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Ultrarapid Freezing (Vitrification) of Human Embryos : A Preliminary Report

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ABSTRACT

Objective To report a simple and economical ultrarapid freezing technique, the so-called vitrification technique, on cryopreservation of cleavage stage human embryos, and to report the first successful pregnancy resulted from this promising technique.

Design Retrospective study.

Setting University hospital.

Subjects Eleven infertile patients with 44 surplus embryos of 2- to 8-cell stage. All embryos were cryopreserved using the vitrification protocol which has been tested previously. The medium was consisted of 5.6 M dimethyl-sulphoxide (DMSO), 0.25 M sucrose and 20% fetal calf serum in phosphate-buffered saline. Two to three minute equilibration of the embryos with the cryoprotectant was carried out at 4 °C. After ultrarapid freezing and thawing, the embryos were morphologically evaluated and selectively transferred into a natural ovulatory cycle with the average number of 2.4 embryos per patient.

Main outcome measures Cryosurvival rates and pregnancy outcome.

Results Seventy percent (31/44) of the frozen-thawed embryos did not degenerate. More than eighty percent (26/31) of these survived embryos with at least 50% intact blastomeres were transferred to 11 patients. Two patients became pregnant, one biochemically and another gave birth to a healthy fullterm baby in January 1995.

Conclusion This is the first series showing that cryopreservation of human embryos by vitrification technique resulted in a successful pregnancy. This simple and easy-to-do ultrarapid freezing technique seems to be more practical than the conventional slow freezing protocol.

Key words : ultrarapid freezing, vitrification

Cryopreservation of human embryos has become a useful assisted reproductive technique. Not only does it increase cumulative pregnancy rates, but also substantially reduce the risk of ovarian hyperstimulation syndrome.[1] Conventionally, the slow cooling protocols require a high-cost programmable freezer and consume at least 2-3 hours.[2] Therefore, a number of rapid cooling protocols have been developed.[3,4] The rapid freezing protocols allow embryos to be equilibrated with specific cryoprotectants for only a few minutes and then plunged directly into liquid nitrogen from temperatures of O°C or above without an aid of any sophisticated machine.[5]

Rapid cooling methods usually require the presence of higher concentrations of cryoprotectants than the slow cooling procedures and have been used successfully to preserve mammalian embryos ranging from the pronuclear to blastocyst stages of development.[6] Among these rapid or ultrarapid cooling methods, vitrification technique has been extensively studied only just recently.[4,7] Basically, vitrification is defined as a physical process by which a highly concentrated solution of cryoprotectants solidifies during cooling without the formation of ice crystals. The solid, called "glass", retains the normal molecular and ionic distribution of the liquid state and can be considered to be an extremely viscous supercooled liquid. Vitrification has certain advantages over freezing because it avoids the damage caused by intracellular ice formation and the osmotic effects caused by extracellular ice formation. The theories behind vitrification as a method for cryopreservation have been described by several workers.[8-11]

Vitrification has been used for mammalian embryos for nearly one decade, but the results were initially very variable, due mainly to cryoprotectant toxicity. Not until the vitrification protocols had been modified, i.e. shorter exposure times and at lower temperatures of equilibration, that constant and successful results were obtained.[7] Recently, a number of very successful vitrification of mouse,[12,13] rabbit,[14,15] sheep[16] and bovine[17] embryos have also been reported. For vitrification of human embryos, a feasibility test has been, for the first time, carried out by our group and very promising results have just been reported.[18] This study is, therefore, a continuing phase of that preliminary report. As a result, a successful pregnancy completed in a healthy fullterm female baby, born in January 1995, was obtained from this innovative assisted reproductive technology.

Materials and Methods

Human embryos

This study was carried out at our centre during the period of 18 months, from January 1993 to June 1994. The protocol was approved by the Departmental Ethics Committee and every patient gave informed consent. From eleven patients with tubal infertility who were scheduled for in vitro fertilization, 44 surplus human embryos of 2 - to 8-cell stage were cryopreserved using the vitrification technique described hereafter. These embryos were in excess at the moment of intrauterine transfer following our routine in vitro fertilization programme.

Cooling procedure

The cooling protocol was nearly the same as that reported previously. Briefly, the cryoprotective solution contained 5.6 M dimethylsulphoxide (DMSO) and 0.25 M sucrose in Dulbecco's phosphate buffered saline (PBS) with 20% fetal calf serum (FCS). The surplus embryos were transferred from growth medium (AO-medium, unpublished data) into the vitrification medium described above (Fig.1), at 4°C, and drawn into a

0.25 ml clear plastic straw. After 2-3 minutes of equilibration, the straw was heat-sealed and quickly plunged into liquid nitrogen.

Warming procedure

The warming protocol was exactly the same as that reported previously. Briefly, the straws were rapidly removed from liquid nitrogen after a storage period of 1-14 months and warmed very rapidly in a waterbath at 37°C for 3-5 seconds. The embryos were then gently transferred into PBS/FCS solution containing 0.5 M sucrose at room temperature for 5 minutes. Thereafter, the embryos were transferred into 0.25 M sucrose in PBS/FCS solution at room temperature for another 5 minutes, before being washed in sucrose-free PBS/FCS solution for the last 5 minutes. After the final wash, the embryos were incubated in growth medium, at 37°C, in an atmosphere of 5% CO_2 in air.

Morphological evaluation

Two to four hours following the post-thaw

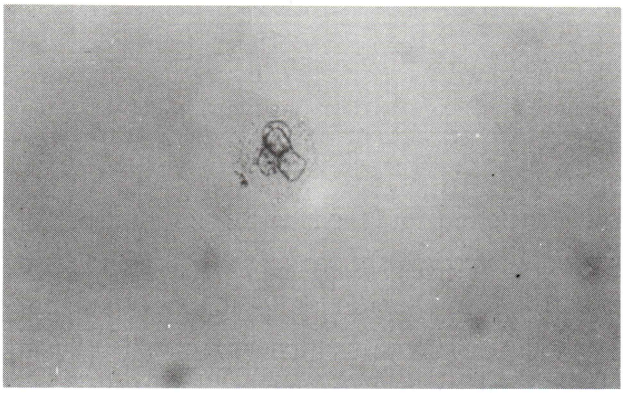

Fig. 1. A 4-cell human embryo showing shrunken cytoplasm following equilibration with vitrification medium at 4°C for 2-3 min (200x).

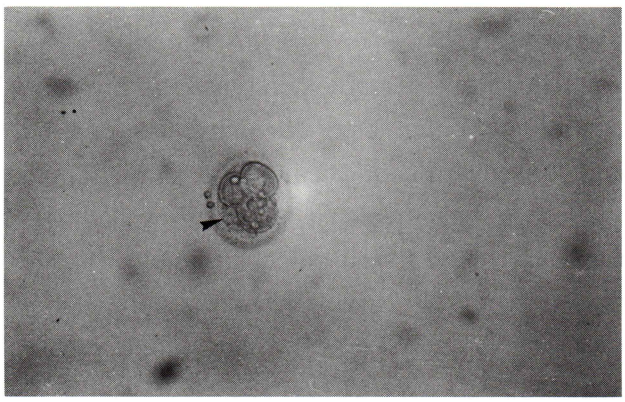

Fig. 3. A frozen-thawed 4-cell embryo with 75%-intact blastomeres. One degenerated blastomere (Arrow-head) is shown while the remainders are still intact (200x).

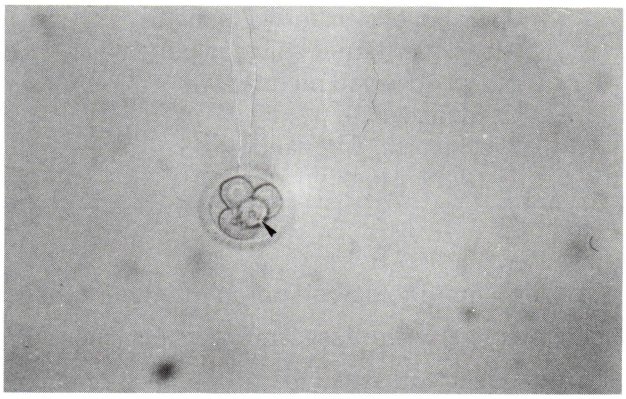

Fig. 2. A frozen-thawed 4-cell embryo with 100%-intact blastomeres. All normal-looking blastomeres are shown. A slightly out-of-focus blastomere is noted (200x).

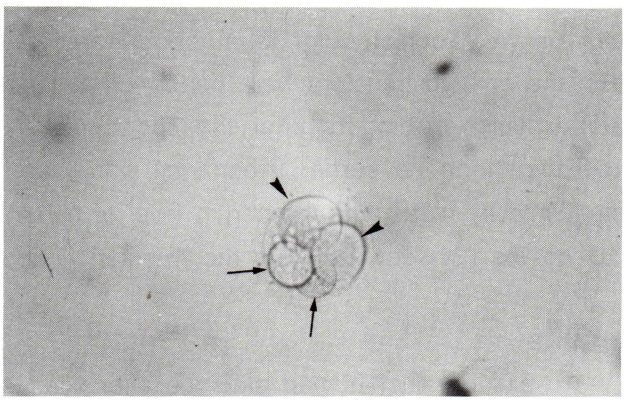

Fig. 4. A frozen-thawed 4-cell embryo with 50%-intact blastomeres. Two degenerative blastomeres showing swelling cytoplasm (Arrow-heads) are clearly seen close to the other two intact blastomeres (Arrows) 200x.

Table 1. Cryosurvival of human embryos vitrified in 5.6 M DMSO and 0.25 M sucrose

	No. of frozen-thawed embryos	%
Degenerated	13 / 44	30
Cryosurvived	31 / 44	70
100% intact blastomeres (Fig. 2)	21 / 31	68
75% intact blastomeres (Fig. 3)	3 / 31	10
50% intact blastomeres (Fig. 4)	2 / 31	6
25% intact blastomeres	5 / 31	16

Table 2. Pregnancy outcome after vitrification of human embryos

No. of patients transfer	11
Total no. of transferred embryos	26
Mean transferred embryos per patient	2.4
No. of achieved pregnancies	2
Pregnancy rate (%)	18
Biochemical pregnancy	1
Delivery (live birth)	1

incubation, morphological parameters of these vitrified embryos were assessed. Although most of the criteria used to assess the appearance of each embryo are subjective in nature, there is substantial evidence that only the normal morphological embryos with the majority of blastomeres intact and with identical blastomere size will implant. Thus, only the "well-looking" frozen-thawed embryos were selected for intrauterine transfer. All of the embryo transfers were performed in the natural cycles, 72 hours after the spontaneous luteinizing hormone surge. Pregnancy was then determined by measuring the serum level of beta-hCG on the 12th day following the transfer.

Results

The survival of human embryos after vitrification are shown in Table 1. Of 44 frozen-thawed embryos, thirty-one (70%) survived, with at least one-fourth of the blastomeres remaining intact. More than eighty percent (26/31) of these survivors, showing at least 50% intact blastomeres, were transferred to 11 patients (mean number of transferred embryos per patient : 2.4, Table 2). Two patients became pregnant (pregnancy rate per transfer : 18%), one unfortunately ended up with biochemical pregnancy while the remainder achieved her 39 weeks' pregnancy and gave delivery to a female baby of 3300 g birthweight in January 1995.

Discussion

The present study confirms our previous feasibility test that high survival rates can be achieved with the vitrification of early human embryos.[18] Furthermore, in the present study we have shown that a high percentage (70%) of the vitrified embryos survived, and a number of these embryos could develop into normal healthy babies.

Only recently have ultrarapid freezing methods been studied and used to cryopreserve mammalian embryos with successful results.[19,20] Initially, the concentrations of the cryoprotective

solutions were low, varying from 2.5 M,[21] 3.0 M,[22] 3.5 M[23] up to 4.5 M[6] ; all of which are not high enough for vitrification to occur. This may explain why the results of ultrarapid freezing in the past were not so satisfactory, since rapid ice-formation, either intracellularly or extracellularly, has enormous detrimental effects, both mechanically and biochemically, to the frozen-thawed cells.[24] In addition, it has also been reported that, chromosomal abnormalities of the rapidly frozen-thawed embryos may be associated with the concentration of cryoprotectant of less than 4.5 M DMSO.[25] Hence, the concentration of the cryo-protectant used in this study was 5.6 M DMSO which was previously tested to be true vitrification medium (data not shown) and also gave satisfactory results in our preliminary report.[18]

In addition to DMSO, 0.25 M sucrose which is an extracellular cryoprotectant was also added to our vitrification media in order to shrink the cell osmotically, thus preventing intracellular ice formation.[26] According to equilibration time, it has been recently demonstrated that a 3- to 5-minute period is required for the 2-4 cell embryos, and 1-2 minutes for those one- and eight-cell stages.[27] Therefore, we have chosen the 2-to 3-minute equilibration time for our vitrification protocol. Since cryoprotectant toxicity definitely occurs at higher temperature and it is required that higher concentration be needed during vitrification, equilibration of the embryos at low temperatures (0°C-4°C) is recommended to avoid such toxicity.[10] This is also the case in our study.

Compared to those of conventional slow freezing methods,[28-30] no difference in the successful results of the ultrarapid freezing of human embryos have been shown in a few recent reports.[31,32] While we recognize the limitations of the present study in comparing the viability of frozen embryos with non-frozen embryos or the viability obtained by other methods, we consider that our vitrification technique is likely to be at least as effective as any other method of cryopreservation and considerably simpler and less expensive. We believe that this ultrarapid freezing technique can be further improved and represents a very interesting alternative for some centres with limited resources in Thailand.

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