Vitrification of Bovine Blastocysts on a Membrane Filter Absorbing Extracellular Vitrification Solution[1)]

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Abstract: *The purpose of this study was to test the hypothesis that an extracellular vitrification solution might be unnecessary after equilibration of bovine blastocysts with a vitrification solution. We assessed hatching in culture and the development to normal calves of bovine blastocysts vitrified on a nitrocellulose membrane filter that absorbed extracellular vitrification solution. Twenty in vitro produced bovine blastocysts were vitrified. All embryos were collected after warming and classified as excellent at the start of culture and all hatched between 48–96 h of culture. Transfer of three vitrified blastocysts derived in vivo given to three recipients resulted in the birth of one healthy calf on December 3, 2004. These results indicate that an extracellular vitrification solution is unnecessary after equilibration of embryos and that this new method of vitrification of embryos on a membrane filter is useful for the cryopreservation of bovine blastocysts.*

Key words: *Vitrification, Bovine blastocyst, IVF, ET*

Introduction

Vitrification has been widely used in the cryopreservation of mammalian oocytes and embryos. Recently various improved vitrification methods for oocytes and embryos have been reported which utilize increased cooling rates through use of a container such as an electron microscope grid [1], open-pulled straw [2], gel-loading tip [3], container-less micro-droplet [4], cryoloop [5] or nylon loop [6]. The most important aspect of these vitrification methods is minimization of the volume of extracellular vitrification solution to obtain a higher cooling rate. However, even if the volume of extracellular vitrification solution is decreased, the vitrification solution is still present around the oocytes or embryos in these methods [1–6]. It is not the extracellular solution, but the embryo itself that we need to vitrify in cryopreservation. Therefore, we hypothesized that the extracellular vitrification solution might be unnecessary after equilibration of embryos with the vitrification solution. The purpose of this study was to test this hypothesis using a membrane filter as a bovine blastocyst container to absorb the extracellular solution. We examined hatching in culture and development to normal calves of bovine blastocysts vitrified on a nitrocellulose membrane filter.

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Materials and Methods

In the present study we used bovine blastocysts which were either produced *in vitro* or derived *in vivo*.

Blastocysts produced in vitro

Cumulus-oocyte complexes (COCs) were collected by ultrasound-guided follicle aspiration from donor heifers and cows. The collected COCs were washed and cultured for 24 h at 39°C under 5% CO_2 in humidified air in 10 mM HEPES-buffered TCM-199 (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 0.3 mM sodium pyruvate, 1.5% 2nd fraction of ultracentrifuged bovine follicular fluid (bFF), 0.5 IU/ml hFSH (containing 2.31 IU/ml LH, Biogenesis, England, UK), 1 μg/ml estradiol-17β (Sigma, Chemical Co., St. Louis, MO, USA), 3 mg/ml polyvinylpyrrolidone (PVP, *Mr* 40000, Sigma) and 65 μg/ml dibekasin sulfate (Meiji-Seika, Tokyo, Japan). The 2nd fractions of bFF were

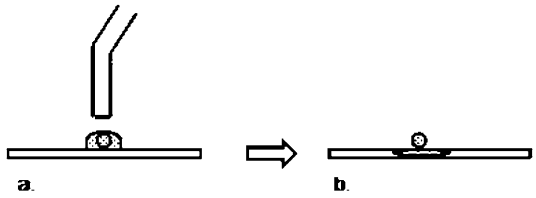

Fig. 1. Vitrification method without an extracellular solution using a nitrocellulose membrane filter. (a) A blastocyst with a small volume of vitrification solution is placed on the membrane filter. (b) Extracellular solution is absorbed by the membrane filter. Then, the membrane filter with the blastocyst is plunged into and stored in liquid nitrogen.

prepared by ultracentrifugation of bFF at 220,000 g, 4° C for 48 h. Frozen-thawed spermatozoa of Japanese Black cattle were suspended in PVP-/BSA-free fertilization medium, referred to as FM [7], supplemented with 10 mM caffeine (sodium benzoate, Sigma), and washed twice by centrifugation at 700 g for 5 min. The washed spermatozoa were resuspended in PVP-/BSA-free FM supplemented with 10 mM caffeine. The sperm suspension was diluted 1:1 with BSA-free FM supplemented with 1.0 mg/ml D-penicillamine (Sigma), 0.5 mM methyl-β-cyclodextrin (Sigma), 1.0 mM citrate (sodium citrate-trisodium salt, Kanto Chemical Co., Tokyo, Japan) and 2 mg/ml PVP. The spermatozoa were preincubated for 4 h at 39° C under 5% CO_2 in humidified air to induce capacitation. After preincubation, 5 μl of the sperm suspension was introduced into 40 μl droplets of FM with 1.0 mg/ml PVP instead of BSA. After 30 min, the eggs were introduced into these droplets for insemination. Six hours after insemination, the eggs were freed from cumulus cells by repeated passage through a pipette with a narrow bore, and cultured in 20% RD (RPMI1640 and Dulbecco's MEM, 1:1 v/v) · mKSOM/aa [8] under humidified 5% CO_2-5% O_2-90% N_2 at 39° C for 144–196 h after insemination. Ninety-six hours after insemination, embryos at the 8-cell stage or later were transferred into the culture medium supplemented with 10 μM β-mercaptoethanol (β-ME, Sigma). After 120–144 h insemination, embryos at the morula stage or later were transferred to fresh culture medium supplemented with 50 μM β-ME.

Collection of blastocysts derived in vivo

Two F_1 (Salers ♂ × Japanese Shorthorn ♀) beef cows from Yakumo Farm of Kitasato University Field Science Center in Hokkaido were used as embryo donors. The blastocysts were collected by flushing the uterine horns of the donor cows with superovulation or spontaneous ovulation on Day 7 (Day of estrus = Day 0).

Vitrification

The basic medium used for vitrification was Whittingham's modified PB1 medium [9] supplemented with 0.1% PVP instead of BSA, 14.8 mM L-proline and 0.2 M trehalose. The basic medium was supplemented with 7% (v/v) ethylene glycol (EG) and 0.5% (v/v) glycerol (G) was used as the equilibration solution. The vitirification solution was the basic medium with 30% (v/v) EG, 0.5% (v/v) G and 0.5 M sucrose. The blastocysts were introduced into 0.2 ml of the basic medium (39° C) in a 35 mm culture dish. Immediately, the culture dish containing blastocysts was stored at 15° C for 10 min. The blastocysts were then transferred into 0.2 ml of the equilibration solution (15° C), and kept for 5 min at 15° C, then transferred into 2 ml of the vitrification solution (4° C). After 20–30 sec, each blastocyst was loaded into a glass capillary with about 5 μl of vitrification solution and placed on a nitrocellulose membrane filter (13 mm in diameter, 8.0 μm pore size, Millipore, Bedford, MA) (see Fig. 1-a). Next the vitrification solution covering the blastocyst was absorbed with the membrane filter (see Fig. 1-b), and the membrane filter with the blastocyst was plunged into liquid nitrogen (LN_2) and placed in a specially ordered a membrane filter container made of stainless steel (see Fig. 2-A, 15 mm in outer diameter, 9 mm in height, Misawa Medical Co., Tokyo, Japan) in LN_2 gas. Thereafter, this container was put in a specially ordered container case (Misawa Medical Co, see Fig. 2-B) and stored in LN_2 for 1–82 days.

Experimental design

Experiment 1: Morphological observation after blastocyst warming

The container was taken out from the container case in LN_2 gas, and the membrane filter with the vitrified blastocyst was taken out from the container with forceps and immediately introduced into 2 ml of Whittingham's modified PB1 (39° C) supplemented with 0.1% PVP, instead of BSA, containing 0.3 M sucrose and kept for 5 min at 39° C. Subsequently, the blastocysts were recovered from the membrane filter by gentle rinsing. The blastocysts were washed and cultured in 20% RD·mKSOM/aa with 1% BSA and 100 μM β-ME under 5% CO_2-5% O_2-90% N_2 with high humidity at 39° C. To assess hatching of blastocysts produced *in vitro*, we

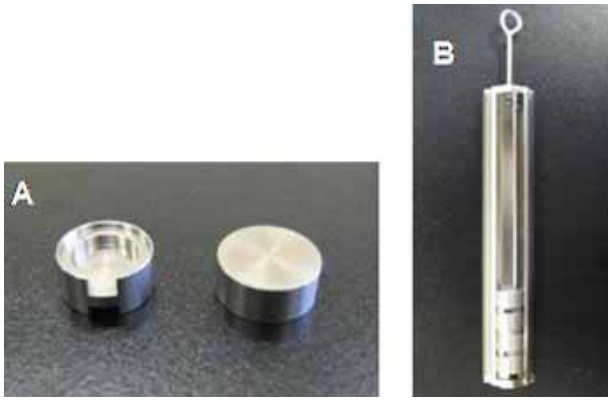

Fig. 2. A membrane filter container (A, left: body, right: lid) and the container case (B) in which 11 membrane filter containers can be placed.

Fig. 3. Morphologic appearance of a bovine blastocyst produced *in vitro* after vitrification/warming.

used BSA instead of PVP, because a significantly higher hatching rate was accomplished (unpublished data). Embryonic morphology was evaluated soon after removal of the vitrification solution from the embryos and the start of culture. The blastocysts were cultured for 96 h to observe hatching.

Experiment 2: Embryo transfer after warming

Twenty-two blastocysts derived *in vivo* were vitrified and stored in LN_2. Three of them were warmed in order of vitrification and recovered. Each blastocyst was transferred non-surgically into the uterine horn ipsilateral to the corpus luteum of each recipient cow on Day 7 of the estrous cycle (day of estrus = Day 0). The recipient cows were synchronized by treatment with a progesterone device (CIDR, InterAg, Hamilton, New Zealand) for eight days.

Results

Experiment 1

All twenty vitrified embryos were recovered after warming. All of them were classified as excellent (see Fig. 3) at the start of culture and all hatched after 48–96 h of culture.

Experiment 2

All three embryos were collected after vitrification/warming and classified as excellent. One of three recipient cows delivered a normal male calf (38.5 kg birth weight) on Day 283, December 3, 2004. The other two recipient cows showed the return of estrus on Day 56 and Day 21, respectively.

Discussion

Bovine blastocysts produced by *in vitro* systems (IVM · IVF · IVEC) usually have low cryotolerance; however, in the present study all the embryos produced *in vitro* and vitrified by our method without extracellular solution were classified as excellent and hatched in culture after cyropreservation and warming. Furthermore, one out of three blastocysts derived *in vivo* developed into a normal calf after transfer. These results clearly show that this vitrification method without an extracellular solution was quite effective for cryopreservation of bovine blastocysts. Recently, successful improved vitrification methods have been reported [1–6]. The common ground for the improvement is the minimization of the vitirification solution in order to increase the rates of cooling and warming during the vitrification and devitrification process. In marked contrast to these vitrification methods, the vitrification method described in this study is characterized by the absence of an extracellular vitrification solution around the embryo. Kuwayama [10] reported that an improvement in the cooling rate and minimization of the toxicity of the extracellular vitrification solution by decreasing the volume of vitrification solution may promote vitrification efficiency. The advantages of the present vitrification method, without vitrification solution, seem to be derived from the absence of extracellular solution around the embryo. The efficiency of vitrification of the embryo itself and minimization of vitrification damage to the embryo are promoted all the better for the absence of extracellular solution around the embryo.

Massip [11] indicated that under the same freezing

protocol, there are differences between species at the same embryo developmental stage and embryos at all developmental stages in the same species do not survive equally. He [11] also reported that some attempts to replace bovine serum / BSA with chemically defined macromolecules for cryopreservation, to avoid sanitary problems, should be noted. However, these macromolecules such as polyvinyl alcohol and sodium hyaluronate [12–16], are not equivalent to serum or BSA. In the present experiment, the vitrification solution that did not contain any serum / BSA and it appears to have been successful in the vitrification of bovine blastocysts. This approach will help prevent diseases transmitted by serum / BSA in the international trade of cryopreserved embryos.

On the basis of the results in the present study, our technically easy, new vitrification method of embryos on a membrane filter that absorbs extracellular vitrification solution could be widely applied to the cryopreservation of mammalian oocytes and embryos.

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