Original Article

# Development of new method and protocol for cryopreservation related to embryo and oocytes freezing in terms of fertilization rate: A comparative study including review of literature

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## Abstract

**Background:** Cryopreservation is basically related to meritorious thin samples or small clumps of cells that are cooled quickly without loss. Our main objective is to establish and formulate an innovative method and protocol development for cryopreservation as a gold standard for clinical uses in laboratory practice and treatment. The knowledge regarding usefulness of cryopreservation in clinical practice is essential to carry forward the clinical practice and research.

**Materials and Methods:** We are trying to compare different methods of cryopreservation (in two dozen of cells) at the same time we compare the embryo and oocyte freezing interms of fertilization rate according to the International standard protocol.

**Results:** The combination of cryoprotectants and regimes of rapid cooling and rinsing during warming often allows successful cryopreservation of biological materials, particularly cell suspensions or thin tissue samples. Examples include semen, blood, tissue samples like tumors, histological cross-sections, human eggs and human embryos. Although presently many studies have reported that the children born from frozen embryos or "frosties, "show consistently positive results with no increase in birth defects or development abnormalities is quite good enough and similar to our study (50–85%).

**Conclusions:** We ensure that cryopreservation technology provided useful cell survivability, tissue and organ preservation in a proper way. Although it varies according to different laboratory conditions, it is certainly beneficial for patient's treatment and research. Further studies are needed for standardization and development of new protocol.

**Key Words:** Autologous bone marrow, cryopreservation, human oocytes, stem cells, transplant, unfertilized oocytes

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## INTRODUCTION

Cryopreservation is a process of reserving whole body tissues and cells by cooling to sub-zero temperatures (77Kor −196°C boiling point of liquid nitrogen). Biological activity, biochemical reactions lead to cell death. Cryopreservation solutions required to preserve and tissue process. However, few of the times low temperature destroys tissues of liver, heart and other body organs. Therefore storage and transplantations samples should be kept carefully. Here we documented the usefulness of cryopreservation in routine practice for patients and future management.

## MATERIALS AND METHODS

### Study design: We performed

A hospital laboratory-based comparative study.

### Study duration

Up-to-date information provided since (2000–2016).

### Releated samples information given unlike

Humanoocyte, ovarian tissue, fresh embryos, frequent infrozenembryotransfer (FRET) cycles, endometrium preparation, embryo, blastocysts, fresh pre-embryos, hematopoietic stem cells (HSCs), umbilicalcordblood (UCB), human spermatozoa, *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI), semen, gamete, follicle, autologous transplantation, human lymphocytes, gonadotrophin releasing hormone analogue (GnRHa), ZPNoocytes, hepatocytes, fluid from the intravascular compartment, bone marrows, and mature blood cells (MBCs).

### Sample preservation

We reviewed through medical literature and summarized recent advances in protocol development and its usefulness.

### Statistical analysis

We have used simple statistics available in excel and SPSS software ver. 17.

## RESULTS

Basically cryopreservatives were used for freezing and measure the viability. Although the frozen unit allowed 72 hr, it should be verified as per constructive maintenance of temperature. Accordingly thawing procedure and donor unit look forward for optimized temperature of cryopreservation. Although different conditions attributed with tissue specific, we should not ignore liquid nitrogen. CCIF3 (Chloro trifluoro methane) is more useful for tissue processing.[1] The ideal conditions –500 mg of tissue be immersed for approximately 7-10 s.[2] Presently we observe liquid nitrogen slushing within a container. Therefore cryogenic storage vials are required for suction. The partial vacuum in the container above the liquid helps to preserve tissues. The samples are dropped into slowly and stored in the container [Table 1].[2,3]

Human oocyte cryopreservation is a novel technology in which a woman's eggs are extracted, frozen, and stored. Later, whenever she is ready for pregnancy, the eggs can be thawed, fertilized, and transferred to the uterus as embryos.[4] Bernardo and Fuller recognized that cryopreservation of unfertilized humanoocytes would make a significant contribution to the treatment of infertility. Successful pregnancies have resulted from cryopreserved oocytes. Studies on the effects of cooling, membrane permeability, cryoprotectant addition and ice formation have been performed on human oocytes by a number of groups. We are trying to improve this technique and developed new concept.[1] A higher fertilization rate (75%) could be achieved after thawing, using 1,2 propanediolas as cryoprotectant. Polyploidy rates of 20% and 40% were observed using dimethyl sulfoxide (DMSO) and 1,2propanediol as cryoprotectants, respectively. Using the ultra-cooling method the survival rate was poor (4%).[5] The paucity of well-controlled studies currently precludes valid comparisons between legal approaches. Legal restrictions on the ability to select embryos from cryopreserved oocytes in countries such as Italy also obscure attempts to assess oocyte cryopreservation objectively.[6] Oocyte cryopreservation is aimed at three particular groups of women: (1) Women diagnosed with cancer who have not yet begun chemotherapy or radiotherapy. (2) Women who are undergoing treatment with assisted reproductive technologies (ARTs) would like to preserve the ir future option to have children either because they donot yet have a partner, or for other personal reasons.[7] (3) Women with a family history of early menopause may go for fertility preservation. With egg freezings they will have a frozen store of eggs, in the likelihood that their eggs are depleted at an early age. The person has the option of using a few eggs for fertilization, and preserving the rest of the unfertilized eggs for future use.[8]

Recent studies focused that the rate of birth defects and chromosomal defects in cryopreserved oocyte are comparable with that of natural conception. The potential advantages associated with the ability of freeze and store human oocytes successfully have been well established. Another way there is a possibility of women, with no medical indications and no immediate plans to conceive, being able to store "young eggs" for potential use at a later date.[9]

**Table 1: Newly developed method and protocol designed for cryopreservation technology as gold standard including treatment and long term follow up**

| OR | DM | CPT | CPV | CT | OR | ST | FT | SV | SF | SR + RM |
|---|---|---|---|---|---|---|---|---|---|---|
| Human oocyte | Egg Freezing (Transvaginal oocyte retrieval) | CPT which replaces most of the water in the cell | Vitrification | Slow cooling | Metaphase-II | Quickly | SlowFrozen | Improved | Asistedre productive Technology | 40 to 50% |
| Ovarian tissue | Standard Freezing method | Glycerol | Fertility preservatio (Vitrification) | Slow cooling | Reimplantation | Overt night Transport | Central CryobankGnRH agonist hCG | Improved | Fertility preservation | 35 to 40% |
| Fresh embryos | Direct Frozen Embryo Transfer | Ethylene Glycerol Freeze Media | Embryo preservation Vitrification | Vitrification vs slow programmable freezing | SPF | IVF | Sub zero temperature | Lower | Ovarian Stimulation Syndrome (OVSS) | 27to 30% |
| Frequent in frozen embryo transfer (FRET) cycles | Cryopreserve embryo | Cryostorage | Vitrification | SPF | IVF | Quickly | Embryo preserve | Improved | Implantation potential | 50 to 70% |
| Endometrium preparation | Cryopreserve embryo | Cryostorage | Vitrification | SPF | IVF Endometrial exposure | Slowly | Blastocystes with exogenous estradiol and progesteron | Excellent | Blastocyst implantation | 98% |
| Embryo | Standard Freezing Method | Cryoprotectant | Tank of liquid nitrogen | Slowly frozen down to −196°C | Glass like solidification | According to HFE ACT 1990, 2008 | Embryo preservation | Good | Fertility | 50 to 75% |
| Blastocysts | Blastocyst Transfer | Premeating (egpropanediol (Non-permeating or extracellular e.g., sucrose) | SF | IVF | Slow Freezing | IVF egg retrievals | Excellent | Blastocyst transfer | Excellent | 99% |
| Fresh pre-embryos | Frozen cycle Transfer | Sucrose | Vitrfication | FT | After biopsy | More quickly | Cryopreserved embryo | Excellent | Fresh embryo | 60 to 70% |
| Hematopoietic stem cells | Standard Freezing method | DMSO10% | Cryopreserved after collection | Membrane stabilizer and bio oxidants | Slowly (CFM) | −80°C | Good | Human umbilical cord blood | Mechanical freezer | 30 to 50% |
| Umbilical cord blood (UCB) | An automated microprocessor controlled rate freezer | DMSO 10% | 1.Cryotubes 2.Aluminium cassette in the chamber of the cell freezer 10% | 1°C to −120°C | Liquid Nitrogen Freezer | Liquid phase of a liquid nitrogen | −60°C to −120°C | Good | Transplantation | 80 to 90% |
| Human Spermatozoa | Conventional freezing techniques (manual and automated) | Human albumin 10% | Direct Swimp Or Density gradient centrifugation | IVF | Slow | GRS | ICSI cycle with fresh cryopreserved | Good | Liquid nitrogen vs computerized program freezer | 40 to60% |
| *In vitro* fertilization (IVF) | Pre-IVF semen analysis | Embryos and thawed | Selection gamete | Liquid nitrogen level | Slow | GRS | Frozen embryos | −196°C | ART | 25 to 50% |

*Contd...*

**Table 1: Contd...**

| OR | DM | CPT | CPV | CT | OR | ST | FT | SV | SF | SR + RM |
|---|---|---|---|---|---|---|---|---|---|---|
| Intracytoplasmic sperm injection (ICSI) | IVF/ICSI procedure | Frozen and thawed epidermal spermatozoa | Injected Oocyte | Normal | 2(PN) | motile | Freshly retired spermatozoa | Good | Pregnancy spermatozoa | 30 to60% |
| Semen | Sperm Banking | Glycerol 10% | Cryopreserved sperm | Thawing at 40°C | ATP content and DNA | Well controlled cooling regime | Slow programming freezing | Vitrification | Potentially improved | 60to85% |
| Gamete | Standard method | Glycerol solution | 4-cell stage embryo | 37°C in a 5% air incubator | Trans cervical | Transfer non frozen | −196°C | Improved | Vitrification | 70 to 80% |
| Follicle | Harvest culture | Mature Oocytes + cryostorage | Frozen and Thawed Ovarian tissue | Most abundant stage | Triple stage process | Isolation of granulosa Oocyte complexes | Vitrification (SF) | Slowly developing | Primordial Follicles | 10 to 15% |
| Autologous Transplantation | DMS | Ovaredonized fresh ovary | −55°C | 0.5°C/min | Ice nucleation at −7°C | Plugned 7 to 15 days | Liquid nitrogen | Improved | Transplantation | 60 to 70% |
| Human lymphocytes | Simple and quick | Cryostorage | Frozen storage | GVH and NLT reaction | Allogeneic lymphocytes (cytotoxic) | Survived Freezing | Frozen and stored | Slowly | Secret complement dependents leucocytes antibodies | 10 to 30% |
| GnRH antagonists | Simple and Rapid | Diluted cryoprotectants (Glycerol) | Egg yolk PH, buffers (TES and TRIS) | Transfered straws | Lebelling and recording | Frozen | −37°C | Up to 24 hours | GHT | 70 to 80% |
| ZPN oocytes | Rapid CFC | Glycerol 5 to 10% | Oocyte bank Cryopreservation | Slow Frozen | Control rate | Slow Freezing | The egg must be dehydrated before freezing | 2-4 weeks | Good | 40 to 60% |
| Hepatocytes | Hepatocytes Cryopreservation protocol | Cryopreservation media | Hepatocyte | Liver cell freezing solution | Less than 10000000 cell | Law density | 37°C | 48 hours preculture | Good | 25 to 50% |
| Fluid from the intravascular compartment | Transcellular compartment | Mg and sulphations | Osmotic equilibrium | Plasma membrane | Intestinal fluid | Fresh embryo | 24 to 36 hours | ICG | Good | 20 to 25% |
| Bone marrows | Liquid culture | Cryo storage | Harvest | Colony Forming cells | Auto lab T | Myeloid | Prolonged | BMT | Good | 40 to 50% |
| Mature blood cells | Simple method | Cryostorage | Extracellular space | MBS | Nucleated cells 1% | BMC product | Mega karyocytic proginetor cells | CD15 + myeloid cells | Good | 30 to 50% |

N.B. Sl: Serial number, OR: Organism/cells or tissue, CPT: Cryoprotectants, CPV: Cryopreserve, CT: Cooling time, OR: Oocyte rate, ST: Storage time, FT: Freezing tolerance, SV: Survivability, SF: Significance, RM: Remarks, GIFT: Gamate intra fallowpian transfer

## DISCUSSION

### Ovarian tissue and transplantation

As per existing literature from 2000 to 2016 we have provided the accountability of cryopreservation.[10] We concluded that advances in reproductive technology have made fertility preservation techniques are all possibility for patients whose gonad function is threatened by premature menopause by treatments such as radiotherapy, chemotherapy, or surgical castration[10] Female cancer patients can access "banking" of gametes before therapy. In addition to transplant patients fresh and frozen ovarian tissues are required for both fertile and infertile women.

Researchers now developed the utility of the tissue banked for the restoration of endocrine and fertility function. In addition to methods such as follicle culture and isolated follicles are required for transplantation in developmental stages. More than 30 transplantations of crypreserved tissue have been reported and six babies have been born, worldwide, following this procedure. Despite these encouraging results, it is essential to optimize the procedure by improving the follicular survival for confirming safety and clinical use.[11]

### Implantation potential and clinical impact (IPCAI)

Potential abilities of human embryos to survive the freezing and thawing process and reflected in their implantation. Cryopreservation affect adversely in the capacity of human embryos to implant. In multiple pregnancies are FRET cycles having no conclusive evidence on the stages of development. Fresh cycle evidence suggested that there are no adverse consequences in the babies born after embryo cryopreservation.[12] Cryopreserved and thawed blastocysts demonstrated a similar pattern of implantation.[13]

### Hematopoietic stem cells (HSC)

Stem cell transplantation represents a critical approach for the treatment of many malignant and nonmalignant diseases. The function of these approaches is the ability to cryopreserve marrow cells for future use. This technique is routinely employed in all autologous settings and is critical for cord blood transplantation.[14] HSC can be stored for prolonged periods at cryogenic temperatures. New techniques currently used are derived from the initial report in 1949 of cryopreservation of bovine sperm in glycerol. In addition to glycerol penetrating cryoprotectant protected the cells, from the injury associated through ice formation. Current cryopreservation techniques with several minor variations suspend cells in an aqueous solution of ions, salts, protein, and other cryoprotectants. Cells are frozen at slow rates and stored generally below −120°C in mechanical freezers or nitrogen refrigerators. These techniques are successful in maintaining HSC viability that is evident from the engraftment of these cells in patients treated with marrow-lethal-conditioning regimens. Basically, the composition of the cryoprotectant solution, cell concentration during freezing, cryoprotectant toxicity, and storage temperatures have not been adequately studied, primarily because lack of appropriate assays for HSC cryosurvival. HSC cryobiology will become an increasingly important subject as new HSC collection and processing techniques are developed. Improved cryosurvival of HSC using modified cryoprotectant solutions may improve engraftment kinetics and decrease cost. It decreases the morbidity of autologous transplantation patients.[15] In cryopreservation, processis of importance for all types of stem cell collection. Particularly, critical for UCB. The actual transplant is harvested at the time of birth and used in the determination of recipient. UCB is usually stored by either public or private cord blood banks (CBBs). Public CBBs are usually nonprofit organizations that offers the donor unit to matching recipients registered in national or international registries.[16] CBBs were stored at donor specimen and unknown recipient for later application. There are 170,000 frozen units in 37 cord blood registries were seen in 21 countries. A total of 3000 units been transplanted till date.[17]

### Human spermatozoa

Freeze human spermatozoa and the possibility of pregnancy followed by intrauterine insemination existed for more than 45 years.[18] Now there have been improvements in the methods of freezing and thawing human spermatozoa. Cryprotective used was glycerol. Minor limitations were observed in the motility recovery. Ultra-structural damage sensitive criteria and objective assessment of morbidity/motility required for their energy status. Additionally damages in the plasma membrane or subcellular elements (like the chromatin stability and chromosomal damage) are needed. Sperm cryopreservation needs different kinds of biochemical environment and physical conditions. Biochemical changes are assessed following different combinations to avoid the increase in osmotic pressure. Hence, we need additional studies to arise from the extensive use of frozen spermatozoa during ARTs, all together with the development to assisted fertilization.[19] New methods of human spermatozoa preservation developed. There is no doubt that the use of frozen-thawed semen has revolutionized domestic animal breeding programs. It allows rapid genetic improvement which facilitated the distribution of germ plasma worldwide.[20] Cryopreservation of male gametes provides increased treatment options in the case of spontaneous or iatrogenic infertility at various developmental stages and ages.[21]

### Safety and effectiveness of tissues in cryopreservation

Human Fertilization and Embryology Authority focused on the safe cryopreservation of gametes and embryos. Human fertilization and embryology authority (HFAEA) focused on the safe cryopreservation of gametes and embryos. There is a need for development of fertility clinics in the UK and India to preserve the spermatozoa. We ensured that containers used for cryopreservation should withstand low temperatures. We can use secondary containers and store in nitrogen vapor as an alternative. A number of issues related to vapor storage, needs a careful consideration.In addition to safety, cost effectiveness of various storage techniques for maintaining gamete and embryo viability should not be ignored.[22]

### Human lymphocytes

Human lymphocytes are cryopreserved through a variety of *invitro* immunologic studies to determine the applicability of using a programmable freezing system, comparing glycerol versus DMSO at varying concentrations on post thaw viability, E-resetting, and immunoglobulin fluorescence including pre-and post-freeze T and Blymphocyte percentages were determined according to generated data on post-thaw. T and B percentages revealed 10% DMSO a nd liquid nitrogen control at 1°C/min as the best condition for lymphocyte preservation.[23]

### Hepatocytes

Hepatocytes are routinely used in human biomonitoring to assess the potential toxic and cryoprotective effects of diet on both DNA damage and tissue repairing.[24] Hepatocytes have been investigated for many years as a method of long-term storage. Unfortunately, an agreed acceptable protocol has not emerged partly due to the susceptibility of hepatocytes to the freeze-thaw process (which is essential nowadays).[25] Cryopreserved human hepatocytes are extensively used now a days for post-thaw viability and determine by trypan blue exclusion, ranged from 55% to 85%. Hepatocytesare cryopreserved from 17 donors post-thaw viability and and found to be stable up to the longest period of 120 days.[26]

### Gonadotropin releasing hormone antagonists

Presently pregnancy associated with blockage of oocytes and GnRHa is a major concern. These oocytes can be used at a later stage. The quality of transferred embryos and the pregnancy rates in the freeze-thaw cycles used carefully.[27] Cryopreserved ZPN oocytescan be replaced after thawing even in the cleavage stage condition.[28]

### Embryo freezing for preventing ovarian hyperstimulation syndrome

Ovarian hyper stimulation syndrome (OHSS) is an iatrogenic potentially life-threatening condition resulting from an excessive ovarian stimulation. Its reported incidence varies from 1% to10% of IVF cycles. That release of vasoactive substances secreted by the ovaries under human chorionic gonadotropin stimulation. Which is plays a key role in triggering this syndrome. Cryopreservation condition are varies in massive shift of fluid from the intravascular compartment. The third space also resulting profound intravascular depletion.[29] Randomized controlled trials (RCT) in human intravenous albumin or cryopreservation of all embryos were used as a therapeutic approach. Women participants were of reproductive age, who were down regulated by GnRH and undergoing superovulation in IVF/ICSI cycles.[30] The interventions compared with cryopreservation (embryo freezing) versus intravenous human albumin administration. Elective cryopreservation of all embryos versus fresh embryo transferbe necessary for primary outcomes. The incidence of moderate to severe OHSS versus nil/mild OHSS, clinical pregnancies/woman varies from countries to countries.[31] In addition to Cochrane menstrual disorders and subfertility group guidelines also clarifying the same.[32] Cryopreservation (embryo freezing) was compared with intravenous human albumin administration through elective cryopreservation having the potential application.[33]

### Hematopoietic stem cell processing (HSCP)

Bone marrow or peripheral blood may be harvested to provide HSC for autologous transplantation and compromise heterogeneous cell populations. HSC are necessary for successful engraftment and constitute. There is a very small fraction of the cells harvested. After collection, the harvested cells usually undergo several new processes to reduce the product volume, remove cells (such as MBCs or tumor cells). In cryopreserving the cells for later reinfusion, granulocytes and red blood cells, survive cryopreservation poorly using freezing techniques designed for HSC. Therefore, bone marrows being cryopreserved must be dependent on MBCs. MBCs are impeding the variety of tumor cells. Purging techniques processed through different study design minimize the loss of HSC. While achieving an appropriate HSC product for the individual patient number of aphaeresis devices and cell washers. Simply the enrichmen to HSC in the harvested cell products were in contrast to tumor cell purging techniques, which were not standardized between the various transplant centers.[34] Liquid nitrogen freezers are not sterile, and both the liquid and vapors phases are potential sources of microbial contamination of hematopoietic progenitor cell components. The low-level contamination by environmental organisms was very common. The occurrence of heavy contamination by potential pathogens such as aspergillus species suggest that monitoring of liquid nitrogen sterility indicating the safe strategies to assess and prevent microbial transmission from liquid nitrogen to HPLC components need further development.[35-37] The clinical evidence of HSCs with UCB/PCB grafts indicated that PCB is one useful resource of HSCs for routine bone marrow reconstitution. The large registry study confirms the potential benefit of using UCBHSCs for allogenic transplants.[38]

### Ovarian tissue and oocyte

Ovarian tissue cryopreservation and oocyte cryopreservation hold promise for future female fertility preservation. Treatment protocols for immature ovarian grafting for puberty and fertility be developed. The data of researchers suggested that follicle depletion leads to premature ovarian failure. It provides valuable information to the study of ovarian transplantation suggesting that these procedures do not produce normal epigenetic marks. These results are highly relevant to the reimplantation question of immature cortex among women.[39]

### Ovarian cryopreservation

Whole ovary tissue was equilibrated at 4°C for 30 mm in cryogenic vials containing Brahma1solution with 10%bovine calf serum and 1.5 M DMSO. These vials were transferred to a programmable freezer (plannerproducts, cryobirysten) and cooled at 2°C/min for seeding. Followed by freezing 0.3°C/min to −40°C and subsequently at 10°C/min to −140°C. Vials were stored in liquid nitrogen, for approximately 2–4 weeks only. Before grafting, tissues were rapidly warmed in air at room temperature for 30 s. Then immersed in water at 30–35°C for 5 min. The ovaries were removed from the cryovials and placed successively in 4 solutions containing decreasing DMSO concentrations. After washing, ovaries were placed in Leibovitz-L15 medium, at room temperature, and grafted within 15 min of thawing. The development and potential impact of ovarian cryopreservation upon assisted fertility and in the restoration of ovarian function of cancer and the menopausal treatment.[40]

### Future of cryopreservation

Advances in the cryopreservation of eggs harvested from hormonal treatment cycles may be used to develop egg banks in the same way as sperm banks are used now. Unfertilized eggs developed into normal healthy children, and the number of successful births can be enhanced greatly. This is a preferred technology in assisted fertility treatment. The use of egg banks allow mothers to delay child bearing until middle age. Recent advances in ovarian cryopreservation and transplantation will make it theoretically possible to restore fertility after menopause. The applications in mammalian cells are virtually limitless and useful tool in a variety of fields such as pediatrics, pediatric surgery, pathology, nuclear medicine, medical oncology, transplant biology, stem cell facilities, and nanotechnology.

### Suggestion and further investigation for future generation

Long-term follow-up studies required special attention to measure the impact of ARTon patients, parents and relatives. Oocyte manufacturing alleviated the burden of age-related infertility problem.

## CONCLUSION

The cryopreservation technology helps female infertility, organ transplantation, and stem cell development through bone marrow transplantation (BMT).

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### Conflicts of interest

There are no conflicts of interest.

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