In vitro growth and maturation of mouse oocyte-granulosa cell complex from cryopreserved ovaries and achievement of pup birth

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Ovarian tissue banking is a feasible strategy for fertility preservation for young women after cancer treatments. Ovarian tissue, after thawing, is used for several options; orthotopic grafting (normal site), autologous heterotopic grafting and collection of ovarian follicles for culture. Recent reports of live birth encouraged clinicians and researchers to apply this technology to premature ovarian failure (POF) resulting from strong cancer therapy. Grafting, however, carries a risk of malignant cell recurrence. For safety, development of a culture method is necessary but optimum culturing conditions for less-developed follicles abundant in the ovary are not well known. In the present article, the current status of ovarian tissue cryopreservation, and *in vitro* oocyte growth and maturation from the preserved ovaries are reviewed. (Reprod Med Biol 2007; **6**: 77–83)

Key words: cancer, fertilization, infertility, premature ovarian failure, vitrification.

INTRODUCTION

ADVANCED ASSISTED REPRODUCTIVE technology (ART) has provided useful therapy for patients suffering from infertility since 1978, when Edwards and Steptoe succeeded in achieving a full-term birth after *in vitro* fertilization (IVF).[1] Effective treatments are now available for tube blockage, sperm factors, immunological factors and unknown factors. Cancer therapy has also advanced, and survival rates of young patients have significantly improved as a result of chemotherapy and/or radiotherapy. However, as a side-effect, ovarian failure as a result of these aggressive treatments is often inevitable. Considering the quality of life (QOL) of young women, ovarian cryopreservation is a feasible method to restore fertility.[2–5] Theoretical options for the use of cryopreserved ovarian tissue are shown in Figure 1. This strategy could also help some patients with severe infertility, such as naturally-occurring premature ovarian failure, if the ovarian tissues can be preserved before deteriorating further. In addition, ovarian tissue banking might be applicable to career women who would like to conceive at older ages.

OVARIAN TISSUE BANKING

THERE ARE SEVERAL options for ovarian tissue banking (Fig. 1). Cryopreserved ovaries are grafted or cultivated to restore fertility. Autografting is a method used to resume the menstrual cycle and achieve natural pregnancy. In fact, two successful births have been reported by this method.[6,7] Alternatively, heterotopic autografting has also been reported.[8,9] The ovarian follicles implanted in the patient's lower abdominal wall grew by hormonal stimulation and recovered eggs were fertilized, cleaved and transferred to the uterus.[8] Regardless of the method, orthotopic or heterotopic, autografting carries a 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 immunodeficient animals. The animals are treated with follicle growth stimulant to recover fertilizable mature eggs. The mature eggs are recovered from the follicles. This method provides

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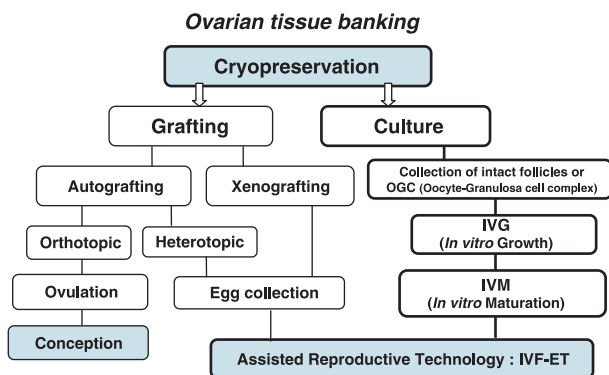
Ovarian tissue banking

Figure 1 Theoretical options for the use of cryopreserved ovarian tissue. Two applications, grafting and *in vitro* culture, are available for cryopreserved ovarian tissue usage. Grafting has two further options, autografting and xenografting. Orthotopic autografting successfully produced two live births in clinical use and heterotopic autografting produced 4-cell-stage embryos. Xenografting provides useful information in research but would not be used for humans. In the culture system, oocyte-granulosa cell complexes are collected from the cryopreserved ovaries and subjected to *in vitro* growth-*in vitro* maturation as well as *in vitro* fertilization and embryo transfer. There is no risk of recurrent malignant cells in this option. (Modified from Oktay *et al*. Fertil Steril 1998, **69**: 1–7)

useful information on ovarian grafting, but would be applied for research purposes only because of potential unknown viral infection as well as ethical problems.[10–12] Hence, a culture system which covers *in vitro* growth (IVG) and maturation (IVM) of oocytes to yield fertilizable eggs is desirable. Practically, oocyte-granulosa cell complexes are collected from the cryopreserved ovaries and cultured for IVG followed by IVM. The mature eggs with metaphase II are used for IVF-ET.

CRYOPRESERVATION

Preimplantation embryo and unfertilized eggs

TWO METHODS ARE available for cryopreservation, slow freezing using a computer-aided programmed freezer and rapid freezing by vitrification. The vitrification method was significantly improved and the embryo survival rate after vitrification was almost 100%. Intensive studies have been carried out to exploit devices and protocols for vitrification, and unfertilized oocytes at the metaphase II have been able to be stored by vitrification.[13–16] In humans, successful live births have been reported from eggs vitrified and warmed before

fertilization.[16–18] This was a surprise because the oocytes at this stage have a dynamic balance of polymerized microtubules attached to chromosomes, increased fluidity of membrane for sperm penetration and biologically active cytoplasm that is preparing for postfertilization events.

Ovarian tissue

Ovarian tissues are quite large compared with unfertilized eggs and pre-implantation embryos. Longer exposure time is needed for permeation of cryoprotectant into the tissues. In contrast, prolonged exposure to the cryoprotectants could cause toxic damage in the cells. Optimum conditions regarding kinds and concentrations of cryoprotectant chemicals, incubation time and temperature should be determined in the ovarian tissues of different animals.

Figure 2 shows the effects of exposure time to cryoprotectant on mouse ovarian histology. Increasing incubation time gave better morphology. Incubation of 2 min showed good morphology in just a thin area of ovarian cortex. With longer incubation times, the morphology became better in the inner tissues. Incubation of 30 min seemed to be enough to maintain comparative morphology to fresh ovarian sections (Fig. 2a,e). These results indicated that cryoprotectants time-dependently permeated and protected the tissues from cryodamage. Vitrification by 30-min immersion in the cryoprotectants showed satisfactory results for mouse ovarian cryopreservation. When applied to large animals, it might be necessary to dissect the tissues into smaller pieces or thinner slices to allow cryoprotectants to permeate into the central portions of the tissue. Tissue dissection is advantageous not only to promote permeation of cryoprotectants, but also to increase occasions for IVG and IVM.

In the literature, histological examinations of grafted ovarian tissues have shown promising results in animals[19,20] and humans.[10,21] In animal experiments, successful pup births from cryopreserved ovaries after grafting have been reported in mice and domestic animals.[22–24] These observations indicate that oocytes included in less-developed follicles could grow, mature and develop into full-term births after cryopreservation.

IN VITRO CULTURE

TO PREVENT MALIGNANT cell reintroduction, development of a culture system is necessary. As the varying stages in ovarian follicles (oocytes) have different requirements, several isolation options are proposed
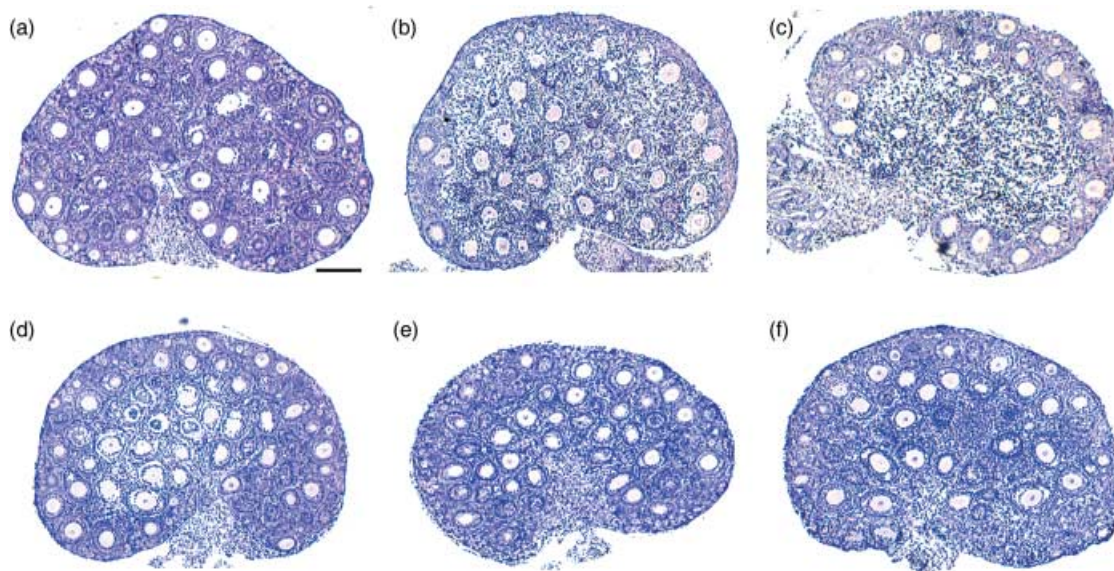
Figure 2 Histological examinations of cryopreserved mouse ovaries during incubation times in cryoprotectant solution before storage in liquid nitrogen. (a) fresh; (b) 2 min; (c) 10 min; (d) 20 min; (e) 30 min; (f) 60 min. (b, c) When the incubation time was short, ovarian tissues just survived in the peripheral regions. (d) The surviving region extended into the medulla in 20-min incubation. (e) The morphology from cryopreserved ovary treated with cryoprotectant for 30 min (a) resembles fresh ovarian tissue. (f) Further incubation, did not change the ovarian morphology compared to 30-min incubation. Bar is 100 μm.

(Fig. 3). First, sufficiently-grown follicles can mature in culture containing adequate supplements such as epithelial growth factor (EGF), follicle-stimulating hormone (FSH) without additional IVG. For treatment of polycystic ovarian syndrome (PCOS), IVM of oocytes obtained by aspiration from small antral follicles is a feasible technique resulting in pregnancies, and many healthy babies are successfully born.[25–28] However, antral follicles in the ovary are small in number and susceptible to cytoskeletal and cell membrane damage by ice crystal formation caused by the surrounding fluid. In contrast, primordial and very small primary follicles are abundant in the ovary and resistant to cryopreservation, but the method to induce them to grow is unknown at the present time. Early preantral follicles are present in young ovaries in considerable numbers and more resistant to cryopreservation than antral follicles. Therefore, preantral follicles might serve as a good source for providing mature oocytes after cryopreservation. As a mouse model experiment, IVG-IVM of this type of follicle is described in the next section.

Mouse

Practically, two methods have been used for IVG in mouse experiments. One is follicle-intact culture,[29,30] and the other is oocyte-granulosa cell complex (OGC) culture.[31,32] The intact follicles are collected by mechanical dissection and the OGC are collected by collagenase treatment in which the basement membrane and theca cell layers are removed. The appearance of intact follicles and OGC are not different under optical microscopic observation (Fig. 4a,b), but optimum conditions are different in the two methods. Higher FSH concentrations and serum factors are necessary in follicle-intact IVG. After IVG, the intact follicle method showed an antral-like structure, whereas the OGC method produced granulosa cell proliferation and spread on the substratum (Fig. 4c,d). The granulosa cells from both methods dispersed and mucified (Fig. 4e,f). The eggs from both methods produced fertilizable eggs and grew into the blastocyst stage and subsequently achieved full-term development.[33]

In the studies using domestic animals, live pup births through IVG-IVM from less-grown oocytes were reported.[34–37] Using cryopreserved ovaries, successful development into the blastocyst stage or pup birth from isolated less-developed oocytes has been documented by many researchers.[38–40] In addition to ovaries, OGC cryopreservation has also achieved live pup birth.[41]

Recently, our laboratory achieved full-term development by implantation of two-cell-stage mouse embryos
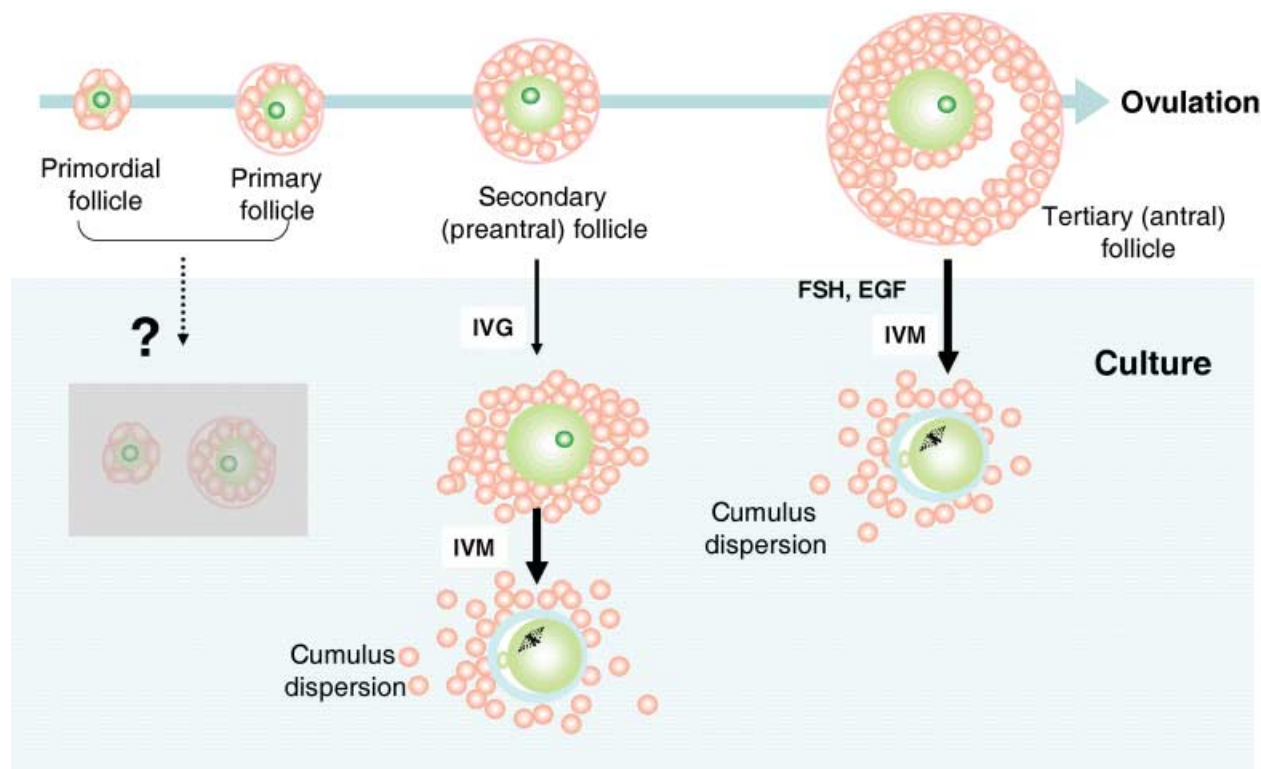
Figure 3 Schematic drawing of *in vitro* growth (IVG) and *in vitro* maturation (IVM). Sufficient information is not currently available on primordial and primary follicles, or gonadotropin-independent phases to trigger growth. The oocytes from full-grown follicles can mature into metaphase II (MII) in IVM culture. In the intermediate stage of secondary (preantral) follicles, the oocytes can grow in adequate IVG conditions and develop into fertilizable eggs.

into host mothers from cryopreserved ovaries through IVG-IVM.[42] Although the success rate (birth/implant) was low, it is similar to that from fresh ovary. This suggests that cryopreservation does not affect oocyte quality and live birth. The IVG conditions should be improved rather than the cryopreservation protocol. Physiological events such as genomic recombination, epigenic modification and cytoplasmic maturation might not adequately occur. Further improvements are necessary for optimum IVG conditions. Information on IVG-IVM conditions from animal models is important for human application because it is essential to resolve ethical, moral and religious issues in the use of human ovaries.

Human

In humans, challenging trials have been reported by several researchers.[43–47] Hovatta *et al.* reported human ovarian tissue recovered from cryopreservation survived 4 weeks in culture conditions.[45] They also showed

tissue culture gave a better result than isolated follicle culture. Insulin, insulin-like growth factors and GDF-9 might have promising effects on follicle survival and growth.[46-48] The information on signal–receptor interactions and growth control mechanism of human folliculogenesis is quite insufficient at this time.

Factors involved in folliculogenesis

Follicle growth is orchestrated by paracrine/autocrine factors in harmony. Various growth factors and oocyte-specific gene expressions have been identified at specific stages of the follicular development, as reviewed in articles[49–51] but information is still insufficient to show the full mechanism of follicular growth. Recent molecular biology provides possible key molecules in early follicular development such as c-kit/kitL, antimüllerian hormone and stem cell factor. In particular, oocyte-specifically expressed TGF-β superfamily including BDF-9 and BMP-15 is being intensively studied. The molecules promote follicle development beyond the primary
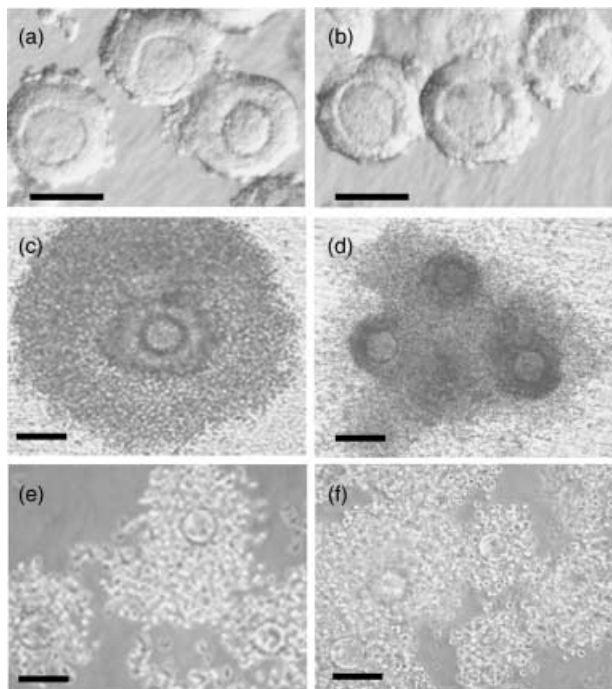
Figure 4 (a) Morphological change of intact follicles and (b) oocyte-granulosa cell complexes (OGC) during *in vitro* growth (IVG). Intact follicles were collected by mechanical dissection and OGC were collected by collagenase-treatment. (c) After 7 day-IVG, intact follicles formed antral-like structures. (d) In the same incubation time, the granulosa cells of OGC proliferated around oocytes. (e, f) After further incubation in *in vitro* maturation, granulosa cells from both cultures were dispersed and mucified with a similar morphology. Bars are 100 μm.

follicle stage. Another interesting factor, FIGLA, which regulates zona pellucida protein expressions by binding upstream of their genes, is involved in primordial follicle formation from germline. The mechanisms of ovarian organization and folliculogenesis have gradually been made clear at the molecular level. These findings encourage us to continue the research of utilizing less-developed oocytes for ovarian tissue banking.

CONCLUSION

VITRIFICATION OF OVARIAN tissues has provided morphologically satisfactory results in several animal species. In mice, growing oocytes collected from pre-antral follicles of vitrified-warmed ovary can develop into live births. Further investigations using non-human primates are needed for application to human use. Continuous efforts will benefit many young women facing premature ovarian failure.

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