Chapter 6

*In vitro* and *in vivo* culture effects on mRNA expression of genes involved in metabolism and apoptosis in bovine embryos
Abstract

Bovine blastocysts produced in vitro differ substantially from their in vivo-derived counterparts with regard to glucose metabolism, level of apoptosis and mRNA expression patterns. An aberrant maternal embryonic transition of transcriptional control due to deficient in vitro culture conditions could be involved in these differences. The aims of the present study were to identify the critical period of culture during which the differences in expression of gene transcripts involved in glucose metabolism are induced and to determine gene transcripts involved in apoptosis that are differentially expressed in in vitro- and in vivo-produced blastocysts. Relative abundances of glucose transporters Glut-1, Glut-3, Glut-4 and Glut-8 and transcripts involved in the apoptotic cascade BAX, BCL-XL, XIAP and HSP-70.1 were analyzed by a semi-quantitative RT-PCR assay in single blastocysts produced in vitro or in vivo for specific time intervals, i.e. prior to or after maternal embryonic transition. Culture environment in vitro vs. in vivo affected expression of glucose transporter transcripts, Glut-3, Glut-4 and Glut-8. However, the critical period during culture responsible for these changes, prior to or after maternal embryonic transition, could not be determined. Furthermore, no effect of culture regimen on the mRNA expression of BAX, BCL-XL and HSP 70.1 could be observed. These data show that expression of XIAP transcripts in expanded blastocysts is affected by in vitro culture. Our findings do not support the hypothesis that maternal embryonic transition is critical in inducing the aberrations in gene expression patterns studied here.

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Submitted for publication
Introduction

*In vitro*-produced bovine embryos differ from their *in vivo*-derived counterparts in numerous parameters, including morphology (Van Soom *et al.*, 1997), metabolism (Khurana and Niemann, 2000), tolerance of low temperatures (Leibo and Loskutoff, 1993), mRNA expression (Niemann and Wrenzycki, 2000) and *in vivo* development (van Wagendonk-de Leeuw *et al.*, 2000). Various studies reported deviations from the normal mRNA pattern in *in vitro*-produced bovine blastocysts versus their *in vivo* counterparts (Wrenzycki *et al.*, 1996; Lequarre *et al.*, 2001; Knijn *et al.*, 2002; Lazzari *et al.*, 2002; Rizos *et al.*, 2002a). However, it is unknown when precisely during *in vitro* culture these molecular deviations are induced. A recent study revealed that the maturation step during *in vitro* production probably is not the critical step and it is rather during the postfertilization period that these differences in quality between embryos cultured *in vitro* or *in vivo* are induced (Knijn *et al.*, 2002; Rizos *et al.*, 2002b).

The earliest stages of development are dependent on maternally-derived messages stored in the oocyte. As development progresses, maternal RNAs and proteins are depleted and embryo-derived mRNAs gain control of development. This shift from dependence on oocyte-derived to embryo-produced messages is referred to as maternal-to-embryonic transition (MET). The onset of MET has been investigated in embryos of a variety of species using α-amanitin, a specific RNA polymerase II inhibitor (for review see Telford *et al.*, 1990). In bovine preimplantation development, a minor embryonic genome activation has been described at the 2-cell stage (Hyttel *et al.*, 1996; Viuff *et al.*, 1996; Memili *et al.*, 1998). Addition of α-amanitin to the culture medium during the earliest stages of development blocks transcription, and in the absence of embryonic transcription the bovine embryos can only survive until the 8- to 16-cell stage (Telford *et al.*, 1990; Memili and First, 1998). An aberrant MET due to deficient *in vitro* culture conditions could be involved in molecular deviations frequently observed in *in vitro*-produced (IVP) blastocysts.

Concomitant with major embryonic genome activation, the metabolism switches from utilization of lactate and pyruvate to glucose as the main energy source (Rieger *et al.*, 1992; Khurana and Niemann, 2000). As the blastocyst forms, glucose consumption increases, requiring specific regulation of glucose transport by the early embryo. Glucose transport across the plasma membranes in mammalian cells is mediated by an active sodium-dependent transport and a passive energy-independent transport via facilitative glucose transporters (Glut). Glut-3 is thought to play a crucial role in the uptake of maternal glucose in the bovine (Augustin *et al.*, 2001) as well as in the mouse (Pantaleon *et al.*, 1997). In the mouse, Glut-8 is critical for blastocyst survival (Pinto *et al.*, 2002). Deviating mRNA expression patterns of Glut-1, Glut-3 and Glut-4 transcripts were found in *in vitro*-produced bovine embryos (Knijn *et al.*, 2002; Lazzari *et al.*, 2002).
A decreased glucose transport was associated with enhanced apoptosis most markedly at the blastocyst stage (Moley et al., 1998; Chi et al., 2000). The level of apoptosis in bovine blastocysts is affected by the culture environment and increased by suboptimal conditions (Gjørret et al., 2003; Knijn et al., 2003). The molecular basis of enhanced apoptosis in in vitro-produced blastocysts has not yet been studied in bovine embryos. Regulation of apoptosis depends upon a cascade comprising pro-apoptotic and anti-apoptotic proteins. The ratio between these proteins determines whether a cell survives or undergoes apoptosis (Oltvai et al., 1993; Wyllie, 1995). Messenger-RNA of BCL-XL, an anti-apoptotic protein, is present in mouse embryos at the 2-cell, 8-cell and blastocyst stages (Exley et al., 1999). Messenger-RNA transcripts for the pro-apoptotic protein BAX are found throughout preimplantation development in the mouse (Exley et al., 1999). Expression of BAX mRNA in bovine blastocysts was affected by the culture method (Rizos et al., 2002a). The extent of apoptosis also seems to be correlated with activity of the X–chromosome-linked Inhibitor of Apoptosis (XIAP) and Heat Shock Protein (HSP 70.1). This relationship has not yet been investigated for bovine embryos.

The goal of the present study was twofold: 1) to identify the critical period of culture during which the differences in expression of gene transcripts involved in glucose metabolism between in vitro- and in vivo-cultured bovine blastocysts are induced, and 2) to determine gene transcripts involved in apoptosis that are differentially expressed by the embryonic genome in in vitro- and in vivo-cultured bovine blastocysts.

**Materials and Methods**

*Experimental design*

The relative abundances of transcripts of the apoptosis-related genes BAX, BCL-XL, XIAP and HSP 70.1 transcripts were analyzed in early blastocysts, blastocysts and expanded blastocysts produced in vitro (In vitro group (1)) or in vivo either after slaughter (In vivo slaughter group (4)) or by non-surgical uterine flushing of the donor animals (In vivo flush group (5)). The relative abundance of the glucose transporter transcripts, Glut-1, Glut-3, Glut-4 and Glut-8 were analyzed in expanded blastocysts produced in vitro (1) or in vivo (5) collected after uterine non-surgical flushing. To identify the critical period of embryo culture in which differences in gene expression are induced, two categories of expanded blastocysts were analyzed, one in vivo developed until 45 h (45 h in vivo group (2)) and the other until after MET (100 h in vivo group (3)) followed by culture in vitro. A schematic drawing of the experimental design is shown in Figure 1.
To investigate if the specific gene transcripts are of maternal origin, the relative abundance of all gene transcripts were analyzed in 8- to 16-cell embryos cultured with or without \( \alpha \)-amanitin.

**Animal treatment**

Experiments were carried out as approved by the Ethical Committee of the Veterinary Faculty of Utrecht University.

Selected heifers were superovulated using a method with a fixed LH surge as described previously (Knijn et al., 2003). Briefly, animals (n=59) were pre-synchronized, and on Day 8 of the synchronized cycle (oestrus = Day 0) follicles larger than 5 mm were ablated by transvaginal ultrasound guided aspiration to synchronize follicle development. On Day 9, the heifers received an ear implant (3 mg norgestomet, Crestar; Intervet International BV, Boxmeer, The Netherlands) for 5 days, without additional administration of norgestomet and oestradiol-valerate. From Day 10 onwards, oFSH (Ovagen; ICP, Auckland, New Zealand) was administered i.m., twice daily with decreasing doses during 4 days (in total 10 mL equivalent to 176 IU NIH-FSH-S1). Prostaglandin (22.5 mg Prosolvin; Intervet International BV) was administered i.m. concomitant with the fifth dose of FSH. Fifty-five h later ear implants were removed and GnRH was administered (1.0 mg Fertagyl in 10 mL saline i.m.; Intervet International BV) to induce the LH surge. Heparinized blood samples were collected from the jugular vein daily during the experimental phase, every 3 h from 24 h after PG admini-
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medium consisting of synthetic oviduct fluid medium (SOF) with BSA as described earlier (van Wagendonk-de Leeuw et al., 2000; Knijn et al., 2003). A maximum of 10 collected embryos per heifer were placed in 20 µL droplets of SOF under oil (Mineral oil for IVF, Reproline Medical GmbH, Rheinback, Germany) at 39°C in humidified air of 5% CO₂, 7% O₂ and 88% N₂. Early blastocysts, blastocysts and expanded blastocysts were collected after 120 h of in vitro culture for the 45 h In vivo group (2) and 72 h for the 100 h In vivo group (3).

**Embryo culture, In vitro group (1)**
Cumulus oocyte complexes (COCs) were aspirated from 3 to 8 mm follicles of ovaries that had been collected at a local abattoir. Those oocytes with a multi-layered compact cumulus investment were used for the experiments. COCs were rinsed once with Hepes buffered M199 (Gibco BRL, Paisley, UK) supplemented with 10% (v/v) fetal calf serum (FCS; Gibco BRL) and once with maturation medium M199 supplemented with 10% FCS, 0.01IU/mL recombinant hFSH (Organon, Oss, The Netherlands), 11.36 µg/ml cysteamine (M-6500, Sigma) and 1% (v/v) penicillin-streptomycin (Gibco BRL). Groups of 35 oocytes were randomly allocated to each well of a 4-well culture plate (Nunc A/S, Roskilde, Denmark) containing 500 µL maturation medium with the above supplements. After maturation for 23 h at 39º C, 5% CO₂ in humidified air, all oocytes were fertilized in vitro.

Procedures for in vitro fertilization (IVF) were performed as described earlier (Parrish et al., 1988) with minor modifications (Izadyar et al., 1996), using semen from the same bull as used for the 45 h In vivo- (2) and 100 h In vivo (3) groups, In vivo slaughter group (4) and In vivo flush group (5). After 21 h of co-incubation (39ºC, 5% CO₂ in humidified air), presumptive zygotes were liberated from cumulus cells by vortexing for 3 min and a maximum of 10 zygotes were placed in 20 µL droplets of SOF and cultured as described above. All cleavage stages were transferred to fresh culture droplets at Day 5 after the start of fertilization. Early blastocysts, blastocysts and expanded blastocysts were collected 165 h after the start of fertilization.

**Total collection of blastocysts**
The number of blastocysts collected at Day 7 po were 73 in the In vitro group (1) (7 early blastocysts (eb), 16 blastocysts (b) and 50 expanded blastocysts (xb)); 86 in the 45 h In vivo group (2) (16 eb, 16 b and 51 xb); 90 in the 100 h In vivo group (3) (7 eb, 21 b and 62 xb); and 67 in the In vivo slaughter group (4) (17 eb, 17 b and 33 xb).
Inhibition of transcription from the embryonic genome
For inhibition of embryonic transcription, presumed zygotes were produced in vitro as described above in two replicates. After fertilization, presumed zygotes were randomly assigned to three groups of 50 each. The first group served as a control without α-amanitin. In the second group 10 mM α-amanitin was added to the culture medium from the start of culture onwards, 24 h after fertilization (d0 group). In the third group, 48 h after the start of fertilization the culture medium was replaced by medium supplemented with 10 mM α-amanitin (d1 group). At 96-100 h after the start of fertilization the embryos were scored and 8- to 16-cell embryos were collected. To ascertain whether the α-amanitin concentration terminated development at major genome activation, two groups of 50 zygotes were cultured, one supplemented with 10 mM α-amanitin at the start of culture and one group without α-amanitin, until Day 7 post-fertilization, and scored for embryonic development.

Storage of embryos
All collected embryos were washed four times in PBS with 0.1% (w/v) PVA and transferred to 0.5 ml Eppendorf vials in a minimum volume (≤ 5 µl) of PBS with 0.1% (w/v) PVA and frozen at -80°C. Analysis of cultured embryos was confined to those in morphological grades I and II (Robertson and Nelson, 1998).

Determination of relative mRNA abundance in individual embryos by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)
Poly (A)⁺ RNA from a single embryo was isolated as previously described (Wrenzycki et al., 1999). Prior to RNA isolation, 1 pg of rabbit globin RNA (Gibco BRL, Gaithersburg, MD) was added as an internal standard. Isolated Poly(A)⁺ RNA was used immediately for reverse transcription which was carried out in a total volume of 20 µl. The reaction mixture consisted of 1x RT buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, Perkin Elmer Biosystems, Vaterstetten, Germany), 5 mM MgCl₂, 1 mM of each dNTP (Amersham, Brunswick, Germany), 50 IU MuLV reverse transcriptase (Perkin Elmer). As negative controls, tubes without RNA samples were analyzed. The RT reaction was carried out in a PTC-200 thermocycler (MJ Research, Watertown, MA) for 10 min at 25°C, 60 min at 42°C, 5 min at 99°C and after finishing immediately put on ice. Immediately after reverse transcription the samples were subjected to polymerase chain reaction (PCR). PCR was performed with embryo equivalents as described in Table 1 and 50 fg of globin RNA (corresponding to 0.05 embryo equivalents) in a final volume of 50 µL, containing 1x PCR buffer (20 mM Tris-HCl, 50 mM KCl, pH 8.4, Life Technologies BV, Eggenstein, Germany), 1.5 mM MgCl₂, 200 µM of each dNTP and 1 µM of each sequence-specific primer (0.5 µM for globin primers). A "hot start" PCR was performed to obtain specific amplification. During the "hot start"
1 IU Taq DNA polymerase (Life Technologies BV) was added at 72°C. The sequences and positions of the primers used, the annealing temperature, the fragment sizes of the expected products, and the sequence references are summarized in Table 1.

Running a linear cycle series established the optimal cycle number at which the transcript was amplified exponentially for the PCR reaction. The PCR program started at 97°C for 2 min, followed by 72°C for 2 min (hot start) and was then followed by a number of cycles depending on the gene (Table 1) of 15 sec at 95°C for denaturation, 15 sec at the annealing temperature of the specific gene (Table 1) and 15 sec at 72°C for primer extension. After these cycles were finished a post dwell of 5 min at 72°C was done followed by cooling to 4°C. A PTC-200 thermocycler was used.

For detection and semi-quantification of RT-PCR products, electrophoresis was performed with 25 µL RT-PCR product that was loaded with 5 µL of 10x loading buffer (0.25% (w/v) xylene cyanol and 25 mM EDTA in 50% (w/v) glycerin). A 2% (w/v) agarose gel in TBE buffer (90 mM Tris, 90 mM borate, 2 mM EDTA, pH 8.3) with 0.2 µg/mL ethidium bromide was used. The concentration of ethidium bromide in the running buffer was the same as in the gel. After running at 100 V for 5 min and at 80 V for 40 min, the fragments were visualised on a 312 nm UV-transilluminator. A CCD camera (Quantix, Photometrics, Munich, Germany) and IP Lab spectrum (IP Lab Gel, Signal Analytics Corporation, Vienna, VA) were used to digitise the image of the gel. Densitometric scanning using a computer-assisted image analysis system (IP Lab Gel) quantified the signal intensity of each band. The relative abundance of the mRNA of the different genes was estimated by dividing the intensity of the band of the gene of interest by the intensity of the globin band from the same sample (Wrenzycki et al., 1999).

Four transcripts were analyzed per blastocyst with 10 to 16 replicates for the glucose transporter genes, 5 to 15 replicates for the apoptosis-related genes, with the exception of BCL-XL in eb of the In vitro group (1) and In vivo slaughter group (4), b in the In vivo slaughter group (4) and xb in the In vivo flush group (5), for which 2 to 4 replicates were analyzed due to the low number of available embryos. The 8- to 16-cell embryos from the α-amanitin experiment were pooled in groups of 3 from which 3 to 5 gene transcripts were analyzed in 4 to 8 replicates per gene.

Relative abundance was calculated on a per cell basis per embryo because the average number of cells per embryo is significantly different between in vitro- and in vivo-derived embryos and among different stages. The results of previous studies were used as a reference to estimate cell numbers in this study (Lazzari et al., 2002; Knijn et al., 2003)(Table 2).
Table 2. Average cell number of total embryos blastocyst stages at Day 7 po in relation to time of development in vivo and culture in vitro

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell number&lt;sup&gt;1&lt;/sup&gt;</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>early blastocyst</td>
<td>blastocyst</td>
<td>expanded blastocyst</td>
</tr>
<tr>
<td>In vitro*</td>
<td>106 ± 10 (3)</td>
<td>114 ± 14 (5)</td>
<td>160 ± 8 (23)</td>
</tr>
<tr>
<td>45 h In vivo*</td>
<td>n.a.***</td>
<td>n.a.</td>
<td>176 ± 8 (30)</td>
</tr>
<tr>
<td>100 h In vivo*</td>
<td>n.a.</td>
<td>n.a.</td>
<td>189 ± 8 (30)</td>
</tr>
<tr>
<td>In vivo*</td>
<td>118 ± 9 (8)</td>
<td>166 ± 11 (18)</td>
<td>181 ± 14 (13)</td>
</tr>
<tr>
<td>In vivo flush**</td>
<td>98 ± 6 (15)</td>
<td>121 ± 9 (15)</td>
<td>158 ± 46 (20)</td>
</tr>
</tbody>
</table>

<sup>1</sup> Values are expressed as the mean ± SEM. Numbers in parentheses are the number of embryos.

* Cell numbers were used from Knijn et al. (2003).

** Cell numbers of Lazarri et al. (2002) were used with results obtained from embryos of donor cows treated for superovulation with eCG.

*** n.a., Not applicable. These stadia were not used for analyses.

Sex determination
For the embryos that were analyzed for the relative abundance of XIAP, a sex determination was performed. DNA was collected from the supernatant of the RNA extraction and prepared using the Microcon YM-100 (42412; Millipore, Eschborn, Germany) columns according to the manufacturer's instructions. Embryonic sex was determined by PCR analysis using bovine-specific and Y-chromosome-specific primers (Table 1) as recently described (Wrenzycki et al., 2002).

Statistical analysis
Relative abundances were analyzed using the SigmaStat 2.0 (Jandel Scientific, San Rafael, CA) software package. After testing for normality (Kollogorov-Smirnov test with Lilliefors correction) and testing for equal variance (Levene Median test), ANOVA followed by multiple paired comparisons using the Tukey test were employed. Relative abundances for XIAP transcripts between male and female embryos were compared using Student t-test. Sex ratio was compared with the expected ratio of 50% using chi-square analysis. Data on the relative abundance are presented as mean ± SEM. Differences of P ≤ 0.05 were considered significant.
<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequence and positions</th>
<th>Annealing temp (°C)</th>
<th>No. cycles and embryo equivalent for blastocyst/8-16 c</th>
<th>Fragment size (bp)</th>
<th>Sequence references (EMBL accession no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Globin</strong></td>
<td>5' (241-260): GCAGCCACGGTGCGAGGTAT 3' (555-657): GTGGGACAGGAGCGTAAAT</td>
<td>60</td>
<td>27/27</td>
<td>257</td>
<td>Cheng et al., 1986 (X04751)</td>
</tr>
<tr>
<td><strong>Glucose transporter-3</strong></td>
<td>5' (1129-1152): CCTTGAGGGATGCTTTTGTTC 3' (1364-1387): CGTGCGCTGAGGGAAGAGCTGCCC</td>
<td>58</td>
<td>34/36</td>
<td>259</td>
<td>Bennett et al., 1995 (I39214)</td>
</tr>
<tr>
<td><strong>Glucose transporter-4</strong></td>
<td>5' (1663-1692): GGAGCGAGAGTGAAACCGACAGAAGACT 3' (1919-1939): CGTGCGCTGAGGGAAGAGCTGCCC</td>
<td>59</td>
<td>39/41</td>
<td>277</td>
<td>Abe et al., 1997 (D63150)</td>
</tr>
<tr>
<td><strong>Glucose transporter-8</strong></td>
<td>5' (184-204): CCTCGCTTCTGTCTGTCA 3' (408-428): CCTCTGTAGAAACTCTCC</td>
<td>58</td>
<td>36/38</td>
<td>244</td>
<td>Augustin et al., unpublished data</td>
</tr>
<tr>
<td><strong>BAX</strong></td>
<td>5' (72-96): CTTTTTGGTTCAGGGTGAGTTCTGCAAGT 3' (281-304): TCCACCGGCACTTTTAAAGGC</td>
<td>63</td>
<td>35/37</td>
<td>232</td>
<td>Reyes and Cockerell, 1996 (U92569)</td>
</tr>
<tr>
<td><strong>BCL-X long</strong></td>
<td>5' (47-72): ATGGAGCCACTGGCAGCAGAAG 3' (329-354): TCCATAACCACTAAGCCTGTTT</td>
<td>60</td>
<td>34/36</td>
<td>307</td>
<td>Amills and Bouzat, unpublished data</td>
</tr>
<tr>
<td><strong>XIAP</strong></td>
<td>5' (83-107): AATATGCAAAATGGAAGCTCTGTGCA 3' (288-314): CTCCCTGCCAGTTAAACTCTCC</td>
<td>52</td>
<td>35/37</td>
<td>229</td>
<td>Gutierrez-Adan et al., unpublished data</td>
</tr>
<tr>
<td><strong>Heat shock protein 70.1</strong></td>
<td>5' (1861-1890): AAGGTTGGCTGGACAGTGCCAGGGAGATGATT 3' (2319-2348): ACTTGGAGAATGAAACAGGAGAGT</td>
<td>59</td>
<td>36/38</td>
<td>488</td>
<td>Gutierrez and Guerrero, 1995 (U0961)</td>
</tr>
<tr>
<td><strong>Y-Chromosome specific</strong></td>
<td>5' CCTCCCCTGTCAACGCCGGCAGATTTCTT 3' TGGCTGACTTGCAGGAGGCGGAGGTTGGG</td>
<td>60</td>
<td>34</td>
<td>210</td>
<td>PCT WO 86/07095</td>
</tr>
<tr>
<td><strong>Bovine specific</strong></td>
<td>5' AGGTGGGATGGATGGTGCTAGTGTCTGAGAAG 3' AAGACCTCGAGAGACCCCTCTTCAACAGGT</td>
<td>60</td>
<td>34</td>
<td>300</td>
<td>PCT WO 86/07095</td>
</tr>
</tbody>
</table>
Results

Effects of in vivo development on relative abundance of genes involved in apoptosis and glucose metabolism

Apoptosis

Representative gel photographs of the semi-quantitative RT-PCR analysis in early blastocysts, blastocysts and expanded blastocysts of BAX, BCL-XL, and HSP 70.1 transcripts developed in vitro or in vivo (Figure 2A), for XIAP in male and female (Figure 2B) and PCR analysis of the sex of the embryo (Figure 2C) are shown in Figure 2.

No effects of in vitro vs. in vivo culture on the relative abundance of BAX and BCL-XL transcripts in eb, b and xb were observed (Figure 3). mRNA expression of HSP 70.1 transcripts was significantly higher in eb, b and xb in the in vivo slaughter group (4), collected after slaughter of the animals, than in the In vitro group (1) and in vivo flush group (5).

To ensure the validity of this finding and to exclude a possible effect of different superovulation procedures, we analyzed the relative abundance of HSP 70.1 in expanded blastocysts that were collected from animals after exactly the same superovulation treatment as used for the animals of the in vivo slaughter group (4), with the sole difference that embryos were collected by non-surgical uterine flushing. Embryos from cows treated with different superovulation protocols did not differ in HSP 70.1 mRNA levels when collected by non-surgical uterine flushing (data not shown).

Figure 2. Photographs of representative agarose gels from semi-quantitative RT-PCR analysis of A. transcripts of BAX, BCL-XL and HSP 70.1 B. transcripts of XIAP in male and female early blastocysts, blastocysts and expanded blastocysts and base pair ladder (bp) C. PCR analysis of the sex of the embryos.
Figure 3. Expression pattern of BAX-, BCL-XL- and HSP 70.1 transcripts in the *In vitro* group (1) (striped bars), *In vivo* slaughter group (4) (dark grey bars) and *In vivo* flush group (5) (black bars) in single early blastocysts, blastocysts and expanded blastocysts. Bars with different superscripts differ significantly.

In early blastocysts the expression patterns of XIAP transcripts were similar in males and females in the *In vitro* group (1) as well as in the *In vivo* flush group (5). In blastocysts, mRNA expression of XIAP was significantly higher in females in the *In vitro* group (1) as well as in the *In vivo* flush group (5). However, expression of XIAP transcripts in expanded blastocysts was different between *in vitro* vs. *in vivo* culture: the relative abundance of XIAP transcripts was similar in male and female *in vitro*-produced expanded blastocysts, but in the *in vivo*-developed embryos the expression of XIAP was significantly higher in female expanded blastocysts than in their male counterparts (Figure 4). Sex ratio in the eb, b and xb of the *In vitro* group (1) and the *In vivo* flush group (5) was similar (data not shown), which is in accordance with earlier studies using the same culture conditions (van Wagtendonk-de Leeuw et al., 2000).

Figure 4. Expression pattern of XIAP transcript in single male (striped bars) and female (black bars) early blastocysts, blastocysts and expanded blastocysts produced *in vitro* and *in vivo*. Bars with different superscripts differ significantly.
Effects of in vitro culture on gene expression in blastocysts

**Glucose metabolism**

A representative gel photograph of the semi-quantitative RT-PCR analysis of glucose transporter 1, 3, 4 and 8 transcripts in expanded blastocysts is shown in Figure 5.

![Gel Photograph](image)

**Figure 5.** Photograph of representative agarose gel from semi-quantitative RT-PCR analysis of transcripts of glucose transporters 1, 3, 4 and 8 in completely in vitro- (1) and in vivo- (5) developed expanded blastocysts.

*In vitro* culture affected mRNA abundance of Glut-3, Glut-4 and Glut-8 in expanded blastocysts (Figure 6). A significant difference for Glut-3, Glut-4 and Glut-8 was observed between *in vitro*-produced and *in vivo*-developed expanded blastocysts. The relative abundance of Glut-1, Glut-4 and Glut-8 transcripts in expanded blastocysts developed *in vivo* until 45 h (2) or 100 h (3) po and further cultured *in vitro* was not different from that of expanded blastocysts produced *in vitro* (1) or *in vivo* (5). Expression of Glut-3 transcripts was significantly higher in the 100 h *in vivo* group (3) compared to the 45 h *in vivo* group (2) and *in vivo* flush group (5).

**Inhibition of transcription from the embryonic genome**

At 96-100 h after the start of fertilization a total of 68, 70 and 54 8- to 16-cell embryos were collected for the control, d0 and d1 groups, respectively. No significant difference in the percentage of embryos developed until the 8- to 16-cell stage was observed between the control, d0 and d1 groups (68%, 70% and 54%, respectively). In the absence of α-amanitin, 28% of zygotes developed to blastocysts. In the presence of α-amanitin no embryo reached the morula/blastocyst stage.

Treatment with α-amanitin at the start of culture as well as 24 h thereafter inhibited transcription from BCL-XL, XIAP, HSP 70.1, Glut-1, Glut-3 and Glut-8.
However, mRNA level of BAX was not affected by treatment with α-amanitin (data not shown). No transcripts for Glut-4 were detectable in 8- to 16-cell embryos.

**Figure 6.** Expression pattern of glucose transporters transcripts in single expanded blastocysts after culture in vitro (1) (grey bars), embryos obtained at 45 h (2) (open bars) and 100 h (3) po (striped bars) from heifers stimulated for superovulation and cultured in vitro until the expanded blastocyst stage, and expanded blastocysts, from heifers stimulated for superovulation, developed entirely in vivo (5) (black bars) and collected by non-surgical uterine flushing. Bars with different superscripts differ significantly.

**Discussion**

The present study investigated the effect of specific periods of in vitro and in vivo culture on mRNA expression of genes involved in apoptosis and glucose metabolism. Expression of gene transcripts involved in apoptosis, BAX, BCL-XL and HSP 70.1 was not affected by in vitro vs. in vivo culture. However, the relative abundance of HSP 70.1 transcripts was altered due to the collection protocol. In in vitro-produced expanded blastocysts the X-chromosome seems to be inactivated prematurely, suggesting that a low mRNA expression of XIAP could possibly be associated with higher levels of apoptosis. Although in vitro culture affected expression of glucose transporter genes, the critical period during which culture induced differential expression could not yet be determined.

The difference previously found in the level of apoptosis between in vitro- vs. in vivo-developed embryos suggests a difference in expression of genes involved in the apoptotic cascade (Gjørret et al., 2003b; Knijn et al., 2003). However, no effect of in vitro vs. in vivo culture on expression of apoptosis-related genes BAX and BCL-XL was observed in this study. A higher BAX expression in blastocysts produced in SOF medium compared to blastocysts developed in vivo has been reported (Rizos et al., 2002a). This could be due to supplementation of the
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culture medium with fetal calf serum. It is known that serum affects the mRNA expression of several genes and thereby affects the quality of embryos (Wrenzycki et al., 1999; Rizos et al., 2003). Messenger-RNA of HSP 70.1 transcripts was increased in all blastocyst stages collected ex-vivo at Day 7. This is in contrast to previous studies in which no differences between blastocysts derived \textit{in vitro} in serum-free medium or \textit{in vivo} were detected (Wrenzycki et al., 2001; Lazzari et al., 2002). By adding a group in which the embryos underwent the same treatment as the \textit{in vivo} slaughter group (4), but the embryos were collected by non-surgical uterine flushing, we conclude that the differences in HSP 70.1 mRNA expression are caused by the collection protocol. This finding confirms that HSP 70.1 is a sensitive indicator of embryonic stress (Wrenzycki et al., 1999) and indicates that even minor manipulations of embryos can lead to alterations in relative abundances.

Expression of XIAP transcripts was higher in female blastocysts irrespective of the production method. Recently it was shown that XIAP mRNA expression was increased in female bovine blastocysts produced \textit{in vitro} compared to their male counterparts (Jimenez et al., 2003). Here, we show that no dosage compensation is observed in \textit{in vivo}-produced female expanded blastocysts. Speculatively, the premature inactivation of the X-chromosome in \textit{in vitro}-produced expanded blastocysts is related to the increased level of apoptosis in \textit{in vitro}-produced expanded blastocysts (Gjørret et al., 2003b). For other X-linked gene transcripts a delay or lack of dosage compensation by \textit{in vitro} culture has been reported (Wrenzycki et al., 2002).

This study has shown that in bovine expanded blastocysts, expression of gene transcripts involved in glucose metabolism is affected by \textit{in vitro} culture. For the first time, it was demonstrated that Glut-8 is actively transcribed by \textit{in vivo}-developed expanded blastocysts and is up-regulated, similar to Glut-3 and Glut-4 transcripts by \textit{in vitro} culture. The post-fertilization period appears critical in determining the quality of the embryo (Knijn et al., 2002), and we hypothesized that the MET \textit{in vitro} vs. \textit{vivo} is crucial for regulating gene transcripts. However, expression of Gluts in embryos derived from the 45 h \textit{in vivo} group (2) and the 100 h \textit{in vivo} group (3) were not significantly different from the \textit{in vivo} group (1) and from the \textit{in vivo} flush group (5), with the exception of Glut-3. This finding suggests that MET \textit{in vitro} or \textit{in vivo} is not likely to be involved in aberrant mRNA expression patterns induced by suboptimum culture conditions. The high expression of Glut-3 transcript in the 100 h group could be due to the shift from \textit{in vivo} to \textit{in vitro} environment as Glut-3 is supposed to be a sensitive marker for embryo environment (Lazzari et al., 2002). In contrast, expression of Glut-1 was not affected by the production method, which is in contrast to results of our earlier study (Knijn et al., 2002) and could be due to the use of embryos of different developmental stages. In the mouse, expression of Glut-8 appears simultaneously with expression of the IGF-1 receptor and coincides with
movement of the embryo from the oviduct to the uterus where 10-fold higher concentrations of IGF-1 and insulin are found (Pinto et al., 2002). A reduced Glut-8 expression, induced by Glut-8 antisense, was associated with increased apoptosis in murine blastocysts and poor pregnancy outcome (Pinto et al., 2002) suggesting that the overall decrease in glucose transport is responsible for apoptosis in early embryos. Possibly, regulation of Glut-8 may be an additional anti-apoptotic mechanism in the blastocyst to maximize glucose utilization at this critical turning point in development, when the embryo switches from using pyruvate as its main energy substrate to using glucose (Wales, 1986; Carayannopoulos et al., 2000).

The α-amanitin treatment effectively blocked embryonic development. Furthermore, it blocked expression of Glut-1, Glut-3, Glut-8, BCL-XL, XIAP and HSP-70.1 transcripts from the embryonic genome and all maternally-derived transcripts were degraded at the 8- to 16-cell stage. These gene transcripts are of embryonic origin and transcribed after MET. The exception was that expression of BAX mRNA was not decreased in 8- to 16-cell stage embryos after treatment with α-amanitin. It is possible that maternally-derived BAX transcripts are stable and not yet degraded in the 8- to 16-cell stage embryo. A possible mechanism to stabilize maternal transcripts was detected in Drosophila where expression of HSP 70.1 seems to be regulated at the level of transcript elongation rather than binding of RNA polymerase II to the promoter (Gilmour and Lis, 1986), and this might hold true for BAX as well.

In conclusion, results of the present study did not show effect of culture regimen on the mRNA expression of BAX, BCL-XL and HSP 70.1. Expression of HSP 70.1 transcripts was up-regulated by the collection protocol. Expression of XIAP transcripts in expanded blastocysts seems to be affected by in vitro culture, suggesting that this could be one of the reasons for increased apoptosis. Culture environment in vitro vs. in vivo affected expression of glucose transporter transcripts, Glut-3, Glut-4 and Glut-8; however, the critical period during culture responsible for this, prior to or after MET, could not yet be determined.

Acknowledgments

We are grateful to Christine Oei, Thea Blankenstein, Henk Heuveling, Klaus-Gerd Hadeler and Doris Herrmann for excellent technical assistance, and to animal handlers for management of the animals. The critical reading of the manuscript by Ms Jane Collins, Boston is highly appreciated. The authors thank Holland Genetics (Arnhem, The Netherlands) for supplying SOF culture medium. Netherlands Organization for Scientific Research supported this study.