Comparison of 1,2-Propanediol and Ethylene Glycol for Cryopreservation of Slow-Cooled Mouse Zygotes and Their Subsequent Development

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Purpose: This study was conducted on cryoprotective activity of ethylene glycol (ETG) and propanediol (PROH) on cleavage rate of mouse zygotes.

Methods: Mouse oocytes were excised from fallopian tube of gonadotropin-treated mice, then inseminated with spermatozoa. After 16.5–17.5 h, zygotes were randomly allocated into three groups; control, toxicity, and frozen. In the latter, zygotes were slowly cooled with ETG and PROH similar to those used for human embryo cryopreservation. The survived zygotes cultured for 120 h and their later stages of development were compared with nonfrozen embryos.

Results: The toxicity test showed that no differences were observed in cleavage rate between exposed and nonexposed embryos. The survival and expanded hatching blastocyst rate of embryos frozen with PROH was significantly better than with ETG (92.8 vs. 58.2% and 68.2 vs. 39.1%, respectively).

Conclusions: ETG does not appear to be a good alternative to the classical PROH for freezing of mouse zygotes.

KEY WORDS: Cryopreservation; in vitro fertilization; mouse; zygote.

INTRODUCTION

Over the past decade, in vitro fertilization and related techniques have become reasonably efficient treatments for infertile patients. The number of supernumerary embryos is continuously increasing, and thus the need for highly efficient cryopreservation tech-

niques is becoming urgent (1). The first pregnancy resulting from the thaw and transfer of cryopreserved preembryos was reported in 1983 by Trounson and Mohr (2), and the first birth was reported in 1984 by Zeilmaker *et al.* (3). The delivery of healthy babies after cryopreservation of early preembryonic stage conceptuses confirms the value of this technique for infertility patients undergoing IVF-ET, and by extrapolation, for the centers that serve these patients (4). To prevent the disturbance in DNA synthesis in interphase stage among cryopreservation, it is better to cryopreserve the zygotes after this stage. Multicell embryos may not be optimal for cryopreservation because of the considerable asynchrony in the second and third cell cycles that results in difficulty in determining the appropriate time for freezing (5).

The type and concentration of cryoprotectant, the temperature at which embryos are transferred to liquid nitrogen, and the age of the embryo when frozen

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are currently considered critical factors in survival rates of frozen–thawed embryos (6). Embryos survive freezing only when suspended in molar concentrations of one or several low-molecular-weight solutes (7). On the basis of the available evidence, partial permeation of the embryo might be required for its protection (8); differences in the permeability of several cryoprotectants have been broadly reviewed by Friedler *et al.* (9).

1,2-Propanediol (PROH) is a cryoprotectant used widely for slow freezing of unicellular embryos in many domestic animals such as rabbits (10), mouse (1,11–13), and human (4,14,15). Good survival and cleavage rates in animals (1,10,11) and human (4,14) and the high expanded blastocyst percentage achieved with frozen–thawed unicellular embryos (1) make this molecule a good cryoprotectant for human embryos slow-freezing.

Ethylene glycol (ETG) is a compound used widely for embryo cryopreservation in many domestic animals such as rabbits (16), cattle (17), and sheep (7), as well in slow-freezing (18) and vitrification procedures (18–20). The low toxicity of ETG (17,18,20) and the normal live births achieved with frozen–thawed embryos in animals (19,20) make this molecule a good candidate for human embryos cryopreservation. Unfortunately, none of the studies on animal models employed freezing–thawing conditions with ETG as a cryoprotectant similar to those used for human embryo cryopreservation with PROH. In the previous studies (1,21), the cryoprotective properties of 1.5 mol/L ETG were compared with those of 1.5 mol/L 1,2-propanediol (PROH) on slowly cooled mouse zygotes and 4-cell stage embryos. Freezing and thawing conditions similar to those described in protocols used for human embryos were used. In our study, the aim was the evaluation of the cryoprotective properties of 1.5 mol/L ETG in mouse zygotes and 2-cell stage embryos, using the same freezing protocol and thawing procedure that led to the higher embryo survival rate described previously (21). The activity of ETG was compared with that of PROH, the classical cryoprotectants used for freezing early human embryos.

MATERIALS AND METHODS

Collection of Mouse Oocyte

Eight to 12-week-old NMRI female mice were superovulated with 10 IU human menopausal gonadotropin (hMG, Manufactured by I.F.Serono S.P.A

125, via Casilina, Italy) and 10 IU human chorionic gonadotropin (hCG, Gonatropin, Teikoku Zoki) given intraperitoneally 48 h apart. Mice were killed by cervical dislocation 13:00–14:30 h after hCG injection, oocytes were excised from the ampullary portion of oviducts into T6 medium (NaCl: 4725, KCl: 110, NaH₂PO₄: 47, MgCl₂: 47, CaCl₂: 196, NaHCO₃: 2100, Na lactated: 2610, Na pyruvate: 30, D glucose: 1000, peniciline G: 63, streptomycin: 50, phenol red: 10; in mg/L), supplemented with 5 mg/mL bovine serum albumin (BSA, fraction V, Sigma, St. Louis, MO). Then cumulus cells were removed by 0.1% hyaluronidase (Sigma, St. Louis, MO) and after washing three times, the oocytes with a polar body were cultured in T6 medium (22) supplemented with 5 mg/mL BSA in 30- μ L drops under mineral oil (M8410; Sigma), at 37°C in a humidified atmosphere of 5% CO₂ and 5% O₂ before insemination.

In Vitro Fertilization

The methods for IVF have been described previously (23). All incubations were carried out at 37°C in a humidified atmosphere of 5% CO₂ in air. Spermatozoa were collected from the cauda epididymis of NMRI male mice and suspended in 300 μ L of T6 medium supplemented with 5 mg/mL BSA, covered with mineral oil. After capacitation of sperm (1.5 h at 37°C under 5% CO₂ and 5% O₂ in air), a 30 μ L of sperm suspension was transferred to new drop of 300 μ L of T6 medium supplemented with 15 mg/mL BSA. The oocytes were then added in this droplet (final sperm concentration 1–2 $\times$ 10⁶/mL in medium). Pronuclear oocytes (the criteria for fertilized oocytes were the presence of two pronuclei and a second polar body) obtained 6–8 h after insemination (24).

After 4–6 h, pronuclear oocytes were washed in T6 medium supplemented with EDTA (100 μ M) and 5 mg/mL BSA and cultured in 30- μ L droplets of the same medium under mineral oil 16.5–17.5 h postinsemination (10 zygotes in a drop). Only morphologically normal zygotes were randomly used and allocated to the control, toxicity, and frozen groups.

Freezing and Thawing Solutions

Freezing and thawing solutions were prepared in PBS (phosphate buffer solution) supplemented with 5 mg/mL BSA with and without sucrose (S1888). The cryoprotectants were 1.5 mol/L ETG (E9129; Sigma) and 1.5 mol/L PROH (P6209; Sigma). Two equilibration solutions were used in three different

equilibration steps for the freezing procedure with each cryoprotectant. Equilibration solutions were 1.5 mol/L ETG and 1.5 mol/L ETG + 0.1 mol/L sucrose; 1.5 mol/L PROH and 1.5 mol/L PROH + 0.1 mol/L sucrose. Three equilibration solutions were used in three different steps for the thawing procedure with each cryoprotectant. The equilibration solutions were 1 mol/L ETG + 0.2 mol/L sucrose, 0.5 mol/L ETG + 0.2 mol/L sucrose and 0.2 mol/L sucrose; 1 mol/L PROH + 0.2 mol/L sucrose, 0.5 mol/L PROH + 0.2 mol/L sucrose, and 0.2 mol/L sucrose.

Toxicity Test

The embryo toxicity of cryoprotectants (ETG and PROH) was tested by exposing the zygotes to freezing and thawing solutions. In toxicity group, all procedures were similar to the frozen group without plunging in liquid nitrogen. The exposed zygotes were washed three times in culture medium and then were placed in drops of T6 medium supplemented with EDTA (100 μ M) and 5 mg/mL BSA for 48 h and then were cultured for further 72 h postinsemination in drops of T6 medium supplemented with 5 mg/mL BSA at 37°C in a humidified atmosphere of 5% CO₂ and 5% O₂ (10 zygotes in a drop). The cleavage rate 24 h later and expanded blastocyst percentage 120 h later were recorded and compared with such data obtained from the control group.

Freezing and Thawing Procedure

The same freezing–thawing procedure was used for the two cryoprotectants. All of the manipulation procedures of oocytes, spermatozoa, freezing, and thawing were conducted at 20°C–22°C (room temperature). The zygotes were exposed to 1.5 mol/L cryoprotectant for 10 min, and then to the solution containing the cryoprotectant with sucrose, again for 10 min. During this last period of equilibration, the zygotes were loaded into 0.25 mL French plastic Straws (I.M.V.L'Aigle, France), prepared as follows: A 3-cm column of the 1.5 mol/L cryoprotectant + 0.1 mol/L sucrose, followed by a short column of bubbles and 5-cm-long column of 1.5 mol/L cryoprotectant + 0.1 mol/L sucrose was drawn into the straw by aspiration. The zygotes were pipetted into this column 10 per straw and followed by a short column of air. After sealing with a plastic plug (Mini-tub, Landshut, Germany) the straws were charged in the freezing machine. A Kryo 10 machine (planer, Middlesex, UK) was used for freezing, and the embryos were cooled

as follows: from 22°C to –7°C at –2°C/min, at –7°C manual seeding, from –7°C to –33°C at –0.3°C/min, from –33°C to –150°C at –50°C/min; the zygotes were then plunged into liquid nitrogen.

For thawing, the straws were held in air for 15 s and then plunged into a water bath at 37°C for 20 s until the ice melted. The cryoprotectants were removed from the zygotes in three steps: 10 min in 1 mol/L cryoprotectant + sucrose, 10 min in 0.5 mol/L cryoprotectant + sucrose, and 10 min in sucrose alone. The zygotes were then washed three times in drops of T6 medium supplemented with EDTA (100 μ M) and 5 mg/mL BSA. After 1-h incubation in a humidified atmosphere of 95% air and 5% CO₂ at 37°C, only morphologically normal zygotes were cultured in drops (30 μ L) of T6 medium supplemented with EDTA (100 μ M) and 5 mg/mL BSA, for 48 h and then were cultured for further 72 h postinsemination in drops of T6 medium supplemented with 5 mg/mL BSA (10 zygotes in a drop).

The experiments were replicated 10 times for control, toxicity, and frozen groups and each time the zygotes were randomly allocated to the groups. In control, toxicity, and frozen groups embryos were observed every 24 h for 5 days and the rate of development of the embryos to a higher stage was recorded daily.

Morphology Assessment and Cell Number Determination

The viability of all treated and untreated zygotes was assessed after 24, 72, 96, and 120 h of culture using phase-contrast microscopy. The following scoring system for development was employed: 2-cell stage (by their ability to cleave to the 2-cell after 24 h of culture), less than morula (uncompacted or partially compacted embryos >2 cells), morula (fully compacted embryos with or without the beginning of a blastocoel), blastocyst (fully expanded blastocyst with a blastocoel cavity larger than two thirds of the embryo volume) which may have initiated herniation of the zona pellucida by the trophectoderm, expanded/hatching blastocyst (initiation or partially completion hatching blastocyst).

Blastocyst cell numbers were determined after 96 h of culture using a modification of Tarkowski's air drying technique (25). Blastocysts of each group were randomly selected and were placed in Acid Tyrod's (pH 2.5) to remove zona pellucida. After washing three times with PBS these were placed on a glass slide, and fixed with 4% paraformaldehyde for 1 h.

After fixation, the slides were washed three times with PBS, the nuclei of blastocysts were stained with Hoechst 33258 (Fluorescent dye; Wako, Osaka, Japan) with 5% wt/vol of DABCO (1,4-diazabicyclo-2, 2, 2-octane, Sigma) in 90% glycerol: 10% 0.2 M Na_2HPO_3 and left for 15 min at room temperature. Observation was carried out under fluorescent microscopy (Model BX50; Olympus, Tokyo Japan). A filter for the wavelength of 330–380 nm was used to search for Hoechst 33258. The number of nuclei in each expanded blastocyst was counted (26).

Statistical Analysis

The significance of differences among treatment group in each experiment was compared with the χ^2 test and data about number of bloomers were analyzed using ANOVA. A test was considered significant if $P < 0.05$.

RESULTS

Toxicity Test

Results of toxicity test for ETG and PROH are shown in Table I. In the ETG and PROH-exposed groups, 76 out of 86 (88.3%) and 98 out of 105 (93.3%) mouse zygotes, respectively, cleaved after 24 h of culture, while in the control group 138 out of 140 (98.6%) cleaved. After a further 72, 96, and 120 h of culture mouse zygotes that exposed with ETG, 69, 66, and 61 embryos, respectively, became morulla, blastocyst, and expanded hatching blastocyst (80.2, 76.7, and 71%, respectively); 89, 84, and 80 embryos (84.7, 80, and 76.2%, respectively) in PROH-exposed group, and 121, 118, and 114 embryos (86.4, 84.3, and 81.4%, respectively) in control group. The differences between the three groups (χ^2 test) were not statistically significant.

Mouse Zygote Freezing

The results of mouse zygote freezing with ETG and PROH are summarized in Table II. A total of 110 zygotes were frozen with ETG in 10 different experiments, 64 survived after thawing (58.2%), 55 cleaved to the 2-cell after 24 h of culture (50.2%). After a further 72, 96, and 120 h of culture 54, 51, and 43, respectively, became morulla, blastocyst, and expanded hatching blastocyst (49.5, 46.4, and 39.1%, respectively).

In the control group, 98.6% of the zygotes (138/140) cleaved after 24 h, and after a further 72, 96, and 120 h of culture 86.4, 84.3, and 81.4%, respectively, became morulla, blastocyst, and expanded hatching blastocyst. The difference in survival and cleavage rates between ETG frozen and control group, and also between ETG and PROH frozen groups were statistically significant (χ^2 ; $P < 0.0001$).

When the zygotes were frozen with PROH, 94.1% survived after thawing (145/154), 87% cleaved to the 2-cell (134/154), and after a further 72, 96, and 120 h of culture 74, 72.7, and 68.2% became morulla, blastocyst, and expanded hatching blastocyst respectively. The difference in survival and cleavage rates between PROH frozen and control group were statistically significant (χ^2 ; $P < 0.0001$ and $P < 0.01$, respectively).

The mean cell numbers of the untreated (control) and frozen blastocysts were not different (Table III).

DISCUSSION

The toxicity test results for ETG and PROH showed that they have a low toxic effect on survival and developmental rates of mouse zygotes, confirming the results reported previously for other animal species (17–19). When the mouse zygotes exposed with ETG and PROH, a short time interval was necessary for the exposed zygotes to restore their initial

Table I. Toxicity Test on Mouse Zygotes Exposed to 1.5 mol/L Ethylene Glycol (ETG) and 1.5 mol/L 1,2-Propanediol (PROH)

Group	Number of zygotes	Morphologically normal after exposure (%)	Developed to 2-cell (%) (24 h)	Developed to morula (%) (72 h)	Developed to blastocyst (%) (96 h)	Developed to expanded/hatching blastocyst (%) (120 h)
ETG	86	86 (100)	76 (88.3)	69 (80.2)	66 (76.7)	61 (71)
PROH	105	105 (95.7)	98 (93.3)	89 (74.7)	84 (80)	80 (76.2)
Control	140	—	138 (98.6)	121 (86.4)	118 (84.3)	114 (81.4)

Note. Values are number (percent).

Table II. Mouse Zygote Freezing with 1.5 mol/L Ethylene Glycol (ETG) and 1.5 mol/L 1,2-Propanediol (PROH)

Group	Number of zygotes	Morphologically normal after freezing (%)	Developed to 2-cell (%) (24 h)	Developed to morula (%) (72 h)	Developed to blastocyst (%) (96 h)	Developed to expanded/hatching blastocyst (%) (120 h)
Slow freezing (ETG)	110	64 ^a (58.2)	55 ^a (50.2)	54 ^a (49.5)	51 ^a (46.4)	43 ^a (39.1)
Slow Freezing (PROH)	154	145 ^b (94.1)	134 ^b (87)	114 ^c (74)	112 ^c (72.7)	105 ^c (68.2)
Control	140	—	138 (98.6)	121 (86.4)	118 (84.3)	114 (81.4)

Note. Values are number (percent).

^a Significance for slow freezing with ETG vs. control (χ^2 ; $P < 0.0001$).

^b Significance for slow freezing with PROH vs. control (χ^2 ; $P < 0.0001$).

^c Significance for slow freezing with PROH vs. control (χ^2 ; $P < 0.01$).

volume after shrinkage, and Emiliani *et al.* (1) suggested a rapid diffusion and a quick equilibration of ETG into the cell through the zona pellucida and the cellular membrane. On the other hand, the excessive shrinkage observed suggested the risk of an intense osmotic stress by exposing the embryos to this ETG concentration.

The present investigation indicated that ETG, by freezing–thawing conditions similar to those used for human embryos, was affected on survival rate of slow-cooled mouse zygotes which was in agreement with Emiliani *et al.* (1) who slow-cooled mouse zygotes using ETG and PROH as a cryoprotectant in freezing–thawing conditions similar to those used for human embryos, and was in disagreement with Shaw *et al.* (21) who used ETG on the survival of slow-cooled mouse early embryos by comparing different thawing conditions which closely resembled those used for freezing human embryos. Shaw and colleagues observed a higher survival rate of embryos when slow warming-up (keeping the straw in air for 15 s) was followed by a fast warming-up (plunging the straws into a water bath at 37°C), at the moment when the straw reached the temperature of –70°C; consequently, this thawing protocol was selected for the Emiliani and present studies. Lower survival rates in

Emiliani and our studies were observed in thawing protocol where embryos warmed up either too rapidly (by direct plunging into water at 37°C) or too slowly (by keeping the straw in air for longer).

Ours and Emiliani's results showed that survival rate of mouse zygotes after freezing–thawing with ETG were 58.2 and 60%, respectively lower than those reported by Shaw (65%). In comparison with Shaw, lower survival rate in Emiliani and our studies, may be due to differences in genetic constitution between the mouse strains used and interlaboratory variations in chemical and physical factors might be a further reason for these observed differences. The different genetic constitution between animal species can strongly influence the resistance of the embryos to freezing, as well as the interaction between embryo and cryoprotectant (1). The present study showed the survival rate of mouse zygotes after freezing–thawing with PROH was 94.1%, which almost corresponds with the percentage of survived zygotes (92%) in Emiliani's study.

EDTA facilitated the development of zygotes from an outbred strain mice through the 2-cell block (27); it can be concluded that EDTA confers its benefit on the embryo during the first 48 h of culture (28). Therefore, we also used EDTA, because it can alleviate the 2-cell block during the first 48 h of culture. In this study, developmental rate of mouse zygotes after freezing–thawing with ETG to 2-cell stage and expanded hatching blastocyst (50.2 and 39.1%, respectively) differed somewhat from those reported by Emiliani *et al.* (82 and 44%, respectively). Although, the rate of development of slow-cooled mouse zygote with ETG to the 2-cell stage in later study higher than our experiment, but expanded hatching blastocyst rate in both studies was decreased. However, expanded hatching blastocyst rate in present and Emiliani's studies was improved when mouse zygotes were frozen with PROH (68.2 and 70%, respectively). In our experience, ETG

Table III. The Number of Cells in the Blastocysts Developed In Vitro From Mouse Zygotes in Control and Slowly Frozen–Thawed with 1,2-Propanediol (PROH) and Ethylene Glycol (ETG) as a Cryoprotectant

Treatment	No. of cells
None (control)	81.1 ± 14.5 (20)
1,2-Propanediol	77.24 ± 13.2 (14)
Ethylene glycol	78.17 ± 13.4 (12)

Note. Values are mean ± SD cells/blastocyst. Numbers of sampled blastocysts appear in parentheses. There was no significant difference in the number of cells between these groups of blastocysts.

did not seem to be a good cryoprotectant for mouse zygote, but both survival rate and expanded blastocyst rate improved when mouse zygotes were frozen with PROH, confirming the results reported previously (1,21). These two later results indicate that different embryonic stages are differently affected by the freezing procedure, and for each embryonic stage optimal results can be achieved using a different cryoprotectant. It has been reported (29) that the exposure time of embryos to the cryoprotectant strongly influences their viability after thawing. The equilibration time in a freezing protocol should in fact be neither too short (in order to allow the substitution of water molecules in the cell), nor too long (in order to avoid toxic effects on the embryos). Different cryoprotectants with different permeability to the cell membrane and toxicity levels can variously influence developmental embryonic stage, characterized by a progressively decreasing blastomere volume and changes in embryonic metabolism (1). The observed decline in survival and expanded blastocyst rate (94.1 and 68.2% for mouse zygotes with PROH compared with 58.2 and 39.1% for mouse zygotes with ETG) confirms previous data (1,21), where a low developmental and survival rate were observed after freezing-thawing with ETG.

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