Successful *In Vitro* Development of Preantral Follicles Isolated from Vitrified Mouse Whole Ovaries

Dong-Hoon Kim†, Jin-Gu No, Jong-Ju Park, Jin-Ki Park and Jae Gyu Yoo

Animal Biotechnology Division, National Institute of Animal Science, RDA, Suwon 441-706, Korea

ABSTRACT

The purpose of this study was to assess follicular viability and competence through *in vitro* culture of preantral follicles isolated from vitrified mouse whole ovaries. Mouse preantral follicles were enzymatically isolated from vitrified-warmed and fresh ovaries and cultured for 10 days followed by *in vitro* oocyte maturation. *In vitro* matured oocytes were fertilized and cultured to the blastocyst stage. Five minutes pre-exposure to vitrification solution of whole ovaries had significantly higher ($p<0.05$) oocyte survival and maturation rates than between 10 min exposure groups. Oocyte diameter was significantly smaller ($p<0.05$) in the 5 and 10 min exposure groups (69.4±2.8 and 67.8±3.1) when compared to that of control group (71.7±2.1). There was no statistical significant difference in blastocyst development rates between vitrification group (8.6%) and the fresh control group (12.0%). The mean number of cells per blastocyst was significantly lower ($p<0.05$) in the vitrification group (41.9±20.2) than in the fresh control group (55.1±22.5). The results show that mouse oocytes within preantral follicles isolated from the vitrified whole ovaries can achieve full maturation, normal fertilization and embryo development.

(Key words : Vitrification, Open straw (OS), Mouse ovary, Preantral follicle)

INTRODUCTION

Cryopreservation of ovaries is a promising technique for long-term fertility preservation for female. This technique would enable to preserve and restore ovarian function in cancer patients who subjected to radiotherapy or chemotherapy. In addition, the technique might be useful for protection of the reproductive potential in valuable and endangered species (Oktay *et al*., 1998).

Cryopreserved ovarian tissues could be used in the autologous transplantation and the production of mature oocytes derived from preantral follicles within ovarian tissues after culture. Pioneering studies on the cryopreservation of ovarian tissue were carried out in rodents. These experiments resulted in the birth of offspring after orthotopic transplantation of frozen-thawed ovarian tissue (Parrott, 1960). Over recent years, significant improvements of cryopreservation technique has been demonstrated successful cryopreservation of ovarian tissues in rat (Aubard *et al*., 1998), rabbit (Daniel *et al*., 1983), sheep (Gosden *et al*., 1994) and human (Gook *et al*., 1998; Oktay *et al*., 1998). In addition, preantral follicles isolated from frozen-thawed mouse tissue could be successfully grown to antral follicles and produced mature oocytes (Newton and Illingworth, 2001).

Slow freezing method was used in most previous studies on cryopreservation of ovarian tissue (Candy *et al*., 1997; Gunasena *et al*., 1997; Newton and Illingworth, 2001). However, it is a time-consuming procedure and requires special equipment like a programmable freezer. Vitrification is an alternative cryopreservation technique might be effective organ cryopreservation method. The vitrification technique is also a convenient tool for cryopreserving small specimens. High concentrations of cryoprotectants are necessary to solidify without the formation of ice crystals during rapid cooling process of vitrification. However, cryoprotectants are very toxic to both oocytes and cells. Cryoprotectant toxicity can be minimized by increasing the cooling rate. Furthermore, the high rates of cooling prevent chilling damage (Rall and Fahy, 1985). The traditional vitrification system using cryovial or cryotube could not achieved good result with mouse ovaries (Newton and Illingworth, 2001). Direct contact between the ovary containing medium and liquid nitrogen could increased cooling rate (Chen *et al*., 2006) and cryotop safeguard the ovarian tissues during rapid cooling process (Hasegawa *et al*., 2004; 2006).

The purpose of this study was to evaluate the developmental potential of preantral follicles in vitrified mou-

* This study was supported by 'Agenda program' of Rural Development Administration, Republic of Korea.

† Corresponding author : Phone: +82-31-290-1632, E-mail: kimdhhj@korea.kr

se ovaries. *In vitro* growth of preantral follicles isolated from vitrified ovaries followed by *in vitro* maturation, fertilization and culture were performed. In addition, these findings were compared to those of fresh controls.

MATERIALS AND METHODS

Collection of Ovaries

Ovaries from 12-day-old ICR mice were dissected free of fat and mesentery. Intact ovaries were transferred into Leibovitz L-15 medium (Gibco-BRL, Grand Island. NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL).

Vitrification and Thawing

Open straw was made by cut at the cotton plug part of 0.25 mL straws with a razor blade.

For vitrification, whole ovaries were exposed to Leibovitz L-15 medium supplemented with 20% FBS, 10% (v/v) dimethyl sulfoxide (DMSO; Sigma, St Louis, MO, USA) and 10% (v/v) ethylene glycol (EG; Sigma) for 5 minutes. The ovaries were subsequently moved to Leibovitz L-15 medium supplemented with 20% FBS, 0.5M sucrose (Sigma), 20% DMSO and 20% EG and equilibrated for 5 or 10 min. Loading of ovaries into these straws was performed using the capillary effect by simple touching a 3- to 5-μl droplet containing the ovaries with the end of the straw. The straws were submerged into liquid nitrogen immediately after loading.

Thawing was performed by placing the end of the straw directly into the 0.5 M sucrose solution (prepared in Leibovitz L-15 medium containing 20% FBS). The straw into the 0.5 M sucrose solution. And then recovered ovaries were sequentially transferred to 0.5, 0.25, 0.125 and 0 M sucrose solution at interval of 5 min. Each step was carried out at room temperature.

Preantral Follicle Isolation

Ovaries were immersed into Leibovitz L-15 medium containing 1 mg/ml collagenase (Type 1A, C-2674; Sigma) and 0.2 mg/ml DNase I (DN-25; Sigma) for 20 min at 37℃ and repeatedly drawn in and out of the pipette until the ovaries were dissociated into individual follicles. The follicles to be cultured were selected by the following criteria: i) intact round follicular structure with two to three layers of granulosa cells; ii) the oocyte had to be visible, round and centrally located within the follicle (Fig. 1).

As a control, preantral follicles from fresh mouse ovary were isolated and cultured in an identical manner to those which had been isolated from vitrified-thawed ovary.

In Vitro Growth (IVG) of Preantral follicles

The IVG medium was αMEM medium supplemented with 100 mIU/ml FSH (Metrodin-HP; Sereno, Aubonne, Switzerland), 10 mIU/ml LH (L-5259; Sigma) and 5% FBS (Kim *et al*., 1999). The follicles were cultured on Transwell-COL membrane inserts (3.0 μm pore size, 24.5 mm diameter; Costar, Cambridge, MA, USA) in 6-well cluster dishes to prevent the loss of structural integrity between the oocyte and granulosa cells. The follicles were cultured for 10 days at 37℃ and 5% CO_2 in air. Half of the medium was changed every 2 days (Fig. 2).

In Vitro Maturation (IVM), Fertilization (IVF) and Subsequent Development

After 10 days of IVG, follicles were allowed to mature for 16~18 h in IVM medium supplemented with 1.5 IU/ml hCG (Profasi; Sereno) (Fig. 2). A part of matured follicles were selected randomly and adherent cumulus cells were removed. The maturation status and the diameter of the oocytes excluding the zona pelluci-

(A) Intact preantral follicle (B) Oocyte damaged preantral follicle (C) Theca and granulosa cells damaged preantral follicle

Fig. 1. Morphology of preantral follicle isolated from vitrified mouse ovary.

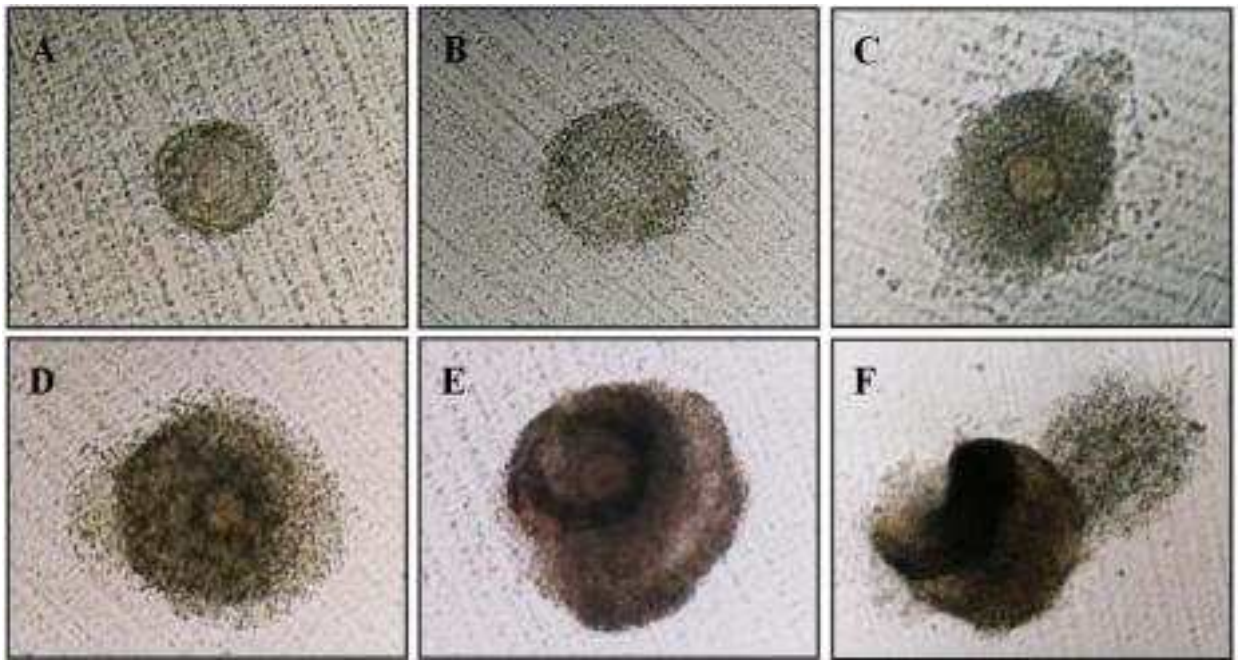

Fig. 2. *In vitro* growth and maturation of preantral follicle isolated from vitrified mouse ovary. An preantral follicle after (A) 2 day, (B) 4 day, (C) 6 day, (D) 8 day and (E) 10 day of the *in vitro* growth. (F) An follicle stimulated with 1.5 IU/ml hCG for maturation.

da were examined with an inverted microscope and the ocular micrometer.

In vitro-matured oocyte-granulosa cell complexes were placed into 50 μl drops of Whittingham's T_6 medium supplemented with 30 mg/ml bovine serum albumin (A-3311; Sigma) under mineral oil. The sperm collected from the cauda epididymis of mature ICR male mice were incubated for 2 h in fertilization medium before insemination. The oocytes were inseminated with spermatozoa with concentration of $1\sim2\times10^6$ / ml. Six hours after the insemination, the fertilized oocytes were cultured in 20 μl drops of KSOM medium supplemented with 10% (v/v) serum substitute supplement (SSS; Irvine, Santa Ana, CA, USA), 1% (v/v) essential amino acids (M-7145; Sigma) and 0.5% (v/v) non-essential amino acids (B-6766; Sigma) under mineral oil. The oocytes were examined the following day, and the number of 2-cell embryos was noted. The 2-cell embryos were further cultured for 4 days until the blastocyst stage.

Assessment of Cell Number of Blastocyst

The cell number of blastocysts (5 days after insemination) was evaluated by fluoresence staining. The blastocysts were fixed in 2% formaldehyde in PBS for 10 min at room temperature. The blastocysts were then placed on slides with a drop of mounting medium consisting of the 3:1 ratio of glycerol versus PBS containing 2.5 mg/ml sodium azide and 2.5 μg/ml Hoechst 33342 (B-2261; Sigma). A cover slip was placed on top of the oocytes and blastocysts, and the edge was sealed with nail polish. The stained blastocysts were examined under a fluorescence microscope.

Statistical Analysis

The statistical significance of the data was analyzed using a Student's *t*-test and chi-square (χ^2) test. A statistical significance was established at the $p<0.05$ level.

RESULTS

Recovery of Preantral Follicles from Vitrified Ovary

Fresh ovaries had three-fold more preantral follicles than vitrified-thawed ovaries ($p<0.005$) (Fig. 3). The periods of exposure to the vitrification solution did not significantly affect the number of harvested preantral follicles. Due to the death of granulosa and theca cells, the collapse of the oocyte within the follicle and the extrusion of denuded oocytes from the follicles were observed.

***In Vitro* Growth and Maturation of Preantral Follicles Isolated from Vitrified Ovary**

Five minutes exposure group (69.8%) in vitrification solution had significantly higher ($p<0.05$) survival rates of vitrified preantral follicles than 10 min exposure group (43.2%). The above value did not differ between the 5 min exposure and fresh group (62.1%) (Table 1).

Table 1. Survival and maturation rates of oocytes within preantral follicles of vitrified mouse ovaries after IVG and IVM

Treatment	No. of follicles cultured	No.(%) of oocytes survived	GV (%)	GVBD (%)	Meta II (%)	Oocyte diameter (μm)
Fresh	116	72 (62.1)[a]	9 (7.8)	19 (16.4)	44 (37.9)[a]	71.7±2.1[a]
5 min	162	113 (69.8)[a]	21 (13.0)	29 (17.9)	63 (38.9)[a]	69.4±2.8[b]
10 min	146	63 (43.2)[b]	3 (2.1)	22 (15.1)	38 (26.0)[b]	67.8±3.1[c]

Values with different superscripts within each column are significantly different (p<0.05).

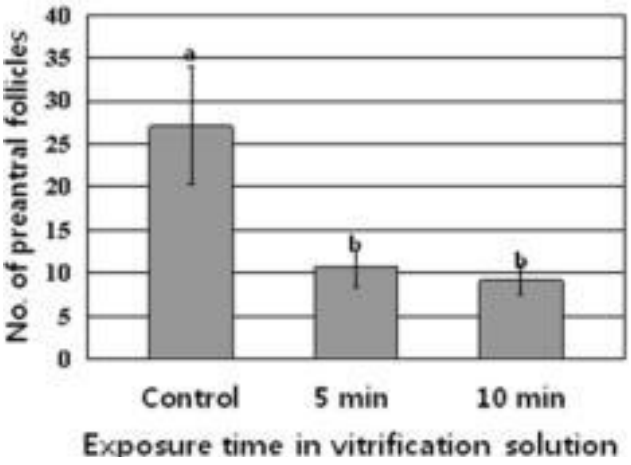

Fig. 3. The number of preantral follicles harvested from vitrified mouse ovary. Values with different superscripts above each column are significantly different (p<0.005).

After *in vitro* maturation, the proportion of oocytes that reached metaphase II stage were significantly higher (p<0.05) in 5 min exposure group (38.9%) in vitrification solution compared to the 10 min exposure group (26.0%). The proportion did not differ between the 5 min exposure and fresh group (37.9%) (Table 1). The diameter of metaphase II oocytes from vitrified groups was significantly smaller (p<0.05) than that of the fresh group (71.7±2.1 μm), and also there was significantly smaller in 10 min exposure group (67.8±3.1 μm) when compared to 5 min exposure group (69.4 ±2.8 μm) (Table 1).

***In Vitro* Development of Oocytes within Preantral Follicles**

After *in vitro* fertilization, developmental rate to blastocyst stage was not different between the vitrification group (8.6%) and the fresh group (12.0%) (Table 2). However, cell numbers of blastocysts derived from vitrification group (41.9±20.2) was significantly lower (p< 0.05) than those of the fresh group (55.1±22.5) (Table 3).

Table 3. Number of cells per blastocyst derived from vitrified mouse ovaries

Treatment	No. of blastocysts examined	No. of cells (Mean±SEM)
Fresh	20	55.1±22.5[a]
Vitrification	13	41.9±20.2[b]

Values with different superscripts within each column are significantly different (p<0.05).

DISSCUSSION

Traditionally, ovarian tissues are cryopreserved using low concentration of cryprotectants and slow freezing techniques that dehydrate the cell during freezing to prevent intracellular crystallization (Cortvrindt *et al*., 1996; Cox *et al*., 1996; Gunasena *et al*., 1997; Sugimoto *et al*., 2000; Liu *et al*., 2001). By using orthotopic grafting after slow freezing, the birth of offspring has been reported in mice (Parrott, 1960; Cox *et al*., 1996; Gunasena *et al*., 1997) and sheep (Gosden *et al*., 1994. However, slow freezing method reduced the survival rates of follicles after culture *in vitro* (Newton and Illingworth, 2001).

Vitrification is an alternative freezing method of freezing in which cells is frozen in high concentrations

Table 2. Development of oocytes within preantral follicles derived from vitirified mouse ovaries

Treatment	No. of oocytes fertilized	No.(%) of embryos developed to			
		2-cell	4-cell	Morular	Blastocyst
Fresh	166	69 (41.6)	47 (28.3)	37 (22.3)	20 (12.0)
Vitrification	151	55 (36.4)	32 (21.2)	24 (15.9)	13 (8.6)

of cryoprotectants, which upon rapid cooling imparts a glasslike state. The complete absence of ice crystal formation eliminates the risk of intracellular freezing and the build up of salts in the extracellular medium (Newton and Illingworth, 2001). In most previous studies, vitrification method using a cryovial or tube has been employed for the cryopresrvation of ovarian tissues (Newton and Illingworth, 2001). However, this method has been reported that the percentages of morphologically normal or viable follicles were significantly lower than those of non-frozen ovary (Chen *et al*., 2006). The cause for this result may be the chilling injury by the insufficient cooling rates of ovarian tissues. Several methods such as use of small amounts of solution have been proposed to overcome this problem (Hasegawa *et al*., 2004; Chen *et al*., 2006; Kim *et al*., 2011; Bos-Mikich *et al*., 2012).

Using open pulled-straw for vitrification resulted in significant improvement in the cryopreservation of oocytes and blastocysts (Vajta *et al*., 1998). With a small amount of vitrification solution and direct contact with liquid nitrogen, the open pulled-straw method achieved high thermal change. However, the open pulled-straw is not appropriate for handling larger size of tissue, such as an ovary. Therefore, we used open straw method that can hold larger size of tissue for vitrification, with direct contact by liquid nitrogen.

It was showed that the birth of mouse pups after orthotopic transplantation of cryopreserved ovary by vitrification (Migishima *et al*., 2003). Mouse pups were also born after transfer of embryos derived from vitrified preantral follicles (dela Pena *et al*., 2002). Recently, it was reported that the birth of mouse pups from mouse oocytes in preantral follicles derived from vitrified-warmed ovaries followed by *in vitro* growth, *in vitro* maturation, and *in vitro* fertilization (Hasegawa *et al*., 2006; Wang *et al*., 2011).

The exposure time to the vitrification solution before vitrification is very important to achieve adequate permeation by the cryoprotectant without incurring toxic damage or the cells vulnerable to excessive volume changes during the return to isotonic conditions after thawing. Therefore, optimization of exposure time in vitrification solution needed for mouse ovaries vitrification procedure. Mouse preantral follicles that were vitrified using vitrification solution containing ethylene glycol and raffinose showed prolonged exposure of preantral follicles to the vitrification solution reduced their developmental capacity (dela Pena *et al*., 2002). In the present study, exposure of mouse whole ovaries to vitrification solution supplemented with 20% DMSO and EG for 5 min after 5 min exposure to 10% DMSO and EG yielded higher follicular survival rate and oocyte maturation rate than 10 min exposure vitrification condition. Therefore, pre-equilibartion in vitrification solution for 5 min was sufficient for the effective permeation of the cryoprotectant.

After *in vitro* fertilization, developmental rate to blastocyst stage was not different between the vitrification group and the fresh group. However, cell numbers of blastocysts derived from vitrification group was significantly lower than those of the fresh group. In the previous study, the blastocyst rates and the number of cells in the blastocyst from vitrified-warmed ovaries was not different from fresh ovaries (Hasegawa *et al*., 2006). In this study, although vitrified-warmed ovaries derived blastocysts had significantly less number than fresh ovary derived blastocyst, the proportion of developing to blastocysts from vitrified-warmed ovaries was as high as fresh ovaries. Although the exact reasons of lower cell number in the blastocyst from vitrified-warmed ovaries followed by *in vitro* growth and maturation is unknown, the *in vitro* culture conditions for preantral follicles and fertilized embryos in the present study is different those of previous studies. Further studies are required to improve the preantral follicle culture conditions for the *in vitro* growth and development of preantral follicles from vitrified-warmed ovaries.

In conclusion, our results indicate that mouse oocytes within preantral follicles isolated from vitrified-thawed whole ovaries can be successfully grown and matured to metaphase II and developed to blastocyst stage after fertilization.

REFERENCES

1. Aubard Y, Newton H, Scheffer G, Gosden RG (1998): Conservation of the follicular population in irradiated rats by the cryopreservation and orthotopic autografting of ovarian tissue. Eur J Obstet Gynecol Reprod Biol 79:83-87.
2. Bos-Mikich A, Marques L, Rodrigues JL, Lothhammer N, Frantz N (2012): The use of a metal container for vitrification of mouse ovaries, as a clinical grade model for human ovarian tissue cryopreservation, after different times and temperatures of transport. J Assist Reprod Genet, Oct 9. [Epub ahead of print].
3. Candy CJ, Wood MJ, Whittingham DG (1997): Effect of cryoprotectants on the survival of follicles in frozen mouse ovaries. J Reprod Fertil 110:11-9.
4. Chen SU, Chien CL, Wu MY, Chen TH, Lai SM, Lin CW, Yang YS (2006): Novel direct cover vitrification for cryopreservation of ovarian tissues increases follicle viability and pregnancy capability in mice. Hum Reprod 21:2794-2800.
5. Cortvrindt R, Smitz J, Van Steirteghem AC (1996): A morphological and functional study of the effect of slow freezing followed by complete *in vitro* matura-

tion of primary mouse ovarian follicles. Hum Reprod 11:2648-2655.
6. Cox CL, Shaw J, Jenkin G (1996): Transplantation of cryopreserved ovarian tissue to adult recipients in mice. J Reprod Fetil 107:315-322.
7. Daniel JC Jr, Fox JM, Richardson P (1983): Return to partial function of rabbit ovaries after cryopreservation in liquid nitrogen. Theriogenology 19:405-412.
8. dela Pena EC, Takahashi Y, Katagiri S, Atabay EC, Nagano M (2002): Birth of pups after transfer of mouse embryos derived from vitrified preantral follicles. Reproduction 123:593-600.
9. Gook DA, Edgar DH, Stern C (1998): Effect of cooling rate and dehydration regimen on the histological appearance of human ovarian cortex following cryopreservation in 1,2-propanediol. Hum Repord 14:2061-2068.
10. Gosden RG, Baird DT, Wade JC, Webb R (1994): Restoration of fertility to oophorectomized sheep by ovarian autografts stored at −196℃. Hum Reprod 9:597-603.
11. Gunasena KT, Villines PM, Critser ES, Critser JK (1997): Live births after autologous transplant of cryopreserved mouse ovaries. Hum Repord 12:101-106.
12. Hasegawa A, Hamada Y, Mehandjiev T, Koyama K (2004): *In vitro* growth and maturation as well as fertilization of mouse preantral oocytes from vitrified ovaries. Fertil Steril 81(Suppl.):824-830.
13. Hasegawa A, Mochida N, Ogasawara T, Koyama K (2006): Pup birth from mouse oocytes in preantral follicles derived from vitrified and warmed ovaries followed by *in vitro* growth, *in vitro* maturation, and *in vitro* fertilization. Fertil Steril. 286(4 Suppl):1182-1192.
14. Kim DH, Chi HJ, Kang HG, Han SW, Lee HT, Chung KS, Lee HJ (1999): Effect of gonadotrophins on *in vitro* growth and maturation of mouse preantral follicles. Korean J Anim Reprod 23(1):53-61
15. Kim GA, Kim HY, Kim JW, Lee G, Lee E, Ahn JY, Park JH, Lim JM (2011): Effectiveness of slow freezing and vitrification for long-term preservation of mouse ovarian tissue. Theriogenology. 75(6):1045-1051.
16. Liu J, Van der Elst J, Ven den Broeke, Dhont M (2001): Live offspring by *in vitro* fertilization of oocytes from cryopreserved primordial mouse follicles after sequential *in vivo* transplantation and *in vitro* maturation. Biol Reprod 64:171-178.
17. Migishima F, Suzuki-Migishima R, Song S-Y, Kuramochi T, Azuma S, Nishijima M, Yokoyama M (2003): Successful cryopreservation of mouse ovaries by vitrification. Biol Reprod 68:881-887.
18. Newton H, Illingworth P (2001): *In-vitro* growth of murine pre-antral follicles after isolation from cryopreserved ovarian tissue. Hum Repord 16:423-429.
19. Oktay K, Newton H, Aubard Y, Salha O, Gosden RG (1998): Cryopreservation of immature human oocytes and ovarian tissue – an emerging technology? Fertil Steril 69:1-7.
20. Oktay K, Nugent D, Newton H, Salha O, Chatterjee P, Gosden RG (1997): Isolation and characterization of primordial follicles from fresh and cryopreserved human ovarian tissue. Fertil Steril 67:481-6.
21. Parrott DMV (1960): The fertility of mice with orthotopic ovarian grafts derived from frozen tissue. J Reprod Fertil 1:230-241.
22. Rall WF, Fahy GM (1985): Ice-free cryopreservation of mouse embryos at −196℃ by vitrification. Nature 313:573-575.
23. Sugimoto M, Maeda S, Manabe N, Miyamoto H (2000): Development of infantile rat ovaries autotransplanted after cryopreservation by vitrification. Theriogenology 53:1093-1103.
24. Vajta G, Holm P, Kuwayama M, Booth PJ, Jacobsen H, Greve T, Callesen H (1998): Open pulled straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. Mol Repord Dev 51:53-58.
25. Wang X, Catt S, Pangestu M, Temple-Smith P (2011): Successful *in vitro* culture of pre-antral follicles derived from vitrified murine ovarian tissue: oocyte maturation, fertilization, and live births. Reproduction 141 (2):183-191.

(Received: 17 November 2012/ Accepted: 14 December 2012)