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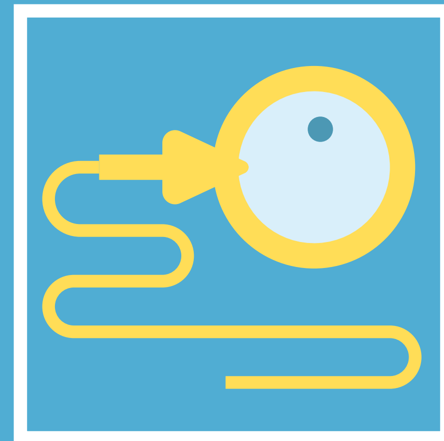

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BACK TO THE FUTURE

Cryopreservation of Oocytes and Embryos in Human Assisted Reproduction

J. Konc[1], S. Cseh[1, 2], E. Varga[1], R. Kriston[1], K. Kanyó[1]

Cryopreservation has become an integral component of assisted reproductive technology. The ability to cryopreserve, thaw, and establish pregnancies with supernumerary preimplantation embryos has become an important tool in fertility treatment. 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. ***J Reproduktionsmed Endokrinol 2005; 2 (4): 251–8.***

Key words: *oocyte, embryo, cryopreservation, vitrification, assisted reproduction, cryobiology*

The use of controlled ovarian stimulation protocols led to the production of large numbers of human oocytes and consequently embryos. In a routine IVF practice, 60 % of stimulated IVF cycles may yield surplus embryos suitable for cryopreservation (CP). In order to avoid the risk of multiple pregnancies without discarding the embryos in excess of the number appropriate for safe transfer embryos are cryopreserved. Thus, the importance of embryo CP procedure has increased and the efforts to study the factors influencing the outcome of freezing have intensified. However, improvements in CP and cryoprotective additives (CPA) have made slow progress since the last 10 years. There is still a large debate on the best stage, protocol, and CPA to freeze. The 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 % of the total of ART babies born in most programs [1].

However, it is unquestionable that successful CP of zygotes and embryos has greatly enhanced the clinical benefits and cumulative conception rate 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 % [2–5]. The increase of the outcome depends on several variables: efficacy of the freezing process, incidence of cycles with CP in the assisted reproductive program, criteria for selection of embryos for freezing, strategy and results of fresh embryo transfers. Results can be expressed as pregnancy rates or delivery rates, as implantation rates or birth rates per transferred or thawed embryos. Other clear benefits include the possibility of avoiding fresh embryo transfer (ET) in stimulated cycles with a potential for ovarian hyperstimulation syndrome or in which factors that may jeopardise implantation are apparent (e.g. bleeding, unfavourable endometrium, polyps or extremely difficult ET). Although, the clinics that offer embryo freezing and long term storage of embryos must also be aware of logistic, legal, moral, and ethical problems which can arise, and ensure that all patients are fully informed and counseled. Both partners must sign comprehensive consent forms, which indicate how long the embryos are to be stored, and define legal ownership in case of divorce or separation, death of one of the partners, or loss of contact between the clinic and the couple. The recently obtained data indicate that the problem connected with long-term management of storage of frozen human embryos had been underestimated. The number of frozen stored embryos is increasing year by year, however, there is a low concern of some patients about their excess embryos and data show that up to 10 % of these patients became "untraceable" after 5 years [1].

Oocyte CP offers more advantages compared to embryo freezing with less ethical, legal, and moral problems (except technical problems): it allows women to delay reproduction until later in life, for example after establishing career, it can help alleviate religious and/or other ethical concerns of embryo storage (see above), it helps to overcome problems for example 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 non-obstructive azoospermia, it makes egg banks possible, eliminating donor-recipient synchronisation problems, and it permits women at risk of becoming sterile to cryopreserve oocytes prior to radiotherapy/chemotherapy. There are relatively few effective clinical options for preserving female fertility, particularly following aggressive chemotherapy and/or radiotherapy treatment protocols. In some European countries, where embryo freezing is forbidden, the oocyte CP means an alternative way out in case of surplus eggs (e.g. Italy).

In 2001, over 625,000 women in the USA were diagnosed with some form of invasive cancer. Approximately 8 %, or 50,000 of these women were under the age of 40 [6]. With current treatment regimens cure rates for some malignancies can exceed 90 %. However, treatments often induce premature ovarian failure, rendering the patient infertile. Embryo banking is a proven method but requires both available sperm and several weeks of preparation. Oocyte banking avoids some of the disadvantages of embryo banking, although efficiency of this procedure is still low. Recently, more encouraging reports on the outcome of the technique have been published. For this reason, a renewed interest in oocyte CP has occurred. With the help of oocyte CP, young cancer patients could store their gametes before undergoing therapies, which may lead to infertility, such as chemotherapy or radiotherapy.

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Basic Principles of Embryo and Oocyte Cryobiology

Since the first success, thousands of babies have been born as a result of the transfer of frozen embryos [1]. Human embryos are now routinely cryopreserved as an adjunct to standard techniques of assisted reproduction. Numerous excellent reviews of embryo CP (human and animal) have been published [3, 7–15]. The procedure used to freeze the first human embryos was the same as the original one used to cryopreserve cleavage-stage embryos of rodents that resulted in the birth of live young [16–18]. The embryos were frozen slowly to about –100 °C, then plunged into liquid nitrogen (LN_2). Several years later, it was found that slow cooling could be interrupted at an intermediate temperature of about –30 °C to –40 °C, if the frozen embryos were thawed rapidly at about 350 °C/minute [19]. Since then, variations on this approach of Willadsen have been widely used to cryopreserve embryos of 15 or more species [8]. A different approach to embryo CP – than that of the traditional method (slow freezing) – is the vitrification when embryos are frozen under "ice-free" conditions [20]. This method has now been adapted to cryopreserve a wide variety of reproductive cells, especially oocytes, which are very sensitive to injury caused by cooling them close to 0 °C.

Traditional Slow Freezing

The traditional slow cooling methods of CP are referred to as equilibrium cooling, and the rapid procedures (vitrification) as non-equilibrium cooling [8, 12, 13, 21–23]. The same fundamental principles operate to determine survival of embryos cryopreserved by both methods. Various factors influence the survival of oocytes and embryos cryopreserved by equilibrium or non-equilibrium cooling procedure [22].

When oocytes or embryos are transferred from an isotonic solution into an non-isotonic solution (e.g. freezing solution), they respond osmotically either by undergoing dehydration due to water loss or by swelling as water enters the cells. For oocytes and embryos, an isotonic solution is approximately 300 mOsm. When the oocyte/embryo is transferred into hypertonic solution (> 300 mOsm), it will dehydrate (water leaves the cell). The rate at which the oocyte/embryo loses water depends on the membrane property of the oocyte/embryo (permeability to water), the temperature coefficient of the permeability, the solution in which the oocyte/embryo is suspended, and the temperature. Furthermore, different stages of embryonic development have different permeability coefficients [5, 24, 25].

In order to avoid the so-called freezing damage during CP of oocytes/embryos, the freezing solution in which the cells are suspended must be supplemented with cryoprotective additives (CPAs). CPAs protect oocytes/embryos against freezing damage. The CPAs may be divided into two groups: intracellular/membrane-permeating (i.e. propylene glycol, DMSO, glycerol, ethylene glycol) and extracellular/membrane-non permeating (i.e. sucrose, glucose, amid, ficoll, proteins, and lipoproteins) [13, 23]. Although, the actual mechanisms responsible for this protection have yet to be determined, much is known about the properties of these CPAs. The permeating CPAs act through different mechanisms: they lower the freezing point of the solution (as well as the amount of ice forming), they interact with the membrane modifications occurring during the CP process (from a relatively fluid state to a relatively rigid state), they prevent the exposition of the oocyte/embryo to high concentration of both intra- and extracellular electrolytes because they link to the electrolytes and partially substitute to the water [22, 26–28]. The permeating CPAs are non-toxic to cells even when present at high concentrations. In this sense, "non-toxic" means that permeating CPA does not kill the cell simply as a result of relatively short exposure to it. However, cells may be damaged or killed when exposed to and/or diluted out of the CPA, generally presented in high concentration in the freezing solution. Such damage is the result of an osmotic shock. Osmotic shock is caused by the higher osmotic pressure of the intracellular solution than of the extracellular solution. As a consequence, water enters the cell more rapidly than an intracellular solute, such as glycerol or propylene glycol, can leave. Depending on the relative permeabilities of the cell to water and to the solute, cell volume may increase to a lytic volume and the cell bursts. There are differences in the permeability among the embryos of different species to permeating CPAs [8, 28–30]. Furthermore, the earlier the stage of development, the less permeable are the embryos [15, 26, 27]. Thus, morulae are more permeable than 8-cell embryos, but 8-cell stage embryos are more permeable than 2-cell embryos, and so on. Most embryos including human ones are less permeable to glycerol than to propylene glycol or ethylene glycol [12, 22]. The result of the difference in permeability is that, if the embryo is much more permeable to one CPA than to another, then it will be much less sensitive to osmotic shock when diluted out of the former compared to the latter. That is the reason why the use of non-permeating solutes such as sucrose or galactose has become an important aspect of the dilution of embryos after thawing [3, 11, 27, 31].

Molecules with big dimension that cannot permeate the cellular membrane represent the non-permeating CPAs. They increase the concentration of extracellular solutes generating an osmotic gradient across the cell membrane, which draws water out of the cell causing the cell to dehydrate before the freezing procedure and prevent the rapid water entering into the cell after thawing during re-hydration/dilution out of the permeating CPAs. In the case of human embryos, with very few exceptions, propylene glycol with low concentration of sucrose is used for CP of early cleavage-stage embryos, and glycerol for blastocyst-stage embryos [1, 3, 11, 31]. However, animal experiments have demonstrated that effective CPAs include glycerol, ethylene glycol, methanol, propylene glycol, as well as DMSO [8, 27]. When oocytes/embryos are exposed to CPAs, they immediately contract osmotically by water loss because of the difference in osmotic pressure between the extracellular and intracellular solutions. At the same time, the CPA begins to permeate the cell by simple diffusion because there is a difference in the concentration of the CPA between extracellular and intracellular solutions. Finally, water begins to reenter the cell to maintain osmotic equilibrium between the extracellular and intracellular solutions. At the end, when equilibrium has been set up, the oocyte/embryo has the same concentration of CPA as that of the solution in which it is suspended, and the osmotic pressure of the cell cytoplasm is the same as that of the suspending medium. Several variables determine how quickly the equi-

librium is established: permeability property of the specific solute (e.g. ethylene glycol enters the cell faster than glycerol), concentration of CPA (the higher the concentration, the faster the CPA will permeate cells), temperature (the higher the temperature the faster the CPA will permeate cell), stage of development and species (see above).

When aqueous/freezing solutions containing CPAs are frozen, water is removed in the form of ice, thus dissolved solutes (CPAs) become increasingly concentrated. As the temperature is decreased more and more water leaves the solution and ice forms, increasing the concentration of the solution of the unfrozen liquid. This process continues until the sample is cooled to the eutectic point of the solution. The eutectic point is the temperature at which the entire system solidifies [15, 27].

In case of the original CP protocol, the embryos are cooled with low cooling rate (0.3–0.5 °C/minute) to very low temperatures of –80 °C to –120 °C. Willadsen (1977) and Willadsen et al. (1978) described a variation on this method in which sheep and bovine embryos were cooled at a rate of 0.3 °C/minute but only to –35 °C before being plunged into liquid nitrogen (LN_2) [19, 32]. Since then, this short protocol becomes the treatment of choice for freezing of domestic animal embryos. Despite the results achieved with animal embryos, the human ones are generally frozen with low cooling rate of 0.3 °C/minute to about –30 °C to –40 °C, then with an increased cooling rate of 10–15 °C/minute they are cooled to a very low temperature of –80 °C to –150 °C before plunging into LN_2 [4, 17, 18, 33–38].

Water and aqueous solution have a strong tendency to cool below their true freezing point before nucleation of ice (ice formation) occurs: this phenomenon is referred to as supercooling. Following ice nucleation and initial crystal growth the temperature rises to its melting/freezing point and remains relatively constant at that temperature during the subsequent phase change to ice (from fluid to ice phase change) ("latent heat plateau"), when the temperature then changes more rapidly to the environment temperature. The tendency of a system to supercool is related to a number of factors including temperature, rate of cooling, volume, exclusion of atmospheric ice nuclei, and purity of particulates. In oocyte/embryo CP, there is a strong tendency for supercooling to occur. To avoid the damaging effects of supercooling on oocytes and embryos ice formation is initiated in a controlled manner. This is commonly referred to as "seeding". Seeding induces ice crystal formation in the solution removing some water from the solution, thus the remaining liquid phase becomes more concentrated. As the temperature is reduced, more ice forms and the residual non-frozen phase becomes increasingly concentrated (hypertonic environment, high osmotic pressure), which helps the cells to become osmotically de-hydrated (see above). The amount of water removed from the cell depends on the rate of cooling. At slow rates of cooling, cells may remain essentially in equilibrium with the external solutions. As the rate of cooling is increased, there is less time for water to leave the cell. The optimal cooling rate results from the balance of these two phenomena. At rates of cooling slower than the optimum, cell death is due to long periods of exposure to hypertonic conditions. At rates of cooling faster than the optimum, cell death is associated with intracellular ice formation, which is inevitably lethal. The optimum rate of cooling depends on several factors: cell volume and surface area, permeability to water, type and concentration of CPAs [24, 39–43]. Controlled ice formation during freezing is a key factor in determining the viability of embryos following freezing and thawing. Data indicate that if nucleation/ice formation is not controlled the recovery of embryos would be expected to be very low. In a series of experiments, samples which were seeded/nucleated below –9 °C had a low survival rate, whilst nucleation at higher subzero temperatures of –5 °C to –7.5 °C resulted in much higher viability [29, 41, 44, 45]. Normal practice is to cool the straw/ampoule containing the embryos to a temperature of approximately –7 °C, hold at this temperature for thermal equilibration, and then initiate ice formation (seeding) in the straw/ampoule by touching the outside of the straw/ampoule with cold forceps etc. The temperature of the straw rises to the melting point of the solution, and then following ice formation the temperature returns to –7 °C. Cellular dehydration then occurs during subsequent slow cooling. By contrast, in a straw/ampoule supercooled to –15 °C spontaneous ice formation again results in a temperature rise followed by rapid rate of cooling to –15 °C. The rapid cooling and large reduction in temperature together (temperature fluctuation) do not permit cellular de-hydration and results in a lethal amount of ice formation killing the cells. Thus, ice nucleation must be controlled in order to obtain high viability of oocytes/embryos in a CP programs [12, 15, 19, 29].

Long term storage of oocytes and embryos requires that they are stored below about –130 °C, the glass transition temperature of water. In practice, the easiest and safest way is to store cryopreserved oocytes/embryos in LN_2 at –196 °C. The data of mouse model experiments indicate that cryopreserved embryos stored in LN_2 will remain "alive" for at least 2000 years [46]. It was concluded, that normal levels of background radiation would not be hazard to the long-term storage of mammalian embryos. Children have been born from embryos that were cryopreserved for more than 8 years [12]. Live mice and sheep have been produced from cryopreserved embryos stored for more than 15 years in LN_2 [47].

In general, warming rate depends on the cooling protocol used [11, 15, 22, 24, 27, 47]. Embryos cooled slowly to subzero temperature of about –30 °C to –40 °C before being rapidly cooled to –196 °C require rapid warming of about 200–350 °C/minute. Embryos cooled slowly to –60 °C or below require slow warming of about 25 °C/minute or less.

The final, very important step of the cryopreservation procedure is the removal of the CPA from the oocyte/embryo. Embryos/oocytes are cryopreserved in CPAs ranging in concentration from 1–8 M (1–2 M in case of traditional slow cooling; 4–8 M in case of vitrification). In general, if oocytes/embryos frozen in permeating CPAs are rapidly diluted (re-hydrated), the oocytes/embryos will be subjected to osmotic shock causing death. A common practice is when the CPAs are diluted out of the frozen oocyte/embryo in a step-wise fashion. Although this method works well in practice, it is very slow and time and work consuming. A much shorter method is to use a non-permeating solute such as the sucrose as an osmotic buffer to lessen the chance of an osmotic shock [1, 11, 15, 27, 30, 48].

Principle of Vitrification

Vitrification is an alternative approach to freezing, which avoids the formation of ice crystals in the intracellular and extracellular space [20]. Vitrification is the solidification of a solution at low temperature without ice crystal formation (vitrify), a process achieved by a combination of a high concentration of CPAs (4–8 mol/l) and an extremely high cooling rate [14, 22, 31, 49–53]. The classical methods of slow embryo freezing are based on a progressive de-hydration of the embryo during cooling process (see above). However, when finally the samples are cooled in LN_2 after sufficient time/de-hydration, the cytoplasm of the cell, together with the extracellular concentrated fraction in which the cells are suspended will turn to a glassy solid without ice formation, will vitrify. In contrast, for vitrification, the cells are de-hydrated mainly just before the cooling process starts, by exposure to the high concentrations of CPAs necessary to obtain a vitrified intracellular and extracellular state. Vitrification can be defined as the solidification of a solution by an extreme elevation in viscosity without crystallisation. The potential risk of vitrification procedure is the high concentration of CPAs that must be used which could be toxic to the cells. However, it is possible to limit CPA toxicity by using a solution of different CPAs, thereby decreasing the relative concentration of each, reducing the length of time and temperature the embryos are exposed to it [14, 22, 52]. The CPAs commonly used for vitrification are composed of permeating (e.g. ethylene glycol, glycerol, DMSO, propylene glycol, acetamid etc.) and non-permeating (e.g. sucrose, trehalose etc.) agents. In some protocols, the vitrification medium is also supplemented with macromolecules such as polyethylene glycol, ficoll or polyvinylpyrrolidone [13, 20, 22, 53]. By increasing the viscosity, the macromolecules support vitrification with lowered concentrations of CPAs. The viscous matrix in which the embryos/oocytes are in also prevents water crystallisation during cooling and warming. In order to achieve the highest cooling speed and successful vitrification special carrier systems have been developed: Open Pulled Straw, Flexipet-denuding pipette, electron microscopy copper grids, cryoloop, Hemi-Straw System [14, 50–52]. Various improvements have been made to the vitrification and it is now possible to cryopreserve many types of embryos with minimal loss of viability, although more precise handling of embryos is required than with the slow freezing method.

Technical Aspects of Human Embryo Cryopreservation

The main purpose of embryo CP is to provide further possibilities for conception in addition to those obtained through the initial cycle using fresh embryos. Data show that women who had transfers of fresh and frozen embryos obtained 8 % additional births by using their cryopreserved embryos [11, 12, 54–57]. Extended storage of embryos did not affect the outcome of thawed cycles [42, 58].

Human embryo freezing was adapted from the application of animal embryo CP techniques. In 1972, Whittingham et al. (1972) obtained the first births from transfer of frozen/thawed mice embryos [16]. Soon after, Wilmut (1972), Wilmut (1972) and Wilmut and Rowson (1973) reported data from successful freezing of mouse and cow embryos and births of offspring from frozen embryos in these species [39, 48, 59]. The embryos were frozen with slow freeze procedure to about –80 °C to –100 °C before plunging to LN_2 and stored at –196 °C and slow thawing was applied. The same protocol led to the first reported pregnancy in humans, which was – unfortunately – terminated by spontaneous abortion in the second trimester [17]. The first births were announced in 1984 [18]. Later studies with animal embryos showed that programmed slow cooling can be interrupted between –30 °C to –40 °C before rapid freezing to the temperature of LN_2 [19, 32]. Nowadays, generally human embryos are cooled with slow rate (0.3–0.5 °C/minute) to about –30 °C to –40 °C, then an increased cooling rate of 10–50 °C/minute is used to about –80 °C to –150 °C before plunging into LN_2. The embryos frozen with the rapid/short protocol must be warmed rapidly to 37 °C or room temperature (RT) [3, 4, 12, 60–64].

Generally, in the first years DMSO was used for cryoprotection of embryos during freezing. Nowadays, DMSO is less frequently used for multicellular embryo freezing and it had been successfully replaced by propylene glycol, ethylene glycol or glycerol [3, 4, 7, 17]. Generally, in the human, propylene glycol is used for freezing of cleavage stage embryos and glycerol for CP of blastocysts [25, 33, 35, 36, 40, 60, 62]. However, ethylene glycol is the most widely used CPA in the CP of cow embryos frozen in very high numbers each year, but is rarely used in human embryo CP with conventional freezing technique [8, 9, 15, 26, 27, 30]. Propylene glycol and DMSO was not found to be an effective CPA for slow cooling of late-stage human embryos [28].

Until now, the preferred stages for human embryo CP were the zygote and early cleavage stages. Blastocyst freezing was abandoned for years, since 25 % of zygotes only were able to reach the blastocyst stage *in vitro* in usual culture media and low pregnancy rates were reported [1, 34, 60, 63, 65]. CP most often concerns zygote and early cleavage stage embryos, and many reports have focused on the main parameters involved in its efficacy [7, 17, 18, 33, 35, 36, 61, 66]. Recently, new embryo culture systems – such as the co-culture on feeder cells and the sequential media – have been developed allowing obtaining good blastocysts with higher rates of 50–60 % [5, 25, 37, 62]. However, the efficacy and results of blastocyst culture, CP and frozen blastocyst transfer reported are still very controversial [34, 38, 67]. Furthermore, human blastocysts obtained using sequential media appear to be half less cryoresistant than the co-cultured ones [4, 7, 10].

Recently, studies have been published focusing on micromanipulation insemination technique (ICSI) that may affect the success of *in vitro* blastocyst formation and freezing/thawing cycles. Lower rate of formation and quality and/or higher sensitivity of ICSI blastocysts to freezing need to be considered [67–69]. Lejeune et al. (1997) reported lower implantation rate for frozen/thawed ICSI embryos (4 %) [70]. However, others found that CP of ICSI-derived zygotes/embryos gives similar results to the freezing of IVF embryos [71–75].

The technical requirements for human embryo freezing are well documented [3, 4, 7, 11–14]. The efficiency of the seeding technique is of major importance [29, 45], and seeding may be impaired in some freezers with automatic systems, thus leading to a failure of the whole freezing programme. The work by Tyler et al. (1996) shows the importance of a rapid handling of freezing containers (vials and especially straws) containing frozen embryos [76]. Expo-

sure times of < 40 s at RT may lead to potentially detrimental temperature changes. They found that a 0.25 ml straw has reached –7 °C, the eutectic point, in 1 minute at RT.

Early cleavage stage embryos are considered surviving when they keep at least half of their initial blastomeres intact after thawing. The survival rate is the percentage of surviving embryos among all frozen/thawed embryos and represents around 75 % of embryos while 50–60 % are totally preserved (100 % of blastomeres survived). Re-expansion of frozen/thawed blastocysts *in vitro* is considered a very good sign of survival (70–80 % of thawed blastocysts). Approximately 10–30 % of frozen embryos, whatever their stage of development at CP, do not survive freezing/thawing. Fresh embryo transfer outcome has a prognostic value and clearly influences the results of frozen embryo transfer [57]. The pregnancy and implantation rates of frozen embryo transfer cycles with zygote and early cleavage stages reported are between 11–22 % and 5–15 % [11, 13, 31, 42]. Recent reports indicate better pregnancy (29–36 %) and implantation (14–16 %) rates with blastocysts [10, 13, 75, 77].

Assisted hatching (partial zona dissection, zona drilling, laser assisted hatching) might enhance the implantation rate of cryopreserved embryos. Check et al. (1996) obtained an increased implantation rate in the groups of embryos with assisted hatching (14 %) compared with the control group (5 %) [78]. The possible explanation is that if freezing leads to zona hardening then assisted hatching may improve the results of CP cycles through assisting implantation.

Early cleavage stage human embryos have been successfully vitrified in DMSO and ethylene glycol based solutions and several pregnancies/deliveries have been reported [64, 79–82]. However, only a few embryos were transferred and the overall pregnancy/delivery rate proved to be very low. In order to increase the cooling speed, several special carrier systems (cryoloop, electro microscope grid, Open Pulled Straw, Flexipet-denuding pipette, electron microscopy copper grids, cryoloop, Hemi-Straw System) have been used to human embryo vitrification with very promising results (pregnancy rate of 25–50 %) [83–88]. The recently obtained data indicate that solutions with ethylene glycol are more widely used, because ethylene glycol is less toxic and more permeable to embryos than DMSO, acetamid, and propylene glycol, and the use of special carrier systems – through increased cooling speed – result in better survival and pregnancy rates after vitrification.

Technical Aspects of Human Oocyte Cryopreservation

The metaphase II (MII) oocyte is extremely fragile and sensitive to low temperature due to its large size (human oocyte is 130 µm), water content, low surface to volume ratio, chromosomal arrangement, and the presence of the spindle [89]. In the mature oocyte, the metaphase chromosomes are lined up by the meiotic spindle along the equatorial plate. It has been well documented that the spindle apparatus is easily damaged by intracellular ice formation during freezing or thawing process [89–93]. In addition, hardening of the zona pellucida can adversely affect the normal fertilisation process [94]. There is a big difference in the sensitivity to CP depending on the maturity status of oocytes. Oocytes frozen at the germinal vesicle (GV) stage survive better than those frozen at the MII stage [92, 95]. However, because of the difficulties connected with the *in vitro* maturation of GV oocytes, the final yield of mature oocytes (MII oocytes) is similar to that obtained with freezing MII oocytes. Variability in survival rates of cryopreserved MII oocytes may be partly attributable to the quality of oocytes and CP protocol used [21, 23, 96, 97]. Increasing the sucrose concentration of the freezing solution, for example, increased the survival rate of MII oocytes in dose dependent manner [23]. Changing the temperature of the equilibration with CPA, the ice nucleation (seeding) and plunging into LN_2, and replacing the sodium with cholin (low sodium medium), or injecting sucrose directly into the cytoplasm of the oocyte improved oocyte survival [45, 73, 97–101].

Vitrification is a new approach for oocyte CP. Vitrification is the process of CP when a very high concentration of CPAs is used in combination with very high cooling speed to solidify (vitrify) the oocyte into a glass-like state without the formation of ice. Although it is relatively successful for embryo CP, the process has not so far been reproducible for oocytes of any species [98]. Recently obtained data show an increase in the post thaw survival rates and fertilisation rates of vitrified human oocytes and even immature human oocytes have been vitrified with success [102–104].

The total number of pregnancies/deliveries from cryopreserved oocytes (slow cooling and vitrification) is about 150 [105]. No increase in the number of abnormal or stray chromosomes in thawed, previously cryopreserved oocytes has been observed [106]. Comparing the incidence of chromosomal abnormalities in human embryos obtained from fresh and frozen oocytes no difference was found [107]. In a follow-up study of 13 children born from frozen oocytes failed to reveal any abnormalities in karyotype, mean age at delivery, mean birth weight, or organ formation [108]. In another study no intellectual and/or developmental deficits were found between the children conceived from cryopreserved oocytes [109]. Despite of the promising results, there are still concerns regarding the potential for chromosomal aneuploidy or other karyotypic abnormalities, organ malformations or other developmental problems in the offspring. We know very little about the potentially detrimental effects of vitrification when compared with conventional CP technique. The recently obtained results with human and animal oocyte CP are very promising and suggest several advantages and potential for clinical application [51, 110, 111]. Although, it is too soon to conclude from the results obtained already that the incidence of chromosomal and developmental abnormalities of children born from cryopreserved oocytes are similar to those born from cryopreserved embryos. Further follow-up studies with adequate numbers of patients involved are needed to clarify this very important question. The current efficiency of the human oocyte CP procedures are too low for clinical application and significantly less than those of standard IVF protocols. However, for patients, who are facing infertility due to chemotherapy/radiotherapy, oocyte CP may be one of the few options available. Thus, the point of view of the Practice Committee of the Society for Assisted Reproductive Technology and the Practice Committee of the American Society for Reproductive Medicine is that (1) oocyte CP holds promise for future female infertility preservation, (2) recent laboratory modifications have resulted in improved

oocyte survival, fertilisation and pregnancy rates from cryopreserved oocytes, (3) no increase in chromosomal abnormalities, birth defects, developmental deficits have been noted in the children born from frozen oocytes, (4) oocyte CP presently should be considered an experimental technique and recommended to cancer patients only and carried out with appropriate informed consent, and (5) oocyte CP should not be marketed or offered as a means to defer reproductive aging [6].

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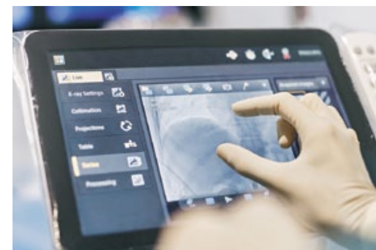

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