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 $1.36 \text{ mol glycerol l}^{-1}$ and $0.25 \text{ mol sucrose l}^{-1}$. *In vitro* survival after thawing was unaffected by dilution rate in $0.25 \text{ mol sucrose l}^{-1}$. 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; Marquant-Le Guienne, 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 cultured to 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 $0.5 \mu\text{g}$ pure porcine follicle-stimulating hormone (FSH) ml^{-1} , $5 \mu\text{g}$ pure porcine luteinizing hormone (LH) ml^{-1} (provided by Beckers, 1987) and $1.0 \mu\text{g}$ oestradiol ml^{-1} (20 complexes per $100 \mu\text{l}$ droplets). After 24 h at 39°C in a humidified atmosphere under 5% CO_2 , cumulus–oocytes 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 $10 \mu\text{g}$ heparin ml^{-1} (Calbiochem, San Diego, CA). To each $50 \mu\text{l}$ fertilization droplet, 1.0×10^5 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 ($1 \text{ embryo } \mu\text{l}^{-1}$) of conditioned medium (CM) prepared according to Eyestone and First (1989) under mineral oil at 39°C in a humidified atmosphere under 5% CO_2 . The conditioning period was 48 h and the medium was stored at -20°C before use. The same batch was

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.

used during these investigations. Cleavage (5–8 cells) was assessed at 48 h after insemination. Media were not changed during the course of incubations. Criteria for normal development consisted of attainment of the expanding blastocyst stage after 7 days of culture (Fig. 1). Development results are expressed as a function of oocytes treated. Only embryos derived from one *in vitro* fertilization that had reached the expanding blastocyst stage with a distinct inner cell mass and blastocoelic cavity were pooled and frozen.

Freeze-thawing and dilution procedures

At the end of the culture period, expanding blastocysts of normal morphology were washed in phosphate-buffered saline (PBS) supplemented with 20% FCS and then frozen by the method of Massip and Van der Zwalm (1984), slightly modified. Briefly, embryos were equilibrated for 10 min in 1.36 mol glycerol l^{-1} , and 0.25 mol sucrose l^{-1} in PBS at room temperature then loaded in 0.25 ml straws (Fig. 2) with a maximum of five embryos per straw.

The proportions of freezing medium and diluent were 1:10. The straws were sealed and placed at $-7^{\circ}C$ in the cooling chamber of a programmable freezing machine (Minicool: Air Liquide France). After 5 min at $-7^{\circ}C$ seeding was induced and, 5 min later, cooling, at $0.3^{\circ}C\ min^{-1}$ was initiated and interrupted at $-25^{\circ}C$ before plunging and storing the straws into liquid nitrogen.

Thawing was done by agitating each straw in a $20^{\circ}C$ water bath for 10–30 s. Two dilution procedures were compared: (a) 1:10, the content of each straw was expelled into a Petri dish and mixed by shaking rapidly; after 10 min at room temperature,

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 χ^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 (2×2 embryos plus 17×1 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

Table 1. Effect of dilution after thawing on survival of bovine blastocysts produced *in vitro*

Dilution	Embryos frozen-thawed (n)	Re-expanded after 24 h		Hatched after 96 h	
		n	%	n	%
(a)* 1:10	40	32	80	13	41
(b)† 1:100	46	38	83	20	53
Total	86	70	81	33	47

*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.

Table 2. Effect of culture conditions after thawing on survival of bovine blastocysts produced *in vitro* (Expt 2a)

Culture conditions	Embryos frozen-thawed (n)	Re-expanded after 24 h		Hatching after 96 h	
		n	%	n	%
Bovine oviduct epithelial cell monolayer	61	50	82 ^a	27	54 ^c
199 + 10% fetal calf serum	55	22	40 ^b	0	0 ^d

Values within rows with different superscripts are significantly different $P < 0.05$.

month. Thirteen recipients (68%) had initiated pregnancy on day 21 (progesterone $> 2 \text{ ng ml}^{-1}$); 9 (47%) showed PAG+ on day 35; and 7 (37%) were confirmed by rectal palpation at 2 months. Pregnancy rate at 2 months was 37% per recipient (7 of 19) and 33% per embryo transferred. Embryonic loss was 31% (4 of 13) at day 35 and 46% (6 of 13) at 2 months. If we consider that all animals would have been pregnant at transfer, the total loss is 63%.

Effects of culture conditions after thawing

Experiment 2a. *In vitro* development of thawed embryos was significantly higher in coculture than in medium alone (82% re-expansion versus 40%, $\chi^2 = 22$, $P < 0.05$). Furthermore, 54% of re-expanded embryos hatched in coculture and none in the absence of cells ($\chi^2 = 32$, $P < 0.05$; Table 2).

Experiment 2b. In this experiment, three groups were considered: M199 + 10% FCS, CM and BOECM. The aim was to determine whether the presence of cells in the culture medium was responsible for the high survival rate after thawing. After 24 h, re-expansion rates in M199 (19%) and BOECM (55%) were lower than in the previous experiment, but the range was similar (Fig. 3). There was no difference between CM and BOECM. After 48 h, no embryo survived in M199. The re-expansion rate was increased by about 5–10% (from 55% to

Fig. 3. *In vitro* survival after thawing of bovine blastocysts placed in different culture conditions for 4 days: TCM199 + 10% fetal calf serum (FCS; $n = 21$), conditioned medium (CM; $n = 31$) and coculture on bovine epithelial cell monolayer (BOECM, $n = 31$). Survival was examined after 24 h (□) and 48 h (▨). Hatching rate (■) was determined after 96 h. ^{a,b,c}Values with different letters differ significantly.

61–65%) both in CM and BOECM. At the end of the culture period, hatching rates were 6% in CM and 41% on BOECM.

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.*,

1990) 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 ($\pm 60\%$), 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 O_2 concentration is lowered to 5%. Our incubation conditions were 5% CO_2 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 blastocoel (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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