OVARIAN CRYOPRESERVATION AND TRANSPLANTATION: BASIC ASPECTS

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Abstract:

Animal studies and studies using human ovarian xenografts in immunodeficient mice have shown that ovarian transplantation is feasible. In this manuscript, we present the first and limited experience with ovarian tissue cryopreservation and transplantation in the human. Despite the progress, more basic studies are needed to improve the survival of primordial follicles during and after the cryopreservation process.

Introduction:

Ovarian tissue banking relies on the principle that the primordial follicles withstand the cryo-toxicity better than the growing follicle (1). A relatively inactive metabolism, lack of zona pellucida, and metaphase spindle grant this privilege to primordial follicle (2). In addition, smaller cell size allows faster penetration of cryoprotectants in the oocyte. It has been possible to isolate primordial follicles (1) but the only practical way of preserving these follicles at the present is to cryopreserve them in the tissue to perform auto-transplantation.

Antral follicles contain oocytes either in prophase-I or metaphase-II stage, and their oocytes are much more liable to cryo-damage (table I). In addition to large cell size and zona pellucida, metaphase II oocytes have an additional disadvantage over prophase I oocytes: they have a cell spindle. Studies have shown that even minor variations in ambient temperature can result in damage to the tubulin proteins in the spindle, albeit some of the damage may be reversed by rewarming (3,4). The spindle is a dynamic structure composed of microtubules which are continuously being assembled at one end and removed at the other in a treadmilling fashion (5). Although the chromosomes can reassemble and align along the spindle equator on rewarming the cell, there is a risk of

chromosomal loss and aneuploidy occurring at the first maturation division (6). It has also been shown that the cytoskeleton of the antral follicle oocytes is damaged by the cryopreservation process, which could lead to significant changes in the organization and trafficking of molecules and organelles (7). In addition, studies have shown that cryopreservation may result in hardening of zona pellucida, preventing natural fertilization (6)

Animal Studies:

Whole-organ and fetal ovarian tissue cryopreservation and transplantation have been a great success in the rodents (8-10). However, human ovary is much larger and fibrous, and therefore better animal models were required before the technique could be applied to humans.

Sheep ovary is similar to human ovary, in that; it has a dense fibrous stroma and relatively high primordial follicle density in the ovarian cortex. Gosden and colleagues have performed a two-part study in the sheep. In the first, frozen-banked strips of ovarian cortical pieces were autotransplanted on the infindibulopelvic ligament (11). Each animal also had a fresh transplant on the opposite site serving as a control. Four months after the transplant, first signs of ovulation were detected. Two pregnancies had occurred, one from a fresh another from a frozen-thawed graft. In the second study, Baird and colleagues have performed autotransplants with frozen tissue in 8 sheep and followed them for up to 22 weeks (12). All the animals resumed cyclicity and showed hormone production. In that study, baseline levels of FSH were elevated but luteal phase

was progesterone measurements were normal. However, serum inhibin-A levels were found to be low in the luteal phase.

Studies Involving Human Ovarian Tissue:

SCID-mice carry a genetic mutation, which results in T-cell and B-cell immune-deficiency (13). This allows xenografts to revascularize and survive in these animals without being rejected. Roger Gosden has adopted this model for human ovarian xenografting. In his earlier studies, both marmoset and sheep ovarian tissues were transplanted under the kidney capsule, and they grew to antral stages (14). In the first study using human tissue, Gosden and colleagues cryopreserved ovarian cortical pieces with various cryoprotectants and grafted into SCID mice (15). After 18 days, the grafts were removed and primordial follicle counts were obtained. With the exception of glycerol, all cryoprotectants (propanediol, ethylene glycole, DMSO) did well and 44-84% of the follicles survived.

After achieving successful results with short-term xenografting, we performed the two long-term studies in SCID mice using human tissue (16,17). In the first study, fresh ovarian tissue was used (16). One mm^3 ovarian pieces from a 17 year-old patient were grafted under the kidney capsules of hypogonadal SCID mice. During the last 6 weeks of 17-weeks grafting period, one group of animals received FSH stimulation. Estradiol levels were measured at the end of 22 weeks, when animals were sacrificed.

In the FSH treated group, up to 5 mm size antral follicles were found, estradiol levels peaked >700 pg/mL and the uteri showed clear signs of estrogenization. Because the animals were oophorectomized, the only source of estrogen was human ovarian xenografts.

In the second study, we grafted frozen-thawed human ovarian tissue into SCID mice (17). Because these animals were not hypogonadal, no FSH was given. Grafts were recovered 22 weeks later. Histological examination showed follicle growth initiation and an abundant number of healthy primordial follicles remaining. Presumably, because no exogenous FSH was given, further follicle development did not occur. However, this study further strengthened the notion that human primordial follicles survive the triple insult of freeze-thaw-transplant in long-term transplants.

Finally, to further confirm and quantify the survival of primordial follicles of freezing process, we have performed viability studies on isolated human primordial follicles (1). 2x2 mm. ovarian cortical pieces were cryopreserved with the slow-freeze process (table-I). After thawing, the tissues were partially digested using collagenase type IA (Sigma, USA) followed by microdissection of primordial follicles. These follicles were then incubated with viability stains. We found that, approximately 70% of follicles survived this process and were viable. Later electron microscopic studies, however, showed that this digestion method might damage the basement membrane of the follicle but not the oocyte is rarely affected (18).

Clinical Trials of Human Ovarian Tissue Cryopreservation and Transplantation

Encouraged by these laboratory studies, we have established a human ovarian transplantation project. In the first-phase, only patients aged<35 years were enrolled, and, malignancy cases were excluded.

In the 32 year-old first patient, ovarian tissue was grafted in the forearm into the brachioradialis muscle, akin to the method used for parathyroid gland transplantation. We also grafted tissue in the broad ligament, adjacent to the uterine artery. Four months after the procedures, ultrasound follow-up indicated that grafts were still intact, and some early antral follicle development was noted in the forearm by high-frequency ultrasound probes. A gradient was detected for estradiol, between the antecubital vein (which the graft drains to) and wrist vein, indicating hormonal production by the graft.

In the second patient, frozen-banked tissue was thawed 8 months after storage, sawn together microscopically to form 2 large grafts, and transplanted laparoscopically in the ovarian fossa (19). The patient is currently under follow-up, however, early findings indicate the normalization of serum testosterone levels and resumption of ovarian function .

Conclusions:

There has been significant progress in ovarian tissue cryopreservation and transplantation in the past 5 years. Now, large-scale human transplantation studies are needed to test the efficacy of this procedure. In addition, research is needed to develop better cryoprotectants and cryopreservation protocols as well as new transplantation techniques to reduce follicle losses that normally occur during the freeze-thaw and grafting process.

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Table I. Comparison of available options for banking follicles, oocyte and ovarian tissue

Cell/Tissue Type	**Size**	**Advantage**	**Disadvantage**
Primordial Follicle	30-50 μm	Least differentiated No ZP No cell spindle	Difficult to isolate Damage to basement membrane Culture not yet possible
Preantral Follicle	60-200μm	Easier to isolate No cell spindle Culture possible	ZP damage More differentiated
Prophase I Oocyte	80-100μm	No spindle Short in vitro maturation	ZP damage Low IVM and fertilization Very few live births
Metaphase II Oocyte	80-100μm	Easy to obtain	ZP damage Cell spindle damage Organelle damage Very few live births
Ovarian Cortex	1 X 1 mm to 1x3 cm strips	Easy to obtain Preserves stroma Can restore fertility Preventive before cancer treatment	Experimental Risk of cancer cell reseeding

ZP: zona pellucida
IVM: in vitro maturation

Table II Protocol for cryopreserving human ovarian tissue

1) Equilibrate 1-3 mm thick slices of ovarian cortex for 30 minutes on ice in buffered medium containing 1.5 M DMSO(Propanediol/Ethylene Glycole) 10% serum and 0.1M sucrose.

2) Load the tissue in cryovials into an automated freezer starting at 0 °C and cool at 2 °C/min to -7 ° C

3) Soak for 10 min before manual seeding

4) Continue to cool at 0.3 ° C/min to -40 ° C

5) Cool at the faster rate of 10 ° C/min to -140 ° C

6) Transfer to liquid nitrogen dewar for storage

7) Thaw at room temperature for 30 seconds than at 37 °C for 2 minutes

8) Wash tissues stepwise in containing progressively lower concentrations of cryoprotectant media (1.5 M, 1.0 M. 0.5 M, 0 M).