Factors affecting the cryosurvival of mouse two-cell embryos

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Summary. A series of 4 experiments was conducted to examine factors affecting the survival of frozen–thawed 2-cell mouse embryos. Rapid addition of 1.5 M-DMSO (20 min equilibration at 25°C) and immediate, rapid removal using 0.5 M-sucrose did not alter the frequency (mean $\pm$ s.e.m.) of blastocyst development *in vitro* when compared to untreated controls ($90.5 \pm 2.7\%$ vs $95.3 \pm 2.8\%$). There was an interaction between the temperature at which slow cooling was terminated and thawing rate. Termination of slow cooling ($-0.3^\circ\text{C}/\text{min}$) at -40°C with subsequent rapid thawing ($\sim 1500^\circ\text{C}/\text{min}$) resulted in a lower frequency of blastocyst development than did termination of slow cooling at -80°C with subsequent slow thawing ($+8^\circ\text{C}/\text{min}$) ($36.8 \pm 5.6\%$ vs $63.9 \pm 5.7\%$). When slow cooling was terminated between -40 and -60°C , higher survival rates were achieved with rapid thawing. When slow cooling was terminated below -60°C , higher survival rates were obtained with slow thawing rates. In these comparisons absolute survival rates were highest among embryos cooled below -60°C and thawed slowly. However, when slow cooling was terminated at -32°C , with subsequent rapid warming, survival rates were not different from those obtained when embryos were cooled to -80°C and thawed slowly ($52.4 \pm 9.5\%$, $59.5 \pm 8.6\%$). These results suggest that optimal cryosurvival rates may be obtained from 2-cell mouse embryos by a rapid or slow thawing procedure, as has been found for mouse preimplantation embryos at later stages. However, for the 2-cell stage, to achieve high survival rates with rapid thawing the temperature at which slow cooling is terminated is greater ($\sim -32^\circ\text{C}$) than values reported for later stages.

Keywords: mouse; 2-cell embryos; freezing rate; thawing rate; development to blastocysts

Introduction

Preimplantation mouse embryos of all stages from the 1-cell to the blastocyst stage are able to survive freezing to -196°C and subsequent warming (see reviews by Whittingham, 1975; Maurer, 1976). However, optimal methods for freezing and thawing differ among embryos at different stages (Schneider & Maurer, 1983; Massip *et al.*, 1984a). While a great deal of information has been generated regarding factors affecting cryosurvival of mouse embryos at the 8-cell stage or later, relatively little is known about optimal freezing and thawing methods for earlier stages. Low temperature storage of later stage embryos works well for several mammalian species and has many practical applications (Whittingham, 1974; Whittingham & Whitten, 1974; Seidel & Seidel, 1981; Leibo, 1986). However, with increasing interest in basic research and applied techniques utilizing early cleavage stage embryos (e.g. in-vitro fertilization, nuclear transfer, gene injection) it is important to develop a more complete understanding of optimized cryopreservation procedures for

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1- and 2-cell mammalian embryos. Optimizing cryopreservation procedures may increase overall efficiency rates as in-vitro culture time, which has been shown to be negatively correlated with development *in vivo* (Bowman & McLaren, 1970a, b; Hahn & Schneider, 1982; Hoppe & Coman, 1983; Massip *et al.*, 1984b), could be eliminated or greatly reduced.

The objectives of this study were to examine the effects of (1) addition and removal of DMSO, and (2) the temperature at which slow cooling is terminated and subsequent thaw rate, on 2-cell mouse embryos.

Materials and Methods

Source of embryos. Female B₆D₂F₁ mice (Jackson Laboratories, Bar Harbor, ME, U.S.A.), aged 6–8 weeks, were maintained on a 14 h light:10 h dark cycle. Females were induced to superovulate with a modification of a protocol previously reported (Whittingham *et al.*, 1972). Each female was injected with 5–7 i.u. PMSG (Organon Pharmaceuticals, West Orange, NJ, U.S.A.) intraperitoneally, followed by i.p. administration of 5–7 i.u. hCG (Organon) approximately 48 h later. The females were paired with adult ICR males (Harlan Sprague–Dawley, Madison, WI, U.S.A.) immediately after administration of hCG and checked for evidence of mating (presence of vaginal plugs) the following morning. Mated females were killed about 40 h after administration of hCG, and 2-cell embryos were flushed from the excised oviducts in a Hepes-buffered modified Tyrode's medium (TALP-Hepes) (Bavister *et al.*, 1983) containing 3 mg bovine serum albumin/ml (BSA; Fraction V). All medium components, for this and other media used in these experiments, were obtained from Sigma Chemical Co., St Louis, MO, U.S.A., unless stated otherwise. The water used to prepare media was from a Milli-Q reagent water system (Millipore, Continental Water Systems, El Paso, TX, U.S.A.). Embryos were pooled across females, washed in fresh TALP-Hepes medium, and randomly assigned to treatments.

Freezing. Embryos were frozen by modifying the technique previously described by Whittingham *et al.* (1972). Embryos were placed directly into TALP-Hepes medium containing 1.5 M-dimethylsulphoxide (DMSO) and 20% fetal bovine serum (FBS) (GIBCO, Grand Island, NY, U.S.A.). Embryos were pipetted into 0.50 ml plastic straws which were heat sealed and allowed to equilibrate with the DMSO for 20 min at room temperature. Straws were placed vertically in the chamber of a programmable biological freezer (Model R204, Planer Products Ltd, Middlesex, U.K.) and (1) cooled at 3°C/min to –5°C, (2) manually seeded and held at –5°C for 10 min, (3) cooled at 0.3°C/min to temperatures between –30.0 and –80.0°C (see experimental designs below), and (4) placed directly into liquid nitrogen (liquid N₂) (–196°C).

Thawing. Embryos were stored in liquid N₂ for 15–30 min, then thawed at +8.0°C/min to room temperature (slow thaw) in the programmable freezer or in a 35–37°C water bath (rapid thaw). Immediately upon thawing, the contents of the straws were emptied into an organ culture dish (Falcon, Becton Dickinson Co., Oxnard, CA, U.S.A.) containing TALP-Hepes medium, 20% FBS, and 0.5 M-sucrose at room temperature for 10 min, and the DMSO removed in a single step as previously proposed (Leibo & Mazur, 1978; Schneider & Mazur, 1984) and described for mouse embryos (Merry *et al.*, 1983; Renard & Babinet, 1984). The embryos were transferred to TALP (bicarbonate-buffered medium; Bavister *et al.*, 1983) medium and placed in culture.

Assessment of embryo viability. Embryos were evaluated by light-microscopy immediately after the removal of DMSO and scored as viable or not viable on the basis of morphological criteria (Exp. 2 only). Embryos judged to be viable had (1) intact zonae pellucidae and (2) both blastomeres with non-granular cytoplasm and intact, distinct vitelline membranes. All recovered embryos were then placed into in-vitro culture in TALP medium (Bavister *et al.*, 1983) at 37°C under 5% CO₂, 95% air and high humidity. The 2-cell embryos were cultured for 72 h and development to the blastocyst stage, used as the end point for each experiment, was assessed.

Statistical analysis. All data from the experiments consisted of frequency data which were analysed by a weighted analysis of variance approach using percentage values after an arcsine transformation. Weighted values were equal to $1/n$ and percentage values equal to 0 or 100 were replaced by $1/(4 \times n)$ or $(n - 1/4n)$, respectively (where n = the total number of observations) (Snedecor & Cochran, 1982). The analyses were performed using the General Linear Models program of the Statistical Analysis System (Spector *et al.*, 1985). A protected LSD was used for comparisons among treatments when appropriate (Steel & Torrie, 1960).

Results

Experiment 1

Methods. This experiment was designed to evaluate the potential loss of embryo survival due to addition and removal of DMSO. Two-cell mouse embryos, 14–41 in each of 5 replicates, were randomly assigned to one of two treatment groups: (1) untreated controls or (2) embryos to which

Table 1. Effect of freezing protocol on percentage recovery, viability and development to blastocyst stage of frozen-thawed 2-cell mouse embryos

Treatment group	Total no. of embryos	% Recovery†	% Viability‡	% Development§
1 (cooled to -40°C , rapid thaw)	111	$82.6 \pm 5.6^*$	$54.0 \pm 5.0^*$	$36.8 \pm 5.6^*$
2 (cooled to -80°C , slow thaw)	112	94.3 ± 2.6	75.3 ± 4.4	63.9 ± 5.7

Values are means $\pm$ s.e.m., based on frequency data averaged over 10 replicates (14–41 embryos/treatment).

†No. recovered/total no. of embryos frozen.

‡No. viable/total no. of embryos frozen.

§No. of blastocysts/total no. of embryos frozen.

* $P < 0.05$ compared with Group 2 value.

DMSO was added and removed as described above. Embryos were exposed to treatments in 0.50 ml plastic straws to simulate a prefreeze situation.

Results. Neither recovery of embryos from the plastic straws nor development of 2-cell embryos to the blastocyst stage *in vitro* differed between the untreated control group ($n = 111$) and the group to which DMSO was added and removed ($n = 238$), ($P > 0.10$) (100.0 ± 0.0 vs $97.3 \pm 1.6\%$ and 95.3 ± 2.8 vs $90.5 \pm 2.7\%$, respectively).

Experiment 2

Methods. This experiment was designed to test the hypothesis that terminating slow cooling at -40°C with subsequent rapid thawing or terminating slow cooling at -80°C with subsequent slow thawing would produce similar survival rates of frozen-thawed 2-cell embryos, comparable to those reported for 8-cell mouse embryos (Whittingham *et al.*, 1979). Two-cell embryos were randomly assigned to one of two treatment groups (1) slowly cooled from -5 to -40°C , plunged into liquid N_2 and rapidly thawed, or (2) slowly cooled from -5 to -80°C , plunged into liquid N_2 and slowly thawed. The numbers of embryos recovered and, the numbers of viable embryos immediately after thawing and development to the blastocyst stage were used as end points.

Results. As shown in Table 1, recovery rates, viability, as estimated by morphological integrity after thawing, and development to the blastocyst stage *in vitro* were all significantly lower in Group 1 than Group 2.

Experiment 3

Methods. This experiment was designed to examine the proposed interaction between the temperature at which slow cooling is terminated and thawing rate and consisted of two separate studies. The first (3a) was a 2×2 factorial design (2 levels of temperature at which slow cooling was terminated and 2 levels of thawing rate). The 20–28 2-cell embryos in each of 5 replicates were randomly assigned to 1 of 4 treatment groups: (1) slowly cooled from -5 to -40°C with subsequent rapid thawing; (2) slowly cooled from -5 to -40°C with subsequent slow thawing, (3) slow cooling from -5 to -80°C with subsequent rapid thawing, or (4) slow cooling from -5 to -80°C with subsequent slow thawing. Study 3(b) was a 2×6 factorial design (2 levels of thawing rate, rapid or slow; 6 levels of temperature at which slow cooling was terminated, -30 , -40 , -50 , -60 , -70 or -80°C) to examine this interaction further. Two-cell embryos were randomly assigned to 1 of the 12 treatment groups formed by this design.

Results. In Exp. 3(a) there was a significant interaction ($P < 0.05$) between the temperature at which slow cooling was terminated and the rate of thawing. When embryos were cooled to -40°C ,

Fig. 1. Percentage (total no. of blastocysts/total no. of embryos frozen) development to the blastocyst stage at 72 h after thawing. In each of the 3 replicates, 20–23 embryos per treatment were used (63 embryos per treatment). Values are mean $\pm$ s.e.m. based on frequency data averaged over the 3 replicates.

survival was higher ($42.5 \pm 7.0\%$) with rapid thawing (Group 1) than with slow thawing ($2.0 \pm 2.0\%$, Group 2). However, when embryos were cooled to -80°C , survival was higher with slow thawing ($53.7 \pm 8.5\%$, Group 4) than with rapid thawing ($12.6 \pm 4.2\%$, Group 3). Recovery rates ranged between 83.6 and 88.0%. There was a main effect ($P < 0.05$) of temperature at which slow cooling was terminated, with fewer embryos cooled to -40°C developing to the blastocyst stage than embryos cooled to -80°C ($22.2 \pm 7.6\%$ in Groups 1 + 2 and $33.1 \pm 8.2\%$ in Groups 3 + 4). Overall, there was no difference in the frequency of blastocyst development between embryos thawed rapidly ($27.5 \pm 6.3\%$ in Groups 1 + 3) or slowly ($27.8 \pm 9.5\%$ in Groups 2 + 4). The results of Exp. 3(b) are presented in Fig. 1. As for Exp. 3(a), there was a temperature at which slow cooling was terminated by thaw-rate interaction ($P < 0.05$). When cooling was terminated above -60°C , survival was enhanced with subsequent rapid thawing. However, when cooling was terminated below -60°C higher survival was observed with subsequent slow thawing. Recovery rates ranged between 82.5 and 96.8%. There was neither a temperature at which slow cooling was terminated nor a thaw-rate main effect ($P > 0.10$).

Experiment 4

Methods. This experiment was conducted to test the hypothesis that terminating slow cooling at temperatures between -32 and -40°C with subsequent rapid warming would provide optimal survival rates similar to embryos slowly cooled to -80°C and thawed slowly. Two-cell mouse embryos were randomly assigned to 1 of 7 treatment groups: (1) untreated controls, (2) cooled to -80°C and thawed slowly, or rapidly thawed after cooling to (3) -32°C , (4) -34°C , (5) -36°C , (6) -38°C or (7) -40°C .

Results. There was a significant main effect of treatment ($P < 0.05$). The frequency of embryo survival (development to the blastocyst stage) was not different ($P > 0.10$) between the groups cooled to -32°C and thawed rapidly or to -80°C and cooled slowly ($52.4 \pm 9.5\%$ in Group 3 and $59.5 \pm 8.6\%$ in Group 2). A greater frequency ($P < 0.05$) of embryos survived cryopreservation in Groups 2 and 3 than in all other treatment groups: Group 4, $26.2 \pm 9.5\%$; Group 5, $11.9 \pm 2.4\%$;

Group 6, $26.2 \pm 6.3\%$; Group 7, $23.8 \pm 4.8\%$. Values for embryos in Groups 2–7 had a lower ($P < 0.05$) frequency of survival than did those in Group 1 (untreated controls, $81.0 \pm 5.7\%$). Recovery rates ranged between 85.7 and 95.2%.

Discussion

The use of cryoprotectants is essential to obtain survival after mammalian embryos are cooled below -20°C (Schneider & Mazur, 1984). The results of Exp. 1 of the present study indicate that a single-step addition of 1.5 M-DMSO combined with its removal in 0.5 M-sucrose does not affect the development of 2-cell mouse embryos to the blastocyst stage. These results are in basic agreement with the generally held view that DMSO and sucrose in concentrations needed for cryoprotection are not toxic to mammalian embryos (Schneider & Mazur, 1984). However, these data are in apparent disagreement with the early report by Whittingham & Wales (1969) which suggested that 16% DMSO might be toxic to 2-cell mouse embryos. These differences may be due to the fact that the present study utilized a lower concentration of DMSO (1.5 M, $\sim 11\%$). These data support the use of 1.5 M-DMSO for the 2-cell stage and are important in the context of examining factors which affect a specific developmental stage since each stage may vary in susceptibility to cryoprotectant concentrations and their rates of addition and removal (Schneider, 1986).

Initial reports investigating the effects of cooling and thawing rates on the survival of mammalian embryos indicated that slow cooling ($\leq 1^{\circ}\text{C}/\text{min}$) to -60°C or below with slow warming ($4\text{--}25^{\circ}\text{C}/\text{min}$) resulted in a higher frequency of survival than with rapid warming ($\sim 250\text{--}\sim 1500^{\circ}\text{C}/\text{min}$) (see Polge & Willadsen, 1978; Whittingham *et al.*, 1979; Rall & Polge, 1984). However, early reports examining factors affecting the cryosurvival of cattle and sheep embryos indicated that relatively high survival rates could be obtained with rapid warming rates if slow cooling was terminated between -30 and -45°C (Willadsen, 1977; Willadsen *et al.*, 1978; Polge & Willadsen, 1978). Subsequently, Whittingham *et al.* (1979), using mouse 8-cell embryos equilibrated with 1.5 M-DMSO at 0°C and frozen in glass test-tubes, critically tested the hypothesis that an interaction exists between the temperature at which slow cooling is terminated and thaw rate. The results of their experiment demonstrated that such an interaction does indeed exist and suggested that embryos survive rapid or slow thawing equally well if slow cooling is terminated between -35 and -40°C (maximum survival at $\sim -40^{\circ}\text{C}$) and at or below -60°C (maximal survival at $\sim -80^{\circ}\text{C}$) respectively. These two approaches to the cryopreservation of embryos have therefore come to be known as 'rapid' and 'slow' freezing methods, respectively, and are generally considered equally efficacious (Rall *et al.*, 1984; Rall & Polge, 1984). However, reports of studies using hamster oocytes (Quinn *et al.*, 1982; Critser *et al.*, 1986) or early cleavage-stage mouse (Quinn & Kerin, 1986) and human (Trounson & Mohr, 1983) embryos have indicated that terminating slow cooling at -40°C with rapid thawing (rapid method) results in lower survival than cooling to -80°C with slow thawing (slow method).

The existence of an interaction between the temperature at which slow cooling is terminated and the thawing rate observed in mammalian embryos is probably representative of differences in degrees of dehydration (Rall *et al.*, 1984; Rall & Polge, 1984) and as such will be affected by blastomere water permeability, surface area/volume ratio, and the activation energy of water permeability (Leibo, 1977, 1980; Mazur *et al.*, 1984). Surface area has been shown to increase with embryo development (Calarco & Epstein, 1973; Burgos *et al.*, 1976; Eager *et al.*, 1976). Water permeability and the activation energy for water permeability have been shown not to differ between unfertilized and fertilized mouse ova (Leibo, 1980) although glycerol permeability increases and its associated activation energy decreases with fertilization in the mouse (Jackowski *et al.*, 1980) and with subsequent development (Mazur *et al.*, 1976). Changes in water permeability and/or its activation energy may also occur amongst embryos at later developmental stages. Collectively, such changes might explain differences among embryo stages within the temperature

at which slow cooling is terminated by thaw-rate interaction. The results of the present experiments are expressed primarily in terms of development to the blastocyst stage based on the total number of embryos frozen rather than recovered. Due to the high recovery rates of unfrozen embryos in Exp. 1, we believe that, at least in general, embryos that are not recovered represent embryos which have undergone extensive damage and are no longer recognizable. Therefore, expressing data on the basis of the number of embryos frozen represents a conservative estimate of survival.

The present results, in part, are generally consistent with early reports examining the survival of frozen-thawed 2-cell mouse embryos. Nakagata & Toyoda (1980) reported relatively high cryo-survival rates of embryos (~80%, based on total number of embryos frozen) after termination of slow cooling at -70°C with subsequent slow ($6\text{--}10^{\circ}\text{C}/\text{min}$) thawing. Shelton (1982) reported relatively low cryosurvival when slow cooling was terminated at -40°C with subsequent rapid thawing (27%) but somewhat higher survival rates (37%) when slow cooling was terminated at -30°C and thawing was rapid.

The termination of slow cooling at temperatures higher than -32°C in conjunction with rapid warming was not studied in the present experiments. Such work may reveal a procedure which will result in still higher rates of cryosurvival. All experiments conducted here utilized 1.5 M-DMSO. Previous reports (Kasai *et al.*, 1981; Miyamoto & Ishibashi, 1981; Seidel *et al.*, 1983; Farrand *et al.*, 1985) have suggested that 3- and/or 4-way interactions may exist among cryoprotectant type, cryoprotectant concentration, temperature at which slow cooling is terminated and thaw rate. The results of these experiments and previous reports using morula- and blastocyst-stage cow embryos (Seidel *et al.*, 1983; Farrand *et al.*, 1985) would suggest the addition of developmental stage as a possible fourth or fifth term in these interactions. Further examination of these multiway interactions may provide additional insight into improving embryo cryosurvival rates.

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