Effects of dilution procedure and culture conditions after thawing on survival of frozen bovine blastocysts produced *in vitro*

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Blastocysts derived from bovine zygotes fertilized and matured *in vitro* and cultured for 7 days in conditioned medium were frozen in 1.36 mol glycerol l⁻¹ and 0.25 mol sucrose l⁻¹. *In vitro* survival after thawing was unaffected by dilution rate in 0.25 mol sucrose l⁻¹. The proportion of blastocysts that re-expanded after 24 h was 81% (70 of 86) and 47% (33 of 70) hatched. Seven pregnancies beyond 2 months resulted from transfer of 21 blastocysts to 19 recipients. Total embryonic loss was 46.2%, of which 31% occurred between days 21 and 35. *In vitro* survival after thawing was influenced by culture conditions, the best being culture with oviduct epithelial cells, where 55–82% of blastocysts re-expanded, of which 41–54% hatched. Conditioned medium also supported re-expansion, but low hatching (6%), whereas M199 plus fetal calf serum allowed only limited re-expansion (19–40%). This behaviour was not a consequence of freezing. It is suggested that blastocysts produced *in vitro* have reduced metabolic activity leading to high embryonic loss before or just at the time of implantation and that oviduct cells create a favourable environment after thawing, allowing hatching *in vitro*.

**Introduction**

Large-scale, low cost production of cattle embryos from ovaries from slaughterhouses by *in vitro* maturation and fertilization of oocytes would permit extensive commercial exploitation of this technology. Tremendous progress has been made during the last 5–10 years (see reviews by First, 1990; Greve and Madison, 1991; Marquart-Le Guenne, 1991) and some commercial ventures have been established (ABC and Ovamass, UK) to produce beef embryos of appropriate genetic quality on an industrial scale (Gordon and Lu, 1990; Gordon, 1991). To be used on farms by the classic embryo transfer method, these embryos have to be matured into the blastocyst stage. This stage is also of interest for research on embryo stem cells from inner cell masses, for example. At present, the yield of transferable embryos produced *in vitro* varies from 10 to 30% of the treated oocytes. Their storage in liquid nitrogen would allow greater flexibility in their use. Successful freezing of blastocysts obtained by a totally *in vitro* technique with subsequent pregnancies or births after transfer have been reported (Lu et al., 1988; Goto et al., 1989; Lu et al., 1990; Reichenbach et al., 1990, 1991; Rorie et al., 1990; Suzuki et al., 1991). *In vitro* and *in vivo* survival has also been tested after freezing and thawing of embryos cultured in different conditions (Rorie et al., 1990; Chen Lu et al., 1991) or frozen–thawed by different methods (Lu et al., 1988; Reichenbach et al., 1991). The objective of the present study was to evaluate the effects of dilution procedure and culture conditions after thawing on survival of frozen bovine blastocysts produced by *in vitro* maturation, fertilization and culture in conditioned medium.

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**Materials and Methods**

**Source of embryos**

Embryos were generated by *in vitro* maturation and *in vitro* fertilization of oocytes from an abattoir according to the method described by Boccart et al. (1991a). Intact cumulus–oocyte complexes were matured in tissue culture medium 199 plus 10% heat-treated fetal calf serum (FCS) supplemented with 0.5 µg pure porcine follicle-stimulating hormone (FSH) ml⁻¹, 5 µg pure porcine luteinizing hormone (LH) ml⁻¹ (provided by Beckers, 1987) and 1.0 µg oestradiol ml⁻¹ (20 complexes per 100 µl droplets). After 24 h at 39°C in a humidified atmosphere under 5% CO₂, cumulus–oocyte complexes with expanded cumulus masses were transferred to fertilization droplets, which consisted of Tyrode's medium supplemented with 6 mg fatty-acid-free bovine serum albumin (Sigma, St Louis, MO) ml⁻¹ and 10 µg heparin ml⁻¹ (Calbiochem, San Diego, CA). To each 50 µl fertilization droplet, 1.0 × 10⁵ Percoll-separated spermatozoa were added. Frozen spermatozoa from the same bull were used throughout the experiments. Cumulus–oocyte complexes were removed from fertilization droplets after 18 h, at which time cumulus cells were discarded by gentle pipetting.

**Embryo culture before freezing**

Zygotes were placed in culture droplets (1 embryo ml⁻¹) of conditioned medium (CM) prepared according to Eyestone and First (1989) under mineral oil at 39°C in a humidified atmosphere under 5% CO₂. The conditioning period was 48 h and the medium was stored at −20°C before use. The same batch was
embryos were washed twice in PBS for 10 min and cultured; and (b) 1:100, the content of each straw was expelled into a Petri dish containing 2 ml of 0.25 mol sucrose l⁻¹ in PBS and, after 10 min, the embryos were treated as in procedure (a).

Embryo culture after thawing

Experiment 1: effect of dilution procedure. The effect of the dilution procedure was investigated by culturing blastocysts in M199 plus 10% FCS on bovine oviduct epithelial cell monolayers (BOECM) in four-well dishes (Nunc, Karmstruf, Denmark) containing five to ten embryos under the incubation conditions described previously.

Experiment 2: effect of culture conditions after thawing. To test the effect of culture conditions, dilution by procedure (a) was used, then embryos from the same pool were allocated to two groups: in Expt 2a, group 1 was cocultured in M199 plus 10% FCS on bovine oviduct epithelial cell monolayers and group 2 was cocultured in M199 plus 10% FCS alone; in Expt 2b, a third group of embryos cultured in conditioned medium was included. Survival was evaluated as re-expansion after 24 h (Expt 2a) and 48 h (Expt 2b) and hatching after 4 days of culture. Some embryos that looked morphologically normal after dilution were transferred nonsurgically to ±1 day asynchronous recipients during natural cycles.

Statistical analysis

Differences between treatments were determined by $\chi^2$ analysis.

Results

Embryo development prior to freezing

Blastocysts used in our experiments were generated by in vitro maturation and fertilization of 1424 oocytes. Zygotes were then cultured for 7 days in conditioned medium. The cleavage rate (eight cells) after 48 h was 56% (804 of 1424) and the yield of blastocysts on day 7 was 19% (275 of 1424). Some of these blastocysts were then placed in different media on irradiated mouse fibroblast feeder layers in an attempt to produce embryonic stem cells, and the rest were frozen.

Effect of dilution procedure after thawing (Expt 1)

With the dilution procedure (a) 80% of frozen–thawed blastocysts re-expanded after 24 h in culture and 41% of them hatched after 4 days compared with 83 and 53% for procedure (b) (Table 1). The differences were not significant.

Twenty-one blastocysts that looked morphologically normal in PBS after thawing were transferred to 19 ± 1 day asynchronous recipients ($2 \times 2$ embryos plus $17 \times 1$ embryo). Pregnancy diagnosis was established by determination of plasma progesterone on day 21, serum pregnancy-associated glycoprotein (PAG) (Zoli et al., 1992) from day 35, both by radioimmunoassay, and by rectal palpation from the second

Fig. 1. Bovine blastocysts selected for freezing after culture for 7 days in conditioned medium of oocytes, matured and fertilized in vitro.

Fig. 2. Diagram of a 0.25 ml straw just before cooling: (■) cryoprotectant solution 1.36 mol glycerol l⁻¹, 0.25 mol sucrose l⁻¹; (□) 0.25 mol sucrose l⁻¹ in phosphate-buffered saline; (□) air; (■) plug; (●) bovine embryo.
Table 1. Effect of dilution after thawing on survival of bovine blastocysts produced in vitro

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Embryos frozen–thawed (n)</th>
<th>Re-expanded after 24 h (n) %</th>
<th>Hatched after 96 h (n) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) 1:10</td>
<td>40</td>
<td>32</td>
<td>80</td>
</tr>
<tr>
<td>(b) 1:100</td>
<td>46</td>
<td>38</td>
<td>83</td>
</tr>
<tr>
<td>Total</td>
<td>86</td>
<td>70</td>
<td>81</td>
</tr>
</tbody>
</table>

*Dilution of the freezing medium (one part) in ten parts of diluent contained in the straw (0.25 mol sucrose l⁻¹ in phosphate-buffered saline).
†Dilution of the content of the straw in 2 ml of diluent.

Discussion

With our experimental conditions using conditioned medium, 19% of treated oocytes reached the blastocyst stage after 7 days of culture. This confirms the results of previous reports (Eyestone and First, 1989; Boccart et al., 1991b; Eyestone et al., 1991; Mermillod et al., 1992) on the efficacy of medium conditioned by oviductal tissue to support embryo development from zygote to blastocyst in vitro. The viability after transfer of such blastocysts has been demonstrated by the birth of calves (Eyestone and First, 1989; Boccart et al., 1991a). In this study, we have shown that they can also be frozen successfully, since, after thawing, 81% re-expanded after 24 h of culture and 47% of them were hatched on day 11 (Table 1). Moreover, seven pregnancies out of 19 transfers are ongoing.

Survival after freezing and thawing was unaffected by the dilution rate in 0.25 mol sucrose l⁻¹ (1 of 10 compared with 1 of 100). The original method was developed to allow direct transfer without dilution by mixing sucrose with glycerol in the freezing medium, and a pregnancy rate of 50% was attained with embryos produced in vivo (Massip et al., 1987). The contribution of sucrose mixed with glycerol is to partially dehydrate the embryo before freezing, by virtue of being a nonpermeating agent. During warming, it prevents osmotic shock. It has also been shown in rabbits (Borland et al., 1976) that, in the presence of sucrose, the blastocyst accumulates Na⁺ and Cl⁻ ions into the blastocoel, the volume of which increases. This transport depends on Na⁺–K⁺-ATPase activity. This activity is inhibited by glycerol (Albers and Koval, 1972), so sucrose is involved directly in ion movements through the cells after thawing. The use of 0.25 or 0.5 mol sucrose l⁻¹ as diluent is not critical for survival, but it may improve the pregnancy rate (Touati et al., 1991).
and, above all, it makes the freezing–thawing procedure safer. However, as sucrose solutions are viscous and may not mix efficiently with cryoprotective agents, it could be better to use galactose solutions of equivalent osmolalities as diluents because monosaccharides have much lower viscosities than disaccharides (McWilliams et al., 1991).

Pregnancy rates after transfer of frozen–thawed bovine blastocysts produced in vitro that were morphologically normal after thawing, are lower than 50% (Lu et al., 1988; Goto et al., 1989; Reichenbach et al., 1991; Suzuki et al., 1991). This indicates some failure in the development of such embryos. The pregnancy rate in this study was 37% and embryonic loss was high (46.2%) and occurred mainly between days 21 and 35, just at the time of or before implantation. Heyman (1985) reported embryonic mortality rates of 22.0, 28.5 and 46.4% after transfer of fresh, frozen and cultured embryos, respectively. In our conditions, in vitro culture and freezing were combined and both affected the viability of the embryo. So, only blastocysts of excellent quality can develop to term. This quality has been stressed as an important factor determining the developmental capacity of blastocysts after transfer or their ability to withstand freezing. It depends on the culture system used (Rorie et al., 1990; Chen Lu et al., 1991; Jiang et al., 1991; Vergos et al., 1991).

Culture conditions have to meet specific requirements at each of the critical steps of embryonic development, i.e. the passage through the block stage, morula compaction, blastocyst formation and hatching, to improve this quality (Ellington et al., 1990).

In vitro survival after thawing was influenced by culture conditions. In Expt 2a, re-expansion rate in M199 was half that in BOECM (40 versus 82%) and hatching was zero compared with 54% (Table 2). The results of Expt 2b (Fig. 3) confirm the results of Expt 2a and show that re-expansion rate is poor in M199 plus FCS and similar in CM or BOECM (±60%), but hatching rate is very low in CM, 6% compared with 40% in BOECM. The same observations were made with nonfrozen blastocysts produced in vitro cultured from day 7 in each of these conditions (Mermillod et al., 1992). This behaviour was therefore independent of the freezing process.

Among the factors that have been shown to affect in vitro development of bovine embryos are medium, protein supplement, gases and the presence or absence of bovine oviduct epithelial cells and their association (Fukui et al., 1991). For example, ova in M199 develop readily in air (20% oxygen) when cells are present, but development is poor in their absence unless O₂ concentration is lowered to 5%. Our incubation conditions were 5% CO₂ in air, and development after thawing was better in M199 plus BOECM than in M199 alone (Table 2). There is, perhaps, an interaction among medium, oviduct, epithelial cells and gases. The cells could mitigate the harmful effects of high oxygen concentrations, probably by reducing the oxygen concentration in the medium or by producing antioxidants, unless they neutralize a toxic effect of serum.

Hatching follows a phase of expansion characterized by high energy requirements, high glutamine and glucose metabolism. Much of the increased metabolic activity in expanded blastocysts is to provide the ATP required by the Na⁺-K⁺ pump for expansion of the blastocoele (Tiffin et al., 1991).

This step perhaps needs low oxygen concentration to allow energy substrate utilization. Khurana and Wales (1989) have demonstrated that a reduced oxygen atmosphere is necessary to promote the use of endogenous glycogen pools in cultured mouse morulae. Thus, hatching would depend on the presence of cells.

The specificity for a reproductive-tract source of cocultured cells is not required since a hatching rate of 54% (47 of 87) was found when nonfrozen in vitro blastocysts were cocultured from day 7 in different media on irradiated mouse fibroblast feeder layers (Delhaise et al., 1991). One of these media was M199 plus 10% FCS plus 10% calf serum in which 64% (18 of 20) of the blastocysts hatched.

However, fibroblasts differ from oviduct cells because embryos cocultured in their presence have a lower rate of development in vivo than those cultured on oviduct epithelial cells (Gandolfi and Moor, 1987). The viability of embryos thus depends on the presence of oviduct cells that secrete specific proteins (Boice et al., 1990; Wegner and Killian, 1991), whereas the action of fibroblast cells could be due, in this particular case, to reduction of the oxygen tension or detoxification.

In conclusion, blastocysts derived from bovine zygotes matured and fertilized in vitro and cultured in conditioned medium can be frozen successfully and give pregnancies after transfer. In vitro survival after thawing is unaffected by dilution rate in 0.25 mol sucrose l⁻¹, but is influenced by culture conditions. Re-expansion of blastocysts is limited in M199 plus FCS and, it increases in conditioned medium, but hatching needs an epithelial cell coculture system. This observation is unrelated to freezing and emphasizes the role of coculture on embryo viability.

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