Successful Pregnancy After the Vitrification of Zygotes Using Commercial Vitrification Solutions and Conventional Straws to Protect Against Infections in Liquid Nitrogen

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Purpose: To report on successful birth after the transfer of postthawed human zygotes that were vitrified using a conventional straw for the purpose of protecting them from infections and a low-toxicity cryoprotectant that is commercially sold.

Methods: A primary infertile couple presented at our IVF program. After being checked for fertilization, the embryos were not transferred to the uterus at that cycle. Instead, all of them were cryopreserved at the 2-pronuclei stage using our original vitrification method. After the vitrification and warming of four zygotes, two embryos were transferred into the uterus.

Results: Twenty-one 2-pronuclei embryos were vitrified in liquid nitrogen. After 2 embryos were thawed and transferred, successful pregnancy was the outcome, and a healthy boy was born at term.

Conclusions: Vitrification is a simple procedure and requires less time than slow freezing. Vitrification of zygotes in a conventional straw seems to be sufficient for viability and works to store the zygotes safely.

KEY WORDS: IVF; straw; safety; vitrification; zygote.

INTRODUCTION

Since the first report of the cryopreservation of mouse embryos more than 30 years ago Whittingham *et al.* (1), the cryopreservation of human oocytes, zygotes, embryos, and blastocysts has become an integral part of infertility treatment. The first pregnancies from cryopreserved human embryos were achieved by Trounson and Mohr in 1983 (2).

On the other hand, ice-free cryopreservation of mouse embryos at -196°C was reported by Rall and Fahy in 1985 (3). Ultra-rapid freezing (vitrification)

is a process that produces a glasslike solidification of living cells that completely avoids ice crystal formation during cooling and warming. Since the first pregnancy following the vitrification of human embryos at the 2-pronuclei stage was reported in 1990 (4), subsequent studies have been undertaken because this process is simple, fast, and less expensive than conventional slow freezing. However, there is a risk of contamination with liquid nitrogen due to the use of the open container as a cryoloop, or by means of the electron microscopic grid, open pulled straw (OPS) or by other means.

In the present case, human pronuclear-stage embryos were successfully vitrified using a conventional straw in a completely closed system. After being warmed, then cultured until day 3, embryos were transferred into the uterus, resulting in a healthy pregnancy.

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MATERIALS AND METHODS

Patient

A 31-year-old woman and her 32-year-old husband presented at our in vitro fertilization (IVF) program with primary infertility for 36 months in January 2003. The patient had tubal infertility, and the husband's semen was normal except for the volume. The ejaculated volume was 0.9 mL, the sperm concentration was $186 \times 10^6/\text{mL}$, the motility was 80%, and the normal morphology of strict criteria was 8% (5).

The ovarian stimulation was performed according to a standard long protocol, with the gonadotrophin-releasing hormone analogues (GnRHa; Busrecur, Fuji, Japan) given three times daily, and human menopausal gonadotropin (HMG; Nikken, Japan) given in a total of 11 ampules, each containing 150 IU of HMG, on the 7th to the 14th cycle day. When the leading follicle had reached a mean diameter of 17 mm on the 16th cycle day, 5000 IU of human chorionic gonadotropin (HCG; Profasi, Serono, Switzerland) was administered. Oval pickup was performed 35 h after the HCG injection. Twenty-eight oocytes were inseminated by both conventional IVF (23 oocytes) and intracytoplasmic sperm injection (ICSI, 5 oocytes), while 21 were fertilized after insemination (19 from conventional IVF and 2 from ICSI). The embryos were not transferred to the uterus in that cycle in order to prevent ovarian hyperstimulation syndrome (OHSS), and all of them were cryopreserved at the 2-pronuclei stage using our original vitrification method.

In the next cycle, the patient was treated for the transfer of cryopreserved embryos by hormone replacement therapy (HRT) with patched estrogen (Estraderm; Nihon Chibagaigi, Japan) at a dose of 2 sheets every other day, for a total of 10 sheets from the 5th to the 14th cycle day. On the 13th cycle day, 4 vitrified zygotes were thawed and cultured for 2 days, and then 2 embryos at the 4- and 5-cell stage were transferred into the patient's uterus on the 15th day (day 3 embryo transfer (ET)). For luteal phase support, the patient was given progesterone by injection (Progeston; Huji, Japan) at a dose of 125 mg every 4 days until the 23rd cycle day. Additionally, patched estrogen (Estraderm) was administered at 2 sheets every other day and progesterone was given internally (Progeston; Huji, Japan) at 30 mg a day until 7 weeks of gestation.

Freezing and Thawing Procedure

Our protocol for vitrification used a new two-step method that was minor modified protocol recommended by KITAZATO Supply (Japan) (6). Two zygotes were vitrified and warmed by using Vitrification Media VT101 and VT102 (KITAZATO Supply, Japan).

The zygotes were equilibrated in a drop of equilibration solution (ES: VT-101) containing 7.5% ethylene glycol (EG) and 7.5% dimethyl sulfoxide (DMSO) and washed in fresh ES several times. During the equilibration process, the 0.25 mL plastic straw (IMV, France) was prepared as follows. Into the straw was drawn thawing solution (TS: VT-102) containing 1.0 M sucrose to about 1/3 the length of the straw, and two small columns of VS were drawn between a breath of air. Ten to 15 minutes later, the zygote volume returned completely, and the zygotes were then exposed to Vitrification Solution (VS: VT-101) containing 15% EG and 15% DMSO and 0.5 M sucrose and washed several times. Into the straw 5 μL VS with 2 zygotes was then drawn, followed by a breath of air and TS to the end. Immediately after heat-sealing the straw, it was placed in the vapor of liquid nitrogen for 3 min, and then plunged into the liquid nitrogen. Thus, zygotes were in the third of three VS columns separated on both sides by a breath of air and by columns of TS. During the procedure, zygotes were exposed to VS for just 1 min.

In the thawing process, the plastic straw was taken out from liquid nitrogen and kept at room temperature for 10 seconds and put into water at 37°C. After the one-step dilution of the cryoprotectant was carried out using TS for 1 min (7), zygotes were placed in the diluent solution (DS: VT-102) containing 0.5 M sucrose for 3 min, the washing solution-1 (WS-1: VT-102) containing TCM-199 for 5 min, and WS-2 (VT-102) for 5 min. Zygotes were cultured in the Blastocyst Assist System Medium-1 (Medicult, Denmark) for another 2 days until day 3. In this case, two straws with two zygotes were warmed.

RESULTS

Four zygotes survived after the warming, and all of them cleaved during the 2 days of culture. Four- and 5-cell stage embryos were transferred into the patient's uterus. A singleton pregnancy was the outcome at 6 weeks gestation with a visible heart beat on ultrasonographic tomography. The patient delivered a healthy boy in the 40th week of gestation.

DISCUSSION

In this study, we vitrified the human zygote in a completely closed container. There are many reports regarding vitrification methods that involve the alternative conventional slow freezing method (8–11).

For successful vitrification and warming, it is important to prevent intracellular ice formation and to expose the zygotes for a short time to cryoprotectant solution that is not too highly concentrated, and to cool/warm rapidly.

It is more important, however, to store patients' embryos safely. It has been reported that sealed cryovials or straws are free from viral contamination, but that cryovials and straws that are not sealed, as well as OPS, have a risk of contamination (12). Liquid nitrogen freezers are not sterile, and both the liquid and vapor phases are potential sources of microbial contamination (13).

To our knowledge, the first successful pregnancy after the vitrification of human embryos was reported in 1990 (4), and in Japan, the first pregnancy after the vitrification of human cleaved embryos was reported in 1996 (14). Recently, pregnancy after the vitrification of human zygotes using OPS was reported in 2002 (15). Gordts *et al.* used 2.5M DMSO as a cryoprotectant. According to the levels of toxicity of permeating cryoprotective agents that were demonstrated by Mukaida *et al.* (16), ethylene glycol was likely the least toxic agent in spite of its high concentration (40%), while DMSO was the fourth of five agents in terms of toxicity (1,2-propanediol, DMSO, Glycerol, ethylene glycol, acetamide). The VS (VT-101) that we used in this study contained 15% DMSO and 15% EG. The use of small quantities of both DMSO and EG allowed it to have a lower toxicity and higher availability. It is well known that when two types of cryoprotective agents are combined they have less toxicity than a single type alone (17). There is a risk of contamination using OPS. Jelinkova *et al.* used OPS that included zygotes that were drawn into a second holding straw, but which previously had been openly plunged once into liquid nitrogen.

The present case report demonstrated that the conventional plastic straw is sufficient for the safe vitrification of human 2PN-stage embryos using low toxicity cryoprotectant that is commercially sold.

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