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Survival Rate of Frozen–Thawed Bovine IVF Embryos in Relation to Exposure Time Using Various Cryoprotectants

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The relationship between cryoprotectant and exposure time on the development of IVF-derived embryos in culture after freezing and thawing was examined. Good to excellent quality Day 7 to 8 blastocysts and expanded blastocysts were suspended in 1.6 *M* propylene glycol (PG), 1.8 *M* ethylene glycol (EG), 1.1 *M* diethylene glycol, and 1.3 *M* ethylene glycol monomethyl ether (EME). In Experiment I, after 30 and 120 min of exposure, embryos were placed directly into culture medium and washed three times. Embryos were cocultured with cumulus cells in culture medium. After a 48-h culture, there were no significant differences in fully expanded, hatching, and hatched rates between the 30- and 120-min exposure to all four cryoprotectants except for the fully expanded rate with PG (55 vs 83%). In Experiment II, to compare the cryoprotective effects of the four cryoprotectants, embryos were exposed to each cryoprotectant for variable times from 10 to 120 min, seeded, and cooled to −30°C at 0.3°C/min, plunged into liquid nitrogen for storage, and then thawed. Survival was measured by coculturing the embryos with cumulus cells in culture medium. There were no significant differences in development to hatched blastocysts of embryos exposed to either PG (27~42%) or EG (37~56%). The number of embryos developing after exposure to EME was similar to that in PG or EG when exposure time did not exceed 40 min (43~49%). Our findings suggest that the toxicity of different cryoprotectants is related to the exposure time and that PG and EG were relatively nontoxic as is exposure to EME for short periods. © 1993 Academic Press, Inc.

Significant progress has been made in the freezing process of mammalian embryos, especially that of bovine embryos (4–7, 10–12). The non-step-wise method for removing the cryoprotectants is becoming more popular than the step-wise method or use of a sucrose solution (7). Recently, some investigators reported on the non-step-wise freezing method for bovine embryos using propylene glycol (PG) and ethylene glycol (EG) as cryoprotectants (2, 13, 17, 18). They observed high survival and pregnancy rates after thawing compared to the step-wise method. On the other hand, the greater permeability of PG and EG than glycerol suggested that the chemical toxicity of PG and EG to the embryo becomes higher (5, 14, 16–18). However, no research about the relationship between the exposure time before freezing and the viability of embryos after thawing with PG and EG has been reported.

In this study, we analyzed the relationship between different times of exposure to ethylene glycol, propylene glycol, diethylene glycol (DEG), and ethylene glycol monomethyl ether (EME) on the survival of *in vitro*-derived embryos after freezing and thawing.

MATERIALS AND METHODS

IVF Bovine Embryos

Cow ovaries were collected from a local slaughterhouse and brought to the laboratory in physiological saline (0.85% w/v sodium chloride) at 25–30°C within 3 h. Follicular oocytes (oocyte–cumulus complexes) were collected by puncturing the follicles 1–7 mm in diameter with an 18-G needle. After the oocytes were washed once with m-PBS (Embryotec; Nihon Zen-

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yaku Co., Fukushima, Japan), they were rinsed two to three times in maturation medium. The maturation medium consisted of 25 m*M* Hepes TCM199 (GIBCO, U.S.A.) that had been supplemented with 5% (v/v %) serum collected from superovulated cows on Day 7 of their cycles (SCS) (8), 0.01 mg/ml follicle stimulating hormone (Denka, Kawasaki, Japan), and 50 μg/ml gentamicin (Sigma, U.S.A.). The oocytes were then cultured in the maturation medium for 21–22 h at 38.5°C in 5% CO_2 in air. After maturation, oocytes were fertilized *in vitro* with a single sample of frozen–thawed semen, and the embryos were transferred to culture medium [25 m*M* Hepes TCM199 supplemented with 5% of SCS, 5 μg/ml insulin (Sigma), and 50 μg/ml gentamicin]. Forty-eight hours after fertilization, embryos were cultured on the cumulus cell layers according to the method previously reported (1, 3).

We used excellent or good quality blastocysts and expanded blastocysts obtained on Day 7 or 8 after *in vitro* fertilization in this experiment. After all embryos were transferred from the culture dishes, the cumulus cell coculture systems were kept in the CO_2 incubator. These cumulus cell coculture systems contained 0.5 ml of fresh culture medium prior to reuse for culture of thawed embryos. In our previous experiments, the hatched rate of these embryos (without freezing and exposure to the cryoprotectants) was 81% (29/36).

Four cryoprotectants, 1.6 *M* PG, 1.8 *M* EG, 1.1 *M* DEG, and 1.3 *M* EME, were used in this experiment. Although the concentrations of the cryoprotectants were different in molar terms, they were selected on the basis of the results of previous experiments using these cryoprotectants (2, 9, 13, 17, 18). Cryoprotectants were dissolved in m-PBS containing 10% SCS and 50 μg/ml gentamicin.

Experiment I

The experiment was conducted to screen the viability of embryos after exposure to each cryoprotectant. Embryos were exposed to each cryoprotectant for 30 and 120 min, while protected from light which affects the development of the embryo (15). After exposure, the cryoprotectant medium containing the embryos was placed directly into the culture medium with a Pasteur pipet by direct rehydration and washed three times with culture medium. Embryos were then transferred into culture wells and were cultured on feeder layers of bovine cumulus cells in TCM199 supplemented with 5% SCS and 5 μg/ml insulin under 38.5°C in 5% CO_2 in air.

Experiment II

Embryos were exposed to each cryoprotectant for 10, 20, 40, 60, 80, or 120 min while protected from light. After various times of exposure, embryos were loaded into 0.25-ml straws and were placed directly into a programmable freezer maintained at 0°C and held for 2 min. They were then cooled from 0°C to −5.5°C at the rate of 1°C/min, seeded at −5.5°C with supercooled forceps, and held at this temperature for 10 min. After seeding, the straws were cooled at a rate of 0.3°C/min to −30°C, and then immediately plunged into and stored in liquid nitrogen. After several days of storage, embryos were thawed by placing the straws in a 30°C water bath, and the contents were drained into a sterile petri dish. The embryos were then transferred with <5 μl of cryoprotectant medium directly into the culture medium by direct rehydration and washed three times. Embryos were cultured on feeder layers of bovine cumulus cells in TCM199 supplemented with 5% SCS and 5 μg/ml insulin under 38.5°C in 5% CO_2 in air.

In Vitro Viability Assessments

Embryos in both experiments were evaluated microscopically at 24-h intervals for 48 h. Viability of embryos were assessed by re-expansion of the blastocoele during a 48-h culture. Also, the fully expanded rate (including the hatching and hatched blasto-

cyst), hatching rate (including the hatched blastocyst), and hatched rate during the 48-h culture were observed.

The experimental design of experiment I and II was a randomized block with three or four replications, respectively.

The data were analyzed by a χ^2 test and the effect of the cryoprotectant on subsequent development into hatched blastocysts at each exposure time was analyzed by an analysis of variance (ANOVA). A probability (P) of 0.05 was considered significant.

RESULTS

Experiment I

Table 1 shows the results of fully expanded, hatching, and hatched embryos after 30 and 120 min of exposure to each cryoprotectant. There were no significant differences in viability between 30 and 120 min of exposure except for the fully expanded rate of PG.

Experiment II

Table 2 shows the results of survival, fully expanded, hatching, and hatched frozen–thawed embryos for each exposure time with PG as a cryoprotectant. The viability of the embryo increased with the exposure time up to 40 min. Exposure treatment for 40 min gave the highest viability. Survival and the fully expanded rate showed a tendency to become significantly lower when the exposure time was longer than 60 min. However, the hatched rate was not significantly different between each exposure time.

Table 3 shows the results of treatment with EG. There were significant differences in the frozen–thawed survival rate between 10 and 40 or 80 min and 40 and 120 min of exposure. There were significant differences in the fully expanded rate between 10 and 40 or 80 min of exposure. Hatching and the hatched rate did not significantly differ with the exposure time.

Table 4 shows the results of treatment with DEG. There were significant differences in the survival rate between 80 and 10, 20, or 60 min of exposure. There was a significant difference in the hatching rate between 10 and 40 min. There were significant differences in the hatched rate between 40 or 80 min and 10 or 60 min.

Table 5 shows the results of treatment with EME. There were significant differences in the survival rate between 10 and 80 or 120 min and 20 or 40 min and 60, 80, or 120 min of exposure time. There were significant differences in the fully expanded rate between 10, 20, 40, or 60 min and 80 or 120 min of exposure. There were significant differences in the hatching rate between 10,

TABLE 1
Viability of Nonfrozen Bovine IVF Embryos in Relation to Exposure Time Using Various Cryoprotectants

Cryoprotectant	Time (min)	No. of embryos	Fully exp No. (%)	Hatching No. (%)	Hatched No. (%)
PG	30	20	11 (55)[a]	4 (20)	4 (20)
	120	23	19 (83)[b]	10 (44)	6 (26)
EG	30	20	16 (80)	10 (50)	8 (40)
	120	23	15 (65)	6 (26)	5 (22)
DEG	30	21	16 (76)	10 (48)	6 (29)
	120	20	13 (65)	10 (50)	7 (35)
EME	30	22	14 (64)	8 (36)	8 (36)
	120	22	19 (86)	9 (41)	8 (36)

[a,b] Values within a column with different superscripts are significantly different ($P < 0.05$, χ^2 test).

Note. PG, propylene glycol; EG, ethylene glycol; DEG, diethylene glycol; EME, ethylene glycol monomethyl ether.

TABLE 2
Viability of Frozen–Thawed Bovine IVF Embryos in Relation to Exposure Time Using Propylene Glycol as Cryoprotectant

	Exposure time (min)					
Embryo development	10 (n = 42)	20 (n = 41)	40 (n = 50)	60 (n = 41)	80 (n = 44)	120 (n = 44)
Survival	$31^{a,b,c,d}$	$33^{a,b,c}$	$41^{a,b,c}$	$28^{a,b,c,d,e}$	$29^{a,d,e}$	$23^{d,e}$
(%)	(74)	(81)	(82)	(68)	(66)	(52)
Fully exp.	$26^{a,b,c,d,e}$	$29^{a,b,c}$	$40^{a,b}$	$25^{a,c,d,e}$	$24^{c,d,e}$	$21^{c,d,e}$
(%)	(62)	(71)	(80)	(61)	(55)	(48)
Hatching	16	22	29^{a}	18	19	15^{b}
(%)	(38)	(54)	(58)	(44)	(43)	(34)
Hatched	17	17	20	11	15	13
(%)	(31)	(42)	(40)	(27)	(34)	(30)

$^{a–e}$ Values within rows with different superscripts are significantly different ($P < 0.05$, χ^2 test).

20, or 40 min and 80 or 120 min of exposure. There were significant differences in the hatched rate between 10, 20, or 40 min and 80 or 120 min of exposure. The number of embryos developing after exposure in EME was similar to that in PG or EG when the exposure time did not exceed 40 min.

Table 6 shows the number and percentage of embryos that had developed into hatched blastocysts with each cryoprotectant. There were no significant differences among each cryoprotectants with the same exposure time except EG and DEG at 80 min.

DISCUSSION

Bovine embryos have been suggested to have greater permeability to PG and EG, and thereby could avoid the osmotic shock when the frozen–thawed embryos were transferred into isosmotic medium without removing these cryoprotectants (5, 9, 13, 14, 16–18). The chemical toxicity of PG and EG to the embryo has also been suggested to be higher because of its full permeation into the cells (16–18). However, the results of Experiment I indicated that exposures as long as 120 min to PG or EG or to DEG or EME were not harmful to embryos.

Suzuki *et al.* (13) froze *in vivo* produced embryos with PG as a cryoprotectant and observed a high survival rate (88.0%) after *in vitro* culture. Goto *et al.* also reported high survival and/or pregnancies of frozen–

TABLE 3
Viability of Frozen–Thawed Bovine IVF Embryos in Relation to Exposure Time Using Ethylene Glycol as Cryoprotectant

	Exposure time (min)					
Embryo development	10 (n = 40)	20 (n = 50)	40 (n = 45)	60 (n = 29)	80 (n = 35)	120 (n = 43)
Survival	37^{a}	42	31^{b}	24	$26^{b,c}$	$38^{a,c}$
(%)	(93)	(84)	(69)	(83)	(74)	(88)
Fully exp.	34^{a}	38	29^{b}	22	22^{b}	33
(%)	(85)	(76)	(64)	(76)	(63)	(77)
Hatching	24	34	26	18	17	24
(%)	(60)	(68)	(58)	(62)	(49)	(56)
Hatched	19	28	19	14	13	21
(%)	(48)	(56)	(42)	(48)	(37)	(49)

$^{a–c}$ Values within rows with different superscripts are significantly different ($P < 0.05$, χ^2 test).

TABLE 4
Viability of Frozen–Thawed Bovine IVF Embryos in Relation to Exposure Time Using Diethylene Glycol as Cryoprotectant

Embryo development	Exposure time (min) 10 (n = 42)	20 (n = 49)	40 (n = 33)	60 (n = 36)	80 (n = 41)	120 (n = 41)
Survival	31^{a}	36^{a}	20	26^{a}	20^{b}	23
(%)	(74)	(74)	(61)	(72)	(49)	(56)
Fully exp.	28	29	16	21	19	22
(%)	(67)	(59)	(49)	(58)	(46)	(54)
Hatching	22^{a}	17	9^{b}	15	13	13
(%)	(52)	(35)	(27)	(42)	(32)	(32)
Hatched	16^{a}	14	5^{b}	13^{a}	6^{b}	8
(%)	(38)	(29)	(15)	(36)	(15)	(20)

^{a,b} Values within rows with different superscripts are significantly different ($P < 0.05$, χ^2 test).

thawed IVF embryos frozen with PG (2). On the other hand, Voelkel and Hu (17, 18) froze *in vivo*-produced embryos with EG as a cryoprotectant and also observed high survival and pregnancies. In previous experiments, embryos were exposed for a short time (10–20 min) to avoid some effects of prolonged exposure to cryoprotectants. However, the results of experiment II indicated that the toxicity of PG and EG, which were related to exposure time, was low. However, the survival and fully expanded rate of embryos with PG and EG as cryoprotectants suggested that EG had a stronger cryoprotective effect on the embryonic cells than PG. On the other hand, the survival and fully expanded rate of embryos using EG as cryoprotectant were significantly lower with 40 and 80 min of exposure. However, they seemed to be unrelated to the length of the exposure time. This suggested that EG was less toxic and it had better cryoprotective properties, similar to PG as a cryoprotectant for direct transfer. Voelkel and Hu (17, 18) suggested that EG is a more effective cryoprotectant than PG for bovine embryos based on both permeability and cryoprotective properties. In our experiments, although the hatched rate of embryos frozen in EG appeared higher than that of embryos frozen in PG, the difference was not significant with each exposure time. The differences between the results reported by Voelkel and Hu (17, 18)

TABLE 5
Viability of Frozen–Thawed Bovine IVF Embryos in Relation to Exposure Time Using Ethylene Glycol Monomethyl Ether as Cryoprotectant

Embryo development	Exposure time (min) 10 (n = 44)	20 (n = 65)	40 (n = 35)	60 (n = 40)	80 (n = 37)	120 (n = 41)
Survival	$38^{a,b,c,d}$	$56^{a,b,c}$	$31^{a,b,c}$	$28^{d,e,f}$	$20^{d,e,f}$	$25^{d,e,f}$
(%)	(86)	(86)	(89)	(70)	(54)	(61)
Fully exp.	32^{a}	51^{a}	28^{a}	26^{a}	15^{b}	17^{b}
(%)	(73)	(79)	(80)	(65)	(41)	(42)
Hatching	28^{a}	36^{a}	20^{a}	17	10^{b}	10^{b}
(%)	(64)	(55)	(57)	(43)	(27)	(24)
Hatched	$21^{a,b}$	$28^{a,b}$	17^{b}	11	8^{c}	6^{c}
(%)	(48)	(43)	(49)	(28)	(22)	(15)

$^{a-f}$ Values within rows with different superscripts are significantly different ($P < 0.05$, χ^2 test).

TABLE 6
Effect of Exposure Time and Cryoprotectant on Subsequent Development *in Vitro* Derived from IVF Bovine Blastocysts into Hatched Blastocysts

Cryoprotectant	Exposure time (min)					
	10	20	40	60	80	120
PG	13/42 (31)	17/41 (42)	20/50 (40)	11/41 (27)	15/48 (31)	13/44 (30)
EG	19/40 (48)	28/50 (56)	19/45 (42)	14/29 (48)	13/35 (37)[a]	21/43 (49)
DEG	16/42 (38)	14/49 (29)	5/33 (15)	13/36 (36)	6/41 (15)[b]	8/41 (20)
EME	21/44 (48)	28/65 (43)	17/35 (49)	11/40 (28)	8/37 (22)	6/41 (15)

[a,b] Values within a column with different superscripts are significantly different ($P < 0.05$, ANOVA).
Note. CP, cryoprotectant; PG, propylene glycol; EG, ethylene glycol; DEG, diethylene glycol; EME, ethylene glycol monomethyl ether.

and ours may be due to the different freezing methods (with different seeding points, cooling rate) or culture methods.

When DEG was used as a cryoprotectant, the proportion of embryo development was high at 10 min of exposure, but these rates generally tended to decrease in relation to the length of the exposure time (Experiment II). In this case, in one trial the proportion of the hatched embryos after 60 min of exposure was high compared with the others. It may be due to some factors involved in the culture condition. Prolonged exposure to DEG was not very toxic to the embryonic cell (Experiment I), but some cellular damage might have occurred during the freezing and thawing process.

High survival, fully expanded, hatching, and hatched rates of embryos were observed with EME as EG until 40 min of exposure. This suggested that EME is an effective cryoprotectant for direct transfer of the bovine IVF embryo as a result of both permeability and cryoprotective properties of EG. However, all these ratios became significantly lower after 60 min of exposure. The findings obtained in Experiment I suggested that some cellular damage occurred while the frozen–thawed process was performed after 60 min of exposure.

In conclusion, the present findings indicated that the toxicity of different cryoprotectants was related to the exposure time and that PG and EG are relatively nontoxic, as is exposure to EME for short periods. Furthermore, EME may be an effective cryoprotectant for direct transfer of the bovine IVF embryo. However, further studies are required to determine the optimal concentration of DEG and EME and the freezing procedure to obtain a high survival rate.

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