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It is essential in every assisted reproductive technology unit to have a well-established frozen/thawed embryo transfer program. Freezing and storing of surplus embryos allows the number of replaced embryos in both fresh and frozen/thawed embryo transfers to be reduced, thereby diminishing the risk of multiple pregnancies, in addition it increases the cumulative pregnancy rates of IVF and ICSI procedures. Nowadays, there is a continuous debate about which cryopreservation method should be used and whether we should be stuck to the conventional cryopreservation techniques or to move toward the new method of vitrification.

In conventional cryopreservation, the cells are suspended in a suitable solution, cooled, stored in liquid nitrogen, warmed to room temperature, and returned to a physiological solution. During each step of this process, cells are at risk for various types of injuries. The primary injury is that caused by the formation of intracellular ice during cooling and warming. To prevent this injury, inclusion of a cryoprotectant is essential for large cells like mammalian embryos. However, the cryoprotectant brings other causes of injuries, i.e. chemical toxicity of the agent and osmotic over-swelling of the cells during removal of the permeated cryoprotectant. During the removal, embryos are usually exposed to a hypertonic solution with sucrose, and embryos can be injured by osmotic over-shrinkage in some cases. In addition, embryos can be dissected physically by a fracture plane during passage through the glass transition temperature. Furthermore, certain types of embryos are injured just by chilling at 20-0°C. In order for embryos to survive cryopreservation, the effect of each of these injuries must be minimized. Slow cooling procedures have also the disadvantage in that they are time consuming and require accurately controlled expensive freezing units, making them unsuitable for use where cost and time is a consideration.

On the other hand, vitrification, in which not only the cells but also the whole solution is solidified without ice crystallization, is relatively simple. It includes two major benefits: the process can be completed in only few minutes and does not require specialized equipments in contrast to conventional slow freezing techniques. Vitrification is a reasonable and effective strategy for preventing the primary cause of injury, the intracellular ice formation. Fracture damage and chilling injury may also be minimized in vitrification. In addition, the survival of embryos is more likely if the embryo treatment is optimized. However, the procedure still might have some disadvantages, solutions for vitrification must include a high concentration of permeating cryoprotectants, which may cause injury through the toxicity of the agents. This could be overcome by applying the ultrarapid vitrification technique using minute tools such as electron microscopic grids, thin capillaries, minute loops, minute sticks, or as micro-drops instead of the conventional vitrification using insemination straws. In that way, a lower concentration of the permeating cryoprotectants is used, thus having a lower toxicity. In addition, the ultrarapid cooling/warming helps to prevent ice formation.

There was also a report of possible embryo infection after exposure to LN2 artificially mixed with high concentrations of virus. Nevertheless, because it is highly unlikely such an adverse environment exists and actual cases of contamination have not occurred in previous surveys, there is hardly any concern in real terms. However, in some countries like USA, legal provisions are beginning to be considered for the future to avoid such a risk. Viral infection mediated by LN2 can be prevented by completely sealing the cryopreservation container prior to immersing the sample in LN2. Kuwayama et al (1) developed a vitrification method for this purpose, the vitritip method, which is able to realize complete sealing of the container along with ultra-rapid cooling and warming rates comparable to the Cryotop method. Kim et al.(2) used pulled straws for oocyte vitrification and they stated that this method provides a simple, rapid and effective strategy for preventing the risk of LN2 contamination during storage.

We still have other causes minimizing the

practical impact of vitrification, the presence of a wide variety of different carriers and vessels in addition to the many different vitrification solutions that have been formulated, which has not helped to focus efforts on perfecting a single approach.

The situation in Germany

The German embryo protection law does not allow embryo selection, but only selection at the pronuclear stage. Furthermore, only as many pronuclear stage zygotes are allowed to be selected as are planned to be transferred in the same cycle. This means that after pre-selection of, for example, three pronucleated zygotes, these three must be transferred on the same or the subsequent day. A second selection process is not allowed. Non-selected pronuclear stage zygotes are allowed to be cryopreserved for a subsequent transfer. The same situation is present in other European countries such as Switzerland and Italy. The idea of these laws was to avoid ethical problems related to cryopreservation of surplus embryos or wastage of embryos, because these have, according to these laws, the status of individual persons. The current situation initiates much interest in developing a refined method of cryopreserving human pronuclear zygotes.

The pronuclear stage appears to be the optimal stage for cryopreservation (3).

The unicellular form and lack of spindle apparatus may account for its high post-thaw survival and implantation potential. This might be also due to the processes during and after the fertilization, such as the cortical reaction and subsequent zona hardening that may give the ooplasmic membrane more stability to cope with the low temperature and osmotic changes. Using this stage for freezing, there are no ambiguities about whether embryos survive thawing because subsequent embryo cleavage essentially proves cellular integrity.

In a study conducted by Schroder et al (4) in Lubeck, All fresh embryo transfer cycles performed from January 1994 until December 1998 in which supernumerary pronucleate zygotes were cryopreserved (n = 557) were analyzed retrospectively, together with data from all subsequent cycles involving transfers of frozen-thawed pronucleate zygotes (n = 420) from January 1994 until June 2001. The additional cumulative pregnancy rate per fresh cycle was 11.5%. This rate depended on the number of embryos per transfer, i.e. 1.9, 8.2 and 13.0% respectively when one, two or three embryos were transferred ($P < 0.05$). A strong correlation was found between the numbers of cryopreserved pronucleate zygotes and pregnancy rates, of 9.3, 10.5 and 17.1% when 1-3, 4-6, or at least 7 pronucleate eggs were available respectively.

In another unpublished data obtained from the same laboratory in Luebeck involving 33 patients, a total of 154 pronuclear stage zygotes were vitrified from which 45 were warmed. After warming, 40 (88,8%) survived, cultured till the embryo stage and transferred to 13 patients. Six pregnancies were obtained (with 1 delivery out of these 6) with a pregnancy rate/ transfer of 46, 15%. When these results are compared with the pregnancy rates of the previous study by Schroder et al using the conventional cryopreservation technique, we can conclude that the low toxicity of ethylene glycol, together with the good survival, cleavage, embryo formation, and pregnancy rates obtained after vitrification of pronuclear zygotes, may satisfy the real need in countries where cryopreservation of later-stage human embryos is not allowed by law or for ethical reasons.

Our results also agree with those obtained by using the minimum volume cooling (MVC) technique using the cryotop carrier system created by Kuwayama. They reported that they performed more than 15,000 cases of virtification over a period of four years on human oocytes, 2PN, 4 cell stage embryos as well as human blastocysts. Survival rates higher than 90% and high pregnancy rates following development of in vitro culture and embryo transfer were obtained regardless of the stage (5). In another study, Liebermann et al. (6), using 5.5 M EG, 1.0 M sucrose, and an FDP (flexipet-denuding pipette) as a carrier for the vitrification, observed 90% of 2PN survival after warming and 82% of 2PN cleavage on Day 2. On Day 3 in the vitrified 2PN group, approximately 80% of embryos cleaved to become an embryo with four or more blastomeres, and 30% of 2PN embryos eventually became blastocysts.

In contrast to the previously mentioned studies,

Uechi et al. (7) by comparing the conventional slow controlled rate freezing and vitrification on two-cell mouse embryos, showed that the implantation rate of blastocysts developed in vitro from vitrified two-cell embryos was significantly lower than that from slow controlled rate frozen embryos (10.2% versus 22.1%). Vitrification may, therefore, exert a more harmful effect than the slow controlled rate freezing in two-cell embryos. The same could be also speculated for one-cell embryos.

In our opinion, despite of the fact that vitrification as a cryopreservation method has had very little practical impact on human-assisted reproduction as a newly employed technique, the reports of successfully completed pregnancies following vitrification at all pre-implantation stages as well as the simplicity of the procedure and the economic advantages, is encouraging for further research and clinical implementation.

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Cryopreservation of different types of living cells including oocytes, embryos even stem cells has become mandatory in infertility treatment and play important role in improving the results. There are different techniques that have developed in that field. All aim to be cell friendly; that is to choose low temperature technology that is likely to minimize cell damage and enhance survival rate of the living material that is stored for infertility treatment.

This debate is to compare slow cooling with vitrification process to evaluate, which is likely to be more suitable for assisted reproductive technology as currently practiced i.e. which of them cause the least damage to the gametes during cryostorage.

Vitrification is a process that produces glass like solidification of living cells that completely avoids ice crystal formation during cooling, and more importantly during thawing, which is fundamental issue in cryopreservation as ice should never be allowed to appear and grow inside the cells or tissue as this leads to damage and death of the living system. This is the same goal of the slow cooling process, as ice crystal formation is very detrimental to the living cells.

Vitrification simply avoid ice crystal formation by cooling the living cells so quickly that ice wouldn't have time to form, that included the use of very high concentration of cryoprotectants to support the cytoplasm. The moment the cytoplasm is sufficiently concentrated the cooling process starts rapidly (1). To achieve this aim vitrification has passed through several steps. Initially the