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# Vitrification of mouse and human blastocysts using a novel cryoloop container-less technique

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**Objective:** To vitrify mouse and human blastocysts with use of the cryoloop procedure and to assess subsequent development.

**Design:** Controlled study of vitrification of mouse and human blastocysts.

**Setting:** Research department of a private assisted reproductive technology unit.

**Patient(s):** Blastocysts that were not suitable to be frozen were donated from patients.

**Intervention(s):** Culture of pronucleate embryos in sequential media to the blastocyst stage.

**Main Outcome Measure(s):** Survival of the vitrification procedure was assessed by reexpansion, hatching, and outgrowth in culture. In addition, the viability of mouse blastocysts was assessed after transfer to pseudopregnant recipients.

**Result(s):** Vitrification of mouse blastocysts did not affect the ability to reexpand, hatch, or outgrow in culture. Furthermore, implantation rates and fetal development were equivalent for nonfrozen and vitrified blastocysts. Vitrified human blastocysts were able to hatch and outgrow in culture at rates similar to nonfrozen controls.

**Conclusion(s):** Cryoloop vitrification was able to cryopreserve mouse and human blastocysts without any reduction in the ability to reexpand and hatch in culture. Furthermore, viability was not reduced by the cryoloop vitrification of mouse blastocysts. (Fertil Steril® 1999;72:1073–8. ©1999 by American Society for Reproductive Medicine.)

**Key Words:** Blastocyst, cryopreservation, culture, viability

With the increasing use of blastocyst culture and transfer as routine treatment in human clinical IVF (1), there is an increased need for a reliable cryopreservation procedure for the human blastocyst. Human blastocysts are currently routinely cryopreserved with use of slow-freezing techniques (2). Such slow-freezing procedures use low concentrations of cryoprotectants and slow controlled rates of cooling usually in the range of 0.1–0.3°C/min to slowly dehydrate the cell during freezing to prevent intracellular crystallization (3–5).

Intracellular crystallization damages both membranes and organelles and is thought to be a major cause for developmental loss after cryopreservation. However, there is a balance between removing the water from the cell because too much dehydration can result in an increase in the intracellular solute concentration to toxic levels. Furthermore, with slow-freezing procedures care must also be taken in the thawing procedure because ice crystals can reform during warming. In addition, the rapid movement of water into the cell can cause the cell to swell. High concentrations of extracellular sugars, such as sucrose, can prevent excessive swelling. Slow-freezing techniques for the cryopreservation of embryos from all species, including humans, results in a reduced ability of blastocysts to both establish and maintain pregnancy after thaw and transfer (6). This loss in developmental competence has been attributed to both ice crystal formation and also chilling sensitivity (7).

An alternative to slow-freezing procedures is vitrification. Vitrification uses high concentrations of cryoprotectants that, when cooled rapidly, solidify without the formation of ice crystals. Furthermore, the high rates of cooling prevent chilling damage (8). However, other

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than a few limited studies (9–11), vitrification has not been widely adopted in a clinical setting. This may be due to the procedure being cumbersome because it is best performed at low temperatures to reduce the toxicity of the high concentrations of cryoprotectants used.

However, recently the success of vitrification procedures has been increased by techniques that substantially increase the cooling rate by either vitrifying on electron microscope grids (12) or in thinly walled straws (13). However, the manipulation and recovery of the oocytes and embryos after these techniques is problematic. Recently, a new vitrification procedure using a cryoloop has been described that enables facile manipulations during both vitrification and warming (14). This technique has been modified from a procedure routinely used for the flash-freezing of protein crystals for data collection at cryogenic temperatures (15, 16). Embryos are suspended on a cryoloop and plunged directly into liquid nitrogen. With use of the cryoloop, hamster and cow oocytes and embryos have been successfully vitrified. The aim of this study is to assess the proficiency of cryoloop container-less vitrification to successfully cryopreserve both mouse and human blastocysts.

## MATERIALS AND METHODS

### Culture Media

Media for embryo culture was G1.2 and G2.2 (IVF Sciences Scandinavian, Gothenburg, Sweden). Media for embryo collection was a HEPES-modification of G1.2 (H-G1.2), and the base medium for cryopreservation and thawing was a HEPES-buffered modification of G2.2 without amino acids and vitamins (H-G2.2). In both cases the media were modified by replacing 20 mM of $NaHCO_3$ with 20 mM of HEPES and adjusted to pH 7.35.

### Animals

Embryos were collected from 4- to 6-week-old F1 (C57BL6×CBa) females. Females were stimulated with 5 IU of pregnant mare gonadotropin (Sigma Chemical Co., St. Louis, MO) and 48 hours later with 5 IU of hCG (Sigma Chemical Co.). After the hCG injection females were placed with males of the same strain, and the following morning the presence of a vaginal plug indicated that mating had taken place. Zygotes were collected at 22 hours after hCG and denuded from surrounding cumulus by incubation in H-G1.2 with 0.5 mg/mL hyalronidase for less than 1 minute. Zygotes were washed twice in H-G1.2 and placed in culture.

### Mouse Embryo Culture

Mouse zygotes were cultured in groups of 10 in 20-$\mu$L drops of medium G1.2 at 37°C in a humidified atmosphere of 5% $CO_2$ in air. After 48 hours of culture, eight-cell embryos were washed three times in medium G2.2 and cultured for a further 48 hours in 20-$\mu$L drops of medium G2.2. Blastocyst development was assessed after 96 hours of culture. All blastocysts that developed were vitrified.

### Human Embryo Culture

The culture system for blastocyst growth was described previously (1). Briefly, after oocyte retrieval, cumulus-enclosed oocytes were incubated in Ham's F-10 supplemented with fetal cord serum (FCS) for insemination. Semen was prepared with a 50-70-95 discontinuous gradient or minigradient method (Pure Sperm, Nidacon, Gothenburg), depending on the initial semen parameters. The resulting pellet was washed in Ham's F-10. For normal insemination, to each oocyte, 50–100,000 sperm/mL were added.

If intracytoplasmic sperm injection (ICSI) was performed, oocytes were denuded with use of hyaluronidase and drawn pipettes. Each mature oocyte was placed in a 6-$\mu$L droplet of phosphate-buffered saline supplemented with 15% FCS. The partner's sperm was placed in a 6-$\mu$L droplet of PVP (IVF Sciences Scandinavian). All droplets were overlaid with Ovoil (IVF Sciences Scandinavian). Intracytoplasmic sperm injection was performed on a Nikon inverted microscope with Narishige micromanipulators. Injected oocytes were then rinsed and placed in tubes of G1.2 until fertilization was assessed.

Fertilization was assessed 15–18 hours after insemination or ICSI. Cumulus and corona cells were removed by dissection with 27-gauge disposable needles in an organ culture dish. Resulting two pronuclear embryos were washed well and subsequently cultured in groups of two to three in G1.2 medium in 1-mL Falcon culture tubes in 5% $CO_2$ in air. After 48 hours of culture embryos were rinsed three times and cultured for a further 48 to 72 hours (1). Blastocysts on day 6 that were not considered of good enough quality to cryopreserve, i.e., not fully expanded or with poor inner cell mass development, were donated for vitrification by the cryoloop method. The HealthONE Institutional Review Board approved this study.

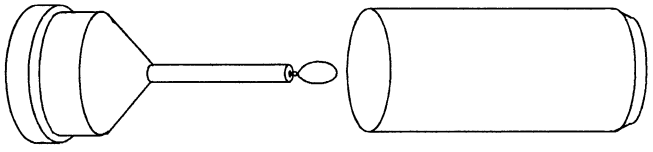
### Vitrification Using the Cryoloop

Cryoloops used for vitrification consisted of a nylon loop (20$\mu$m wide; 0.5–0.7 mm in diameter) mounted on a stainless steel pipe inserted into the lid of a cryovial (Fig. 1). The loops were purchased mounted and epoxied into vials (Hampton Research, Laguna Niguel, CA). A metal insert on the lid enables the use of a handle with a small magnet for manipulation of the loop if desired.

Blastocysts were vitrified with use of a two-step loading with cryoprotectants. Initially, blastocysts were placed in cryoprotectant solution I which contained 10% dimethylsulphoxide (DMSO) and 10% ethylene glycol for 2 minutes before being transferred to solution II, which contained 20% DMSO and 20% ethylene glycol, 10 mg/mL ficoll molecular weight ([MW] 400,000) and 0.65 M sucrose for approximately 20 seconds. These concentrations of cryoprotectants and length of exposure were previously demonstrated to be optimal for the vitrification of both rodent and domestic animal embryos using the cryoloop procedure (14).

**FIGURE 1**

Illustration of the cryoloop used for the vitrification of blastocysts. The nylon cryoloop is attached to the lid of a cryovial by a stainless steel tubing. For vitrification, the blastocysts are placed on the cryoloop that had been coated with a thin film of cryoprotectant solution. Blastocysts on the cryoloop are placed into the cryovial, which is submerged and filled with liquid nitrogen and the vial is sealed.



*Lane. Vitrification of blastocysts. Fertil Steril 1999.*

**TABLE 1**

Effect of cryoloop vitrification of mouse blastocysts on reexpansion and outgrowth.

| Treatment | Study group | |
|---|---|---|
| | Control | Vitrified blastocysts* |
| Reexpansion (%) | — | 100 |
| Hatching (%) | 87.5 | 95.5 |
| Attachment (%) | 78.1 | 85.9 |
| ICM outgrowth (means ± SEM) | 2.21 ± 0.10 | 2.17 ± 0.09 |
| Trophectoderm outgrowth (means ± SEM) | 2.00 ± 0.09 | 2.14 ± 0.09 |

* There was no statistically significant difference between control and vitrified blastocysts for any parameter measured.

*Lane. Vitrification of blastocysts. Fertil Steril 1999.*

While blastocysts are in cryoprotectant solution I, the cryoloop is dipped into cryoprotectant solution II to create a thin film on the loop. The blastocysts were then transferred from solution II onto the film of cryoprotectant on the cryoloop. The cryoloop containing the blastocyst was then plunged into the cryovial, which is submerged and filled with liquid nitrogen. By previously submerging the cryovial under liquid nitrogen, the loop containing the blastocysts could be plunged into the cryovial containing liquid nitrogen and sealed under liquid nitrogen in one motion (14). The vials were stored in standard canes.

Blastocysts were thawed using a two-step dilution with sucrose. With the cryovial submerged under liquid nitrogen, the vial was opened, and the loop containing blastocysts was removed from the liquid nitrogen and placed directly into a well of the base medium (H-G2.2) containing 0.25 M sucrose. The blastocysts immediately fell from the loop into the thaw solution. Blastocysts were moved from this solution after 2 minutes and transferred to base medium containing 0.125 M sucrose for an additional 3 minutes. Subsequently, blastocysts were washed twice in the base medium for 5 minutes and were then returned to culture.

After vitrification, mouse and human blastocysts were cultured in medium G2.2 for 6 hours to assess reexpansion before assessment of blastocyst outgrowth. A 6-hour incubation was chosen because this is the normal time period used in our clinic for the assessment of thawed blastocysts before transfer.

## Assessment of Blastocyst Outgrowth

Both mouse and human blastocysts were assessed for outgrowth as a marker of subsequent viability. Blastocysts were transferred to medium G2.2 supplemented with 10% fetal cord serum to assess blastocyst attachment and outgrowth. Blastocysts were cultured in four-well plates (Nunclon, Denmark) previously coated with 0.1% gelatin in 500-$\mu$L drops at 37°C in 5% $CO_2$ in air for 48 hours. Blastocyst hatching and attachment were assessed after 24 hours and outgrowth assessed after an additional 24 hours of culture. Outgrowth of inner cell mass (ICM) and trophectoderm was given a score between 0 and 3 based on the amount of outgrowth, where 0 was no growth and 3 was extensive growth as described and illustrated by Spindle and Pederson (17).

## Assessment of Blastocyst Viability

Viability of mouse blastocysts after vitrification was assessed by transfer to pseudopregnant recipients. After warming, blastocysts were cultured for 6 hours in medium G2.2 before transfer. All reexpanded blastocysts after the 6-hour period were pooled, and blastocysts for transfer randomly selected. Six blastocysts were transferred to each uterine horn. On day 15 of pregnancy, implantation, fetal development, and fetal weights were assessed. Noncryopreserved blastocysts served as the control.

## Statistical Analyses

Differences in hatching, attachment, and viability after vitrification were assessed by $\chi^2$ analysis with Yates correction. Data for outgrowth of both the ICM and trophectoderm were initially subjected to a Kolmogorov-Smirnov test to determine the normality of the data. An *F* test was then used to determine whether the two groups of data had equal variances. Once the normality and equal variances were established, differences in outgrowth were assessed by Student's *t*-test.

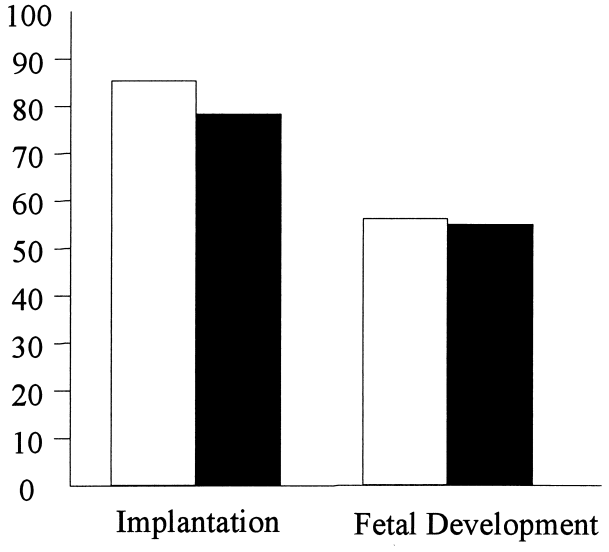
# RESULTS

## Mouse Blastocyst Vitrification

A total of 160 mouse blastocysts were vitrified with the cryoloop. After vitrification, 100% of these blastocysts were able to reexpand in culture. There was no difference in the ability of vitrified blastocysts to hatch and attach in culture

**FIGURE 2**

Effect of cryoloop vitrification of mouse blastocyst viability. Implantation per blastocyst transferred (%) and fetal development per blastocyst transferred. Control, blastocysts not cryopreserved (*open bars*). Vitrified blastocysts (*closed bars*). N = 60 embryos transferred per treatment group.




*Lane. Vitrification of blastocysts. Fertil Steril 1999.*

compared with control embryos (Table 1). Similarly, there was no difference in the ability of either the ICM or trophectoderm to outgrow in culture between the control and vitrified blastocysts (Table 1).

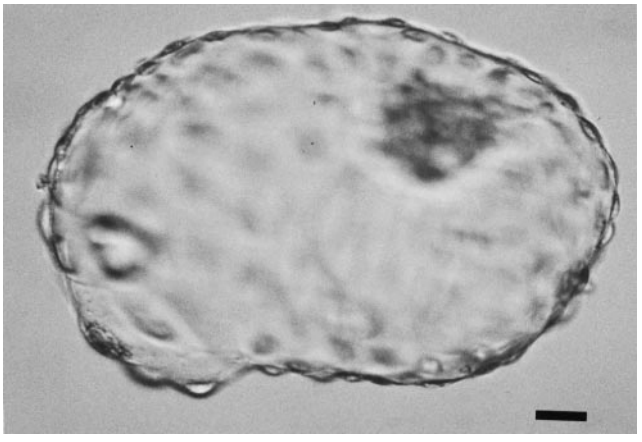
After vitrification, 60 blastocysts were transferred to pseudopregnant recipients, and their viability was compared with sibling control blastocysts that were not cryopreserved. There was no difference in the ability of vitrified blastocysts to implant and develop to a fetus compared with control blastocysts (Fig. 2). Resultant fetal weights were also similar for blastocysts that were vitrified (0.245 ± 0.021 g) compared with control blastocysts (0.250 ± 0.017 g). All fetuses resulting from both vitrified and control blastocysts were morphologically normal. In addition, a recipient female that received eight vitrified blastocysts (four per uterine horn) was allowed to litter. All pups born were morphologically normal.

## Human Blastocyst Vitrification

Eighteen human blastocysts between minimally to semiexpanded were vitrified with the cryoloop. Of these, 11 (83.3%) reexpanded in culture. Figure 3 demonstrates a vitrified and thawed blastocyst that had reexpanded after 24 hours of culture. Ability to hatch in culture and outgrowth of the ICM and trophectoderm were similar for blastocysts that were vitrified and control blastocysts that were not cryopreserved (Table 2).

**FIGURE 3**

Micrograph of human blastocyst 24 hours after cryoloop vitrification. Blastocyst was fully expanded and hatched from the zona pellucida. Bar = 30 μm.



*Lane. Vitrification of blastocysts. Fertil Steril 1999.*

## DISCUSSION

This study demonstrates that both mouse and human blastocysts can be successfully vitrified by suspension on a small nylon loop and plunging directly into liquid nitrogen. Blastocysts from both the mouse and human were capable of reexpansion and hatching in culture after vitrification with the cryoloop. Furthermore, mouse blastocysts had equivalent implantation and fetal development rates compared with control blastocysts that were not cryopreserved.

With the development of sequential media based on the physiology of the reproductive tract and changing physiol-

**TABLE 2**

Effect of cryoloop vitrification on human blastocysts on reexpansion and outgrowth.

| | Study group | |
|---|---|---|
| Treatment | Control (n = 12) | Vitrified blastocysts* (n = 18) |
| Reexpansion (%) | — | 83.3 |
| Hatching (%) | 63.6 | 73.3 |
| Attachment (%) | 36.0 | 60.0 |
| ICM outgrowth (means ± SEM) | 2.0 ± 0.2 | 1.7 ± 0.2 |
| Trophectoderm outgrowth (means ± SEM) | 1.7 ± 0.2 | 2.0 ± 0.2 |

* There was no statistically significant difference between control and vitrified blastocysts for any parameter measured.

*Lane. Vitrification of blastocysts. Fertil Steril 1999.*

ogy of the developing embryo it is now possible to grow viable blastocysts in culture (1, 18). However, a potential limitation to blastocyst culture and transfer is the ability to reliably cryopreserve human blastocysts.

The first report of human blastocyst freezing dates back to 1985 (19). In this study blastocysts were cryopreserved with use of a slow-freezing protocol with the cryoprotectant glycerol. However, subsequent larger trials of blastocyst freezing reported disappointing rates of survival (approximately 50% and 60%), low pregnancy rates (9% and 7%), and high rates of fetal loss (32%) (2, 20). Another report of 500 cycles of cryopreservation of blastocysts grown with use of coculture with Vero cells using a slightly modified protocol reported survival rates of approximately 80% and an implantation rate of 13% (21). However, this implantation rate of frozen blastocysts was only approximately half of that reported for the transfer of fresh blastocysts (21).

It is thought that the use of vitrification would be a preferred method of cryopreservation over slow-freezing procedures because of the lack of ice crystal formation. Furthermore, vitrification is substantially better for cells that have high chilling sensitivities such as bovine oocytes and embryos (12, 13). Vitrification of oocytes and embryos using the cryoloop has advantages over conventional vitrification procedures in that the open system lacking any thermoinsulating layer, coupled with the small volume of $<1$ $\mu$L, results in both rapid and uniform heat exchange during cooling. High rates of cooling prevent chilling injury to sensitive cells (12). The rapid cooling rate obtained with the cryoloop substantially reduces the exposure time to the cryoprotectants, thereby reducing their cytotoxicity. A HEPES-buffered medium was used with the cryoloop procedure to prevent perturbations in intracellular pH during vitrification. Even small perturbations in intracellular pH of embryos result in a loss in developmental competence (22, 23). Furthermore, cryopreservation results in a decrease in pH transport systems in embryos (Lane, unpublished observations). Therefore, a HEPES-buffered medium is considered to be advantageous for this procedure.

The ultimate test of the viability of embryos after cryopreservation is the ability to establish and maintain a pregnancy, resulting in normal fertile young. In this study the implantation and fetal development rates of mouse blastocysts vitrified with the cryoloop were similar to that of fresh blastocysts. However, for the human blastocysts that were used in this study it was not possible to transfer to the mother. Therefore, an in vitro viability marker was used to assess developmental competence.

Outgrowth of the ICM has been correlated with subsequent fetal development (24). Therefore, although ETs were not performed in the human, the equivalent ICM outgrowth in the vitrified and control blastocysts is encouraging because these blastocysts were viable after thawing. It has also been suggested that the cryopreservation of embryos may result in a hardening of the zona pellucida. However, with cryoloop vitrification there was no difference in the ability of blastocysts to hatch in vitro in either the mouse or the human. The high success rates of reexpansion and hatching after vitrification was despite the blastocysts in this study being of poor quality. Quality of the blastocysts before cryopreservation is likely to affect survival rates and implantation rates after thawing. An initial study indicated that the day of appearance of the blastocysts has a significant impact on the subsequent implantation rate of blastocysts after thawing (25).

Vitrification using the cryoloop was previously used to successfully vitrify cleavage stage hamster embryos, resulting in the first reported live birth of hamster pups after cryopreservation by any procedure (14). Similar to this study, the viability of hamster blastocysts vitrified at the two-cell stage with the cryoloop was similar to that of fresh embryos. Cryoloop vitrification was also used to vitrify bovine oocytes, cleavage stage embryos, and blastocysts. Vitrification with the cryoloop did not reduce the ability of bovine embryos to reexpand and hatch in culture (14).

The demonstrated versatility of cryoloop vitrification to vitrify different stages of development from several different species makes it an exciting development in cryobiology of assisted reproductive technology. Vitrification with the cryoloop provides a technique that is both successful and easy to perform and would be widely applicable to all mammalian gametes and embryos.

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