

CRYOPRESERVATION OF HUMAN EMBRYOS USING A SHORTER COOLING PROGRAM / ONE STEP REMOVAL OF CRYOPROTECTANT DURING THAWING

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ABSTRACT

A simplified cryopreservation technique in which there is a shorter cooling program and one step method of removing the cryoprotectant has been successfully employed in the cattle industry. A modification of this procedure was attempted using human embryos. Normally human cryopreservation using the cryoprotectant 1,2 propanediol has been most successful when the freezing occurs at the zygote or early embryo stage. In contrast, with cattle this simpler technique is most effective in cryopreservation of late morula to early blastocyst embryos. The results showed that 59% of even 4 cell human embryos had at least 2 cells appearing intact after thawing as did an average of 60% of 2, 3, 5 and 6 cell embryos ($p < .003$, Chi-square analysis). A total of 14 viable pregnancies resulted from 167 transfers of cryopreserved embryos (8.4% per transfer). Since frequently poor quality embryos were cryopreserved (the better embryos are transferred on the retrieval cycle) it is difficult to compare pregnancy rates with programs that may freeze the better embryos and transfer in another cycle.

INTRODUCTION

Several babies have been born following the intrauterine placement of cryopreserved-thawed embryos (Trounson et al, 1983; Cohen et al, 1985; Testart et al, 1986). Most successful human programs are reported after the use of 1,2 propanediol (PROH) as a cryoprotectant (Lassalle et al, 1985); zygotes/early cleaved embryos are preferred for cryopreservation (Cohen et al, 1988; Fehilly et al, 1985). A method with a shorter cooling program and a single step removal of cryoprotectant during thawing has been used successfully for cryopreservation of bovine late morula to early blastocyst embryos (Leibo, 1984). The present study was designed to see if this technique could be successfully modified for cryopreservation of human embryos.

MATERIALS/METHODS

Cryopreservation: 1.5 M 1,2 propanediol in Dulbecco's PBS was used with 3mg/ml bovine serum albumin (BSA). The cryoprotectant removal solution was 1.08 M sucrose in PBS with 3mg/ml BSA; the Buffer solution was PB1: PBS with 3mg/ml BSA. Two and 3 day embryos are transferred to PB1 to equilibrate

in the phosphate buffered media for 5 to 10 minutes. They were transferred to cryoprotectant solution for 12-20 minutes. Each embryo was loaded into a 1/4 freezing straw followed by an air bubble. The rest of the straw was filled with the sucrose solution. The straws were suspended in the bath of a Bio Cool Ethanol bath rate controlled freezer set at -6.0°C . The straws were seeded above/below the embryo column. Ice crystals grew into the embryo column. After 15 minutes the freezing program was initiated. The straws, cooled at $-0.4^{\circ}\text{C}/\text{minute}$ to -35°C , held at this temperature for 15 minutes, then plunged into liquid nitrogen.

Thawing: Each straw was thawed at room temperature for 2 minutes. The straw was shaken like a fever thermometer to mix the sucrose with the embryo. The straw was incubated at 37°C for 3 minutes, and cooled in room temperature water for 1 to 2 minutes and the embryo was expelled from the straw. It was transferred to PB1 for 10 minutes then placed into culture media for 2-18 h before transferring to the patient.

Ovarian Stimulation: Anovulatory patients were stimulated with clomiphene citrate or hMG and given hCG (10,000 IU) 70 h before embryo transfer. Ovulatory patients had the embryo transfer on the fourth day of the progesterone rise of a natural cycle.

RESULTS

There were 124 of 210 (59%) 4 cell embryos that had at least 2 cells appear intact after thawing. Similarly, 81 of 133 (61%) of 2, 3, 5, and 6 cell embryos survived. Interestingly, 188 of 320 (59%) 2, 4, 5, and 6 cell embryos with blastomeres of uneven size and little or no fragmentation also demonstrated preservation of at least 50% of their blastomeres. The only difference in survival was found comparing even vs uneven 3 cell embryos where only 6 of 21 (29%) of uneven ones survived compared to 17 of 23 (74%) of even 3 cell embryos ($p < 0.003$ using Chi-square analysis). There were 14 clinical pregnancies with fetal viability demonstrated by sonography out of 167 transfers of frozen embryos (8.4% per transfer). During the same 2-year time period there were 88 viable pregnancies from 640 transfers of non-cryopreserved embryos (14% per transfer).

DISCUSSION

Typical methodology for cryopreservation requires ice nucleation induction at -5°C to -7°C and the embryos are cooled at $-0.3^{\circ}\text{C}/\text{min}$ down to -30°C or as low as -80°C . The step wise addition and removal of cryoprotectant is accomplished by transferring the embryo into increasing and decreasing concentrations of cryoprotectant. 0.1m sucrose is often added to the cryoprotectant solution and 0.2 mm sucrose is used after thawing to aid in the cryoprotectant removal. With the present method, there is a slow cooling program which starts at -6°C and cools at $-0.4^{\circ}\text{C}/\text{min}$ and thus takes less time than

other methods. The entire freezing process including the addition of cryoprotectant is accomplished in 2 h. Upon thawing the cryoprotectant is removed from the embryo in one step by the addition of 1.08 mM sucrose. The thawing process including the removal of cryoprotectant is accomplished in 6 minutes.

The conclusion from this larger series was slightly different from previously presented data (Baker et al, 1990). Only one third of 2 and 6 cell even embryos survived whereas there were no differences in the expanded data between any cell numbers except uneven 3 cell embryos. Present studies are now ongoing to randomly compare this simplified technique to more common methods within our program so that adequate comparisons can be made. The pregnancy rate per transfer of cryopreserved-thawed embryos may be biased somewhat low because sometimes only poor quality embryos were available for cryopreservation; the normal procedure is to transfer up to 5 good looking embryos during the retrieval cycle and only cryopreserve the remainder.

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