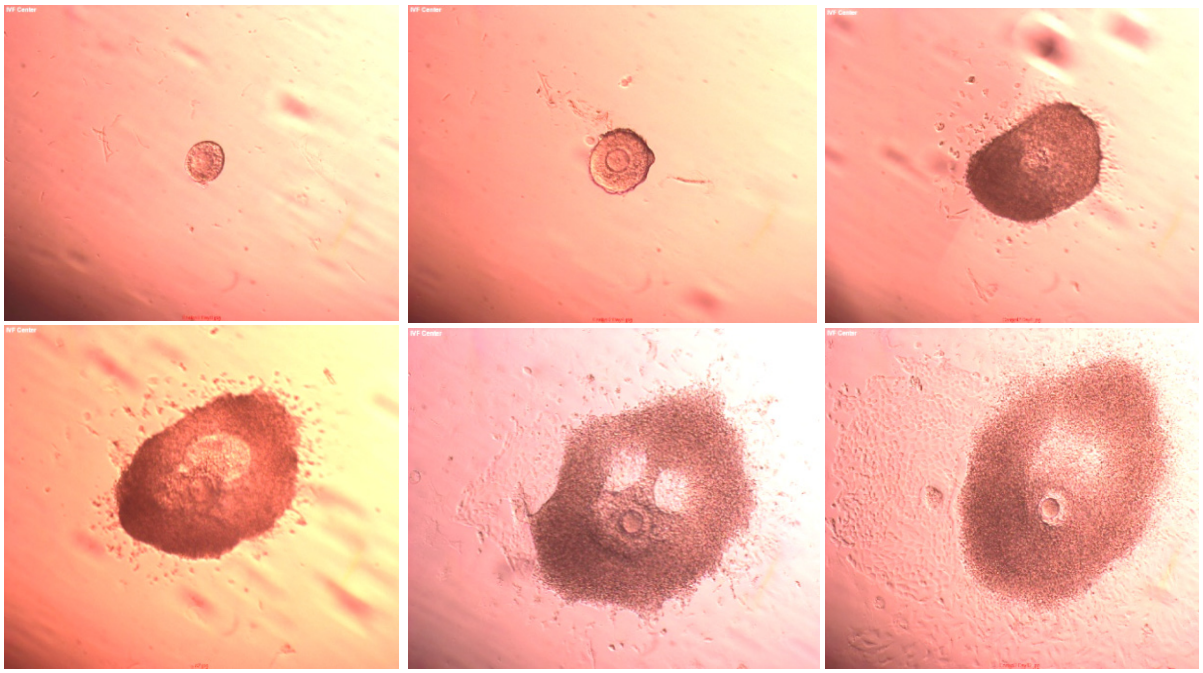

Figure 3. Preantral follicle growth the in non-vitrified [*groups (A-E)?] and the control group [*(F)?] (day 0, 4, 6, 8, 10 and 12)

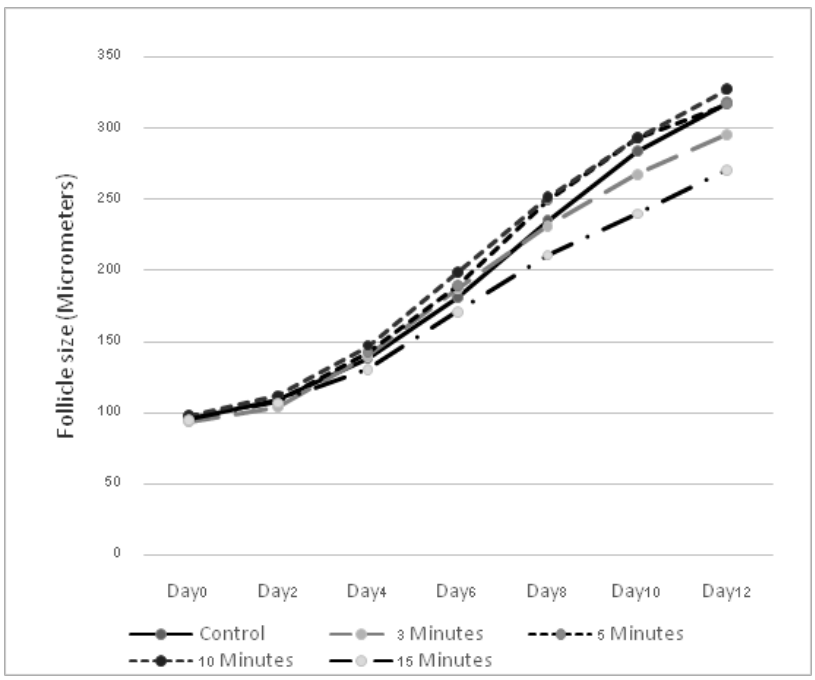

Figure 4. Growth rate of preantral follicles from control and vitrified/warmed mouse ovarian tissues that were exposed to vitrification solution (V2) for different time periods

time of ovarian tissues to vitrification solution on preantral follicle viability and developmental competency after warming. We chose mouse ovarian tissue for this study because it was easy to acquire and adequate numbers of preantral follicles could be obtained from the

tissues. In contrast, donated human ovarian specimens were scarce and most of them had few preantral follicles, as they often came from ageing females who had previously undergone gynecological surgery for benign or malignant conditions.

We found that 90% of the follicles from the non-vitrified controls were viable, which is comparable to other studies[17]. The spontaneous demise of some preantral follicles in our study could be due to two factors. First, the process of follicular atresia is known to be a part of normal follicular development, and hence the loss of some follicles through this natural process is inevitable. The second factor could be due to our suboptimal culture conditions. Fatehi *et al*[17] showed that during preantral follicular growth, cavitation was observed in almost all follicles. However, in this study, we did not find any antral formation in the follicles. Unlike the natural state where follicles in the ovary are growing in a three-dimensional organization, our culture system provided only a two-dimensional support for growth. It is possible that a culture system with a three-dimensional scaffold or an alginate encapsulation, or a multi-step culture system that provides more natural physiological conditions may solve this problem[15].

Our study confirmed that appropriate exposure time to vitrification solution was important. Too short an exposure time to vitrification solution could limit the penetration of cryoprotectants into the entirety of the ovarian tissues, while overly long exposure to the vitrification solution could exert a toxic effect on the cells.

For growth rate assessment, post vitrified/warmed preantral follicles from the 5- and 10- minute V2 exposure groups had a growth rate comparable to that of non-vitrified controls throughout the entire 12 days of in vitro culture. The growth rates of follicles from the 3- and 15-minute V2 exposure groups were lower than the controls and showed a significant difference on day 12 of the culture. This indicates that both under- and over-exposure of ovarian tissues to vitrification solution not only adversely affects the survival rates of follicles but also their subsequent development. Our study suggests that over-exposure could be more detrimental than under-exposure in terms of immediate survival and subsequent growth of the follicles.

From our data, we found that exposure of mouse ovarian tissues to V2 for 5 and 10 minutes was the optimal time in terms of survival and growth rate.

There are many limitations to our study. We used the survival rate and subsequent growth rate of the follicles as the end points in our study. However, it is possible that follicles could have survived cryopreservation but partially been damaged to the extent that they lost the ability to undergo normal development. The diameter of the follicles as measured in our study reflected only granulosa-thecal cell proliferation and expansion but did not represent true development of the follicles as we observed no atrial formation inside these follicles. Also, we did not evaluate other components of the vitrified/warmed ovarian tissues such as steroid hormone production by the granulosa cells. Ideally, investigation should include the number of metaphase II oocytes, fertilization rate, blastocyst formation rate as well as implantation and live birth rate as part of the monitoring of the success of ovarian tissue cryopreservation. However, due to limitations of skills, knowledge and time, we were not able to perfect our culture system to achieve those outcomes. Additionally, one should be very cautious in applying the results of this study to human ovarian tissue cryopreservation because there is much dissimilarity between them, e.g., mouse ovaries have a looser structure than human ovaries. In addition, appropriate exposure time to cryoprotectants depends on the permeability of the tissues. Tissue size, thickness and density could also have a direct impact on the permeability of cryoprotectants. All these factors should be considered in future experiments.

Acknowledgments

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ผลของระยะเวลาการสัมผัสกับน้ำยาแช่แข็งในการแช่แข็งเนื้อเยื่อรังไข่หนูแบบเนื้อแก้ว

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วัตถุประสงค์ เพื่อเปรียบเทียบอัตราการอยู่รอดและอัตราการเจริญเติบโตของ preantral follicles ในเนื้อเยื่อรังไข่หนูที่ผ่านการแช่แข็งแบบเนื้อแก้ว เมื่อสัมผัสกับน้ำยาแช่แข็ง (Vitrification Solution 2: V2) ในระยะเวลาที่แตกต่างกัน

วิธีการศึกษา รังไข่ของหนูตัวเมียแต่ละข้างจะถูกตัดครึ่งและถูกนำมาแบ่งออกเป็นสี่กลุ่มการศึกษา โดยทั้งสี่กลุ่มการศึกษาจะผ่านการแช่แข็งแบบเนื้อแก้ว โดยแช่ในน้ำยา V2 ในระยะเวลาที่ต่างกัน คือ 3, 5, 10 และ 15 นาที หลังจากละลายออกมาเนื้อเยื่อรังไข่หนูจะถูกนำมาแยกเอาเฉพาะส่วน preantral follicles ที่ขนาดมากกว่า 70 µm มาเพาะเลี้ยงเดี่ยวในหยดน้ำยาเลี้ยงตัวอ่อนเป็นเวลา 12 วัน เปรียบเทียบกับกลุ่มควบคุมซึ่งเนื้อเยื่อรังไข่หนูไม่ได้ผ่านการแช่แข็ง ทำการวัดขนาดและสังเกตการอยู่รอดของ Preantral follicles วันเว้นวัน ตั้งแต่วันที่ 0 ถึง วันที่ 12

ผลการศึกษา ในกลุ่มควบคุมมีอัตราการอยู่รอดของ preantral follicles อยู่ที่ร้อยละ 90.94 ในกลุ่มทดลองที่เนื้อเยื่อรังไข่หนูผ่านการสัมผัสน้ำยา V2 นาน 5 และ 10 นาที มีอัตราการอยู่รอดสูงที่สุด คือ ร้อยละ 73.2 และ 72.17 ตามลำดับ (p=0.759) ส่วนเนื้อเยื่อรังไข่หนูที่สัมผัสน้ำยา V2 นาน 3 และ 15 นาทีมีอัตราการอยู่รอดที่ร้อยละ 65.37 และ 56.55 ตามลำดับ ซึ่งต่ำกว่ากลุ่มที่สัมผัสน้ำยา V2 นาน 5 และ 10 นาทีอย่างมีนัยสำคัญทางสถิติ (p<0.001) ในแง่อัตราการเจริญเติบโตขนาดเริ่มต้นของ Preantral follicles ในแต่ละกลุ่มไม่มีความแตกต่างกัน (p=0.069) และเฉพาะ preantral follicles ที่รอดชีวิตเท่านั้นที่จะถูกนำมาวัดขนาด พบว่าในทุกกลุ่มทดลองและกลุ่มควบคุม preantral follicles จะมีการโตอย่างต่อเนื่องตลอด 12 วัน โดยไม่มีความแตกต่างกันในวันที่ 0 ถึงวันที่ 8 แต่ในวันที่ 10 ขนาดของ peantral follicles ในกลุ่มที่สัมผัสน้ำยา V2 นาน 15 นาที จะเล็กกว่ากลุ่มควบคุมอย่างมีนัยสำคัญ (p<0.001) และในวันที่ 12 ขนาดของ preantral follicles ในกลุ่มที่สัมผัสน้ำยา V2 นาน 3 และ 15 นาที จะเล็กกว่ากลุ่มควบคุมอย่างมีนัยสำคัญ (p=0.005 และ p<0.001 ตามลำดับ) ในขณะที่ขนาดของ Preantral follicles ในกลุ่มที่สัมผัสน้ำยา V2 นาน 5 และ 10 นาที มีขนาดใกล้เคียงกับกลุ่มควบคุมตลอดช่วง 12 วัน

สรุปผลการศึกษา เนื้อเยื่อรังไข่หนูที่สัมผัสกับน้ำยาแช่แข็ง V2 นาน 5 และ 10 นาทีมีอัตราการอยู่รอดของ Preantral follicles ดีที่สุด และมีอัตราการเจริญเติบโตของ preantral follicles ใกล้เคียงกับกลุ่มควบคุมมากที่สุด การสัมผัสกับน้ำยาแช่แข็งน้อยเกินไปหรือนานเกินไปมีผลเสียต่ออัตราการรอดและอัตราการเจริญต่อของ Preantral follicles **เชียงใหม่เวชสาร 2559;55(4):143-51.**

คำสำคัญ: เนื้อเยื่อรังไข่ ระยะเวลาการสัมผัส น้ำยาแช่แข็งแบบเนื้อแก้ว