In-vitro development of refrozen mouse embryos

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To evaluate the effects of sequential, repetitive freezing on their in-vitro development, mouse embryos at the eight- to 16-cell stage were subjected to one of five treatments. They were (i) cultured as unfrozen controls, (ii) frozen once and cultured, (iii) subjected to two consecutive freeze–thaw cycles, (iv) frozen and thawed, and then cultured for 18–30 h before being frozen a second time, and (v) frozen three times in succession without being cultured. To assess their functional survival after freezing and thawing, all embryos were cultured in vitro to the hatched blastocyst stage in Whitten’s medium. In one experiment, hatched embryos that developed after one, two or three cycles of freezing and thawing were stained with Hoechst 33342 to determine their mean cell number. More embryos of the culture control group and the once-frozen group developed into hatching blastocysts than those of the refrozen groups. There was no difference in the second post-thaw rate of in-vitro development for embryos refrozen with the culture-refreeze or direct-refreeze procedure. Furthermore, there was no difference among in-vitro development rates for embryos frozen two or three times. However, among those embryos subjected to repeated cycles of freezing and thawing that did not survive, there was a considerable amount of damage to their zona pellucidae. Furthermore, frozen mouse embryos had fewer cells per embryo at the time of hatching than the unfrozen embryos. Nevertheless, these results demonstrate that mouse embryos can survive even three successive freeze–thaw cycles yet still be capable of in-vitro development.

Key words: cryopreservation/freezing/mouse embryos/refreeze

Introduction

Since the first reports of successful freezing of mouse embryos (Whittingham et al., 1972; Wilmut, 1972), substantial progress has been made in improving procedures to cryopreserve mammalian embryos. Factors known to affect the cryopreservation of embryos include the effectiveness of various cryoprotectants, equilibration times in cryoprotectants, cooling and warming rates, temperatures at which slow cooling ends and rapid cooling begins, and dilution methods for embryos of various mammalian species (see reviews by Schneider and Mazur, 1984; Leibo, 1986, 1992; Niemann, 1991; Fahning and Garcia, 1992; Rall, 1992). Live births resulting from the transfer of frozen–thawed embryos have been reported for at least 20 mammalian species (reviewed by Schiewe, 1991; Rall, 1996). Most cryobiological studies have been designed to develop methods to prevent cellular damage during the freeze–thaw process. The primary objective of embryo cryopreservation is to obtain the highest possible post-thaw survival rates, and in-vitro or in-vivo development rates comparable to those of unfrozen control embryos. Pregnancy rates similar to those produced with unfrozen embryos have been reported with frozen–thawed embryos of mice (Shaw et al., 1991; Rall and Wood, 1994), sheep (McGinnis et al., 1992) and cattle (Willadsen et al., 1977; Renard et al., 1980).

If it were known that embryos can survive more than one freeze–thaw cycle, cryopreserved embryos could be thawed and their cells used for various micromanipulation procedures, such as nuclear transfer, genetic screening by DNA analysis, and embryo sexing. Although many types of genetic analysis can now be performed within a few hours, the type, sensitivity and reliability of such analyses continue to dramatically improve at a rapid pace. Therefore, if embryos that have already been frozen could be thawed and frozen again as their cells were being used or analysed, this would substantially increase the utility of such cryopreserved embryos. In the case of both non-domestic and domestic species, many academic institutions and animal breeding organizations have amassed large numbers of embryos cryopreserved over the past 10–15 years. The capability to analyse such embryos retrospectively, yet refreeze them so as to have time to make the most productive use of the embryos, would also increase their value.

The ability to refreeze embryos could be used together with various techniques of assisted reproduction. Previously frozen embryos resulting from in-vitro fertilization (IVF) could be thawed, biopsied for sexing or genetic analysis, then refrozen for transfer at a later date. The ability to refreeze once-frozen samples of embryos of laboratory or domestic species would be useful should a straw or ampoule be thawed that contained more embryos than were needed.

In the case of human embryos, retrospective genetic analysis of embryos will be especially important. The possibility and necessity for such retrospective analyses of cryopreserved embryos are increasing as new molecular probes become
Embryo survival was assessed immediately after thawing by overall morphology. Those containing <50% degenerate or extruded blastomeres, or with no more than a slight crack in the zona were classified as morphologically viable. Those missing a zona, or having a large crack, or containing ≥50% degenerate or extruded cells were scored as non-viable. Functional survival was based on the ability of the embryos to develop into expanded and hatched blastocysts in droplets of Whitten’s medium (Whitten, 1971) under silicone oil at 39°C in a humidified atmosphere of 5% CO₂ in air.

Embryo freezing procedure
Embryos to be frozen were placed into 0.7 M glycerol prepared in mPBS solution for 7 min at 20–25°C, and then into 1.4 M glycerol in mPBS for an additional 7 min. Groups of 20–25 embryos were pipetted into 0.25 ml plastic artificial insemination straws (# A201, IMV, l’Aigle, France) containing 100 ml of freezing medium, followed by an air bubble and an additional 50 ml of freezing medium to fill the straw, which was then heat-sealed.

The straws were placed into a controlled-rate freezer (Model R204; Planer Products Ltd, Sunbury-on-Thames, UK) at −6°C, and the medium seeded to induce ice formation. About 5 min later, the straws were cooled to −33°C at 0.3°C/min, held 10 min, and then plunged directly into liquid nitrogen (LN₂). After ~24 h in storage, the straws were warmed in air at 20–25°C for 2 min. The embryos were expelled from the straws and the cryoprotectant removed using a four-step dilution procedure. To do this, the embryos were sequentially rinsed and held for 5 min in 0.90 M glycerol + 0.3 M sucrose in mPBS, 0.45 M glycerol + 0.3 M sucrose in mPBS, 0.3 M sucrose in mPBS, and in mPBS alone.

Embryo staining procedure
Within 4–8 h after they hatched, blastocyst-stage embryos were stained with Hoechst 3342 (Sigma) using a procedure similar to that of Pursel et al. (1985), differing only in that counterstaining with Trypan blue was eliminated. The Hoechst 3342 stock solution was prepared by dissolving 1 mg stain/ml sterile water and was stored in the dark. The working solution consisted of 0.75 ml of 2.3% sodium citrate dihydrate, 0.25 ml ethanol and 10 ml of the Hoechst 3342 stock solution. One to three embryos in mPBS were pipetted onto glass slides coated with silicone (Sigmacote; Sigma), rinsed with sodium citrate solution for 30 s, the excess solution removed, then fixed and stained with Hoechst 33342 (Sigma) using a procedure similar to that of Pursel et al. (1984), differing only in that counterstaining with Trypan blue was eliminated. The Hoechst 3342 stock solution was prepared by dissolving 1 mg stain/ml sterile water and was stored in the dark. The working solution consisted of 0.75 ml of 2.3% sodium citrate dihydrate, 0.25 ml ethanol and 10 ml of the Hoechst 3342 stock solution. One to three embryos in mPBS were pipetted onto glass slides coated with silicone (Sigmacote; Sigma), rinsed with sodium citrate solution for 30 s, the excess solution removed, and then fixed and stained with Hoechst 3342 for 18–30 h. Of those that developed into blastocysts on a given day, were pooled. Without intentional selection, about 20–25% degenerate or extruded cells were included in the sample. The embryos were expelled from the straws and the cryoprotectant removed using a four-step dilution procedure. To do this, the embryos were sequentially rinsed and held for 5 min in 0.90 M glycerol + 0.3 M sucrose in mPBS, 0.45 M glycerol + 0.3 M sucrose in mPBS, 0.3 M sucrose in mPBS, and in mPBS alone.

Embryo methods
For each replicate of an experiment, embryos were collected from several superovulated females at ~80 h after HCG injection by standard methods, as described by Hogan et al. (1994). All of the collected embryos of excellent quality, usually numbering about 100 on a given day, were pooled. Without intentional selection, about 20–25 embryos were distributed into one of four undesignated groups, and each group of embryos was then assigned to a given treatment by drawing a treatment number from a hat. The experiments were repeated either once (experiment 3) or twice (experiments 1 and 2), so that a total of about 40 or 60–80 embryos were used for each treatment.

Materials and methods

Experimental animals
Colonized ICR female and male mice (Harlan Sprague Dawley, Indianapolis, IN, USA) were maintained on a 12-h light and 12-h dark cycle (lights activated at 0700 h). Prepubertal female mice (4–6 weeks old) were treated by i.p. injection of 10 IU of pregnant mare serum gonadotrophin (Sigma Chemical, St Louis, MO) at 1300 hours, followed by an i.p. injection of 10 IU human chorionic gonadotrophin (HCG; Sigma) 48 h later. Immediately after HCG injection, each female was placed with a fertile male (5–7 months of age) and examined ~19 h later (day 1) for the presence of a vaginal plug.

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**Table I.** Morphological survival and development of mouse embryos frozen once or twice, either immediately after thawing or after having been cultured for 18–30 h until they developed into blastocysts (experiment 1)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>1st Post-thaw</th>
<th>2nd Post-thaw</th>
<th>Expanded blastocyst (%)</th>
<th>Hatched blastocyst (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Frozen–Control</td>
<td>62</td>
<td>56 (90.3)</td>
<td>—</td>
<td>47 (75.8)(^{A,a})</td>
<td>36 (58.1)(^{B,a})</td>
</tr>
<tr>
<td>B. Cultured–Refrozen</td>
<td>78</td>
<td>73 (93.6)</td>
<td>48 (77.4)*</td>
<td>31* (50.0)^ {A,b}</td>
<td>17* (27.4)^ {B,b}</td>
</tr>
<tr>
<td>C. Direct–Refrozen</td>
<td>62</td>
<td>56 (90.3)</td>
<td>43 (69.4)</td>
<td>26 (41.9)^ {A,b}</td>
<td>18 (29.0)^ {B,b}</td>
</tr>
<tr>
<td>D. Cultured–Control</td>
<td>62</td>
<td>—</td>
<td>—</td>
<td>54 (87.1)^ {A,a}</td>
<td>36 (58.1)^ {B,a}</td>
</tr>
</tbody>
</table>

\(^{A, a, b}\) Means with different capitalized superscripts within rows, or with different lower case superscripts within columns are significantly different (\(\chi^2\), \(P < 0.05\)).

*Note that 62 embryos were frozen a second time.

**Experiment 2**

With one minor modification, this experiment was a repeat of experiment 1. The modification was in the Cultured–Refrozen group, in which all embryos in group B (\(n = 75\)) were cultured in Whitten’s medium for only 18 h before refreezing, rather than waiting until all embryos reached the blastocyst stage. As noted, this required from 18 to 30 h in experiment 1. In the second experiment, consisting of 75 embryos/group, the embryos in the three other groups (group A, the Frozen–Control group; group C, the Direct–Refrozen group; group D, the Cultured–Control group) were treated as described for experiment 1.

**Experiment 3**

The objective of this experiment was to determine if mouse embryos could survive three freeze–thaw cycles using the Direct–Refrozen procedure. In addition, shortly after the time of zona hatching for embryos subjected to one, two or three freeze–thaw cycles, the mean number of cells per embryo was determined. We emphasize that embryos subjected to the Direct–Refrozen procedure were not permitted time to develop before being frozen a second or third time. In this experiment (\(n = 40\) embryos per group), embryos in group A were frozen once (Frozen–Control), embryos in group B were frozen twice (Direct–Refrozen I), embryos in group C were frozen a total of three times (Direct–Refreeze II) and embryos in group D served as a non-frozen control (Cultured–Control). As each set of embryos hatched in culture, and within <8 h of hatching, the embryos were mounted on glass slides and stained, so that their total cell numbers could be counted.

**Statistical analysis**

All statistical analyses were performed with the aid of a Microstat software package (Ecosoft, Inc.). Comparisons among treatment groups for the number and percentages of embryos developing in vitro to the expanded- and hatching-blastocyst stages were conducted using \(\chi^2\) analysis with a contingency table and a Fisher’s exact probability test. In addition, the overall treatment effect for the stained embryos in experiment 3 was determined using ANOVA, and comparisons among treatment groups for mean cell numbers per embryo were evaluated using Fisher’s least significant difference test.

**Results**

**Experiment 1**

Based on their morphology, 90% of the embryos in group A frozen once, 94% of those in group B refrozen after culture, and 90% of those in group C frozen a second time immediately after being thawed were considered as potentially viable (Table I). These results were based on embryos classified as potentially viable after cryoprotectant removal and embryo rehydration, except in group C, in which embryos were not removed from the straw until the second post-thawing. Embryos in group C were observed and evaluated through the wall of the transparent straw with the aid of an inverted microscope (\(\times 200\) magnification) during the 2 min warming period. There was no difference in the first post-thaw morphological survivals among all frozen treatment groups (range 90–93%). During the post-thaw culture period (18–30 h), embryos were selected without intentional bias for refreezing from the embryos originally assigned to group B. After 30 h of culture in Whitten’s medium, 63 of 78 embryos (81%) had developed into blastocysts in vitro. To keep the same number across treatment groups, 62 of these embryos were selected for refreezing in group B. After the second freeze–thaw cycle, 77% of the Cultured–Refrozen embryos in group B and 69% of the Direct–Refrozen embryos in group C were classified as viable. There was no significant difference in the second post-thaw survival rates between groups B and C (77 versus 69%). Among the non-survivors in the two twice-frozen embryo groups (groups B and C), however, there were notable differences in the amount of zona pellucida damage. In group B, six of 14 embryos (43%) from the Cultured–Refrozen group had marked zona pellucida damage compared with 15 of 19 embryos (79%) of those in the Direct–Refrozen group (group C).

Significantly more once-frozen embryos in group A (76%) and unfrozen control embryos in group D (87%) developed into expanded blastocysts (\(P < 0.05\)) compared to 50% for the embryos in the Cultured–Refrozen group (group B) and 42% for the embryos in the Direct–Refrozen group (group C). The same percentage, 58%, of the once-frozen embryos in group A and the controls in group D, developed into hatched blastocysts. Compared to those two groups of control embryos, only half of the Cultured–Refrozen embryos in group B and the Direct–Refrozen embryos in group C hatched.

**Experiment 2**

After one freeze–thaw cycle and removal of the cryoprotectant, the morphological survival rates of groups A, B and C were 95, 92 and 96% respectively (Table II). Furthermore, there was little difference (64 versus 71%) after the second thawing between the viability of Cultured–Refrozen embryos compared to the

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Table II. Morphological survival and development of mouse embryos frozen once or twice, either immediately after thawing or after having been cultured for 18 h (experiment 2)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>1st Post-thaw</th>
<th>2nd Post-thaw</th>
<th>Expanded blastocyst (%)</th>
<th>Hatched blastocyst (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Frozen–Control</td>
<td>75</td>
<td>71 (94.7)</td>
<td>—</td>
<td>60 (80.0)a</td>
<td>53 (70.7)a</td>
</tr>
<tr>
<td>B. Cultured–Refrozen</td>
<td>75</td>
<td>69 (92.0)</td>
<td>48 (64.0)</td>
<td>50 (66.7)b</td>
<td>50 (66.7)b</td>
</tr>
<tr>
<td>C. Direct–Refrozen</td>
<td>75</td>
<td>72 (96.0)</td>
<td>53 (70.7)</td>
<td>46 (61.3)b</td>
<td>39 (52.0)b</td>
</tr>
<tr>
<td>D. Culture–Control</td>
<td>75</td>
<td>—</td>
<td>—</td>
<td>69 (92.0)c</td>
<td>61 (81.3)a</td>
</tr>
</tbody>
</table>

a,b,c Means with different lower case superscripts within columns are significantly different ($\chi^2$, P < 0.05).

Table III. Morphological survival and development of mouse embryos frozen once, twice, or three times immediately after thawing (experiment 3)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>1st Post-thaw</th>
<th>2nd Post-thaw</th>
<th>3rd Post-thaw</th>
<th>Expanded blastocyst (%)</th>
<th>Hatched blastocyst (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Frozen–Control</td>
<td>40</td>
<td>34 (85.0)</td>
<td>—</td>
<td>—</td>
<td>28 (70.0)a</td>
<td>25 (62.5)a</td>
</tr>
<tr>
<td>B. Direct–Refrozen I</td>
<td>40</td>
<td>36 (90.0)</td>
<td>25 (62.5)</td>
<td>—</td>
<td>21 (52.5)b</td>
<td>16 (40.0)b</td>
</tr>
<tr>
<td>C. Direct–Refrozen II</td>
<td>40</td>
<td>36 (90.0)</td>
<td>26 (70.0)</td>
<td>20 (50.0)</td>
<td>16 (40.0)b</td>
<td>9 (22.5)b</td>
</tr>
<tr>
<td>D. Culture–Control</td>
<td>40</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>36 (90.0)c</td>
<td>31 (77.5)a</td>
</tr>
</tbody>
</table>

a,b,c Means with different lower case superscripts within columns are significantly different ($\chi^2$, P < 0.05).

to the Direct–Refrozen group. Of the unfrozen cultured embryos in group D, 92% developed into expanded blastocysts in vitro, a significantly higher percentage than for any of the once- or twice-frozen groups. However, more once-frozen embryos in group A (80%) developed into expanded blastocysts in vitro compared to 67% for the Cultured–Refrozen embryos or 61% for the Direct–Refrozen embryos. In addition, more unfrozen embryos in group D (81%) and once-frozen embryos in group A (71%) developed into hatched blastocysts during in-vitro culture compared to 67 and 52% for twice-frozen embryos in groups B and C respectively. There was no significant difference in the development rates to the expanded or hatched blastocyst stages between the embryos of these two freeze–thaw treatment groups.

Experiment 3

In the third experiment, 85% of the embryos in group A, and 90% in groups B and C were considered viable after one freeze–thaw cycle (Table III). After the second cycle, 63 and 70% of the Direct–Refrozen embryos in groups B and C respectively were judged morphologically viable, and after three freeze–thaw cycles, 50% of the embryos in group C were considered viable.

After being cultured, a significantly higher percentage of the unfrozen embryos developed into expanded blastocysts in vitro than in any of the frozen treatment groups. There was no statistical difference in developmental rates to expanded blastocysts between the once-frozen embryos in group A compared to the twice-frozen embryos in group B (70% versus 53%). However, more once-frozen embryos in group A (70%) developed to expanded blastocysts when compared with the embryos frozen three times in group C (40%). In addition, significantly more non-frozen embryos in group D (78%) and once-frozen embryos in group A (63%) hatched in culture compared to twice-frozen embryos in group B (40%) or those thrice-frozen in group C (23%).

Within <8 h after hatching, control or treated embryos were mounted on slides and stained to determine their cell numbers. The results in Figure 1 show the mean number of cells/embryo of embryos that developed from 8- to 16-cell embryos that were frozen one, two or three times, compared to control embryos which were not frozen. Points with different letters are significantly different.
the controls, but significantly more than after three cycles of freezing, the latter group of embryos containing ~85 cells/embryo.

Discussion

The results of these experiments demonstrate that 8- to 16-cell mouse embryos can be successfully refrozen either immediately after thawing, or after having been cultured to the blastocyst stage. In all experiments and treatment groups in which embryos were frozen twice, there was no difference in the second post-thaw morphological survival and in vitro development rates between the two refreezing methods used. There was, however, a notable difference in the amount of zona damage when the Direct–Refrozen and Cultured–Refrozen embryo groups in both experiments 1 and 2 were compared. Of the Direct–Refrozen embryos (group C) in both experiments 1 and 2, 30 of 41 embryos (73%) were classified as damaged as manifested by damage to their zonae, compared with only 19 of 41 embryos (46%) in the Cultured–Refrozen groups (group B).

The results of this study also suggest that excellent quality embryos, frozen and thawed at the 8- to 16-cell stage, often do not develop in vitro without full protection of an intact zona pellucida. For early stage embryos, it is thought that the zona pellucida helps to maintain cellular integrity of the blastomeres. Artificially induced alterations in the integrity of the zona pellucida may affect subsequent embryo cleavage, compaction, blastocyst formation and zona hatching of human embryos (Cohen, 1992). Bielanski (1987) noted that only 31% of mouse morulae frozen without a zona pellucida survived 24 h in vitro compared to 78% of those frozen with an intact zona. Moreover, to survive in vitro after transfer, early precompaction-stage embryos of several species appear to require an intact or nearly intact zona pellucida following micromanipulation, such as those of the rabbit (Moore et al., 1968), pig (Moore et al., 1969), sheep (Trounson and Moore, 1974) and mouse (Brounson and McLaren, 1970; Modlinski, 1970). In contrast, it has been reported that zona-free bovine embryos can survive cryopreservation (Blakewood et al., 1986).

The zona pellucida may not be necessary for development of bovine embryos beyond the compacted morula stage (Hoppe and Bavister, 1983; Farrand et al., 1985). Yet, the zona pellucida may protect preimplantation-stage embryos against hostile uterine environments (Cohen, 1992) and against invasion by pathological organisms (Singh, 1987). Furthermore, a broken zona pellucida after cryopreservation of bovine morulae and blastocysts has been interpreted to be an indicator of some damage to the embryo (Farrand et al., 1985).

It was difficult to determine at which point in the standard freeze–thaw cycle most cellular and/or zona damage occurred. The freezing and warming of cells imposes a concatenation of stresses, including equilibration with a cryoprotectant, cooling, warming, dilution and rehydration (Leibo, 1992). Any one of these might have caused embryo damage. The criterion used in this study to classify embryos as ‘not viable’ after thawing was primarily that of zona damage. It is believed that damage to the zona pellucida of mouse and bovine embryos may be due to thermally induced fracturing due to temperature fluctuations that occur during cooling and warming (Lehn-Jensen and Rall, 1983; Rall and Polge, 1984; Rall and Meyer, 1989).

Many years ago, it was noted that the incidence of cracked zonae is considerably higher when bovine embryos are warmed rapidly at >750°C/min, compared to that when they are warmed at ~175°C/min (Leibo, 1983). In a detailed study of this phenomenon, Rall and Meyer (1989) observed that zona damage to bovine embryos frozen slowly in plastic straws can be greatly reduced if low warming rates are used. The low freezing and warming rates used in the present study produced a low incidence of zona damage in once-frozen compared to twice-frozen embryos. Furthermore, in the refrozen treatment groups, more embryos were damaged during the second freeze–thaw cycle than in the first cycle. Additional cycles of freezing apparently exert a greater thermal stress on embryos than the initial freeze–thaw cycle. Perhaps, the time in culture permitted the zonae to ‘recover’ from the thermal stresses of freezing and thawing.

The directly refrozen embryos in this study were warmed from ~196°C in air at a rate of ~150°C/min, held at room temperature until thawed, and then immediately cooled again to ~6°C, all within a time span of 2 min. Direct refreezing of embryos resulted in higher rates of zona damage compared to culturing and then refreezing them. Repetitive osmotic fluctuations resulting from sequential freezing and thawing of the suspending solutions, as well as extreme changes in temperature within a short period of time, may have contributed to the higher rate of zona damage in this treatment group.

In the present study, mouse embryos subjected to repeated freezing without intervening time in culture between each cycle after thawing contained lower mean cell numbers at hatching compared to unfrozen embryos. The adjusted regression coefficient of mean cell numbers as a function of the number of freeze–thaw cycles was 0.94, suggesting that freezing damage was cumulative. That is, the results plotted in Figure 1 imply that each cycle of freezing caused a constant amount of damage. If the embryos were not permitted to ‘recover’ in culture, then about 10–20% of their cells were damaged by each successive freeze–thaw. Consider the functional survival based on development into hatched blastocysts in experiment 3. Normalized to the Cultured–Control embryos, survival of the once-frozen embryos was 62.5/77.5 = 80.6%. Thus, three cycles of freezing ought to yield a cumulative survival of 81×81×81%, or 53%. The mean number of cells in hatched blastocysts after three cycles of freezing was 85, compared to 145 cells in the unfrozen blastocysts, or 58%. That is, blastocysts resulting from three cycles of freezing contained 58% of the cell number of unfrozen controls.

The results of in-vitro development for the non-frozen cultured embryo groups in this study (group D) are comparable with those of previous reports describing development rates (El-Shershaby and Hinchliffe, 1974; Mohr and Trounson, 1982) and mean cell numbers at hatching (Copp, 1978) of mouse embryos. It has been suggested that a decreased cell number per embryo at hatching does not always indicate a
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decline in the further in-vitro or in-vivo developmental capacity of bovine embryos (Loskutoff et al., 1993).

It has been shown that refrozen embryos retain full functional capability, as assessed by post-thaw pregnancy rates in mice (Leibo et al., 1991). In the latter study, a similar number of embryos were lost during the first and second freeze–thaw cycles. In addition, Fields et al. (1991) reported a post-thaw viability rate of 95% and in-vitro development rate of 69% following freezing, biopsy and refreezing of mouse embryos. An analogous study in this laboratory has shown that bovine embryos can also withstand repeated cycles of freezing (Vitale, 1993). Bovine embryos at day –7 to 7.5 frozen twice or three times exhibited hatching rates of 37 and 30% respectively when co-cultured on a bovine oviduct cell monolayer.

The ability to refreeze in-vitro derived human embryos might be used as an adjunct to micromanipulation procedures, especially in cases in which patients have limited numbers of frozen embryos. Recent reports confirm that precompaction-stage mouse and human embryos can be successfully biopsied for genetic screening and embryo sex determination prior to replacement (Handyside et al., 1990; Kola and Wilton, 1991; Grifo, 1992; Levinson et al., 1992). Hence, frozen embryos could be thawed, biopsied, and then refrozen for transfer at a later date. The total number of freeze–thaw cycles that a single embryo can tolerate without losing its developmental capacity has yet to be determined. Also, the need to study the developmental competence of refrozen embryos in vivo is now apparent, with further research needed to determine if refrozen mammalian embryos will yield acceptable pregnancy rates and live births.

Acknowledgement

This manuscript was approved for publication by the Director of the Louisiana Agricultural Experiment Station as manuscript number 93-11-7149.

References


Received on March 8, 1995; accepted on October 30, 1996