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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. © 1996 Academic Press, Inc.

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,

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Me_2SO) 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 nonpermeating 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 m*M* 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 a culture dish (35 mm diameter, Falcon, Becton Dickinson Co., Ltd., Oxnard, CA). Thereafter, the COCs were placed in the maturation medium, covered with mineral oil (Squibb, E.R. Squibb and Sons, Inc: Princeton, NJ), and cultured for 21–22 h at 38.5°C in 5% CO_2 in air.

In Vitro Fertilization and Culture

Samples of frozen semen from a single bull were thawed in a water bath at 30 to 35°C, washed by centrifugation at 500*g* for 5 min with the medium of Brackett and Oliphant (BO medium; 5) supplemented with 5 m*M* caffeine (caff-BO). The sperm pellet was resuspended in caff-BO supplemented with 1% bovine serum albumin (BSA, Initial Fractionation by Cold Alcohol Precipitation, Sigma) and 20 μg/ml heparin to give a sperm concentration of 5×10^6/ml. A 100-μl droplet of the sperm suspension was incubated under mineral oil for 1 h at 38.5°C with 5% CO_2 in air prior to insemination. Oocytes matured *in vitro* were transferred into sperm droplets (20–30 oocytes/droplet) for insemination. After a sperm–oocyte incubation of 5 h, the oocytes were washed two to three times and transferred to the culture medium (TCM 199, Earle's salts, L-glutamine, 2200 mg/liter sodium bicarbonate, 25 m*M* HEPES buffer, Gibco) supplemented with 5% SCS, 5 μg/ml 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 (LN_2). 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 LN_2 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 CO_2 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 LN_2 for storage. Warming was carried out in a 30°C water bath and the contents were drained directly into mPBS

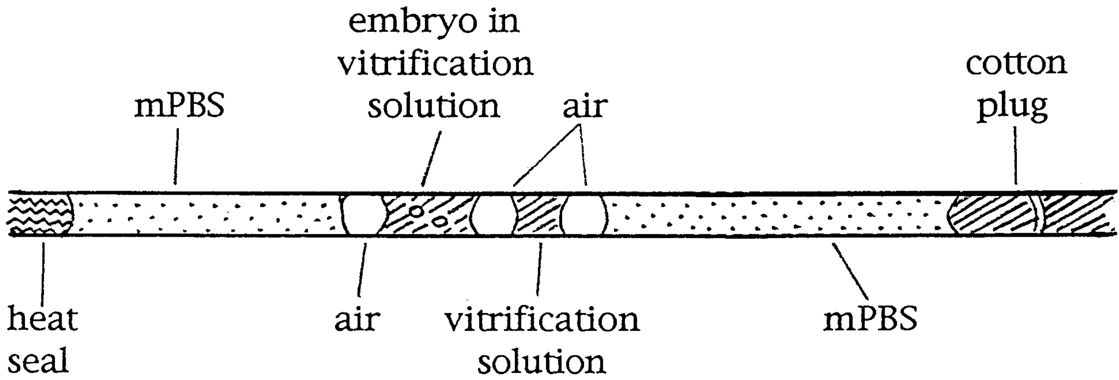

FIG. 1. Configuration of the solution in a 0.25-ml plastic straw loaded with embryos before cooling. Columns of mPBS, small volume of vitrification solution, vitrification solution with embryo, and mPBS were sequentially aspirated into the straw to yield four liquid chambers separated by 5-mm air bubbles.

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 LN_2 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 χ^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 1

Development (at 24–48 h) of IVF Bovine Blastocysts Exposed to the Vitrification Solution Containing 40% EG + 11.3% Trehalose + 20% PVP at 5 and 20°C for 5, 10, 15, and 20 min

Temperature (°C)	Time (min)	Development (%)
5	5	18/18 (100.0)e
	10	20/24 (83.3)d
	15	20/27 (74.0)cd
	20	14/22 (63.6)bc
20	5	10/18 (55.5)b
	10	12/23 (52.1)b
	15	13/29 (44.8)b
	20	3/29 (10.3)a
ANOVA	Temperature	**
	Time	**
	Temperature × Time	*

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

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

Note. Values within columns with different letters differ significantly (a,b, $P < 0.05$; A,B, $P < 0.01$; χ^2 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 (χ^2 test) among the embryo development and hatching rates.

TABLE 3
Development and Hatching Rates of Vitrified IVF Bovine Embryos Using 40% EG + 11.3% Trehalose + 20% PVP and Rehydrations by Different Concentration of Trehalose Solutions

Percentage trehalose	No. of embryos (n)	No. developed (%)	No. hatched (%)
0	20	16 (80.0)	14 (70.0)
11.3	28	21 (75.0)	18 (64.3)
22.7	41	34 (82.9)	27 (65.9)
34.0	29	23 (79.3)	20 (69.0)

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_r average 30,000). PVP is a large, interface-seeking molecule. Many years ago, it was suggested that PVP coats the cells immediately following thawing, giving them mechanical protection and stability against osmotic stresses (30). The mechanism of protection of the large polymer is not clear; perhaps it prevents osmotic injury during the rapid removal of extracellular water (6, 37). It may coat the cells and protect the cell membrane from denaturation (33). Loss of lipoprotein from the membrane makes it permeable to cations and therefore liable to osmotic lysis on thawing. A coating of polymer may either prevent the denaturation or stabilize the membrane against subsequent osmotic stress (26). It has been shown that the inclusion of a macromolecule in solutions facilitates vitrification (12) by increase the tendency of the solutions to supercool. The presence of high concentrations of endogenous macromolecules in the dehydrated cytoplasm of the embryos ought to facilitate intracellular glass (35). Macromolecules may also preventing devitrification during warming (17).

The development rates varied between 75.0 and 82.9% with rehydration in different concentrations of trehalose (Table 3). Dobrinsky *et al.* (10) observed a maximum 71% of IVM-IVF Day 6 bovine morulae/early blastocysts developing to blastocysts after vitrification (25% glycerol + 25% propylene glycol + 0.4% BSA) with dilution in 0.3 *M* sucrose. Yang *et al.* (51) vitrified Day 7 IVF bovine blastocysts using ethylene glycol or propylene glycol in combination with glycerol. They obtained hatching rates ranging from 51 to 80%. In the present experiments, hatching rates ranged from 64.3 to 70.0% using only one

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 from 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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REFERENCES

1. Agca, Y., Monson, R. L., Northey, D. L., Schaefer, D. M., and Rutledge, J. J. Postthaw survival and pregnancy rates of biopsied, sexed and vitrified bovine IVF embryos. *Theriogenology* **43,** 153 (1995). [Abstract]
2. Bank, H., and Maurer, R. R. Survival of frozen rabbit embryos. *Exp. Cell Res.* **89,** 188–196 (1974).
3. Barnet, R. E. The effect of dimethylsulfoxide and glycerol on $Na^{+}K^{+}$ ATP-ase and membrane structure. *Cryobiology* **15,** 227–229 (1978).
4. Borland, R. M., Biggers, J. D., and Lechene, C. P. Kinetic aspects of rabbit blastocoel fluid accumulation: An application of electron probe microanalysis. *Dev. Biol.* **50,** 201–211 (1976).
5. Brackett, B. G., and Oliphant, G. Capacitation of rabbit spermatozoa *in vitro. Biol. Reprod.* **12,** 260–274 (1975).
6. Bricka, M., and Bessis, M. Sur la conservation des erythrocytes par congelation a basse temperature en presence de polyvinyl–pyrrolidone et de dextran. *C.R. Sciences Biol.* **149,** 875 (1955).
7. Carvalho, R. V., Del Campo, M. R., Plante, Y., and Mapletoft, R. J. Effects of stage of development on sex ratio and survival after freezing of day 7 bovine IVF embryos. *Theriogenology* **43,** 183 (1995). [Abstract]
8. Cseh, S., Kreysing, U., Lucas-Hahn, A., and Niemann, H. Direct rehydration of IVM and IVC bovine embryos frozen in ethylene-glycol. *Theriogenology* **43,** 190 (1995). [Abstract]
9. Dinnyes, A., Carolan, C., Loneragan, P., Solti, L., Massip, A., and Mermillod, P. *In vitro* survival of *in vitro* produced (IVP) bovine embryos frozen or vitrified by techniques suitable for direct transfer. *Theriogenology* **43,** 197 (1995). [Abstract]
10. Dobrinsky, J. R., Stice, S. L., Phillips, P. E., Duby, R. T., and Robl, J. M. Development of IVM-IVF bovine embryos following vitrification dilution treatments. *Theriogenology* **37,** 202 (1992). [Abstract]
11. Doebbler, G. F. Cryoprotective compounds: Review and discussion of structure and function. *Cryobiology* **3,** 2–11 (1966).
12. Fahy, G. M., McFarlane, D. R., Angell, C. A., and Meryman, H. T. Vitrification as an approach to cryopreservation. *Cryobiology* **21,** 407–426 (1984).
13. Hasler, J. F., Henderson, W. B., Hurtgen, P. J., Jin, Z. Q., McCauley, A. D., Mower, S. A., Neely, B., Shuey, L. S., Stokes, J. E., and Trimmer, S. A. Production, freezing and transfer of bovine IVF embryos and subsequent calving results. *Theriogenology* **43,** 141–152 (1995).
14. Ishimori, H., Saeki, K., Inai, M., Nagao, Y., Itasaka, J., Miki, Y., Seike, N., and Kainuma, H. Vitrification of bovine embryos in a mixture of ethylene glycol and dimethyl sulfoxide. *Theriogenology* **40,** 427–433 (1993).
15. Ishimori, H., Saeki, K., Itasaka, J., Miki, Y., Nozaki, N., Seike, N., and Kainuma, H. Direct transfer of

vitrified bovine embryos.*Theriogenology* **39,** 238 (1993). [Abstract]

16. Kasai, M., Niwa, K., and Iritani, A. Effects of various cryoprotective agents on the survival of unfrozen and frozen mouse embryos. *J. Reprod. Fertil.* **63,** 175–180 (1981).
17. Kasai, M., Komi, J. H., Takakamo, A., Tsudera, H., Sakurai, T., and Machida, T. A simple method for mouse embryo cryopreservation in a low toxicity vitrification solution, without appreciable loss of viability. *J. Reprod. Fertil.* **89,** 91–97 (1990).
18. Kobayashi, S., Pollard, J. W., and Leibo, S. P. Survival of *in vitro* derived bovine embryos frozen rapidly in ethylene glycol, PVP and galactose. *Cryobiology* **30,** 630 (1993). [Abstract]
19. Kobayashi, S., Tomita, M., Pollard, J. W., and Leibo, S. P. Survival of cryopreserved porcine embryos vitrified in ethylene glycol plus polyvinyl pyrrolidone. *Theriogenology* **41,** 228 (1994). [Abstract]
20. Krag, K. T., Koehler, I. M., and Wright, R. W. Trehalose: A non-permeable cryoprotectant for direct freezing of early stage murine embryos. *Theriogenology* **23,** 200 (1985). [Abstract]
21. Kuwayama, M. Vitrification of IVMFC bovine embryos at various developmental stages and of different quality. *Theriogenology* **43,** 257 (1995). [Abstract]
22. Leeuw, A. M. W., Daas, J. H. G., and Rall, W. F. Pregnancy rates in a comparative field trial of vitrification and one-step dilution or conventional slow freezing and three-step dilution of bovine embryos are similar. *Theriogenology* **41,** 326 (1994). [Abstract]
23. Leibo, S. P. Fundamental cryobiology of mouse ova and embryos. *In* ''The Freezing of Mammalian Embryos'' (K. Elliot and J. Whelan, Eds.), Ciba Foundation Symp. No. 52 (new series), pp. 69–92. Elsevier, North Holland, Amsterdam, 1977.
24. Leibo, S. P. Cryobiology: Preservation of mammalian embryos. *In* ''Genetic Engineering of Animals: An Agricultural Perspective'' (J.W. Evans and A. Hollaender, Eds.), pp. 251–272. Plenum, New York/London, 1986.
25. Leibo, S. P., and Oda, K. High survival of mouse zygotes and embryos cooled rapidly or slowly in ethylene glycol plus polyvinylpyrrolidone. *Cryo-Letters* **14,** 133–144 (1993).
26. Lovelock, J. E. The denaturation of lipid-protein complexes as a cause of damage by freezing. *Proc. R Soc. London Ser. B* **147,** 427 (1957).
27. Massip, A., Van Der Zwalmen, P., Scheffen, B., and Ectors, F. Pregnancies following transfer of cattle embryos preserved by vitrification. *Cryo-Letters* **7,** 270–273 (1986).
28. Massip, A., Van Der Zwalmen, P., and Ectors, F. Recent progress in cryopreservation of cattle embryos. *Theriogenology* **27,** 69–76 (1987).
29. Matsuoka, K., Sakata, S., Ichino, K., Shimaya, S., Katagihara, T., and Suzuki, T. Ultra-rapid freezing of *in vitro* produced bovine embryos. *Theriogenology* **43,** 274 (1995). [Abstract]
30. Meryman, H. T. Review of Biological Freezing. *In* ''Cryobiology'' (H. T. Meryman, Ed.), pp. 1–106. Academic Press, London/New York, 1966.
31. Miyamoto, H., and Ishibashi, T. Survival of frozen–thawed mouse and rat embryos in the presence of ethylene glycol. *J. Reprod. Fertil.* **50,** 373–375 (1977).
32. Miyamoto, H., and Ishibashi, T. The protective action of glycols against freezing damage of mouse and rat embryos. *J. Reprod. Fertil.* **54,** 427–432 (1978).
33. Nash, T. The chemical constitution of compounds which protect erythrocytes against freezing damage. *J. Gen. Physiol.* **46,** 167 (1962).
34. Niemann, H. Cryopreservation of ova and embryos from livestock: Current status and research needs. *Theriogenology* **35,** 109–124 (1991).
35. Rall, W. F. Factors affecting the survival of mouse embryos cryopreserved by vitrification. *Cryobiology* **24,** 387–402 (1987).
36. Rall, W.F., and Fahy, G.M. Ice-free cryopreservation of mouse embryos at −196°C. *Nature* **313,** 573–575 (1985).
37. Rinfret, A. P. Some aspects of preservation of blood by rapid freeze–thaw procedures. *Fed. Proc.* **22,** 94–101 (1963).
38. Robertson, J. L., Minhas, B. S., and Randall, G. W. Ultrarapid freezing of mouse embryos with DMSO and trehalose. *Theriogenology* **31,** 250 (1989). [Abstract]
39. Saha, S., Boediono, A., and Suzuki, T. Vitrification of IVF bovine embryo with PVP. *Proc. Jpn. Soc. Anim. Reprod.* **I-57,** 57 (1993). [Abstract]
40. Saha, S., Boediono, A., and Suzuki, T. Toxicity due to vitrification of IVF bovine embryos with polyvinylpyrrolidone. *Proc. Jpn. Soc. Vet. Sci.* **VIII-7,** 219 (1994). [Abstract]
41. Saha, S., Takagi, T., Boediono, A., and Suzuki, T. Direct rehydration of *in vitro* fertilised bovine embryos after vitrification. *Vet. Rec.* **134,** 276–277 (1994).
42. Saha, S., Rajamahendran, R., Boediono, A., Cece, S., and Suzuki, T. Viability of bovine blastocysts obtained after 7, 8 or 9 days of culture following vitrification and one-step rehydration. *Theriogenology* **43,** 311 (1995). [Abstract]
43. Semple, M. E., Betteridge, K. J., and Leibo, S. P. Cryopreservation of *in vitro*-derived bovine embryos produced in a serum-free culture system. *Theriogenology* **43,** 320 (1995). [Abstract]
44. Seregi, J., Cseh, S., and Treuer, A. The first successful transfer of frozen embryos derived from

IVMFC of bovine oocytes in Hungary. *Theriogenology* **43,** 321 (1995). [Abstract]

45. Smorag, Z., Heyman, Y., Garnier, V., and Gajda, B. The effect of sucrose and trehalose on viability of one- and two-cell rabbit embryos. *Theriogenology* **33,** 741 (1990).
46. Szell, A., and Shelton, J. N. Role of equilibration before rapid freezing of mouse embryos. *J. Reprod. Fertil.* **78,** 699–703 (1986).
47. Thonon, F., Lens, H., Touati, K., Ectors, F.J., Delval, A., Beckers, J.F., and Ectors, F. A step-wise procedure for cryoprotectant equilibration improves the survival rate after thawing of *in vitro* produced embryos. *Theriogenology* **43,** 338 (1995). [Abstract]
48. Whittingham, D. G. Survival of mouse embryos after freezing and thawing. *Nature* **233,** 125–126 (1971).
49. Whittingham, D. G., Leibo, S. P., and Mazur, P. Survival of mouse embryos frozen to −196°C and −269°C. *Science* **178,** 411–414 (1972).
50. Willemsen, D., Palma, G. A., and Wolf, E. Vitrification of *in vitro*-produced bovine embryos after culture in medium supplemented with insulin-like growth factor-I (IGF-I). *Theriogenology* **43,** 352 (1995). [Abstract]
51. Yang, N. S., Lu, K. H., Gordon, I., and Polge, C. Vitrification of bovine blastocysts produced *in vitro*. *Theriogenology* **37,** 326 (1992). [Abstract]