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Cryopreservation of two pronuclear stage zygotes

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Abstract

The German embryo protection law (Embryonenschutzgesetz, ESchG) 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 Swizerland and Italy. it is illegal to cryopreserve an oocyte after fusion of the pronuclei (PN). 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 following article will discuss that issue in details.

Keywords: Cryopreservation; Two pronuclear stage zygote.

1. Introduction

A well-established frozen embryo transfer (FET) programme is essential in every assisted reproductive technology (ART) unit. Cryopreservation programs may also increase the cumulative pregnancy rates of IVF and ICSI procedures [1,2].

Freezing and storing of surplus embryos also allows the number of replaced embryos in both fresh and frozen embryo transfers to be reduced, thereby diminishing the risk of multiple pregnancies [3,4]. In addition, if the woman has a risk of developing ovarian hyperstimulation syndrome all embryos can be cryopreserved [5]. However, careful consideration of all clinical and embryological factors influencing the outcome of FET is a prerequisite for a successful programme.

Cryopreservation of human embryos has been introduced into clinical IVF in order to preserve supernumerary embryos for a later transfer. Human embryos at different developmental stages have been frozen with variable success rates. The pronuclear stage appears to be the optimal stage for cryopreservation [6]. The unicellular form and lack of spindle apparatus may account for its high post-thaw survival and implantation potential. Using this stage for freezing, there are no ambiguities about whether embryos survive thawing because subsequent embryo cleavage essentially proves cellular integrity. In addition, in some European countries, the freezing of cleaved stage embryos is illegal as in Germany, Switzerland and Italy, thus limiting the choice to freezing of either unfertilized oocytes or pronuclear stage zygotes.

Several protocols of freezing have been formulated depending on the embryo cellular stage, type of cryoprotectant and speed of cooling.

Conventional (slow) freezing of human pronuclear zygotes has been the most widely used method of storage

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up until now [6,7] with variable results [7–9]. Slow cooling procedures have 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. Different freezing protocols that are faster and cheaper and achieve higher survival and development rates after freezing and thawing than do conventional freezing procedures have been reported.

The rapid procedure for freezing human pronuclear embryos such as that reported by Trounson et al. [10] has been reported by a few IVF groups with variable results [11,12].

However, there have been several recent reports of the successful cryopreservation of human pronuclear zygotes by direct plunging into liquid nitrogen (vitrification) [13–15]. This method is now an object of intensive investigation in a number of laboratories, taking into account that the protocol of vitrification includes two major benefits: the complete process can be completed in only minutes in contrast to a long time for the conventional method, and this method does not require specialist equipment, in contrast to conventional slow freezing techniques.

Prior to the successful vitrification of human pronuclear zygotes, vitrification of fertilized animal oocytes was developed by an effective protocol for the vitrification of mouse oocytes, which involved direct plunging into liquid nitrogen [16–18]. Since then, several publications on the vitrification of animal oocytes at the pronuclear stage have emerged, in which the ability of cells of transgenic mice [19] and rabbits [20] to develop after cryopreservation was evaluated. Subsequent protocols for the vitrification of human pronuclear zygotes were based on the data provided by these studies.

1.1. Slow freezing technique

1.1.1. Preparation of oocytes

Following oocyte retrieval, the cumulus and corona radiata are removed mechanically under a stereomicroscope, after exposure to 0.5% hyalouronidase solution (Sigma Company, Deisenhofen, Germany) for 30 s. IVF or ICSI are performed as previously described by Al-Hasani et al. [21].

Pronucleate zygotes must have an intact zona pellucida and healthy cytoplasm with two distinct pronuclei clearly visible. When pronuclei start to migrate before syngamy the mitochondreal system is highly vulnerable to temperature fluctuation leading to possible scattering of the chromosomes. Ludwig et al. [22] recently published a new scoring system for zygotes at the PN stage. This score is based on the fact that a faster developmental process after fertilization demonstrates a better quality of the zygotes and resulting embryos. Their score included not only the morphological appearance of the pronuclei, but also the further development up to the PN membrane breakdown and first cleavage division. This last item (PN membrane breakdown and first cleavage division within 24–26 h post oocyte retrieval) constituted 2/5 of the maximum score. Scoring was done at 16–18 h post ICSI according to (i) the position of the PN, (ii) the alignment of nucleoli at the junction of the two pronuclei, and (iii) the appearance of the cytoplasm.

In Germany, however, this item cannot be included, since only selection at the PN stage is allowed and supernumerary PN zygotes must be cryopreserved at the PN stage or discarded.

1.1.2. Freezing and thawing procedures

The supernumerary Zygotes of the collecting cycles are cryopreserved 18 h after the IVF or ICSI procedure. Ham's F-10 (Biochrom Company, Berlin, Germany) supplemented with 20% human umbilical cord serum is used as freezing solution. The cryoprotectants 1,2-propanediol (PROH) and sucrose are used at concentrations of 1.5 and 0.1 mol/L, respectively [23]. Pronuclear stage embryos are then equilibrated in two steps (first step: 1.5 mol/L PROH, second step: 1.5 mol/L PROH and 0.1 mol/L sucrose) at room temperature, each for 10 min. A CTE-880 biological freezer (Cryo Technik Company, Erlangen, Germany) working with an open freezing system and self-seeding was used for cryopreservation. Up to three 2PN zygotes are transferred with medium to each ministraw (Cryo Technik Company). The ministraws are cooled slowly from room temperature to −33 °C. They should be kept at −33 °C for 30 min and then they are plunged directly into liquid nitrogen for storage [23].

The thawing procedure begins with the direct transfer of ministraws to a 30 °C water bath, for 30 s. After this, the cryoprotectants are diluted in four steps, using different solutions: first, with 1 mol/L PROH and 0.2 mol/L sucrose; second, with 0.5 mol/L PROH and 0.2 mol/L sucrose; third, with 0.2 mol/L sucrose; and finally with Ham's F-10 medium alone. Each step should last 5 min [7,24].

Pronuclear stage zygotes are then cultured in Ham's F-10 for 2–3 h and then inspected for survival under both a stereomicroscope (magnification 50) and an inverted microscope (magnification 200–400).

1.2. Ultra-rapid freezing technique

The zygotes are again first exposed to a cryoprotectant, equilibration prior to freezing is carried out as described in the slow freezing method. Zygotes are then drawn up into plastic straws, also electron microgrids can be used as a physical support, before they are plunged directly into liquid nitrogen after 2–4 min.

For thawing, the straw is gently expulsed into a phosphate buffered solution containing 20% fetal calf serum and 0.25 mol/L sucrose for 10 min at room temperature. The zygotes are then placed in culture and incubated for 2 to 4 h before transfer into the recipient uterus.

As freezing solution EFS30 is often used consisting of 30% Ethylene Glycol, 18% Ficoll, 0.5 mol/L sucrose, 10% fetal bovine serum with added modified Dullbecco's

phosphate buffered saline, supplemented with sodium pyruvate (0.33 mmol/L), glucose (5.6 mmol/L), penicillin G (0.0375 g/L) and streptomycin(0.025 g/L).

1.3. Vitrification

The physical definition of vitrification is the solidification of a solution (water is rapidly cooled and formed into a glassy, vitrified state from the liquid phase) at low temperature, not by ice crystallization but by extreme elevation in viscosity during cooling [25]. This method combines the use of concentrated solutions with rapid cooling in order to avoid ice formation. The samples reach low temperature in a glassy state which has the molecular structure of a viscous liquid and is not crystalline.

Today, human pronuclear zygotes can be cryopreserved successfully by vitrification [13–15,26]. The efficacy of a rapid freezing method using the electron microscope copper grid or the Flexipet denuding pipette (FDP) for human PN embryos has already been reported [13,14].

With respect to survival, cleavage on Day 2, and blastocyst formation, a high survival and cleavage rate of multi-pronuclear zygotes was also documented. Liebermann and Tucker [26], using 5.5 M EG, 1.0 M sucrose, and an FDP 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.

More recently, successful pregnancies after vitrification of human zygotes have been reported [15,27]. It is stated that the pronuclear stage is well able to withstand the vitrification and warming conditions. Probably, this might be 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. Finally, the low toxicity of EG, together with the good survival, cleavage, blastocyst 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.

2. Comparison of the different cryopreservation techniques

Because of the low water permeability and a low surface to volume ratio of the two pronucleate zygotes, a slow cooling rate may be advantageous. At slow cooling rates the compositional changes in the intracellular solution can follow those in the extracellular solution. Intracellular freezing is avoided because the water content of the zygote has approached the equilibrium water content before reaching the homogenous nucleation temperature. On the other hand, the slow freezing method requires expensive equipment and is time consuming.

With the ultra-rapid freezing method the need for a computer controlled freezing apparatus is avoided and the time required for freezing and thawing is greatly reduced. However, the extreme toxicity of the high concentration of the cryoprotectant solution is the main disadvantage of this method. Van den Abbel et al. [28] compared a slow controlled rate freezing procedure with a rapid cooling procedure using one-cell human embryo. They showed that slow controlled rate freezing is more efficient than rapid cooling.

Vitrification can be an alternative to the conventional slow freezing protocol with advantages of the lack of the ice crystal formation and ease of operation. The method also has the advantage of taling only a few seconds to cool embryos. Furthermore, it does not require a controlled rate cooling apparatus. However, Uechi et al. [29] by comparing the conventional slow controlled rate freezing and vitrifcation 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.

To date, vitrification as a cryopreservation method has had very little practical impact on human-assisted reproduction. This may be due to the wide variety of different carriers and vessels that have been used for vitrification. Second, many different vitrification solutions have been formulated, which has not helped to focus efforts on perfecting a single approach. On the other hand, the reports of successfully completed pregnancies following vitrification at all pre-implantation stages is encouraging for further research and clinical implementation.

3. Assessment of embryo survival

Since the only criteria to evaluate whether the zygotes survived the freezing /thawing procedure is if they retain their pre-freeze morphology (e.g. PNs existence, have no obvious damage to the zona pellucida and oolemma, if their cytoplasm is clear and re-expands to its original volume after rehydration etc.), thus only if they cleaved in culture after 16–24 h, they are appropriate for intrauterine transfer.

4. Preparation of transfer cycles

The success of frozen embryo transfer requires synchronization of the endometrium to enable it to receive embryos which have arisen from a different menstrual cycle. Transfer

of frozen thawed embryos may take place in a natural cycle or alternatively in a programmed cycle with comparable pregnancy rates of 15–20%, respectively [30].

In a natural cycle the patients are monitored for the onset of endogenous luteinizing hormone (LH) surge (day 14) and the transfer of the embryo is performed on Day 17. Alternatively, in programmed cycles, the endometrium is exogenously stimulated with sequential estrogen and progesterone following down-regulation of the hypophysis with a gonadotrophin releasing hormone agonist (GnRHa) to prevent premature luteinization after pituitary down-regulation the patient is given an estrogen preparation from cycle day one onwards to mimic the proliferative phase. Estrogen may be administered as an oral preparation, skin patches, vaginal preparation or as subcutaneous implants [31,32]. This is followed by concomitant administration of progesterone to imitate the luteal phase. Progesterone may be administered as i.m. progesterone in oil injections or as tablets given orally or vaginally.

Lassale et al. [33] claimed that GnRH agonist therapy adversely affects oocyte quality and freezing outcome, but this could not be confirmed by others [34,35]. However, recent studies showed that suppression with GnRH agonist for endometrial preparation is not necessary as pregnancy and implantation rates are similar with or without GnRH a down-regulation. Also, the procedure is simpler, less expensive and more convenient to the patient if performed without GnRH agonist [36,37].

Embryo transfer of frozen thawed zygotes is performed after a twenty-four hour period of culture at cleavage stage. Up to three cleaving embryos are transferred, according to the German Embryo Protection Law.

Before transfer, attention should be paid to the degree of fragmentation and the regularity of blastomeres, each embryo being graded as 1, 2 or 3 (modified grading according to Veeck, 1991) [38]. The grade of each embryo is multiplied by the number of blastomeres, to produce a quality score. The total score of all embryos transferred is accepted as the cumulative embryo score (CES) [39]. It is important to clarify that this is the scoring system we follow, as there are various ways to score the cleaved embryos.

Clinical pregnancies are defined by the presence of positive fetal heartbeats. In these cases, the administration of progesterone is continued up to week 12 of gestation.

5. The effect of stimulation protocol

It is known that ovarian stimulation protocols used in collection cycles may possibly be involved in the success of cryopreservation [40–42]. Furthermore, concerns have been raised recently about the possible impact of GnRH-antagonists on the quality of oocytes, embryo development and implantation [43]. Although these questions are mainly related to fresh cycles, the quality of oocytes may also affect the outcome of freeze-thaw cycles. Consequently, these concerns raise additional interest in the cryopreservation outcome of embryos or oocytes.

Nikolettos et al. [44] in a retrospective study, compared the cryopreservation outcome of 2PN zygotes obtained by cetrorelix and triptorelin depot. They reported 3.26% implantation rate for the cetrorelix group and 3.73% for the triptorelin group, as well as pregnancy rates of 8.33 and 10.25%, respectively. They concluded that there was no negative effect of cetrorelix on viability, implantation potential or pregnancy outcome.

Kol et al. [45] analysed the outcome of freeze-thaw cycles with oocytes obtained with the use of six different doses of ganirelix, in a multiple dose schedule. Even though there was a negative effect of too high doses of ganirelix on implantation in the fresh cycle, there was a good pregnancy rate in subsequent freeze-thaw cycles. They concluded that high dosages of ganirelix in the collecting cycles do not adversely affect the potential of embryos to establish clinical pregnancy in freeze-thaw cycles.

In another retrospective study conducted by Byron et al. [46] they evaluated the outcome of frozen-thaw cycles with oocytes obtained either during a multiple dose protocol of cetrorelix, or after the use of a gonadotrophin-releasing hormone (GnRH) agonist. A total of 101 subfertile couples were included. These couples had a total of 222 transfers of frozen-thawed pronuclear zygotes after IVF/intracytoplasmic sperm injection (ICSI) treatment. According to the stimulation protocol during various cycles, four groups were established:

1. cetrorelix/recombinant FSH (recFSH) (69 cycles);
2. cetrorelix/human menopausal gonadotrophin (HMG) (10 cycles);
3. GnRH-agonist/recFSH (71 cycles); and
4. GnRH-agonist/HMG (72 cycles).

The transfer cycles were mildly stimulated with transdermal estradiol. No statistically significant difference was seen among the four groups regarding post-thaw survival rate, cumulative embryo score, implantation rate and pregnancies.

From all the previous studies, it could be concluded that frozen-thawed pronuclear zygotes obtained with the use of GnRH antagonists give satisfactory implantation and pregnancy rates, similar to those obtained with a GnRH-agonist. These results do not depend on the gonadotrophins (HMG or recFSH) used in the collecting cycle.

6. Is there a difference between in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI)?

Only few studies investigated the effect of cryopreservation on human embryos with perforated zonae. Al-Hasani et al. compared cryopreservation of pronuclear stage human zygotes obtained either after classical in vitro fertilization or

after intracytoplasmic sperm injection [47]. After ICSI or IVF, three fertilized oocytes from each patient were kept in culture for a further 24 h before embryo transfer. The surplus zygotes were cryopreserved using the 'open freezing system' and 1,2-propanediol and sucrose as cryoprotectants. A cohort of 817 and 1626 zygotes in pronuclear stage were frozen after IVF and ICSI, respectively. Of these, 333 and 744 zygotes have been thawed, of which 78 and 76.5% were morphologically intact zygotes after IVF and ICSI respectively. From the 204 (ICSI) and 89 (IVF) zygote transfers performed, 34 (17%) and 18 (20%) pregnancies were established. Both groups showed a similar abortion rate of approximately 20%. They concluded that pronuclear stage zygotes resulting from ICSI can be successfully frozen/thawed and the survival and pregnancy rates achieved are comparable to those for zygotes obtained after IVF.

Aytoz et al. [48] compared the obstetric outcome and the pregnancies after the transfer of fresh and cryopreserved embryos obtained by ICSI. They found a lower implantation rate in the frozen ICSI group, indicating a decreased vitality of the cryopreserved ICSI embryos. Furthermore, they reported a higher miscarriage rate in the frozen ICSI group.

7. Conclusions

Cryopreservation of human embryos has been introduced into clinical IVF in order to preserve supernumerary embryos for a later transfer. Human embryos at different developmental stages have been frozen with variable success rates. The pronuclear stage appears to be the optimal stage for cryopreservation.

Several protocols of freezing have been formulated for cryopreservation of human pronuclear zygotes among which the conventional (slow) freezing has been the most widely used method of storage up to the present.

Other methods for freezing of the pronuclear zygotes have been postulated like ultra-rapid freezing technique and vitrification.

The refinement of vitrification in the last few years leads this technique to be the method of choice for cryopreservation of human zygotes and all stages of embryonic development. This conclusion can be drawn because of the high success rates after thawing in these different stages as well as the simplicity of the procedure and the economic advantages.

For future research it will be important to establish a cryopreservation regimen which allows survival not only for the zygotes but also of the associated somatic cell components of the tissues.

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