Normal Calves Obtained after Direct Transfer of Vitrified Bovine Embryos Using Ethylene Glycol, Trehalose, and Polyvinylpyrrolidone

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In the present study, IVF bovine embryos were vitriﬁed using as the cryoprotectants, ethylene glycol plus trehalose plus the polymer, polyvinylpyrrolidone (PVP). In Experiment I, toxicity of the vitriﬁcation solution (VS) containing 20% PVP was tested in relation to temperature and exposure time. One hundred percent embryo development was observed with treatment at 5°C for 5 min, whereas only 55.5% embryos were developed when the treatment was carried out at 20°C for 5 min. In Experiment II, embryos were vitriﬁed using one of the three treatments (Treatment A, 40% ethylene glycol (EG); treatment B, 40% EG + 11.3% trehalose; and treatment C, 40% EG + 11.3% trehalose + 20% PVP and rehydrations) was performed directly in mPBS. Highest development (84.1%) and hatching rate (68.2%) were obtained when embryos were vitriﬁed with the vitriﬁcation solution used in treatment C. In Experiment III, embryos were vitriﬁed as in Experiment II (treatment C). The development and hatching rates were compared after rehydration in different rehydration solutions. No signiﬁcant difference was observed among the development and hatching rates when rehydration was carried out in different concentrations of trehalose. Five vitriﬁed-warmed bovine embryos were transferred directly to five recipients and three recipients gave birth to three normal calves.

Vitriﬁcation has attracted attention as an embryo cryopreservation technique because it is easy, quick, and economical (36). This procedure offers considerable promise for simplifying and improving the cryopreservation of cells because controlled-rate freezing equipment is not required and potential injury associated with formation of ice in the suspension is eliminated (35). Leibo (24) suggested that vitriﬁcation procedures will have important practical as well as fundamental implications for embryo preservation. Niemann (34) concurred, saying that vitriﬁcation is reliable under ﬁeld or laboratory conditions. The vitriﬁcation procedure is effective not only as a simpliﬁcation of the freezing protocol but also because it yields high survival of in vitro matured, fertilized, and cultured (IVMFC) bovine embryos after thawing (21). The ﬁrst successful vitriﬁcation of bovine embryos was achieved by Massip et al. (27).

Age and time of blastocyst formation are important factors for successful freezing of bovine IVMFC embryos. The reduced survival rates for Day 9 to 10 blastocysts have been shown to be accompanied by a lower cell number. The most appropriate age and developmental stages for freezing of IVMFC bovine embryos in ethylene glycol were the Day 7 and 8 blastocysts (7, 8, 13, 18, 21, 43). Saha et al. (42) reported that Day 7 IVF bovine blastocysts survive vitriﬁcation and direct dilution after warming.

Sucrose as well as other carbohydrates such as trehalose are effective in preserving the structural and functional integrity of membranes at low water activities (28). During gradual equilibration of embryos in the cryoprotectant solution, water is withdrawn and substantial amounts of cryoprotectant enter the embryonic cells; at the same time nonpermeating carbohydrates such as trehalose or sucrose also dehydrate the embryonic cells, a process which is very important for successful vitriﬁcation (41). In the ultrarapid freezing technique involving vitriﬁcation, the addition of trehalose to dimethyl sulfoxide (DMSO,
Me₄SO) offers significantly better results than the addition of sucrose (38). Trehalose has a greater stabilizing effect on cell membranes than sucrose (45). In vitro produced embryos can be successfully cryopreserved using ethylene glycol and trehalose or sucrose as a cryoprotectant (29).

Ethylene glycol and other glycols were first shown to protect mouse and rat embryos from freezing damage by Miyamoto and Ishibashi (31, 32). Glycol derivatives have strong and numerous hydrogen-bonding sites (11). Polyvinylpyrrolidone (PVP) was first introduced as a cryoprotectant by Bricka and Bessis in 1955 (6) and the successful freezing of mouse embryos by using PVP was reported in 1971, although the embryos did not survive if they were held at −79°C for more than 30 min (48). One year later, Whittingham et al. were able to explain the limitations of the previous report (49). No survival was obtained with rabbit embryos frozen in the presence of PVP, glycerol, or sodium acetate (2). Recently, Leibo and Oda (25) observed that it was possible to freeze mouse zygotes and embryos slowly or rapidly using ethylene glycol plus PVP, the actual result being dependent on the ethylene glycol concentration, the warming rate, and the dilution method. Saha et al. (39, 40) used PVP (0, 5, 10, and 20%) with 40% EG and 0.3 M trehalose to vitrify IVF bovine, Day 7–9 blastocysts and expanded blastocysts. Kobayashi et al. (18, 19) used PVP to freeze bovine and porcine embryos.

In this study, our objectives were, first, to vitrify IVF bovine embryos very simply with only one type of cryoprotectant, second, to determine the efficacy of trehalose as a non-permeating cryoprotectant and polyvinylpyrrolidone as a macromolecular component, and third, to assess the survival of vitrified embryos after direct transfer to recipients.

MATERIALS AND METHODS

Oocyte Collection and In Vitro Maturation

Ovaries were obtained from a local slaughter house and were 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 30–32°C. Cumulus–oocyte complexes (COCs) were aspirated from small antral follicles (<5 mm in diameter) with an 18-gauge needle attached to a 5-ml syringe containing modified PBS. After collection, COCs were washed twice with the maturation medium (TCM 199, Earle’s salts, L-glutamine, 2200 mg/liter sodium bicarbonate, 25 mM HEPES buffer, Gibco, Grand Island, NY) supplemented with 5% superovulated cow serum (SCS), 0.01 mg/ml follicle-stimulating hormone (FSH, Denka Pharmaceutical Co., Kawasaki, Japan) and 50 µg/ml gentamycin sulfate (Sigma Chemical., St. Louis, MO) in 1% insulin, and 50 µg/ml gentamycin for development.
Embryo Evaluation

After 48 h of insemination, the culture medium was changed and the embryos were cultured in the same plate until Day 7. Only excellent and good quality embryos which had developed into blastocysts and expanded blastocysts (diameter, approximately 200–230 μm) on Day 7 (IVF = Day 0) were used for the experiments.

Preparation of Vitrification Media

First, modified phosphate-buffered saline (mPBS) was prepared by adding 10% (v/v) superovulated cow serum, 0.3% (w/v) BSA, and 50 μg/ml gentamycin to simple Dulbecco’s phosphate-buffered saline (DPBS, Gibco). Before being placed in the final vitrification solution, the embryos were put into two other media. The first was made up of mPBS supplemented with 10% EG and the second was made up of 10% ethylene glycol with additional 11.3% trehalose. Three vitrification solutions (VS) were prepared by adding 40% EG alone or 40% EG + 11.3% trehalose or 40% EG + 11.3% trehalose + 20% PVP (PVP, Denka Pharmaceutical Co., Kawasaki, Japan) in DPBS.

Vitrification Test of Media

The vitrification solutions were tested for visual evidence of vitrification. Three different types of solutions were put into separate 0.25-ml plastic straws and plunged directly into liquid nitrogen (LN2). After 5 min the straws were put in a 30°C water bath. Throughout cooling and warming, the medium inside the straws remained glassy and transparent.

Preparation of Rehydration Solutions

Four solutions were used to dilute the cryoprotectant solutions after the embryos had been exposed to them; these contained 0, 11.3, 22.7, and 34.0% trehalose in mPBS.

Vitrification of Embryos

The embryos were put into 10% EG for 5 min and then into 10% EG + 11.3% trehalose for 5 min at room temperature. Then the embryos were placed in one of the vitrification solutions, which was kept on an ice block, for 1 min. They were then introduced into 0.25-ml plastic straws (Fig. 1) and plunged in a horizontal orientation directly into LN2 for storage. For warming, the straws were placed in a water bath at 30°C. The embryos were transferred to the rehydration solution for 2–3 min depending on experiment, washed with mPBS two to three times, and finally put into culture medium and placed in the CO2 incubator.

Evaluation of Warmed Embryo

The appearance of the embryos was evaluated once immediately after warming and then at 24-h intervals for at least 3 days. The development rate was assessed by the reexpansion of the blastocoel.

The experiments were divided into four parts:

Experiment I. Embryos were suspended in the vitrification solution containing 40% EG in 11.3% trehalose and 20% PVP. After being held for 5, 10, 15, and 20 min at 5 or 20°C, embryos in the vitrification solution were transferred to 34.0% trehalose for 2 min, washed in mPBS and culture medium, and then cultured. The development of the embryos was assessed by their ability to develop into expanded and hatched blastocysts during 72 h of culture.

Experiment II. After having assessed the toxicity of the vitrification solution, we proceeded to the next step. First, the embryos were put in 10% EG and 10% EG + 11.3% trehalose for 5 min at room temperature. Then embryos were placed in three different types of the vitrification solution, namely, solutions containing 40% EG (treatment A), 40% EG + 11.3% trehalose (treatment B), or 40% EG + 11.3% trehalose + 20% PVP (treatment C), each for 1 min. The embryos were then introduced into 0.25-ml plastic straws and plunged directly into LN2 for storage. Warming was carried out in a 30°C water bath and the contents were drained directly into mPBS.
embryo in v invitritification solution mPBS cotton plug heat seal air vitrification solution

Fig. 1. Configuration of the solution in a 0.25-ml plastic straw loaded with embryos before cooling. Columns of mPBS, small volume of vitrification solution, vitrification solution with embryo, and mPBS were sequentially aspirated into the straw to yield four liquid chambers separated by 5-mm air bubbles.

for rehydration. After rehydration, the embryos were washed and cultured.

Experiment III. In this experiment, the embryos were transferred sequentially into 10% EG and then 10% EG + 11.3% trehalose, for 5 min each, at room temperature (22–25°C). The embryos were then put into the precooled vitrification solution containing 20% PVP, loaded into straws, and finally cooled in LN2 as in experiment II (treatment C). Warming was carried out in a 30°C water bath and the contents were drained into 11.3, 22.7, and 34.0% trehalose solution and also directly into mPBS for rehydration. Finally, all the embryos were washed and cultured.

Experiment IV. Five vitrified and rewarmed compacted bovine morulae (in vivo derived, Day 7), using 40% EG + 11.3% trehalose + 20% PVP, were transferred directly to five recipients (one embryo per recipient) without removing the embryos from the straws in which they were cryopreserved. It should be noted that whereas we used in vitro derived embryos to define “optimum conditions” for vitrification, we used in vivo derived embryos to test ultimate developmental capability of embryos cryopreserved by vitrification in EG + trehalose + PVP.

The data from Experiment I was analyzed by Duncan’s multiple range test and the data from Experiments II and III were analyzed by the $\chi^2$ test.

RESULTS

The data in Table 1 show that the development of embryos exposed to EG + trehalose + PVP decreased both with increased temperature of exposure and increased duration of

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time (min)</th>
<th>Development (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>5</td>
<td>18/18 (100.0)e</td>
</tr>
<tr>
<td>10</td>
<td>20/24 (83.3)d</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>20/27 (74.0)c</td>
<td></td>
</tr>
<tr>
<td>20/27 (74.0)c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>14/22 (63.6)bc</td>
<td></td>
</tr>
<tr>
<td>12/23 (52.1)b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13/29 (44.8)b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3/29 (10.3)a</td>
<td></td>
<td></td>
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<tr>
<td>ANOVA</td>
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<td>Temperature</td>
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<td>Time</td>
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<tr>
<td>Temperature × Time</td>
<td>*</td>
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</tr>
</tbody>
</table>

Note. Mean separation within columns by Duncan’s multiple range test at $P < 0.05$ (Significant at *$P < 0.05$, **$P < 0.01$, respectively).
TABLE 2
Vitrification of IVF Bovine Embryos Using Three Types of Vitrification Solutions and Direct Rehydration in mPBS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of embryos (n)</th>
<th>No. developed (%)</th>
<th>No. hatched (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40% EG</td>
<td>51</td>
<td>27 (52.9)a,A</td>
<td>10 (19.6)a,A</td>
</tr>
<tr>
<td>40% EG + 11.3% trehalose</td>
<td>40</td>
<td>30 (75.0)b</td>
<td>17 (42.5)b</td>
</tr>
<tr>
<td>40% EG + 11.3% trehalose + 20% PVP</td>
<td>82</td>
<td>69 (84.1)B</td>
<td>56 (68.2)B</td>
</tr>
</tbody>
</table>

Note. Values within columns with different letters differ significantly (a,b, P < 0.05; A,B, P < 0.01; χ² test).

exposure. The rate of decrease (development vs time) was higher at 20 than at 5°C. The results are significantly different between 5 and 20°C within each group of time of exposure (P < 0.05).

In Table 2, the results are shown for the experiment in which rehydration was performed directly in mPBS; the highest embryo development (84.1%) was achieved by treatment C, followed by treatment B (75.0%) and treatment A (52.9%). Again, the hatching rate was highest (68.2%) with treatment C. With treatments A and B, the hatching rates were 19.6 and 42.5%, respectively. The embryo development and hatching rates were significantly different (P < 0.05, P < 0.01).

According to the results shown in Table 3, 82.9% embryo development was obtained when rehydration was performed in 22.7% trehalose followed by embryo development rates of 80.0, 79.3, and 75.0% when the rehydration solutions contained 0, 34.0, and 11.3% trehalose, respectively. But a higher rate of hatching of blastocysts was observed when rehydration solutions contained 0% trehalose followed by 34.0, 22.7, and 11.3% trehalose, respectively. Statistical analysis showed no significant difference (χ² test) among the embryo development and hatching rates.

Five vitrified and rewarmed in vivo bovine embryos were transferred to five recipients and three recipients gave birth to three normal calves.

DISCUSSION

In this study, vitrification of bovine embryos was performed in a very simple way with a single type of cryoprotectant. The cryoprotectant used was ethylene glycol, which has the advantage compared to other cryoprotectants of having higher permeability and lower toxicity to embryos (16, 17). In this experiment, trehalose was chosen in place of sucrose as a nonpermeating agent because of its reported improved effectiveness (20, 38, 45). The concentration of trehalose used was 11.3% (0.3 M), a concentration reported to yield better results than other concentrations (10).

It was clear that embryo development decreased with both increased temperature and increased duration of exposure (Table 1). Kasai et al. (17) observed 98% embryo development of mouse embryos with EFS (EG + Ficoll + sucrose) solution at 20°C for 5 min. In this experiment, we obtained 100.0, 83.3, 74.0, and 63.9% embryo development with the vitrification solution (40% EG + 11.3%
trehalose + 20% PVP) at 5°C for 5, 10, 15, and 20 min, respectively. These results are significantly better than those obtained when similar treatments were done at 20°C (55.5, 52.1, 44.8, and 10.3%, respectively). Perhaps the increased permeability of the embryo membrane to the cryoprotectant at the higher temperature and the prolonged exposure to the cryoprotectant caused more cryoprotectant to enter and thereby cause damage (16, 23).

When embryos were vitrified with three types of solution in treatments A, B, and C, and rehydrations were carried out directly in mPBS, only embryos that had been vitrified by treatment C exhibited a higher development and hatching rate (Table 2). Ishimori et al. (15) observed 20, 73, or 85% development (at 24 h) using Day 7 in vivo bovine blastocysts that were vitrified after exposure in a mixture of EG + DMSO in PBS. In this experiment, we obtained 84.1% development using Day 7 IVF bovine blastocysts and expanded blastocysts with 1 min in 40% EG + 11.3% trehalose + 20% PVP in DPBS. Using this technique, the development and hatching rates were very high even after direct rehydration in mPBS. The gradual addition of 11.3% trehalose (treatment B) and 20% PVP (treatment C) with 40% EG (treatment A) increased the percentage of developed and hatched embryos.

The nonpermeating carbohydrate may reduce toxicity that is associated with the macromolecular component by causing the embryos to shrink rapidly and thereby reducing the amount of cryoprotectant in the cells (17). The addition of nonpermeating agents reduces cryoprotectant permeation (17, 46). When the embryos are exposed to the cryoprotectant plus a nonpermeating carbohydrate solution, only the cryoprotectant itself permeates the cells. The extra osmolality created by the nonpermeating carbohydrate causes dehydration, which reduces the formation of intracellular ice (46). On thawing, the presence of a nonpermeating agent in the medium restricts water movement across the membranes, preventing cell lysis during diffusion of the cryoprotectant out of the embryo (28). Also, the nonpermeating agent is involved in active ion transport through the trophectoderm (4). This active transport is controlled by the Na⁺K⁺ATP-ase system which is inhibited by some cryoprotectants (3).

In this study, a macromolecular component had been added to ethylene glycol (cryoprotectant) and trehalose (sugar) in a vitrification mixture. The macromolecule was polyvinylpyrrolidone (M, average 30,000). PVP is a large, interface-seeking molecule. Many years ago, it was suggested that PVP coats the cells and protect the cell membrane from denaturation (33). Loss of lipoprotein EG/DMSO in PBS. In this experiment, we obtained 84.1% development using Day 7 IVF bovine blastocysts and expanded blastocysts with 1 min in 40% EG + 11.3% trehalose + 20% PVP in DPBS. Using this technique, the development and hatching rates were very high even after direct rehydration in mPBS. The gradual addition of 11.3% trehalose (treatment B) and 20% PVP (treatment C) with 40% EG (treatment A) increased the percentage of developed and hatched embryos.

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NORMAL CALVES OBTAINED AFTER DIRECT TRANSFER

Type of cryoprotectant (ethylene glycol) with Day 7 IVF bovine blastocysts and expanded blastocysts. However, in this case, even after direct rehydration of vitrified embryos in mPBS, the development rate (80.0%) and the hatching rate (70.0%) did not differ significantly from the other rehydration solutions; perhaps, the high concentration of PVP was helping to prevent osmotic stress on the embryo during rehydration. Based on this observation we suggest that PVP is very useful for direct rehydration.

Finally, to prove the effectiveness of PVP in practice, five vitrified embryos were transferred directly to five recipients; three pregnancies (60%) were achieved. There are other reports in which pregnancy rates of 40% (14), 31% (15), and 44.5% (22) were obtained in similar experiments. From our experiments we conclude that it is possible to vitrify IVF bovine blastocysts very simply, effectively, and successfully using only one type of cryoprotectant (ethylene glycol) with trehalose as a nonpermeating cryoprotectant and PVP as a macromolecule. By this technique it is possible to rehydrate the vitrified embryo directly in mPBS in the laboratory and it is also possible to transfer directly to the recipient under field conditions.

Developments continue in the in vitro maturation, culture, freezing, and vitrification of bovine oocytes and embryos (1, 7, 9, 43, 44, 47, 50). Additional research is needed to design IVC system that will reduce problems that appear to exist in some IVF systems (13) and to achieve success after one-step addition and one-step dilution methods of vitrification.

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