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Survival of rapidly frozen hatched mouse blastocysts

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Summary

The objective of the present study was to examine the effect of rapid freezing on the *in vitro* and *in vivo* survival of zona-pellucida-free hatched mouse blastocysts. Hatched blastocysts were rapidly frozen in a freezing medium containing either ethylene glycol (EG) or glycerol (G) in 1.5 M or 3 M concentration. Prior to freezing, embryos were equilibrated in the freezing medium for 2 min, 10 min, 20 min or 30 min at room temperature. To freeze them, embryos were held in liquid nitrogen vapour [~1 cm above the surface of the liquid nitrogen (LN_2)] for 2 minutes and then immersed into LN_2. After thawing, embryos were transferred either to rehydration medium (DPBS + 10% foetal calf serum + 0.5 M sucrose) for 10 minutes or rehydrated directly in DPBS supplemented with foetal calf serum. *In vitro* survival of embryos frozen with EG was higher than those frozen with G. The highest survival was obtained with 3 M EG and 2 min or 10 min equilibration prior to freezing, combined with direct rehydration after thawing. Frozen blastocysts developed into normal foetuses as well as unfrozen control ones did, with averages of 30% (control), 26% (EG) and 15% (G). The results show that hatching and hatched mouse blastocysts can be cryopreserved by a simple rapid freezing protocol in EG without significant loss of viability. Our data indicate that the mechanical protection of the zona pellucida is not needed during freezing in these stages.

Keywords: Cryopreservation, Hatched blastocyst, Ethylene glycol, Glycerol, Mouse embryo

Introduction

The first report on successful cryopreservation of embryos was published more than 30 years ago (Whittingham, 1971). Since then, the attention of cryobiologists has mostly been focused on freezing oocytes and embryos with an intact zona pellucida (ZP). The culture conditions are optimized for unhatched embryos with intact ZP. Moreover, the presence of the ZP is required in the breeding-animal embryo trade in order to minimize the risk of disease transmission. Thus, the freezing protocols are designed and investigated accordingly. However, in the pioneer studies, the first calf (Wilmut & Rowson, 1973), lamb (Willadsen *et al*., 1976) and piglets (Kashiwazaki *et al*., 1991) from frozen embryos were produced from hatched blastocysts.

Recently, more attention has been paid to the cryobiology of embryos in later developmental stages such as hatching and hatched blastocyst. In human *in vitro* fertilization (IVF), to lower the incidence of multiple gestations and to improve implantation rates, the embryos are cultured to the blastocyst stage and transferred at the hatching or hatched stages (Khorram *et al*., 2000; Gardner *et al*., 1998; Yoon *et al*., 2001). Moreover, it is known that cryopreserved embryos undergo physicochemical changes that could inhibit hatching, thus contributing to unsuccessful conceiving (Ouhibi *et al*., 2000). Consequently, in the past few years, interest has increased in the freezing of hatched blastocysts.

In human IVF, a few reports have recently been published on successful transfer of frozen-thawed hatched blastocysts using a propanediol–sucrose method (Quintans *et al*., 2001). Shaw *et al*. (1995) examined the survival of mouse expanded and hatched

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blastocysts after conventional freezing with propanediol and ethylene glycol (EG) using different thawing procedures. Expanded and hatched blastocysts were treated together in the same groups with different freezing–thawing protocols. Results showed that mouse blastocysts in late developmental stages can be successfully cryopreserved by conventional slow freezing, but no separate experiment was carried out on ZP-free embryos.

In another study, Shaw *et al*. (1991) used simple rapid freezing to cryopreserve different preimplantation-stage mouse embryos, including hatching blastocysts with 4.5 M dimethyl sulfoxide and found that the developmental stage of embryo at the time of freezing and equilibration time influenced the post-thaw survival *in vitro* and *in vivo*. The *in vitro* survival of hatching blastocysts equilibrated at 0 °C for 3 or 6 min and 10–60 min was 40% and 40–60%, respectively. The 3 min equilibration at room temperature (RT) resulted in 20% survival. The ratio of the normal foetuses in a 15-day autopsy was 52%.

The most complex experiment on the cryopreservation of hatched mouse blastocysts was performed by Zhu *et al*. (1996). Embryos were equilibrated for 1.5 min or 2 min in the one-step method or for 5–10 min and 0.5 min in the two-step method, and then vitrified in two vitrification solutions containing EG or glycerol (G), Ficoll and sucrose. The highest *in vitro* survival rate with EG (65%) was obtained using the one-step method with embryos that had already hatched. The results were better with G (89–94%), obtained by two-step method. The highest *in vivo* survival rate (40–44%) was achieved using embryos transferred to recipients on day 3 or day 4 of pseudopregnancy.

The objective of our study was to examine the effect of rapid freezing with EG or G on the *in vitro* and *in vivo* survival of ZP-free mouse blastocysts. The influence of different equilibration time before freezing and different rehydration methods after thawing was also studied.

Materials and methods

Animals

CB6F1 (Charles River, Germany) mice were housed under standard conditions (22 ± 2 °C; 12 h dark/12 h light; water and food *ad libitum*).

Embryo production

Females were superovulated by intraperitoneal injection of 10 international units of PMSG (Sigma) followed 46 h later by 10 IU of human chorionic gonadotropin (hCG) (Sigma). After 6 hours of hCG administration, females were mated with fertile males in monogamous pairs. One- to two-cell-stage embryos (day 0 and day 1) were harvested by flushing the oviduct with FertiCult flushing medium (FertiPro, Belgium). Embryos were cultured at 37 °C in a thermostat with 5% CO_2 and maximal humidity in air. Embryos between one-cell and compact morula stages were cultured in G 1.2 medium (Vitrolife, Göteborg) under Ovoil (Vitrolife, Göteborg). Then, the embryos were transferred and cultured in G 2.2 (Vitrolife, Göteborg, Sweden) under paraffin oil until the hatching/hatched blastocyst stage.

Freezing and thawing of embryos

Hatching and/or hatched blastocysts on day 5 (140–142 h after hCG injection; day 0 = plug formation) were randomly allocated to different treatment groups, including a control group. Embryos were equilibrated in DPBS + 10% foetal calf serum (FCS) + 0.25 M sucrose containing either 1.5 M or 3.0 M EG (Group I) or 1.5 M or 3.0 M G (Group II) for 2 min, 10 min, 20 min and 30 min at RT. Then, the embryos were loaded in freeze medium into 0.25 ml straws (7 or 8 embryos per straw) and held 1 cm above the surface of liquid nitrogen in LN_2 vapour for 2 minutes. Finally, the embryos were immersed into LN_2 (Cseh *et al*., 1997; Takahashy & Kanagawa, 1990).

For embryo thawing, the straws were air-thawed for 20 seconds and then dipped into a 22–24 °C water bath for 2 minutes. After thawing, the embryos were transferred from the freeze medium to rehydration medium (DPBS + 10% FCS + 0.5 M sucrose). After 10 minutes of rehydration, embryos were rinsed three times in DPBS supplemented with 10% FCS [washing medium (WM)] at RT. Half of the embryos were rehydrated directly in WM (10 minutes) at RT. Finally, the embryos were transferred and cultured in G2.2, as above, for 24 hours.

Assessment of *in vitro* and *in vivo* survival

Embryo quality was examined just after thawing and 12 h and 24 h later. Embryo survival was evaluated by morphological appearance: integrity of the blastomeres, re-expansion of the blastocoel and intactness of the blastomeres were the signs of survival. Untreated hatched blastocysts kept in DPBS for 40 minutes in RT were used as controls.

For *in vivo* evaluation, frozen and thawed embryos were cultured in G 2.2 for 1–2 hours with 5% CO_2 and maximal humidity in air. Then, the embryos (7–12 embryos per animal) with re-expanded blastocoel were transferred to day 3 pseudopregnant recipients. Untreated hatched blastocysts were transferred as controls. The recipients were exterminated at 18–19 days of pregnancy.

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Table 1 *In vitro* and *in vivo* survival of ZP-free mouse blastocysts frozen rapidly in EG

No.	Cryoprotectant concentration	Equilibration time	Rehydration time	*In vitro* survival				*In vivo* survival	
				n	At thawing	12 h	24 h	Pregnancies[1]	Offspring[2]
1	3 M	30 min	10 min	66	64 (97%)^{a}	62 (94%)^{a}	62 (94%)^{a}	3/4	7/27 (26%)
2	3 M	20 min	10 min	68	64 (94%)^{a}	60 (88%)^{a}	57 (84%)^{**a}	2/4	6/23 (26%)
3	3 M	10 min	10 min	76	71 (93%)^{a}	67 (88%)^{a}	65 (85%)^{**a}	2/3	6/19 (31%)
4	3 M	2 min	direct	63	62 (98%)^{a}	60 (95%)^{a}	60 (95%)^{a}	2/3	6/20 (30%)
5	3 M	10 min	direct	58	57 (98%)^{a}	57 (98%)^{a}	57 (98%)^{a}	3/4	7/27 (26%)
6	1.5 M	2 min	direct	63	38 (60%)^{*b}	37 (59%)^{*b}	36 (57%)^{*b}	3/3	7/23 (30%)
7	1.5 M	10 min	direct	63	29 (46%)^{*b}	27 (43%)^{*b}	27 (43%)^{*b}	2/4	5/21 (24%)
Control		Kept in DPBS for 40 minutes		78	–	76 (97%)	76 (97%)	3/3	8/27 (30%)

* Values are significantly different from the control group ($p < 0.01$).
** Values are significantly different from the control group ($p < 0.05$).
Values marked ^{a,b} are different from each other.
[1] This column shows the number of recipients that became pregnant, followed by the total number of recipients.
[2] This column shows the number of offspring followed by the number of blastocysts implanted into those animals that became pregnant.

Statistical analysis

Analysis was performed by ANOVA, when it was inappropriate, by logistic regression; the survival rates were compared with the control by the χ^2 test.

Results

In vitro and *in vivo* results of the experiments with EG are summarized in Table 1. A total of 457 embryos rapidly frozen with EG were recovered after thawing. Embryos frozen in 1.5 M EG showed significantly reduced survival compared with the control ones (57% and 43% vs 97%; $p < 0.01$) and with those frozen with 3 M EG (57% and 43% vs 94%, 84%, 85%, 95% and 98%; $p < 0.01$). There was no significant difference amongst the groups where the concentration of the cryoprotectant was 3 M or 1.5 M. The equilibration and rehydration times only showed significant effect on the survival in combination with the concentration of the cryoprotectant, but not on their own. No significant difference was noticed between the survival rates at thawing and 12 and 24 hours after thawing at each of the groups. 230 embryos were transferred to 25 recipients, 17 of which became pregnant. In the *in vivo* studies, no groups showed significant difference from the control.

In vitro and *in vivo* results of the experiments with G are summarized in Table 2. A total of 503 embryos frozen with G were recovered after thawing. The survival in all of the groups was significantly reduced compared to the control ($p < 0.01$). The cryoprotectant concentration of 1.5 M proved to reduce the survival rate significantly, whereas the rehydration time and

Table 2 *In vitro* and *in vivo* survival of ZP-free mouse blastocysts frozen rapidly in glycerol

No.	Cryoprotectant concentration	Equilibration time	Rehydration time	*In vitro* survival				*In vivo* survival	
				n	At thawing	12 h	24 h	Pregnancies[1]	Offspring[2]
1	3 M	30 min	10 min	84	34 (40%)^{a}	34 (40%)^{a}	32 (38%)^{a}	2/3	4/22 (18%)
2	3 M	20 min	10 min	76	40 (53%)^{a}	40 (53%)^{a}	39 (51%)^{a}	2/3	4/20 (20%)
3	3 M	10 min	10 min	88	40 (45%)^{a}	37 (42%)^{a}	36 (41%)^{a}	32/3	5/19 (26%)
4	3 M	2 min	direct	40	9 (23%)^{b}	7 (18%)^{b}	7 (18%)^{b}	0/3	0/0 (0%)
5	3 M	10 min	direct	80	35 (44%)^{a}	34 (43%)^{a}	32 (40%)^{a}	2/3	3/25 (12%)
6	1.5 M	2 min	direct	72	54 (75%)^{c}	51 (71%)^{c}	15 (21%)^{b}	1/3	1/10 (10%)
7	1.5 M	10 min	direct	63	9 (14%)^{b}	10 (16%)^{b}	9 (14%)^{b}	2/3	3/14 (21%)
Control		Kept in DPBS for 40 minutes		78	–	76 (97%)	76 (97%)	3/3	8/27 (30%)

Values marked ^{a,b,c} are different from each other.
[1] This column shows the number of recipients that became pregnant, followed by the total number of recipients.
[2] This column shows the number of offspring followed by the number of blastocysts implanted into those animals that became pregnant.

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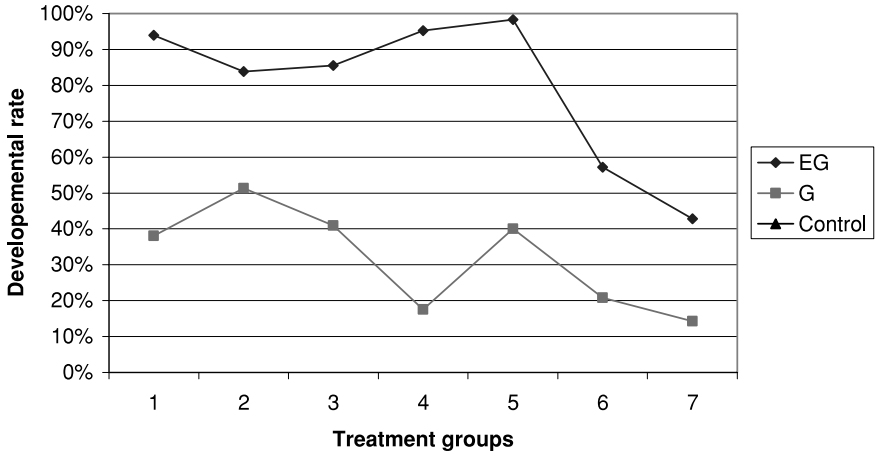

Figure 1 *In vitro* development (24 h) of zona-free embryos rapidly frozen with EG or G. Numbers on the X axis correspond to the groups in Table 1 and Table 2.

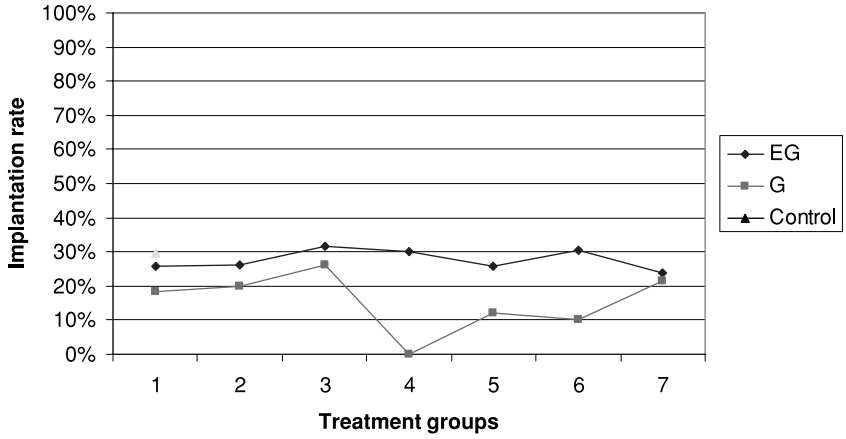

Figure 2 *In vivo* development of zona-free embryos rapidly frozen with EG or G. Numbers on the X axis correspond to the groups in Table 1 and Table 2.

equilibration time alone had no significant effect. In the *in vivo* studies, 190 embryos were transferred to 21 recipients, of which 11 became pregnant. No result was significantly different from that of the control group and there was no significant difference within the treated groups. Comparing the *in vitro* survival rates, there was a significant difference between EG and G. Figure 1 shows the differences between the two cryoprotectants for each group. There was, however, no significant difference between the different groups frozen with EG and G in the *in vivo* survival rates (Fig. 2).

Discussion

Rapid freezing is a simple and effective way to cryopreserve embryos. In quick freezing, the concentration of the cryoprotectant is higher than in controlled freezing but lower than the level required for vitrification, so the toxicity is reduced (Fahy, 1986). The quick-freezing method used in this study combines dehydration with non-penetrating sucrose together with the penetrating EG or G (Leibo, 1989). In the freezing experiments, only blastocysts that hatched early were used, because the viability of the delayed embryos is lower and the freezing might reduce it further.

Comparing the two cryoprotectants, EG was superior to G. The best results were obtained with direct rehydration of embryos cryopreserved in 3 M EG (98% and 95%). However, these results did not show a significant difference from the survival of the other groups cryopreserved in 3 M EG. The results indicate that a 2 min equilibration provides enough time for EG to penetrate the cells, and that the toxicity of 3 M EG is negligible, because the 2 min and 30 min equilibrations gave similar survival rates

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(95% and 94%). The results support the hypothesis that permeability increases as development proceeds (Mazur *et al*., 1976): EG penetrates hatched blastocysts faster than it does blastocysts with an intact ZP. Zhu *et al*. (1996) found that 5 min of RT equilibration of hatched blastocysts in 40% EG solution reduced the survival rate to 6%. It might be that the extremely high concentration of EG penetrating too rapidly into the cells causes more expressed intracellular toxicity then a moderate concentration of 3 M. The significant drop in the survival rate when the concentration of EG was reduced to 1.5 M (43% and 54%) indicates that the optimal concentration for rapid freezing of hatching/hatched blastocysts in EG is 3 M.

Compared with the control group, all groups of embryos frozen with G showed a significantly reduced survival. These results contrast with the findings of Zhu *et al*. (1996), who found that vitrification with G was more effective than with EG, although the concentration of the cryoprotectant in the vitrification solution was much higher than for rapid freezing. According to our results, the equilibration time did not affect the survival rates, indicating that the toxicity of G might play a similar role in the poor survival rates in each of the groups. However, because Zhu *et al*. (1996) showed that a 5 min equilibration in 40% G at RT (which resulted in 68% survival vs 6% for those equilibrated in 40% EG) was less toxic than EG, our results also mean that the 3 M concentration was not optimal for freezing ZP-free blastocysts in G. The best result with G was obtained with a 20 min equilibration, which supports the principle that, owing to its higher molecular weight, G penetrates the cells more slowly.

Rapid freezing of hatched blastocysts with dimethyl sulfoxide equilibrated at 0 °C gave similar results as our 3 M EG, but RT equilibration did not – significantly reduced survival was experienced in this case (Shaw *et al*., 1991).

Transferring hatched blastocysts to day 3 pseudo-pregnant recipients, we used 2 days of asynchrony in order to give the embryo more time to resume the normal developmental potential (Zhu *et al*., 1996; Landa, 1982; Tsunoda *et al*., 1982). Our results with EG were better than those obtained with G, although the differences were not significant. Zhu *et al*. obtained higher implantation rates with vitrified embryos transferred on days 3 and 4, suggesting that vitrification might be less stressful than freezing (Zhu *et al*., 1996). However, the pregnancy results obtained in this study are lower than those found in the literature. The poorer pregnancy rates could be explained by technical difficulties connected with housing of the animals and inexperience of the person doing the transfer.

From the present study, it can be concluded, that ZP-free hatched mouse blastocysts can be successfully cryopreserved by a simple rapid freezing method in 3 M EG-based freezing medium, using as short as 2 min equilibration time (which is conveniently enough to load the blastocysts into straws and seal them) at RT. The results also indicate that the mechanical protection of the ZP is no longer needed during freezing in these stages.

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