REVIEW ARTICLE

Cryopreservation of Oocytes and Ovarian Tisuue

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Introduction

Dr. Cline's Christian in collaboration with Dr. Jeffery Boldt, a colleague at Community North Hospital in Indianapolis, developed a new method for storing frozen unfertilized human eggs for future use. It is estimated that there are over one million frozen embryos worldwide and most will be discarded. Attempts at freezing unfertilized eggs, eventually thawing them, fertilizing them with the husbands' sperm and then transferring the embryos derived from these frozen eggs to the uterus resulting in a successful pregnancy have been tried for years. Most attempts were unsuccessful as the eggs did not survive the freeze-thaw process. However, Drs. Cline and Boldts' new method for freezing and storing human eggs has been successful.

In late 2000, the first pregnancy from a frozen egg was announced and Emma was born in July, 2001. If frozen eggs are no longer wanted, they may be discarded with no moral/ethical issues. The success enjoyed by realizing pregnancies and babies from frozen eggs has led to the idea of "fertility preservation". If a woman is to be treated for cancer by chemotherapy or radiation, and the ovaries will be permanently damaged with this treatment in less than thirty days, eggs can be stimulated to develop in the ovaries and egg retrieval as an outpatient is done. Then cryopreservation of the eggs can be done prior to initiating cancer therapy. As far as it is known, there is no limit in the length of time that eggs can be frozen and then thawed and successfully fertilized in vitro.

The Oocyte Freezing and Storage (1-2)

The human oocytes can be stored as either denuded individual oocytes at metaphase-II (M-II) or cumulus enclosed at germinal vesicle stage immature oocytes. The protocol of freezing depends upon the stage of nuclear maturity of oocytes. When mature MII oocytes are harvested for oocytes, the gametes are commonly denuded prior to freezing to confirm their nuclear status. However, it would be physiologically far more appropriate to freeze immature oocytes with intact germinal vesicles along with their cumulus cells for better in vitro maturation after thawing.

Mature Metaphase II Oocytes

At this stage oocytes have undergone nuclear and cytoplasmic maturation, the first polar body has been extruded and chromosomes are condensed and are arranged on the delicate MII spindle. Freezing of M II oocytes have been tried (1-5) but results in humans have largely been disappointing and to date only few dozen live births using cryopreserved oocytes have been reported. Several explanations have been offered for these poor results:

(a) Mature oocytes have a short fertile life
(b) They are very sensitive to chilling and have little capacity for recuperating from the cryoinjury before fertilization.

Other recent studies (6, 7) gave improved results with vitrification as an alternative to slow freezing protocols.

Immature Germinal Vesicle Oocytes

Germinal Vesicle stage oocytes are full sized but their chromatin is at diplotene stage of first prophase and they do not have spindle apparatus unlike mature M-II stage oocytes. They require a period of maturation to induce the required nuclear and cytoplasmic changes before they are capable to undergo fertilization and support early embryo development.

Effects of Cryopreservation on Oocytes (1-2)

1. Injury to Chromosomes and Meiotic Spindle

Meiotic spindle is made up of fragile fibers originating from the centriole at the opposing poles and extending to the chromosomes. A loss of microtubules during freezing could separate chromosomes and cause aneuploidy. Gook *et al* (5) showed in 1994 that normal fertilization can be achieved in cryopreserved oocytes. He further suggested that reasonable integrity is preserved after cryopreservation. Karyotyping and DNA staining has

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proved that the chromosomal integrity is not lost and there is no damage to the spindles. The most probable reason for this is that chromosomes are anchored via the associated kinetochores and are not free to move in the cytoplasm. Chromosomal loss from the spindle is minimal in human oocytes freezing/ thawing and fertilization. Therefore, the belief and doubts that oocyte cryopreservation would result in a high rate of aneuploid embryos is unfounded for human oocytes.

2. Injury to Cytoskeleton

Cytoskeleton is constituted of a complex fibrillary structure. The cytoskeleton maintains and modifies the form, allowing the movement of cytoplasmic organelles, membrane proteins. Vincent *et al* (8) showed that cryoprotectant DMSO produces notable damage in the microfilaments of murine oocytes, which is directly proportional to its concentration. Van Blerkom and Davis (9) also showed the injury to cytoskeleton produced by ice-crystals or cryoprotectants in frozen/thawed oocytes. It has been established that the alteration in cytoskeleton are directly proportional to the concentration of the cryoprotectants, their exposure time and the temperature at which they are added to the culture medium.

Injury to Cortical Granules

Cortical granules in mature oocytes are aligned immediately under the oolemma. The zona reaction that takes place after the movement of these granules to the periphery of the cytoplasm is responsible for block of polyspermy. Van Blerkom and Davis noticed that the premature exocytosis of cortical granules might lead to sudden zona hardening and as a consequence, to a reduction of the fertilization rate (IVF) (9). However to the contrary Gook *et al* (5)observed that the cryoprotectants and the lower temperature does not reduce the release of cortical granules as he observed an elevated number of granules in frozen thawed oocytes.

Zona Pellucida

A common character of all mammalian oocytes is the presence of a layer external to the oolemma known as Zona pellucida, which is made up of glycoprotein. It has been suggested by various workers that due to formation of ice crystals, which may get trapped during freezing/ thawing process, may damage the ZP during cryopreservation.The zona pellucida performs multiple important functions, which are only partly understood. The best known once is; it has receptors for the sperms, it induces zona reaction, blockage of polyspermy and physical protection for delicate embryo.

Survival/Fertilization Rates-Frozen/Thawed Oocytes

Various scientists (1,2,5) concerning the survival of human oocytes at thawing have reported consideration variations in results. The results of Chen (1), who reported 76% survival rates, are classified among the highest. He has frozen the best quality MII oocytes. Al-Hasani *et al* (2) reported 25% survival rates with supernumery oocytes, which were not always the best quality. Gook *et al* (5) reported 48% and 95% survival rates.

The often reduced percentage of fertilization and the rather higher incidence of anomalies and fertilization in cryopreserved oocytes have been related to possible damage of the zona pellucida and cortical granules, which interferes with the correct interaction with the spermatozoa ICSI has been proposed as a solution for this problem.

Safety Aspects of Cryopreservation

1. On Mature Oocytes (Metaphase II stage)

Presence or absence of the cumulus granulosa cells during the freeing process may have a direct impact on metaphase II oocyte survival after thawing. Although not proven it is thought that the cumulus cells offer some protection against sudden osmotic changes and stresses induced by the rapid influx of CPAs. M-II oocytes are vulnerable to cryoinjury because the meiotic spindle, on which the chromosomes have become aligned, is actually temperature sensitive. Oocyte freezing can therefore increase the incidence of aneuploidy after extrusion of the second polar body through non-disjunction of sister chromatids.

This duplication of the cytoskeletal architecture may also lead to abnormal cytokinesis, retention of the second polar body and alterations in the organization and trafficking of the molecules and organelles (10). While the deleterious effects on the cytoskeleton resulting from chilling may be avoided by cryopreservation of GV oocytes, the difficulties associated with in-vitro maturation and extended culture appear to counteract the potential benefits of freezing oocytes at this stage. Furthermore, chilling reduces the development capabilities of GV oocytes, quite apart from damaging the meiotic spindle (11). Few pregnancies have been achieved after in vitro maturation (IVM) of human oocytes (12, 13). At present the cryopreservation of GV stage oocytes offer little or no advantages over freeze store of MII oocytes. In addition to the cytogenetic impact of cryopreservation, there is an increased risk of parthenogenetic activation

of the oocytes after thermal shock and exposure to cryoprotective agents (CPA). Although precise mechanism by which activation is initiated is unknown recent evidence suggests that CPA exposure promotes the passive influx of Ca++ across the plasma membrane , possibly by stimulation the release of Ca++ from storage sites in mitochondria and endoplasmic reticulum. Furthermore, calcium fluxes are known to activate intracellular phospholipases, protease, ATPases and endonucleases, which may result in altered plasma membrane integrity, Denaturation of cytosolic proteins and chromosomal fragmentation, all of which can lead to irreversible cell injury and apoptosis.

Parthenogenetic Activation: The thermal shock in the form of heat and cold could lead to parthenogenetic activation. Gook *et al* (5) observed that 27% of the fresh and 29% aged cryopreserved human oocytes underwent parthenogenesis respectively. Parthenogenetic activation depends upon the type of cryoprotectant used.

2) On Primordial Oocytes

These GV stage oocytes appear to be less vulnerable to cryoinjury than MII stage oocytes as they are smaller, lack zona pellucida and cortical granules and are relatively metabolically inactive and undifferentiated. Additionally they are more tolerant to insults such as immersion in CPAs and cooling to very low temperatures as their small size makes them less susceptible to damage induced by water movements into and out of the cells during freezing and thawing. Further, the primordial oocytes have more time to repair sub-lethal damage to organelles induced due to cryopreservation during their prolonged growth phase after thawing.

3) On Ovarian Tissue

Although storage of pieces of ovarian cortex as an attractive alternative to mature oocyte freezing, there are a number of technical problems associated with the cryopreservation of ovarian tissue compared to storing isolated oocytes. Tissues respond very differently to ice formation than cell suspensions. Cells in tissues are usually loosely packed and they also have interacting connections with each other and have basement membranes and traversed by blood vessels and capillaries. Changes in extracellular ice surrounding the tissues during the freezing process and recrystallization during warming of the tissues are both hazardous. On the basis of animal experiments, it is likely that fertility restoration will be achieved by heterotopic or orthotopic autographting (14-17). Xenografting has also been successfully used as a research tool to investigate the developmental potential of frozen-thawed human ovarian tissues (18).

Applications of Oocyte and Ovarian Freezing

- Improve the efficiency of IVF
- Alternative to embryo freezing
- Oocyte preservation for patients with ovarian hyper stimulation syndrome
- Oocyte donation programme
- The treatment of congenital infertility disorders
- Prevent fertility loss through surgery
- Treatment of premature ovarian failure (POF)

Recent Advances in This Field

For women who undergo retrieval of more than 20 oocytes in IVF/ICSI, the clinical outcome of fresh embryo transfer cycle, such as fertilization rate and clinical pregnancy rate, are not influenced by oocyte cryopreservation and embryo cryopreservation. There is no significant difference in the clinical pregnancy rate (per embryo transfer cycle) between frozen-thawed oocyte group and frozen-thawed embryo group. Compared with embryo cryopreservation, oocyte cryopreservation has obvious advantages in fertility preservation and oocyte donation (19).

In the case of patients who are facing infertility due to cancer therapy, oocyte cryopreservation may be one of the few options available. Ovarian tissue cryopreservation can only be recommended as an experimental protocol in carefully selected patients. In ovarian tissue transplantation, more research is needed in order to enhance the revascularization process with the goal of reducing the follicular loss that takes place after tissue grafting. These technologies are still investigational, although tremendous progress has been made. The availability of such treatment will potentially lead to its demand not only from patients with cancer but also from healthy women who chose to postpone childbearing until later in life and therefore wish to retain their fertility (20).

The Human Oocyte Preservation Experience (HOPE) Registry is an initiative of EMD Serono which aims to systematically track the outcomes of oocyte cryopreservation cycles and validate the efficacy and safety of techniques to freeze and thaw oocytes. Beginning in November 2008 (ASRM, San Francisco, CA), the registry will be performed as a national Phase IV observational 5-year study (including 3 years of enrolment and 2 years follow-up of babies born) in the

USA and will enrol approximately 400 women of reproductive age who have thawed frozen oocytes for subsequent use through in-vitro fertilization (IVF) and embryo transfer (ET). Data relating to controlled ovarian stimulation protocols, freezing and thawing of oocytes, culture and transfer of resulting embryos, implantation rates, pregnancy outcomes and information on child health and development after birth and at 12 months will be recorded. It is anticipated that the HOPE Registry will provide answers to various unresolved questions from healthcare providers and their patients who use oocyte cryopreservation to preserve fertility, such as oocyte cryopreservation and thawing techniques and other factors associated with successful cycle outcomes (21).

Conclusion

Oocyte cryopreservation is an emerging technology with promising future, which still requires much developmental work to improve the survival rates and developmental potential of frozen-thawed oocytes. The potential ethical benefits, the convenience and lower costs which will result from successful programme of mature or immature oocyte freezing will surely stimulate continuing efforts to improve protocols and clinical practice as this approach may well transform assisted reproductive technologies (ART).

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