Temporal sensitivity of bovine embryos to culture environment after fertilization and the implications for blastocyst quality

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The aim of this study was to examine the temporal sensitivity of bovine embryos to culture environment after fertilization to determine which period, if any, is most critical in determining blastocyst quality. Bovine zygotes produced *in vitro* were divided into six groups and cultured either *in vitro* (in synthetic oviductal fluid, SOF), *in vivo* (in the ewe oviduct) or in a combination of both systems. Development to the blastocyst stage, the ability of the blastocysts to withstand cryopreservation and the relative abundance of several gene transcripts were examined. Culture in SOF for either 2 or 4 days, followed by subsequent culture in the ewe oviduct, resulted in a significantly lower yield of blastocysts than did all other methods, the effect being most marked in embryos that were cultured in SOF for 4 days. In contrast, culture *in vivo* for the first 2 or 4 days

after fertilization followed by culture *in vitro* did not have such a marked effect on blastocyst development. Blastocysts produced after culture in the oviduct for 6 days had the highest rates of survival over 72 h after warming (100% survival at 24 h; > 95% survival at 72 h). The embryos that spent the last 4 days of culture *in vivo* also had relatively high rates of survival (100% at 24 h, 73.7% at 72 h). Blastocysts produced entirely in SOF had very low rates of survival after vitrification, with < 40% viable at 24 h and < 20% survival at 72 h. Blastocysts derived from embryos that spent the first 2 days *in vivo* and the last 4 days *in vitro* had the lowest rates of survival (6.7%), whereas those that spent the last 2 days only in SOF had intermediate rates of survival (40.6%). These differences were reflected in the relative abundance of transcripts for the *Bax* gene.

Introduction

Although the success of *in vitro* maturation and fertilization in cattle is high (approximately 80%), most cleaved oocytes fail to reach the blastocyst stage (Lonergan *et al.*, 1999a; Rizos *et al.*, 2002a). Using the bovine model, Rizos *et al.* (2002a) showed that, despite the fact that the major fall off in development occurs between the two-cell stage and the blastocyst stage, which would implicate the culture environment after fertilization, it is actually the intrinsic quality of the oocyte itself that is the key factor in determining its ability to reach the blastocyst stage.

McEvoy (2000) stated that attainment of the blastocyst stage is more a reflection of past achievement than a guarantee of future fitness. Therefore, in addition to increasing the success rate of embryo production *in vitro* (that is, the number of oocytes that develop to the blastocyst stage), it is essential that the embryos that do reach this stage *in vitro* are of the highest quality possible to ensure optimal rates of pregnancy after transfer. The quality

of blastocysts produced *in vitro* continually lags behind that of blastocysts produced *in vivo* and this can be linked to differences between them. Embryos produced *in vitro* tend to have darker cytoplasm and a lower buoyant density (Pollard and Leibo, 1994) as a consequence of their higher lipid content (Abd El Razek *et al.*, 2000), a more fragile zona pellucida (Duby *et al.*, 1997), reduced expression of intercellular communication devices (Boni *et al.*, 1999), and a higher incidence of chromosome abnormalities (Viuff *et al.*, 1999; Slimane *et al.*, 2000) than do their *in vivo*-produced counterparts.

Within *in vitro* systems, modifications of the embryo culture environment after fertilization can have profound effects on gene expression in the embryo (Wrenzycki *et al.*, 1999, 2001; Rief *et al.*, 2002; Rizos *et al.*, 2002b, 2003) that, in turn, can have serious implications for the normality of the blastocyst. By culturing *in vitro*-produced bovine zygotes *in vivo* in ewe oviduct it has been shown that it is possible to increase markedly the quality of the resulting blastocysts, measured in terms of cryotolerance, to a level similar to that of embryos produced entirely *in vivo* (Enright *et al.*, 2000; Rizos *et al.*, 2002a). Conversely, the culture of *in vivo*-produced

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bovine zygotes *in vitro* resulted in blastocysts of low cryotolerance (Rizos *et al.*, 2002a), indicating that the culture period after fertilization (from day 1 to day 7) is the most critical period for determining blastocyst quality. The detrimental effects of culture under suboptimal conditions *in vitro* are mediated through modifications of gene expression in the embryo. These detrimental effects can be seen as blastocysts incapable of withstanding cryopreservation or establishing a pregnancy, or, as has been shown in ruminants, the production of offspring of abnormal size (Young *et al.*, 1998). Production of offspring of abnormal size is directly linked to *in vitro* culture after fertilization (McEvoy *et al.*, 1998) and has very serious implications for human assisted reproduction, in which there is a trend towards transferring blastocyst stage embryos instead of early stage embryos, which necessitates a longer period of culture *in vitro* in potentially detrimental conditions (Gardner and Lane, 1997; Gardner *et al.*, 2000).

Several major developmental events occur during the 6-day window between zygote and blastocyst formation in cattle. These include the first cleavage division, the timing of which is of critical importance in determining the subsequent development of the embryo (Lonergan *et al.*, 1999), the activation of the embryonic genome at the 8–16-cell stage (Camous *et al.*, 1986; Kopecny *et al.*, 1989; Memili and First, 2000), compaction of the morula on day 5, which involves the establishment of the first intimate cell–cell contacts in the embryo (Boni *et al.*, 1999), and blastocyst formation on days 6–7, involving the differentiation of two types of cell, the trophectoderm and the inner cell mass (Watson, 1992). Clearly, any modifications of the culture environment, which could affect any, or all, of these processes, could have a major effect on the quality of the embryo. The aim of the present study was to isolate these developmental events, using the ewe oviduct model, and to identify the period of culture after fertilization (that is, the period of early embryo development from day 1 to day 7) that is most critical in determining blastocyst quality, measured in terms of the blastocyst cryotolerance and the relative abundance of certain gene transcripts.

Materials and Methods

Experimental design

The design of Expt 1 is shown (Fig. 1). Zygotes produced *in vitro* were divided into six groups and cultured either *in vitro* (in synthetic oviduct fluid (SOF), which is known to produce poor quality blastocysts), *in vivo* (in ewe oviduct, which is known to produce excellent quality blastocysts) or in a combination of both systems, according to the scheme outlined (Fig. 1). A group of *in vivo*-derived blastocysts from superovulated heifers were used as a control. The groups used were: group 1: culture in SOF for 6 days; group 2: culture in SOF for

2 days followed by culture in ewe oviduct for 4 days; group 3: culture in SOF for 4 days followed by culture in ewe oviduct for 2 days; group 4: culture in ewe oviduct for 2 days followed by culture in SOF for 4 days; group 5: culture in ewe oviduct for 4 days followed by culture in SOF for 2 days; group 6: culture in ewe oviduct for 6 days; and group 7: *in vivo*-produced blastocysts after superovulation and embryo recovery.

On the basis of the results of Expt 1, a second experiment was performed to confirm the poor development observed in groups 2 and 3. Presumptive zygotes were cultured in: (i) SOF for 6 days (group 1 from Expt 1); or (ii) SOF for 4 days followed by ewe oviduct for 2 days (group 3 from Expt 1). In addition, a sham group of embryos was removed from the incubator, transported to the surgery alongside group 3 and then replaced in the incubator in SOF.

Oocyte collection and *in vitro* maturation (IVM)

Cumulus–oocyte complexes (COCs) were obtained by aspirating follicles from the ovaries of slaughtered heifers. After four washes in PBS supplemented with 36 µg pyruvate ml⁻¹, 50 µg gentamycin ml⁻¹ and 0.5 mg BSA ml⁻¹ (Sigma, St Louis, MO), groups of up to 50 COCs were placed in 500 µl maturation medium in four-well dishes (Nunc, Roskilde) and cultured for 24 h at 39°C under an atmosphere of 5% CO₂ in air with maximum humidity. The maturation medium was TCM-199 supplemented with 10% fetal calf serum (FCS) and 10 ng epidermal growth factor (EGF) ml⁻¹ (Lonergan *et al.*, 1996).

Sperm preparation and *in vitro* fertilization (IVF)

For IVF, COCs were washed four times in PBS and then in fertilization medium before they were transferred in groups of up to 50 into four-well dishes containing 250 µl fertilization medium (Tyrode's medium with 25 mmol bicarbonate l⁻¹, 22 mmol sodium lactate l⁻¹, 1 mmol sodium pyruvate l⁻¹ and 6 mg fatty acid-free BSA ml⁻¹). In addition, 10 µg heparin–sodium salt ml⁻¹ (184 U mg⁻¹ heparin; Calbiochem, San Diego, CA) was added. Motile spermatozoa were obtained by centrifugation of frozen–thawed semen (Dairygold A.I. Station, Mallow) on a discontinuous Percoll (Pharmacia, Uppsala) density gradient (2.5 ml 45% (v/v) Percoll over 2.5 ml 90% (v/v) Percoll) at 700 g for 8 min at room temperature. Viable spermatozoa collected at the bottom of the 90% fraction were washed in Hepes-buffered Tyrode's medium and pelleted by centrifugation at 100 g for 5 min. The spermatozoa were counted in a haemocytometer and diluted in the appropriate volume of fertilization medium to give a concentration of 2 × 10⁶ spermatozoa ml⁻¹. A 250 µl aliquot of this suspension was added to each fertilization well to obtain a final concentration of 1 × 10⁶ spermatozoa ml⁻¹. The plates

Fig. 1. Design of Expt 1. Blastocysts were produced after culture of *in vitro*-matured–*in vitro*-fertilized zygotes in: group 1: synthetic oviductal fluid (SOF) for 6 days; group 2: SOF for 2 days followed by culture in ewe oviduct for 4 days; group 3: SOF for 4 days followed by culture in ewe oviduct for 2 days; group 4: ewe oviduct for 2 days followed by culture in SOF for 4 days; group 5: ewe oviduct for 4 days followed by culture in SOF for 2 days; or group 6: ewe oviduct for 6 days. An additional group of blastocysts (group 7) was derived *in vivo* after superovulation and embryo recovery.

were incubated for 20 h at 39°C under an atmosphere of 5% CO₂ in air with maximum humidity. Semen from the same bull was used for all experiments.

Embryo culture after fertilization

At approximately 20 h after insemination, presumptive zygotes were denuded by gentle vortexing and washed four times in PBS before they were randomly assigned to treatment. For *in vitro* culture (IVC), zygotes were transferred to 25 µl culture droplets of SOF (25 embryos per droplet; Lonergan *et al.*, 1999b) under mineral oil (Sigma). FCS (10%) was added 24 h after placement in culture. The dishes were incubated in an atmosphere of 5% CO₂, 5% O₂ and 90% N₂ at 39°C.

For *in vivo* culture, presumptive zygotes or embryos produced after IVM–IVF were surgically transferred to ligated ewe oviducts (approximately 100 per oviduct). Two ewes (that is, four oviducts) were used per group. Ewes were used without regard to stage of cycle; however, a vaginal progestagen pessary was inserted to prevent the ewes from entering oestrus during the period of

transfer and recovery. Transfer was performed at 20 h after insemination (groups 4, 5 and 6), 3 days after insemination (group 2) or 5 days after insemination (group 3). Embryos were recovered from the ewes at day 3 after insemination (group 4), day 5 after insemination (group 5) or day 7 after insemination (groups 2, 3 and 6) by flushing the oviduct with 20 ml PBS. For some groups, the embryos were placed in SOF + 10% FCS for further culture (see Fig. 1).

Cleavage was assessed at 48 h after insemination (or at recovery from ewe oviduct) and blastocyst development was recorded on days 6–8 (day 0 = day of IVF) and expressed as a fraction of the total number of embryos recovered. Day 7 blastocysts were used for the cryotolerance and transcript abundance studies. The remaining embryos were maintained *in vitro* until day 8.

In vivo production of blastocysts

In vivo-derived blastocysts collected from heifers were used as a control. The protocol used for the *in vivo* production of embryos was as described by Rizos *et al.*

Table 1. Specific semi-quantitative RT-PCR conditions

cDNAs	RT-PCR primers	Annealing temperature (°C)	Number of embryos per RT-PCR	Number of PCR cycles
β -actin	5'-GAGAAGCTCTGCTACGTCG 3'-CCAGACAGCACCGTGTGG	60	1	29
Bax	5'-TGCAGAGGATGATCGCAGCTGTG 3'-CCAATGTCCAGCCCATCATGGTC	59	1	29
L37	5'-TCATCGTTTGGAAAGCGTCGGAATA 3'-GCTCGCTTGGGTTTAGGTGTTGTC	59	1	25
S3a	5'-TTGGACTGGTGGCTCGTATC 3'-AGATCCGCCGAGAAGATGATG	59	1	28

(2002a). Beef cross heifers were synchronized using a controlled internal drug releasing (CIDR) device (InterAg, Hamilton) for 8 days. At day 3 before CIDR removal, the heifers received 2 ml (15 mg) of prostaglandin $F_{2\alpha}$ analogue (PG; Prosolvin; Intervet, Dublin). The heifers were checked for standing oestrus (= day 0). The dominant follicle was ablated by transvaginal aspiration on day 8 of the oestrous cycle. Beginning on day 10, the animals were superovulated with a total of 180 mg FSH (Folltropin; Vetrepahm Canada Inc., London, ON) given as injections twice a day over 4 days on a decreasing dose schedule. Luteolysis was induced with 15 mg PG given on day 12. Heifers were inseminated with frozen-thawed semen at 48 h and 60 h after PG injection. The same semen batch as was used for IVF was used for artificial insemination. Day 7 embryos were recovered by non-surgical flushing at day 9 after PG administration.

Blastocyst vitrification

The ability of the blastocyst to withstand cryopreservation was used as a tool to assess quality (Rizos *et al.*, 2001, 2002a, 2003). Blastocysts were vitrified using the open pulled straw (OPS) method described by Vajta *et al.* (1998) in a final solution containing 20% (v/v) ethylene glycol and 20% (v/v) DMSO. Warmed blastocysts were cultured in 25 μ l droplets of M199 + 10% FCS in the presence of a granulosa cell monolayer (Rizos *et al.*, 2001) and were examined at 24, 48 and 72 h after warming. Survival was defined as re-expansion of the blastocoel and its maintenance for 24, 48 and 72 h, respectively. The rate of hatching was also recorded and was expressed as a percentage of the embryos surviving at 72 h after warming.

RNA extraction from blastocysts for analysis of transcript abundance

Total RNA was prepared from each group of pools of ten blastocysts using an RNeasy mini column (Qiagen, Hilden) following the manufacturer's instructions. The blastocysts were washed three times in PBS and immediately snap-frozen in a minimal volume (approximately

2 μ l) of PBS in liquid nitrogen and stored at -80°C until use. After lysis of blastocysts in lysis buffer (Qiagen) and isolation on the Qiagen minicolumn, the RNA (isolated from the group of ten blastocysts) was resuspended in 30 μ l RNase-free water and stored at -80°C .

Semi-quantitative RT-PCR

Cell death inducer gene (*Bax*) and ribosomal proteins S3a and L37 were selected for determining the relative abundance of transcripts in the blastocysts derived from the different groups. *Bax* belongs to the group of pre-apoptotic proteins that initiate mitochondrial dysfunction and localize to the endoplasmic reticulum (Scorano *et al.*, 2003). Ribosomal protein S3a is essential for initiation of translation (Westermann *et al.*, 1981; Cramton and Laski, 1994), its expression prevents apoptosis (Song *et al.*, 2002), and up- and downregulation of S3a expression, coordinated in sequence, can induce apoptosis (Naora *et al.*, 1998a). In contrast, L37 ribosomal protein gene was chosen because L37 is expressed constitutively even during the transition from quiescence to active proliferation or terminal differentiation (Su and Bird, 1995). The β -actin gene expression was used as an internal standard (Rizos *et al.*, 2003).

The platinum quantitative RT-PCR ThermoScript one-step system (Life Technologies, Rockville, MD) was used for multiplication of RNA molecules. This system takes advantage of high temperature reverse transcription by ThermoScript plus reverse transcriptase directly coupled to an automatic hot-start PCR. The equivalent of one blastocyst (3 μ l RNA sample) was used in RT-PCR for each transcript according to the manufacturer's instructions. RT-PCR was performed with specific primer pairs (Table 1) within a previously determined linear range of amplification. The PCR reaction volume was 25 μ l and consisted of ThermoScript Reaction Mix (Life Technologies), sense primer (0.2 $\mu\text{mol l}^{-1}$), antisense primer ($\mu\text{mol l}^{-1}$), RNase (20 U; Promega, Madison, WI) and ThermoScript plus/platinum *Taq* enzyme mix (Life Technologies). RT-PCR was performed as follows: cDNA synthesis at 60°C for 30 min, pre-denaturation at 95°C for 5 min, followed by a specific number of cycles of

Table 2. Effect of culturing *in vitro*-produced bovine zygotes *in vitro*, in synthetic oviductal fluid (SOF), *in vivo* in ewe oviduct or in a combination of both systems, on cleavage and development to the blastocyst stage

Culture system	Number of zygotes or embryos transferred	Number of embryos recovered (%)	Number of embryos cleaved (%)	Blastocyst yield		
				Day 6 (n) (%)	Day 7 (n) (%)	Day 8 (n) (%)
SOF 6 days	354	–	292 (82.5)	55 (15.5)	94 (26.5) ^{ab}	107 (30.2) ^a
SOF 2 days + ewe 4 days*	400	318 (79.5)	329 (82.2)	–	12 (3.8) ^c	26 (8.2) ^b
SOF 4 days + ewe 2 days	334	135 (40.4)	267 (79.9)	–	0 (0) ^d	0 (0) ^c
Ewe 2 days + SOF 4 days	400	327 (81.7)	257 (78.6)	36 (11.0)	66 (20.2) ^a	74 (22.6) ^a
Ewe 4 days + SOF 2 days	400	274 (68.5)	–	32 (11.7)	85 (31.0) ^b	89 (32.5) ^a
Ewe 6 days	400	298 (74.5)	–	–	72 (24.2) ^{ab}	86 (28.9) ^a
Significance			ns	ns	$P < 0.05$	$P < 0.05$

*SOF 2 days + ewe 4 days: zygotes were cultured for 2 days in synthetic oviductal fluid followed by 4 days in ewe oviduct.

^{a–d}Values in the same column with different superscripts are significantly different ($P < 0.05$). ns: not significant.

denaturation (95°C for 30 s), annealing (59 or 60°C for 30 s), extension (72°C for 45 s), and final extension at 72°C for 5 min. Products of the semi-quantitative specific RT-PCR (10 µl) were separated by electrophoresis on a 1.5% (w/v) agarose gel, blotted on Hybond N+ nylon membrane (Amersham Biotech, Little Chalfont) and hybridized with specific radioactive probes (³²P) diluted in a Church buffer (Church and Gilbert, 1984) at 65°C overnight. The membranes were washed under stringent conditions twice with 2 × SSC, 0.1% (w/v) SDS buffer at 65°C for 15 min and once with 0.2 × SSC, 0.1% (w/v) SDS buffer at 65°C for 15 min. Hybridized signals were detected in BAS 2500 (Fuji, Kanagawa) and quantified using the AIDA programme (Raytest, Straubenhardt). Negative control tubes were always prepared in which RNA was omitted.

Statistical analysis

Differences in the rate of cleavage and the proportion of immature oocytes reaching the blastocyst stage, and rates of blastocyst survival after vitrification and warming were compared using chi-squared analysis. The data of semi-quantitative mRNA expression were analysed using SigmaStat version 3.00 (Jandel Scientific, San Rafael, CA) software package. One way repeated measures ANOVA (followed by pairwise multiple comparison procedures, Holm-Sidak method) was used for the analysis of differences in mRNA expression. $P < 0.05$ was considered significant.

Results

Development

Rates of embryo recovery and developmental data from Experiment 1 are shown (Table 2). The mean rate of recovery of embryos from ewe oviduct was 69.9% (1352/1934). There were no differences in rates of cleavage between any of the groups. Similarly, blastocyst yield on

day 6 was not different between the groups in which it was assessed.

Consistent with the authors' previous observations, there was no difference in blastocyst yield when culture took place either *in vitro* or *in vivo* for the entire period (30.2 versus 28.9%, respectively). Culture in SOF for either 2 or 4 days, followed by subsequent culture in ewe oviduct, resulted in a significantly lower yield of blastocysts than did all other treatments, the effect being most marked in the group of embryos cultured in SOF for 4 days, in which all embryos were degenerated on recovery. In contrast, culture *in vivo* for the first 2 or 4 days after fertilization followed by culture *in vitro* did not have such a marked effect on blastocyst development (Table 2).

The results of Expt 2 (Table 3) confirmed those of Expt 1. There were no differences in the rate of cleavage between the three groups of embryos (81.4–85.0%). Culture in SOF for 4 days followed by culture in ewe oviduct for 2 days resulted in no blastocyst development despite the fact that embryos from the sham transfer group developed as well as the control embryos.

Cryotolerance

In terms of embryo quality, as measured by cryotolerance, blastocysts produced *in vivo* (control), as well as those produced after culture in the oviduct for 6 days, had the highest rates of survival over 72 h after warming (100% survival at 24 h, >95% survival at 72 h; Fig. 2). The embryos that spent the last 4 days of culture *in vivo* also had relatively high rates of survival (100% at 24 h, 73.7% at 72 h). Consistent with previous observations, blastocysts produced entirely in SOF had very low rates of survival after vitrification, with <40% viable at 24 h and <20% survival at 72 h. Blastocysts derived from embryos that spent the first 2 days *in vivo* and the last 4 days *in vitro* had the lowest rates of survival (6.7%), whereas those that spent the last 2 days only in SOF had intermediate rates of survival (40.6%).

Table 3. Effect of culturing *in vitro*-produced bovine zygotes *in vitro*, in synthetic oviductal fluid (SOF), *in vivo* in ewe oviduct or in a combination of both systems, on cleavage and development to the blastocyst stage

Culture system	Number of zygotes or embryos transferred	Number of embryos recovered (%)	Number of embryos cleaved (%)	Blastocyst yield		
				Day 6 (n) (%)	Day 7 (n) (%)	Day 8 (n) (%)
SOF 6 days	43	–	35 (81.4)	4 (9.3) ^a	8 (18.6) ^a	9 (20.9) ^a
SOF 4 days + ewe 2 days*	147	132 (40.4)	125 (85.0)	0 (0) ^b	0 (0) ^b	0 (0) ^b
Sham transfer	100	100 (100)	82 (82.0)	14 (14.0) ^a	27 (27.0) ^a	29 (29.0) ^a
Significance			ns	$P < 0.001$	$P < 0.001$	$P < 0.001$

*SOF 4 days + ewe 2 days: culture for 4 days in synthetic oviductal fluid followed by 2 days in ewe oviduct.

^{ab}Values in the same column with different superscripts are significantly different ($P < 0.001$). ns: not significant.

Fig. 2. Survival of bovine blastocysts after vitrification and warming and subsequent culture for 72 h. Blastocysts were produced after culture of *in vitro*-matured-*in vitro*-fertilized zygotes in: group 1 (◆): synthetic oviductal fluid (SOF) for 6 days ($n=31$); group 2 (■): SOF for 2 days followed by culture in ewe oviduct for 4 days ($n=19$); group 4 (●): ewe oviduct for 2 days followed by culture in SOF for 4 days ($n=30$); group 5 (▲): ewe oviduct for 4 days followed by culture in SOF for 2 days ($n=32$); or group 6 (○): ewe oviduct for 6 days ($n=31$). An additional group of blastocysts (group 7; △) was derived *in vivo* after superovulation and embryo recovery. An additional group of blastocysts (group 7) was derived *in vivo* after superovulation and embryo recovery ($n=6$). (No blastocysts were available from Group 3: culture in SOF for 4 days followed by culture in the ewe oviduct for 2 days). ^{a-d}Values with different superscripts at the same time-point differ significantly ($P < 0.05$).

These differences in rates of blastocyst survival were reflected in the proportion of blastocysts hatching by 72 h after vitrification and warming. All of the blastocysts

produced *in vivo* and > 80% of those cultured in ewe oviduct for 6 days hatched, compared with only 3.2% of those cultured in SOF for 6 days. Culture *in vivo* for the last 4 days resulted in significantly higher rates of hatching (42.1%) compared with culture in SOF for the same period (0%).

Relative abundance of transcripts

The amount of β -actin amplification products obtained from the different experimental groups was very similar with minimal variations quantified (Fig. 3). Thus, β -actin amplification was used as an internal standard control of RT-PCR. Values for Bax (Fig. 4), L37 ribosomal protein (Fig. 5) and S3a ribosomal protein (Fig. 6) expression were obtained by dividing the value for the specific product by the corresponding β -actin value; the largest sample signal was then set at 100%.

High transcript abundance for Bax correlated with longer periods of culture *in vitro* in SOF. Bax mRNA was expressed strongly in blastocysts produced *in vitro* in SOF (Fig. 4); transcript abundance was significantly higher than after culture in the ewe oviduct for 6 days (group 6) or in embryos produced entirely *in vivo*. There was no difference in transcript abundance between blastocysts produced by culture for 6 days in ewe oviduct or those derived *in vivo*.

Transcripts for the ribosomal gene L37 were expressed strongly in all experimental groups. There was no correlation between culture environment and mRNA expression. L37 behaved as a housekeeping gene.

S3a mRNA was expressed weakly in the blastocysts of all experimental groups. The variation in expression was high even among samples in individual groups. There was no correlation between culture environment and expression.

Discussion

The main findings of the present study were: (i) blastocysts produced after culture of *in vitro*-matured/*in vitro*-fertilized zygotes in ewe oviduct are of superior quality to

Fig. 3. Semi-quantitative RT-PCR of bovine β -actin. RT-PCRs were performed with the equivalent of one blastocyst from each group. Group 1: synthetic oviductal fluid (SOF) for 6 days; group 4: ewe oviduct for 2 days followed by culture in SOF for 4 days; group 5: ewe oviduct for 4 days followed by culture in SOF for 2 days; group 6: ewe oviduct for 6 days; and group 7: blastocysts were derived *in vivo* after superovulation and embryo recovery. The mean $\pm$ SD was obtained from three independent RT-PCRs from different batches of blastocysts. The values obtained for group 1 were set at 100% and those of other groups were expressed relative to this value. There were no significant differences between groups.

those produced after culture *in vitro*; (ii) such blastocysts show temporal sensitivity in terms of quality depending on both the duration and the timing of culture in an optimal (*in vivo*) or suboptimal (*in vitro*) environment; and (iii) the temporal sensitivities as manifested in terms of cryotolerance are reflected in the patterns of gene expression.

Comparative studies on the effects of various media constituents have generally focused on the percentage of embryos that develop to the blastocyst stage. Although it is reasonable to use such developmental parameters as markers of the efficacy of culture systems, it is likely that in some cases the effects of a given culture method may not manifest themselves during the relatively short period of *in vitro* culture, or that the effect may be on less tangible parameters than blastocyst yield, such as blastocyst quality. For this reason, we have combined measurement of developmental competence (cleavage and blastocyst development) with two qualitative measures, namely the ability of the blastocyst to withstand cryopreservation (Rizos *et al.*, 2002a,c, 2003) and the pattern of gene expression within the blastocyst (Rizos *et al.*, 2002b, 2003).

Fig. 4. Semi-quantitative RT-PCR of bovine *Bax*. RT-PCRs were performed with the equivalent of one blastocyst from each group. Group 1: synthetic oviductal fluid (SOF) for 6 days; group 4: ewe oviduct for 2 days followed by culture in SOF for 4 days; group 5: ewe oviduct for 4 days followed by culture in SOF for 2 days; group 6: ewe oviduct for 6 days; and group 7: blastocysts were derived *in vivo* after superovulation and embryo recovery. The mean $\pm$ SD was obtained from three independent RT-PCRs from different batches of blastocysts. The values for *Bax* were divided by the corresponding β -actin value (housekeeping gene, internal standard) and expressed relative to the highest sample signal set at 100%. ^{a-c}Bars with different superscripts are significantly different ($P < 0.05$).

In agreement with previous observations (Enright *et al.*, 2000; Rizos *et al.*, 2002a), there was no difference in blastocyst yield between culture in SOF for 6 days versus culture *in vivo* for 6 days. In terms of blastocyst quality, the results were also consistent with previous observations; culture in ewe oviduct for 6 days resulted in blastocysts of high quality with cryotolerance similar to that of *in vivo*-derived blastocysts. In contrast, culture in SOF for 6 days resulted in blastocysts of low cryotolerance, with $< 40\%$ surviving at 24 h after warming, $< 20\%$ surviving at 72 h and only a very low percentage hatching (3.2%).

The results of the present study indicate that bovine embryos show temporal sensitivity to the culture environment after fertilization, which is manifested in terms of the quality of the blastocysts produced. In terms of embryo quality, as measured by cryotolerance, blastocysts produced *in vivo* (control), as well as those produced after culture in oviduct for 6 days, had the highest rates of survival over 72 h after warming (100% survival at 24 h, $> 95\%$ survival at 72 h). The embryos that spent the last 4 days of culture *in vivo* also had relatively high rates of survival (100% at 24 h, 73.7% at 72 h). Consistent with previous observations, blastocysts produced entirely

Fig. 5. Semi-quantitative RT-PCR of bovine L37 ribosomal protein. RT-PCRs were performed with the equivalent of one blastocyst from each group. Group 1: synthetic oviductal fluid (SOF) for 6 days; group 4: ewe oviduct for 2 days followed by culture in SOF for 4 days; group 5: ewe oviduct for 4 days followed by culture in SOF for 2 days; group 6: ewe oviduct for 6 days; and group 7: blastocysts were derived *in vivo* after superovulation and embryo recovery. The mean $\pm$ SD was obtained from three independent RT-PCRs from different batches of blastocysts. The values for L37 were divided by the corresponding β -actin value (housekeeping gene, internal standard) and expressed relative to the highest sample signal set at 100%. There were no significant differences between groups.

in SOF had very low rates of survival after vitrification, with <40% viable at 24 h and <20% survival at 72 h. Blastocysts derived from embryos that spent the first 2 days *in vivo* and the last 4 days *in vitro* had the lowest rates of survival (6.7%), whereas those that spent the last 2 days only in SOF had intermediate rates of survival (40.6%). These data support the contention that some windows of embryo development are more predisposed to aberrant programming than others.

The data on cryotolerance were supported by observations of the relative abundance of gene transcripts in the blastocysts derived from the different groups. In particular, the relative abundance of transcripts for the cell death promoter, *Bax*, was significantly altered by modifying the culture environment. Consistent with previous observations (Rizos *et al.*, 2002b), the expression of *Bax* was significantly higher in blastocysts derived from SOF than in those derived from culture in ewe oviduct or entirely *in vivo*. The ranking of the relative abundance of this transcript followed the same pattern as the cryotolerance results. Gjorret *et al.* (2001) reported that apoptosis is more frequent in blastocysts produced

Fig. 6. Semi-quantitative RT-PCR of bovine S3a ribosomal protein. RT-PCRs were performed with the equivalent of one blastocyst from each group. Group 1: synthetic oviductal fluid (SOF) for 6 days; group 4: ewe oviduct for 2 days followed by culture in SOF for 4 days; group 5: ewe oviduct for 4 days followed by culture in SOF for 2 days; group 6: ewe oviduct for 6 days; and group 7: blastocysts were derived *in vivo* after superovulation and embryo recovery. The mean $\pm$ SD was obtained from three independent RT-PCRs from different batches of blastocysts. The values for S3a were divided by the corresponding β -actin value (housekeeping gene, internal standard) and expressed relative to the highest sample signal set at 100%. ^{ab}Bars with different superscripts are significantly different ($P < 0.05$).

in vitro than those produced *in vivo*. Consistent with these observations, a higher incidence of apoptosis has been reported in blastocysts produced *in vitro* derived from late-cleaving zygotes than in those that cleave earlier (Byrne *et al.*, 1999). Distortions of apoptosis in the blastocyst may lead to either early embryonic death or the formation of anomalies in the fetus that produce early abortions (Brill *et al.*, 1999).

In contrast, expression of mRNA for ribosomal protein L37 behaved as a housekeeping gene; no correlation was found between embryo culture environment and pattern of gene expression. This finding is perhaps not surprising as ribosomal protein L37 shows constitutive expression in various mouse embryonic tissues; L37 is expressed constitutively even during transition from quiescence to active cell proliferation or terminal differentiation (Su and Bird, 1995).

Ribosomal protein S3a is located at the 40S ribosomal subunit and is essential for initiation of translation (Westermann *et al.*, 1981; Cramton and Laski, 1994). There is increasing evidence that ribosomal proteins are capable of extra-ribosomal functions. In many tumour cells, in which expression of ribosomal protein S3a is much higher than in normal cells, apoptosis could be induced by inhibiting S3a expression (Naora *et al.*, 1998b).

In stably transfected cell lines, up- and downregulation of S3a expression, coordinated in sequence, can actively induce apoptosis (Naora *et al.*, 1998a). In bovine blastocysts produced in different culture environments, no correlation was found between S3a expression and treatment group. Although there were significant differences between some of the groups, these differences were not large and probably have no physiological importance.

There is some evidence in the literature to support the contention that embryos have temporal sensitivities to the culture environment. Although culture of ovine embryos with serum for 5 days resulted in oversized fetuses at day 125 of gestation, short-term exposure to serum either for the first 2 days or during the last 2 days of culture had no effect. Negrin-Pereira *et al.* (1997) performed an experiment involving culture for 2 days in either SOF plus serum or in co-culture, followed by culture for a further 3 days in the same or the alternative system. Their observations indicated that the developmental ability of the zygotes did not appear to be affected by the culture system used in the first 2 days, but the system used subsequently had a significant impact. In contrast, the results of the present study indicate that embryos cultured in SOF for 2 or 4 days before transfer to the sheep oviduct may be more sensitive to a change in their environment than those cultured *in vivo* for the initial 2 or 4 days and then cultured *in vitro*. This difference is difficult to explain but may relate to the vastly different culture environments in the two studies (ewe oviduct in this study versus co-culture in the study by Negrin Pereira *et al.*, 1997). In another study from the present authors' group, it was shown that blastocysts produced in co-culture with granulosa cells had a higher cryotolerance than did those produced in SOF plus serum (Rizos *et al.*, 2001); however, exposure to co-culture cells was necessary from day 3 onwards to acquire the improved cryotolerance.

Such evidence of temporal sensitivities of embryos has in part led to the production and commercialization of sequential media (Menezo *et al.*, 1998). In addition, in human assisted reproduction, there is a move away from the transfer of early (day 2–3) embryos towards the transfer of blastocysts to: (i) identify the more developmentally competent embryos, as the poorer ones will not reach this stage; and (ii) to allow the transfer of fewer embryos, resulting in fewer multiple pregnancies (Menezo *et al.*, 1998; Gardner *et al.*, 2000). As shown in the present study, modification of the culture environment or inadequate culture conditions during the period of culture immediately after fertilization can result in severe consequences in terms of the normality of the blastocysts.

In conclusion, the results of the present study indicate that the conditions of embryo culture can have a marked effect on the quality of the resulting blastocyst and that bovine embryos show clear temporal sensitivity to their culture environment. The reduced cryotolerance of blastocysts generated *in vitro* is accompanied by alteration in mRNA expression of the *Bax* gene.

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