Effect of carrier system on the yield of human oocytes and embryos as assessed by survival and developmental potential after vitrification

J. Liebermann and M. J. Tucker

Georgia Reproductive Specialists, 5445 Meridian Mark Road Suite 270,
Atlanta, Georgia, 30342, USA

The present study was undertaken to examine the effect of a cooling regimen during vitrification on survival and morphological appearance of human oocytes after warming and the developmental potential of day 3 embryos. Aged human oocytes that had failed to fertilize and human embryos derived from abnormally fertilized zygotes that showed one pronucleus or three or more pronuclei were used as models in this study. In the first part of the study, 928 aged human oocytes that had failed to fertilize were vitrified. The viability of oocytes after vitrification using the hemi-straw system was slightly higher than it was using the cryoloop after warming (85.4% (410 of 480) versus 80.6% (361 of 448)), but the difference was not significant. In the second part of the study, 266 embryos were vitrified. The survival of day 3 embryos after vitrification

was improved by using a mixture of ethylene glycol and dimethyl sulphoxide in combination with the hemi-straw system method rather than the cryoloop method (89.7% (122 of 136) versus 83.8% (109 of 130)), but the difference was again not significant. The potential for development up to compaction on day 4 in embryos vitrified using the hemi-straw system method was significantly higher (37.7%) than it was in embryos vitrified using the cryoloop (29.4%; χ^2 , $P = 0.002$). The hemi-straw system and cryoloop methods of vitrification are both successful, easy to perform, and demonstrate the ability of both carriers to vitrify different stages of development (oocytes and day 3 embryos). In addition, the success of both methods appears to be related to the rate of cooling and the lower concentration of cryoprotectant used.

Introduction

As early as 1985, ice-free cryopreservation of mouse embryos at -196°C by vitrification was reported (Rall and Fahy, 1985) as a possible alternative approach to cryostorage. Many later studies were undertaken to reduce the time of the cryopreservation procedure, and also to try to eliminate the use of expensive programmable freezing equipment. One way to achieve these goals is to use vitrification techniques and protocols that allow oocytes and embryos to be placed in the cryoprotectant and then plunged directly into liquid nitrogen. The definition of vitrification is the solidification of a solution at a low temperature without the formation of ice crystals, by increasing the viscosity, using high cooling rates from 15 000 to 24 000 $^{\circ}\text{C min}^{-1}$ (Fahy *et al.*, 1984; Martino *et al.*, 1996; Arav and Zeron, 1997; Vajta *et al.*, 1997). The key aim of any vitrification technique and protocol is to achieve high rates of cooling in association with high concentrations of cryoprotectant. However, this is problematic and technically difficult to achieve (Rall, 1987). Cryoprotective agents are essential for the cryopreservation of cells. There is a practical limit of attainable cooling speed, and a

biological limit on the concentration of cryoprotectant tolerated by cells during vitrification. A critical concentration of cryoprotectant is required for vitrification; variation in concentration can lead to either osmotic or chemical toxicity. Therefore, a balance between maximizing the cooling rate and minimizing the cryoprotective concentration is important. The high concentration of cryoprotectant used for vitrification and the known biological and physicochemical effects of cryoprotectants make the toxicity of these agents a key limiting factor in cryobiology.

The present authors have found that a high cooling rate is an important factor in improving the effectiveness of oocyte vitrification. High cooling rates allow reduction of the concentration of the cryoprotectants used and in this way reduce their toxicity. Furthermore, a reduction in the amount of cryoprotectants required decreases their toxic and osmotic effects. The volume of the vitrification solution is minimized by using special carriers during the vitrification process. These carriers include: the open pulled straw (OPS; Vajta *et al.*, 1998; Chen *et al.*, 2000a,b; Hurtt *et al.*, 2000; Oberstein *et al.*, 2001), the flexipet-denuding pipette (FDP; Liebermann *et al.*, 2002; J. Liebermann and M. J. Tucker, unpublished), micro-drops (Papis *et al.*, 2000), electron microscope copper grids (EM; Hong *et al.*, 1999; Park *et al.*, 1999; Chung *et al.*, 2000; Park *et al.*, 2000), the

E-mail: jliebermann@t-online.de

hemi-straw system (Kuwayama and Kato, 2000; Vandervorst *et al.*, 2001), nylon mesh (Matsumoto *et al.*, 2001) and the cryoloop (Lane *et al.*, 1999a,b; Oberstein *et al.*, 2001; Yeoman *et al.*, 2001; J. Liebermann and M. J. Tucker, unpublished). These carriers or vessels have all been used to achieve higher cooling rates. Vitrified mouse (Chen *et al.*, 2000a), bovine (Vajta *et al.*, 1998; Hurtt *et al.*, 2000), equine (Hurtt *et al.*, 2000) and human oocytes can survive cryopreservation by vitrification (Hunter *et al.*, 1995; Chen *et al.*, 2000b; Chung *et al.*, 2000). Good survival, cleavage and blastocyst formation rates after vitrification of human oocytes and multipronuclear zygotes have been reported (Chung *et al.*, 2000; Park *et al.*, 2000; Liebermann *et al.*, 2002), as have successful pregnancies and deliveries after using vitrification techniques and protocols for human oocytes, day 3 embryos and blastocysts (Kuleshova *et al.*, 1999; Yokota *et al.*, 2000; Yoon *et al.*, 2000; El-Danasouri and Selman, 2001; Mukaida *et al.*, 2001; Jelinkova *et al.*, 2002; Selman and El-Danasouri, 2002). Until now, direct comparison of different cooling regimens using the hemi-straw system and the cryoloop for human oocytes and embryos has not been reported.

Despite the fact that vitrification protocols for human oocytes, embryos and blastocysts are entering the mainstream of human-assisted reproductive technologies, so far vitrification as a cryopreservation method has had very little practical impact on human assisted reproduction, and oocyte cryopreservation is still considered experimental. However, reports of successfully completed pregnancies after vitrification of oocytes will encourage further research. Clearly, the lower survival rate of oocytes after cryopreservation in general, and vitrification in particular, means alternative approaches must be explored.

In the present study, aged human oocytes that had failed to fertilize and human embryos derived from abnormally fertilized zygotes that showed one pronucleus or three or more pronuclei were used as a model to determine any possible influence arising from variations in the vitrification technique and protocol, and cooling regimen in terms of survival and developmental potential. The aim of the present study was to compare the effects of vitrification using either hemi-straw system or the cryoloop as carrier on the viability of human oocytes (Expt 1) and the developmental potential of day 3 embryos up to the stage of compaction (Expt 2).

Materials and Methods

Human oocytes and embryos

The local ethics committee approved this investigation. Inseminated oocytes that had failed to fertilize ($n = 928$), and abnormally fertilized zygotes ($n = 266$) that showed one pronucleus or three or more pronuclei at the time of the fertilization check (for the presence and number of pronuclei) after standard IVF or intracytoplasmic sperm injection (ICSI) were obtained from patients who gave their

informed consent. The percentage of degeneration observed in the vitrified oocytes that represented background loss owing to the nature of the oocytes that had failed to fertilize was assessed by collecting data from July to December 2001 from 810 oocytes from IVF procedures as a control group. The rate of degeneration, the rate of atretic oocytes, and the rate of fractured zona, empty zona and dead oocytes 24 h after insemination were estimated. The rate of degeneration of control oocytes 24 h after insemination was 1.6% of the oocytes (13 of 810); of the 13 assessed oocytes, about 0.6% were empty zona and 0.7% of all oocytes were dead.

The oocytes that had failed to fertilize and abnormally fertilized zygotes were separated from the normally fertilized zygotes and used for vitrification in this study. The selected oocytes and zygotes from different patients were pooled, and then randomly assigned to groups that would undergo either the hemi-straw system or cryoloop vitrification technique. The oocytes or zygotes from the two groups were vitrified simultaneously. Thus, the possible influence of individual patient variation was excluded or minimized and it could be assured that the two experimental groups of oocytes and zygotes were identical in nature and only differed in terms of the vitrification technique used. Afterwards, the warming of the oocytes was also performed simultaneously.

The recovered zygotes were placed for culture in IVC-1 (In-Vitro Care, San Diego, CA), supplemented with 10% human serum albumin (HSA) in 20 μ l drops under mineral oil (SAGE BioPharma, Bedminster, NJ) at 37°C in a humidified atmosphere of 5% CO₂ in air until day 3. On day 3, embryos at the 6–8 blastomere stage of development (average number of cells before vitrification in each experimental group was eight) were pooled and simultaneously vitrified. Day 3 embryos with > 50% fragmentation were omitted from the vitrification procedures. The embryos were warmed simultaneously and then cultured from day 3 to day 4 in CCM (Vitrolife, Gothenburg).

Vitrification and dilution solutions

Chemicals for precooling, vitrification and dilution were purchased from Sigma (St Louis, MO) and prepared with Dulbecco's phosphate-buffered saline (DPBS) without calcium or magnesium (In-Vitro Care) with 20% serum substitute supplement (SSS) (Irvine Scientific, Santa Ana, CA). The 0.25 ml straws used for the hemi-straw system were obtained from IVM (L'Aigle), and the cryoloop (20 μ m thickness; 0.5–0.7 mm in loop diameter) from Hampton Research (Laguna Niguel, CA).

Before vitrification using the hemi-straw system or cryoloop, the oocytes were placed in DPBS with 20% SSS at room temperature for 10 min to cool down. The vitrification procedure was initiated in culture dishes (Nalge Nunc International, Rochester, NY) prepared with one droplet of 100 μ l holding medium (HM: DPBS with 20% SSS), one droplet of 100 μ l pre-cooling solution (VS1) and one droplet

of 100 μ l vitrification solution (VS2) and an additional 20 μ l droplet of VS2. A six-well tissue culture plate (Genesis Instruments, Elmwood, WI) was prepared with 1 ml of each dilution solution for warming. The solutions for dilution after warming were prepared using DPBS with 20% SSS and different concentrations of sucrose.

Cryoloop vitrification technique

The vitrification method using the cryoloop was based on the method described by Lane *et al.* (1999a,b). The oocytes or embryos were placed in VS1, and then transferred into VS2. The nylon loop was dipped into VS2 to create a thin, filmy layer of the VS2 by surface tension on the nylon loop. After a short exposure to VS2, the oocytes or embryos were placed on to the nylon loop pre-loaded with the thin film of VS2, using a flexipet-denuding pipette (end hole 140 μ m; Cook IVF, Spencer, IN;) and kept there by surface tension. A metal insert on the lip of a cryovial enables the use of a stainless steel rod with a small magnet for manipulation of the nylon loop at low temperatures. The loaded nylon loop was plunged into liquid nitrogen, and then screwed into the cryovial that had previously been submerged in liquid nitrogen, using the stainless steel rod. The vials were attached in standard canes for storage in liquid nitrogen.

Hemi-straw system vitrification technique

The hemi-straw system vitrification procedure was reported first by Vandervorst *et al.* (2001). The end of a 0.25 ml straw was cut with a sharp scalpel, so that the end was open (about 1 cm in length), and it was easy to pipette a small droplet (< 1.0 μ l) onto the open inner face of the straw. The oocytes or embryos were pretreated briefly with VS1. After pre-equilibrating, the oocytes or embryos were placed in VS2, and then transferred with a minimum amount of VS2 into the 20 μ l droplet of VS2, and loaded in as small a volume as possible (< 1.0 μ l) on to the inner surface of the open edge of the 0.25 ml straw. The straw was then plunged vertically into liquid nitrogen, and placed into a prelabelled 0.5 ml cryostraw (Cryo Bio Systems, L'Aigle) held under liquid nitrogen. The cryostraw was then plugged with a plastic colour-coded plug (Cryo Bio Systems) before cryostorage.

Experimental design

In pre-trials, the influence of the temperature of the vitrification solution during the loading process (25°C versus 37°C) on the survival potential of the oocytes was tested (J. Liebermann and M. J. Tucker, unpublished). It was decided for all protocols to use a loading temperature near room temperature (25°C). In all protocols, oocytes or embryos were vitrified by the use of a two-step loading process with cryoprotectants. The protocols with the concentrations and properties of each vitrification solution, and the time spent in each solution, are summarized (Table 1).

Experiment 1: oocyte vitrification protocol

After cooling for 10 min, oocytes were first equilibrated in VS1 containing a mixture of 10% ethylene glycol, 10% (v/v) dimethyl sulphoxide (DMSO) and 1 mg polyethylene glycol ml⁻¹ (average molecular weight 8000) for 60 s at 25°C and vitrified by exposure for 20 s to vitrification solution (VS2) containing 20% ethylene glycol, 20% (v/v) DMSO 1 mg polyethylene glycol ml⁻¹, 10 mg Ficoll ml⁻¹ (average molecular weight 400 000) and 0.65 mol sucrose ml⁻¹.

After storage for 10–14 days, the straws and cryoloops were taken out of the liquid nitrogen. The cryoprotectants were removed by warming the oocytes and diluting them using a four-step dilution with sucrose on a stage at 37°C. In brief, warming was carried out at 37°C by submerging the oocytes in 1 mol sucrose l⁻¹. After 2 min, oocytes were transferred to 0.5 mol sucrose l⁻¹ for 3 min, and then to 0.25 mol sucrose l⁻¹ for 5 min and finally to 0.125 mol sucrose l⁻¹ for a further 5 min. After warming, the recovered oocytes were placed in culture in IVC-1 for 24 h, and then evaluated for cryosurvival.

Experiment 2: embryo vitrification protocols

Embryos were first equilibrated in VS1 containing a mixture of 10% EG and 10% (v/v) DMSO for 4 min at 25°C and vitrified after exposure for 20 s to 20% ethylene glycol, 20% (v/v) DMSO and 0.4 mol sucrose l⁻¹ (VS2).

After storage for 10–14 days, the embryos in both carriers were simultaneously warmed and diluted using a three-step dilution with sucrose on a stage at 37°C. In brief, embryos were submerged in a solution of 1 mol sucrose l⁻¹. After 5 min, they were transferred into 0.25 mol sucrose l⁻¹ for 3 min, and finally into 0.125 mol sucrose l⁻¹ for 2 min.

Evaluation of the viability of vitrified–warmed human oocytes and embryos

After warming, the recovered oocytes and embryos were placed in 20 μ l drops under mineral oil at 37°C in a humidified atmosphere of 5% CO₂ in air for the next 24 h. IVC-1 was used as culture medium for the oocytes, whereas the embryos were incubated in CCM.

After 24 h, oocytes were evaluated for cryosurvival. The number of oocytes with fragmented cytoplasm, indistinct oolemma or damaged zona pellucida, and those that degenerated during the 24 h culture period were recorded as not surviving after vitrification.

Two hours after warming, embryos were assessed to determine their integrity and the number of surviving blastomeres. Surviving embryos were defined as having half or more of their blastomeres intact after warming. Therefore, the survival rate was judged as the percentage of embryos that were morphologically intact ($\geq$ 50% intact blastomeres) after removal of the cryoprotectant. In addition, after culture for 24 h, the embryos were checked for further cleavage and compaction.

Table 1. Summary of the protocols of each experiment regarding concentration and properties of the vitrification solution, time in each solution, carrier used and type of oocyte examined

	Holding medium (HM)	Vitrification solution 1 (VS1)	Vitrification solution 2 (VS2)	Carrier
Oocytes	20% SSS in DPBS (10 min)	10% EG, 10% DMSO, 1 mg PEG ml ⁻¹ (60 s)	20% EG, 20% DMSO, 1 mg PEG ml ⁻¹ , 10 mg Ficoll ml ⁻¹ , 0.65 mol sucrose l ⁻¹ (30 s)	CL/HSS
Embryos	–	10% EG, 10% DMSO (4 min)	20% EG, 20% DMSO, 0.4 mol sucrose l ⁻¹ (30 s)	CL/HSS

DPBS: Dulbecco's phosphate-buffered saline; SSS: serum substitute supplement; EG: ethylene glycol; DMSO: dimethyl sulphoxide; PEG: polyethylene glycol; CL: cryoloop; HSS: hemi-straw system.

Table 2. Survival after warming of human oocytes after 24 h culture in cryoloop or hemi-straw system

	Vitrification technique	
	Cryoloop	Hemi-straw system
Number of oocytes	448	480
Oocyte survival (%)	361 (80.6%)*	410 (85.4%)*
Overall survival (%)	771 of 928 (83.0%)	

* $P = 0.07$, not significant, chi-squared test comparison of cryoloop versus hemi-straw system

Statistical analysis

Statistical analysis was carried out by means of a chi-squared test. Statistical significance was defined as $P < 0.05$.

Results

The survival rates of oocytes after warming using the cryoloop or the hemi-straw system are shown (Table 2). In Expt 1, 928 aged human oocytes that had failed to fertilize were subjected to vitrification. The rate of survival of oocytes after 24 h of culture was slightly lower in the cryoloop group than in the hemi-straw system group (80.6% versus 85.4%), but the difference was not significant. Overall 83.0 % of oocytes survived (771 of 928).

The survival rate after warming of day 3 embryos with more than half of the blastomeres intact was slightly lower in the cryoloop group (83.8%) compared with the survival in the hemi-straw system group (89.7%), but the difference was not significant (Table 3). In general, the survival rate of vitrified embryos after 2 h of culture was 86.8% (231 of 266). A reasonable rate of further cleavage and compaction occurred in the cryoloop group (29.4%; 32 of 109), which was significantly different from that in the hemi-straw system group (37.7%; 46 of 122; χ^2 ; $P = 0.002$; Table 3). Overall, 78 of 231 of the day 3 embryos became compacted (33.8%).

Discussion

Vitrification, as a simple cryopreservation method of directly submerging a cell in liquid nitrogen after brief exposure to a cryoprotectant agent, is substantially less

Table 3. Survival after warming of human day 3 embryos after 2 h culture, and cleavage behaviour after 24 h using cryoloop and hemi-straw system

	Vitrification technique	
	Cryoloop	Hemi-straw system
Number of embryos	130	136
Survival ($\geq 50\%$ of intact blastomeres)	109 (83.8%)*	122 (89.7%)*
Overall embryo survival ($\geq 50\%$ of intact blastomeres)	231 of 266 (86.8%)	
Day 4 (≥ 8 blastomeres and stage of compaction; %)	32 (29.4%)**	46 (37.7%)**
Overall compaction rate (%)	78 of 231 (33.8%)	

* $P = 0.07$, not significant, chi-squared test comparison of cryoloop versus hemi-straw system (survival); ** $P = 0.002$, chi-squared test comparison of cryoloop versus hemi-straw system (development potential).

harmful to cells such as oocytes and embryos that have high chilling sensitivities than are conventional cryopreservation methods using traditional slow-freezing (Martino *et al.*, 1996; Vatja *et al.*, 1997). The advantage for the cells of vitrification over conventional cryopreservation procedures is that the 'open system' lacks an insulating layer around the sample. The vapour surrounding the cells can create effective insulation that reduces temperature transfer, resulting in a decreased cooling rate and possibly crystallization. The volume of the vitrification solution should be minimized as much as is practical to ensure that the cells are surrounded with liquid nitrogen and not vapour. Thus, the duration of any coating vapour is reduced and the rate of cooling is increased. However, the direct and rapid contact with liquid nitrogen, coupled with as small a volume of the vitrification solution as is practical, allows higher cooling rates to be achieved, because conductive heat transfer in liquid is much faster than it is in vapour. Furthermore, the high rate of cooling has the positive effect of reducing the concentration of cryoprotectant and the exposure time. Thus, exposure to the toxic effects of the cryoprotectants is also decreased (J. Liebermann and M. J. Tucker, unpublished).

The results of the present study indicate a high rate of

survival after warming of morphologically normal aged oocytes that had failed to fertilize and day 3 embryos when vitrification protocols with high rates of cooling are used. Furthermore, very promising results were obtained using a mixture of 20% ethylene glycol and 20% (v/v) DMSO. Although human embryo cryopreservation has become a well-established process in assisted human reproduction, oocyte cryopreservation, regardless of some success with this approach (Van Uem *et al.*, 1987; Chen, 1988; Polak *et al.*, 1998; Tucker *et al.*, 1998a,b; Yang *et al.*, 1998), is still considered experimental, and is thought to be of limited clinical use. Unlike all stages of human preimplantation embryos, oocytes are more vulnerable to the cryopreservation procedures involving ice crystallization because of the reduced permeability of the cytoplasmic membranes of oocytes (Ruffing *et al.*, 1993). In addition, cryopreserved cells just after warming are more sensitive to osmotic swelling than are fresh cells (Pedro *et al.*, 1997).

Conventional cryopreservation protocols for human oocytes have resulted in various types of injury (for example, damage of intracellular lipid droplets, and the cytoskeleton), leading to low overall rates of success of about 1% implantation per egg frozen (Mandelbaum *et al.*, 1998). In addition, the developmental potential of 'slow-rate' frozen human oocytes after fertilization, up to cavitation and blastocyst formation, is only 5.6% (Cobo *et al.*, 2001). In contrast, a recent study showed the beneficial effect of microinjected trehalose on the survival of human oocytes after conventional cryopreservation (Eroglu *et al.*, 2002). About 66% of oocytes in the intracellular trehalose group survived cooling to -60°C , whereas all oocytes in the control group degenerated when cooled to -60°C . However, similar survival rates (approximately 65%) have been reported after vitrification of human oocytes using high concentrations of cryoprotectant agent (Hunter *et al.*, 1995; Kuleshova *et al.*, 1999; Chen *et al.*, 2000b). Kuwayama and Kato (2000) described a minimum volume cooling (MVC) technique that is comparable to the hemi-straw system used in the present study. With a small number of human oocytes, a high survival rate after warming of 91% (60 of 64) was achieved by using 30 or 40% ethylene glycol + 1.0 or 0.5 mol sucrose l^{-1} (Kuwayama and Kato, 2000). Vitrification of human oocytes that had failed to fertilize using the cryoloop and a mixture of 20% ethylene glycol and 20% (v/v) DMSO without added macromolecules such as polymers resulted in survival after warming of 81.1% (574 of 708; J. Liebermann and M. J. Tucker, unpublished) indicating that increased rates of cooling are beneficial for oocyte vitrification. It has been reported previously that increased rates of cooling result in more successful vitrification of animal oocytes (Arav *et al.*, 2000; Hochi *et al.*, 2001; Isachenko *et al.*, 2001).

Experience with carriers such as flexipet-denuding pipette (FDP) with or without liquid nitrogen slush (Liebermann *et al.*, 2002) and cryoloop led the present authors to search for new types of carrier. It was necessary to find materials with rapid heat transfer that also support

the process of uniform heat exchange. The hemi-straw system has been used in only a few studies and with a small number of cells, but still with very good results. This system is very simple, allowing a very fast rate of cooling by direct contact with liquid nitrogen; it is easy to handle for any embryologist; it is low cost and easy to store in any commercially available storage system (Vandervorst *et al.*, 2001). In contrast to the nylon loop technique, in which the oocytes or embryos are vitrified by placing them on to a thin film on a small nylon loop, with the hemi-straw system, the oocytes or embryos are placed in a very small droplet ($\leq 1.0 \mu\text{l}$) on an open edge of a cut 0.25 ml cryostraw. The results of Expt 1 of the present study demonstrate that the hemi-straw system and the cryoloop both have a beneficial effect on survival after warming, and achieve consistent rates of survival of between 80 and 85%. Furthermore, the high rate of cooling attained using the hemi-straw system or cryoloop allows an apparently beneficial reduction in the concentration of the cryoprotectant. Kuleshova *et al.* (1999) achieved a rate of survival after warming of human oocytes of approximately 65%, using the OPS and 40% ethylene glycol as vitrification solution. In the present study, 40% ethylene glycol was replaced by a mixture of 20% ethylene glycol and 20% (v/v) DMSO. Thus the toxicity of the cryoprotectant was reduced, possibly accounting for the higher survival rate after using the cryoloop or the hemi-straw system for vitrification. Results of the present study indicate that using the hemi-straw system has a more positive effect on the survival after warming than using the cryoloop. One reason for this may be that cells loaded on the carrier build a more effective viscous matrix for encapsulation of these cells in the hemi-straw system, and also prevent crystallization during cooling and warming. These observations were supported by Kuwayama and Kato (2000), who reported three efficient vitrification techniques, one of which they recommended for clinical use: the vitrification technique of placing a fine drop on the inner surface of a straw, similar to the method used for hemi-straw system in the present study.

Reports of human embryo vitrification are rare. El-Danasouri and Selman (2002) reported successful pregnancies and deliveries after vitrification of day 3 human embryos using the OPS. The structure of the OPS covers the vitrification solution containing the embryos, so that contact between the vitrification solution and the liquid nitrogen is not as direct as when using the cryoloop or hemi-straw system. The results showed a negative correlation between stage of development and survival: eight-cell embryos showed a higher survival rate (79.2%; 62 of 78) than did embryos with fewer than six cells (21.1%; 11 of 53) after vitrification (El-Danasouri and Selman, 2002). The results recorded in Expt 2 of the present study show a survival rate of human day 3 embryos with an average of eight blastomeres of between 84 and 90%. This finding supports previous reports in which high survival rate of eight-cell human embryos using 40% ethylene glycol were documented (Otha *et al.*, 1996; Mukaida *et al.*, 1998). Nevertheless, it is important to note that in the present study the ethylene

glycol concentration was only 20%, combined with 20% (v/v) DMSO. It is possible that this improved success is a result of a higher cooling rate obtained by increasing the speed of thermal conduction using the cryoloop or the hemi-straw system. Despite the fact that a promising rate of survival after warming was achieved, overall only about 34% of the surviving embryos had the developmental potential to reach the stage of compaction. Significantly more embryos compacted in the hemi-straw system group than in the cryoloop group. The morphological grade of the embryos was not dependent on the carrier system used. The findings of the present study support a previous report (Kuwayama and Kato, 2000) in which significantly higher rates of cleavage and blastocyst formation of bovine embryos were obtained with the MVC method than using plastic straws and open pulled straws. However, the compaction rate of day 3 embryos after warming reported in this study needs to be improved, although it should be noted that these embryos were obtained after abnormal fertilization, and may not have the same cryobiological properties and potential for development as embryos obtained after normal fertilization (Noto *et al.*, 1991; Joris *et al.*, 1999).

In conclusion, the present study demonstrates that using the cryoloop or the hemi-straw system, which enabled ultra-rapid cooling rates to be achieved, in combination with a low concentration of a mixture of ethylene glycol and DMSO as cryoprotectant agents can effectively improve survival rates of human oocytes and day 3 embryos after warming after vitrification. The hemi-straw system may be a superior method to the cryoloop, particularly as it achieves a higher rate of compaction for day 3 embryos after warming. The vitrification techniques described here appear to be a good alternative to classical slow-freezing protocols. Therefore, the results presented in this study are encouraging, and, together with the more convenient protocols of vitrification in general, should generate further research to develop this technique and increase its clinical use and efficiency.

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