

Successful fertility preservation following ovarian tissue vitrification in patients with primary ovarian insufficiency

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STUDY QUESTION: Is ovarian tissue cryopreservation using vitrification followed by *in vitro* activation (IVA) of dormant follicles a potential approach for infertility treatment of patients with primary ovarian insufficiency (POI)?

SUMMARY ANSWER: Our vitrification approach followed by IVA treatment is a potential infertility therapy for POI patients whose ovaries contain residual follicles.

WHAT IS KNOWN ALREADY: Akt (protein kinase B) stimulators [PTEN (phosphatase with TENsin homology deleted in chromosome 10) inhibitor and phosphatidylinositol-3-kinase (PI3 kinase) stimulator] activate dormant primordial follicles *in vitro* and ovarian fragmentation disrupts the Hippo signaling pathway, leading to the promotion of follicle growth. We treated POI patients with a combination of ovarian vitrification, fragmentation and drug treatment, followed by auto-transplantation, and reported successful follicle growth and pregnancies.

STUDY DESIGN, SIZE, DURATION: Prospective clinical study of 37 infertile women with POI between 12 August 2011 and 1 November 2013. We enrolled 10 new patients since the previous publication.

PARTICIPANTS/MATERIALS, SETTING, METHODS: POI patients were originally selected based on a history of amenorrhea for more than 1 year and elevated serum FSH levels of >40 mIU/ml ($n = 31$) but this was later changed to >4 months, age <40 years and serum FSH levels of >35 mIU/ml ($n = 6$) (mean 71.8 ± 30.8 , range 35.5–197.6) so as to include patients with a shorter duration of amenorrhea. Under laparoscopic surgery, ovariectomy was performed and ovarian cortices were dissected into strips for vitrification. Some pieces were examined histologically. After warming, two to three strips were fragmented into smaller cubes before culturing with Akt stimulators for 2 days. After washing, ovarian cubes were transplanted beneath the serosa of Fallopian tubes under laparoscopic surgery. Follicle growth was monitored by ultrasound and serum estrogen levels. After oocyte retrieval from mature follicles, IVF was performed.

MAIN RESULTS AND THE ROLE OF CHANCE: Among 37 patients, 54% had residual follicles based on histology. Among patients with follicles, 9 out of 20 showed follicle growth in auto-grafts with 24 oocytes retrieved from six patients. Following IVF and embryo transfer into four patients, three pregnancies were detected based on serum hCG, followed by one miscarriage and two successful deliveries. For predicting IVA success, we found that routine histological analyses of ovarian cortices and shorter duration from initial POI diagnosis to ovariectomy are valid parameters.

LIMITATIONS, REASONS FOR CAUTION: Although our findings suggest that the present vitrification protocol is effective for ovarian tissue cryopreservation, we have not compared the potential of vitrification and slow freezing in follicle growth after grafting. We chose the serosa of Fallopian tubes as the auto-grafting site due to its high vascularity and the ease to monitor follicle growth. Future studies are needed to evaluate the best auto-grafting sites for ovarian tissues. Also, future studies are needed to identify biological markers to indicate the presence of residual follicles in POI to predict IVA treatment outcome.

WIDER IMPLICATIONS OF THE FINDINGS: In POI patients, ovarian reserve, namely the pool of residual follicles, continues to diminish with age. If one ovary is cryopreserved at an earlier stage of POI, patients could undergo additional non-invasive infertility treatments before the final decision for the IVA treatment. Furthermore, in the cases of unmarried POI patients, cryopreservation of ovarian tissues allows their fertility preservation until they desire to bear children.

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Introduction

Ovarian tissue cryopreservation followed by auto-transplantation is a promising method for fertility preservation in women undergoing gonadotoxic treatments (von Wolff *et al.*, 2009; Donnez and Dolmans, 2010; Kolp and Hubayter, 2011; Andersen *et al.*, 2012; Grynberg *et al.*, 2012; Jeong *et al.*, 2012; Gamzatova *et al.*, 2014). This procedure could also be useful for patients suffering from progressive ovarian dysfunction. In contrast to oocyte freezing that requires the storage of mature oocytes obtained from pre-ovulatory follicles, ovarian tissue cryopreservation is suitable for prepubertal girls by preserving their immature oocytes in early ovarian follicles. Following ovarian transplantation and follicle growth, early follicles could develop into pre-ovulatory follicles to allow the generation of mature eggs for fertility treatment. Ovarian tissue cryopreservation also has the advantage of storing a large number of immature oocytes inside early follicles. After the first successful live birth from a patient with Hodgkin's lymphoma following orthotopic transplantation of cryopreserved ovarian tissues (Donnez *et al.*, 2004), several reproduction centers have reported success in restoring fertility in cancer survivors, resulting in live births (Meirow *et al.*, 2005). In all these cases, slow freezing protocols were used for the cryopreservation of ovarian tissues (Donnez *et al.*, 2004).

Following the earlier use of slow freezing for tissue preservation, rapid advances in the vitrification approach have led to successful cryopreservation of preimplantation embryos and mature oocytes (Katayama *et al.*, 2003; Kuwayama *et al.*, 2005; Kuwayama, 2007). The advantages of the vitrification approach include no ice crystal formation that is known to cause physical and mechanical injury of cells. In addition, the vitrification procedure is attractive, because it is a quick and easy way to cryopreserve ovarian tissues and does not require special and expensive equipment. Many studies comparing slow freezing and vitrification of ovarian tissues have been conducted [reviewed in Amorim *et al.* (2011)]. However, the use of divergent vitrification protocols led to conflicting outcomes. Thus, it is becoming important to establish optimal vitrification protocols for the cryopreservation of ovarian tissues for infertility treatments.

Ovarian functions decrease with age, characterized by a diminishing number of follicles and menstrual cycle cessation. In patients with primary ovarian insufficiency (POI), early exhaustion of ovarian follicles is evident due to genetic, immunological, iatrogenic or other causes. POI affects 1% of women and is characterized by high circulating FSH levels together with amenorrhea before 40 years of age (Nelson,

2009). These patients are infertile due to a lack of follicle growth and ovulation; oocyte donation is the only treatment option. Although menstrual cycles cease in these patients, some of them still contain residual small ovarian follicles not producing enough circulating estrogens and progesterone to modulate uterine functions (De Vos *et al.*, 2010).

Our earlier report demonstrated the ability of PTEN (phosphatase with TENsin homology deleted in chromosome 10) inhibitors and phosphatidylinositol-3-kinase (PI3 kinase) stimulators to activate dormant murine and human primordial follicles *in vitro* (Li *et al.*, 2010). We further demonstrated that ovarian fragmentation disrupts the Hippo signaling pathway in the ovary, leading to increased production of downstream CCN growth factors and the promotion of follicle growth (Kawamura *et al.*, 2013). We combined ovarian cryopreservation, fragmentation and *in vitro* activation (IVA) drug treatment (the PTEN inhibitor and the PI3K activator), followed by auto-transplantation, as infertility treatment for POI patients and reported successful follicle growth and pregnancies (Kawamura *et al.*, 2013).

Here, we provide detailed clinical information on our POI patients and protocols of ovarian tissue cryopreservation using vitrification for the successful generation of mature oocytes in POI patients as a potential approach for infertility treatment and update the outcome of an expanded clinical study. We also demonstrated that histological analyses of ovarian cortices after ovariectomy and patient's history of shorter durations from POI diagnosis to ovariectomy are reliable parameters to predict the success of IVA.

Materials and Methods

Patients

For present clinical studies, we obtained informed consent from patients and approval from the Human Subject Committee of St. Marianna University and Japan Society of Obstetrics and Gynecology.

POI patients were originally selected based on a history of amenorrhea for more than 1 year and elevated serum FSH levels of >40 mIU/ml ($n = 31$) but these criteria were later changed to amenorrhea for more than 4 months, age <40 years and serum FSH levels of >35 mIU/ml ($n = 6$; mean 71.8 ± 30.8 , range 35.5–197.6) so as to include patients with a shorter duration of amenorrhea. For measurement of anti-Müllerian hormone (AMH) levels, Active MIA/AMH EIA kit or AMH Gen II ELISA kit (Beckman Coulter, Brea, CA, USA) was used according to the manufacturer's protocol. Because the assay for AMH has been changed from Active MIA/AMH EIA to AMH Gen II ELISA, Data obtained by Active MIA/AMH

EIA in earlier patients were converted into the unit (ng/ml) for AMH Gen II ELISA with a multiplication factor of 0.14. The detection limit of AMH Gen II ELISA and Active MIA/AMH EIA were 0.16 ng/ml and 1.0 pmol/l, respectively. Presence of endometriosis was determined based on the pelvic observation during laparoscopic surgery for ovariectomy (Re-AFS: stage I-III). A total of 37 patients were enrolled and received ovariectomy for ovarian tissue cryopreservation, followed by IVA for infertility treatment (Supplementary data, Table S1).

Ovarian tissue cryopreservation

Under laparoscopic surgery, ovariectomy was performed to remove one or both ovaries without using electrocautery hemostasis to avoid damage to residual follicles. After removal, ovaries were immediately cut in half to expose the medulla (Fig. 1A, left panel). The ovary was immersed in modified-HTF medium supplemented with 10% Serum Substitute Supplement (SSS; IrvineScientific, Santa Anna, CA, USA) on a warm plate at 37°C. The majority of small residual follicles were located within a 1–2 mm thickness from the surface of the ovarian cortex in POI patients (Kagawa et al., 2009). Therefore, the medulla was removed by dissection with small scissors before thin layers of ovarian cortices (1–2 mm thickness) were prepared (Fig. 1A, middle panel) and cut into small strips (0.5–1 × 0.5–1 cm, 1–2 mm thickness; Fig. 1A, right panel). This step maximized the surface to mass ratio and the strips were suitable for loading onto the stainless needles of the Cryosupport (Fig. 1B, left panel). For cryopreservation of ovarian tissues, a vitrification method was used as previously described with modifications (Hashimoto et al., 2010; Suzuki et al., 2012). Ovarian strips were washed in TCM199 medium (Life Technologies, Foster City, CA, USA) supplemented with 20% (v/v) SSS (WS medium) and then sequentially exposed to three different vitrification solutions at room temperature. The strips were first equilibrated in TCM199 medium containing 10% (v/v) ethylene glycol (EG, Wako Pure Chemical Industries, Tokyo, Japan) and 20% (v/v) SSS for 5 min, followed by equilibration in the

TCM199 medium containing 20% (v/v) EG and 20% (v/v) SSS for 5 min. Subsequently, strips were transferred into the TCM199 medium containing 35% (v/v) EG, 5% (w/v) polyvinylpyrrolidone (Sigma-Aldrich, St. Louis, MO, USA), and 0.5 mol/l sucrose (Wako Pure Chemical Industries) for equilibration before the final step of vitrification. Within 15 min, ovarian strips were loaded on the Cryosupport (Fig. 1) consisting of four fine stainless needles and a cryogenic vial (BD Bioscience, San Jose, CA, USA) before direct immersion vertically into liquid nitrogen with a minimum volume of media. After putting the frozen strips individually into cryogenic vials, ovarian tissues were stored in liquid nitrogen until use. After successful vitrification, cortical strips became transparent in appearance (Fig. 1C, upper device), whereas failed vitrification was characterized by the crystalline appearance of a white cryohydrate (white arrow, Fig. 1C, lower device).

For tissue warming, ovarian strips loaded on the Cryosupport were immersed in 5 ml of TCM199 medium containing 20% SSS and 0.8 mol/l sucrose before warming at 37°C for 1 min. To remove cryoprotectant, ovarian strips were incubated in TCM199 containing 20% SSS and 0.4 mol/l sucrose for 3 min, followed by incubation in the WS medium for 5 min twice at room temperature. Warmed strips were kept in modified-HTF medium supplemented with 10% SSS until the next step.

Videos demonstration of the ovarian tissue preparation, vitrification and warming steps are shown in Supplementary Materials.

Histological analyses of residual follicles in ovarian cortices

To identify residual follicles in ovarian cortices of POI patients, 10–20% of the volume of the cortices was subjected to histological analysis. Pieces of ovarian cortex were fixed in the Bouin's solution for 2 h, embedded in paraffin, serially sectioned at a thickness of 4 μm and then stained with hematoxylin and eosin (H&E). Follicles were detected under a light microscope (Olympus, Tokyo, Japan). If we could detect some follicles in sections, we terminated

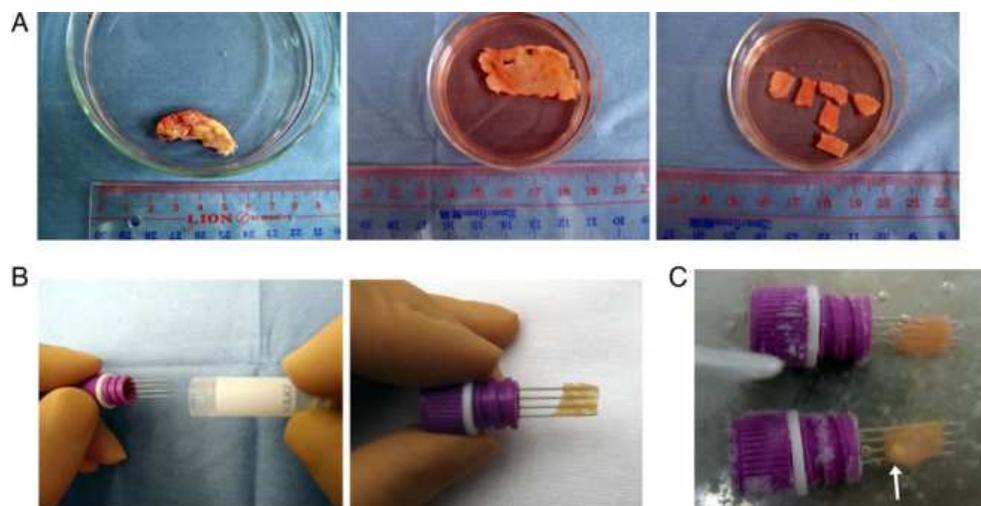


Figure 1 Vitrification of ovarian tissues from POI patients. After ovariectomy under laparoscopic surgery, ovarian cortices were dissected into small strips (0.5–1 × 0.5–1 cm) for vitrification using CryoSupport. **(A)** Preparation of ovarian cortical tissues for vitrification. Representative images were obtained from a patient with POI. Left panel: an ovary before dissection of medulla; middle panel: an ovary after dissection of medulla; right panel: small ovarian strips ready for vitrification. **(B)** The 'CryoSupport' device used for vitrification. Left panel: the CryoSupport composed of four stainless needles inserted into the cap of a cryogenic vial; right panel: an ovarian cortical strip from a POI patient placed on the CryoSupport. Owing to its thin thickness (1–2 mm), the cortical strip appeared transparent. **(C)** Appearance of ovarian cortical strips of POI patients after the initial vitrification procedure. Upper device: successful vitrification is characterized by the transparent appearance of a cortical strip; lower device: failed vitrification is characterized by the crystalline appearance of the white cryohydrate (arrow).

the analyses without examining remaining sections. Absence of residual follicles was decided after observation of all sections.

IVA: ovarian fragmentation, Akt stimulation and auto-transplantation

After patients recovered from the first surgery, two to three pieces of cryopreserved ovarian strips were warmed at 2 days before the second laparoscopic procedure. Ovarian strips were further fragmented into smaller cubes ($1-2\text{ mm}^3$) using a fine scalpel. Six to nine ovarian cubes were put on cell culture inserts (Millicell Cell Culture Insert, 12 mm, polycarbonate, $3.0\ \mu\text{m}$; Merck Millipore, Darmstadt, Germany). They were treated with $30\ \mu\text{M}$ of bpV(hopic), a PTEN enzyme inhibitor (Merck Millipore), and $150\ \mu\text{g/ml}$ of 740YP (Tocris, Bristol, UK), a PI3K stimulator, for 24 h followed by incubation with 740YP alone for another 24 h in Dulbecco's Minimum Essential Medium/F12 medium containing 10% (v/v) human serum albumin (Mitsubishi Tanabe Pharma, Tokyo, Japan), $0.05\ \text{mg/ml}$ ascorbic acid, 1% (v/v) antibiotic/antimycotic solution (Life Technologies) and $0.3\ \text{IU/ml}$ recombinant FSH (GONAL-f; Merck Serono, Tokyo, Japan) at 37°C under a 5% CO_2 atmosphere (Hashimoto *et al.*, 2010). Bioactivities of the PTEN inhibitor and PI3K activating peptide have been pretested using murine models as previously described (Li *et al.*, 2010). For auto-transplantation, ovarian cubes were repeatedly washed for three times in warmed (37°C) culture media immediately before transplantation. Transplantation of ovarian cubes beneath the serosa of one or both Fallopian tubes was performed under the second laparoscopic surgery. The underside of serosa in Fallopian tubes was selected as the grafting site due to high vascularization, convenience for transvaginal ultrasound monitoring, and ease for oocyte retrieval from pre-ovulatory follicles. After cutting the serosa and making a pouch between serosa and Fallopian tube, $\sim 20-100$ ovarian cubes were inserted (Fig. 2A, upper and middle panels) followed by closure of the serosa using sutures (Kawamura *et al.*, 2013). Alternatively, the wound was covered by an oxidized regeneration cellulose (Interceed; Johnson & Johnson, Tokyo, Japan) to avoid cube loss from the graft site (Fig. 2A, lower panel).

Monitoring of follicle growth and IVF-embryo transfer

After auto-transplantation of ovarian cubes beneath the serosa of the Fallopian tubes, follicle growth in grafted sites was monitored weekly or biweekly by transvaginal ultrasound together with serum estrogen and gonadotrophin levels to detect growing antral follicles. It has been reported that suppression of endogenous gonadotrophins is important in the induction of folliculogenesis and ovulation in patients with POI likely by restoring the responsiveness of residual follicles to exogenous gonadotrophin stimulation and by mitigating the desensitization to gonadotrophins (Menon *et al.*, 1983; Nelson *et al.*, 1994; Ishizuka *et al.*, 1997). We treated patients with $0.625-1.875\ \text{mg}$ estrogen (Premarin; Pfizer Inc.) to suppress elevated endogenous gonadotrophins before exogenous gonadotrophin stimulation. When antral follicles were detected, monitoring frequency increased to every 2–3 days and follicle growth was promoted by injecting $150-300\ \text{IU}$ recombinant FSH (Merck Serono) daily until the follicle reached $>16\ \text{mm}$ in diameter or serum estradiol levels elevated to $>200\ \text{pg/ml}$. In some cases, $0.25\ \text{mg}$ of a GnRH antagonist, cetrorelix acetate (Cetrotide, Merck Serono), was administered daily when the growing follicle reached $\sim 14\ \text{mm}$ in diameter to avoid the premature LH surge. When follicle reached $>16\ \text{mm}$, oocyte maturation was induced by a single injection of $10\ 000\ \text{IU}$ hCG (Gonotropin; Asuka Pharma). A few patients received hCG when follicles reached 13–15 mm due to their personal scheduling reasons. At 36 h later, oocytes were aspirated from follicles using ultrasound-guided, transvaginal retrieval via a 19–20 G needle. After oocyte retrieval, IVF was performed by using ICSI

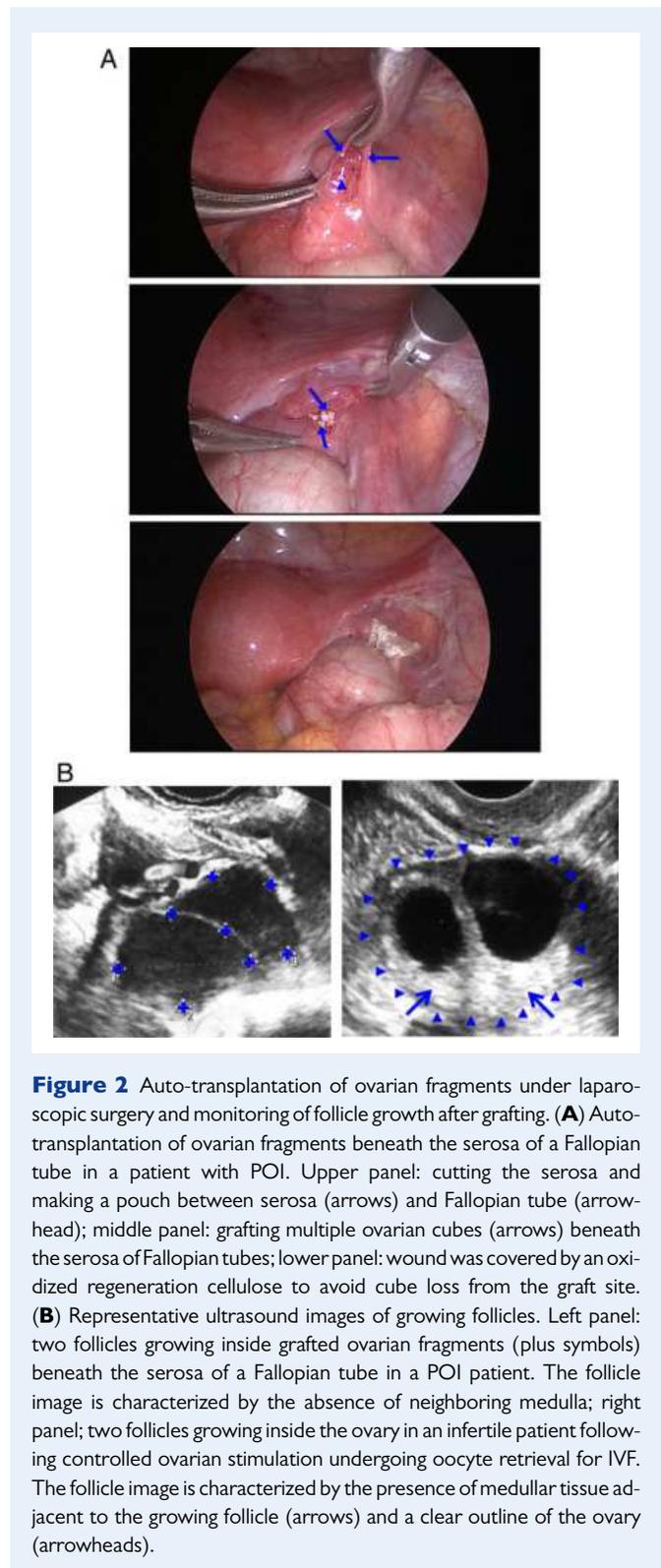


Figure 2 Auto-transplantation of ovarian fragments under laparoscopic surgery and monitoring of follicle growth after grafting. **(A)** Auto-transplantation of ovarian fragments beneath the serosa of a Fallopian tube in a patient with POI. Upper panel: cutting the serosa and making a pouch between serosa (arrows) and Fallopian tube (arrowhead); middle panel: grafting multiple ovarian cubes (arrows) beneath the serosa of Fallopian tubes; lower panel: wound was covered by an oxidized regeneration cellulose to avoid cube loss from the graft site. **(B)** Representative ultrasound images of growing follicles. Left panel: two follicles growing inside grafted ovarian fragments (plus symbols) beneath the serosa of a Fallopian tube in a POI patient. The follicle image is characterized by the absence of neighboring medulla; right panel: two follicles growing inside the ovary in an infertile patient following controlled ovarian stimulation undergoing oocyte retrieval for IVF. The follicle image is characterized by the presence of medullar tissue adjacent to the growing follicle (arrows) and a clear outline of the ovary (arrowheads).

before culturing injected oocytes in the fertilization medium (Quinn's Advantage® Fertilization HTF Universal Medium; CooperSurgical, Trumbull, CT) for 16 h. ICSI was used to increase fertilization success.

Fertilized oocytes were transferred to the cleavage medium (Global®, LifeGlobal, Tokyo, Japan) and cultured for one more day. Early preimplantation embryos at Day 2 of culture were cryopreserved by vitrification using

Cryotop (Kitazato BioParma; Kuwayama, 2007) and stored in liquid nitrogen until use. To improve embryo implantation of POI patients, embryos were cryopreserved and then transferred following hormone supplementation of patients with estrogens (Premarin and Estrana TAPE; Hisamitsu Pharmaceutical) and then progesterone (Progestone; Fuji Pharmaceutical). The dosage and duration of estrogen and progesterone treatments were determined based on the uterine endometrial status of individual patients. After embryo transfer, patients were treated with 10 mg of oral progesterone daily (Provera; Pfizer Inc.) and 125 mg of i.m. injection of progesterone weekly (Proge depot; Mochida Pharmaceutical) for 6 weeks to support early pregnancy. Establishment of pregnancy was determined by ultrasound and by measuring serum hCG levels. After the patient became pregnant, development of fetus was monitored by routine prenatal checkups.

Statistical analyses

Statistical analysis was carried out by using a non-parametric Kruskal–Wallis test to evaluate differences of time from diagnosis of POI to ovariectomy and Fisher's exact test to evaluate differences of the frequencies of AMH concentrations above a threshold (0 pg/ml) between the groups. Fisher's exact test was used to compare the proportion of patients with endometriosis. Results for age at ovariectomy are presented as mean \pm standard deviation, whereas data for time from diagnosis of POI to ovariectomy are expressed as median (quartiles).

Results

Vitrification of ovarian tissues from POI patients

The numbers of ovarian strips prepared from POI patients were limited when compared with those from 'normal' ovaries of cancer patients for fertility preservation (POI: mean 7.2 ± 5.4 strips, range: 1.5–25 per ovary, $n = 37$; cancer patient: mean 19.2 ± 5.1 strips, range: 11–30 per ovary, $n = 18$).

Auto-transplantation of ovarian cubes and monitoring of follicle growth

In patients with follicle growth in grafts beneath Fallopian tubes, growing follicles in the graft (Fig. 2B, left panel, plus signs) were detected based on their unique morphology characterized by the absence of a neighboring medulla. This is in direct contrast to the routine detection of growing follicles inside the ovary of infertile patients undergoing oocyte retrieval for IVF following controlled ovarian stimulation. For these follicles inside the ovary (Fig. 2B, right panel), adjacent medullar structure (arrows) and the outline of ovary (arrowheads) could be detected.

Histological assessment of residual follicles in ovarian cortices for vitrification

In our previous study (Kawamura et al., 2013), follicle growth after IVA in POI patients was only detected in patients with residual follicles based on histological analyses of ovarian cortices. After enrolling 10 more patients, we re-evaluated if this approach could be a reliable parameter to predict the IVA outcome. In general, we used 10–20% of each ovarian strip to evaluate residual follicles. Although total numbers of residual follicles were different among patients, we could find a few primordial, primary and secondary follicles in sections from some patients (Fig. 3A, left upper and lower panels). In contrast, 46% of patients showed the absence of residual follicles even after extensive histological analyses

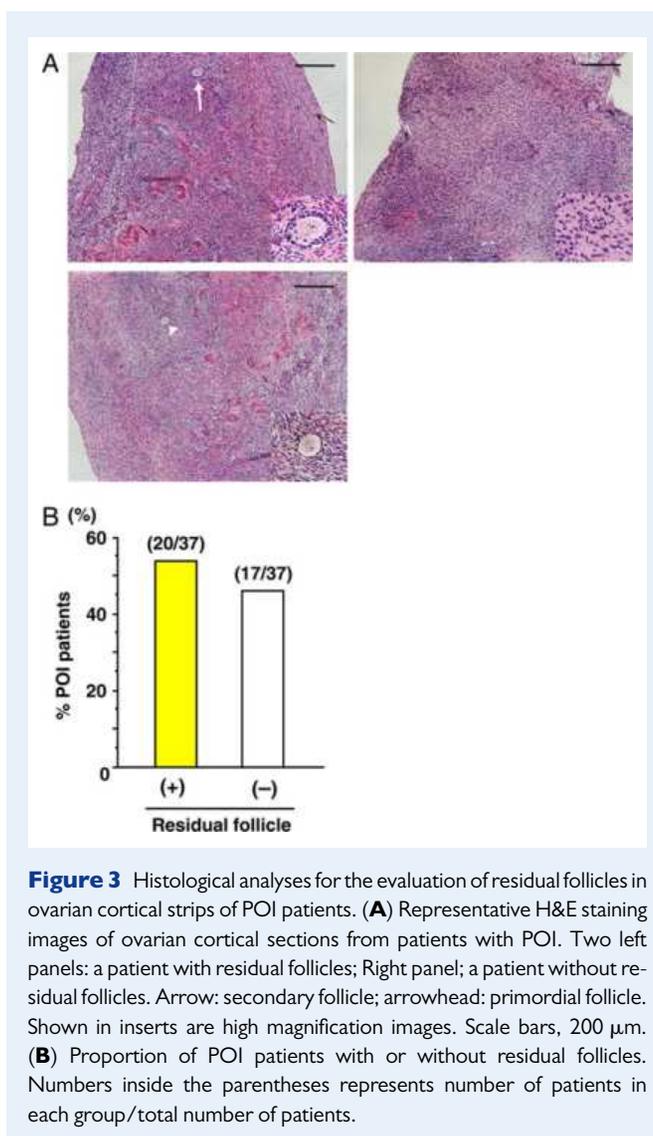


Figure 3 Histological analyses for the evaluation of residual follicles in ovarian cortical strips of POI patients. (A) Representative H&E staining images of ovarian cortical sections from patients with POI. Two left panels: a patient with residual follicles; Right panel: a patient without residual follicles. Arrow: secondary follicle; arrowhead: primordial follicle. Shown in inserts are high magnification images. Scale bars, 200 μ m. (B) Proportion of POI patients with or without residual follicles. Numbers inside the parentheses represents number of patients in each group/total number of patients.

(Fig. 3A, right panel showing the presence of connective tissue cells alone in the ovarian cortex). Ratios of patients with or without residual follicles are shown in Fig. 3B. For 17 patients without residual follicles based on histological analyses, we performed auto-transplantation following patients' requests. However, none of these patients showed follicle growth during \sim 1 year of observation after transplantation. Thus, it is becoming clear that our histological analyses appear to be a valid parameter to predict potential IVA success.

Successful follicles growth after IVA followed by auto-transplantation

By adding 10 new patients to the cohort of 27 patients in a previous paper (Kawamura et al., 2013), we now describe findings on 37 POI patients. Among them, ovaries from 20 out of 37 contained residual follicles based on the histological analyses. In patients with follicles, 9 out of 20 showed follicle growth in auto-grafts with 24 oocytes retrieved from six patients. Following IVF and embryo transfer into four patients, pregnancies were detected in three patients based on serum hCG, followed by one miscarriage and two successful deliveries. In addition to the first

healthy male baby born after IVA treatment (Kawamura *et al.*, 2013), a female baby (birthweight, 2970 g; and Apgar score, 8 at 1 min/9 at 5 min; blood pH level of umbilical artery, 7.27) was delivered at 38 weeks and 2 days of pregnancy by caesarian section. Physical features of the baby are normal, together with normal placenta (443 g) and umbilical cord. Checking the transplantation sites of ovarian fragments indicated no abnormal growth in the transplanted site beneath the Fallopian tubes.

Based on the clinical data and patient history, we attempted to identify additional parameters to predict IVA success. As shown in Table I, patient age at ovariectomy for vitrification was similar in all groups, but the duration from the diagnosis of POI to ovariectomy was significantly shorter in those patients with residual follicles that responded to IVA treatment. Among 37 POI patients, only nine patients showed detectable levels of serum AMH and patients with positive IVA outcomes had the highest median serum AMH levels (Table I). Among patients containing residual follicles, those responded to IVA treatment by showing follicle growth have median of serum AMH levels almost 2.3 times higher than those not responding to IVA treatment (Table I). However, there was no difference of the frequencies of AMH concentrations above a threshold (0 pg/ml) between groups with histological presence and absence of residual follicles (20 versus 29.4%), thus suggesting serum AMH levels are a secondary parameter for predicting IVA success when compared with screening using histological analyses. Interestingly, we frequently detected endometriosis in patients with residual follicles and responded to IVA treatment by showing follicle growth (Table I).

Discussion

We successfully cryopreserved functional early ovarian follicles using the vitrification protocol described above based on successful follicle growth after IVA and auto-grafting. Enrolling 10 more patients in addition to those described in our previous publication (Kawamura *et al.*, 2013), we demonstrated that histological analyses of ovarian cortices and the patients' history of duration from POI diagnosis to ovariectomy are associated with the IVA outcome.

Although we have not compared vitrification versus slow freezing for ovarian tissue cryopreservation here, our findings suggest that the present vitrification protocol is effective for ovarian tissue preservation. Cryopreservation of ovarian tissues is becoming an important option for fertility preservation in cancer patients. Its advantages include its

availability to prepubertal girls and the storage of a large number of immature oocytes; moreover, cryopreservation of ovarian tissue minimizes the delay needed for freezing of mature oocytes or embryos. Ovarian tissue vitrification can be scheduled immediately, whereas substantial times are required for ovarian stimulation to obtain mature oocytes and embryos (Loren *et al.*, 2013). For cancer patients, ovarian tissue cryopreservation allows chemo- or radiation therapies to be started without delay. In the cases of POI, ovarian reserve, namely the pool of residual follicles, continues to diminish with age (Nelson, 2009). This is consistent with our findings showing that a shorter duration of amenorrhea from POI diagnosis to ovariectomy is associated with better responses to the IVA treatment. If one ovary is cryopreserved at an earlier age, patients could undergo additional non-invasive infertility treatments before the final decision for the IVA treatment. In addition, cryopreservation of ovarian tissues allows sufficient time for histological analyses to evaluate the presence or absence of residual follicles before a decision is made for auto-transplantation. It also provides the option to schedule the second surgery more conveniently.

The present approach represents a successful cryopreservation of ovarian tissues using vitrification for the generation of functional mature oocytes for infertility treatment. Based on our experience, it is important to avoid failure of vitrification during the step of cryoprotectant equilibration. This failure can be minimized by preparing thin layers of ovarian strips (Amorim *et al.*, 2011). For the vitrification of POI ovaries, special care should also be taken for tissue preparation due to the frequent presence of uneven ovarian surfaces caused by ovarian shrinkage, especially in advanced POI cases.

For transplantation of cryopreserved ovarian tissues, one could select the original site (orthotopic) for grafting, including the peritoneum of the ovarian fossa and the remaining ovary. Orthotopic site has the advantages of using the original niche for follicular development and the possibility for natural conception. At present time, more than a dozen of live births have been reported after auto-transplantation of cryopreserved ovarian tissues at orthotopic sites (Donnez *et al.*, 2004). For heterotopic grafting, the subcutaneous site is frequently chosen due to the involvement of minimal invasive surgical intervention and easy access for oocyte retrieval (Demeestere *et al.*, 2009). However, heterotopic grafting was less effective for successful pregnancy. Recently, one live birth was reported after grafting cryopreserved ovarian tissues at pockets on the rear side of the broad ligament, following promotion of neoangiogenesis by adding platelet-rich plasma containing high concentration of

Table I Possible parameters to evaluate successful follicle growth after IVA treatment.

Residual follicles at histology and growth after transplantation (n)	Age at ovariectomy (y)	Time (y) from diagnosis of POI to ovariectomy	Number of patients with AMH before ovariectomy (pg/ml)*	% patients with endometriosis (n)
None (17)	37 ± 4.7 (28–43)	7.5 (2.5–12.1) ^a	5 (196)	6 (1) ^b
Present but no growth (11)	37 ± 4.9 (31–48)	5.0 (3.0–5.5)	1 (170)	0 (0)
Present and growth (9)	39 ± 4.4 (29–42)	1.0 (0.5–4.7) ^a	3 (390)	44 (9) ^b

Data for age at ovariectomy are mean ± SD (range), whereas data for time from diagnosis of POI to ovariectomy are median (quartiles). For anti-Müllerian hormone (AMH), the number of patients with detectable AMH and median of serum AMH levels among these are shown.

*Data (pM) obtained by Active MIA/AMH EIA were converted into the unit (ng/ml) used for the AMH Gen II ELISA by a multiplication factor of 0.14 but are shown as pg/ml for clarity. Statistical analysis was carried out by using a non-parametric Kruskal–Wallis test to evaluate differences of time from diagnosis of POI to ovariectomy and Fisher's exact test to evaluate differences of the frequencies of AMH concentrations above a threshold (0 pg/ml) between the groups. Fisher's exact test was used to compare the proportion of patients with endometriosis. ^{a,b}Values in the same column with different superscripts are significantly different ($P < 0.05$).

pro-angiogenic growth factors (Callejo et al., 2013). We chose the serosa of Fallopian tubes as the auto-grafting site due to its high vascularity and the ease to monitor follicle growth. Because we observed minimal bleeding after surgical cutting of POI ovaries during removal, we hypothesized that the remaining ovary is not optimal for the revascularization of transplanted tissues. Future studies are needed to evaluate the best auto-grafting sites for ovarian tissues.

As indicated earlier (Kawamura et al., 2013), we started with bilateral ovariectomy for IVA treatment but subsequently recommended the removal of one ovary because only small numbers of vitrified ovarian strips are needed for the IVA procedure and many patients expressed the intention to retain one ovary. Following cutting ovarian strips into cubes (Hippo signaling disruption) and Akt stimulation using IVA drugs, multiple antral follicles were detected in 45% of POI patients containing residual follicles. Twenty-four oocytes were retrieved from six patients and 15 embryos were derived from six patients. Among them, embryo transfer was performed for four patients and ongoing efforts are being made to generate more oocytes and embryos for the remaining patients. Three patients became pregnant based on elevated serum hCG levels. Among these three patients, one had a miscarriage and two gave birth. Although two healthy babies were born and the first baby showed normal development during 22 months after birth, more follow-up studies are required to ensure the safety of the present IVA procedure.

It is well-known that the quality of oocytes decreases with age (Broekmans et al., 2009) and it is important to note that the present IVA treatment mainly promotes follicle growth and is unlikely to correct any age-related decline in egg quality. For POI patients, who are candidates for IVA treatments, cryopreservation of ovarian tissues at a younger age is highly recommended for the minimization of age-related increases in genetic defects in oocytes.

The semi-quantitative histological analyses show the strongest association with IVA outcome. For non-invasive approaches to predict the presence of residual follicles, our data suggested that high levels of serum AMH are associated with a better IVA outcome. However, some patients with undetectable AMH levels could still respond to the IVA treatment. In the human ovary, AMH expression was negligible in primordial follicles, low in granulosa cells of primary follicles but highest in granulosa cells of secondary, pre-antral and small antral follicles ≤ 4 mm in diameter. In larger antral follicles, AMH expression gradually disappeared (Weenen et al., 2004). Because the IVA treatment presumably promotes the development of primordial, primary and secondary follicles, patients with residual primordial and primary follicles could also respond to the IVA treatment. Thus, our findings indicated that serum AMH is not the best predictor for treatment outcomes.

A shorter duration from diagnosis of POI to ovariectomy is also associated with the likelihood of follicle growth. For the treatment of symptoms of estrogen deficiency, POI patients often received cyclic estrogen and progesterone replacement, leading to the induction of artificial menses. As a result, it is sometimes difficult to know the exact duration of amenorrhea after POI diagnosis. Thus, in this study, we used the duration from the diagnosis of POI to ovariectomy as one of the parameters.

We also found prevalent endometriosis in patients responding to IVA treatment. Vascular endothelial growth factor (VEGF) is the most prominent pro-angiogenic factor shown to promote angiogenesis in endometriosis (Taylor et al., 2002). Of interest, promotion of neovascularization at ovarian transplantation sites by creating peritoneal wounds before

grafting (Donnez et al., 2004) and by using platelet-rich plasma containing pro-angiogenic growth factors (Callejo et al., 2013) have been found to enhance ovarian grafting success. One can speculate that VEGF derived from endometriosis might contribute to a better outcome of IVA. However, the number of patients under investigation here is too low and future expansion of IVA treatments is needed to evaluate if inclusion of angiogenic factors is useful. Also, future studies are needed to identify additional markers to indicate the presence of residual follicles in POI and to predict IVA treatment outcome using non-invasive approaches. Different oocyte-derived factors, including GDF-9, BMP15 and R-spondin2 (McGrath et al., 1995) are potential candidates due to the larger size of oocytes when compared with small numbers of surrounding somatic cells in pre-antral follicles. Development of ultra-sensitive assays are needed to detect the presumably low 'leakage' of these secreted factors into the systemic circulation.

Supplementary data

Supplementary data are available at <http://humrep.oxfordjournals.org/>.

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Authors' roles

K.K. designed the study and wrote the manuscript. N.S., N.Y., S.T., Y.S., M.T., S. H., Y.M. and K.K. conducted the studies. N.S. participated in critical discussions and manuscript editing.

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Conflict of interest

None of the authors has a conflict of interest.

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