Vitrification of bovine blastocysts obtained by *in vitro* culture of oocytes matured and fertilized *in vitro*

M. Kuwayama,¹ S. Hamano¹ and T. Nagai^{2*}

¹Tokyo Bio-Technology Center, Livestock Improvement Association 3-21-10 Higashi Shinagawa, Shinagawa-ku, Tokyo 140, Japan; and ²National Institute of Animal Industry, Tsukuba Norindanchi, Ibaraki 305, Japan

Summary. Two experiments were conducted to assess the viability of bovine blastocysts obtained by *in vitro* fertilization of oocytes matured *in vitro* (IVM–IVF) and cryo-preserved by vitrification. In Expt 1, the optimal concentrations of glycerol and 1,2-propanediol in the basic medium (modified TCM199) for cooling and warming without formation of ice crystals were determined by plunging the solution into liquid nitrogen and then warming it in a water bath at 15°C; when both glycerol and 1,2-propanediol were present in the solution (>45% v/v), vitrification of the medium was observed. In Expt 2, IVM–IVF blastocysts were equilibrated to the mixture of glycerol and 1,2-propanediol (0% to 45%) at 15°C in a stepwise manner as follows: (i) in one step, for 18 min to the final vitrification solution; (ii) in two steps, for 8 min in the first step and 10 min in the second step; (iii) in four steps, for 4 min in the first three steps and 6 min in the last step; (iv) in eight steps, for 2 min in each step, but 4 min in the last step; and (v) in 16 steps, for 1 min in each step, but 3 min in the last step. After removal of cryoprotectants, the blastocysts were cultured for 24 h *in vitro*. The survival rates for the embryos equilibrated in 1, 2, 4, 8 and 16 step(s) were 56, 89, 100, 100 and 100%, respectively. The blastocysts equilibrated in 1, 2, 4, 8 and 16 steps were vitrified by plunging the straws containing them into liquid N₂, thawed and cultured *in vitro*. Higher survival rates were obtained for blastocysts equilibrated in 4, 8 and 16 steps (79, 82 and 87%, respectively) than for those in one (0%) or two (10%) steps. Ten blastocysts that survived after vitrification were transferred to ten recipients and six of these became pregnant. These results indicate that vitrification can be used for cryo-preservation of blastocysts obtained by *in vitro* culture of IVM–IVF bovine follicular oocytes.

Keywords: *in vitro*; vitrification; fertilization; embryo transfer; cow

Introduction

The procedure for successful vitrification of mammalian embryos has been the subject of investigation, since Rall & Fahy (1985) showed that mouse embryos can be successfully cryopreserved by vitrification. Scheffen *et al.* (1986) reported survival of mouse blastocysts after vitrification using a simple vitrification solution consisting of 25% glycerol and 25% 1,2-propanediol.

However, in cattle, *in vivo* blastocysts did not survive after vitrification even though the solution used by Scheffen *et al.* (1986) and Massip *et al.* (1986) was used. Van der Zwalm *et al.* (1989) reported that blastocysts did survive after vitrification, but only when they were equilibrated in three steps, in a glycerol sucrose solution instead of the solution used by Scheffen *et al.* (1986); the survival rate of blastocysts after vitrification was not high (57.1%).

*Reprint requests.

Procedures of *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) of bovine follicular oocytes have progressed to the extent that embryos can now be obtained by these techniques (Lu *et al.*, 1987; Xu *et al.*, 1987; Aoyagi *et al.*, 1990; Eyestone & First, 1989; Hamano & Kuwayama, 1992). IVM–IVF embryos have been successfully frozen and thawed, resulting in pregnancies after embryo transfer (Goto *et al.*, 1988; Kuwayama & Hamano, 1991). There are no reports, however, on vitrification of the IVM–IVF blastocysts. In the present study, the viability of bovine blastocysts after vitrification was examined *in vitro* and *in vivo*.

Materials and Methods

Experiment 1: vitrification assays

The basic medium for the vitrification solution was TCM199 (Earl's salts; Gibco, Grand Island, USA, modified with Hepes at 20 mol l^{-1} and 0.35 g sodium bicarbonate l^{-1}) supplemented with 20% calf serum (CS; Gibco, Grand Island, USA). Because in a preliminary experiment, when IVM–IVF blastocysts were cultured for 24 h in phosphate-buffered saline (PBS; Nissui Pharmaceutical Co., Ltd, Tokyo, Japan) or in modified TCM199 (the basic medium) supplemented with 20% CS, the survival rate was higher ($P < 0.05$) with modified TCM199 (96/104: 92%) than with PBS (79/100: 79%). As a cryoprotectant, a mixture (equal volume) of glycerol and 1,2-propanediol was used. Transparent plastic straws (0.25 ml ; IMF, France) were filled with vitrification solution containing the mixture (10–60%), sealed and plunged into liquid N_2 . The amorphous state (Boutron & Kaufman, 1979) of the vitrification solution was checked by direct visual inspection as described by Scheffen *et al.* (1986). The straws were then warmed in a water bath at 15°C . Devitrification occurring during warming was evident as whitening of the solution, giving the straw a milky aspect (Scheffen *et al.*, 1986).

Experiment 2: survival assays

Source of blastocysts. Blastocysts were obtained by IVM–IVF of bovine follicular oocytes using the method described by Hamano & Kuwayama (1992).

Collection of oocytes. Ovaries were obtained from a slaughterhouse and transported to the laboratory in 0.9% saline containing 0.1 mg kanamycin ml^{-1} at 37°C . All visible follicles (2–5 mm in diameter) on the ovarian surface were aspirated with a 5 ml syringe with an 18-gauge needle.

Oocyte maturation in vitro. Cumulus–oocyte complexes were washed three times and cultured in a CO_2 incubator (2% CO_2 in air with high humidity at 38.5°C) for 20–21 h in droplets of maturation medium in culture dishes ($35 \text{ mm} \times 15 \text{ mm}$, Nunclon 153066; Inter-med, Denmark). The maturation medium used was TCM199 (Earl's salts with 25 mmol Hepes l^{-1} ; Gibco, USA) supplemented with 5% fetal bovine serum (Filtron Pty Ltd, Victoria) and antibiotics.

Sperm capacitation. Sperm capacitation was induced by the method described by Parrish *et al.* (1986). After thawing of frozen semen in a waterbath at 37°C , the semen was suspended in 10 ml of Brackett & Oliphant (BO) medium (Brackett & Oliphant, 1975) containing 10 mmol caffeine l^{-1} (Sigma, USA) and washed twice by centrifugation for 5 min at 800 g . The concentration of live spermatozoa was adjusted to $2 \times 10^7 \text{ cells ml}^{-1}$ and the sperm suspension was diluted twofold with BO medium containing 10 mg bovine serum albumin ml^{-1} (Fraction V; Wako Pure Chem., Japan) and $10 \mu\text{g}$ heparin ml^{-1} (Novo-heparin; Kodama Ind., Japan).

In vitro fertilization and culture. After maturation culture, the cumulus–oocyte complexes were washed by pipetting several times in the medium and introduced into the drops of sperm suspension. They were cultured together for 5 h; the maturation medium from which the oocytes had been removed, although it still contained cumulus cells, was kept in a CO_2 incubator.

All the inseminated oocytes, still surrounded by cumulus cells with attached motile spermatozoa, were subsequently introduced into the maturation medium that had been kept with the cumulus cells.

Forty-eight hours after insemination, the oocytes were removed from the cumulus cell masses by pipetting and then examined for their cleavage stages; four- to eight-cell embryos were further cultured with a cumulus cell monolayer in the same dish.

Embryos that had developed to the blastocyst (Fig. 1) and expanding blastocyst stage after 7 days of coculture with the cells were used for the experiments.

Survival assay after stepwise equilibration. IVM–IVF blastocysts were equilibrated to the mixture of glycerol and 1,2-propanediol (0 to 45%) at 15°C in a stepwise manner as follows: (i) in one step, for 18 min to the final vitrification solution; (ii) in two steps, for 8 min in the first step and 10 min in the second step; (iii) in four steps, for 4 min

Fig. 1. Bovine blastocyst that was cultured with a monolayer of cumulus cells from the original oocytes for 7 days after *in vitro* fertilization of *in vitro* matured oocytes; $\times 300$.

in the first three steps and 6 min in the last step; (iv) in eight steps, for 2 min in each step, but 4 min in the last step; and (v) in 16 steps, in 1 min in each step, but for 3 min in the last step. Removal of cryoprotectants was carried out by exposing the embryos to a solution of 1 mol sucrose l^{-1} in modified TCM199 in a one-step method (Scheffen *et al.*, 1986). The embryos were then washed twice in the basic medium and cultured with a monolayer of cumulus cells for 24 h as described above. If the blastocysts expanded again after culture, the embryos were considered to have survived.

Survival assay after vitrification. The embryos that were equilibrated in the stepwise manner (1, 2, 4, 8 and 16 steps) as described above were loaded into a small column of the solution in 0.25 ml transparent straws. All the straws were suspended above the liquid N_2 ($-40^\circ C$) for 30 s before plunging and storing for 3–7 days. Some embryos were also frozen by a one-step method (Leibo, 1984) that was known to be effective for cryopreservation of IVM–IVF embryos (Kuwayama & Hamano, 1991). After thawing, they were used as control embryos for the embryo transfer.

Thawing was carried out by gentle shaking of the straw in a waterbath at $15^\circ C$ for 8 s. The contents of the straw were emptied into a solution of 1 mol sucrose l^{-1} in modified TCM199 and the embryos were placed on the surface of the medium and exposed therein for 10 min at $15^\circ C$ to remove the cryoprotectants. The embryos were then washed twice in the basic medium and cultured with a monolayer of cumulus cells for 24 h as described above. If the blastocysts expanded again after culture, they were considered to have survived (Fig. 2).

The embryos that had survived after vitrification using 16-step equilibration were loaded in 0.25 ml straws in the basic medium and transferred nonsurgically into ten synchronous recipients. Pregnancy was diagnosed by ultrasonic scanning when the cattle had passed 60 days without showing oestrus.

Statistical analysis

Data were analysed by χ^2 tests.

Results

Experiment 1

When the vitrification solution contained glycerol and 1,2-propanediol, the concentration of cryoprotectant allowing the content of the straw to remain transparent during cooling as well as during warming was $>45\%$. Thereafter, this concentration of cryoprotectants (45%) was used in Expt 2.

Fig. 2. Bovine blastocyst, from oocyte matured and fertilized *in vitro*, that was equilibrated in 16 steps to 22.5% glycerol and 22.5% 1,2-propanediol in modified TCM199, cryopreserved by vitrification and then cultured with a monolayer of cumulus cells for 24 h; $\times 300$.

Experiment 2

Higher survival rates were obtained for blastocysts equilibrated in four (100%), eight (100%) and 16 steps (100%) than for those in one (57%) or two steps (89%) ($P < 0.05$, Table 1).

Table 1. *In vitro* survival^a of bovine blastocysts after equilibration^b in a stepwise manner

Equilibration procedure (number of steps)	Number of blastocysts examined (number of trials)	Number (%) of blastocysts survived <i>in vitro</i>
1	50 (3)	28 (56) ^c
2	65 (3)	58 (89) ^d
4	50 (3)	50 (100) ^c
8	50 (3)	50 (100) ^c
16	61 (3)	61 (100) ^c

^aAfter equilibration and removal of cryoprotectants, blastocysts were cultured for 24 h.

^bBlastocysts were equilibrated in a stepwise manner to 22.5% glycerol 22.5% and 1,2-propanediol in modified TCM199.

^{c,d}Values with different superscripts are significantly different ($P < 0.05$).

When blastocysts were equilibrated in four, eight and 16 steps, the survival rate after vitrification was significantly higher (79, 82 and 82%, respectively) than when equilibrated in one (0%) or two steps (10%) ($P < 0.05$, Table 2).

When ten embryos that had survived after vitrification using 16-step equilibration and developed to the expanding blastocyst stage were transferred into ten synchronous recipients, six (60%) were considered to be pregnant after diagnosis using ultrasonography 60 days after embryo

Table 2. *In vitro* survival^a of bovine blastocysts vitrified after equilibration^b in a stepwise manner

Equilibration (number of steps at 15°C)	Number of blastocysts frozen-thawed (number of trials)	Number (%) of blastocysts survived
1	30 (3)	0 (0) ^c
2	30 (3)	3 (10) ^d
4	38 (3)	30 (79) ^c
8	38 (3)	31 (82) ^c
16	38 (3)	33 (87) ^c

^aAfter vitrification and removal of cryoprotectants, blastocysts were cultured for 24 h.

^bBlastocysts were equilibrated in a stepwise manner to 22.5% glycerol and 22.5% 1,2-propanediol in modified TCM199.

^{c,d,e}Values with different superscripts are significantly different ($P < 0.05$).

transfer (Table 3). Five (55%) of nine recipients that received the embryos frozen-thawed by the one-step method (Kuwayama & Hamano, 1991) became pregnant. The pregnancy rates were not significantly different between the two groups of embryos.

Table 3. Results of bovine embryo transfer^a of blastocysts after cryopreservation by vitrification or by one-step method

Method of cryopreservation	Number of blastocysts transferred	Number (%) of pregnancies
Vitrification ^b	10	6 (60)
One-step ^c	9	5 (56)

^aBlastocysts that survived after cryopreservation were co-cultured for 24 h with a monolayer of cumulus cells and then transferred to synchronous recipients.

^bBlastocysts were equilibrated in 16 steps to 22.5% glycerol and 22.5% 1,2-propanediol in modified TCM199 and then plunged into liquid N₂.

^cBlastocysts were equilibrated to 10% glycerol in modified TCM199 for 10 min, cooled and frozen in liquid N₂.

Discussion

Goto *et al.* (1988) reported the first successful cryopreservation of bovine IVM-IVF blastocysts, but they did not include details on the survival rate of blastocysts after thawing. In a previous experiment, we reported that high proportions of IVM-IVF blastocysts cryopreserved by conventional methods survived after thawing, when they had been treated at 15°C in modified TCM199 before cooling (Kuwayama & Hamano, 1991). In the present study, all the blastocysts were therefore exposed to the cryoprotectants in modified TCM199 at 15°C before vitrification.

Our results indicate that bovine IVM-IVF blastocysts can be successfully cryopreserved by vitrification resulting in normal fetuses after *in vitro* culture and embryo transfer. On the other hand, *in vivo* blastocysts did not survive after vitrification using the same solution as Scheffen *et al.* (1986) (Massip *et al.*, 1986). The reason for the better survival rate of blastocysts in this study is not

clear, but osmotic injury during equilibration has been suggested to be lethal (Massip *et al.*, 1989). In the present study, when blastocysts were equilibrated in one or two steps before vitrification, drastic distortion of zona pellucida and over-shrinkage of blastocysts occurred just after exposure to the solution, resulting in low percentages (0–10%) of survival after vitrification, even if 56% (one step) and 89% (two steps) expanded again after removal of cryoprotectants without vitrification. By contrast, high proportions of blastocysts expanded again after vitrification when they were equilibrated in four, eight and 16 steps. In the 16-step method in particular, no distortion of zona pellucida occurred, shrinkage of blastocysts was slight and 87% of vitrified blastocysts expanded again. These results suggest that osmotic injury caused by water outflow might be avoided by increasing equilibration steps, resulting in high survival rates.

In a preliminary experiment, reducing the duration of exposure (to 3 or 10 min instead of 18 min) of blastocysts to the final vitrification solution in one step did not improve the survival of blastocysts after vitrification. Furthermore, when blastocysts that had been equilibrated in 16 steps for 18 min were exposed to the final vitrification solution for an additional 5 min, a high percentage of blastocysts expanded again after vitrification (data not shown). Thus, it is suggested that the vitrification solution used in the present study is not as toxic to blastocysts as suggested by Massip *et al.* (1986).

Pregnancies following transfer of *in vivo* embryos that were equilibrated in two steps and vitrified by the method used by Scheffen *et al.* (1986) have been reported in cattle (Massip *et al.*, 1986; Douchi *et al.*, 1990), but only late morulae and early blastocysts survived after vitrification, while blastocysts did not (Massip *et al.*, 1986). Van der Zwalmen *et al.* (1989) reported that bovine *in vivo* blastocysts can survive after vitrification, but only when they are equilibrated in three steps, in a glycerol sucrose solution instead of the solution used by Massip *et al.* (1986), though the survival rate of the blastocysts after vitrification was not high (57%) (Van der Zwalmen *et al.*, 1989). When IVM–IVF blastocysts instead of *in vivo* blastocysts were vitrified by the methods of Massip *et al.* (1986) and Van der Zwalmen *et al.* (1989), the same results as they reported were obtained (data not shown). It therefore appears that the improvement in the survival of blastocysts reported by Van der Zwalmen *et al.* (1989) is due to an additional equilibration which reduces osmotic injury.

It has been shown that a mixture of 25% glycerol and 25% 1,2-propanediol in phosphate-buffered saline is the least toxic combination for mouse embryos, keeping the vitrification solution in an amorphous state (Scheffen *et al.*, 1986). It can be concluded that 22.5% glycerol 22.5% 1,2-propanediol in modified TCM199 is effective not only for keeping the vitrification solution in an amorphous state but also for vitrification of bovine IVM–IVF blastocysts, when they are gradually equilibrated to the final vitrification solution.

We thank E. Fuku for useful advice and M. Takahashi, N. Takenouchi, K. Endo and M. Sato for excellent technical assistance.

References

- Aoyagi, Y., Fukui, Y., Iwazumi, Y., Urakawa, M. & Ono, H. (1990) Effects of culture systems on development of *in vitro* fertilized bovine ova into blastocysts. *Theriogenology* **34**, 749–759.
- Brackets, B.G. & Oliphant, G. (1975) Capacitation of rabbit spermatozoa *in vitro*. *Biology of Reproduction* **12**, 260–274.
- Boutron, P. & Kaufman, A. (1979) Stability of the amorphous state in system water-1,2-propanediol. *Cytobiology* **16**, 557–568.
- Douchi, O., Takamura, H. & Imai, K. (1990) Transfer of bovine embryos cryopreserved by vitrification. *Japanese Journal of Animal Reproduction* **36**, 69–72.
- Eyestone, W.H. & First, N.L. (1989) Culture of early bovine embryos with oviduct tissue or in conditioned medium. *Journal of Reproduction and Fertility* **85**, 415–420.
- Goto, K., Kajihara, Y., Kosaka, S., Koba, M., Nakanishi, Y. & Ogawa, K. (1988) Pregnancies after co-culture of cumulus cells with bovine embryos derived from *in-vitro* fertilization of *in-vitro* matured follicular oocytes. *Journal of Reproduction and Fertility* **83**, 753–758.
- Hamano, S. & Kuwayama, M. (1992) Effect of medium supplemented with bovine calf serum on the development of bovine oocytes matured and fertilized *in*

- vitro*. *Japanese Journal of Reproduction Technology* **14**, 6–16.
- Kuwayama, M. & Hamano, S.** (1991) Cryopreservation of bovine blastocysts developed from *in vitro* fertilized oocytes matured *in vitro*. *Japanese Journal of Animal Reproductive Technology* **13**, 165–171.
- Leibo, S.P.** (1984) A one-step method for direct non-surgical transfer of frozen-thawed bovine embryos. *Theriogenology* **21**, 767–789.
- Lu, K.H., Gordon, I., Gallagher, M. & McGovern, H.** (1987) Pregnancy established in cattle by transfer of embryos derived from *in vitro* fertilization of oocytes matured *in vitro*. *Veterinary Record* **121**, 259–260.
- Massip, A., Van der Zwalm, P., Scheffen, B. & Ectors, F.** (1986) Pregnancies following transfer of cattle embryos preserved by vitrification. *Cryo-Letters* **7**, 270–273.
- Massip, A., Van der Zwalm, P., Scheffen, B. & Ectors, F.** (1989) Some significant steps in the cryopreservation of mammalian embryos with a note on a vitrification procedure. *Animal Reproduction Science* **19**, 117–129.
- Parrish, J.J., Susko-Parrish, J.L., Leibfried-Rutledge, M.L., Crister, E.S., Eyestone, W.H. & First, N.L.** (1986) Bovine *in vitro* fertilization with frozen thawed semen. *Theriogenology* **25**, 591–600.
- Rall, W.F. & Fahy, G.M.** (1985) Ice-free cryopreservation of mouse embryos at -196°C by vitrification. *Nature* **313**, 573–575.
- Scheffen, B., Van der Zwalm, P. & Massip, A.** (1986) A simple and efficient procedure for preservation of mouse embryos by vitrification. *Cryo-Letters* **7**, 260–269.
- Van der Zwalm, P., Touati, K., Ectors, F.J., Massip, A. & Ectors, F.** (1989) Vitrification of bovine blastocysts. *Theriogenology* **31**, 270 (abstract).
- Xu, K.P., Greve, T., Calleses, H. & Hyttle, P.** (1987) Pregnancy resulting from cattle oocytes matured and fertilized *in vitro*. *Journal of Reproduction and Fertility* **81**, 501–504.

Received 30 July 1991