Deleterious Effect of Equilibration Temperature on the Toxicity of Propanediol During Cryopreservation of Mouse Zygotes

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Purpose: Our aim was to determine the effect of temperature and incubation time on the toxicity of propanediol on mouse zygotes.

Method: Zygotes were pooled and randomly allocated to one of the treatment groups. Zygotes were incubated in PBS or PBS containing 1.5M propanediol at 37°C or room temperature (RT) for up to 30 min, washed, and cultured. Similarly, zygotes were incubated at 4°C or RT for 30 min and either washed and cultured or frozen-thawed, washed, and cultured. Zygotes were examined at 24, 48, and 96 hr of culture and embryo quality was determined.

Results: Exposure of zygotes to 1.5 M propanediol at 37°C significantly impaired first cleavage and blastocyst formation compared to exposure at RT ($P < 0.0007$). Incubation of zygotes for more than 5 min at 37°C significantly reduced embryo development ($P < 0.00001$). Exposure to propanediol at 4°C results in similar embryo development before and after freeze-thawing compared to exposure at RT.

Conclusions: Propanediol is toxic to mouse zygotes in a temperature- and time-dependent fashion. Cryopreservation of zygotes after exposure at 4°C appears to be no better than after exposure at RT.

KEY WORDS: Mouse zygote; propanediol; toxicity; equilibration temperature.

INTRODUCTION

Improvement in the success of cryopreservation of human embryos will result in overall higher pregnancy rates and lower risk of multiple pregnancy. The ability of cryoprotectants such as glycerol, dimethylsulfoxide (DMSO), and propanediol (PROH) to protect mamma-

lian cells successfully depends on their water solubility, permeation, and cytotoxicity. Cytotoxicity of a cryoprotectant depends on factors such as the type of cell, temperature and duration of exposure, composition of the medium, and concentration of the cryoprotectant (1,2). Techniques to cryopreserve embryos at the one- to eight-cell, morula, and blastocyst stages have been described for many species including human (2). Although freezing can be performed at any stage, there appears to be a recent preference to freeze human embryos at the one-cell or blastocyst stage. One-cell human zygotes have been successfully frozen and thawed using PROH with (3) and without (4) sucrose during freezing and thawing.

We use a cryopreservation technique for zygotes using 1.5 M PROH (4) in our IVF program. In this technique zygotes are handled at room temperature (RT). However, in-depth animal studies to optimize this freezing technique have not been reported. The effect of equilibration temperature and time on the survival of either human or animal embryos using this technique (4) was not reported. It is common for human in vitro laboratories to handle oocytes, zygotes, and embryos on heated microscope stages or enclosed chambers (isolettes) at 37°C (5). At a busy IVF clinic, therefore, zygotes may be exposed to PROH at high temperatures, particularly when a limited number of isolettes is available for embryo handling.

This study evaluated the potential effect of failing to control for equilibration temperature during the initial PROH exposure by investigating the effect of equilibration temperature and time on the toxicity and survival of mouse zygotes.

MATERIALS AND METHODS

One-cell zygotes obtained from B₆C₃F₁ mice were exposed to either 1.5 M PROH (Sigma Chemical Co., St. Louis, MO) in phosphate-buffered saline (PROH-

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PBS) at RT ($20 \pm 2^\circ\text{C}$) and 37°C or PBS only at RT (control) for 30 min. The PROH was washed off the zygotes by incubating them for 5 min each in 1, 0.5, and then 0 M PROH-PBS. The zygotes were subsequently cultured in a modified Ham's F10 medium (GIBCO BRL, Grand Island, NY) without hypoxanthine, phosphates, and transition metals containing 5 mM glucose (Mahadevan *et al.*, unpublished observations) and embryo development was assessed at 24, 48, and 96 hr under an inverted microscope. The effect of time was then investigated by comparing embryo growth after incubating zygotes in PROH-PBS for various periods of time at RT or 37°C and cultured as above.

In another experiment mouse zygotes were exposed to PROH-PBS at RT or 4°C for 30 min. In this study 0.3 ml of the cooled PROH-PBS (at 4°C) was transferred to cryovials (at RT). The vials were placed in the refrigerator (at 4°C) for approximately 30 min before the embryos were exposed to the PROH-PBS solution. After 30 min of incubation PROH was washed off in steps and cultured as above. In addition, to assess the effect of equilibration temperature on cryosurvival, zygotes were exposed to PROH-PBS at 4°C or RT and frozen-thawed (4). After 30 min of incubation at RT, the vials incubated at RT for 10 min were transferred to a freezing machine and freezing was initiated. When the freezing chamber temperature was 4°C , the vials incubated at 4°C were also transferred and freezing was completed. After thawing the zygotes were cultured and assessed at 24, 48, and 96 hr.

RESULTS

Equilibration temperature and time of zygote exposure to PROH-PBS had a significant effect on initial cleavage and subsequent development to blastocysts. When zygotes were exposed to PROH-PBS at RT or 37°C for 30 min, 83.8% (57/68) and 31.3% (21/67) cleaved at 24 hr, respectively ($P < 0.000001$). After 96 hr in culture, only 16.4% (11/67) of the zygotes developed to blastocysts when exposed at 37°C , whereas 61.8% (42/68) developed when exposed at RT ($P < 0.000001$). Incubation of zygotes at 37°C for more than 5 min significantly impaired embryo development to blastocysts ($P < 0.00001$; Table I). Embryo development after 5 min of exposure to PROH at 37°C (62.3%) was not significantly lower than when exposed for 1 min (74.2%; $P = 0.2026$).

Incubation of mouse zygotes at RT for up to 30 min did not significantly impair further embryo development (Table II; $P = 0.6898$). Exposure of mouse

zygotes to 1.5 M PROH-PBS at 4°C or RT resulted in similar embryo development up to blastocyst stage (Table III; $P = 0.5285$). Cryosurvival of zygotes exposed to PROH at 4°C or RT was similar (Table IV) in terms of cleavage to two cells at 24 hr (58.4% vs 53.6%; $P = 0.7338$) or formation of blastocysts at 96 hr (48.8% vs 42.7%; $P = 0.2436$).

DISCUSSION

Techniques to cryopreserve embryos at the one- to eight-cell, morula, and blastocyst stages have been described for many species including human (2). Although human embryos can be frozen at any stage, there appears to be a preference to freeze at the one-cell or blastocyst stage. One-cell human zygotes have been successfully frozen and thawed using PROH with and without sucrose during freezing and thawing (3,4). However, very few in-depth studies to optimize these freezing techniques have been done.

Permeability of a cryoprotectant such as PROH is important for cell survival in certain cell cryopreservation techniques. However, the same cryoprotectants can also cause toxicity under certain conditions (6). Permeation of a cryoprotectant into a cell depends on the concentration, temperature, and time of equilibration. Kasai *et al.* (7) reported that the survival rate of mouse embryos depended on the equilibration time in DMSO at 0 and 20°C . However, survival was higher when equilibrated in ethylene glycol or glycerol at 20 than at 0°C . This indicates that although toxicity may be lower at a lower temperature, cryosurvival may be paradoxically lower after freeze-thawing due to reduced permeation of cryoprotectants (1,7). Renard *et al.* (8) reported that highly permeable PROH was not toxic to rabbit two-cell embryos when exposed for up to 30 min at RT. This was found to be true also for one-cell mouse zygotes at RT and 4°C in this study.

Bovine pregnancy rates decreased when ambient temperatures were exceeded during the thawing of embryos (9). It was hypothesized that embryos may be sensitive to sucrose in the cryopreservation medium. Sucrose toxicity to mouse embryos during exposure at 25 and 36°C compared to 20°C was confirmed by Szell and Shelton (10). Whatever the cause may be, exposure to cryoprotectants such as DMSO (11) and PROH (12) has been shown to damage the microtubular structure of mouse oocytes in a temperature-, concentration-, and time-dependent manner. It is possible that a similar mechanism may explain the temperature- and time-dependent toxicity of PROH on mouse

Table I. Effect of Equilibration Time in 1.5 M Propanediol at 37°C on Embryo Growth

Time (min)	No. of zygotes	Number (%) of embryos developed to			
		At 24 hr, ≥ 2 -cell	At 48 hr, ≥ 8 -cell	At 96 hr	
				$\geq$ Morula	Exp. blast.
1	62	60 (96.8)	48 (77.4)	59 (95.2)	46 (74.2)
5	61	54 (88.5)	41 (67.2)	48 (78.7)	38 (62.3)
10	67	45 (67.2)	28 (41.8)	31 (46.3)	21 (31.3)
15	66	29 (43.9)	12 (18.2)	22 (33.3)	9 (13.6)
30	63	26 (41.3)	3 (4.8)	3 (4.8)	3 (4.8)
PBS control	58	51 (87.9)	45 (77.6)	51 (87.9)	44 (75.9)
		$P < 0.000001$	$P < 0.0001$	$P < 0.00001$	$P < 0.00001$

Table II. Effect of Equilibration Time in 1.5 M Propanediol at Room Temperature on Embryo Growth

Time (min)	No. of zygotes	Number (%) of embryos developed to			
		At 24 hr, ≥ 2 -cell	At 48 hr, ≥ 4 -cell	At 96 hr	
				$\geq$ Morula	Exp. blast.
PBS control	52	49 (94.2)	46 (88.5)	47 (90.4)	38 (73.1)
10	49	47 (95.9)	45 (91.8)	45 (91.8)	36 (73.5)
15	57	48 (84.2)	52 (91.2)	45 (79.0)	36 (63.2)
20	45	42 (93.3)	44 (97.8)	40 (88.9)	33 (73.3)
30	53	46 (86.8)	48 (90.6)	42 (79.3)	39 (73.6)
		$P = 0.1662$	$P = 0.5516$	$P = 0.1545$	$P = 0.6898$

Table III. Comparison of Exposure of Mouse Zygotes to 1.5 M Propanediol at Room Temperature or 4°C on Embryo Development

Temperature	No. of zygotes	Number (%) of embryos developed to			
		At 24 hr, ≥ 2 -cell	At 48 hr, ≥ 4 -cell	At 96 hr	
				$\geq$ Morula	Exp. blast
RT (a)	82	67 (81.7)	67 (81.7)	65 (79.3)	61 (74.4)
4°C (b)	82	74 (90.2)	65 (79.3)	65 (79.3)	58 (70.7)
PBS only (control) (c)	62	54 (87.1)	54 (87.1)	52 (83.9)	49 (79.0)
avsb		$P = 0.2763$	$P = 0.4685$	$P = 0.7379$	$P = 0.5285$
avsc					$P = 0.6518$
bvsc					$P = 0.3492$

Table IV. Effect of Exposure of Mouse Zygotes to 1.5 M Propanediol at Room Temperature or 4°C on Cryosurvival

Temperature	No. of zygotes	Number (%) of embryos developed to			
		At 24 hr, ≥ 2 -cell	At 48 hr, ≥ 4 -cell	At 96 hr	
				$\geq$ Morula	Exp. blast.
RT (22°C) (a)	220	118 (53.6)	114 (51.8)	112 (50.9)	94 (42.7)
4°C (b)	209	122 (58.4)	114 (54.6)	117 (56.0)	102 (48.8)
PBS control (not frozen)	107	95 (88.8)	95 (88.8)	93 (86.9)	88 (82.2)
avsb		$P = 0.7338$	$P = 0.3392$	$P = 0.3392$	$P = 0.2436$

zygotes. Whether toxicity is due to PROH or some contaminant in PROH is unknown.

Toxicity of 1.5 M PROH on mouse zygotes incubated at various temperatures has been determined. PROH was highly toxic at 37°C compared to incubation at 4°C or RT. Even incubation at 37°C for 10 min significantly reduced blastocyst formation. This toxicity was not observed after 1 min, and only a nonsignificant reduction occurred at 5 min. However, an equilibration time of up to 30 min at RT did not influence blastocyst formation in vitro. These findings in this study agree with the report by Van der Elst *et al.* (12) in mouse oocytes that 1.5 or 3 M PROH is toxic at 37°C within a few minutes, in terms of morphological survival. However, in our study mouse zygotes, unlike mouse oocytes, are less sensitive to the toxicity of 1.5 M PROH at RT (12). Exposure and incubation of zygotes at 4°C with or without freezing and thawing appeared not to improve cryosurvival compared to RT. This is in contrast to the improved survival of mouse oocytes and mouse zygotes exposed to 1.5 or 3 M PROH at 0°C compared to exposure at RT (12,13). Recently, Van der Elst *et al.* (13) reported that even at RT, 3 and 4.5 M PROH was toxic to mouse zygotes within 20 and 10 min, respectively. However, at 4°C, 3 and 4.5 M PROH was not toxic to mouse embryos even after 30 min of exposure. For practical reasons, at a busy IVF clinic handling embryos at 4°C is more convenient than at 0°C. Although little permeation of cryoprotectants occurs at 0–4°C (1, 14), studies by Miyamoto and Ishibashi (15) indicate that equilibration of eight-cell mouse embryos and morulas in 2 M PROH for 20 to 30 min at 0°C results in optimal postthaw survival. Optimal cryopreservation conditions resulting in maximal zygote survival after freezing and thawing deserve further study.

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