Article

Vitrification of human 8-cell embryos, a modified protocol for better pregnancy rates

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Abstract

Human day 3 embryos were vitrified in modified ethylene glycol-based protocol. The present study reports a post-thaw survival rate of 95.3%, which was significantly higher than that of the conventional slow-freezing protocol (60.0%, $P < 0.05$). The implantation and pregnancy rates were also higher (14.9% and 35.0%) than that in the slow-freezing protocol (4.2% and 17.4%). On average, three embryos were transferred per patient. Out of the 40 transfer cycles, five deliveries and nine ongoing pregnancies were achieved. Four ended with delivery of a singleton and one a miscarriage. Ethylene glycol is a good cryoprotectant to preserve human 8-cell embryos because of its low toxicity as shown by the high survival rate, and vitrification is a promising alternate to the conventional slow-freezing method.

Keywords: *pregnancy outcome, rehydration, survival rate, vitrification*

Introduction

Cryopreservation of surplus human embryos by a slow-freezing protocol has been a widely established procedure and an integral part of every human IVF clinic. Vitrification is now regarded as a potential alternative to the conventional slow-freezing method. It has the advantage of preventing ice crystal formation by a short exposure to high concentrations of cryoprotectant with low water content, and eliminating the use of expensive equipment (Rall and Fahy, 1985; Scheffen *et al*. 1986; Trounson *et al*., 1987). The main drawback of this method is the exposure of the embryos to a high concentration of cryoprotectant which may have a detrimental effect (Fahy *et al*., 1984). This can be minimized by allowing a very short exposure of the embryos to the cryoprotectants i.e. 30–40 s (Kasai *et al*., 1990; Shaw *et al*., 1992), combination of one or more cryoprotectant (Kuleshova *et al*., 1999), and using a cryoprotectant which has a lesser toxic effect on the embryos.

Ethylene glycol has been widely used as a cryoprotectant in both slow freezing and vitrification methods due to its high permeation ability (Newton *et al*. 1998) and low toxicity compared with other cryoprotectants (Emiliani *et al*., 2000; Kasai and Mukaida, 2004). This study reports successful pregnancies using a modified protocol of vitrification with an ethylene glycol-based freezing solution.

Materials and methods

The vitrification protocol was explained to all couples and they signed the required consent form, approved by the institutional ethics committee. A total of 436 day 3 embryos, with an average of four embryos in each vial, were vitrified in an ethylene glycol-based solution. Gamete (Vitrolife, Gothenburg, Sweden) was used as the base media for all freezing and thawing solutions (Gamete is a bicarbonate HEPES-buffered medium containing human serum albumin).

Patients

In this prospective study, which was conducted between May 2004 and June 2005 (13 months), the excess embryos after embryo transfer of 164 patients, were randomly divided and cryopreserved using two protocols i.e. vitrification and slow freezing. The method used was a modified version of the vitrification protocol reported by Danasouri and Selman (2001). The slow-freezing method was a modified version of that of Testart *et al.* (1986) using a commercial kit (Vitrolife).

Vitrification procedure

After one wash in HEPES-buffered medium, embryos were placed in 10% ethylene glycol solution (v/v; Sigma Aldrich, St Louis, MO, USA) using a Pasteur pipette, and incubated for 5 min at 37°C. Embryos were then transferred to 40% ethylene glycol in 0.6 mol/l sucrose (Sigma Aldrich) solution for 30 s. The embryos were then immediately loaded onto a nylon loop (made in the Biomechanical Department, Krishna IVF Clinic) with a thin film of vitrification solution (3–5 μl), and directly plunged into a cryovial (also made in the Biomechanical Department, Krishna IVF Clinic) containing liquid nitrogen. These cryovials were closed tightly and placed in liquid nitrogen storage tanks. Embryos were stored for 6 months.

Thawing procedure

For frozen–thawed embryo transfer, patients were subjected to a controlled cycle using gonadotrophin-releasing hormone (GnRH) analogue (Lupride 4; manufactured by Sun Pharmaceuticals India Ltd., Mumbai, India) and oestrogen replacement (Progynova; manufactured by Cadila Healthcare Ltd., Ahmedabad, Gujarat, India). Vitrified embryos were then thawed by placing the cryoloop directly into 1 mol/l sucrose solution for 2.5 min at 37°C. Embryos were then transferred through different concentrations of sucrose solution i.e. 0.5 mol/l, 0.25 mol/l, and 0.125 mol/l for 2.5 min in each step at 37°C. The embryos were finally placed in G2 media (Vitrolife) for culturing in the CO_2 incubator for 3–4 h before embryo transfer. In both vitrification and freezing procedures, a temperature of 37°C was maintained using a hot plate (Isachenko and Nayudu, 1999).

Statistical analysis

Post-thaw survival and implantation rates of embryos in the vitrification and slow freezing protocols were compared by chi-squared analysis. Patient's age and the number of embryos transferred per cycle were compared using Student's *t*-test. A *P*-value of <0.05 was considered statistically significant.

Results

In both the vitrification and slow-freezing groups in 164 patients, there was no significant difference in the mean age of patients and the mean number of embryos transferred in the vitrifcation (31.30 ± 4.5 and 3.02) and slow freezing (30.20 ± 3.1 and 3.13) groups respectively (**Table 1**). The post-thaw survival rate of embryos in the vitrification group (95.3%, $P < 0.05$) was significantly higher than for embryos in the slow-freezing group (60.0%). The implantation rate of 14.9% ($P < 0.05$) in the vitrification group was significantly higher than the rate of 4.2% in the slow freezing, and the number of clinical pregnancies was higher in the vitirifcation group (35.0%) than in the slow-freezing group (17.4%).

Discussion

Vitrification is a process of rapid cooling of a liquid with a high concentration of cryoprotectant to achieve a glass-like solid state. This approach avoids the formation of ice crystals and it takes only a few seconds to freeze the embryos. The rapid freezing minimizes chilling injury and osmotic shock to the embryo.

Ideal characteristics of any cryoprotectant are low toxicity and high permeation ability. Ethylene glycol has proven to be a stable cryoprotectant, with less toxicity, high permeation ability (Valdez *et al.*, 1997) and a wide acceptance as a principal cryoprotectant in both slow-freezing and vitrification methods. Chi *et al.* (2002) suggested that ethylene glycol diffuses into and leaves the embryos very rapidly due to its low molecular weight; hence embryos do not undergo osmotic shock during freezing and thawing procedures, compared with other cryoprotectants (Chi *et al.*, 2002). In the present study it was also observed that the better growth progression of embryos after thawing indicates a low chemical toxicity of ethylene glycol (**Figure 1**). Further the observed embryo survival rate of 95.3% is in agreement with the result of a previous study by Kasai and Mukaida (1994) using an ethylene glycol-based solution.

Mukaida *et al.* (1998) reported a survival rate of 81% and a pregnancy rate of 5.5% using an ethylene glycol-based solution (Mukaida *et al.*, 1998). Another study by Danasouri and Selman (2001) reported a low survival rate of 79.2% but higher pregnancy rates of 30.5% (Danasouri and Selman, 2001) in comparison with Mukaida *et al.* 1998) who used an ethylene glycol-based solution. The present study reports an overall embryo survival rate of 95.3% and a pregnancy rate of 35.0% using an ethylene glycol-based modified vitrification protocol. Good pregnancy rates were achieved by the transfer of multiple embryos leading to singleton pregnancies. Due to the lower survival and implantation rates of embryos from the conventional slow-freezing protocol, the transfer of three embryos was advocated by this clinic. With the higher survival and implantation rates achieved with the present vitrification protocol, however, transfer of fewer embryos is being considered by the Krishna IVF Clinic ethics committee. This is in line with moves in many countries to limit the numbers of embryos transferred in order to avoid multiple pregnancies (De Neubourg and Gerris, 2003; Gerris, 2005).

In the present study the protocol described by Danasouri and Selman (2001) was modified by decreasing the exposure time during freezing from 10 min to 5 min in 10% ethylene glycol, and increasing the rehydration steps, i.e. 1 M, 0.5 M, 0.25 M, 0.125 M sucrose solution, with an exposure time of 2.5 min in each step, from two steps to four. The shorter exposure time during freezing (Vander Elst *et al.*, 1995; Walker *et al.*, 2004) and increase number of thawing steps may have had a role in the better survival of embryos.

Table 1. Comparison between vitrification and slow-freezing protocols in human 8-cell embryos

Parameters	*Vitrification*	*Slow freezing*
No. attempted cycles	84	80
Mean (± SD) age (years)	31.30 ± 4.5	30.20 ± 3.1
No. embryos frozen	436	420
No. embryos thawed	127	120
No. embryos surviving (%)	121 (95.3)[a]	72 (60.0)[b]
Mean no. embryos transferred	3.025 ± 0.158	3.130 ± 0.344
No. transferred cycles	40	23
No. clinical pregnancies (% per embryo transfer)	14 (35.0)	4 (17.4)
No. embryos implanted (% of surviving embryos)	18 (14.9)[a]	3 (4.2)[b]
No. deliveries	4	2
No. miscarriages	1	1
No. ongoing pregnancies	9	1

[a,b]Numbers within rows with different superscripts are significantly different ($P < 0.05$).

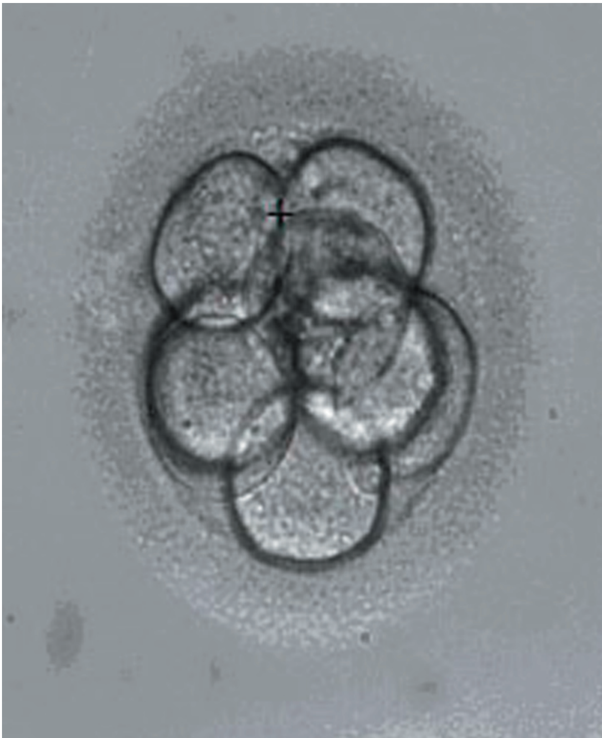

Figure 1. The vitrified human 8-cell embryo 4 h after thawing.

In the present study, it was observed that embryos exposed to 10% ethylene glycol shrank rapidly and expanded close to their initial size within 3 min. Therefore, an exposure of 5 min was thought to be sufficient, as further exposure may be detrimental to the embryos. Further, during thawing the number of changes in sucrose concentration was increased to four so as to prevent rapid movement of water into the cell and cause osmotic swelling and degeneration. These changes may have played a role in increasing the post-thaw embryo quality and survival. One aspect which needs to be focused on during the vitrification procedure is viral contamination from liquid nitrogen. Recent studies by Bielanski *et al.* (2000, 2003) reported the possible transmission of pathogens to bovine embryos vitrified and stored in liquid nitrogen (Bielanski *et al.*, 2000, 2003). However proper sealing of straws and cryovials containing embryos has been considered an effective measure against contamination during storage. An alternative preventive step described by Vajta *et al.* (1998) against contamination is liquid nitrogen filtration and the application of accessory protective storage containers (Vajta *et al.*, 1998). A straw in straw method for successful storage of vitrified blastocysts, reported by Lieberman *et al.* (2002) and Vanderzwalmen *et al.* (2003) is also an additional measure to

reduce the viral contamination from liquid nitrogen (Lieberman *et al.*, 2002; Vanderzwalmen *et al.*, 2003). Cross-contamination may be prevented by storing embryos from known infectious patients in separate liquid nitrogen tanks. Though liquid nitrogen contamination has not been reported in human embryology, preventive measures should be taken as suggested by Vajta *et al*. (1998) and further studies are required in this aspect. In conclusion, cryopreservation by a vitrification method is a simple, inexpensive and efficient method of freezing human embryos because it greatly simplifies the freezing process and eliminates ice crystal formation. Further optimization and modification of vitrification protocols may improve the survival rates, establishing vitrification as the procedure of choice for human embryo cryopreservation.

References

Bielanski A, Bergeron H, Lau PCK, Devenish J 2003 Microbial contamination of embryos and semen during long term banking in liquid nitrogen. *Cryobiology* **46**, 146–152.

Bielanski A, Nadin Davis S, Sapp T, Lutze Wallace C 2000 Viral contamination of embryos cryopreserved in liquid nitrogen. *Cryobiology* **40**, 110–116.

Chi HJ, Koo JJ, Kim MY *et al*. 2002 Cryopreservation of human embryos using ethylene glycol in controlled slow freezing. *Human Reproduction* **17**, 2146–2151.

Danasouri DVM, Selman H 2001 Successful pregnancies and deliveries after a simple vitrification protocol for day 3 human embryos. *Fertility and Sterility* **76**, 400–402.

De Neubourg D, Gerris J 2003 Single embryo transfer – state of the art. *Reproductive BioMedicine Online* **7**, 615–622.

Emiliani S, Bergh MVD, Vannin AS *et al*. 2000 Comparison of ethylene glycol, 1-2-propanediol and glycerol for cryopreservation of slow cooled mouse zygotes, 4 cell embryos and blastocysts. *Human Reproduction* **15**, 905–910.

Fahy GM, MacFarlane, DR, Angell CA, Meryman HT 1984 Vitrification as an approach to cryopreservation. *Cryobiology* **21**, 407–426.

Gerris JM 2005 Single embryo transfer and IVF/ICSI outcome: a balanced appraisal. *Human Reproduction Update* **11**, 105–121.

Isachenko EF, Nayudu PL 1999 Vitrification of mouse germinal vesicle oocytes: effect of treatment temperature and egg yolk on chromatin and spindle normality and cumulus integrity. *Human Reproduction* **14**, 400–408.

Kasai M, Mukaida T 2004 Cryopreservation of animal and human embryos by vitrification. *Reproductive BioMedicine Online* **9**, 164–170.

Kasai M, Komi JH, Takakamo A *et al*. 1990 A simple method for mouse embryo cryopreservation in a low toxicity vitrification solution, without appreciable loss of viability. *Journal of Reproduction and Fertility* **89**, 91–97.

Kuleshova LL, MacFarlane DR, Trounson AO 1999 Sugar exerts a major influence on the vitrification properties of ethylene glycol based solution and have a low toxicity to embryos and oocytes. *Cryobiology* **38**, 119–130.

Lieberman J, Tucker M, Gardner DK *et al*. 2002 Blastocyst development after vitrification of multipronuclear zygotes using the Flexipet denuding pipette. *Reproductive BioMedicine Online* **4**, 146–150.

Mukaida T, Wada S, Takahashi K *et al*.. 1998 Vitrification of human embryos based on the assessment of suitable conditions for 8-cell mouse embryos. *Human Reproduction* **13**, 2874–2879.

Newton H, Fisher J, Arnold JPR *et al*. 1998 Permeation of human ovarian tissue with cryoprotective agents in preparation of cryopreservation. *Human Reproduction* **13**, 376–380.

Rall WF, Fathy GM 1985 Ice free cryopreservation of mouse embryos at 196°C by vitrification. *Nature* **313**, 573–575.

Scheffen B, Vanderzwalmen P, Massip A 1986 A simple and efficient procedure for preservation of mouse embryos by vitrification. *Cryo Letters* **7**, 260–269.

Shaw PW, Fuller BJ, Bernard AG, Shaw RW 1992 Vitrification of mouse oocytes using short cryoprotectant exposure: effect of varying exposure times on survival. *Molecular Reproduction and Development* **33**, 210–214.

Testart J, Lassalle B, Belaisch-Allart J *et al*. 1986 High pregnancy rate after early human embryo freezing. *Fertility and Sterility* **46**, 268–272.

Trounson A, Peura A, Kirby C 1987 Ultra rapid freezing: a new low cost and effective method of embryo cryopreservation. *Fertility and Sterility* **48**, 843–849.

Vajta, G, Lewis IM, Kuwayama M *et al*. 1998 Sterile application of the open pulled straws (OPS) vitrification method. *Cryo Letters* **19**, 389–392.

Valdez CA, Abas Mazni O, Takahashi Y *et al*. 1992 Successful cryopreservation of mouse blastocysts using a new vitrification solution. *Journal of Reproduction and Fertility* **96**, 793–802.

Vander Elst JV, Abbeel EV, Steirteghem ACV 1995 The effect of equilibration temperature and time on the outcome of ultra rapid freezing of 1 cell mouse embryos. *Human Reproduction* **10**, 379–383.

Vanderzwalmen P, Bertin G, Debauche C *et al*. 2003 Vitrification of human blastocysts with the Hemi Straw carrier: application of assisted hatching after thawing. *Human Reproduction* **18**, 1504-1511.

Walker DL, Tummon IS, Hammitt DG *et al*. 2004 Vitrification versus programmable rate freezing of late stage murine embryos: a randomized comparison prior to application in clinical IVF. *Reproductive BioMedicine Online* **8**, 558–568.

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