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The future of human ovarian cryopreservation and transplantation: fertility and beyond

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Objective: To review the current progress in ovarian cryopreservation and transplantation and to discuss the obstacles with the clinical application of this technique.

Design: The literature on ovarian cryopreservation and transplantation was reviewed to facilitate understanding and predict future directions. The studies related to this topic were identified through MEDLINE and other bibliographic databases, focusing on the most recent developments.

Conclusion(s): The experimental evidence for low-temperature storage of ovarian tissue is encouraging. Although restoration of fertility with cryopreserved ovarian grafts has been successful in various animals, there are uncertainties about the optimum use of stored ovarian tissue in humans. Autotransplantation appears to be promising, but the potential risk of transmitting malignant cells in women with cancer is of great concern. The maturation of primordial follicles with xenotransplantation is encouraging, but the efficacy and the safety of this method need further investigation. Furthermore, the quality of oocytes that have been matured in a host animal is unknown. The development of in vitro culture systems for oocyte maturation is still in its infancy. There are many issues to be resolved in ovarian transplantation before the full clinical use of this emerging technique. Most of all, there is an urgent need to optimize the freeze/thaw procedure and to find the means to protect grafts from ischemia-reperfusion injury. Nevertheless, ovarian transplantation should prove to be clinically useful for women at risk for premature ovarian failure. (Fertil Steril® 2001;75:1049–56. ©2001 by American Society for Reproductive Medicine.)

Key Words: Transplantation, cryopreservation, antifreeze protein, ovarian tissue, fertility, cancer

The recent surge of interest in ovarian tissue banking reflects the importance and the need of fertility conservation for many women facing premature ovarian failure, particularly those with cancer. The emergence of new technologies can also create confusion and false expectations. Therefore, it would be beneficial to obtain a better understanding by reviewing and updating current studies related to this emerging technology. The concept of ovarian transplantation is not new, but the clinical application of this technique in conjunction with modern reproductive technologies should be viewed as innovative.

Ovarian transplantation has a long history, dating back to the 18th century. However, it was not until the turn of the 20th century that widespread interest in reproductive organ transplantation was generated. As concepts emerged in endocrinology, experimental transplantation offered novel opportunities for investigators to test hormonal secretion and action (1). Despite many attempts at allogeneic ovarian transplantation in the 1900s, no clear clinical benefit was realized, primarily because of immune reactions. The concept of autotransplantation was also not applicable in practice because of the absence of long-term organ storage techniques. A breakthrough occurred in 1948 when the first cryoprotectant, glycerol, was discovered at the Audrey Smith's laboratory in London. The development of freezing methods using cryoprotectants led to crude, but valuable, pioneering work on the transplantation of cryopreserved gonadal tissue in the 1950s (2, 3). Over the next 30 years, however, there was no further progress in this field. In the 1990s, the recognition of the potential clinical application of this technology in reproductive medicine and animal sciences rekindled interest in ovarian cryopreservation and transplantation.

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To date, successful cryopreservation and transplantation of ovarian tissue has been achieved in various animals. Cryopreservation of mouse ovarian tissue was found to produce good results, with restoration of fertility after transplantation (4–6). Furthermore, Candy et al. reported the restoration of normal reproductive life span after grafting cryopreserved mouse ovaries (7). In large animals, restoration of fertility in oophorectomized sheep by ovarian autografts stored at −196°C has been demonstrated (8). Ovarian tissue from marmoset monkeys, which had been frozen and grafted into immunodeficient mice, demonstrated that the total number of follicles and the proportion of normal follicles were similar in fresh and frozen grafts, and estrogen activities were restored after transplantation of frozen grafts (9).

Recently, it has been reported that ovulation occurred in autografted human ovarian tissue after gonadotropin stimulation (10). In fact, multiple clinical centers worldwide have been storing human ovarian tissue, even though it has not yet proven to be clinically effective. When methods have been proven to be effective and healthy children have been conceived using oocytes derived from frozen/thawed ovarian tissue, it is likely that the applications will grow from strictly medical indications to those with social implications.

CRYOPRESERVATION

Freezing Injury

When isolated cells or tissues are frozen, a number of biological and chemical changes take place. To minimize injury from freezing, an understanding of the nature and cause of these alterations is required. Cooling retards the process of degradation and therefore preserves the structure of cells. However, it can destroy their function irreversibly when it goes below the freezing temperature without cryoprotective agents. Although cells frozen and stored at the temperature of liquid nitrogen (−196°C) can maintain their viability for many years, the rate at which they are frozen and thawed is crucial for survival. The rate of temperature change controls the transport of water across the cell membrane and hence, indirectly, the probability of intracellular freezing (11). Each cell has an optimal cooling rate, although absolute survival at that cooling rate is extremely low unless a cryoprotectant is present to reduce damage.

The main causes of freezing injury are intracellular ice crystal formation and salt deposits. Significant freezing injury can occur during the thawing (reexpansion) phase because of changes in the composition of the surrounding milieu, possibly mediated by temporary leakage of the plasma membrane (12). Although cryoprotectants are imperative for the successful freezing, many cryoprotectants can be cytotoxic. This cytotoxic property is enhanced by prolonged exposure times at temperatures above 0°C (13, 14).

Cryoprotective Agents

Cryoprotection of cells can be achieved by many different chemicals, such as alcohols, amines, sugars, and proteins. An effective cryoprotectant depends on a number of key properties: high water solubility to depress the freezing point, high permeability to minimize the osmotic gradient, and low toxicity. Although many compounds have some of these properties, dimethyl sulfoxide (DMSO), 1,2-propanediol (PROH), ethylene glycol (EG), and glycerol are most commonly used for the freezing of living cells. The efficiency of these four cryoprotectants has been compared by freeze-thawing and subsequently xenografting human ovarian tissue into immunodeficient mice (15). The experiment showed that DMSO, PROH, and EG were much more effective than glycerol, based on histological criteria. Glycerol, although chemically less toxic, may not be as effective for ovarian cryopreservation because of slower penetration and higher osmotic stress.

The toxicity of cryoprotectants depends on the inherent characteristics of the chemical itself, duration of exposure, and temperature. Rapid permeation of cells with cryoprotectants at low temperatures is desirable to minimize the toxicity. Takagi et al. (16) found in a bovine embryo study that the toxicity of different cryoprotectants is related to the exposure time. Multicellular tissues require longer exposure time to reach equilibration than single cells or preimplantation embryos; consequently, the risk of toxicity to the tissue will be increased. It has been found that PROH and glycerol penetrated the tissue significantly slower than either EG or DMSO at 4°C (17).

Antifreeze Proteins

The discovery of cryoprotective agents and the birth of modern cryobiology, despite a short history, have significantly affected the course of long-term storage of tissues and cells in various scientific disciplines. As a matter of fact, many animals and plants in nature have been using cryoprotective agents for a long time in an evolutionary process to protect freezing injuries and to survive in a freezing climate and environment. For instance, the larvae of a parasitic wasp, *Bracon cephi*, accumulate 25% glycerol in their body fluids during the autumn and can then tolerate winter temperatures as low as −50°C.

Another fascinating example is the production of antifreeze proteins by some species of polar fish and overwintering insects. Antifreeze proteins are mainly glycopeptides and probably act by an adsorption–inhibition mechanism to prevent the growth of ice crystals (18). It has been reported that an antifreeze protein from the common yellow mealworm beetle, *Tenebrio larvae*, has higher activity than fish antifreeze proteins (19). Natural antifreeze proteins may prove to be beneficial for vitrification (ultra-rapid cooling in the presence of high concentration of cryoprotectants to eliminate ice crystal formation). Rubinsky et al. (20) reported that the addition of fish antifreeze glycopeptides to

vitrifying solutions increases postthaw viability in cultured immature pig oocytes and embryos and appears to preserve cell membrane structural integrity.

Antifreeze proteins have been found not only in animals but in plants as well. Researchers in York have isolated antifreeze proteins from the taproot of cold-acclimated carrots (*Daucus carota*). This protein inhibits the recrystallization of ice and exhibits thermal-hysteresis (antifreeze) activity (21). Although antifreeze proteins appear to reduce freezing injury and improve cell viability, the practical value and application of antifreeze proteins needs further investigation.

THE FUTURE OF OVARIAN TRANSPLANTATION

Clinical Applications

The original impetus for storing ovarian tissue came from the threat posed to ovarian function by the use of potentially sterilizing chemotherapeutic drugs and/or abdominal radiation. In particular, there are concerns about the reproductive health of children and young women undergoing gonadotoxic treatment for malignancy or other conditions (e.g., SLE). It has been estimated that the proportion of childhood cancer survivors in the adult population will approach 1 in 250 by the year 2010 (22). According to the National Cancer Data Base, the 5-year survival rate of children with solid tumors increased from 27% in 1960 to 70% in 1990 (23).

Ovaries are susceptible to cytotoxic treatment, especially to alkylating agents and ionizing radiation (24, 25). Because the ovary serves both endocrine and reproductive functions, its damage may result in a number of clinical problems, including amenorrhea, menstrual irregularity, loss of libido, failure to develop secondary sex characteristics, infertility, and premature ovarian failure. It has been reported that 42% of patients with cancer treated with combined modality reached menopause by age 31, compared with 5% of controls (26, 27). The number of young women and children who lose ovarian function by high-dose chemotherapy and/or total body irradiation has been increasing. There are also cases of premature ovarian failure caused by genetic, infectious, and autoimmune factors, which could also potentially benefit from the development of ovarian cryopreservation and transplantation technology.

Fertility Conservation Options

There are several potential options for preserving fertility for the patient who is facing premature ovarian failure (Table 1), including oocyte cryopreservation, embryo cryopreservation, and cryopreservation of ovarian cortical tissue. Although mature oocytes can be successfully cryopreserved in the mouse (28, 29), the success rate of human oocyte cryopreservation is still limited (30–33). Mature human oocytes are extremely sensitive to temperature change and have little capacity for repairing cellular damage. Cryoprotectant exposure and/or ice crystal formation during the freeze/thaw procedure can lead to depolarization of the meiotic spindle, disruption of chromatid separation during meiosis, and the potential induction of aneuploidy (34, 35). Furthermore, zona hardening can occur as a result of the premature release of cortical granules from the ooplasm (36).

TABLE 1

Fertility conservation options for cancer patients.

Chemoprotection
Transposition of gonads
Cryopreservation of gametes
Cryopreservation of gonads
Cryopreservation of embryos

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The cryopreservation of embryos is a well-established technique with a relatively good efficiency. However, it has limited application in cancer patients because it requires IVF, with its lengthy protocol, and is only an option for patients who have a partner or are willing to accept fertilization by donor sperm. Obviously, it is not suitable for prepubertal children.

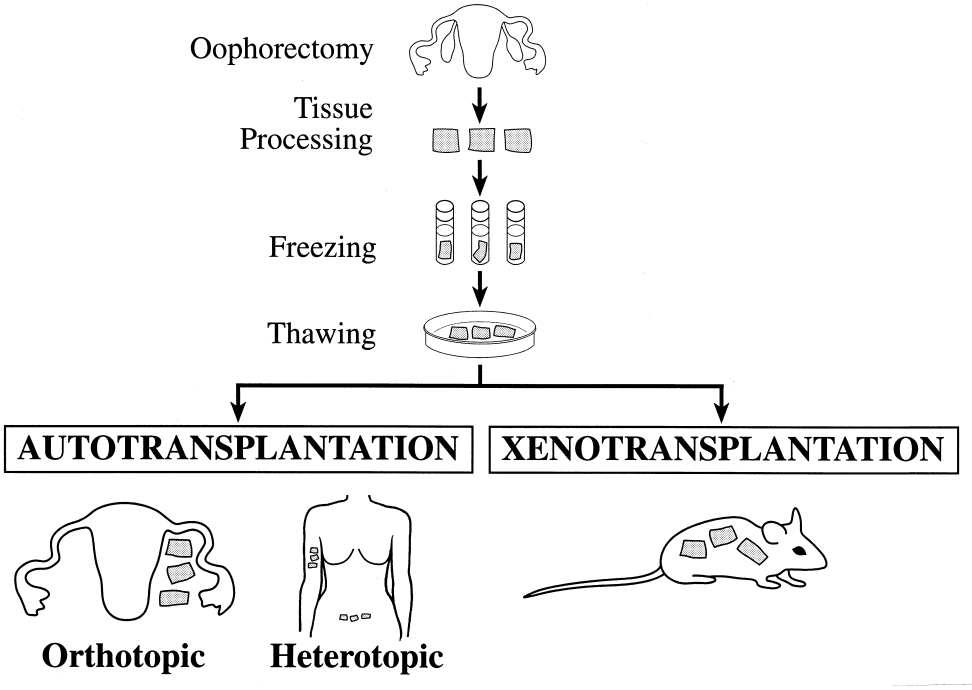
Cryopreservation of ovarian tissue has several potential advantages over both oocyte and embryo freezing. In this procedure, hundreds of immature oocytes are cryopreserved without the necessity of ovarian stimulation and delay in initiating cancer treatment (37). It offers the potential for the restoration of natural fertility with less ethical dilemma. The technology involves freezing immature primordial follicles in situ in the ovarian cortex. This is of great benefit because immature oocytes are relatively quiescent, smaller, and lack zona pellucida and cortical granules. These properties make them far more tolerant to freezing and thawing injuries than mature oocytes. Furthermore, primordial follicles have more potential to repair sublethal damage to organelles and other structures during their prolonged growth phase.

Collection of Ovarian Tissue

A major advantage of ovarian cryopreservation is that this technique does not delay cancer treatment, unlike cryopreservation of embryos or oocytes. However, the patient needs to undergo a surgical procedure. In most cases, ovarian tissue can be collected using a laparoscopic technique, either laparoscopic oophorectomy or multiple ovarian biopsy (38). For an experienced laparoscopic surgeon, there would be little difference between these two procedures in terms of either operation time or the degree of surgical difficulty. The choice of surgical procedure can be determined by the risk of premature ovarian failure and the prognosis after the cytotoxic cancer treatment. It is also important to consider the fact that a significant follicular loss occurs with freezing, thawing, and grafting. We do not know how well and how

FIGURE 1

Options for development of immature oocytes in cryopreserved ovarian tissues include autotransplantation and xenotransplantation. An orthotopic site or a heterotopic site (e.g., intramuscular, subcutaneous) can be considered for autotransplantation. Xenotransplantation of human ovarian tissue into immunodeficient animals can prevent immunological rejection phenomena. Oocyte retrieval and in vitro fertilization will be required for heterotopic autotransplantation as well as for xenotransplantation.



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long a given frozen/thawed ovarian section will function after transplantation in humans. For the time being, storing a relatively large amount of ovarian cortex with unilateral oophorectomy would be a more secure way to preserve fertility.

The Maturation of Oocytes in Stored Ovarian Tissue

At present, there are still uncertainties about the optimal use of stored ovarian tissue. Theoretically, there are three promising methods that can be applied to restore fertility.

Autotransplantation

Autotransplantation appears, at least in concept, to be a simple and practical method for maturation of oocytes in stored ovarian tissue. Cryopreserved ovarian tissue can be autografted either orthotopically or heterotopically (Fig. 1), but as yet, we do not know which is the more practical and effective approach. The theoretical advantage of orthotopic grafts is the restoration of normal reproductive function and natural conception after transplantation. However, the expected relatively short life span of frozen-thawed ovarian grafts is a concern. Where few follicles remain and early graft exhaustion is expected, it may be more reasonable to use a heterotopic site such as subcutaneous tissue and recover oocytes for an IVF procedure (39).

Although autografting into an orthotopic or heterotopic site seems most promising, the clinical application for cancer patients is problematic because of the potential risk of transmission of microscopic metastatic disease. Shaw et al. has reported that ovarian grafts from AKR mice can transfer lymphoma to recipient animals (40). The risk of transferring cancer cells depends on the disease type, activity, stage, and the mass of malignant cells transferred. Attempts to confirm the safety of ovarian tissue based on the absence of malignant cells by light microscopy may not be sufficient (41).

A recent study using a NOD/SCID xenograft model tested the safety of cryopreserved human ovarian tissue from cancer patients for transplantation (42). Although this study suggested that ovarian tissue transplantation in Hodgkin's disease patients was safe, it did not exclude the risk of cancer transmission in other types of cancer (especially hematogeneous or systemic neoplasms). Hence, it is necessary to

develop screening methods to detect minimal residual disease (MRD) in ovarian tissue to eliminate the risk of cancer cell transmission with transplantation.

Currently, molecular genetic techniques such as nested polymerase chain reaction (PCR), flow cytometry, fluorescence in situ hybridization (FISH), and cytogenetics have been applied to detect MRD before autologous peripheral stem cell or bone marrow transplant. The detection of specific gene translocation and immunoglobulin gene rearrangement in leukemia has been successful using PCR amplification (43). MRD in B-cell lymphoma can be detected by a PCR-mediated RNase protection assay (44).

Xenotransplantation

Transplantation of frozen-thawed ovarian tissue into an animal host with subsequent maturation and collection of oocytes can offer considerable advantages to cancer survivors. With this technique, the possibility of cancer transmission and relapse can be eliminated because cancer cells cannot penetrate the zona pellucida, and some technical difficulties of in vitro growth and maturation of primordial follicles can be bypassed. Additionally, this technique can be applied to patients at high risk for hyperstimulation syndrome (e.g., polycystic ovary syndrome) or to patients for whom hormonal replacement therapy is contraindicated such as those with breast cancer.

It has been demonstrated not only that human ovarian tissue survives in the SCID mouse but that the follicles can grow to antral-secretory stages (45). Revascularization of the graft is crucial for the survival of ovarian follicles after transplantation. Therefore, the richly vascularized subcapsular region of the kidney has been a favored site for xenografts in rodents. Weissman et al. demonstrated, however, that after subcutaneous transplantation of human ovarian cortex into NOD/SCID mice, exogenous gonadotropin stimulation resulted in follicle growth in 19/37 (51%) of the grafts, including the development of antral follicles (46). Furthermore, a recent study showed that growth of antral follicles to 3–5 mm in xenografted human ovarian tissue enabled oocyte retrieval (47). We have demonstrated follicle maturation and subsequent corpus luteum formation in human ovarian tissue xenografted into the subcutaneous space of SCID mice (48). There are many advantages of subcutaneous xenografts, including the simplicity of the procedure, easy and convenient monitoring of follicle development, and direct access for follicle aspiration. Nevertheless, the efficiency of revascularization in subcutaneous tissue appears to be inferior to other more vascular sites such as muscle.

Theoretically, in vivo animal culture systems for the growth and maturation of human primordial follicles should be easier to manage than in vitro culture systems. Developing an in vitro culture system contains multiple variables that can affect oocyte integrity, as it takes more than 6 months for the development of primordial follicles to the preovulatory stage in humans (49). It is unknown whether conditions for the growth and maturation of human oocytes in an animal host are comparable to those in situ. It is also of concern that animal pathogens can be transmitted to human tissue with xenografting.

TABLE 2

Unresolved issues in human ovarian transplantation.

Optimization and standardization of a freeze–thaw method
Metabolic injury
Ischemia-reperfusion injury
The most practical and effective graft site(s)
The quality of oocytes matured in a graft
The efficacy of frozen–thawed grafts for fertility restoration and hormonal function
The safety issue (especially an effective screening method for cancer cells)

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In Vitro Maturation

In vitro growth and maturation of human primordial follicles, followed by IVF, is an attractive and desirable option, but it is technically challenging because of the length of the growing phase and lack of knowledge about the optimal conditions for growth and maturation of human oocytes. The feasibility of maturation of primordial follicles in vitro, which are capable of successful fertilization, has been demonstrated in a mouse model (50). Wandji et al. (51) reported that primordial follicles from nonhuman primates could survive and develop to the secondary stage in vitro with serum-free culture conditions. It is also encouraging that preantral follicles isolated from cryopreserved ovine tissue can be grown up to antral stages (52).

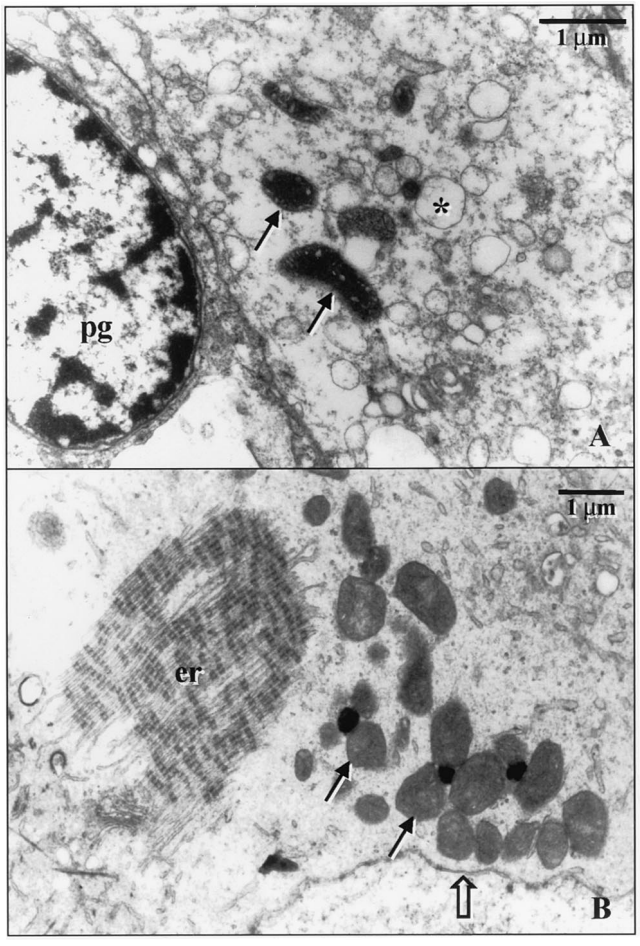
The ability to completely grow and mature human immature oocytes in vitro, however, will not be available until the development of an optimal culture system, which depends on the acquisition of a full understanding of the signal and control mechanism of follicle growth. Recently, the feasibility of mouse primordial follicle maturation by combination of in vivo autotransplantation and in vitro culture was demonstrated (53). This two-step strategy for oocyte maturation needs to be investigated in other species.

Priorities for Research

There are several urgent issues that need to be resolved for the clinical application of ovarian transplantation to be successful (Table 2). Despite the existence of acceptable ovarian cryopreservation techniques, there is a need for more information about optimal dehydration times, cooling and thawing rates, and identification of the most effective cryoprotectant. In addition, it may be useful to explore the efficacy and feasibility of an ultra-rapid freezing method (vitrification). Distinctive ultrastructural changes in frozen/

FIGURE 2

TEM findings of primordial follicles from fresh and cryopreserved human ovarian tissue. (**A**), Evidence of cryoinjury to a frozen-thawed primordial oocyte can be seen in the cytoplasm and in the mitochondria. Extensive vacuolation (*) as well as decreased density of mitochondria (*solid arrows*) are evident. However, intact cell membranes in the oocyte and the pre-granulosa cell (pg) are noted (×10,000). Scale bar = 1 μm. (**B**), Morphologically normal primordial follicle in nonfrozen tissue showing abundant mitochondria (*solid arrows*), intact endoplasmic reticulum (er), and nuclear membrane (*open arrow*; ×7,500). Scale bar = 1 μm.



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thawed ovarian tissue can be detected by transmission electron microscopy (TEM), even in the absence of apparent tissue damage by light microscopy (Fig. 2). These findings indicate the need to investigate the structural and molecular consequences of this process in more detail.

The most crucial factor for tissue survival is the degree of ischemic-reperfusion injury after the transplantation. It has been reported that more primordial follicles die of ischemia than of freezing injury (15, 54, 55). In Aubard's experiment using a sheep autograft model, only 5% primordial follicles appeared to have survived after autografting (56). Ovarian tissue is endowed with abundant genes for angiogenic factors, and it has been reported that vascular ingrowth was accompanied by a 40- to 60-fold increase in expression of the genes encoding two angiogenic factors, vascular endothelial growth factor (VEGF), and transforming growth factor-β1 (TGF-β1; 57). Even with this physiologic advantage, the problem of ovarian transplantation without a vascular anastomosis is still hypoxic tissue damage that occurs while waiting for the revascularization that takes about 48 hours.

It is necessary, therefore, to find a way to facilitate angiogenesis or to protect from ischemic damage. Perhaps it can be achieved by manipulating VEGF gene expression. It appears that expression of the VEGF gene in the ovary is regulated by gonadotropins (58). Another approach would be to alleviate hypoxic tissue damage with antioxidant treatment during the avascular ischemic period of ovarian transplantation. Indeed, it has been suggested that vitamin E treatment improved the survival of follicles in ovarian grafts (59).

The optimal site for transplantation is still unknown. The ideal transplantation site should provide the most optimal conditions for tissue survival and the most practical environment for follicle access. Furthermore, oocyte quality in follicles matured in vivo or in vitro should be evaluated and tested vigorously before attempting fertilization and embryo development in a clinical setting.

The safety of autotransplantation of human ovarian tissue is a crucial issue for cancer patients. Although disease transmission with ovarian transplantation is unlikely, it would be unethical to recommend this procedure without an indication of safety. There is an urgent need to develop screening methods for the detection of MRD in ovarian tissue before transplantation. Molecular genetic techniques such as nested PCR, flow cytometry, and FISH need to be tested for their ability to detect MRD in ovarian tissue.

Ethical and Legal Considerations of Clinical Applications

The clinical application of ovarian cryopreservation and transplantation contains potential legal and ethical issues, as has happened with other new reproductive technologies.

This is the beginning of a new technology that has numerous unresolved ethical and legal issues, including clinical indications, safety, age limits, time limit for storage, and tissue custody. Defining clinical indications and resolving safety issues will be an ongoing effort, along with improvement of the technology. In addition, there is a high possibility of extending the application of the technology beyond the present indication.

It would be an ethical dilemma if a woman requesting use of her preserved ovarian tissue has reached the customary age of menopause in her culture (56). Is it ethical to harvest

and freeze ovarian tissue without a certainty of success in transplantation? Women electing ovarian preservation at the time of surgery (oophorectomy) for other indications will incur no further risk from the operative procedure. Women facing treatment for cancer, on the other hand, have to undergo oophorectomy before chemotherapy, which can be burdensome for many patients (59).

Although engaging in ovarian banking may be a personal choice of the patients who desperately want to preserve fertility with no other option, it is also the responsibility of the medical and scientific communities to prevent misunderstanding and misuse of this emerging technology. There are many reasons, after all, to proceed with care in developing this technology.

SUMMARY

The procedure for collection and storage of ovarian tissue is straightforward, and the recent experimental evidence is encouraging. Standardization and optimization of the freezing and thawing method, however, need to be established. At present, there are uncertainties about the optimum use of the stored ovarian tissue when it is acquired. Autotransplantation seems to be promising, but restoration of natural fertility in humans with this method may not be as easy as in animals.

The potential risk of reintroducing malignant cells with autotransplantation can be minimized or even eliminated by developing screening methods that can detect minimal residual disease in ovarian tissue. Although the results of maturation of primordial follicles with xenotransplantation are encouraging, the efficacy and the safety of this method should be further elucidated. Furthermore, the quality of oocytes matured in a host animal is not known.

The main hurdle with ovarian transplantation is ischemia-reperfusion injury during revascularization. It is crucial to find a way to minimize this hypoxic injury, which can significantly influence the future success of ovarian transplantation. Like other new reproductive technologies, it is inevitable that ethical and legal considerations will arise with clinical use. Nevertheless, ovarian transplantation may prove to be a clinically useful technique to restore fertility and endocrine function for women who face iatrogenic or natural menopause.

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