Cryopreservation and Transplantation of Reproductive Organs

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I. Brief History of Reproductive Organ Transplantation

Transplantation of reproductive organs has been considered by physicians and scientists alike for many years. Indeed, the first recorded transplantation is attributed to the 18th century Scottish anatomist and surgeon John Hunter, who grafted chicken testis and ovaries. However, the first successful testicular transplantation was by the Goettingen biologist, Berthold in capons. There were numerous attempts and interests in testicular transplantation during the early part of the 20th century, mainly aiming for boosting male hormone level with a desire to rejuvenate. In contrast, the current concept of testicular transplantation is restoration of fertility. Sperm cryobanking can preserve male fertility, but this cannot serve prepubertal boys. Therefore, male germ cell transplantation technique (Brinster and Avarbock, 1994) or testicular transplantation (Schlatt et al. unpublished data) warrants further investigation.

Paul Bert published his pioneering experiments on ovarian transplantation in rabbit in 1863. Although initial results were disappointing and somewhat dampened interest in this technique, many investigators continued their experiments on ovarian transplantation

In 1895, the first human ovarian transplantation was performed by Robert Morris, a New York surgeon, in a woman suffering from ovarian failure (Morris, 1985). 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 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 (Parkes & Smith, 1992; Deanesly, 1954). In 1960, restoration of fertility to ovariectomized mice was demonstrated by isografting of frozen/thawed ovarian tissue (Parrott, 1960). However, interest in this technology waned and there was no further progress over the next 30 years because of no immediate applications at the time.

In 1994, Gosden et al. reported successful restoration of fertility by autografting of frozen/thawed ovarian tissue in sheep (Gosden et al., 1994). Recently, four case reports, which showed the restoration of endocrine functions after autotransplantation of fresh or frozen/thawed ovarian tissue in humans, have been published (Oktay & Karlikaya, 2000; Oktay et al., 2001; Radford et al., 2001; Callejo et al., 2001).

Uterine transplantation in animals had been attempted in the 1900s. Successful pregnancies were repor-

ted in sheep following autotransplantation of the reproductive organ (Zhordana & Gotsiridze, 1964) and in dogs by vascular anastomosis (Ersalan et al., 1966). However, allogeneic transplantation, which can be used as a potential treatment for reversing sterility in women with no uterus, was not successful due to graft rejection (Yonemoto et al., 1969). Recently, there was a case report of transplantation of the human uterus, which was not very convincing (Fageeh et al., 2002). As the risks of surgical complications and the problems with immune reactions are still too high, allogeneic uterine transplantation in humans should not be pursued until it shows clear benefits over the risks.

II. Basics of Cryopreservation

1. General Principles

The largest portion of living cells is composed of water which is crucial for biological function. Cooling retards the process of degradation, but causes irreversible destruction of function. The lethal cell damage with cooling is mainly due to intracellular ice crystal formation and increased salt concentrations. Supercooling and the presence of solutes in the extracellular medium prevent ice formation at temperatures above -5℃ (Mazur, 1984). While the temperature can often fall -15℃ before spontaneous ice formation occurs, this degree of supercooling should be avoided. Therefore, the solution with sample is usually "seeded" manually to induce ice formation at temperature between -5℃ and -9℃.

The most potentially damaging phase of the cryopreservation is the subsequent cooling to -40℃. Above -10℃ extracellular crystals induced by seeding are too large to penetrate the narrow cellular membrane channels and nucleate internal ice formation (Mazure, 1963). Optimal freezing conditions allow water to be removed from the cell at a rate which prevent supercooling. The rate of cellular dehydration is determined by three parameters: the surface area to volume ratio; the hydraulic conductivity of the plasma membrane (Lp); the activation energy (Ea) of the permeability constant. Optimal cooling rates thus depend upon the cell type and size. As ovarian tissue is composed of the various cell population, optimal freezing of ovarian tissue can be problematic.

2. Cryoprotectant (CPA)

Successful cryopreservation procedures require the inclusion of cryoprotectants. The role of cryoprotectants is to protect the cell from damages by ice crystals and high concentrations of solutes. The cryoprotectant must be water soluble, easily permeable to the cell (to minimize the osmotic gradient) and relatively non-toxic. The colligative properties of the cryoprotectants provide cryoprotection by depressing the freezing point, thus reducing the amount of ice formed at a given temperature and minimizing the build up of salts concentration. In addition, cryoprotectants have the ability to reduce the toxic effects of high concentrations of salts (salt buffering).

On the other hand, most of cryoprotectants are cytotoxic and may potentially damage the cell with prolonged exposure or exposure to a high concentration. The exact nature of CPA toxicity is unknown but has been suggested to involve membrane lipid solubilization and protein denaturation (Merryman, 1971).

The osmotically induced volume changes with CPA are potentially damaging if not kept within tolerant limits. As a rule of thumb, the volume swing should be restricted to ±40% for mammalian cells.

The equilibration of isolated cells with cryoprotectant is rapid, thus duration of the exposure time required is short and the risks of toxic damage are low. The cryopreservation of muticellular tissue, however, is more difficult. As diffusion is slower, the exposure time should be prolonged in order to reach an adequate concentration of the CPA at the central area of the tissue sample. The cells at the periphery of the sample, however, can be damaged by excessive exposure to the CPA. It is important to balance adequate permeation of the tissue against the toxic effects of overexposure. The NMR spectroscopy study to compare the permeation rate of the different CPAs revealed the ideal method for equilibration human ovarian cortex with the cryoprotectant prior to cooling is to treat the tissue to 1.5 M ethylene glycol or dimethyl sulfoxide for approximately 30 mins at 4℃ (Newton et al., 1998).

III. Ovarian Cryopreservation: A New Strategy for Fertility Preservation

The recent resurgence of interest in gonadal tissue banking is the results of increased long term survivals of young cancer patients with aggressive modern cancer treatment and advances in reproductive technologies. When the cancer is controlled, quality of life becomes a major issue. To many of these young people, fertility is an important quality of life issue. Unfortunately, aggressive cancer treatments can cause gonadal failure. Indeed, most of the patients undergoing hematopoietic cell transplant will lose fertility due to high dose of alkylating agents and/or ionizing radiation. Where the risk of gonadal failure is high with chemotherapy and radiotherapy, it is wise to attempt to safeguard the fertility before treatment. In men, semen cryopreservation can preserve male fertility. To date, there is still no good safeguard for women which is comparable to sperm banking in men. Nevertheless, a new strategy, involving transplantation of stored ovarian tissue, will prove to be effective for reinstating fertility for women facing premature ovarian failure.

IV. Ovarian Cryopreservation; Current Clinical Indications

As demand for the procedure is accelerating, it seems appropriate to set a guideline for ovarian cryopreservation to protect patients and minimize its misuse. We must first think whether the patient can benefit from this procedure. All patients should be provided with counseling in which the experimental nature of the technique is explained. Since there is no proven method for using the banked tissue to restore fertility to date, this technique should only be offered to the women who are at high risk of losing fertility and with good long-term prognosis.

The safety of autotransplantation of human ovarian is a crucial issue for cancer patients. Although our recent study showed the safety of ovarian grafting in lymphoma patients, the safety of other cancers has not been confirmed (Kim et al., 2001). At present, the type of malignancy and the prognosis are prime considerations to determine the candidates for this procedure. Ovarian cryopreservation for patients with sys-

temic or disseminated malignancies should be discouraged until reliable cancer screening methods become available or *in vitro* culture techniques can be perfected.

V. How to use Cryopreserved Ovarian Tissue

After many attempts by many investigators for many years, storing ovarian tissue permanently in liquid nitrogen has become a practical technique for fertility conservation. At present, however, there are still uncertainties about the optimal use of stored ovarian tissue when it is required. Theoretically, there are three strategies for restoring fertility using frozen-thawed ovarian tissue: autotransplantation, xenotransplantation and *in vitro* maturation. At present, autotransplantation either to the orthotopic site or heterotopic site appears to be a practical method and can be applied clinically without too many ethical and technical problems. This technique should not be applied to the cancer patients who are at high risk of ovarian metastasis. When the screening method for microscopic malignant cells in ovarian tissue is developed in the future, autotransplantation will be used more liberally to women with cancer.

Xenotransplantation can be useful for cancer patients because it eliminates the risk of cancer cell transmission that can occur with autotransplantation. It has been demonstrated that human primordial follicles can grow to antral follicles in the host animal (Weissman et al., 1999). Our study also showed follicle maturation and subsequent corpus luteum formation in human ovarian tissue xenografted into the subcutaneous space of immunodeficient mice (Kim et al., 2000). The most desirable strategy would be developing an effective culture system which can support the growth and maturation of immature oocytes *in vitro*. The culture techniques and media presently available are inadequate to sustain the long period of follicle development.

VI. Priorities for Research

There are several urgent issues that need to be resolved for the clinical application of ovarian transplantation to be successful (Table 1). Despite the existence of acceptable ovarian cryopreservation techniques, there is a need for more information about optimal dehydration times, cooling and thawing rates, and

Table 1. Unresolved issues in human ovarian transplantation

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

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/thawed ovarian tissue can be detected by transmission electron microscopy (TEM), even in the absence of apparent tissue damage by light microscopy. 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 (Newton et al., 1996; Candy et al., 1997). Ovarian tissue is endowed with abundant genes for angiogenic factors. 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 (Dissen et al., 1994). 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 (Nugent et al., 1998).

Theoretically, transplantation by vascular anastomosis can prevent ischemic injury. Recently, the successful transplantation of the whole intact ovary in rats after storage in liquid nitrogen has been reported (Wang et al., 2002). This breakthrough may lead to further development in transplantation medicine as well as reproductive medicine.

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