Developmental and molecular correlates of bovine preimplantation embryos

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Abstract

Expression of embryonic genes is altered in different culture conditions, which influence developmental potential both during preimplantation and fetal development. The objective of this study was to define the effects of culture conditions on: bovine embryonic development to blastocyst stage, blastocyst cell number, apoptosis and expression patterns of a panel of developmentally important genes. Bovine embryos were cultured in vitro in three culture media containing amino acids, namely potassium simplex optimization medium (KSOMaa), Charles Rosenkrans 1 (CR1aa) and synthetic oviductal fluid (SOFaa). Apoptosis in blastocysts was determined by TUNEL assay and expression profiles of developmentally important genes were assayed by real-time PCR. In vivo-produced bovine blastocysts were used as controls for experiments determining gene expression patterns. While the cleavage rates did not differ, embryos cultured in SOFaa had higher rates of development to blastocyst stage \( (P < 0.05) \). Mean cell numbers and percentages of apoptotic cells per blastocyst did not differ among the groups. Expression of the heat shock protein 70 (Hsp70) gene was significantly up-regulated in both CR1aa and KSOMaa when compared with SOFaa \( (P < 0.001) \). DNA methyltransferase 3a (Dnmt3a) expression was higher in embryos cultured in CR1aa than in those cultured in SOFaa \( (P < 0.001) \). Expression of interferon tau (Ifn-t) and insulin-like growth factor II receptor (Igf-2r) genes was significantly up-regulated in KSOMaa when compared with CR1aa \( (P < 0.001) \). Gene expression did not differ between in vivo-derived blastocysts and their in vitro-derived counterparts. In conclusion, SOFaa supports higher development to blastocyst stage than KSOMaa and CR1aa, and the culture conditions influence gene expression.


Introduction

Cattle are among the most economically important species for US agriculture. Both the dairy and beef industry rely heavily on a healthy reproductive system for more efficient product generation. In vitro maturation of bovine oocytes followed by in vitro fertilization (IVF) and culture for the production of cows around the world is rapidly increasing. Thousands of IVF-generated embryos are transferred to recipient cows annually to generate offspring of higher genetic merit. In addition to providing an inexpensive and readily available source of preimplantation bovine embryos, these methods have significant value in studying the basic biological events occurring during oocyte maturation, fertilization and early embryonic development.

Preimplantation-stage embryos can develop in different media whose compositions range from simple balanced salt solutions and carbohydrates (e.g. Charles Rosenkrans 1 (CR1), synthetic oviductal fluid (SOF) and potassium simplex optimization medium (KSOM)) to very complex constituents, such as tissue culture medium (TCM)-199, with further supplementation of serum and/or a feeder layer of somatic cells (Krischer et al. 1999, Niemann & Wrenzycki 2000, Summers & Biggers 2003). Major developmentally important events take place during development of embryos from post-fertilization to the blastocyst stage. These include zygote formation, first cleavage division, embryonic genome activation (EGA), compaction of the morulae (compacted embryos with more than 16 blastomeres) and blastocyst formation (Lonergan et al. 2003). Early embryonic development after fertilization depends heavily on stored maternal mRNAs and translated proteins in mature oocytes. EGA is an essential event initiating as early as the one-cell zygotic stage in the cow and...
increasing gradually as embryonic development advances. In the absence of proper EGA, the developing embryo dies because it can no longer support essential developmental functions (Memili & First 1998, 1999, Latham & Schultz 2001). It was shown that although the primary factor influencing the percentages of immature oocytes developing to the blastocyst stage was the quality of oocytes itself, the most important step of the embryo production system affecting the quality of blastocysts, determined in terms of the resistance against cryopreservation and relative abundance of gene transcripts, was the post-fertilization culture conditions (Lonergan et al. 2003, Rinaudo & Schultz 2004, Wrenzycki et al. 2004). Therefore, a suboptimal in vitro culture environment can seriously affect the developmental potential of in vitro-produced embryos. Furthermore, culture conditions have been previously shown to change the expression patterns of a number of genes during mammalian embryogenesis (Wrenzycki et al. 2001, Lazzari et al. 2002, Rizos et al. 2002, Rinaudo & Schultz 2004). The number of genes expressed at the blastocyst stage is limited as compared to >25 × 10³ genes expressed in the adult. However, the genes that are expressed during the blastocyst stage are expected to have vital roles in the development to the blastocyst stage and beyond. This is because a number of critical events take place during early embryogenesis. This stage consists of more than just dividing the cytoplasm, changing length of cell cycles and replicating DNA until the first cell differentiation occurs (Latham & Schultz 2001, Hamatani et al. 2004). In fact, early embryogenesis is as important as the other phases of developmental biology since the embryo dies if the EGA fails to occur properly. Available data demonstrate that the expression of various gene transcripts differs between embryos developed in vivo or in vitro (Knijn et al. 2002, Lonergan et al. 2003, Gutierrez-Adan et al. 2004). Altered expression of embryonic genes in different culture conditions may influence the developmental potential both during preimplantation and fetal development (Fernandez-Gonzalez et al. 2004). In vivo-produced embryos are the most reliable control group when comparing gene expression patterns in developing embryos since any in vitro embryo production system is not optimized enough to mimic in vivo conditions (Niemann & Wrenzycki 2000).

The effects of culture media gene expression in bovine blastocysts have been studied by Wrenzycki et al. (2001) and by Tesfaye et al. (2004). However, the genes studied in these reports were different, and the studies did not compare gene expression profiles in parallel culture media. The experiments proposed here are aimed at comparing currently used embryo culture media containing amino acids (namely KSOMaa, CR1aa and SOFaa) on their ability to support embryonic development as determined by comparing the rates of embryonic development (number of embryos reaching blastocyst stage), mean cell number, percentages of apoptotic cells and the expression patterns of some developmentally important genes at the blastocyst stage. In the present study, we characterize expression of glucose transporter-1 (Glut-1), heat shock protein 70 (Hsp70), interferon tau (IF-τ), DNA methyltransferase 3a (Dnmt3a), desmosomal glycoprotein desmocollin III (Dclll), insulin-like growth factor II receptor (Igf-2r) relative to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). These chosen genes involve metabolism (Glut-1), stress (Hsp70), maternal recognition of pregnancy (IF-τ), DNA methylation (Dnmt3a), compaction and cavitation (Dclll), and growth factor signaling (Igf-2r). In the present study, in vivo control blastocysts were only used for determining the expression patterns of a panel of developmentally important genes.

Materials and Methods

All the chemicals used in the present study were purchased from Sigma unless otherwise stated.

In vivo blastocysts

Bovine in vivo blastocysts frozen in 0.25 ml straws (one blastocyst/straw) were provided by North American Breeders, Inc. (Berryville, VA, USA). Straws were immersed in a water bath at 36°C for 15 s. After thawing, the contents of the straws were poured into PBS solution containing 5% glycerol and 0.55 M sucrose and kept for 3 min. Blastocysts were then collected and transferred into PBS solution containing 0.55 M sucrose for 3 min. Blastocysts were transferred into TL-HEPES. Then, groups of ten blastocysts were rinsed three times in saline and transferred into 500 μl centrifuge tubes with a minimum amount of saline. Samples were quickly frozen and kept at −80°C for RNA isolation.

In vitro maturation (IVM)

Bovine oocytes were collected from 2–8 mm follicles of bovine ovaries obtained from a local abattoir; collection was carried out by aspiration using an 18-gauge needle attached to a vacuum system. Only oocytes with several layers of cumulus cells and homogenous cytoplasm were selected for use in the present study. Oocytes were washed three times in TL-HEPES and matured in tissue culture medium (TCM) -199, Gibco/Invitrogen) supplemented with 0.2 mM pyruvate, 0.5 μg/ml follicle-stimulating hormone (FSH; Sioux Biochemicals, Sioux City, IA, USA), 5 μg/ml luteinizing hormone (LH; Sioux Biochemicals), 10% fetal calf serum (FCS, Gibco/Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco/Invitrogen). Ten oocytes were matured in 50 μl maturation drops covered with mineral oil for 24 h at 39°C in a humidified incubator with 5% CO₂. This culture condition was the same for IVF and in vitro culture (IVC).
**IVF**

Matured oocytes were washed twice in TL-HEPES and groups of ten oocytes were transferred to 44 μl fertilization drops under mineral oil. The fertilization medium was glucose-free TALP supplemented with 0.2 mM pyruvate, 6 mg/ml fatty acid-free BSA (BSA-FAF), 100 U/ml penicillin and 100 μg/ml streptomycin. Frozen sperm from a previously tested bull was used for the fertilization of in vitro-matured oocytes. A density gradient system using Percoll was used for the separation of live spermatozoa in frozen–thawed semen (Parrish et al. 1995). A straw of frozen sperm was thawed at 36 °C for 1 min, and then carefully layered on top of the Percoll gradient system. Sperm was diluted to 5 × 10⁶ sperm cells/ml in TL-HEPES, which produced 2 × 10⁶ spermatozoa/ml in fertilization drops. Fertilization of matured oocytes was completed by adding 2 μl diluted sperm, 2 μl of 5 μg/ml heparin and 2 μl PHE solution (20 μM penicillamine, 10 μM hypotaurine, 1 μM epinephrine) into the 44 μl fertilization drops. Oocytes and sperm were co-cultured for 18 h in the incubator (Leibfried & Bavister 1982).

**IVC**

Eighteen hours after insemination, cumulus cells were removed by vortexing the embryos in a 1.5 ml Eppendorf tube at high speed for 3 min. Twenty-five cumulus-free presumptive zygotes were transferred from 4 ml embryos were stored at 4 °C until the TUNEL assay. The embryos were then incubated at room temperature in a humidified chamber and washed twice in PBS. The embryos were then incubated with 100 μl DNase buffer for 5 min; 100 μl DNase buffer containing 20 U/ml DNase I were added to the embryos previously assigned as negative and positive controls, and 100 μl DNase buffer was added to the remaining embryos. All embryos were incubated at 37 °C for 30 min, and then washed four times in 100 μl double distilled water by transferring embryos from one drop to the next. Thereafter, the embryos were exposed to 100 μl equilibration buffer for 10 min. Subsequently, 51 μl of prepared rTdT reaction buffer (45 μl equilibration buffer, 5 μl nucleotide mix, 1 μl rTdT enzyme) were added to the ten embryos. For negative control slides, 1 μl autoclaved deionized water was used in place of rTdT enzyme. Embryos were incubated in the dark at 37 °C for 1 h. The reaction was completed by incubating the embryos in 2 × SSC solution for 15 min at room temperature and washing twice in PBS. The embryos were then incubated in 50 μg/ml of RNase A (Sigma) in 10 mM Tris–HCl (pH = 7.5) and 15 mM NaCl at 37 °C for 40 min. Finally, embryos were stained with 1 μg/ml propidium iodide for 15 min and washed with deionized water to eliminate extra stain. After the last wash, the embryos were mounted with a DABCO anti-fade solution and covered with a coverslip. Assessment of total and TUNEL-positive nuclei was determined by evaluating each embryo under an epifluorescent microscope (Nikon, Japan) equipped with a 450–490 nm excitation filter, a 520 nm dichroic mirror and a 520 nm barrier emission filter, using a × 40 objective. The apoptotic nuclei with fragmented DNA were stained yellowish green, while normal nuclei appeared orange-red in color.

**Table 1** Compositions of KSOMaa, CR1aa and SOFaa media in bovine embryo culture.

<table>
<thead>
<tr>
<th>Components</th>
<th>KSOMaaa</th>
<th>CR1aa</th>
<th>SOFaa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inorganic salts (mM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>94.97</td>
<td>114.60</td>
<td>107.70</td>
</tr>
<tr>
<td>KCl</td>
<td>2.50</td>
<td>3.08</td>
<td>7.16</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>25.00</td>
<td>26.18</td>
<td>25.07</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>0.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>1.71</td>
<td></td>
<td>1.17</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td></td>
<td></td>
<td>0.49</td>
</tr>
<tr>
<td>Other components</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na lactate (60% syrup; mM)</td>
<td>21.9</td>
<td>2.52</td>
<td>3.3</td>
</tr>
<tr>
<td>t-Lactate (mg/ml)</td>
<td>—</td>
<td>0.55</td>
<td>—</td>
</tr>
<tr>
<td>Na pyruvate (mM)</td>
<td>0.6</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>BSA (fatty acid-free) (mg/ml)</td>
<td>3.0</td>
<td>3.0</td>
<td>8.0</td>
</tr>
<tr>
<td>d-Glucose (mM)</td>
<td>0.2</td>
<td></td>
<td>—</td>
</tr>
<tr>
<td>EDTA (mg/ml)</td>
<td>3.0</td>
<td></td>
<td>—</td>
</tr>
<tr>
<td>Penicillin (µg/ml)</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Streptomycin (µg/ml)</td>
<td>50</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Amino acids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BME amino acid solution (µL/ml)</td>
<td>10</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>MEM non-amino acid solution (µL/ml)</td>
<td>20</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>t-Glutamine (mg/ml)</td>
<td>1.46</td>
<td>1.5</td>
<td>—</td>
</tr>
</tbody>
</table>

aFrom Specialty Media; bRosenkraus et al. (1993); cWithout t-glutamine.

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Molecular dynamics of bovine embryonic development

897

Isolation of total RNA and cDNA synthesis

Similar to in vivo blastocysts, in vitro-produced blastocysts were first transferred into TL-HEPES from culture media and groups of ten blastocysts were rinsed three times in 0.9% NaCl, and finally transferred into 500 µl centrifuge tubes with a minimal amount of saline. After transferring into centrifuge tubes, samples were immediately frozen and kept at −80°C until RNA isolation. Total RNA was isolated from a pool of ten in vivo- or in vitro-produced blastocysts using an RNeasy Micro Kit (Qiagen) according to the manufacturer’s instructions. The quality and quantity of total RNA isolated from blastocysts were estimated using a Bioanalyzer 2100 RNA 6000 picochip kit (Agilent, Palo Alto, CA, USA). Total RNA (8 ng) was used for cDNA synthesis using the first-strand cDNA synthesis kit for RT-PCR (AMV, Roche Applied Sciences, IN, USA) according to the manufacturer’s protocol. Samples were incubated for 10 min at 25°C, 60 min at 42°C and then at 99°C for 5 min.

Primer and Taqman probe design

All PCR primers and probes were designed using Primer Premier 5 software (Premier Biosoft International, Palo Alto, CA, USA). The primers and probes were carefully designed to avoid amplification of genomic DNA. The sequences and positions of the primers and TaqMan probes used, and the fragment size and the sequence references of the expected PCR products are shown in Table 2.

Table 2 Sequences of real-time PCR primers and TaqMan probes from bovine genome.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer and TaqMan probe sequences and positions (5′ → 3′)</th>
<th>Fragment size (bp)</th>
<th>Sequence references (accession no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose transporter-1 (Glut-1)</td>
<td>CCAAGGATCTCTCAGGAGGACAG (1688–1709) TCTTCTGGCAGCATCTGCTGG (1797–1776) FAM-GATAGATCTCGACAGAGGGGCTGCTT-TAMRA (1734–1758)</td>
<td>110</td>
<td>M60448</td>
</tr>
<tr>
<td>Interferon tau (IF-t)</td>
<td>TCCATGGAGATGCTGCTCGCATG (433–453) TGTTGAGGCCCACAGCA (535–517) FAM-AGCAGCTGCTGCTGCGACAGCA-TAMRA (475–498)</td>
<td>103</td>
<td>X65539</td>
</tr>
<tr>
<td>DNA methyltransferase 3a (Dnmt3a)</td>
<td>TGATCTTTCCATGCTCGAACCT (2062–2083) GAAAGGCGCAGGCTCCTCC (2185–2166) FAM-TGAGTTCACCGCAAGCAGGCA-TAMRA (2125–2150)</td>
<td>124</td>
<td>AY271298</td>
</tr>
<tr>
<td>Desmocollin III (DeIII)</td>
<td>CCTCTGTGATTGTACTAACCCCG (2006–2028) GAAGTATGGCAAGGAGGATCC (2078–2097) FAM-TGCTCGAGGGAGTGGGAGCGACCT-TAMRA (2042–2062)</td>
<td>92</td>
<td>L33774</td>
</tr>
<tr>
<td>Insulin-like growth factor II receptor (Igf-2r)</td>
<td>CAGGTCTTGCACAGTCCTGATGA (4935–4957) TCTTCAACGGAGGATCAGC (5071–5051) FAM-AGACGGGATGCTGCTGGAACGCA-TAMRA (4998–5022)</td>
<td>137</td>
<td>J03527</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)</td>
<td>AAGGCTACATACATCTTCAA (178–197) CCACATGATACGCACCGGAT (253–230) FAM-AGGGATCTGCAACACATCAAGG-TAMRA (200–225)</td>
<td>76</td>
<td>U85042</td>
</tr>
</tbody>
</table>

All of the primer sets for each gene were sequenced and confirmed.
Real-time quantitative PCR

Real-time quantitative PCR was performed to assess transcripts of Glut-1, Hsp70, IF-τ, Dnmt3a, DcIII and Igf-2r relative to the housekeeping gene GAPDH. Each cDNA sample was analyzed in duplicate using the LightCycler instrument (Roche). Quantitative assessment of RNA amplification was detected with the TaqMan probes, which are specific for the targeted genes. The TaqMan probe contains two dyes, a reporter and a quencher dye, and the cross point (Ct) values are constant in all in vitro and in vivo groups. A new software tool was used, named REST (relative expression software tool), and compared all samples of each group. The mathematical model used is based on the PCR efficiencies and the crossing point deviation between the samples (Pfaffl et al. 2002).

Statistical analysis

In vitro-embryo production experiments were repeated at least three times. Developmental rates to the different stages were determined from the number of fertilized oocytes. Mean developmental percentages of fertilized oocytes to various developmental stages, and mean cell numbers and mean percentages of apoptotic cells per blastocyst, were calculated as estimated marginal means using the SPSS statistical program (SPSS 10.0 for Windows; SPSS, Inc., Chicago, IL, USA). Significant differences were determined by one-way ANOVA using the SPSS program. In cases of more than two groups, ANOVA was followed by multiple pair-wise comparisons using Tukey’s test.

Results

Development of embryos in culture media

In the present study, a total of 538, 518 and 503 oocytes were used for KSOMaa, CR1aa and SOFaa groups respectively. Cleavage rates in KSOMaa, CR1aa and SOFaa groups were 79, 78 and 80% respectively; developmental rates to eight-cell, morulae and blastocyst stage are all summarized in Table 3. There was no statistical difference between groups until the blastocyst stage. The blastocyst development rate of the SOFaa group was significantly higher (P < 0.05) than those of KSOMaa and CR1aa groups (32.77 vs 22.67 and 23.08% respectively).

Cell numbers and apoptotic cell ratios

On day 8 of IVC, 19, 27 and 39 blastocysts were used to determine the mean cell numbers and the percentages of TUNEL-positive nuclei for the KSOMaa, CR1aa and SOFaa groups respectively. Mean cell numbers for KSO-Maa, CR1aa and SOFaa groups were 109.3, 101.0 and 90.1 respectively. Cleavage rates in KSOMaa, CR1aa and SOFaa groups were 109.3, 101.0 and 90.1 respectively; developmental rates to eight-cell, morulae and blastocyst stage are all summarized in Table 3. There was no statistical difference between groups until the blastocyst stage. The blastocyst development rate of the SOFaa group was significantly higher (P < 0.05) than those of KSOMaa and CR1aa groups (32.77 vs 22.67 and 23.08% respectively).

Table 3 Development of bovine embryos cultured in three different culture media.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of fertilized oocytes</th>
<th>Number of two cell embryos (%)</th>
<th>Number of eight cell embryos (%)</th>
<th>Number of morulae (%)</th>
<th>Number of blastocysts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KSOMaa</td>
<td>538</td>
<td>426 (79.15)</td>
<td>249 (46.65)</td>
<td>215 (39.92)</td>
<td>119 (22.16)</td>
</tr>
<tr>
<td>CR1aa</td>
<td>518</td>
<td>402 (77.53)</td>
<td>248 (48.31)</td>
<td>205 (39.88)</td>
<td>120 (23.41)</td>
</tr>
<tr>
<td>SOFaa</td>
<td>503</td>
<td>403 (80.16)</td>
<td>248 (50.17)</td>
<td>222 (42.28)</td>
<td>164 (32.85)</td>
</tr>
</tbody>
</table>

Values with different superscripts in the same column are significantly different (P < 0.05).
Expression patterns of some developmentally important genes

The Bioanalyzer gel image of the total RNAs for KSOMaa, CR1aa, SOFaa and in vivo control groups is shown in Fig. 1. The figure shows that the integrity of the total RNA was solid enough to proceed with further analysis of mRNAs. The dynamics of gene expression in different culture conditions is shown in Figs 2 and 3. The expression of the Hsp70 gene was significantly up-regulated in both CR1aa and KSOMaa when compared with SOFaa (P < 0.001) (Fig. 2A) while expression of the Dnmt3a gene was significantly up-regulated in CR1aa when compared with SOFaa (P < 0.001) (Fig. 2B). There was no significant difference between in vivo control and in vitro cultured blastocysts for any of the gene transcripts (Fig. 2C–F) determined in the present study. The expression of IF-τ and Igf-2r genes was significantly up-regulated in KSOMaa when compared with CR1aa (P < 0.001) (Fig. 3). There was no significant difference between CR1aa and KSOMaa for Glut-1, Hsp70, Dnmt3a and DclIII gene transcripts (Fig. 3).

Discussion

The preimplantation period of mammalian embryogenesis is notable for many critical events that affect the genome both before and after implantation. These include extensive changes in DNA methylation, changes in chromatin structure and histone acetylation–methylation, changes related to genomic imprinting and changes in the array of transcription factors, all of which, over the course of several cell divisions, lead to the establishment of a nuclear structure that is able to support embryogenesis and carry out the developmental program (Latham & Schultz 2001). It has been known that bovine early-stage embryos and blastocysts are transcriptionally active (Memili & First 1999), and embryo culture conditions influence gene expression (Wrenzycki et al. 2005). However, parallel studies focusing on the influence of the most commonly used culture media (CR1aa, KSOMaa and SOFaa) on gene expression in the same experiment have not been characterized.

In the present study, the development rate to blastocyst stage in SOF was significantly higher than that in CR1 and KSOM media (P < 0.05). However, the ingredients of these media differed slightly. While KSOM and CR1 have glutamine, SOF does not have it. There are recent reports investigating the inhibitory and teratological effects of glutamine on embryos (Devreker & Hardy 1997, Summers et al. 2005). Also, the concentration of BSA in SOF is higher than that in CR1 and KSOM. These differences could have an effect on the embryo development data in our study.

In an effort to achieve a higher development rate in bovine in vitro embryo production systems, researchers have used different in vitro culture strategies such as the use of somatic cell co-culture, cell-conditioned media and complex media containing serum (Bavister 1995). Recently, the use of somatic cell co-culture and cell-conditioned media has declined because of the extra work involved in cell culture production and the improvements in defined culture conditions. Culture media used in bovine in vitro embryo production have undergone dramatic improvement. Even though semi-defined or mostly defined culture media are used by some, they do not completely mimic in vivo conditions (Wrenzycki et al. 2005). The most commonly used culture media in bovine embryo production are SOFaa, KSOMaa and CR1aa. To our

Table 4 Mean cell numbers of day-8 bovine blastocysts developed in three different culture media.

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Mean cell number blastocyst (± S.D.)</th>
<th>%apoptotic cell number blastocyst (± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KSOMaa</td>
<td>19</td>
<td>109.3 ± 29.5</td>
<td>1.25 ± 1.0</td>
</tr>
<tr>
<td>CR1aa</td>
<td>27</td>
<td>101.0 ± 26.0</td>
<td>1.91 ± 1.9</td>
</tr>
<tr>
<td>SOFaa</td>
<td>39</td>
<td>114.0 ± 28.7</td>
<td>1.87 ± 1.7</td>
</tr>
</tbody>
</table>

Figure 1 Total RNA was isolated from pools of ten blastocysts developed in KSOMaa, CR1aa, SOFaa and in vivo. RNA (1 μl) was loaded for each lane in the Bioanalyzer 2100 RNA 6000 picochip (Agilent). Lane L, size markers; RNA from control blastocysts in KSOMaa (lane 1), CR1aa (lane 2), SOFaa (lane 3) and in vivo (lane 4). The ratio of the sharp bands of 28S and 18S ribosomal RNA below the 4000 and 2000 molecular weight markers, respectively, demonstrate the integrity of the total RNA isolated.
knowledge there are no studies comparing these three culture media side by side. Developmental data in the present study showed that SOFaa medium produced significantly higher numbers of blastocyst-stage embryos than the other two media ($P < 0.05$); even though there were no significant differences in developmental rates to two-cell, eight-cell and morulae stages among the three groups. TUNEL-positive nuclei of day-8 blastocysts derived from KSOMaa, CR1aa and SOFaa did not differ significantly (Table 4). However, other developmentally important genes could be affected.

Advances in the field of molecular biology have provided new ways to evaluate the quality of embryos (Wrenzycki et al. 2005). Determination of the expression patterns of genes relevant to early embryonic development provides an opportunity to assess the quality of the embryos produced in vitro and a chance to optimize in vitro embryo production systems. Real-time PCR is one of

![Figure 2](https://www.reproduction-online.org)

**Figure 2** Relative abundance of various developmentally important gene transcripts in bovine blastocysts obtained from SOFaa (solid bars), CR1aa (open bars), KSOMaa (gray bars) culture media and in vivo controls (stippled bars). Expression ratios of Hsp70, Dnmt3a, Glut-1, IF-$\tau$, DcIII and Igf-2r genes are demonstrated in panels A, B, C, D, E and F respectively. *Significant differences when compared with SOFaa ($P < 0.001$).

![Figure 3](https://www.reproduction-online.org)

**Figure 3** Relative abundance of IF-$\tau$, Igf-2r, Glut-1, Hsp70, Dnmt3a and DcIII gene transcripts in bovine blastocysts obtained from CR1aa (solid bars) and KSOMaa (open bars) culture media. *Significant differences when compared with CR1aa ($P < 0.001$).
the most reliable methods to compare some developmentally important gene transcripts (Gutierrez-Adan et al. 2004, Tesfaye et al. 2004, Miyazaki et al. 2005). The effects of in vitro culture conditions on gene expression have been mostly studied in later developmental stages such as morulae and blastocyst stages (Wrenzycki et al. 2005). Our study analyzed the expression of a panel of developmentally regulated genes in blastocysts cultured in KSOMaa, CR1aa and SOFaa, and supported the hypothesis that in vitro conditions affect the patterns of embryonic gene expression in bovine (Niemann & Wrenzycki 2000) and murine embryos (Ho et al. 1994, 1995). Others have reported that there are differences between the expression profiles of some specific genes in embryos derived in vitro and in vivo (Wrenzycki et al. 1996, Rizos et al. 2002, Lonergan et al. 2003). However, in the present study, there was no significant difference between the in vivo control group and in vitro groups (CR1aa, KSOMaa and SOFaa). In our study, frozen/thawed in vivo blastocysts were used. Freezing and thawing could potentially have an effect on the mRNA expression levels, and there is no comparative study examining alterations of mRNA amount in relation to freezing and thawing. However, we did not see any RNA degradation in our RNA samples. In addition, we checked transcript levels relative to the housekeeping gene GAPDH, and thus, we expect that the effects of freezing and thawing would not change our results. It has been reported that there was a significant difference between the metabolism pathways of in vitro-produced and in vivo-developed bovine embryos (Khurana & Niemann 2000). However, in the present study the expression of Glut-1 did not differ significantly among the four groups, which reflects the fact that this isoform might not play a crucial role in embryonic glucose uptake from the environment. It has been shown that environmental stressors, such as free oxygen radicals and increased temperature, induce gene expression prior to major activation of the gene expressions in bovine embryos (Edwards & Hansen 1996, 1997, Edwards et al. 1997). In the present study, the expression of the Hsp70 gene in CR1aa and KSOMaa culture media was significantly different from that in SOFaa (P < 0.001) (Fig. 2A). Our results indicated that the use of SOFaa culture media may create more sensitivity in response to heat shock.

It has been shown that while IF-α mRNA was found in blastocysts, it was absent in morulae (Hernandez-Ledezma et al. 1993, Wrenzycki et al. 1999). IF-α is exclusively secreted by the trophodermal cells of blastocysts and primarily functions as a factor responsible for maternal recognition of pregnancy in cattle (Roberts et al. 1992). It also plays a crucial role in placentaion. IF-α mRNA was expressed at higher levels in hatched blastocysts cultured in the presence of poly vinyl alcohol (PVA) than those cultured when serum was present (Wrenzycki et al. 1999). Similarly, Rizos et al. (2003) reported a significantly higher level of IF-α transcript in blastocysts produced in the absence of serum. It was hypothesized that deviation from normal placentation is one of the major causes of pregnancy loss after transferring in vitro-produced embryos (Hasler et al. 1995, Wells et al. 1999, Hill et al. 2000). It is commonly believed that a higher amount of IF-α mRNA is an indicator of poor-quality blastocysts (Wrenzycki et al. 2001). In the present study, the expression of the IF-α gene in KSOMaa culture media was significantly different from CR1aa (P < 0.001) (Fig. 3). Our results indicated that the bovine blastocysts cultured in KSOMaa might have higher implantation rates.

Methylation of DNA is an essential process in early embryonic mammalian development. It has important roles in regulation of transcription, X chromosome inactivation, cell differentiation and imprinting, and it usually causes gene silencing (Kaneda et al. 2004). The expression of the Dnmt3a gene in CR1aa was significantly different from that in SOFaa (P < 0.001) (Fig. 2B). The use of CR1aa culture media might affect global gene methylation, which may result in different transcription patterns in further development.

Desmosomal glycoprotein DcIII has a role in compaction and cavitation during blastocyst formation. In the present study, there was no significant difference in the expression of DcIII among the four groups. The use of different culture media had no effect on blastocyst formation. Expression of Igf-2r is detectable at all stages of embryonic development in bovine embryos (Watson et al. 1992, Yoshida et al. 1998, Yaseen et al. 2001). It has been postulated that insulin-like growth factors play a role as a survival factor in preimplantation bovine embryos (Yaseen et al. 2001). In the present study, the expression of the Igf-2r gene in KSOMaa culture media was significantly different from that in CR1aa (P < 0.001) (Fig. 3). This result indicates that the KSOMaa culture media has a positive effect on blastocyst development. In conclusion, SOFaa supports higher development to blastocyst stage than KSOMaa and CR1aa, and the in vitro culture conditions influence gene expression. The results of this study showed that the culture conditions alter the expression of a panel of genes that might affect their developmental potential. However, further studies are needed to examine genome-wide transcriptomes of embryos cultured in different media by DNA microarrays, and to study fetal development by embryo transfer into recipient cows.

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