VIABILITY OF BOVINE BLASTOCYSTS OBTAINED AFTER 7, 8 OR 9 DAYS OF CULTURE IN VITRO FOLLOWING VITRIFICATION AND ONE-STEP REHYDRATION

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ABSTRACT

This study examined morphological appearance, viability and hatching rates in relation to the total cell number following vitrification of in vitro produced bovine blastocysts and expanded blastocysts. In Experiment 1, embryos obtained after 7, 8 or 9 d of culture were pooled and equilibrated in either 10% ethylene glycol (EG) or 10% EG plus 0.3M trehalose in Dulbecco's phosphate buffered saline (DPBS) supplemented with 10% calf serum and 0.6% BSA for 5 min each, at room temperature, and then vitrified together in precooled vitrification solutions consisting of 40% EG (Treatment 1), 40% EG plus 0.3M trehalose (Treatment 2), 40% EG plus 0.3M trehalose and 20% polyvinylpyrrolidone (PVP, Treatment 3) in DPBS. The embryo viability and hatching rates of Treatment 1 (19 and 3%) differed significantly (P<0.05) from those of Treatment 2 (56 and 31%) and Treatment 3 (70 and 43%). There was a significant difference (P<0.05) in embryo viability between Treatment 2 (31%) and Treatment 3 (43%). In Experiment 2, Day 7, 8 and 9 embryos were vitrified separately, with higher viability and hatching rates in Experiment 1 than in Experiment 2. The viabilities of Day 7 (87%), 8 (71%) and 9 (46%) embryos differed significantly (P<0.05). Again, there were significant differences (P<0.01) among the hatching rates of Day 7 (75%), 8 (38%) and 9 (9%).

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embryos. The total cell number of hatched blastocysts was then determined by differential fluorochrome staining. The total cell number of Day 7, 8 and 9 embryos differed significantly (P<0.05).

Key words: vitrification, ethylene glycol, trehalose, polyvinylpyrrolidone, embryo aging

INTRODUCTION

Currently, vitrification along with the conventional freezing procedures is widely used for the cryopreservation of embryos and oocytes. Several researchers have tried various vitrification solutions to preserve embryos of species ranging from the mouse to the cow. The efficacy of various vitrification and rehydration solutions has also been investigated (21, 23, 27, 32, 40-42, 44-47).

Determinations in the field of cryobiology are still dependent primarily on visual or morphological examination and on pregnancy rates after the transfer of cryopreserved embryos. To achieve optimal success after cryopreservation research into the physical and chemical changes that an embryo must endure if it is to survive and develop normally are needed (26). The mechanism by which a freeze-thaw cycle kills mammalian cells is poorly understood, although a variety of mechanisms have been proposed such as thermal shock or cold shock injury (15, 35), osmotic dehydration (36), denaturation of vital cellular structures by increased dissolved electrolyte concentration (33, 34) and recrystallization (49). Moreover, not only the biochemical aspects of cryoprotectant toxicity need to be understood but also the basis for the detrimental effects of cryoprotectants (8-14).

There are several studies on the success of cryopreservation in relation to the age and time of blastocyst formation (3-6, 17, 18, 38, 47, 54). As we know the developmental rate of in vitro produced embryos on Day 9 after insemination was lower than that of on Day 7 or 8 (52). In other words, the later developing blastocysts are of poorer quality as judged by the lower cell number (20).

In this study our objectives were to examine 3 types of vitrification solutions and to select the best one for the vitrification of in vitro produced bovine blastocysts and expanded blastocysts obtained after 7, 8 or 9 d of culture.
MATERIALS AND METHODS

Ovaries were obtained from local slaughterhouse and brought to the laboratory within 3 h of collection in Ringer’s solution supplemented with penicillin-G (100 IU/ml) and streptomycin (0.2 µg/ml) at 30 to 32°C. Cumulus-oocytes complexes (COCs) were aspirated from small antral follicles (< 5 mm in diameter) with an 18-g needle and a 5-ml syringe with DPBS supplemented with 0.3% BSA. After collection, the COCs were washed twice with the maturation medium (25 mM-Hepes TCM-199 with Earles salt, Gibco, Grand Island, NY, USA) supplemented with 5% superovulated cow serum (SCS), 0.01 mg/ml FSH (Denka Pharmaceutical Co., Kawasaki, Japan) and 50 µg/ml gentamycin sulfate (Sigma Chemical Co., St. Louis MO, USA) in culture dish (35 mm diameter, Falcon, Becton Dickinson Co., Ltd., Oxnard, CA, USA). Thereafter, the COCs were placed into the maturation medium, covered with paraffin oil and cultured for 21 to 22 h at 38.5°C in 5% CO₂ in air.

Frozen semen from a single bull were thawed in water bath (30 to 35°C), washed with Brackett and Oliphant’s medium (BO medium; 1) supplemented with 5 mM-Caffeine (Caff-BO) by centrifugation at 500 g for 5 min. The sperm pellet was resuspended in Caff-BO supplemented with 1% bovine serum albumin (BSA, Sigma) and 20 µg/ml heparin for a sperm concentration of 5x10⁶/ml. A 100 µl- aliquot of the sperm suspension under mineral oil was incubated for 1 h at 38.5°C in 5% CO₂ in air prior to insemination. Oocytes matured in vitro were transferred into sperm droplets (20 to 30 oocytes/droplet) for insemination. After incubation for 5 h the oocytes were washed 2 to 3 times and transferred to the culture medium (TCM-199) supplemented with 5% SCS, 5 µg/ml insulin and 50 µg/ml gentamycin for development using the cumulus cell co-culture system.

Experiment 1

The blastocysts and expanded blastocysts developed on Day 7, 8 and 9 of culture (insemination = Day 0) were utilized. The embryos were kept in 10% ethylene glycol in DPBS supplemented with 10% calf serum and 0.6% BSA (mPBS; equilibration solution-I, ES-I) for 5 min, then in 10% ethylene glycol plus 0.3M trehalose in mPBS (equilibration solution II, ES-II) for 5 min. Finally, the embryos were kept in vitrification solution containing 40% EG (Treatment 1), 40% EG + 0.3M trehalose (Treatment 2), or 40% EG + 0.3M trehalose + 20% polyvinylpyrrolidone (PVP, Treatment 3) in DPBS for 1 min. The first 2 media (10% EG and 10% EG plus 0.3M trehalose) were at room
temperature but the third vitrification solution was pre-cooled on ice. The embryos were placed in 0.25-ml plastic straws which were immediately immersed horizontally in liquid nitrogen (LN₂) for storage. The vitrified embryos were warmed in a water bath at 30°C for 10 to 20 sec, and the contents were expelled directly into 2 ml of mPBS. The embryos were washed 2 to 3 times in fresh mPBS, and their immediate post-warmed viability was assessed morphologically. The embryos were then cultured in medium, and their viability or hatching was recorded visually by re-expansion or hatching of the vitrified-warmed embryos at 24 to 48 h.

Experiment 2

The blastocysts and expanded blastocysts developed on Days 7, 8 and 9 were vitrified separately in a way similar to that in Experiment 1, with 1 of the 3 media yielding higher embryo viability and hatching. Random in vitro produced blastocysts and expanded blastocysts from Day 7, 8 or 9 were considered as controls.

The total cell number of hatched blastocysts was then determined by differential fluorochrome staining (19, 53). The hatched blastocysts were rinsed in fresh culture medium and incubated in DPBS containing propidium iodine (PI, 10 µg/ml) and bisbenzimide (10 µg/ml) for 30 min in an incubator at 38.5°C and 5% CO₂ in air. The embryos were washed in DPBS supplemented with 0.3% BSA and pressed on to a glass slide and then examined under a fluorescence microscope. This resulted in vital (bisbenzimide-positive) nuclei fluorescing blue and nonvital nuclei fluorescing pink (PI-positive). Total cell numbers for 20 embryos were determined for each treatment. If the embryos did not hatch after 72 h of culture they were treated with 0.25% pronase (Pronase E, Sigma) in TCM-199 for 4 to 6 min to dissolve the zona pellucida.

RESULTS

The results of Experiment 1 are presented in Figure 1. Embryo viability and hatching in Treatment 1 (34/175, 19% and 6/175, 3%) differed significantly (P<0.01) from those of Treatment 2 (47/84, 56% and 26/84, 31%) and Treatment 3 (137/195, 70% and 83/195, 43%). There was a significant difference (P<0.05) in embryo viability between Treatment 2 (56%) and Treatment 3 (70%).

In Figure 2, the results of the first part of Experiment 2 are shown. The viabilities of Day 7 (70/80, 87%), Day 8 (51/71, 71%) and Day 9 (30/65, 46%) embryos differed significantly (P<0.05). Again,
there were significant differences (P<0.01) among the hatching rates of Day 7 (60/80, 75%), 8 (27/71, 38%) and 9 (6/65, 9%) embryos.

![Bar graph showing viabilities of in vitro produced bovine blastocysts and expanded blastocysts following vitrification with different treatments.](chart)

Figure 1. Viabilities of in vitro produced bovine blastocysts and expanded blastocysts following vitrification with 40% EG (Treatment 1), 40% EG + 0.3M trehalose (Treatment 2) or 40% EG + 0.3M trehalose + 20% PVP (Treatment 3) and one-step rehydration (ab, P<0.05; AB, P<0.01, Chi-square test).

The results of the second part of Experiment 2 are tabulated in Table 1. The total cell numbers for each day of development differed among the embryos (P<0.05), and the percentage of live cells on Days 7 and 8 and in control embryos differed significantly (P<0.05) from that of Day 9 embryos.

**DISCUSSION**

Three types of cryoprotectant media were tested for vitrifying embryos. The optimal medium would allow for high embryo viability and hatching. These 2 characteristics (high embryo viability and hatching) were observed with 40% ethylene glycol, 0.3M trehalose and 20% PVP (Treatment 3), and 40% ethylene glycol plus 0.3M trehalose (Treatment 2), while 40% ethylene glycol alone (Treatment 1) yielded lower rates of viability and hatching. The better results in Treatments 2 and 3 might be due to the addition of a nonpermeating carbohydrate (NPC), trehalose and trehalose plus a macromolecular component, PVP.
The molecular weight of ethylene glycol is lower (62.07) than that of glycerol (92.10), propylene glycol (76.10) and DMSO (78.13). Therefore, sufficient permeation of ethylene glycol into embryos for vitrification takes place in a shorter period of time than that for other cryoprotectants, and thus removal of ethylene glycol from the embryos during dilution is faster than with other vitrification solutions, resulting in a decrease in the rate of embryo toxicity (29).

Trehalose reduces toxicity in association with PVP by causing embryos to shrink rapidly (21). When the embryos are exposed to cryoprotectant plus trehalose solution, only the cryoprotectant permeates the cells, the extra osmolarity created by trehalose causes dehydration, which reduces the occurrence of intracellular ice formation (24, 25, 32, 50, 51). Trehalose maintains a high osmotic pressure in the extracellular medium during removal of the cryoprotectant. This agent also prevents osmotic shock due to diffusion of cryoprotectant out of the embryos after warming. Upon warming, the presence of trehalose in the cryoprotectant restricts water movement across the membranes, preventing lysis of embryonic cells during diffusion of the

Figure 2. Viabilities of in vitro produced bovine blastocysts and expanded blastocysts obtained after 7, 8 or 9 d of culture following vitrification using 40% EG + 0.3M trehalose + 20% PVP and one-step rehydration (ab, P<0.05; ABC, P<0.01, Chi-square test).

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cryoprotectant out of the embryo (32). In this study, along with ethylene glycol (cryoprotectant) and trehalose (sugar, as a nonpermeating agent), a macromolecular component (PVP) was tried: PVP is a large, interface-seeking molecule, and it is suggested that PVP coats the cells immediately following warming, giving them mechanical protection against osmotic stresses. The mechanism of protection of the large polymer PVP (molecular weight = average 30,000) is not clear (2, 43). Perhaps it prevents osmotic injury during the rapid removal of extracellular water, or the extracellular polymers coat the cells and protect embryo membrane from denaturation (37). Loss of lipoprotein from the membrane made it permeable to cation and to osmotic lysis on warming. A coating of polymer may either prevent denaturation or stabilize the membrane against subsequent osmotic stress (28).

Table 1. Assessment of embryo total cell number in relation to embryo age after vitrification using 40% EG + 0.3M trehalose + 20% PVP

<table>
<thead>
<tr>
<th>Day in culture</th>
<th>Total no. of cells</th>
<th>Live : dead cell ratio</th>
<th>Percentage of live cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>128±24</td>
<td>125:3</td>
<td>98a</td>
</tr>
<tr>
<td>8</td>
<td>120±29</td>
<td>117:3</td>
<td>97a</td>
</tr>
<tr>
<td>9</td>
<td>109±11</td>
<td>103:6</td>
<td>95b</td>
</tr>
<tr>
<td>Control</td>
<td>122±11</td>
<td>119:3</td>
<td>98a</td>
</tr>
</tbody>
</table>

Differences in means within columns were estimated by Duncan's multiple range test (abcP<0.05).

Day 7, 8 and 9 blastocysts and expanded blastocysts were vitrified separately to analyze the effect of aging, with the cryoprotectant medium in Experiment 1 having the better results (40% EG + 0.3M trehalose + 20% PVP). Higher embryo viability and hatching were obtained when Day 7 embryos were used (Figure 2). Viability and hatching rates of Day 7, 8 and 9 embryos differed significantly (P<0.05). Higher viabilities of Day 7 in vitro produced bovine blastocysts and expanded blastocysts than that of Day 8 or 9 might be due the younger stages of the embryos at Day 7 and to the maximum total cell numbers (Table 1). The lower survival rate for Day 9 to 10 blastocysts was shown to be accompanied by a lower cell number (4). Embryos on Day 7 survive better than on Day 8 or 9. The younger embryos are more vigorous, thus they are more likely to survive (17,
22). Moreover, Day 7 blastocysts result in higher pregnancy rates upon transfer than Day 8 or 9 embryos (18, 38). It is observed that forceful contractions of the whole cell mass in the bovine blastocyst after freezing and thawing leads to a disturbance in the hatching process. These are due to the damage of some structures between the trophoblast cells of the embryos during freezing and thawing (31), and may account for some of the disturbance to the normal hatching process of Day 8 embryos (38).

The biochemical state of the intact fresh cells influences their capacity to withstand the rigors of freezing and thawing. Younger cells are biochemically different from older cells; the younger cells are less dense, contain more K+ and less Na+ and are more resistant in hypotonic media (7). It has been observed that the levels of glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase and phosphohexose isomerase are markedly higher in young than in old cells (30).

The shape of each inner cell mass (ICM) from fresh expanded and hatched blastocysts are sharply defined, but the ICM cells from frozen blastocysts are partially distorted. The cell-to-cell contact of the ICM from frozen blastocysts are less tight than that from fresh blastocysts. This suggest that the cell-to-cell contact is lost during freezing and thawing (19). The viability of ICM of frozen-thawed embryos tends to be lower than that of fresh embryos irrespective of the cryoprotectant used (53). In the present experiment importance was given to the whole embryo rather than only to the ICM. The trophectoderm is specialized for attachment and invasion of the uterine endometrium during implantation (16); therefore it is also equally important in the developmental process. There was a significant difference (P<0.05) observed among the total cell numbers for each day of culture (Table 1). The cell number therefore, may be an important factor in the successful development of younger stage embryos (4, 7, 20, 22, 38, 52). In Experiment 2 a trial was conducted to assess live to dead cell ratios of post vitrified-warmed embryos on Days 7, 8 or 9 using differential fluorochrome staining (19, 53). The results showed a significantly (P<0.05) higher number of live to dead cells in Day 7 and 8 embryos and in the controls than in Day 9 embryos (Table 1). But it is possible that propidium iodide may not reach cells of the inner cell mass due to various types of junctional specializations between trophectodermal cells and ICM cells, and between trophectodermal and ICM cells. Thus it may be possible that there is a population of dead cells that are not stained and ultimately not counted. To avoid this possibility further investigation is needed (Figure 3).
From this experiment it may be concluded that the vitrification solution containing 40% ethylene glycol plus 0.3M trehalose and 20% PVP is the best among the three vitrification solutions tested to obtain high percentage of embryos viability and embryos hatching. Our results suggest that to obtain best results, in vitro produced bovine embryos must be vitrified on Day 7 rather than Day 8 or 9.

Figure 3. Day 7 (A) and Day 9 (B) in vitro produced bovine vitrified embryo after differential fluorochrome staining (arrows indicate dead cells).

Finally, as we know, the greatest challenge for successful vitrification is the formulation of a vitrification solution and effective equilibration procedure to achieve the necessary degree of cellular dehydration and avoid significant toxic or osmotic injury (41, 42). Generally, the choice of vitrification solution (composition, concentration) and equilibration conditions (time, temperature) depend on the permeability characteristics of the cells in question (40, 42). In addition to some recent developments (3, 6, 48) additional research is necessary to achieve better success (39).

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