Osmotic shock of fertilized mouse ova

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Summary. The effect of osmotic changes on fertilized mouse ova was studied by measuring their survival, defined as development into hatching blastocysts, after exposure to various concentrations of ethanediol (ethylene glycol). In addition, a Boyle–van't Hoff plot was derived from exposing ova to hypotonic and hypertonic solutions ranging from 0.1 to 2.8 osmol. Volume of ova was inversely proportional to osmolality over this range. Extrapolation of this relationship yielded a nonosmotic volume of the ova of 22.5%. Eighty-five per cent or more of the ova survived exposure to this wide range of concentrations and developed into blastocysts. The rate of development of ova exposed to anisotonic solutions was the same as that of controls. Ova underwent osmotic shock when abruptly diluted out of concentrated solutions of ethanediol with an isotonic solution. Their survival was highly dependent on the ethanediol concentration with which they had equilibrated before dilution, and the manner, rate and temperature of dilution. The longer the exposure to ethanediol the greater was the sensitivity of the ova to osmotic shock, reflecting permeation of ethanediol into the ova. Osmotic shock could be alleviated by dilution at a high temperature, and prevented by the use of sucrose as an osmotic buffer at 37°C. Identification of the variables that influence osmotic shock of ova will be helpful in the systematic study of their cryopreservation.

Keywords: osmotic shock; ova; survival; osmometry; cryoprotectant; mouse

Introduction

The cryopreservation of mammalian ova and embryos has become a routine procedure. Thousands of live young, especially of mice and cows, have been born after having been cryopreserved as embryos. To survive freezing and thawing, however, ova and embryos must be suspended in molar concentrations of compounds referred to as cryoprotectants. A variety of compounds act as cryoprotectants, including methanol, (formula weight (form. wt) = 32.0; first described by Rall *et al.*, 1984), ethanediol (form. wt = 62.1; Miyamoto & Ishibashi, 1978), propanediol (form. wt = 76.1; Renard & Babinet, 1984), dimethyl sulfoxide (form. wt = 78.1; Whittingham *et al.*, 1972), glycerol (form. wt = 92.1; Whittingham *et al.*, 1972), and di- and triethylene glycol (form. wt = 106.1, 150.2, Miyamoto & Ishibashi, 1978). These compounds share several common features including low molecular weight, apparent lack of toxicity, and the ability to permeate ova and embryos.

Cryoprotection requires exposure of ova to these solutes before freezing, but subsequent development demands their removal after thawing. Relatively little is known about the effects of

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exposure and removal of cryoprotectants on viability of ova. Only a few systematic studies of the permeability of ova and embryos to such compounds have been published (Jackowski *et al.*, 1980; Leibo, 1983; Szell & Shelton, 1986a, b; Shaw *et al.*, 1990). Three recent developments in the field of cryopreservation of ova and embryos have prompted a detailed examination of osmotic phenomena of fertilized mouse ova.

First, there is growing interest in cryopreservation of both fertilized and unfertilized ova rather than cleavage-stage embryos. Cryopreserved zygotes can be used in the production of transgenic animals (Leibo *et al.*, 1991). Because fertilized ova are generally considered to be more sensitive than embryos to physical and chemical anomalies, they provide a more discriminating bioassay for quality control in the practice of human *in vitro* fertilization (Quinn *et al.*, 1984; Pabon *et al.*, 1989; Leibo, 1990). Cryopreservation of human unfertilized ova rather than cleavage-stage embryos provokes fewer ethical and legal objections (AMA Board, 1990).

Second, the cytoskeleton and meiotic or mitotic spindle of ova have been shown to be particularly sensitive to cooling and to chemical alteration by exposure to cryoprotectants (Johnson & Pickering, 1987; Pickering & Johnson, 1987; Vincent *et al.*, 1987, 1989, 1990; Johnson, 1989; Pickering *et al.*, 1990; Van der Elst *et al.*, 1988), observations with important cytogenetic implications. Third, high concentrations of cryoprotectants are frequently used for cryopreservation by rapid cooling (Kasai *et al.*, 1980; Renard & Babinet, 1984; Takeda *et al.*, 1984; Szell & Shelton, 1986a, b; Trounson *et al.*, 1987, 1988) and by vitrification (Rall & Fahy, 1985; Rall, 1987; Massip *et al.*, 1986; Scheffen *et al.*, 1986). Vitrification refers to the phenomenon by which an aqueous solution becomes a glass, or vitrifies, upon cooling to subzero temperatures, rather than crystallizes, or freezes.

Vitrification of a solution requires that it is cooled rapidly, that the solute is present at a high concentration or that both these conditions occur. But the high concentrations of cryoprotectants used for rapid freezing of ova and embryos render them extremely sensitive to osmotic shock. Cells exhibit osmotic shock if (i) the difference in osmotic pressures between the intracellular and extracellular solutions is sufficiently large to cause the cells to expand beyond their critical volumes, or (ii) the permeability of the cells to water and to solutes is sufficiently different so that water enters the cells faster than the solute can escape from the intracellular space or (iii) both these conditions occur. Osmotic shock is responsible for membrane lysis when a cell is rapidly diluted out of a hypertonic solution into an isotonic one. Osmotic shock is an often-overlooked aspect of cell cryopreservation that may be responsible for cell damage (Leibo, 1978, 1984) and has been described for biological specimens as diverse as erythrocytes (Jacobs *et al.*, 1935), bacteriophages (Anderson *et al.*, 1953; Leibo & Mazur, 1966) and fetal pancreatic cells (Mazur *et al.*, 1976). A systematic investigation of osmotic shock of fertilized mouse ova exposed to ethylene glycol was performed as a prelude to a study of their cryopreservation.

Materials and Methods

Animals and superovulation

Animals were housed in facilities of the Baylor Animal Program according to guidelines established by the National Institutes of Health (USA), and their care and use were reviewed and approved by the Animal Use Committee of Baylor College of Medicine. Six- to twelve-week-old female B6C3F₁ mice (Charles River Laboratories, Inc., Wilmington, MA) were induced to superovulate by i.p. injection of 5.0–7.5 iu pregnant mares' serum gonadotrophin (MSG; Sigma Chemical Corp., St Louis, MO) and of 5.0–7.5 iu human chorionic gonadotrophin (hCG; Sigma) 46–48 h later. The hormones were prepared in sterile saline (0.165 mol NaCl l⁻¹) and stored at -70°C until used. The female mice were mated with fertile B6D2F₁ males immediately after the hCG injection, and were killed by cervical dislocation approximately 16–18 h later.

Fertilized ova and their survival

Ova fertilized *in vivo* were collected by slitting the ampullae of excised oviducts, and incubating the ova clots in 0.03% hyaluronidase for 3–5 min at room temperature to remove adherent cumulus cells. The ova were rinsed in

sterile M2 solution, a Hepes-buffered solution (Hogan *et al.*, 1986); ova from six to ten females were pooled for each experiment, and 250–300 ova were used per experiment. Stocks of M2 solution were prepared and stored in the frozen state at -70°C until used, since the stock solutions are completely stable for six months or more at that temperature. All solutions were prepared using analytical grade inorganic reagents. Bovine serum albumin (BSA), phenol red, lactate, pyruvate and antibiotics were purchased from GIBCO BRL (Grand Island, NY) and Sigma Chemical Co. (St Louis, MO). For some experiments, zona-free ova were prepared by incubation in 0.5% pronase in M2 for about 5 min at about 20°C until the zonae just dissolved (Hogan *et al.*, 1986).

Ova survival was assayed by rinsing treated ova two or three times in sterile M2 solution, and then incubating them in 0.010 ml CZB medium (Chatot *et al.*, 1989) in microtest plates (either Nunc Inc., Naperville, IL or Sarstedt, Inc., Princeton, NJ) under silicone oil (Dow Corning Corp., Midland, MI; Medical Grade Fluid 200) in an atmosphere of 5% CO_2 :5% O_2 :90% N_2 at 37°C for five days. A 'survivor' was an ovum that developed into an expanded blastocyst and exhibited partial or complete hatching after five days in culture. Survival experiments were repeated once or twice, and survival was expressed as the mean $\pm$ standard error.

Rate of development of treated ova

The subtle effects of osmotic stress were determined by measuring the rates of development of fertilized ova exposed to either a hypertonic (2.5 osmol, galactose in M2 solution) or a hypotonic (0.05 osmol, made by dilution of M2) solution. Controls consisted of ova exposed only to isotonic M2 solution. Ova were collected approximately 22–24 h after hCG injection of the donors and made cumulus-free as described above and were handled in groups of 20 to 25. Each group was rinsed twice in about 3 ml of 2.5 osmol, or 0.05 osmol, of M2 solution, and held in the same solution at room temperature for 15 min. The treated ova were recovered, rinsed twice in fresh M2, and cultured as described above. Approximately every 8–12 h, the developing embryos were examined microscopically, and the number of blastomeres of each embryo was counted. After about 50, 60, 76 and 118 h of culture, approximately 10–20 embryos per group were fixed with freshly prepared methanol:acetic acid (3:1), stained with 4% Giemsa, and the total number of cells per embryo was counted. The results are expressed below as the mean number of cells per embryo for each set as a function of time in culture, time 'zero' referring to about 24 h after hCG injection.

Ova volume measurements

Osmotic expansion or contraction was measured by suspending zona-free and zona-intact ova in various tonicities of M2 at about 21°C for 15 min, and their cross-sectional areas were measured, from which their volumes were calculated by previously published methods (Leibo, 1980). Briefly, the ova were photographed, the photomicrographs enlarged about 18-fold and traced with an electronic image analyser (Carl Zeiss, Inc., Thornwood, NY; ZIDAS Model). Hypertonic solutions of M2 were prepared by the addition of galactose, and hypotonic solutions by the dilution of M2 with distilled water. Osmolalities of these anisotonic solutions were measured with a calibrated osmometer (Precision Systems, OSMETTE A Model 5002). Ova volume in isotonic M2 solution at an osmolality of 0.280 osmol was taken to be 100%. Volumes of ova in anisotonic solutions were plotted relative to 100% as a Boyle-van't Hoff graph of % relative volume versus (Osmolality) $^{-1}$.

Osmotic expansion was measured by suspending groups of three to seven zona-free ova in five hypotonic solutions of M2. Survival of ova in these solutions was measured by suspending large groups of ova in each concentration of hypotonic M2 (three sets of about 20 ova per set per concentration) for 15 min at room temperature, rinsing them in isotonic M2 solution and culturing them. Osmotic contraction was measured by suspending zona-intact ova in M2 solutions made hypertonic with added galactose. Volumes and survival of ova were determined as described for the hypotonic solutions.

Osmotic shock procedure

In a few experiments, the ova were diluted out of concentrated ethanediol solutions contained in glass tubes. In all the others, osmotic shock was performed in a similar way to the procedure that is commonly used to recover ova and embryos cryopreserved in plastic straws (Renard *et al.*, 1982; Chupin *et al.*, 1984; Leibo, 1984; Leibo *et al.*, 1990). Rinsed ova were pipetted from M2 solution into approximately 3 ml of solutions of the test concentrations of the cryoprotective agent (CPA) at room temperature of 20 – 22°C . Groups of 15–25 ova were then pipetted into a 0.015 ml of the same test CPA that had already been aspirated into a 0.25 ml plastic straw (1.4 mm i.d. $\times$ 110 mm long) commonly used for artificial insemination of cattle (no. A201, Instruments de Médecine Vétérinaire, L'Aigle, France). The straw also contained about 0.14 ml of the test diluent solution separated from the CPA by a 10 mm long air bubble. The dilution was made by shaking the straw to dislodge the air bubble to mix the contents. The variables of these experiments were the concentration of the CPA, the duration of exposure of ova to the CPA before dilution, the temperature of the solutions when they were mixed to effect the dilution, and the composition of the diluent solution. The CPA was 1,2-ethanediol (ethylene glycol) at concentrations of 1.0 – 6.0 mol l^{-1} . All CPA solutions were prepared in M2 solution containing 0.4% (wt/vol) BSA.

In the first series of osmotic shock experiments, the ethanediol concentration was varied from 1.0 – 6.0 mol l^{-1} and the dilution was effected at different temperatures. The ova were exposed to the ethanediol solutions in straws at

approximately 21°C for 10 min. The straws were then placed into stirred baths at 0°, 10°, 20° or 37°C for 1 min before shaking them vigorously. Dilutions of ova at 0° or 37°C were repeated once or twice, so that a total of about 35–60 ova were exposed to each ethanediol concentration. Dilutions at 10° and 21°C were only performed once, with totals of about 20–30 ova exposed to each concentration. Temperature measurements made with 30 gauge copper–constantan thermocouples inserted into the straws and connected to a millivolt recorder showed that the contents reached thermal equilibrium in less than 5 s. In a second series, the ova were pipetted into 0.2 ml ethanediol contained in 10 mm × 75 mm glass tubes. After 10 min at room temperature, the tubes were transferred to baths at 0°, 21° or 37°C for 1 min, and 1.8 ml of M2 solution previously equilibrated at each temperature was rapidly pipetted into respective tubes to dilute the ova suspensions tenfold. Diluted ova were recovered, rinsed in M2, and cultured for 5 days. One set of controls consisted of ova held in M2 solution for 15 min and diluted with M2 at each temperature. Another set of controls consisted of ova pipetted into M2 in tubes, cooled to 0°C and then diluted with 1.8 ml of M2 after 1, 2, 5, 10, 15 or 30 min at 0°C. In a third series of experiments, ova were pipetted into 3.0 or 4.0 mol ethanediol l⁻¹ in straws, and held for 1, 2, 5, 10 or 15 min. The straws were cooled to 0° or warmed to 37°C before being shaken vigorously to mix the contents. The diluents contained in the straws were either M2 solution or 1.0 mol sucrose l⁻¹ prepared in M2. This latter solution has been demonstrated to act as an osmotic buffer to prevent osmotic shock of cryopreserved embryos (Leibo & Mazur, 1978; Leibo, 1984; Schneider & Mazur, 1984).

Results

The osmometric measurements (Fig. 1) show that ova behave as perfect osmometers over the range of 2.78–0.1 osmol, shrinking or swelling in a linear fashion as a function of (Osmolality)⁻¹ = 0.36–10. Survival of ova exposed to tonicities of 0.1–2.1 osmol and normalized to that of control ova held in isotonic M2 solution (taken to be 100%) was about 85–98%. Compared with the controls, 80% of zona-free ova exposed to an M2 solution of only 0.05 osmol at room temperature for 15 min developed into hatching blastocysts. However, in that dilute M2 solution, the ova collapsed somewhat, swelling to a volume of about 290% of control ova; extrapolation of the linear portion of the plot indicates that they should have swollen to a volume of about 440% of the isotonic volume.

Although more than 80% of ova survived exposure to extremely anisotonic solutions and developed into blastocysts, it is possible that there might be more subtle damage caused by these treatments. To examine this possibility, the rate of development of ova exposed to solutions of 2.5 and 0.05 osmoles was measured. The results (Fig. 2) show that the cell doubling time of ova exposed either to extremely hypertonic or extremely hypotonic solutions was virtually identical to that of ova held in isotonic M2. These data also show that under the culture conditions used, from the two-cell to the early morula stage, the embryos divided about once every 13 h. After 60 h of culture, however, the cleavage rate decreased to about once every 20 h.

The extent to which ova tolerate rapid dilution out of concentrated ethanediol solutions was estimated by mixing ova in ethanediol solutions of various concentrations contained in 0.25 ml straws with M2 solution within the straw. The controls consisted of ova suspended in M2 solutions in straws and handled in the same way as the treated samples; more than 95% of control ova developed into hatched or hatching blastocysts. Ova suspended in 3.0 mol ethanediol l⁻¹ or less and diluted at 10°C or higher were only slightly affected by rapid dilution out of these concentrated solutions (Fig. 3). When diluted at 0°C, however, about 25% of the ova were damaged even when diluted out of only 2.0 mol ethanediol l⁻¹, and the percentage of damaged ova increased with increasing concentrations of ethanediol. Even when diluted at the higher temperatures, an increasing fraction of the ova were damaged when abruptly diluted out of ethanediol solutions of 4.0 mol l⁻¹ or greater. Only about 10–15% of the ova survived when diluted out of 6.0 mol ethanediol l⁻¹, regardless of the dilution temperature.

Mixing the two columns of liquid in a straw is an inefficient way to dilute the concentrated ethanediol solutions. Dilutions were therefore performed in glass tubes rather than in straws. Ova diluted by this much more efficient method of mixing were far more sensitive to dilution out of less concentrated ethanediol solutions. The results (Fig. 4) show that, when diluted out of 2.0 mol ethanediol l⁻¹, about 50% of the ova survived dilution at 0°C, about 60% at 21°C, and about 80% at 37°C. But 20% or less survived dilution out of 4.0 mol ethanediol l⁻¹, and only a few per cent out

Fig. 1. (a) Boyle-van't Hoff plot of fertilized mouse ova compared with their survival after exposure to both hypertonic and hypotonic solutions. Volumes were measured as described in the text. Ova exposed to hypotonic solutions were made zona-free by treatment with pronase. The volumes (●) are the means $\pm$ SEM of three to seven ova per solution. Normalized survivals are shown for zona-intact (■) and for zona-free (▼) ova exposed to hyper- and hypotonic solutions for 15 min at room temperature. The survivals, shown as the means $\pm$ SEM, were normalized to control ova (both zona-free and zona-intact) held in M2 solution. Each point is based on 64–74 ova. Inset (b) shows data for ova suspended in 2.78–0.79 osmolal solutions.

of $6.0 \text{ mol ethanediol l}^{-1}$, regardless of the dilution temperature. We determined whether ova would be damaged simply by being held at 0°C by diluting samples of ova that had been held at 0°C for up to 30 min with M2. Approximately 80% of the ova survived in samples held for 1, 2, 5, 10, 15 and 30 min. The respective number of survivors of the total number treated were: 37 of 45, 31 of 38, 36 of 43, 34 of 40, 39 of 48 and 30 of 42.

Dilutions made in straws were compared with those made in glass tubes (Table 1). The ethanediol concentrations were obtained by interpolation from the survival curves (Figs 3 and 4). The results show that ova withstood dilution from higher concentrations of ethanediol when diluted at higher temperatures. However, they also indicate that the efficiency of dilution within straws must be rather low, since a tenfold dilution in tubes yielded 50% survival at considerably lower ethanediol concentrations than did comparable dilutions in plastic straws. Despite this apparent inefficiency, we examined osmotic shock under these conditions in more detail because of the practicality of an 'in straw dilution' method to recover frozen-thawed ova.

The results (Fig. 5) show the survival of ova suspended in $3.0 \text{ mol ethanediol l}^{-1}$ for 1–15 min, and then diluted with M2 at 0° or 37°C or with sucrose at 37°C . Dilution of ova at 0°C had already been demonstrated to be the most damaging procedure. The graph of survival versus exposure time is a manifestation of the acquisition of sensitivity of the ova to osmotic shock under these conditions. The results show that ova in $3.0 \text{ mol ethanediol l}^{-1}$ at room temperature became maximally sensitive to dilution at 0°C after 10 min. Dilution of the ova at 37°C significantly reduced the extent of the osmotic shock. When the ova were diluted out of $3.0 \text{ mol ethanediol l}^{-1}$ at 37°C with $1.0 \text{ mol sucrose l}^{-1}$, 90% of them survived and developed into hatching blastocysts even after 15 min of exposure to $3.0 \text{ mol ethanediol l}^{-1}$. These results demonstrate that there are few, if

Fig. 2. Rate of development of fertilized ova after 15 min exposure to hypertonic (▲, 2.5 osmol), hypotonic (■, 0.05 osmol), or isotonic (●) M2 solution. The mean numbers of blastomeres of 63 embryos exposed to M2, or 78 embryos exposed to hypertonic solution, or 57 embryos exposed to hypotonic solutions were counted at 8, 21 and 32.5 h after the embryos were placed into culture. After 48 h, small groups of embryos (6–24) in each set were removed from culture, fixed, stained and the cells counted.

Fig. 3. (a) Survival (mean $\pm$ SEM) of ova diluted with M2 within plastic straws after exposure to 1.0–6.0 mol ethanediol l⁻¹ at room temperature for 10 min. One minute before mixing them, the straws were placed into a 0°C (●) or 37°C (▲) bath, and then immediately shaken vigorously to mix the contents. Controls consisted of ova handled as the treated samples, except that they were exposed only to and diluted with M2 solution. Each treatment was repeated once or twice. Each point is based on 37–59 ova. (b) Survival (mean $\pm$ SEM) of ova diluted at 10°C (●) and 21°C (▲), as described above. These experiments were performed only once. Each point is based on 19–30 ova.

any, toxic effects of 3.0 mol ethanediol l⁻¹ on the *in vitro* developmental capability of fertilized mouse ova.

Analogous results were obtained with 4.0 mol ethanediol l⁻¹ (Fig. 6). Ova in 4.0 mol ethanediol l⁻¹ at room temperature became maximally sensitive to dilution at 0°C after 10 min of exposure;

Fig. 4. Survival of ova diluted in glass tubes after exposure to 1.0–6.0 mol ethanediol l⁻¹ at room temperature for 10 min. The tubes were placed into baths at 0° (■), 21° (●) or 37°C (▲) for 1 min and then the ova were diluted rapidly tenfold with M2 equilibrated at each temperature. Each point is based on 26–46 ova.

Table 1. Comparison of survival of ova diluted in glass tubes and straws

Dilution temperature (°C)	Ethanediol concentration of 50% survival after dilution in	
	Tubes	Straws
0	2.07	3.85
21	2.78	4.48
37	3.41	4.37

Fig. 5. Survival of ova diluted out of 3 mol ethanediol l⁻¹ within straws after various times of exposure. The ova in 3 mol ethanediol l⁻¹ within straws were held at room temperature for 1–15 min, and cooled or warmed to 0° or 37°C before being diluted. The dilutions were with M2 at 0° (■), M2 at 37° (▲) or sucrose at 37°C (●). Each experiment was repeated once or twice, and the data are shown as the means ± SEM. The values shown for '0 exposure time' are for the controls held in M2. Each point is based on 18–64 ova.

dilution of the ova at 37°C significantly reduced the osmotic shock. Dilution of the ova with 1.0 mol sucrose l^{-1} at 37°C yielded 85% survival even after 15 min of exposure to 4.0 mol ethanediol l^{-1} , again suggesting that even 4.0 mol ethanediol l^{-1} is not toxic to fertilized mouse ova. The survival of mouse ova diluted out of ethanediol at various times were compared (Fig. 7).

Fig. 6. Survival of ova diluted out of 4 mol ethanediol l^{-1} as described above. After various times, the ova were diluted with M2 at 0°C (■), at 37°C (▲), or with sucrose at 37°C (●). Survivals are shown as the means $\pm$ SEM. Each point is based on 28–67 ova.

Fig. 7. Comparison of the survival of mouse ova diluted out of ethanediol solutions after various times of exposure. (Data are redrawn from Figs 5 and 6.) (a) Dilution at 0°C with M2 out of 4 mol (▲) or 3 mol ethanediol l^{-1} (●); (b) dilution at 37°C with M2 out of 4 mol (▲) or 3 mol (●) ethanediol l^{-1} ; (c) dilution at 37°C with sucrose out of 3 mol (●) and 4 mol (▲) ethanediol l^{-1} .

Discussion

The purpose of these experiments was to characterize the biological responses of fertilized mouse ova when diluted out of concentrated solutions of the cryoprotectant, ethanediol. The ultimate goal is to devise a procedure for cryopreservation of ova that would yield high survival, yet would incorporate a simple dilution method. The experiments were conceived by analogy with a method that has been successfully used to cryopreserve cleavage-stage bovine and human embryos (Leibo, 1984; Freedman *et al.*, 1988) and unfertilized hamster ova (Leibo *et al.*, 1990).

The Boyle–van't Hoff plot shows that mouse ova behave as 'perfect osmometers' over a very broad range of osmolalities. In hypertonic solutions (> 0.280 to 2.78 osmol) at about 21°C, the ova contracted osmotically as nearly perfect spheres. Extrapolation by a least-squares fit of all of the volume data, except for those in the 50 mosmol solution, yielded a nonosmotic volume of fertilized ova of 22.5% (Fig. 1). It should be noted that the relationship of volume versus (Osmolality) $^{-1}$ was

plotted from both zona-free and zona-intact ova. The nonosmotic volume of 22.5% agrees well with the previously reported values of 18% for mouse ova (Leibo, 1980), 21.6% for hamster ova (Shabana & McGrath, 1988), 14.9% and 20.1% for eight-cell and blastocyst mouse embryos (Mazur & Schneider, 1986), 16.3% for bovine morulae/blastocysts (Mazur & Schneider, 1986) and 17% for bovine oocytes (Myers *et al.*, 1987). Remarkably, in a 0.05 osmol solution the ova swelled to a volume 2.9 times that of their isotonic volume. Nevertheless, the rate of development of ova exposed briefly to that hypotonic solution was the same as that of control ova (Fig. 2). The overall survival of zona-free ova exposed to such a dilute solution was about 80% of that of the control, zona-free ova. Similar observations have been reported for eight-cell and blastocyst-stage embryos (Mazur & Schneider, 1986). We used diluted M2 solution for hypotonic exposure, whereas Mazur & Schneider used diluted phosphate-buffered saline. The use of M2 may partially explain the higher survival of ova compared with that of cleavage-stage embryos after exposure to hypertonic solutions. We found that about 90% of the ova withstood exposure to solutions of about 2100 mosmol (7.5 times isotonic), whereas Mazur & Schneider found that survival began to decrease in hypertonic solutions of about 1200 mosmol (four times isotonic) and 20% or less survived exposure to 1800 mosmol. Their solutions were made hypertonic with NaCl, whereas we used galactose. Recent results have shown that the development of ova is unaffected by exposure to galactose concentrations up to 1.7 mol l^{-1} (McWilliams *et al.*, 1991). Again, the cleavage rate of embryos developing from hypertonic-exposed ova was the same as the controls.

The observations that ova can contract or swell to volumes of 30–290% of their isotonic volume without appreciable effects on their developmental capacity has important cryobiological implications. It has been observed experimentally (Lehn-Jensen & Rall, 1983; Leibo *et al.*, 1984; Leibo, 1986; Myers *et al.*, 1987) that ova and embryos contract osmotically during equilibrium freezing to volumes of 30–40% of their isotonic volume. These experimental observations confirm the theoretical response of ova and embryos, as calculated by Mazur *et al.* (1984), Mazur & Schneider (1986), Mazur (1990), and Shabana & McGrath (1988). The present survival measurements indicate that osmotic shrinkage alone is unlikely to be the sole factor responsible for the death of slowly cooled ova. Furthermore, they suggest that even extremely hypertonic solutions can be used as efficacious osmotic buffers to recover cryopreserved ova from concentrated CPA solutions.

The manner in which the ova were diluted out of concentrated ethanediol had a significant effect on their survival. When the mixing was done in glass tubes (Fig. 4, Table 1), the ova were more damaged when diluted out of less concentrated solutions than when the mixing was performed in straws. The viscosity of concentrated solutions is high, and such solutions cannot be mixed efficiently within a narrow tube. However, the purpose of these experiments was to identify the factors that influence osmotic shock of ova, not to identify efficient methods to damage them. It is clear that the inefficiency of mixing solutions within a straw reduces the osmotic shock, undoubtedly by reducing the rate at which the ova are diluted out of the concentrated CPA. The reduced rate of dilution, therefore, effectively 'protects' the ova against osmotic shock. This protective effect of an 'in straw dilution' may prove especially efficacious for dilution of ova out of very concentrated solutions used for rapid cooling methods of cryopreservation. These data also suggest that dilution out of a hypertonic solution may be more critical to ova survival than any inherent toxic consequences of exposure to concentrated solutions. This suggestion is supported by data from the dilution experiments (Figs 5 and 6) in which 85% or more of ova survived dilution out of 3 and 4 mol ethanediol l^{-1} with isosmotic sucrose. In other words, high survival of ova could be achieved as long as osmotic shock was prevented.

When viewed in a comparative fashion (Fig. 7), the results of the dilution experiments illustrate several general features of osmotic shock. The higher the concentration of the permeating solute, the more sensitive are the ova to rapid dilution with an isotonic salt solution (Fig. 7a). More ova survive dilution out of 3 mol ethanediol l^{-1} than out of 4 mol ethanediol l^{-1} , but the ova become maximally sensitive to dilution out of 3 or 4 mol ethanediol l^{-1} at about the same time, suggesting that the rate of entry of ethanediol into the ova is the same with both concentrations. This is

consistent with the observations of Mazur *et al.* (1974), who measured the permeability of bovine erythrocytes to glycerol. The absolute intracellular concentration of the permeating solute depends on the extracellular concentration, but the rate of entry does not (Leibo, 1976). Comparison of the results of dilution of the ova out of 3 or 4 mol ethanediol l^{-1} with an isotonic solution at 37°C (Fig. 7b) shows that high temperature must increase the rate of efflux of the ethanediol, since it is known that the temperature coefficient of water permeability of mouse ova is rather low (Leibo, 1980). That is, raising the temperature of dilution has only a slight effect on the rate at which water enters the ova, but clearly has a significant effect on the rate at which ethanediol flows out of the ova. Finally, it has been known for some time that sucrose can act as an osmotic buffer to obviate osmotic shock of ova and embryos diluted out of concentrated CPA solutions (Leibo & Mazur, 1978). The results of these experiments further substantiate this phenomenon; when diluted with sucrose at 37°C, 85% or more of 441 fertilized mouse ova survived and developed into hatching and hatched blastocysts after exposure to 3 or 4 mol ethanediol l^{-1} for up to 15 min at room temperature (Fig. 7c). This means that ova can be exposed to concentrated solutions of ethanediol without damaging them either by some inherent toxic effect of ethanediol or by osmotic shock resulting from the dilution itself. These results prepare the way for a detailed examination of cryopreservation of fertilized mouse ova in this cryoprotective additive.

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