

Human Embryos and Oocytes Cryopreservation in Human Assisted Reproductive Technology-A Case Study

Bipasha Ahmed^{1*}, Angel², Smita Sharma³

¹Fellow, International Fertility Center, Delhi, India

²Senior Embryologist, International Fertility Center, Delhi, India

³Fellow, International Fertility Center, Delhi, India

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*Corresponding author: Bipasha Ahmed

Abstract

Case Report

Human embryo cryopreservation represents an indispensable extension of in-vitro fertilization (IVF) programmes as long as they are based upon the recovery of a large number of oocytes. By then, 86% of stored embryos had been thawed for transfer to patients. The most widely used procedures include the cryopreservation of human zygotes or embryos in early cleavage, using 1, 2-propanediol and sucrose as cryoprotectants. Both sperm and embryo cryopreservation have become routine procedures in human assisted reproduction and oocyte cryopreservation is being introduced into clinical practice and is getting more and more widely used. Embryo cryopreservation has decreased the number of fresh embryo transfers and maximized the effectiveness of the IVF cycle. The data shows that women who had transfers of fresh and frozen embryos obtained 8% additional births by using their cryopreserved embryos. Oocyte cryopreservation offers more advantages compared to embryo freezing, such as fertility preservation in women at risk of losing fertility due to oncological treatment or chronic disease, egg donation, and postponing childbirth, and eliminates religious and/or other ethical, legal, and moral concerns of embryo freezing. In this review, the basic principles, methodology, and practical experiences as well as safety and other aspects concerning slow cooling and ultrarapid cooling (vitrification) of human embryos and oocytes are summarized.

Keywords: Cryopreservation, IVF, ICSI, Sperm, Oocytes, Embryos, Malignancies.

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INTRODUCTION

The first successful mouse embryo cryopreservation (CP) was reported independently from each other by two research groups in 1972 [1-3]. One year later, the birth of the first calf from frozen embryo was published [4]. The first human pregnancy from frozen embryo was achieved with the same procedure used successfully for CP of mouse and cow embryos; however, it was terminated by spontaneous abortion in the 2nd trimester [5]. Since then, both sperm and embryo CP have become routine procedures in human assisted reproduction (AR) and oocyte CP is being introduced into clinical practice and is getting more and more widely used. Embryo CP has decreased the number of fresh embryo transfers and maximized the effectiveness of the IVF cycle. Similarly, embryo CP is a crucial tool in cases of cancelled embryo transfer (ET) due to ovarian hyperstimulation risk, endometrial bleeding, elevated serum progesterone levels on the day of triggering, or any other unplanned events. There is still a large debate on the best stage, protocol/procedure,

and cryoprotective additives (CPA) to use. The average potential of a frozen stored embryo to become a living child lies in the order of 4%, and babies born from cryopreserved embryos do not represent more than 8–10% of the total number of babies born from AR [6]. However, it is unquestionable that successful CP of zygotes/embryos has greatly enhanced the clinical benefits and cumulative conception rates possible for couples following a single cycle of ovarian stimulation and IVF. Results expressed as the augmentation of the delivery rate per oocyte harvest vary greatly in the literature, between 2% and 24% [7]. The data shows that women who had transfers of fresh and frozen embryos obtained 8% additional births by using their cryopreserved embryos [8, 9]. The metaphase II (MII) oocyte has a very special structure (i.e., large size, very sensitive to low temperature, extremely fragile, high water content, low surface to volume ratio, presence of the spindle and other cell organelles, not optimal plasma membrane permeability to CPA and water, etc.) that leads to complex difficulties associated with its CP. The spindle is crucial for the events following

fertilization in the completion of meiosis, second polar body formation, migration of the pronuclei, and formation of the first mitotic spindle. However, oocyte CP offers more advantages compared to embryo freezing: (1) fertility preservation in women at risk of losing fertility due to oncological treatment, premature ovarian failure, or chronic disease; (2) it can help alleviate religious and/or other ethical, legal, and moral concerns of embryo storage; (3) it helps to overcome problems such as when the husband is unable to produce a viable sperm sample or when spermatozoa cannot be found in the testis at a given moment in case of nonobstructive azoospermia; (4) it makes “egg banks and/or egg donations” possible by eliminating donor-recipient synchronization problems; and (5) it allows women to postpone childbirth until a later time/age (e.g., after establishing a career, etc.). The latter is called social freezing when the oocytes are cryopreserved for nonmedical purposes. For about 10 years, in parallel with the technical improvement of oocyte freezing, the possibility of egg storing for nonmedical purposes is more extensively discussed and more commonly accepted by the general population and expert committees in the USA and Europe.

CASE REPORT

The aim of the social freezing is to prevent age-related fertility decline which is widely promoted by fertility centers and the lay (unacademic) press throughout the world. Research place: International Fertility Center, New Delhi, India during from Jun to December-2019. It is a fact that the best reproductive performance/ability of women is around their 20–30 years of age. Embryo cryopreservation has decreased the number of fresh embryo transfers and maximized the effectiveness of the IVF cycle. The data shows that women who had transfers of fresh and frozen embryos obtained 8% additional births by using their cryopreserved embryos. Oocyte cryopreservation offers more advantages compared to embryo freezing, such as fertility preservation in women at risk of losing fertility due to oncological treatment or chronic disease, egg donation, and postponing childbirth, and eliminates religious and/or other ethical, legal, and moral concerns of embryo freezing. Afterwards pregnancy rates decline relatively fast from 35 years and miscarriage rates rise exponentially. After the age of 35 years, chances of becoming pregnant are very low. However, it is a worldwide tendency that women decide to give birth in their elder ages, as compared to earlier/20–30 years ago. Data of our patients having frozen cycle indicate that the average age (n = 3601) increased from 31.8 to 35.4 in the last 10 years. In the case of almost 70% of the frozen cycles the patients were between 31 and 40 years old and 7.5% of them were >41. The “age effect” is detectable in the frozen embryo survival rate which slowly but continuously decreased in the last 10 years as the average age of the patients increased by 4 years without doing any modification in the freezing process (89% versus 81%; $P < 0.0001$). The number of

successful frozen cycles is significantly lower over 30 years and there is a strong significant difference over 35 compared with under 30 years of age ($P < 0.01$ and $P < 0.0002$). The success rate of embryo/oocyte CP depends on several variables: efficacy of the freezing process, carriers used for vitrification (open versus closed), frequency of cycles with CP in the assisted reproductive program, the criteria for selection of embryos/oocytes for freezing, and the results of fresh embryo transfers. In order to do so the freezing solution, in which the cells are suspended, must be supplemented with cryoprotective additives (CPA). Exposure to CPA supports the dehydration of the cell and reduces intracellular ice formation. The CPA may be divided into two groups: intracellular/membrane-permeating (i.e., propylene glycol/PG/, DMSO, glycerol/G/, and ethylene glycol/EG/) and extracellular/membrane-no permeating compounds (i.e., sucrose, trehalose, glucose, amid, ficoll, proteins, and lipoproteins). The permeable CPA displaces water via an osmotic gradient and partly occupies the place of the intracellular water, while the extracellular CPA increases the extracellular osmolarity generating an osmotic gradient across the cell membrane supporting the dehydration of the cell before CP.

DISCUSSION

In this review, we summarize recent results including our own experiences concerning oocyte and embryo CP. Results can be expressed as survival rates (but it is not enough alone, retention of normal physiological function of the cell organelles is essential), implantation rates, pregnancy rates, or delivery rates per transferred or thawed embryos or harvested oocytes [12]. A Short Overview of the Basic Principles and Methodology of Slow Cooling and Vitrification: The traditional slow cooling methods for CP are referred to as equilibrium cooling, and the rapid/ultrarapid procedures (vitrification) as nonequilibrium cooling [13-15]. Various factors influence the survival of embryos and oocytes cryopreserved by equilibrium or nonequilibrium cooling procedures [8, 16].

Traditional Slow Cooling of Embryos and Oocytes

The greatest challenge during the CP of embryos and oocytes is to prevent the formation of ice crystal and toxic concentrations of solutes, which are the two main causes of cell death associated with CP, while maintaining the functionality of intracellular organelles and the viability of the embryo/oocyte. In order to do so the freezing solution, in which the cells are suspended, must be supplemented with cryoprotective additives (CPA). Exposure to CPA supports the dehydration of the cell and reduces intracellular ice formation. The CPA may be divided into two groups: *intracellular/membrane-permeating* (i.e., propylene glycol/PG/, DMSO, glycerol/G/, and ethylene glycol/EG/) and *extracellular/membrane-no permeating* compounds (i.e., sucrose, trehalose,

glucose, amid, ficoll, proteins, and lipoproteins). The permeable CPA displaces water via an osmotic gradient and partly occupies the place of the intracellular water, while the extracellular CPA increases the extracellular osmolarity generating an osmotic gradient across the cell membrane supporting the dehydration of the cell before CP. At the same time, it prevents the rapid entry of water into the cell after thawing during rehydration/dilution out of the permeating CPA [8, 13–15]. Dehydration of the cell mainly depends on the permeability properties of the cell membrane. There are differences in permeability among the embryos of different species to water and permeating CPA. Embryos usually are less permeable to G than to PG or EG. Furthermore, the earlier the stage of development, the less permeable are the embryos [15–17]. The permeability properties of immature and mature oocytes differ and can vary by 7-fold between individual human MII oocytes [18, 19]. This difference in membrane permeability may have a strong impact on the outcome of slow freezing of oocytes but can be controlled by the elevation of the concentration of the no permeable CPA and the environmental temperature [20, 21]. By having the concentration of no permeating CPA increased (sucrose: 0.2 and 0.3 M) higher survival rates were reported, and the overall fertilization rates of frozen-thawed oocytes appeared to be similar to those of fresh oocytes [20, 22–28]. Prior to slow cooling, dehydration of the embryos/oocytes is carried out by exposure to a mixture of permeable and no permeable CPA (duration: 10 minutes). In the case of human embryos/oocytes, with very few exceptions, low concentration of PG (1.5 M) and sucrose (0.1–0.25–0.5 M) is used for early cleavage stage embryos and oocytes and G for blastocyst stage embryos. In case of the original successful CP protocol mouse and cow embryos were cooled with a slow cooling rate (between minus 0.3°C–0.5°C/min) to very low temperatures of minus 80°C–120°C [1–5]. Therefore, the duration of the procedure was very long (several hours). Willesden [29] and Willesden et al. [30] described a variation of this method in which sheep and bovine embryos were cooled slowly at a rate of 0.3°C/min, but only to minus 30–35°C before being plunged into liquid nitrogen (LN₂) [29, 30]. With this modification the duration of the CP process was dramatically shortened (1.0–1.5 hours). Since then, this short protocol has become the treatment of choice for freezing of domestic animal embryos. Despite the excellent results achieved with animal embryos, human embryos are generally frozen with a low cooling rate of 0.3°C/min to about minus 30°C to 40°C, followed by an increased cooling rate of minus 50°C/min to a temperature of minus 80°C–150°C before being plunged into LN₂ [7, 8]. During slow cooling, the dehydration process is thought to continue until minus 30°C, after which any remaining water is super cooled [14]. During the slow cooling phase ice nucleation (seeding) is induced manually between –5 and –8°C (close to the true freezing point of the solution). Embryos/oocytes cooled slowly to sub-zero

temperatures of minus 30°C to 40°C before being rapidly cooled to minus 196°C require rapid warming/thawing in warm water of 25°C–37°C [13, 17]. Rapid thawing is followed by removal of the CPA from the embryo/oocyte. Rehydration of the cells is carried out in decreasing concentrations of permeating CPA, generally in the presence of increased concentrations of no permeating CPA.

Vitrification (Ultrarapid Cryopreservation) of Embryos and Oocytes

Vitrification (i.e., a glass-like state) is an alternative approach to embryo/oocyte CP which has been recently described as a revolutionary technique; however, the first successful embryo vitrification was published in the middle of the 1980s [32]. Vitrification is different from slow freezing in that it avoids the formation of ice crystals in the intracellular and extracellular space [34]. Vitrification is the solidification of a solution by an extreme elevation in viscosity at low temperatures without ice crystal formation, a process achieved by a combination of a high concentration of CPA (4–8 mol/L) and an extremely high (ultrarapid) cooling rate [15, 33–35]. In contrast to slow freezing (when dehydration of the embryos/oocytes starts during the equilibration in the freezing solution prior to slow cooling and continues during slow cooling to minus 30–35°C), during vitrification, cells are dehydrated mainly before the start of the ultra-rapid cooling by exposure to high concentrations of CPA, which is necessary to obtain a vitrified intracellular and extracellular state afterwards. In order to further increase the cooling rate (>10,000°C/min) necessary for successful vitrification, the volume of the solution in which the embryos/oocytes are vitrified has been recently dramatically decreased (0.1–2 µL). To achieve this, special carrier systems (open versus closed) have been developed such as open pulled straws, Flexipet-denuding pipettes, Cryotop, electron microscopy copper grids, cryoloops, or the “Hemi-Straw” system [15]. Similarly to slow freezing, rapid thawing is required for the optimal survival of vitrified embryos/oocytes, followed by stepwise rehydration using similar techniques employed after slow cooling. Blastocyst freezing was abandoned for years, since only 25% of zygotes were able to reach the blastocyst stage in vitro in usual culture media, and overall low pregnancy rates were reported. Recently, new embryo culture systems—such as the coculture on feeder cells and the sequential media—have been developed making it possible to obtain good quality blastocysts in 50–60% of the cases [36]. Therefore, the importance of blastocyst CP increased in the last 8–10 years. Early cleavage stage embryos are considered surviving CP when they keep at least half of their initial blastomeres intact after thawing. The moderate loss of cells did not significantly influence implantation. In an early, large multicentre study with 14 000 cleavage stage slow frozen and thawed embryos it was determined that 73% of the

embryos had at least half of their initial blastomeres still intact and the results showed clinical pregnancy and implantation rates of 16 and 8.4%, respectively, after transfer. In another study of over 300 single frozen embryo transfers of Day 2 embryos at the 4-cell stage and the embryos lost only a single blastomere during freezing/thawing (25%) similar implantation equivalent with fully intact frozen embryos and also with fresh embryos was obtained [25].

Practical Experiences with Human Oocyte Cryopreservation Using Slow Freezing or Vitrification

Since the first successes achieved in the field of human oocyte CP many changes have been introduced into the slow cooling procedure. Increasing the sucrose concentration both in the slow freezing and vitrification solutions (from 0.1 M to 0.3 M) increased the rate of dehydration and the survival and fertilization rates of MII oocytes in a dose-dependent manner [20, 22–28]. Changing the temperature of the equilibration with CPA, ice nucleation (seeding) and plunging embryos into LN₂, replacing sodium with choline (low sodium medium), or injecting sucrose directly into the cytoplasm of the oocyte all improved oocyte survival [31]. These results indicate that there is still room to improve the outcome of slow freezing of oocytes. Slower development relative to fresh controls, both with respect to timing of the first cleavage division and the developmental stage reached on Day 2, has been observed in oocytes slowly cooled in 0.3 M sucrose [24]. Konc et al. [22] reported comparable fertilization rates (fresh: 83%; frozen: 76%) but significantly slower development in the cryopreserved group, although implantation rates per embryo and oocyte were similar (fresh: 18% and 11%; frozen: 15% and 7%) [22]. Their results show a very pronounced difference in the cell stage on Day 2 between the frozen and fresh groups of oocytes ($P < 0.05$) as they found slower embryo development in the frozen oocyte cycles relative to fresh cycles. In the frozen group 64% of the embryos remained in the 2-cell stage and only 17% were in the 4-cell stage on Day 2. In contrast, in the fresh group on Day 2 66% of embryos were already in 4-cell stage and only 25% of them were in the 2-cell stage. Their results indicate that by observing the response of the individual oocytes the spindle does not always reform in its original position within the oocyte. After thawing and culturing the oocytes, they were able to visualize the spindle in 84.3% of the oocytes. However, they found that in half of the oocytes (53.1%) in which the spindle was rebuilt/visualized it was detected in a new location, not at the initial place, indicating that the spindle and the polar body move relative to each other [38]. The most widely used vitrification solution consists of a mixture of permeating (2.7 M EG and 2.1 M DMSO) and no permeating CPA (0.5 M sucrose). Comparing the results of slow freezing and vitrification we have to take into consideration that most of the published data generated by oocyte vitrification was obtained mainly

by open systems and from oocyte donation programmes in which the egg donors were fertile and generally young women.

Safety and Other Aspects of Oocytes and Embryo Cryopreservation

The total number of children born worldwide after the fertilization of frozen and thawed oocytes is more than 1500 [39-41]. Studies indicate that pregnancies and infants conceived after oocyte CP do not present with increased risk of adverse obstetric outcomes or congenital anomalies [42]. No increase in the number of abnormal or stray chromosomes has been observed in the thawed oocytes [43]. In addition, no difference was found when comparing the incidence of chromosomal abnormalities in human embryos obtained from fresh and frozen oocytes [43, 44]. The follow-up study of 13 children born from frozen oocytes failed to reveal any abnormalities in karyotype or organ formation, mean age at delivery, and mean birth weight [45]. In another study no intellectual and/or developmental deficits were found in children conceived from cryopreserved oocytes [37, 45-47]. Despite the promising results, there are still concerns regarding the possibility of chromosomal aneuploidies or other karyotypic abnormalities, organ malformations or other developmental problems in offsprings; therefore, further follow-up studies with adequate numbers of patients involved are needed to clarify this very important question. For patients, who are facing infertility due to chemotherapy/radiotherapy, oocyte CP is one of the few options available to keep their fertility potential [40, 48]. At present, spermatozoa and embryos/oocytes are commonly frozen/stored in LN₂ using straws/vials and newly developed open or closed carriers used for vitrification. Since the freezing container may leak or shatter during freezing, the potential for contamination of liquid nitrogen represents a real danger, especially in case of the “open carriers” developed for embryo/oocyte vitrification with ultrarapid cooling. The occurrence of cross-contamination during LN₂ storage of biological material and subsequent cross-infection of patients has previously been demonstrated [49]. Viruses have previously been found to survive direct exposure to LN₂, including vesicular stomatitis virus, herpes simplex virus, adenovirus, and papilloma virus [50]. There is also evidence of contamination of LN₂ by other microorganisms, including a wide range of bacterial and fungal species [51]. Given the strength of the evidence of LN₂ contamination by microbes and cross-infection in certain situations the possibility of contamination or cross-contamination during reproductive cell CP should be taken seriously.

CONCLUSION

Human embryo cryopreservation offers an efficient solution to the problem of supernumerary embryos whatever their developmental stage: zygote, cleaved embryos, and blastocyst. Thanks to this

flexibility, clinics could be able to choose the time to cryopreserve their embryos and the various strategies of transfer. Improvements will be difficult, even though 20-30% of cryopreserved embryos still fail to survive thawing. Human oocyte cryopreservation has practical application in preserving fertility for individuals prior to cancer treatments. While the efficiency of oocyte and embryo freezing technology has increased over time, there is still room for improvement, since even under ideal circumstances the clinical pregnancy rate from frozen embryo transfer is approximately two-thirds of that from the fresh transfer of embryos. Thus, studies connected with cryopreservation of human oocytes and embryos are very important to the expansion of effective clinical services. This review gives a summary of the theoretical and technical aspects of oocyte and embryo cryopreservation.

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