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REVIEW ARTICLE

Human Oocyte Banking; Clinical Applications and Limitations

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Throughout the last two decades, significant advances have been made in oocyte cryopreservation and over 150 babies have been born from fertilized frozen-thawed oocytes. Oocyte cryopreservation is analogous to semen cryopreservation for men. It is potentially the best way to preserve female fertility and offers important benefits to unmarried women or young girls at risk of losing ovarian function as a result of surgery or cancer chemotherapy, women who work with toxins or teratogens, and women who are concerned about age-related infertility. Many women today are involved with education or developing their careers and for majority of them, being married and having children may not be feasible until they are in their late 30s and early 40s. In all of these cases, oocyte cryopreservation may be an appropriate solution. On the other hand, cryopreserved oocytes of infertile couples that have completed their families could be an important source of oocyte donation to help other couples. In addition, oocyte cryopreservation presents an attractive alternative to embryo storage, which is often fraught with religious, ethical, and legal complications. While oocyte cryopreservation success has increased overtime, the pregnancy rates remain low and there is still concern about the safety of the procedure.

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Introduction

The successful cryopreservation of cells from freezing injury was an accidental discovery. It was in 1948 that Polge, Smith, and Parkes, following a mistake in labeling of a solution in a refrigerator, successfully cryopreserved some fowl semen in a mixture of glycerol and albumin rather than in the intended solution of levulose1. Since then attempts at cryopreservation have included tissues from several organ systems, but the most vigorously pursued has been reproductive tissue and cells. The continuous development in cryopreservation of viable reproductive materials has had a huge impact on experimental reproductive sciences and infertility treatment in recent years. At the moment, ongoing reproductive research is mainly focused on reducing multiple preg-

nancies, pre-implantation genetic diagnosis and preserving fertility through ovarian tissue or oocyte cryopreservation.

Ovarian tissue cryopreservation

The ovarian cortex contains a heterogeneous population of cells including thousands of primordial or resting follicles containing oocytes. Cryopreservation of ovarian tissue, especially in the case of cancer, has several potential advantages. In this procedure, a large number of immature oocytes are cryopreserved without the need for ovarian stimulation, which may delay cancer therapy. Since immature oocytes are smaller and lacking a zona pellucida and cortical granules, they are more tolerant to freezing and thawing damage compared to mature oocytes. It is assumed that frozen-thawed ovarian tissue can restore its endocrine function when transplanted. Following thaw, ovarian tissue can be transplanted back into its anatomic pelvic location (autologous transplant) or xenografted to immune deficient mice with further IVF and embryo transfer2. Disadvantages of autologous transplants include possible difficulty in monitoring the follicles with ultrasound in cases where the implanted tissue is high in the pelvis. Moreover, the risk of possible reimplantation of malignant cells with the thawed cryopreserved ovary has been raised following experimental animal observations. It has been shown, at least in an animal model, that ovarian autotransplantation may cause the exposure of ovarian autoantigens and altered immunogenic proteins to the immune system. This exposure results in the formation of anti-ovarian antibodies, which in turn may interfere with ovarian function and inhibit successful reproduction3. The major disadvantage for both autologous and xenografted transplants is that longevity of the transplants is unknown. In addition, with xenografts, if oocytes are used to create embryos for IVF, there is the possible risk of transfer of animal pathogens to humans. Because of the latter concern, xenotransplants are now illegal in Canada. Although ovarian tissue freezing does not require ovarian stimulation, so far there has been no reassurance that oocytes obtained from thawed ovarian tissue can be matured in vitro, or are fertilizable.

Oocyte cryopreservation

Oocyte cryopreservation is analogous to semen cryopreservation. It is potentially the best way to preserve female fertility. Cryopreserved oocytes of infertile couples that have completed their families could be an important source of oocyte donation to help other couples. In addition, oocyte cryopreservation presents an attractive alternative to embryo storage, which is often fraught with religious, ethical, and legal complications[4].

Methodological improvements have made egg freezing more efficient with an increase in survival, fertilization and pregnancy rates. The changes in methodology have attempted to address the following problems: during cooling to ultra-low temperatures, cells are exposed to a number of stresses consisting of intra- and extra-cellular ice formation, uncontrolled dehydration, gas bubble formation, increases in viscosity, and altered solute and ion concentrations. All of these factors are believed to potentially contribute to different forms of cell damage[5]. One approach to oocyte cryopreservation involves a slow cooling/rapid thawing method using a propanediol (PROH) and sucrose as cryoprotectants, similar to the methodology used for embryo cryopreservation. On the other hand, some investigators have reported improved survival, fertilization and live birth rates using vitrification as a freezing method[6]. Vitrification, derived from *vitreous* meaning resembling glass, is a result of high cooling rates associated with high concentrations of cryoprotectant leading to minimal ice crystal formation and reduced intracellular damage. Despite interest in the potential benefits of vitrification as an alternative laboratory approach to long-term oocyte preservation, there is little agreement on how safe and ap-

plicable this procedure is. Vitrification has mostly been studied in animals and requires validation from more extensive experimental studies in humans. Frozen-thawed oocytes can be fertilized by conventional insemination. However, intracytoplasmic sperm injection (ICSI) has become the accepted means of insemination of oocytes post-thaw in order to avoid reduction in sperm penetration of the zona pellucida due to premature cortical granule release or general hardening of the zona following freezing and thawing. Our previous data indicate that the incidence of complete failed fertilization after ICSI is rare, and the possible contributing factors to failed fertilization of thawed oocytes include their starting number and the availability of viable sperm for injection7.

Recently, we performed a pilot prospective study with randomization of oocytes from patients undergoing ICSI into cryopreserved and fresh groups[8]. The objective of our study was to evaluate the efficacy of oocyte cryopreservation with respect to oocyte freeze/thaw survival rate, oocyte fertilization rate, embryo quality and pregnancy rate. Ten patients, 36 years of age or younger, with at least 10 antral follicles on ultrasound, were enrolled. The women underwent *in vitro* fertilization with intracytoplasmic sperm injection using a long luteal phase GnRH-agonist protocol.

Oocytes were retrieved 34-36 hours after administration of hCG. Cumulus cells were mechanically stripped and the metaphase 2 (MII) stage oocytes were randomly divided into two equal groups. One group of oocytes was preincubated for 4 hours prior to undergoing ICSI. The other group of oocytes was cryopreserved using a slow cooling protocol. The oocytes were stored in liquid nitrogen for two hours and then thawed and underwent ICSI after 2 to 3 hours preincubation in culture. The resultant embryos from both groups were assessed serially in the usual fashion and graded at 68 hours after ICSI. Only embryos resulting from cryopreserved oocytes were transferred. The number of embryos transferred (ET) was dependent on number of available embryos, patient age and embryo quality. Natural progesterone was administered until menstruation or the first ten weeks following a positive β-hCG. Good quality embryos from fresh oocytes were frozen at 72 hours for subsequent transfer in another cycle.

Our preliminary data showed that oocyte cryopreservation resulted in an overall survival rate for good quality oocytes of 67%. The fertilization rate for ICSI using frozen oocytes (that have survived) was approximately 70% similar fertilization rate to the fresh oocytes. However the number of blastomeres and the embryo quality on day-3 was superior in embryos from fresh oocytes compared to the frozen oocytes (Fig. 1). The implantation potential of the embryos derived from oocyte cryopreservation did not seem to be reduced significantly as the live birth of healthy twins resulted from the study.

Potential clinical applications of oocyte cryopreservation

Oocyte cryopreservation has potential clinical applications. The following paragraphs are a brief, non-exhaustive discussion of some of the clinical situations that may benefit from application of oocyte cryopreservation technology.

Oocyte donation program

Oocyte donation makes pregnancy feasible in virtually any woman with a normal uterus regardless of age, or the absence of ovaries or ovarian function. A woman's reproductive age now has been artificially extended. Women with premature ovarian failure, women of post-reproductive age, and patients with recurrent failure of IVF treatment may give birth by using donated oocytes fertilized in vitro and transferred to their uteri[9]. The main problem with this technique is difficulty in recruiting donors and the shortage of donor oocytes. Oocyte cryopreservation pro

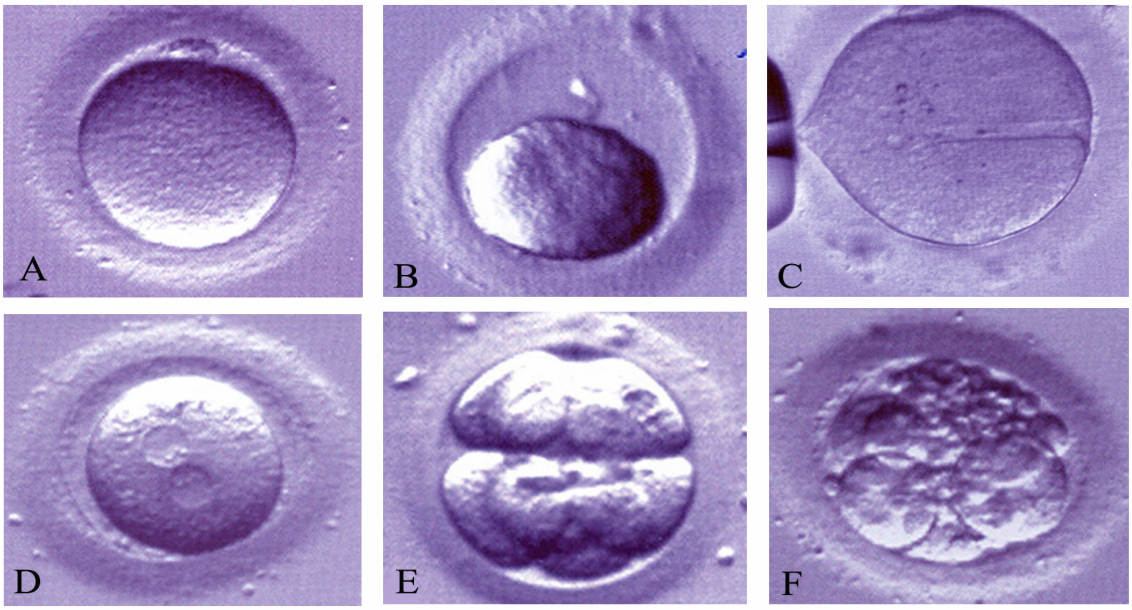

Fig. 1. Human mature (metaphase-II) oocyte before freezing (A), and after exposure to cryoprotectant (B). The frozen oocyte thawed and underwent ICSI (C), and a fertilized frozen-thawed oocyte at two pronuclear (2PN) stage (D). Grade-I quality day-3 embryo resulting from a fresh oocytes (E), and a day-3 embryo with fragmentation and poor quality resulting from a cryopreserved oocyte (F).

vides an opportunity for infertile patients looking for donor eggs. The oocyte donation cyces are usually successful even though there may be cases in which synchronization of the menstrual cycles of the donor and recipient is problematic. Oocyte cryopreservation would simplify donor/recipient oocyte cycles by avoiding the need for cycle coordination.

Due to the good quality of embryos resulting from young fertile donors, lower numbers of embryos are needed to establish successful pregnancies in recipients. Therefore, the majority of embryos will be kept frozen and create a future dilemma for the couple and fertility centers regarding how to deal with these surplus embryos. Oocyte cryopreservation would allow patients to have more options for donor selection, a greater number of available oocytes for their treatment cycle, and convenience in timing their treatment. The utilization of frozen oocytes may decrease the cost for recipient couples and help eliminate the excess number of frozen embryos resulting from the hyperstimulation of young donors. As the frozen oocytes can be stored for extended period of time, utilization of frozen oocytes may help decrease the risk of communicable disease by testing the donor prior to freezing and then again six months post freezing. A possible disadvantage for this option is a potential second freezing cycle for the remaining supernumerary embryos if better than expected oocyte survival rates after thawing occur during a donor cycle.

Split Freezing and Thawing

In vitro fertilization procedures usually generate more embryos than can be used at one time, and extra embryos are typically frozen for possible use later. This is mainly because ovarian stimulation protocols during

IVF are designed to produce a large number of eggs so that the best embryos for transfer can be selected. Embryo freezing is an important tool to reduce the number of multiple gestations and to increase the cumulative pregnancy rate for infertility patients. Given recent trends toward improved pregnancy rates per transfer, the trend of chronic stockpiling of cryopreserved excess embryos needs to be addressed [10]. There are three basic options for handling excess embryos. The embryos may be discarded, donated anonymously to other infertile couples or donated to scientific research. This decision is often too difficult to make for couples and as a result clinics are left to deal with "abandoned" embryos or embryos involved in a "custody battle." Embryo cryopreservation is also a religious consideration whereas oocyte cryopreservation may help alleviate this dilemma. Oocyte cryopreservation allows a minimal number of oocytes to be fertilized ensuring a small number of embryos for fresh transfer. Excess oocytes can be fertilized in subsequent cycles thereby reducing the financial, emotional and physical burdens of IVF. The potential disadvantages for this approach include the variable survival rate of oocytes following thawing, the necessity of ICSI, and a limited choice of embryos for transfer which may affect pregnancy success following embryo transfer.

Patients facing ovarian Loss

Oncology patients: Intensive modern cancer treatment has resulted in an increased number of long-term survivors. For those young cancer survivors, later effects of treatment on quality of life, in particular fertility can be a very important issue. Unfortunately, high doses of chemotherapy and radiotherapy are gonadotoxic and a large proportion of childhood and young cancer patients will lose their fertility after cancer therapy [11]. For female cancer patients there has been no promising procedure comparable to routine sperm banking for male cancer patients. In order to preserve fertility potential, several options are currently available, many of which should be considered as experimental. Ovarian transposition out of the radiation field may considerably reduce the radiation dose and should be considered for patients younger than 40 years of age [12]. Another option is to undergo therapy and resign oneself to the utilization of egg donation if cured. Embryo freezing following IVF is a well established approach which can be offered prior to cancer treatment, but it is only an option for patients who have a partner or are willing to accept fertilization by donor sperm aiming for subsequent transfer should the patient survive her disease. Embryo freezing is not always a suitable option as it needs expensive and time-consuming treatment and requires ovarian stimulation, which sometimes is contraindicated in the case of steroid-dependent cancers [13]. When the cancer treatment cannot be delayed for ovarian stimulation or the tumor is hormone sensitive, then collection of immature oocytes from unstimulated ovaries is particularly useful. The oocytes are matured in-vitro and either fertilized and cryopreserved as embryos or vitrified as mature oocytes. Cryopreservation of ovarian tissue has also become an emerging option but the main problem is the lack of a successful method to mature oocytes in vitro at the present time. We are then left with oocyte cryopreservation that may offer the oncology patient an opportunity to save her eggs prior to initiation of her treatment. Oocyte cryopreservation is an ideal technique for fertility preservation in single women as it does not require a partner, and avoids legal, regulatory and religious dilemmas. The experimental nature of egg and ovarian tissue freezing should be fully explained to cancer patients, along with the uncertainty of the ultimate legal consequences or problems concerning use of any such frozen genetic material by patients or surviving family members [14].

Absence of the uterus following hysterectomy for uterine or cervical carcinoma or a physically unfit patient will determine the

need for a gestational carrier. Frozen oocytes are thawed, fertilized in the laboratory with the husband's sperm and the resulting embryo is then transferred into the gestational carrier's uterus in a synchronized menstrual cycle. Gestational carrier has been shown to be an effective treatment for several medical conditions including patients with Mayer-Rokitansky-Kuster-Hauser syndrome.[15]

Premature ovarian failure: Premature ovarian failure may occur in women with genetic abnormalities (such as Turner's syndrome), after chemotherapy or radiotherapy for malignant diseases, in severe or recurrent ovarian disease (such as cysts, benign tumors or endometriomas), or after removal of the ovaries to treat endometriosis, ovarian pain or genital cancer. For women at risk of premature ovarian failure, oocyte freezing now is a viable possibility for preserving their fertility [16].

Delaying childbearing

Women who delay childbearing into their late 30s and early 40s risk infertility, miscarriage, complicated pregnancies, and ultimately immense heartbreak. Experimental data and clinical observations suggest that delaying childbearing influences the biology of the mother-fetus relationship, with a negative effect on fetal development and predisposition to severe diseases such as type 1 diabetes [17]. Nulliparous women aged 40 or over have a higher risk of operative delivery (cesarean, forceps, and vacuum deliveries: 61%) than do younger nulliparous women (35%) [18]. This increase occurs in spite of lower birth weight and gestational age and may be explained largely by the increase in other complications of pregnancy. The increased frequency at which women are having their first child at age 40 or over may reflect career choices that involve delaying childbirth until the fifth decade of life. The potential indications for egg "banking" could be expanded to include women considering delaying childbearing. The link between advancing reproductive age and a qualitative and quantitative wane in ovarian function is well appreciated. Frequently, patients suffering from age related problems have to make a difficult choice between low pregnancy rates utilizing their own eggs and markedly higher chances of success with donor oocytes. This often causes great agony for the patient over "time lost". With the potential to cryopreserve eggs, patients anticipating a delay in childbearing may choose to store their gametes during their reproductive prime for autologous use in the twilight of their reproductive years. Such women should be informed of potential disadvantages for this approach. Banking oocytes to extend the reproductive age is not a guaranteed procedure as the survival rate of frozen oocytes are variable and several egg retrieval to obtain a large number of mature eggs are required. Given the current pregnancy rate for oocyte cryopreservation, the success rate barely exceeds the pregnancy rate for women in their early 40s, and the important question is whether oocyte freezing is efficient enough to be used electively to delay child bearing? Therefore, these women need to make a difficult decision whether to undergo several egg retrievals, at high cost, to achieve enough oocytes for freezing to make the probability of conceiving realistic, or stay hopeful that their chance of pregnancy will still be reasonable when in their 40s.

Limitations of oocyte cryopreservation

Regardless of the promising results, the oocyte freezing technique is still at a very early stage. The likelihood of success cannot be predicted, but it is probably less than with conventional IVF and cryopreservation of embryos. We still don't know for sure if there are unrecognized risks involved, or an increase in miscarriage, birth defects, or chromosomal abnormalities in the resulting offspring. Zona pellucida hardening and spindle damage are the problems associated with

metaphase II oocyte cryopreservation. The cryopreservation of germinal vesicle-stage oocytes has been undertaken as a means of circumventing the problem of spindle damage in mature oocytes. One of the main disadvantages of immature oocyte cryopreservation is the fact that in vitro maturation is required post-thaw and a small percentage of frozen-thawed immature eggs mature in culture.

Oocyte quality is a critical variable to guarantee oocyte survival after thawing and the survival rate increases when oocytes have better quality. Moreover, oocyte morphology has been demonstrated to affect fertilization rate, embryo quality or implantation after ICSI19. Poor oocyte quality is considered a major determinant in failed or impaired fertilization20. In our recent study, we have shown that some young infertility patients and donors produce lower quality oocytes and embryos compared to their older counterparts. In this group of women the average quality of embryos and the number of surplus embryos for cryopreservation was significantly lower compared to women in their early 30s. This is a conundrum as we generally assume that young patients or donors are perfect candidates for producing the healthiest eggs. Perhaps accelerated oocyte atresia, known to reduce the ovarian follicular pool in infants and children, is still occurring in some of the young IVF patients. This may result in a portion of the retrieved oocytes having already initiated a cell-death program or apoptosis, leading to poor embryo quality and a lower clinical pregnancy rate.[21]

Thus cryopreservation of oocytes, especially in very young female patients, remains a controversial technique especially when the number of oocytes to be frozen is also small. An increased number of oocytes retrieved or several oocyte-freezing cycles will provide more peace of mind and may guarantee a larger number of oocytes that survive and fertilize after thaw, therefore, increasing the chance of selecting healthy embryos for transfer.

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