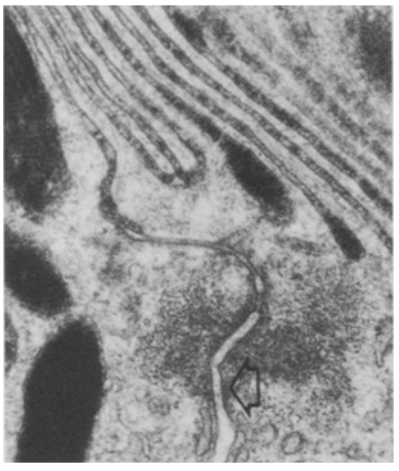

Figure 6. Black reaction product of intraocularly injected microperoxidase does not penetrate tight junctions of hyperplastic RPE; extracellular space on choroidal side of RPE (arrow) does not contain tracer. × 42,000.

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## The effects of time of equilibration with cryoprotectants at 0 °C prior to freezing on the survival of mouse embryos frozen by the two-step method

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*Summary.* Mouse embryos were frozen by the two-step method after equilibration for 0.1–60 min with cryoprotectants at 0 °C. No survival or a very low survival was obtained after equilibration for only 0.1 min. The morulae showed the highest survival rates when equilibration time was 5–30 min with 2 M DMSO, 20–30 min with 2 M glycerol, 5–10 min with 2 M ethylene glycol and 20–30 min with 2 M propylene glycol, respectively.
*Key words.* Mouse embryos; two-step freezing; cryoprotectant; equilibration time; permeation.

Mammalian embryos have been preserved at low temperatures only if the cryoprotectant is present in the suspending solution during cooling[1]. The cryoprotectants such as dimethyl sulfoxide (DMSO) or glycerol used for embryo freezing are able to penetrate cells and the amount of permeation is reflected to some extent by the duration of exposure prior to freezing. Although most workers have exposed embryos to cryoprotectants for about 10–20 min prior to initiating freezing of embryos, the mechanisms by which these compounds protect embryos against freezing damage remain obscure[1]. It is suggested that DMSO probably need not permeate 8-cell mouse embryos to protect them against freezing damage[2–4].

A two-step cooling procedure is useful in the study of various factors influencing the recovery of cells following freezing and thawing[5]. The present experiments were designed to examine the effect of equilibration time with various cryoprotectants, prior to freezing, on the survival of mouse embryos frozen by the two-step method.

*Materials and methods.* Female ICR mice, aged 4–6 weeks, were induced to superovulate by the i.p. injection of 5 IU of pregnant mare serum gonadotropin followed 48 h later by 5 IU of human chorionic gonadotropin (hCG). They were mated with males of the same strain. The 8-cell and morula stage embryos were flushed from the reproductive tracts with a modified Dulbecco's phosphate-buffered saline (PBS)[6] at 67–70 and 77–79 h after the injection of hCG, respectively.

The 10–25 embryos were pipetted into 10 × 100 mm glass tubes containing 0.1 ml PBS and cooled to 0 °C. The cryoprotectant in 0.15 ml PBS was added to samples at 0 °C in a single addition. The cryoprotectants used were DMSO, glycerol, ethylene glycol and propylene glycol and the concentration of cryoprotectant was usually 2 M. The samples were equilibrated for 0.1–60 min at 0 °C after the addition of cryoprotectant and then transferred without ice-seeding from 0 °C into constant temperature ethanol baths at either −20 °C (for embryos for which DMSO was the cryoprotectant) or −40 °C (for embryos with other cryoprotec-

tants). After 10 min in the baths they were plunged into liquid nitrogen for up to 3 days before thawing.

All samples were thawed rapidly in a 40 °C water bath. Immediately upon thawing, embryos were pipetted into PBS containing the same concentration of cryoprotectant plus 0.5 M sucrose and were kept there for 3 min at room temperature before transfer into PBS with 0.5 M sucrose. A few minutes later, the embryos were washed twice in fresh PBS. Survival was assayed by counting the number of embryos developing into blastocysts in vitro using standard culture methods[7]. Experiments were replicated 3–6 times and data were analyzed for statistical significance using the $\chi^2$ test. To examine the further development of morulae frozen after equilibration for 1 and 20 min with 2 M glycerol, the embryos which had developed to blastocysts after being cultured for 36–40 h were transferred into the uterine horns (5–10 embryos per horn) of females on Day 4 of pseudopregnancy. The recipients were allowed to give birth to litters.

*Results and discussion.* The results are presented in the table. The equilibration time of mouse embryos with cryoprotectant at 0 °C was important. No survival or a very low survival (8–9%) was obtained after equilibration for only 0.1 min with DMSO. The highest survival rates (62–65%) for 8-cell embryos were found after equilibration for 10–20 min with 2 M DMSO ($p < 0.01$). The highest survival rates (52–60%) for morulae were obtained with equilibration for 5–30 min with 2 M DMSO and for 5–20 min with 3 M DMSO ($p < 0.05$). Prolonged equilibration (60 min) with DMSO appeared to be detrimental.

When morulae were frozen in the presence of glycerol, ethylene glycol or propylene glycol, no embryos, or a very low proportion of the embryos, were protected after equilibration for 0.1 min at 0 °C. The highest survival rates were obtained after equilibration for 20–30 min (75–77%) with 2 M glycerol, for 5–10 min (44–47%) with 2 M ethylene glycol and for 20–30 min (49–55%) with 2 M propylene glycol, respectively ($p < 0.05$).

As a further test of viability, 102 embryos that survived freezing after 1 min equilibration were transferred to 14 recipients and 8 females gave birth to 38 (37%) normal young. One hundred and twenty-one embryos that survived freezing after 20 min equilibration were transferred to 16 recipients and 8 females gave birth to 40 (33%) normal young. Therefore, no difference in the potential for survival in vivo was found between embryos that developed in vitro with 1 min equilibration with 2 M glycerol and embryos that developed in vitro with 20 min equilibration. The present results suggest that the cryoprotectant has to permeate the mouse embryos to protect them against freezing damage and permeation of the cryoprotectant into mouse embryos may enhance their survival.

The optimum equilibration time of morulae with ethylene glycol was shorter than that of embryos with propylene glycol. This result is consistent with that of a previous study[8] in which 8-cell mouse embryos were cooled slowly after ice-seeding. It was, however, suggested at that time that protection against freezing damage by DMSO or ethylene glycol probably does not require permeation of the cryoprotectant into 8-cell mouse embryos[2–4, 8]. In those experiments[2–4, 8], following equilibration with the cryoprotectant the samples were transferred to a −3.5 to −6 °C bath, held for 2 min, ice-seeded and 5 min later cooled slowly (0.3–1.8 °C/min); it took about 7 min from the ceasing of equilibration to the initiating of the continuous slow cooling. It seems reasonable, therefore, to assume that, in contrast to our former assumption, DMSO and ethylene glycol did enter the blastomeres during these 7 min and also during the interval of slow cooling even at high subzero temperatures. Further experiments are needed to find out whether a compound that protects embryos against freezing damage must permeate them to exert its beneficial effect.

The effects of equilibration time with various cryoprotectants at 0 °C, prior to freezing, on the survival of mouse embryos frozen by the two-step method

| Cryoprotectant | Equilibration time at 0 °C (min) | % Survival 8-cell | Morula |
|---|---|---|---|
| DMSO (2 M) | 0.1 | 0 (69) | 9 (82) |
| | 1 | 9 (77) | 26 (78) |
| | 5 | 31 (81) | 52 (75) |
| | 10 | 65 (82) | 57 (84) |
| | 20 | 62 (77) | 55 (67) |
| | 30 | 43 (75) | 55 (77) |
| | 60 | 25 (72) | 7 (72) |
| DMSO (3 M) | 0.1 | | 8 (79) |
| | 1 | | 10 (83) |
| | 5 | | 60 (84) |
| | 10 | | 58 (86) |
| | 20 | | 52 (77) |
| | 30 | | 38 (80) |
| | 60 | | 4 (76) |
| Glycerol (2 M) | 0.1 | | 2 (81) |
| | 1 | | 22 (77) |
| | 5 | | 55 (82) |
| | 10 | | 52 (67) |
| | 20 | | 75 (84) |
| | 30 | | 77 (79) |
| | 60 | | 33 (75) |
| Ethylene glycol (2 M) | 0.1 | | 0 (58) |
| | 1 | | 23 (64) |
| | 5 | | 47 (72) |
| | 10 | | 44 (66) |
| | 20 | | 30 (68) |
| | 30 | | 28 (71) |
| | 60 | | 10 (63) |
| Propylene glycol (2 M) | 0.1 | | 0 (65) |
| | 1 | | 0 (58) |
| | 5 | | 3 (66) |
| | 10 | | 13 (77) |
| | 20 | | 55 (74) |
| | 30 | | 49 (65) |
| | 60 | | 30 (63) |

Figures in parentheses represent the number of embryos recovered after thawing.

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