Effects of culture system and protein supplementation on mRNA expression in pre-implantation bovine embryos

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Assisted reproduction technologies have made great progress during the last 15 years in most mammalian species, including humans. Growing evidence indicates that bovine pre-implantation development is a superior model for investigating early human development than the mouse. The purpose of this study was to investigate the effects of two basic culture systems [tissue culture medium (TCM) with 5% CO2 in air or synthetic oviduct fluid (SOF) with 7% O2, 88% N2, 5% CO2] and various protein supplements (serum, bovine serum albumin or polyvinyl alcohol) on the relative abundance of a set of developmentally important gene transcripts in bovine morulae and blastocysts and to compare the results with those for their in-vivo-derived counterparts. The basic culture system including the basic medium composition and oxygen tension had profound effects on the amounts of specific transcripts in bovine embryos, whereas the ‘protein source’ had only weak effects. Significant differences (P ≤ 0.05) in the relative abundance of specific gene transcripts were detected between in-vivo and in-vitro-derived embryos, especially at the morula stage. More differences were found between embryos produced in the TCM system and in-vivo-derived embryos than between SOF-generated embryos and their in-vivo counterparts. No differences were found in the relative abundance of gene transcripts in embryos generated under chemically defined conditions in the two different laboratories. It is concluded that the SOF system provides an environment in which pre-implantation development of bovine embryos is more similar to that occurring in vivo than in the TCM system.

Key words: bovine/culture system/gene expression/pre-implantation embryo/protein supplementation

Introduction

Reproductive biotechnologies have made great progress during the last 15 years. In-vitro production (IVP) of bovine embryos has emerged as a reliable alternative method to conventional ovulation induction techniques and an important tool to study pre-implantation embryo development (Bavister, 1995). In humans, the number of patients undergoing assisted reproduction technologies has increased significantly since the first successful IVF (Ménézo et al., 2000). Little research has focused on the human embryo, and most of the employed culture media are analogous with those in other mammalian species, mostly the mouse. However, there is growing evidence that the bovine is a better model for the human embryo, i.e. with regard to timing of genome activation, intermediate metabolism, and interaction with the culture medium (Andriesz et al., 2000; Ménézo et al., 2000; Neuber and Powers, 2000; Niemann and Wrenzycki, 2000).

A variety of embryo culture systems has been developed for the production of viable embryos (Bavister, 1995; Ménézo et al., 2000). Usually the basic culture media are supplemented with serum or bovine serum albumin (BSA), which are complex undefined mixtures (Gardner and Lane, 1993) and result in highly variable developmental rates. These problems make comparisons between different laboratories difficult (Bavister, 1995). To circumvent these problems and to standardize culture media, the ‘undefined’ components are replaced by synthetic macromolecules, such as polyvinyl alcohol (PVA), resulting in chemically defined media (cattle: Pinyopumminr and Bavister, 1991; Eckert and Niemann, 1995; Keskintepe and Brackett, 1996; human: Desai et al., 1997; Gardner et al., 1998; Jones et al., 1998). In human assisted reproduction technologies, the composition of an improved culture medium has recently gained greater interest for the prolonged culture of human embryos to the blastocyst stage. Transfer of blastocyst stages increases pregnancy rates and avoids multiple pregnancies as fewer embryos need to be transferred per patient (Gardner and Lane, 1997; Bavister and Boatman, 1999; Ali et al., 2000).

However, despite the recent improvements in bovine IVP, in-vitro generated embryos display a number of marked differences compared to their in-vivo counterparts (for review see Thompson, 1997; Holm and Callesen, 1998; Niemann and Wrenzycki, 2000). Moreover, extended in-vitro culture seems to be associated with the ‘large calf syndrome’ (Kruip and
denDaas, 1997). It has been postulated that persistent alterations from the normal expression pattern of developmentally important genes contribute to the phenomenon (Nieman and Wrenzycki, 2000).

Indeed, differences in the expression pattern of developmentally important genes have been determined between in-vivo- and in-vitro-derived bovine embryos (Wrenzycki et al., 1996, 1998; Eckert and Niemann, 1998). Recently, it has been reported that the quantitative expression pattern of developmentally important genes in pre-implantation bovine embryos is altered by the presence of serum in tissue culture medium (TCM)199 as basic culture medium (Wrenzycki et al., 1999).

This widely used IVP system has been replaced more and more by the SOF system (synthetic oviduct fluid; Tervit et al., 1972). The effects of different culture systems on mRNA expression in bovine embryos have not been previously investigated.

Here we report for the first time the effects of two different culture systems containing either serum, BSA or PVA on the relative abundance of a set of developmentally important gene transcripts involved in compaction and cavitation: gap junction protein connexin43 (Cx43), desmosomal protein plakophilin (Plako), desmosomal glycoproteins, desmocollins II and III (Dc II, III), cell adhesion protein E-cadherin (E-cad), tight junction protein zonula occludens (ZO-1); metabolism: glucose transporter-1 (Glut-1); RNA processing: poly(A) polymerase (PolyA); stress: heat shock protein 70.1 (Hsp); and maternal recognition of pregnancy: interferon tau (IF τ) in bovine morulae and blastocysts employing a semi-quantitative reverse transcription–polymerase chain reaction (RT–PCR) assay. The relative abundance of these transcripts in the in-vitro generated embryos was compared with that of in-vivo produced counterparts. Furthermore, the relative abundance of this set of marker genes was analysed in embryos grown under defined conditions, but generated in two different laboratories. The results of these experiments could serve as a model for studying the effects of culture conditions on genomic activities of human embryos.

Materials and methods

**IVP of bovine embryos**

Bovine embryos were produced as described recently (Wrenzycki et al., 1999). Briefly, ovaries from a local slaughterhouse were transported in Dulbecco’s phosphate buffered saline (PBS, number D6650; Sigma Chemical Co., St Louis, MO, USA) at 25–30°C to the laboratory. Cumulus–oocyte complexes (COC) were isolated via slicing (Eckert and Niemann, 1995). Category I COC (with a homogeneous evenly granulated cytoplasm possessing at least three layers of compact cumulus-cells) and category II COC (with less than three layers of cumulus cells or partially denuded but also with a homogeneous evenly granulated cytoplasm; Pavlok et al., 1992) were pooled in TCM air [TCM 199 containing l-glutamine and 25 mmol/l HEPES (Sigma) supplemented with 22 µg/ml pyruvate, 350 µg/ml NaHCO₃, 50 µg/ml gentamicin and either 10% heat-inactivated (30 min at 56°C) oestrous cow serum (OCS, collected on the first day of standing oestrus), 0.1% BSA (fraction V, number A9647, Sigma) or 0.1% PVA (polyvinyl alcohol, number P8136, Sigma)].

For oocyte maturation in vitro, TCM 199 containing l-glutamine and 25 mmol/l HEPES served as basic medium. One ml was supplemented with 22 µg pyruvate, 2.2 mg NaHCO₃, 50 µg gentamicin, 1 µg oestradiol-17β (Serva, Heidelberg, Germany), 0.5 µg FSH (Folltropin®, Vetephrum, London, Ontario, Canada), 0.06 IU human chorionic gonadotrophin (HCG) (Ekluton®, Vemie, Kempen, Germany) and either 10% OCS, 0.1% BSA (fraction V) or 0.1% PVA. COC were divided in groups of 20–25, transferred into 100 µl maturation drops under silicone oil and cultivated in a humidified atmosphere composed of 5% CO₂ in air at 39°C for 24 h.

Following in-vitro maturation, COC were rinsed in fertilization medium (fert-TALP supplemented with 6 mg/ml BSA) and fertilized in fert-TALP containing 10 µmol/l hypotaurine (Sigma), 1 µmol/l epinephrine (Sigma), 0.1 IU/ml heparin (Serva) [HHE] and 6 mg/ml BSA. Frozen semen from one bull with proven fertility in IVF was used. For IVF, semen was prepared by the modified ‘swim-up’ procedure (Parrish et al., 1986, 1988). Briefly, semen was thawed in a waterbath at 37°C for 1 min. After swim-up separation in sperm-TALP containing 6 mg/ml BSA for 1 h, the semen was washed twice by centrifugation at 350 g and 36°C for 10 min before being resuspended in fert-TALP supplemented with HHE and BSA. The final sperm concentration added per fertilization drop was 1×10⁶ spermatozoa/ml. Fertilization was initiated during a 19 h coincubation under the same temperature and gas conditions as described for maturation.

For in-vitro culture, two different basic culture media, either TCM199 or SOF, were employed. Each was supplemented with either 10% OCS, 0.1% (TCM: fraction V) or 0.8% (SOF: fatty acid free (FAF)-BSA, number A7030, Sigma) BSA or 0.1% PVA. Presumptive zygotes were transferred into 200 µl drops of TCM199 or 30 µl of SOF. For culture in SOF all of the adhering cumulus cells were removed by repeated pipetting. Culture in TCM199 was maintained in a humidified atmosphere composed of 5% CO₂ in air at 39°C, whereas for culture in SOF 7% O₂, 88% N₂ and 5% CO₂ (Air Products, Hattingen, Germany) in modular incubator chambers (ICN Biomedicals Inc., Aurora, OH, USA) were used. No differences were detected between the developmental rates and the transcriptional parts. Furthermore, the relative abundance of this set of marker genes in bovine embryos has not been previously investigated.

Here we report for the first time the effects of two different culture systems containing either serum, BSA or PVA on the relative abundance of a set of developmentally important gene transcripts involved in compaction and cavitation: gap junction protein connexin43 (Cx43), desmosomal protein plakophilin (Plako), desmosomal glycoproteins, desmocollins II and III (Dc II, III), cell adhesion protein E-cadherin (E-cad), tight junction protein zonula occludens (ZO-1); metabolism: glucose transporter-1 (Glut-1); RNA processing: poly(A) polymerase (PolyA); stress: heat shock protein 70.1 (Hsp); and maternal recognition of pregnancy: interferon tau (IF τ) in bovine morulae and blastocysts employing a semi-quantitative reverse transcription–polymerase chain reaction (RT–PCR) assay. The relative abundance of these transcripts in the in-vitro generated embryos was compared with that of in-vivo produced counterparts. Furthermore, the relative abundance of this set of marker genes was analysed in embryos grown under defined conditions, but generated in two different laboratories. The results of these experiments could serve as a model for studying the effects of culture conditions on genomic activities of human embryos.

**Production of bovine embryos in vivo**

Holstein Friesian donor cows were superovulated with a single i.m. injection of 3000 IU pregnant mares’ serum gonadotrophin (Intergonan®, Intervet, Tönisvorst, Germany) between days 9 and 13 of the oestrous cycle followed by Cloprostenol (Estrumate®, Essex, Munich, Germany) 48 h later. When oestrus was detected 48 h later,
the donors were inseminated twice at an interval of 12 h with semen from the same bull as used for IVF. On day 7 after insemination, morula and blastocyst stages were recovered by non-surgical flushing of the uterine horns with 300 ml PBS plus 1% newborn calf serum (NBCS; Serva) employing established procedures. Only embryos with morphological grades I and II (Robertson and Nelson, 1998) were included in this study. They were handled as described above.

**Determination of the relative abundance of developmentally important gene transcripts in bovine embryos**

Poly(A)^+RNA was isolated from pools of embryos (20–30) as described recently (Wrenzycki et al., 1999) and was used immediately for reverse transcription (RT) which was carried out in a total volume of 20 µl using 2.5 µmol/l random hexamers (Perkin–Elmer, Vaterstetten, Germany) to get the widest array of cDNA. The reaction mixture consisted of 1×RT buffer (50 mmol/l KCl, 10 mmol/l Tris-HCl, pH 8.3, Perkin–Elmer), 5 mmol/l MgCl₂, 1 mmol/l of each dNTP (Amersham, Brunwick, Germany), 20 IU RNase inhibitor (Perkin–Elmer) and 50 IU MuLV reverse transcriptase (Perkin–Elmer). The mixture was overlaid with mineral oil to prevent evaporation. The RT reaction was carried out at 25°C for 10 min, 42°C for 1 h followed by a denaturation step at 99°C for 5 min and flash cooling on ice. The reaction mixture was diluted to get a final concentration of 0.5 embryo equivalents/µl and 50 fg globin RNA/µl. Usually PCR was performed with cDNA equivalents corresponding to two embryos with the exception of poly(A) poly-merase/E-cadherin (one embryo equivalent) and plakophilin (four embryo equivalents) from different pools of embryos generated in different IVF runs as well as 50 fg of globin RNA in a final volume of 100 µl of 1×PCR buffer (20 mmol/l Tris-HCl, pH 8.4, 50 mmol/l KCl, Gibco BRL, Eggenstein, Germany), 1.5 mmol/l MgCl₂, 200 µmol/l of each dNTP, 1 µmol/l of each sequence specific primer (globin: 0.5 µmol/l) using a PTC-200 thermocycler (MJ Research, Watertown, MA, USA). To ensure specific amplification, a ‘hot start’ PCR was employed by adding 1 IU Tag DNA polymerase (Gibco) at 72°C. PCR primers were designed from the coding regions of each gene sequence using the Oligo® program (Plymouth, MN, USA). The sequences and positions of the primers used, the annealing temperatures, the fragment sizes and the sequence references from most of the expected PCR products have been published recently (Wrenzycki et al., 1999). The primer pairs to detect transcripts of the E-cadherin gene [forward primer (position 1486–1515): 5’-CTCAAGCTCGCCG-ATAACCGAACAAGAC-3’; reverse primer (position 1785–1814): 5’-CTTGTATTGAGCCAGCTGCAAGGGCCT-3’] and the ZO-1 gene [forward primer (position 5922–5921): 5’-CACAGTTTGGC-ACAGCCTCTGAGTTTGAC-3’; reverse primer (position 6350–6379): 5’-TAAAACCTGCCAAAACAGTGCTTCCCAGCC-3’] were designed from the mouse (Ringwald et al., 1987; accession number X06339) and the human sequence (Willott et al., 1992; accession number L14837), resulting in fragments of 332 bp and 524 bp. In size employing an annealing temperature of either 55°C or 64°C respectively. The E-cadherin fragment shares a homology of 86% with the mouse sequence and the ZO-1 amplicons show a homology of 81%, 81% and 82% with the human sequence. Whether the three fragments found for ZO-1 are new splice variants of this gene as detected in canine cells (Gonzalez-Mariscal et al., 1999) warrants further experiments.

The PCR programme employed an initial step of 97°C for 2 min and 72°C for 2 min (hot start) followed by 30 cycles (globin: 27 cycles) of 15 s each at 95°C for DNA denaturation, 15 s at different temperatures for annealing of primers, and 15 s at 72°C for primer extension. The last cycle was followed by a 5 min extension at 72°C and cooling to 4°C.

As negative controls, tubes were always prepared in which RNA or reverse transcriptase was omitted during the RT reaction (data not shown).

The RT–PCR products were subjected to electrophoresis on a 2% agarose gel in 1×TBE buffer (90 mmol/l Tris, 90 mmol/l borate, 2 mmol/l EDTA, pH 8.3) containing 0.2 μg/ml ethidium bromide. Further ethidium bromide in the same concentration was added to the running buffer. The image of each gel was recorded using a CCD camera (Quantix®, Photometrics, München, Germany) and the IP Lab Spectrum programme (Signal Analytics Corporation, Vienna, VA, USA). The intensity of each band was assessed by densitometry using an image analysis programme (IP Lab Gel). The relative amount of the mRNA of interest was calculated by dividing the intensity of the band for each developmental stage by the intensity of the globin band for the corresponding stage. Experiments were repeated with at least five separate embryo batches each derived from different IVF runs for each mRNA.

For each pair of gene-specific primers, semilog plots of the fragment intensity as a function of cycle number were used to determine the range of cycle number over which linear amplification occurred and the number of PCR cycles was kept within this range (Wrenzycki et al., 1999). The linear range was observed from 25–33 amplification cycles (data not shown). The fixed cycle number was used to prove that the amount of added RNA provided a proportional output of RT–PCR product (data not shown). Since the total efficiency of amplification for each set of primers during each cycle is not known, such an assay can only be used to compare relative abundance of one mRNA among different samples (Temeles et al., 1994).

The RNA recovery rate was estimated as the ratio between the intensity of the globin band with and without RNA preparation procedure, starting with an equivalent of 50 fg in the PCR reaction. On average, 46% of poly(A)-tailed RNA was recovered using our Dynabead oligo(dT) mRNA isolation method which corresponded well with other published yields (Shim et al., 1996). After optimization, the RT–PCR assay was sensitive enough to detect specific bovine mRNA from tongue epithelium at amounts from 0.5–5 ng of total RNA and from 0.5 blastocyst equivalents (data not shown). This corresponds well with the total amount of RNA typically found in bovine oocytes and blastocysts (2.4–5.3 ng; Bilodeau-Goezeels and Schultz, 1997).

The increase in the amounts of a PCR product could not be attributed to an increased length of the poly(A) tail which results in higher efficiency of reverse transcription (Temeles and Schultz, 1997) as in our protocol the RT reaction was primed with random hexamers. Furthermore, the use of random hexamer primers rather than oligo dT minimizes the effects of sequence complexity, mRNA secondary structure and distance of the primer sequences from the poly(A) tail (Noonan and Roninson, 1988). In addition, the use of random hexamers has been demonstrated to increase the sensitivity of the reaction by 2.4-fold (Bernardi et al., 1996). Considerable variation had been found between bovine and human single oocytes or embryos with regard to mRNA expression (DeSouza et al., 1998). Recently, it has been shown that mRNA patterns can differ even among single blastomeres from the same embryo (Kruttszel et al., 1998). To eliminate this variability pools of embryos were used in these experiments.

**Experimental design**

In the first experiment the relative abundance of 10 specific gene transcripts in bovine morulae and blastocysts produced either in vitro or in vivo were compared. Factors compared in the in-vitro embryos were two different culture systems containing either serum, BSA or PVA. To gain insight into potential laboratory variability with respect to transcript levels, in-vitro matured and fertilized embryos cultured in the SOF system containing PVA (defined, simple culture system), but generated in the laboratory at UGA (Keskiintepe and Brackett, 1996;
significantly increased in serum-supplemented medium compared to those supplemented with BSA and PVA. In addition, a significantly higher proportion of blastocysts was found in medium supplemented with BSA than with PVA [OCS: 30.1 ± 1.9% (424/1415) versus BSA: 23.9 ± 1.2% (348/1479) versus PVA: 15.9 ± 1.2% (403/2486) P < 0.05].

Relative abundance of 10 specific gene transcripts in bovine IVP embryos compared to their in-vivo counterparts

Representative gel photos of mRNA expression in bovine blastocysts cultured in either TCM199 (A) or SOF (C), each supplemented with serum compared to in-vivo-derived counterparts (B), are shown in Figure 2.

Experiment 1

The relative abundance of gene transcripts analysed in bovine morulae and blastocysts is summarized in Figures 3 and 4. Figures 3A and 4A only show transcripts for which significant differences related to the basic culture medium were found, whereas significant differences due to the protein source are summarized in Figures 3B and 4B. At the morula stage, the relative abundance of Hsp and Dc III transcripts was significantly (P < 0.05) higher in embryos cultured in the TCM system than in embryos cultured in the SOF system or generated in vivo. No significant differences were found between the SOF and in-vivo-derived embryos. For Dc II, Plako, Glut-1 and E-cad transcripts, significantly (P < 0.05) higher relative levels were detected for in-vivo-generated embryos than in the two culture systems (Figure 3A). Furthermore, for in-vivo-derived morulae a significantly (P < 0.05) higher relative abundance of Glut-1 and Plako transcripts was observed compared with all three different supplementations (Figure 3B).

At the blastocyst stage, the number of differences decreased as compared with morulae. The relative abundance of Hsp transcripts was significantly increased (P ≤ 0.05) in TCM-derived embryos compared to their SOF- and in-vivo-generated counterparts. The relative amount of Plako transcripts was significantly higher (P ≤ 0.05) in in-vivo generated blastocysts than in the two culture groups, whereas the relative amount of IF τ was significantly lower (P ≤ 0.05) in the in-vivo group compared to both culture groups (Figure 4A). At the blastocyst stage, protein supplementation was shown to affect only two out of the 10 analysed genes in this study. Embryos grown in the presence of serum displayed a significantly higher (P ≤ 0.05) level of Hsp transcripts than their in-vivo-generated counterparts.

Results

Developmental rates in vitro

The developmental rates of bovine embryos generated in the two different systems (either TCM or SOF), each supplemented with either serum, BSA or PVA are summarized in Figure 1. No significant differences were detected for the two basic culture media with regard to cleavage rate [TCM: 71.1 ± 1.6% (1028/1442) versus SOF: 65.4 ± 2.7% (732/1120), not significant (NS)], developmental rates to the morula [TCM: 30.8 ± 2.3% (1272/4199) versus SOF: 31.5 ± 3.4% (488/1593) NS] and blastocyst [TCM: 21.7 ± 1.8% (758/3496) versus SOF: 21.5 ± 2.0% (417/1884) NS] stage. Furthermore, no significant difference was found for the cleavage rate in terms of protein supplementation [OCS: 71.5 ± 5.3% (535/727) versus BSA: 66.4 ± 2.9% (505/738) versus PVA: 65.0 ± 2.3% (720/1097) NS]. However, a significantly higher percentage of morulae was obtained in serum-enriched medium than in medium supplemented with BSA and PVA [OCS: 41.0 ± 2.7% (722/1800) versus BSA: 30.1 ± 1.2% (528/1771) versus PVA: 23.2 ± 2.2% (510/2221) P < 0.05]. Development to the blastocyst stage was also significantly increased in serum-supplemented medium compared to those supplemented with BSA and PVA. In addition, a significantly higher proportion of blastocysts was found in medium supplemented with BSA than with PVA [OCS: 30.1 ± 1.9% (424/1415) versus BSA: 23.9 ± 1.2% (348/1479) versus PVA: 15.9 ± 1.2% (403/2486) P < 0.05].

Statistical analysis

Data were analysed using the SigmaStat 2.0 (Jandel Scientific, San Rafael, CA, USA) software package. After testing for normality (Kolmogorov–Smirnov test with Lilliefors correction) and testing for equal variance (Levene median test) parametric analysis of differences in the means between two or more populations were tested using analysis of variance (ANOVA) with the main effects being basic culture system and protein supplementation and their interactions followed by multiple pairwise comparisons using Tukey’s test (experiment 1). An ANOVA followed by multiple pairwise comparisons using Tukey’s test was employed to determine differences between embryos generated in two different laboratories (experiment 2). Differences of P ≤ 0.05 were considered to be significant.
Figure 2. Representative gel photographs of a semi-quantitative reverse transcription–polymerase chain reaction (RT–PCR) analysis of developmentally important gene transcripts in vitro [employing TCM (Figure 2A) or SOF (Figure 2C), both supplemented with serum] or in vivo (Figure 2B) produced bovine blastocysts. Each lane represents the RT–PCR product derived from polyA⁺ RNA from the equivalent of two embryos [poly(A) polymerase, polyA and E-cadherin, E-cad: one embryo]. The RNA recovery rate was estimated as the ratio between the intensity of the fragment with (globin/H11001 prep) and without preparation (~ prep) procedure, starting with an equivalent of 50 fg in the PCR reaction. The bands presenting Dc III as well as Cx43 mRNA did not reproduce well as only weak signals were obtained.

Hsp = heat shock protein 70.1, Glut-1 = glucose transporter-1, Dc II and III = desmocollins II and III, Cx43 = gap junction protein connexin43, IF τ = interferon tau, Plako = desmosomal protein plakophilin, ZO-1 = zonula occludens.

counterparts. This phenomenon was also observed when comparing BSA- and PVA-generated embryos with the in-vivo ones. The relative abundance of IF τ transcripts was significantly increased (P ≤ 0.05) in serum-and PVA-derived blastocysts compared to in-vivo generated embryos (Figure 4B).

Experiment 2
Morulae and blastocysts generated in the two different laboratories employing a largely identical IVP system did not reveal any significant differences in the relative abundance of the developmentally important gene transcripts tested in this study. However, significant differences (P < 0.05) were consistently found in the relative amount of Glut-1 and Dc II transcripts at the morula stage and Hsp, IF τ and Plako transcripts at the blastocyst stage between in-vitro generated embryos and their in-vivo generated counterparts (Figure 5).

Discussion
With the availability of a highly sensitive technology, such as RT–PCR, and a functional IVP system, it is now feasible to analyse gene expression in detail in species other than the mouse and to obtain more information on the control of gene expression during pre-implantation development. Results of this study demonstrate for the first time that it is predominantly the basic culture system that has profound effects on the relative abundance of a set of marker genes and that it is more important than the various supplements with regard to effects on amounts of transcript of early bovine embryos. In addition, evidence is provided that a chemically defined simple medium can eliminate laboratory variability at the mRNA level. The basic culture media tested in the present study are widely used for bovine IVP and differ in essential parameters (Bavister, 1995). TCM belongs to the so-called ‘complex’ media and was designed for cell culture in general, whereas SOF is a ‘simple’ medium, specifically designed for the demands of the pre-implantation embryo (Tervit et al., 1972). Similar approaches have been used to formulate media for the in-vitro culture of human embryos up to the blastocyst stage and these media have resulted in improved embryo development (Desai et al., 1997; Gardner et al., 1998; Jones et al., 1998).

The data presented here indicate that despite a similar rate of development in the two basic culture systems profound differences existed at the transcriptional level. The morula stage obviously was more susceptible to these alterations and could potentially serve as a sensitive indicator when testing culture media in their suitability for in-vitro production of pre-implantation embryos. On the other hand, results of this study and others (Bavister, 1995) show that serum supplementation leads to a higher proportion of blastocyst stages. The current findings question the commonly used criterion to evaluate the efficiency of a specific IVP system by the ‘maximum number of blastocysts’ and should encourage implementation of a more suitable parameter to characterize the quality of embryos generated in a specific culture system. Interestingly, in this
study no differences were found in embryos generated in two different laboratories employing the same chemically defined, simple culture system despite the use of an in-vitro maturation (IVM) and IVF system with minor differences. This indicates that culture of embryos in a defined system can reduce the variability normally found when embryos are cultured in the presence of different serum supplements.

Determination of the relative abundance of developmentally important gene transcripts is a relatively new tool to assess the developmental competence and viability of oocytes and embryos. The set of marker genes analysed in the present study characterizes several important physiological compartments and functions indicative for compaction and cavitating, metabolism, RNA processing, stress and maternal recognition of pregnancy. The novel finding of this study was that at the morula stage, the basic culture system was shown to exert strong effects on mRNA expression patterns, with more genes being differentially expressed between TCM- and in-vivo- than SOF- and in-vivo-derived embryos. However, most of the differences were considerably diminished at the blastocyst stage. Thus it seems that the culture system itself is a more important determinant of the physiological expression pattern than the protein source. However, it cannot be ruled out that other gene transcripts could respond in a different manner. Embryos generated in the SOF system seem to be more similar to their in-vivo counterparts with regard to gene expression patterns than those generated in the TCM system. This may be attributed to the reduced O2 tension of the SOF system via the reduction of deleterious effects of reactive oxygen species on early development (Johnson and Nasr-Esfahani, 1994). A similar low oxygen tension has been found in the genital tract of mammalian species (Fischer and Bavister, 1993). Culture of human oocytes and embryos for the first 2 or 3 days of development under either 20% or 5% O2 concentrations revealed no significant differences between the two groups in rates of fertilization, pregnancy and implantation. However, culture under 5% O2 resulted in a significantly higher blastocyst rate and a higher cell number of these blastocysts (Dumoulin et al., 1999). The current results support the findings that environmental stresses, such as free oxygen radicals, induce expression of specific genes such as Hsp (Edwards and Hansen, 1996, 1997; Edwards et al., 1997). Hsp transcripts were strongly increased in embryos grown in TCM or in the presence of serum. The transcriptional activity of Hsp70.1 was 15 times higher in in-vitro than in in-vivo murine embryos (Christians et al., 1995), indicating that Hsp is a sensitive indicator of stress caused by suboptimal culture conditions. Similarly, Hsp protein was also up-regulated in in-vitro-produced bovine blastocysts following heat shock (Kawarsky and King, 1998).

Developmental stage and quality of bovine embryos were shown significantly to affect the expression of IFτ (Hernandez-Ledezma et al., 1993). A negative relationship was found between early IFτ production and developmental competence (Kubisch et al., 1998). Results of the present

Figure 3. Effects of the basic culture system [either TCM (black bars) or SOF (open bars)] on relative mRNA abundance (mean ± SEM) in bovine morulae compared to their in-vivo (lined bars)-generated counterparts (A) or supplements [either oestrous cow serum (OCS) (black bars), BSA (grey bars), PVA (open bars) or in-vivo (lined bars)-produced embryos, B]. Bars with different superscripts within each gene transcript differ significantly (a, b, c: P ≤ 0.05).
study show that IF \( \tau \) transcription is significantly increased \((P \leq 0.05)\) in in-vitro-generated blastocysts compared with in-vivo-derived embryos. This finding was independent of the culture system or protein source and supports the hypothesis that a high amount of IF \( \tau \) mRNA is an indicator of poor quality of the bovine embryo.

With regard to the other genes whose expression was investigated in this study, Glut-1 mRNA was significantly reduced in in-vitro-produced embryos compared to those generated \textit{in vivo}. Similar findings were reported for mouse embryos (Morita \textit{et al.}, 1994). Expression of genes involved in compaction and cavitation was higher in in-vivo-generated embryos compared to in-vitro-produced ones. This supports the previous observation that compaction may be less pronounced in in-vitro-produced morulae (Prather and First, 1993).

The present findings could contribute to the understanding of the large offspring syndrome (Kruip and DenDaas, 1997). However, the suggestion that persistent alterations of the normal gene expression pattern are responsible for this phenomenon has not been yet verified. The observed deviations from the normal mRNA expression patterns seen for IVP embryos may reflect epigenetic changes specifically in the pattern of methylation, as has recently been demonstrated for the murine H19 gene (Doherty \textit{et al.}, 2000; Niemann and Wrenzycki, 2000). Aberrant expression patterns of imprinted genes have been implicated in embryonic and fetal abnormalities (Moore and Reik, 1996) and are associated with various human diseases (Tesarik and Mendoza, 1996). Presumably, current in-vitro culture systems can lead to either persistent silencing or enhanced expression of a particular gene throughout critical phases of fetal development. One possible mechanism could be that in-vitro culture conditions alter the rate of degradation of mRNA and the degraded products affect methylation and thus expression. Another intriguing possibility is that culture conditions induce the expression of cell death genes as shown for murine, human and bovine embryos (Hardy \textit{et al.}, 1989; Jurisicova \textit{et al.}, 1998; Byrne \textit{et al.}, 1999) and thus increase early embryonic and fetal losses (Niemann and Wrenzycki, 2000).

**Figure 4.** Effects of basic culture system [either TCM (black bars) or SOF (open bars)] on relative mRNA abundance (mean ± SEM) in bovine blastocysts compared to their in-vivo (lined bars) counterparts (Figure 4A) or supplements [either OCS (black bars), BSA (grey bars), PVA (open bars) or in-vivo-produced (lined bars) embryos, Figure 4B]. Bars with different superscripts within each gene transcript differ significantly (a, b, c: \(P \leq 0.05\)).

**Figure 5.** Detection of gene transcripts in bovine morulae and blastocysts generated in two different laboratories (laboratory 1: black bars, laboratory 2: open bars) employing similar embryo culture systems (SOF medium supplemented with PVA) and in-vivo-generated ones (lined bars). Values are shown as mean ± SEM. Bars with different superscripts within each gene transcript differ significantly (a, b, c: \(P \leq 0.05\)).
Human fetal development can similarly be affected by IVF procedures, i.e. the incidence of babies that are too small for their gestational age was significantly greater in singleton IVF pregnancies than in the normal obstetric population (Wang et al., 1994; Tanbo et al., 1995). Therefore, the findings of this study might also have strong implications for the application of human assisted reproduction technologies. Basic research needs to be carried out to gain a better understanding of the underlying physiological mechanisms of assisted reproduction technologies and to ensure the delivery of normal and healthy offspring free of long-term side-effects (Benagiano and Rowe, 1995; Ménézo and Dale, 1995; Seamark and Robinson, 1995; Walker et al., 1996). For pre-implantation mouse embryos it was shown that an optimized culture system is correlated with an mRNA expression pattern that is identical to that found in the in-vivo-derived counterparts (Ho et al., 1994, 1995). Recent technical advances allow the simultaneous monitoring of thousands of genes via cDNA microassays. This technology has already found clinical application in the diagnosis of various forms of cancer (Alizadeh et al., 2000), for the characterization of embryonic stem cells (Kelly and Rizzino, 2000) or auto-immune diseases (Rogge et al., 2000) and should soon also be available for pre-implantation embryos.

In conclusion, it was shown in this study that the SOF system provides a better environment for pre-implantation development of bovine embryos than the TCM system. However, the differences still found between SOF- and in-vivo-derived embryos emphasize the need for further research to optimize in-vitro culture systems for bovine (and human) embryos to ensure normal fetal development and offspring. The results of this study may also have strong implications for the application of assisted reproductive technologies in humans and could serve as a model for studying the effects of culture conditions on the transcriptional activities of human embryos.

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