—Mini Review—

Ovarian Tissue Banking

Akiko Hasegawa[1]*, Nahoko Mochida[2], Hiroyuki Kasumi[1],
Shinji Komori[1, 2] and Koji Koyama[1, 2]

[1]Laboratory of Developmental Biology and Reproduction, Institute for Advanced Medical Sciences, Muikogawa-cho 1-1, Nishinomiya, Hyogo 663-8501, Japan
[2]Department of Obstetrics and Gynecology, Hyogo College of Medicine, Hyogo 663-8501, Japan

Abstract: *Ovarian tissue cryopreservation is an effective method for protecting against infertility as well as preservating endangered animal species. The technique is particularly sought after as a strategy against ovarian failure caused by aggressive chemotherapy in young women with cancer. There are two uses for cryopreserved ovarian tissues after thawing: grafting and culture. Grafting carries the risk of reintroduction of disease. This article describes the status of ovarian cryopreservation in humans and the other animals and also details the successful birth of a pup from preantral follicle oocytes derived from a mouse cryopreserved ovary followed by* in vitro *growth,* in vitro *maturation and* in vitro *fertilization-embryo transfer (IVF-ET).*

Key words: *Vitrification, IVG/IVM, Fertilization, Infertility, Cancer*

Introduction

As an ovary contains a large amount of oocytes at various stages, it is a good resource for reproduction therapy. Ovarian tissue cryopreservation is an attractive method for the preservation of endangered animal species and for protection against human infertility.

Recent advanced therapy has achieved high survival rates of young women suffering from cancer. However, the patients inevitably become infertile due to premature ovarian failure (POF) caused by aggressive chemotherapy and/or radiotherapy. The development of low-temperature storage of ovarian tissues is a valuable addition to resources for restoring fertility, from the viewpoint of quality of life (QOL). Futhermore, this technique could be applied to naturally occurring POF and variation of life-style in which career women would prefer to conceive at older ages. The cryopreservation of ovarian tissues has been termed "ovarian tissue banking" [1–4].

Received: February 16, 2007
Accepted: February 22, 2007
*To whom correspondence should be addressed.
e-mail: zonapel@hyo-med.ac.jp

Application of Ovarian Tissue Cryopreservation to Cancer Patients

When a woman with cancer takes an adjuvant therapy, she will be informed about her future reproductive prospects and receive counseling [5]. If she wants to restore her fertility and has a partner, cryopreservation of fertilized embryos would be recommended, because embryo cryopreservation is a well-established treatment in assisted reproductive medicine. When the patient does not have a partner, oocyte cryopreservation may be applicable. In both cases ovarian tissue cryopreservation is an alternative option when the cancer therapy is urgent, because it takes at least 2 weeks to recover fertilizable oocytes from a patient.

Basically there are two main problems to solve before undertaking ovarian tissue banking. One is to determine the optimum conditions of the cryopreservation protocol. The second is to select the method for use after thawing: grafting or *in vitro* culture (Fig. 1). Grafting carries the risk of reintroduction of malignant cells, while for the *in vitro* growth method there is little knowledge of culture conditions at present. More theoretical and practical examinations are necessary to develop effective ovarian tissue banking. Currently, two cases successful live births after surviving cancer treatments have been reported as described below.

Successful Live Births

The two baby births were achieved after grafting [6, 7]. The first case was a woman with Hodgkin's

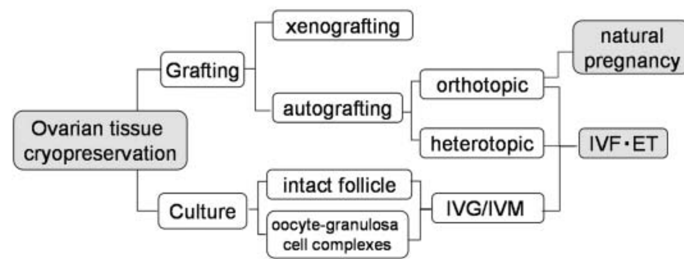

Fig. 1. Theoretical options for the use of cryopreserved ovarian tissue. Two applications, grafting and culture, are available for cryopreserved ovarian tissues. Grafting has two further options, xenografting and autografting. In xenografting, the recovered ovarian tissues are transplanted to immuno-deficient animals that do not reject human tissues. This method provides useful information for research but should not be used for humans. Orthotopic autografting has successfully resulted in two live births. In the culture system intact follicles or oocyte-granulosa cell complexes (OGCs) are collected from the cryopreserved ovaries and subjected to IVG/IVM to induce oocyte growth and maturation. Natural pregnancy is expected only by orthotopic autografting, the other methods are applied to IVF-ET (modified from Oktey *et al.*, Fertil Steril 1998, 69: 1-7).

lymphoma. In her case, the cortex of the left-side ovary was removed by biopsy. It was cut into small pieces to allow the cryoprotectants to permeate and then frozen by slow freezing. Five years later, thawed tissue pieces were transplanted to the original site, but ovarian function did not recover. Two more years after that, the remaining fragments of the cryopreserved ovarian tissues were transplanted into the right-side ovary. The menstrual cycle resumed 5 months after the operation and natural pregnancy occurred 4 months later.

The second case was a woman who had POF after high-dose chemotherapy for non-Hodgkin's lymphoma [7]. After 2 years of chemotherapy, the patient remained free from disease and thawed cryopreserved ovarian tissue was transplanted. Eight months later, her ovarian function recovered as assessed by inhibin B, anti-müllerian hormone and FSH levels. Regular IVF was performed and a live birth was achieved. The report of this case does not describe the cryopreservation protocol.

In both cases, the possibility that the outcome of pregnancy was due to the restoration of remaining natural ovarian function contributing to successful birth cannot be excluded. However, successful live births from grafted cryopreserved ovaries have also been reported in animal studies [8–10]. Hence, cryopreservation and grafting is a promising protocol for restoring female fertility.

Heterotopic autografting has also been reported [11, 12] in humans. Ovarian follicles implanted in a patient's lower abdominal wall grew after hormonal stimulation and the recovered eggs were fertilized and transferred to the patient's uterus [12]. Regardless of whether orthotopic or heterotopic, autografting carries the risk of reintroduction of malignant cells to patients who have recovered from cancer. Xenografting is an alternative method in which human ovaries are grafted to immuno-deficient animals [13–15]. The animals are then treated with follicle growth stimulant to recover fertilizable mature eggs. This method provides useful information on ovarian grafting but should be used for research purposes only because of the potential for unknown viral infections as well as ethical issues.

Vitrification

Ovarian tissues contain a lot of oocytes/follicles at heterogenous stages. It seems to be difficult to store every oocyte/follicle in a good condition under cryopreservation. Moreover, survival of granulosa cells is also important for supporting oocyte growth in the follicle. Membrane permeation and sensitivity to

cryoprotectants are different among cell types and oocyte/follicle stages. Antral follicles are usually small in number in the ovary and H_2O remaining in the surrounding fluid forms ice-crystals which cause cell damage. Resting primordial oocytes/follicles are resistant to cryopreservation but it is difficult to induce their growth after thawing. The intermediate stage of follicles, preantral or secondary follicles seems to be the most suitable as the target stage of follicles for cryopreservation.

Previously, slow freezing was generally used for cryopreservation of tissues as well as embryos. Nowadays a vitrification method is mainly used which yields a high success rate in embryo cryopreservation. Even oocytes at metaphase II of meiosis can be stored at low temperatures by the vitrification method in animals [16–18] and humans [19–21]. These results encouraged us to apply the vitrification techniqe to ovarian tissues and improve the conditions of cryopreservation.

Mouse ovarian tissues are useful for experiments, because the growth of a considerable number of follicles occurs synchronously depending on the age of the mice. We used 12-day-old mouse ovaries that contain a lot of homogeneous preantral follicles to determine the optimum conditions for vitrification/warming. After incubation for 30 min in an equilibration medium composed of 7.5% ethylene glycol (EG) and 7.5% dimethylsulfoxide (DMSO), the ovaries were further incubated in the vitrification medium composed of 15% EG, 15% DMSO and 0.5 M sucrose for 2, 10 and 30 min. Then, the treated ovaries were immediately plunged into liquid nitrogen. Warming was carried out in two steps at sucrose concentrations of 1.0 M and 0 M. The vitrified ovaries were incubated in each step for 10 min at 37°C. After warming, the ovaries were cultured in alpha-MEM (minimum essential medium) for 60 min and subjected to histological examination.

Figure 2 shows a histological comparison of incubation times of ovaries in vitrification medium. The morphology was improved in a manner which was time dependent. Thirty-minute incubation in the vitrification medium is enough for mouse ovarian tissue (size 1 mm^3) cryopreservation. Excess exposure to the cryoprotectants would be toxic to the ovarian tissues. In larger animals, dissection may be necessary for sufficient cryoprotectant permeation and good preservation of ovarian tissue. Successful auto-grafting and follicle development from grafted cryopreserved ovarian tissues were reported in animal experiments [22–24].

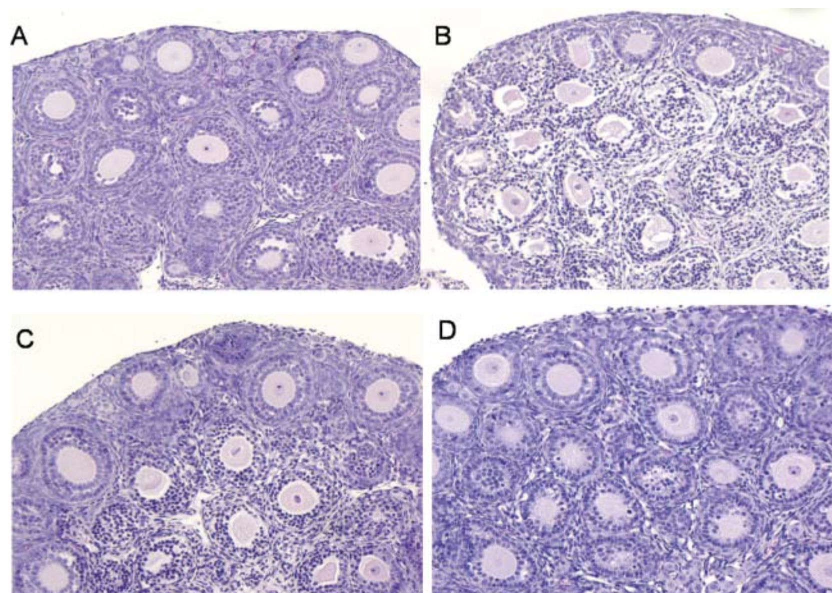

Fig. 2. Histological examinations of cryopreserved mouse ovaries during incubation in vitrification medium (A: fresh; B: 2 min; C: 10 min; D: 30 min). When the incubation time is short, ovarian tissues survived just in the peripheral regions (B). The surviving region extended into the medulla in 10-min and 30-min incubations (C and D). The morphology of cryopreserved ovaries treated with cryoprotectant for 30 min (D) resembles fresh ovarian tissue (A).

In Vitro Growth (IVG) and Maturation (IVM) of Mouse Ovarian Follicles

Auto-grafting carries the risk of the reappearance of cancer. As a safer strategy, *in vitro* culture is an option. IVM is usually used for naturally grown oocytes in the ovary. These oocytes are capable of maturing into fertilizable eggs under IVM conditions supplemented with EGF (epithelial growth factor) and FSH. Successful live births have been reported in POF patients [25–28]. On the other hand, IVG is still being experimentally investigated, because various factors are involved in the follicle growth [29–31]. Although successful births in domestic animal experiments have been reported in the literature, the experimental conditions used by the individual researchers were quite diverse and the success rates have been low [32–34].

In mouse experiments, two isolation methods, mechanical [35, 36] and enzymatic [37, 38] have been used for *in vitro* growth culture. Eppig's group succeeded in obtaining pups conceived from primordial follicle oocytes by OGC culture combined with organ culture [39]. We conducted a similar experiment to try to produce live pups from preantral follicles in vitrified/warmed mouse ovaries [40]. The ovaries were taken from 16-day-old mice and preantral oocyte-granulosa cell complexes (OGCs) were collected by collagenase treatment. The collected OGCs were cultured for 7 days in IVG medium, alpha-MEM containing ITS (insulin, transferrin and selenium), fetuin, estradiol and BSA (bovine serum albumin), and subsequently cultured in IVM medium further supplemented with EGF and FSH.

The maturation rates of the follicles/oocytes and developmental rates of the presumed embryos were examined in vitrified and warmed ovaries. The ovaries were treated the same as fresh ovaries for collection of OGCs. The results are summarized in Fig. 3. OGC maturation was assessed by granulosa cell dispersion and mucification. Although the granulosa cell maturation rate was lower in the cryopreserved group than in the fresh ovary group, fertilization and subsequent embryo development rates were similar in the two groups. The oocytes in the OGCs that reached maturation stage could be fertilized and the fertilized eggs grew to the blastocyst stage with rates similar to the fresh ovary group.

The success rate of live-pup birth, however, was low from both the fresh and cryopreserved groups compared with *in vivo* matured oocytes [40]. This result indicates that vitrification does not have negative effects on the developmental quality of oocytes, and that the low success rate of pup birth is dependent on the culture process rather than on vitrification. Genomic recombination, epigenic modification and cytoplasmic maturation may not adequately occur under culture conditions. Actually more than 50% of the transplanted

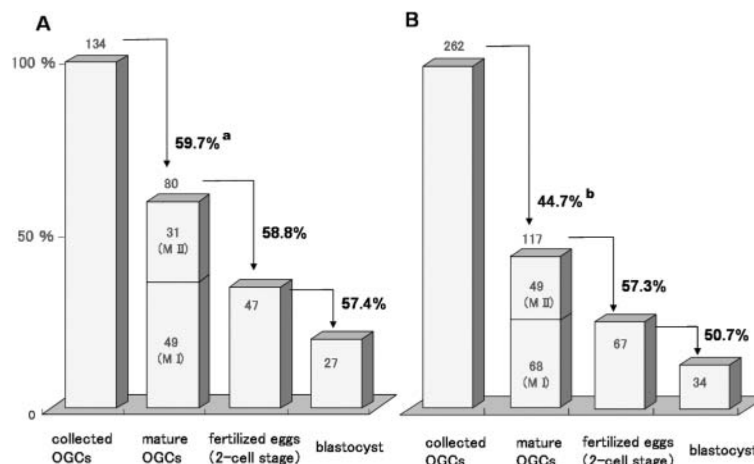

Fig. 3. Comparison of successful rates of OGC maturation, egg fertilization and embryo development between fresh (A) and cryopreserved ovaries (B). The granulosa cell maturation rate, which was assessed by dispersion and mucification, was lower (b) in the cryopreserved group than in the fresh group (a). $p<0.05$ (χ^2 test). Fertilization and embryo development, however, occurred at levels similar to the fresh group.

embryos ceased further growth after implantation and were absorbed in the uterus during gestation.

Conclusion

Intensive studies of cryopreserved ovarian tissues including human ovarian tissues have been performed by many researchers [41–44]. These efforts have been helpful for the development of the optimum IVG condition for human use. However, development of IVG for humans is still at the trial stage [45–48]. Further theoretical and practical examinations are necessary to determine the optimum conditions for IVG. Consideration of ethical issues and studies using non-human primates should be carried out before IVG is used in human reproductive therapy.

Acknowledgements

This research work was supported in part by the High-Tech Research Center Project for Private Universities matching fund subsidy from the Japanese Ministry of Education, Culture, Sports, Science and Technology, 2004–2008 and a Grant-in-Aid for Science Research (No. 18390453) from the Japan Society for the Promotion of Science.

References

1) Newton, H. (1998): The cryopreservation of ovarian tissue as a strategy for preserving the fertility of cancer patients. Hum. Reprod. Update, 3, 237–247.
2) Oktay, K. and Sonmezer, M. (2004): Ovarian tissue banking for cancer patients: fertility preservation, not just ovarian cryopreservation. Hum. Reprod., 19, 1924–1925.
3) Kim, S.S. (2006): Fertility preservation in female cancer patients: current developments and future. Fertil. Steril., 85, 1–11.
4) Fabbri, R. (2006): Cryopreservation of human oocytes and ovarian tissue. Cell Tissue Bank, 7, 113–122.
5) Sonmezer, M. and Oktay, K. (2006): Fertility preservation in young women undergoing breast cancer therapy. Oncologist, 11, 422–434.
6) Donnez, J., Dolmans, M.M., Demylle, D., Jadoul, P., Pirard, C., Squifflet, J., Martinez-Madrid, B. and van Langendonckt, A. (2004): Livebirth after orthotopic transplantation of cryopreserved ovarian tissue. Lancet, 364, 1405–1410.
7) Meirow, D., Levron, J., Eldar-Geva, T., Hardan, I., Fridman, E., Zalel, Y., Schiff, E. and Dor, J. (2005): Pregnancy after transplantation of cryopreserved ovarian tissue in a patient with ovarian failure after chemotherapy. N. Engl. J. Med., 353, 318–321.
8) Migishima, F., Suzuki-Migishima, R., Song, S.Y., Kuramochi, T., Azuma, S., Nishijima, M. and Yokoyama, M. (2003): Successful cryopreservation of mouse ovaries by vitrification. Biol. Reprod., 68, 881–887.
9) Gosden, R.G., Baird, D.T., Wade, J.C. and Webb, R. (1994): Restoration of fertility to oophorectomized sheep by ovarian autografts stored at –196 degrees C. Hum. Reprod., 9, 597–603.
10) Salle, B., Demirci, B., Franck, M., Rudigoz, R.C., Guerin, J.F. and Lornage, J. (2002): Normal pregnancies and live births after autograft of frozen-thawed hemi-ovaries into ewes. Fertil. Steril., 7, 403–408.
11) Newton, H., Aubard, Y., Rutherford, A., Sharma, V. and Gosden, R. (1996): temperature storage and grafting of human ovarian tissue. Hum. Reprod., 11, 1487–1491.
12) Oktay, K., Buyuk, E., Veeck, L., Zaninovic, N., Xu, K., Takeuchi, T., Opsahl, M. and Rosenwaks, Z. (2004): Embryo development after heterotopic transplantation of cryopreserved ovarian tissue. Lancet, 363, 837–840.
13) Oktay, K., Newton, H. and Gosden, R.G. (2000): Transplantation of cryopreserved human ovarian tissue results in follicle growth initiation in SCID mice. Fertil. Steril., 73, 599–603.
14) Gook, D.A., McCully, B.A., Edgar, D.H. and McBain, J.C. (2001): Development of antral follicles in human cryopreserved ovarian tissue following xenografting. Hum. Reprod., 16, 417–422.
15) Sato, Y., Terada, Y., Utsunomiya, H., Koyanagi, Y., Ito, M., Miyoshi, I., Suzuki, T., Sasano, H., Murakami, T., Yaegashi, N. and Okamura, K. (2003): Immunohistochemical localization of steroidogenic enzymes in human follicle following xenotransplantation of the human ovarian cortex into NOD-SCID mice. Mol. Reprod., 65, 67–72.
16) Begin, I., Bhatia, B., Baldassarre, H., Dinnyes, A. and Keefer, C.L. (2003): Cryopreservation of goat oocytes and in-vivo derived 2- to 4-cell embryos using the cryoloop (CLV) and solid-surface vitrification (SSV) methods. Theriogenology, 59, 1839–1850.
17) Abe, Y., Hara, K., Matsumoto, H., Kobayashi, J., Sasada, H., Ekwall, H., Rodriguez-Martinez, H. and Sato, E. (2005): Feasibility of a nylon-mesh holder for vitrification of bovine germinal vesicle oocytes in subsequent production of viable blastocysts. Biol. Reprod., 72, 1416–1420.
18) Fujihira, T., Kishida, R. and Fukui, Y. (2004): Developmental capacity of vitrified immature porcine oocytes following ICSI: effects of cytochalasin B and cryoprotectants. Cryobiology, 49, 286–290.
19) Fuchinoue, K., Fukunaga, N., Chiba, S., Nakajo, Y., Yagi, A. and Kyono, K. (2004): Freezing of human immature oocytes using cryoloops with Taxol in the vitrification solution. J. Assist. Reprod. Genet., 21, 307–309.
20) Yoon, T.K., Kim, T.J., Park, S.E., Hong, S.W., Ko, J.J., Chung, H.M. and Cha, K.Y. (2003): Live births after vitrification of oocytes in a stimulated in vitro fertilization-embryo transfer program. Fertil. Steril., 79, 1323–1326.
21) Katayama, K.P., Stehlik, J., Kuwayama, M., Kato, O. and Stehlik, E. (2003): High survival rate of vitrified human oocytes results in clinical pregnancy. Fertil. Steril., 80, 223–224.

22) Sugimoto, M., Maeda, S., Manabe, N. and Miyamoto, H. (2000): Development of infantile rat ovaries autotransplanted after cryopreservation by vitrification. Theriogenology, 53, 1093–1103.
23) Kagabu, S. and Umezu, M. (2000): Transplantation of cryopreserved mouse, Chinese hamster, rabbit, Japanese monkey and rat ovaries into rat recipients. Exp. Anim., 49, 17–21.
24) Salle, B., Demirci, B., Franck, M., Rudigoz, R.C., Guerin, J.F. and Lornage, J. (2002): Normal pregnancies and live births after autograft of frozen-thawed hemi-ovaries into ewes. Fertil. Steril., 7, 403–408.
25) Cha, K.Y., Han, S.Y., Chung, H.M., Choi, D.H., Lim, J.M., Lee, W.S., Ko, J.J. and Yoon, T.K. (2000): Pregnancies and deliveries after in vitro maturation culture followed by in vitro fertilization and embryo transfer without stimulation in women with polycystic ovary syndrome. Fertil. Steril., 73, 978–983.
26) Child, T.J., Phillips, S.J., Abdul-Jalil, A.K., Gulekli, B. and Tan, S.L. (2002): A comparison of in vitro maturation and in vitro fertilization for women with polycystic ovaries. Obstet. Gynecol., 100, 665–670.
27) Chian, R.C., Buckett, W.M., Abdul-Jalil, A.K., Son, W.Y., Sylvestre, C., Rao, D. and Tan, S.L. (2004): Natural-cycle in vitro fertilization combined with in vitro maturation of immature oocytes is a potential approach in infertility treatment. Fertil. Steril., 82, 1675–1678.
28) Le Du, A., Kadoch, I.J., Bourcigaux, N., Doumerc, S., Bourrier, M.C., Chevalier, N., Fanchin, R., Chian, R.C., Tachdjian, G., Frydman, R. and Frydman, N. (2005): In vitro oocyte maturation for the treatment of infertility associated with polycystic ovarian syndrome: the French experience. Hum. Reprod., 20, 420–424.
29) Eppig, J.J. (2001): Oocyte control of ovarian follicular development and function in mammals. Reproduction, 122, 829–838.
30) Epifano, O. and Dean, J. (2002): Genetic control of early folliculogenesis in mice. Trends Endocrinol. Metab., 13, 169–173.
31) Choi, Y. and Rajkovic, A. (2006): Genetics of early mammalian folliculogenesis. Cell Mol. Life Sci., 63, 579–590.
32) Yamamoto, K., Otoi, T., Koyama, N., Horikita, N., Tachikawa, S. and Miyano, T. (1999): Development to live young from bovine small oocytes after growth, maturation and fertilization in vitro. Theriogenology, 52, 81–89.
33) Brown, B.W. and Radziewic, T. (1998): Production of sheep embryos in vitro and development of progeny following single and twin embryo transfers. Theriogenology, 49, 1525–1536.
34) Hirao, Y., Itoh, T., Shimizu, M., Iga, K., Aoyagi, K., Kacchi, M., Hoshi, H., and Takenouchi, N. (2004): In vitro growth and development of bovine oocyte-granulosa cell complexes on the flat substratum: effects of high polyvinylpyrrolidone concentration in culture medium. Biol. Reprod., 70, 83–91.
35) Spears, N., Boland, N.I., Murray, A.A. and Gosden, R.G. (1994): Mouse oocytes derived from in vitro grown primary ovarian follicles are fertile. Hum. Reprod., 9, 527–532.
36) Cortvrindt, R., Smitz, J. and Van Steirteghem, A.C. (1996): In-vitro maturation, fertilization and embryo development of immature oocytes from early preantral follicles from prepuberal mice in a simplified culture system. Hum. Reprod., 11, 2656–2666.
37) Eppig, J.J. and Schroeder, A.C. (1989): Capacity of mouse oocytes from preantral follicles to undergo embryogenesis and development to live young after growth, maturation, and fertilization in vitro. Biol. Reprod., 4, 268–276.
38) Hasegawa, A., Hamada, Y., Mehandjiev, T. and Koyama, K. (2004): In-vitro growth and maturation as well as fertilization of mouse preantral oocytes from vitrified ovaries. Fertil. Steril., 81, 824–830.
39) Eppig, J.J. and O'Brien, M.J. (1996): Development in vitro of mouse oocytes from primordial follicles. Biol. Reprod., 54, 197–207.
40) Hasegawa, A., Mochida, N., Ogasawara, T. and 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., 86 (Suppl 4), 1182–1192.
41) Segino, M., Ikeda, M., Aoki, S., Tokieda, Y., Hirahara, F. and Sato, K. (2003): In vitro culture of mouse GV oocytes and preantral follicles isolated from ovarian tissues cryopreserved by vitrification. Hum. Cell, 16, 109–116.
42) Cecconi, S., Capacchietti, G., Russo, V., Berardinelli, P., Mattioli, M. and Barboni, B. (2004): In vitro growth of preantral follicles isolated from cryopreserved ovine ovarian tissue. Biol. Reprod., 70, 12–17.
43) Newton, H. and Illingworth, P. (2001): In vitro growth of murine pre-antral follicles after isolation from cryopreserved ovarian tissue. Hum. Reprod., 16, 423–429.
44) dela Pena, E.C., Takahashi, Y., Katagiri, S., Atabay, E.C., and Nagano, M. (2002): Birth of pups after transfer of mouse embryos derived from vitrified preantral follicles. Reproduction, 123, 593–600.
45) Abir, R., Franks, S., Mobberley, M.A., Moore, P.A., Margara, R.A. and Winston, R.M. (1997): Mechanical isolation and in vitro growth of preantral and small antral human follicles. Fertil. Steril., 68, 682–688.
46) Hovatta, O., Wright, C., Krausz, T., Hardy, K. and Winston, R.M. (1999): Human primordial, primary and secondary ovarian follicles in long-term culture: effect of partial isolation. Hum. Reprod., 14, 2519–2524.
47) Louhio, H., Hovatta, O., Sjoberg, J. and Tuuri, T. (2000): The effects of insulin, and insulin-like growth factors I and II on human ovarian follicles in long-term culture. Mol. Hum. Reprod., 6, 694–698.
48) Hreinsson, J.G., Scott, J.E., Rasmussen, C., Swahn, M.L., Hsueh, A.J. and Hovatta, O. (2002): Growth differentiation factor-9 promotes the growth, development, and survival of human ovarian follicles in organ culture. J. Clin. Endocrinol. Metab., 87, 316–321.