ELSEVIER

Animal Reproduction Science 101 (2007) 225–240

ANIMAL REPRODUCTION SCIENCE

www.elsevier.com/locate/anireprosci

Developmental potential of bovine oocytes cultured in different maturation and culture conditions

Hakan Sagirkaya [a,1], Muge Misirlioglu [a], Abdullah Kaya [c], Neal L. First [b], John J. Parrish [c], Erdogan Memili [a,*]

[a] *Department of Animal & Dairy Sciences, Mississippi State University, 4025 Wise Center, Box 9815, MS 39762, USA*
[b] *Department of Biological Sciences, Mississippi State University, MS 39762, USA*
[c] *Department of Animal Sciences, University of Wisconsin-Madison, Madison, WI, USA*

Received 9 May 2006; accepted 7 September 2006
Available online 18 October 2006

Abstract

Diverse groups of chemicals in culture media are needed for successful bovine oocyte maturation and embryo development during which dramatic cytoplasmic and nuclear reprogramming events take place. In vitro embryo production (IVP) procedures frequently include supplements such as serum and/or co-culture with various types of somatic cells. However, the presence of undefined serum in culture media introduces a variation from batch to batch, increases viral or prion contamination risk, and leads to problems during fetal development. The aim of the present study was to investigate the possibility of using chemically defined-synthetic serum substitute (SSS) in place of fetal calf serum (FCS) during maturation and long-term culture to stimulate in vitro maturation (IVM), fertilization (IVF) and subsequent embryo development. In Experiment I, the effect of the protein source on in vitro maturation was tested by maturing oocytes in culture media supplemented with 10% FCS (Control Group), 10% SSS (Group I) and 10% SSS + 10 ng/ml epidermal growth factor (EGF) (Group II). In Experiment II, effects of SSS on both oocyte maturation and embryo development during in vitro culture (IVC) were tested by maturing oocytes in media supplemented with 10% FCS (FCS Group) or 10% SSS + 10 ng/ml EGF (SSS Group), followed by IVF and IVC in SOF media supplemented with 10% FCS and 10% SSS on day 4 for FCS and SSS Groups, respectively. Even though rates for cleavage and development to blastocyst stage were not different, blastocyst cell numbers were higher in Group II containing SSS and EGF. The SSS supplementation group had higher apoptotic nuclei as compared to the FCS Group in Experiment II. Transcripts for heat shock protein 70 (Hsp70), interferon tau (IF-τ), DNA methyltransferase 3a (Dnmt3a), desmosomal glycoprotein desmocollin III (DcIII) and insulin-like growth factor II receptor (Igf-2r) were altered in different culture conditions in Experiment I. However, only glucose transporter-1 (Glut-1) mRNA was different in the SSS and FCS Groups in the

* Corresponding author. Tel.: +1 662 325 2937; fax: +1 662 325 8873.
E-mail address: em149@ads.msstate.edu (E. Memili).
[1] Present address: Uludag University, Veterinary Faculty, Gorukle, Bursa, Turkey.

0378-4320/$ – see front matter
doi:10.1016/j.anireprosci.2006.09.016

second experiment. In summary, SSS and EGF in maturation medium and replacement of FCS with SSS alone in culture medium on day 4 of IVC support oocyte maturation and embryo development in vitro. However, significance of culture condition induced changes on the genome-wide abundance of messenger ribonucleic acid and the significance of the apoptotic nuclei during fetal development still remain to be determined.

Keywords: Early development; Gametogenesis; Oocyte development; Embryo; Developmental biology

1. Introduction

In vitro maturation of bovine oocytes, followed by in vitro fertilization for the production of bovine embryos in the laboratory, is rapidly increasing (Zeuner et al., 2003). These in vitro techniques have important values both in studying the basic biological events occurring during oocyte maturation, fertilization and early embryonic development, and in providing an inexpensive and readily available source of preimplantation bovine embryos. Consistently successful and reliable oocyte maturation (both cytoplasmic and nuclear maturation) would dramatically improve the efficiency of preimplantation embryonic development as well as fetal development. However, current methods include supplements such as serum and/or co-culture with various types of somatic cells because of the limited understanding of embryo metabolism and growth requirements (Mastromonaco et al., 2004). This causes undefined and variable culture conditions. One of the most commonly used protein sources in the culture media has been serum, which has a biphasic effect. Including serum in the culture media can inhibit early cleavage divisions, while it can improve the development later in culture (Lonergan et al., 1999; Thompson et al., 1998). Its beneficial effect is especially important during the development of morulae into blastocyst (Gordon, 1994). In addition, it was reported that the effectiveness of serum in in vitro embryo production (IVP) might change considerably from one batch to another (Kane, 1987) and ingredients of serum, such as amino acids, hormones, growth factors, cytokines, vitamins and many other substances exhibit wide variations (Gordon, 1994). Even though a higher development rate to the blastocyst stage is obtained from media supported with serum, these variations could cause some alterations in the ultrastructure of embryos, impaired compaction, abnormal blastulation, large calf syndrome, aberrant mRNA expression profiles, and greater incidences of stillbirths and deaths after birth (Abe et al., 1999; Holm et al., 2002; Wrenzycki et al., 1999, 2004). Moreover, bovine-derived sera or proteins have recently been avoided especially in human IVP systems because of the appearance of bovine spongiform encephalopathy (BSE) and a viral or prion contamination risk. Therefore, even more completely defined culture conditions supporting high developmental rates are important not only to obtain consistent results but also to eradicate the contamination risk of BSE and other diseases. Because of these reasons, there has been a trend to use more defined proteins, such as bovine serum albumin (BSA), human serum albumin (HSA) and synthetic serum preparations instead of undefined natural serum preparations like fetal calf serum (FCS) and estrus cow serum (OCS) (Chanson et al., 2001; Russell et al., 1997; Sagirkaya et al., 2004).

Morphological characteristics such as appearance of cumulus cells and cytoplasm, oocyte size and time of polar body extrusion are related to the ability of oocytes to be fertilized and develop into viable embryos (Dominko and First, 1997). However, these are not reliable enough to act as the sole criteria for the evaluation of embryo developmental potential in vivo. Recently, there

has been a tendency to use mRNA expression patterns as a criterion to predict the developmental potential of embryos in vivo (Rizos et al., 2002; Wrenzycki et al., 2004). Recent studies show that the relative abundance of several mRNA transcripts affected by the choice of culture medium and the type of protein supplement played a critical role in preimplantation development (Rizos et al., 2002; Wrenzycki et al., 1999, 2001a, 2004; Sagirkaya et al., 2006). Knijn et al. (2002) compared the relative abundance of mRNA expressions for six developmentally important genes in bovine blastocysts produced in vitro from oocytes obtained from 3 to 8 mm follicles, preovulatory follicles before LH surge and preovulatory follicles 24 h after the LH surge. While the first two groups were matured in vitro in the presence of serum, the third group was an in vivo matured control group. However, there is a need for a comparative study on development and mRNA patterns of embryos cultured in vitro in more defined media including the in vitro maturation (IVM), fertilization (IVF) and culture (IVC) steps.

The aim of this study was to investigate the effect of protein source (FCS versus serum substitute supplement, SSS) during maturation and long-term culture on the development of in vitro produced embryos. Firstly, the effect of protein source on in vitro maturation was tested in Experiment I. After determining the effect on maturation, the effect of protein source supplementation on day 4 of the culture was examined in Experiment II. The effect of protein source was determined by comparing the rates of embryonic development (proportion of embryos developed to blastocyst stage), mean cell number, percentages of apoptotic cells and mRNA expression patterns of a panel of developmentally important genes at the blastocyst stage. The genes assayed in the present study are glucose transporter-1 (Glut-1), heat shock protein 70 (Hsp70), interferon tau (IF-τ), DNA methyltransferase 3a (Dnmt3a), desmosomal glycoprotein desmocollin III (DcIII) and insulin-like growth factor II receptor (Igf-2r). Expression patterns of these genes were determined relative to that of housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). These mRNAs were chosen to obtain the maximum possible understanding about important steps in early embryo development and because the proteins of these genes are involved in metabolism (Glut-1), stress (Hsp70), maternal recognition of pregnancy (IF-τ), DNA methylation (Dnmt3a), compaction and cavitation (DcIII) and growth factor signaling (Igf-2r).

2. Materials and methods

2.1. Reagents

In the present study, all chemicals were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise stated.

2.2. In vitro maturation

Bovine ovaries were obtained from a slaughterhouse and used for aspiration of follicles 2–8 mm in diameter to collect oocytes. Only oocytes with several layers of cumulus cells and homogenously granulated cytoplasm were used in this study. Selected oocytes were washed three times in TL-HEPES. Immature bovine oocytes were matured in three different protein supplementation conditions. Tissue Culture Medium (TCM-199, Gibco/Invitrogen, Grand Island, NY) supplemented with pyruvate (0.2 mM), FSH (0.5 μg/ml) (Sioux Biochemicals, Sioux City, IA), LH (5 μg/ml) (Sioux Biochemicals), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Gibco/Invitrogen) was used as the base medium for all of the groups. In Experiment I, oocytes were matured in TCM-199 medium supplemented with 10% FCS (Gibco/Invitrogen) as a Control

Group and Groups I and II were supplemented with 10% serum substitute supplement (SSS, Irvine Scientific, Santa Ana, CA), consisting of 6% total protein (84% human serum albumin, 16% alpha and beta globulins) in physiological saline which is more defined than serum or 10% SSS + epidermal growth factor (EGF, 10 ng/ml), respectively. The EGF at 10 ng/ml concentration has previously been shown to improve embryonic development (Vigneron et al., 2004; Oyamada et al., 2004). Following 4 days in culture, all treatments were supplemented with 10% FCS. In Experiment I, a total of 439, 395 and 415 oocytes were used for Control Group, Group I and Group II, respectively. However, in Experiment II, oocytes were matured in TCM-199 medium supplemented with 10% FCS (FCS Group) or 10% SSS + EGF (10 ng/ml) (SSS Group). These treatments were maintained with their respective protein supplementation on day 4 of in vitro culture. In Experiment II, a total of 574 and 644 oocytes were used for FCS and SSS Groups, respectively. For both experiments, 10 oocytes were matured in 50 μl drops covered with mineral oil for 24 h at 39 °C in 5% CO_2 in a humidified tissue culture incubator. The same culture conditions were used throughout all IVP procedures in Experiments I and II.

2.3. In vitro fertilization

After a 24 h maturation period, oocytes were rinsed twice in TL-HEPES and then groups of 10 oocytes were transferred into 44 μl fertilization drops covered with mineral oil. The fertilization medium was glucose-free TALP supplemented with pyruvate (0.2 mM), fatty-acid-free BSA (BSA-FAF, 6 mg/ml), penicillin (100 U/ml) and streptomycin (100 μg/ml). Frozen–thawed semen from a single bull was used for the fertilization of oocytes. Percoll density gradient system was used for the separation of the motile fraction of the frozen–thawed semen (Parrish et al., 1986, 1995). Percoll (500 μl at 90% concentration) was transferred to the bottom of a 1.5 ml Eppendorf centrifuge tube, and 0.5 ml of 45% Percoll was placed on top with a great caution. Frozen sperm was thawed at 35 °C for 1 min, and then carefully layered onto the Percoll gradient. The tube loaded with sperm sample and Percoll layers was then centrifuged at 700 × *g* for 10 min at room temperature. The supernatant was removed carefully without disrupting the pellet containing live sperm cells. After the pellet was resuspended, sperm concentration was determined using a hemocytometer. Sperm were then diluted to 50×10^6 sperm cells/ml in TL-HEPES, which would produce a 2×10^6 spermatozoa/ml final concentration. The fertilization procedure was completed by adding 2 μl of diluted sperm, 2 μl heparin (5 μg/ml) and 2 μl of PHE solution (20 μM penicillamine, 10 μM hypotaurine, 1 μM epinephrine in final concentration) into the 44 μl fertilization drops containing oocytes, respectively. Oocytes and sperm were cultured together for approximately 18–19 h in the incubator (Leibfried and Bavister, 1982; Parrish et al., 1986, 1995).

2.4. In vitro culture

Post-insemination (hpi 18 h), the cumulus cells were removed by vortexing the embryos in a 1.5 ml Eppendorf tube at the highest speed for 3 min. Cumulus-free presumptive zygotes were washed three times in TL-HEPES and transferred into 50 μl drops of embryo culture medium known as synthetic oviduct fluid, SOF (Specialty Media, NJ) under mineral oil (25–30 presumptive zygotes per each 50 μl drop). The SOF medium was supplemented with pyruvate (0.4 mM), BSA-FAF (8 mg/ml), 100× MEM (20 μl/ml), 50× BME (10 μl/ml), penicillin (100 U/ml) and streptomycin (100 μg/ml) on the day of use. We previously demonstrated that proportion of embryos developing to blastocyst stage were greater in SOF medium as compared to those in other

common media such as KSOM and CR1 (Sagirkaya et al., 2006). In addition, supplementation on day 4 of culture until completion on day 8 of IVC were 10% FCS in Experiment I, and 10% FCS and 10% SSS (FCS and SSS Groups) in Experiment II. Developmental data were recorded after 48, 96, 120 and 192 hpi culture periods for 2-cell, 8-cell, morulae and blastocyst stage embryos, respectively. Fertilization time was considered as 0 h. On day 8, after recording the blastocyst stage embryos, they were either fixed in 4% formaldehyde for staining purposes or frozen in saline for the determination of amounts of mRNA of a panel of some developmentally important genes.

2.5. Blastocyst staining for total cell number

Day 8 blastocysts were fixed in 4% formaldehyde after washing three times in 1% polyvinylpyrrolidone (PVP) in PBS overnight. Embryos were placed in 1% Triton X-100 overnight and finally stained with Hoechst 33342. Embryos were then mounted on slides and covered with a cover slip. The total cell numbers of blastocysts from three groups of Experiment I were determined by counting the number of nuclei under an epifluorescent microscope. The total cell numbers of blastocysts were visualized by a Nikon epifluorescent microscope with a 40× fluor objective (Tokyo, Japan) equipped with a 365 nm excitation filter, a 400 nm barrier filter, and a 400 nm emission filter.

2.6. Detection of DNA fragmentation by terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assay

In Experiment II, day 8 blastocysts were used to determine the mean cell numbers and percentages of TUNEL-positive nuclei for FCS ($n = 34$) and SSS ($n = 29$) Groups. The TUNEL assay was applied according to the modified method of Fedorcsak and Storeng (2003) using the DeadEnd™ Fluorometric Apoptosis Detection Kit (Promega, Madison, WI). Briefly, blastocysts were removed from culture media and washed three times in 100 μl drops of 1% PVP in PBS, and then fixed with 4% formaldehyde for approximately 1 h. Fixed embryos were stored at 4 °C until the TUNEL assay was performed. On the day of the TUNEL assay, the embryos were transferred from 4 °C to room temperature and washed three times in 100 μl drops of 1% PVP in PBS and then permeabilized in 50 μl of 0.5% Triton X-100 for 30 min at room temperature in a humidified chamber and rinsed twice in PBS. Then, the embryos were incubated with 100 μl of DNase buffer for 5 min. Meanwhile, 100 μl of DNase buffer containing DNase I (20 U/ml) was added to the embryos previously assigned as positive and negative controls for TUNEL assay, and 100 μl DNase buffer was added to the other embryos. All embryos were incubated at 37 °C for 30 min, and then they were washed four times with 100 μl of double distilled water by transferring embryos from one drop to another. Thereafter, the embryos were exposed to 100 μl equilibration buffer for 10 min. Subsequently, 10 embryos were transferred into 50 μl of prepared rTdT reaction buffer (45 μl of equilibration buffer, 5 μl of nucleotide mix and 1 μl of rTdT enzyme). For negative control slides, rTdT enzyme was replaced with 1 μl autoclaved deionized water. All embryos were incubated for 1 h at 37 °C in the dark. The reaction was terminated by incubating the embryos with 2XSSC solution for 15 min at room temperature and then they were washed twice in PBS. The embryos were then incubated with RNase A (50 μg/ml) in Tris (10 mM, pH 7.5) and NaCl (15 mM) for 40 min at 37 °C. Finally, embryos were stained with propidium iodide (1 μg/ml) for 15 min and washed with deionized water to remove excess stain. After the last wash, the embryos were mounted with an antifade solution (DABCO) and covered with a coverslip for evaluation. Assessment of TUNEL-positive and

total cell numbers was accomplished by evaluating each embryo under an epifluorescent microscope (Nikon, Japan) equipped with a 450–490 nm excitation filter, a 520 nm barrier emission filter and a 520 nm dichroic mirror, using a 40× objective. The apoptotic nuclei with fragmented DNA were observed as yellowish green, while normal nuclei appeared as an orange-red color.

2.7. Total RNA isolation and cDNA synthesis

In vitro produced blastocysts were transferred into TL-HEPES. Then, groups of 10 blastocysts were washed three times in saline (0.9% NaCl) and transferred into 500 μl centrifuge tubes with a minimum amount of saline. Following the transfer into centrifuge tubes, samples were snap frozen and kept at −80 °C until the use for the RNA isolation. Total RNA was isolated from two independent replicates of all in vitro produced blastocysts which were collected in a pool of 10 blastocysts using RNeasy Micro Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Quality and quantity of total RNA obtained from blastocysts were estimated using the Bioanalyzer 2100 RNA 6000 picochip kit (Agilent, Palo Alto, CA). Total RNA (8 ng) was used for cDNA synthesis using a first strand cDNA synthesis kit for RT-PCR (AMV) (Roche Applied Sciences, IN, USA) according to the manufacturer's protocol. Samples were incubated at 25 °C for 10 min, 42 °C for 60 min and then at 99 °C for 5 min.

2.8. Design of the primers and TaqMan probes

Primer Premier 5 software (Premier Biosoft International, Palo Alto, CA) was used to design all PCR primers and probes. The primers and probes were specifically designed to avoid any detection of amplification from genomic DNA. The positions and sequences of the primers and TaqMan probes, fragment size and the sequence references of the expected PCR products are shown in Table 1.

2.9. Real-time quantitative PCR

Real-time quantitative PCR was performed to assess amounts of mRNA for Glut-1, Hsp70, IF-τ, Dnmt3a, DcIII and Igf-2r. Each cDNA sample was analyzed in duplicate by using the LightCycler™ instrument (Roche Applied Sciences) according to previously established methods (Sagirkaya et al., 2006). Quantitative assessment of RNA amplification was detected with TaqMan probes, specific for the targeted genes. The RT-PCR reactions were performed in a total volume of 10 μl according to the manufacturer's manuals for Hybridization probes Master Mix (Roche Applied Sciences). The concentrations of TaqMan probes and primers (Tibmolbiol, Adelphia, NJ) were 0.2 and 0.3 μM, respectively. Probe and primer sequences used for RT-PCR are listed in Table 1. The cycling parameters were 2 min at 95 °C for denaturation, 50 cycles of 5 s at 95 °C, 20 s at 60 °C for amplification and quantification. GAPDH mRNA was evaluated to adjust the amount of total RNA in each sample with GAPDH probe and primer set. Amounts of mRNA for the developmentally important genes were determined relative to the transcripts of housekeeping gene GAPDH. In RT-PCR reactions the same initial amounts of target molecules were used, and the C_p values (22.80 ± 0.02) of GAPDH mRNA were constant in all groups.

Table 1
Sequences of real-time PCR primers and TaqMan probes

Genes	Primer and TaqMan probe sequences and positions (5′ → 3′)	Fragment size (bp)	Sequence references (accession no.)
Glucose transporter-1 (Glut-1)	CCAAggATCTCTCAgAgCACAg (1688–1709) TTCTTCTggACATCACTgCTgg (1797–1776) FAM-gATAgATCTCAgCAgAgCCgggCCT-TAMRA (1734–1758)	110	M60448
Heat shock protein 70 (Hsp70)	GACAAGTGCCAGGAGGTGATTT (1870–1891) CAGTCTGCTGATGATGGGGTTA (1986–1965) FAM-AGCACAAGAGGAAGGAGCTGGAGCA-TAMRA (1934–1958)	117	U09861
Interferon tau (If-τ)	TCCATgAgATgCTCCAgCAgT (433–453) TgTTggAgCCCAgTgCAgA (535–517) FAM-AgCACTCgTCTgCTgCCTggAACA-TAMRA (475–498)	103	X65539
DNA methyltransferase 3a (Dnmt3a)	TgATCTCTCCATCgTCAACCCT (2062–2083) gAAgAAggggCggTCATCTC (2185–2166) FAM-TgAgTTCTACCgCCTCCTgCATgATg-TAMRA (2125–2150)	124	AY271298
Desmocollin III (DcIII)	CCTCTGTGATTGTACTAACCCCG (2006–2028) GAAGTATGGCAAGGATCGCC (2078–2097) FAM-TGCTCGACGGAGTGCGGACGT-TAMRA (2042–2062)	92	L33774
Insulin-like growth factor 2 receptor (Igf-2r)	CAggTCTTgCAACTggTgTATgA (4935–4957) TTgTCCAgggAgATCAgCATg (5071–5051) FAM-AAgAgCgTCATCAgCTTCgTgTgCA-TAMRA (4998–5022)	137	J03527
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	AAGGCCATCACCATCTTCCA (178–197) CCACTACATACTCAGCACCAGCAT (253–230) FAM-AGCGAGATCCTGCCAACATCAAGTGG-TAMRA (200–225)	76	U85042

RT-PCR products of each gene were confirmed by sequencing (data not shown).

2.10. Statistical analysis

The IVP trials were repeated at least three times. Developmental rates to different embryonic stages were calculated from the number of oocytes used for fertilization. Developmental rates of fertilized oocytes to different developmental stages, cell numbers and the amount 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). Significant differences were evaluated by one-way ANOVA of SPSS program. In case of more than two groups, ANOVA was followed by multiple pair-wise comparisons using the LSD test. Differences at $p < 0.05$ were considered significant.

Statistical analysis of mRNA expression patterns of the developmentally important genes was performed using a new software tool, relative expression software tool (REST©, 384-beta version May 2005), running in Microsoft Excel. The software combines mRNA quantification and normalization into a single computation. REST© is founded on an efficiency corrected mathematical model for data analysis. It computes the relative expression ratio on the basis of the PCR efficiency (E) and crossing point deviation (ΔCP) of the investigated mRNAs. REST© uses the pair-wise fixed reallocation randomization test to compute significance of the results (Pfaffl, 2001; Pfaffl et al., 2002). Differences at $p < 0.001$ were considered as significant. The software for statistical analyses is an established method and directly analyzes the RT-PCR results.

3. Results

3.1. Experiment I

3.1.1. Embryonic development and blastocyst cell number

Developmental rates to 2-cell, 8-cell, morulae and blastocyst stages are summarized in Table 2. There was no significant difference among groups at any stage. On day 8 of IVC, 14, 10 and 16 blastocysts were used to determine the mean cell numbers for Control Group, Group I and Group II, respectively. Mean cell numbers for Control Group, Group I and Group II were 122.3, 90.0 and 105.6, respectively (Table 3). There was only a significant difference between Control Group and Group I ($p < 0.05$).

3.1.2. Gene expression

The Bioanalyzer gel image of the total RNAs for Experiments I and II is shown in Fig. 1, which indicates that the total RNA quality was sufficient to perform further analysis of mRNAs. Dynamics of mRNA patterns in Experiment I are shown in Fig. 2. In Experiment I, amount of

Table 2
Development of embryos derived from oocytes matured in three different maturation conditions

Group[a]	No. of fertilized oocytes	No. of 2-cell embryos (%)	No. of 8-cell embryos (%)	No. of morulae (%)	No. of blastocyst (%)
Control	439	335 (76.15)	210 (48.40)	169 (39.09)	127 (29.10)
Group I	395	296 (75.65)	185 (47.12)	146 (38.25)	110 (28.52)
Group II	415	323 (78.39)	211 (51.03)	164 (39.75)	120 (29.15)

[a] Oocytes were matured in TCM-199 supplemented with 10% FCS (Control Group), 10% SSS (Group I) and 10% SSS + 10 ng/ml EGF (Group II). Developing embryos in SOF medium were supplemented with 10% FCS on day 4 of IVC in all groups.

Table 3
Mean cell numbers of day 8 blastocysts developed from oocytes matured in three different maturation conditions

Group	No. of blastocysts	Mean cell number ± S.E.M./blastocyst
Control	14	122.3 ± 10.7[a]
Group I	10	90.0 ± 5.0[b]
Group II	16	105.6 ± 8.5[a,b]

Values with different superscripts (a and b) in the same column are significantly different ($p < 0.05$).

mRNA of Glut-1 gene was significantly down-regulated in Group II when compared to Control Group ($p < 0.001$) (Fig. 2A). Expression of Hsp70 gene was significantly down-regulated in Group II when compared to Group I ($p < 0.001$) (data not shown). IF-τ gene was significantly down-regulated in Groups I and II when compared to Control Group ($p < 0.001$) (Fig. 2C). Dnmt3a mRNA was less in both Groups I and II when compared to Control Group ($p < 0.001$). Also, Group II had less Dmnt3a mRNA when compared to Group I (data not shown) ($p < 0.001$). Messenger RNA of DcIII gene was significantly less in Groups I and II when compared to Control Group ($p < 0.001$) (Fig. 2E). Igf-2r gene transcription was significantly less in Group II when compared to Control Group ($p < 0.001$) (Fig. 2F) and Group I (data not shown) ($p < 0.001$).

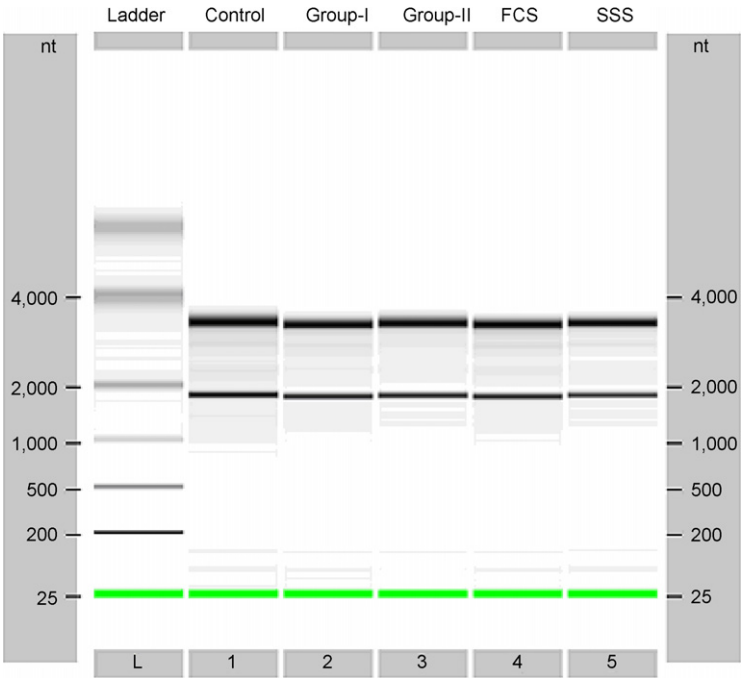

Fig. 1. Bioanalyzer gel image of the total RNAs. Total RNA was isolated using RNeasy Micro Kit (Qiagen, Valencia, CA), and 1 μl of RNA was loaded for each lane in the Bioanalyzer 2100 RNA 6000 picochip (Agilent, Palo Alto, CA). Lane L size markers, RNA from Control (Lane 1), Group I (Lane 2), Group II (Lane 3), FCS (Lane 4) and SSS blastocysts (Lane 5). Sharp bands of 28S and 18S ribosomal RNA below the 4000 and 2000 molecular weight markers, respectively, demonstrate the integrity of total RNA isolated.

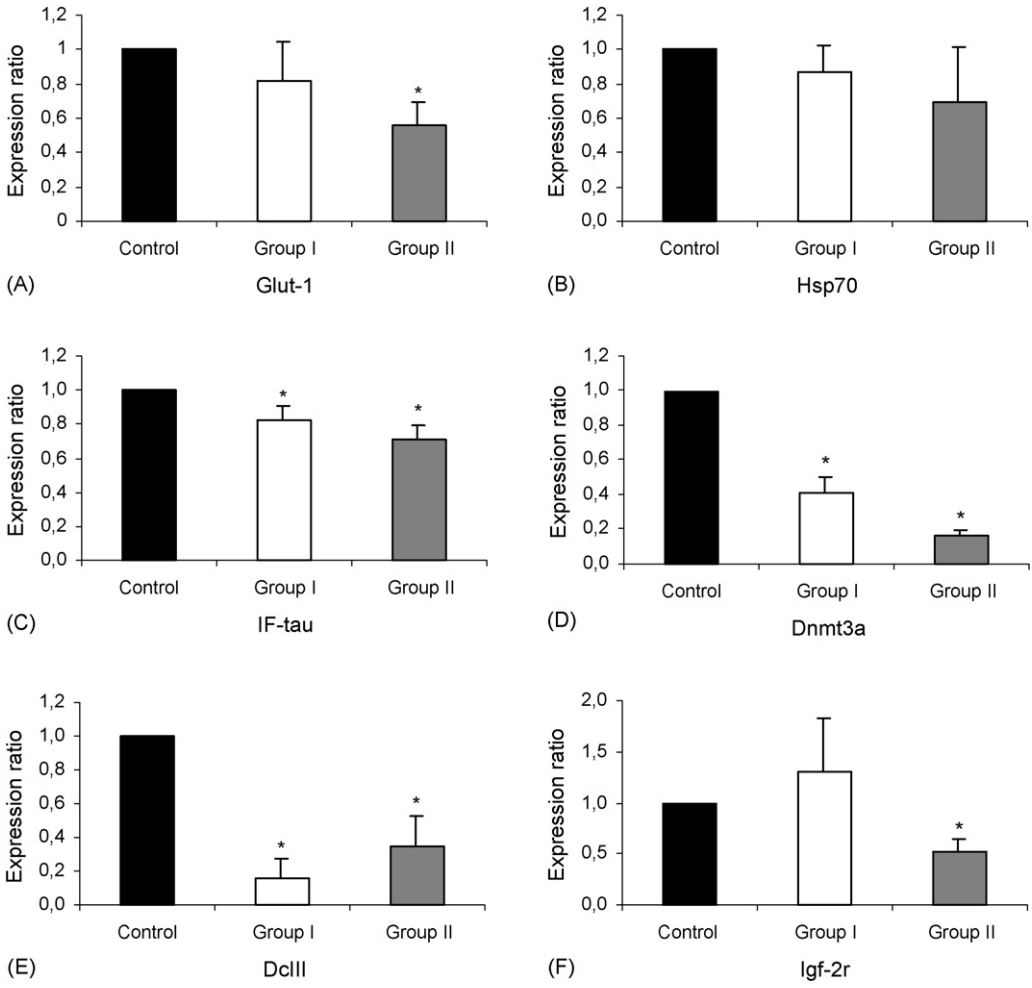

Fig. 2. Relative abundance of various developmentally important gene transcripts in bovine blastocysts obtained from Control Group (solid bars), Group I (open bars) and Group II (gray bars). Expression ratio of Glut-1, Hsp70, IF-τ, Dnmt3a, DcIII and Igf-2r genes are demonstrated in (A–F), respectively. Asterisk (*) shows significant differences when compared to Control Group ($p < 0.001$).

3.2. Experiment II

3.2.1. Embryo development

Developmental data regarding the development to 2-cell, 8-cell, morulae and blastocyst stage embryos are shown in Table 4. There was no statistically significant difference between the developmental rates of embryos in the two groups at any stage.

3.2.2. Cell numbers and apoptosis

The mean cell numbers of day 8 blastocysts of FCS and SSS Groups did not differ significantly (105.8 versus 104.1, respectively). However, a significant difference was found between the per-

Table 4
Development of embryos derived from oocytes matured in media supplemented with FCS or SSS

Group[a]	No. of fertilized oocytes	No. of 2-cell embryos (%)	No. of 8-cell embryos (%)	No. of morulae (%)	No. of blastocyst (%)
FCS	574	393 (70.1)	222 (40.0)	193 (35.1)	145 (26.6)
SSS	644	480 (75.6)	268 (41.8)	238 (36.9)	173 (27.8)

[a] Oocytes were matured in TCM-199 supplemented with 10% FCS and 10% SSS + 10 ng/ml EGF, and developing embryos in SOFaa medium were supplemented with 10% FCS and 10% SSS on day 4 of IVC for FCS and SSS Groups, respectively.

Table 5
Mean cell numbers and percentages of apoptotic blastomeres of day 8 blastocysts from FCS and SSS Groups

Group	*n*	Mean cell number ± S.E.M./blastocyst	Percentages of apoptotic blastomeres/blastocyst ± S.E.M.
FCS	34	105.8 ± 4.0	4.23 ± 0.6[a]
SSS	29	104.1 ± 6.0	8.67 ± 1.0[b]

Values with different superscripts (a and b) in the same column are significantly different ($p < 0.01$).

centages of TUNEL-positive nuclei of FCS and SSS Groups (4.23% versus 8.67%, respectively, $p < 0.01$) (Table 5).

3.2.3. Gene expression

Surprisingly in Experiment II, there were no significant differences among the relative mRNA abundance of the studied genes between FCS and SSS (Fig. 3A–F).

4. Discussion

In the present study, the effects of the FCS replacement with SSS in the maturation media alone, and in the maturation media as well as the culture media were investigated in terms of different evaluation procedures for assessing the quality of developing blastocysts. In the present study the aim was to investigate the effects of replacement of FCS (Control Group) with SSS only (Group I) or SSS + EGF (Group II) on in vitro maturation in Experiment I. Developing embryos were all treated in the same way to observe the effect of serum replacement in the maturation medium and supplemented with 10% FCS on day 4 of in vitro culture. Results of Experiment I showed that SSS and EGF combination might be successfully used instead of serum in the maturation medium. Then, in Experiment II embryos were produced by using FCS (FCS Group) or SSS + EGF (SSS Group) in the maturation medium and developing embryos were, respectively, supplemented with 10% FCS or 10% SSS on day 4 of in vitro culture to see the effect of FCS replacement in the maturation and culture media. In terms of cleavage rates results of the present study did not differ significantly in both experiments (Table 2). However, mean cell number of blastocysts developed from Group I was significantly less than the Control Group in Experiment I (Table 3), which supported previous work showing the beneficial effect of EGF in the absence of serum (Lonergan et al., 1996; Mtango et al., 2003; Oyamada et al., 2004; Sagirkaya et al., 2004; Sakaguchi et al., 2000). In Experiment II, there was no significant difference between the developmental results

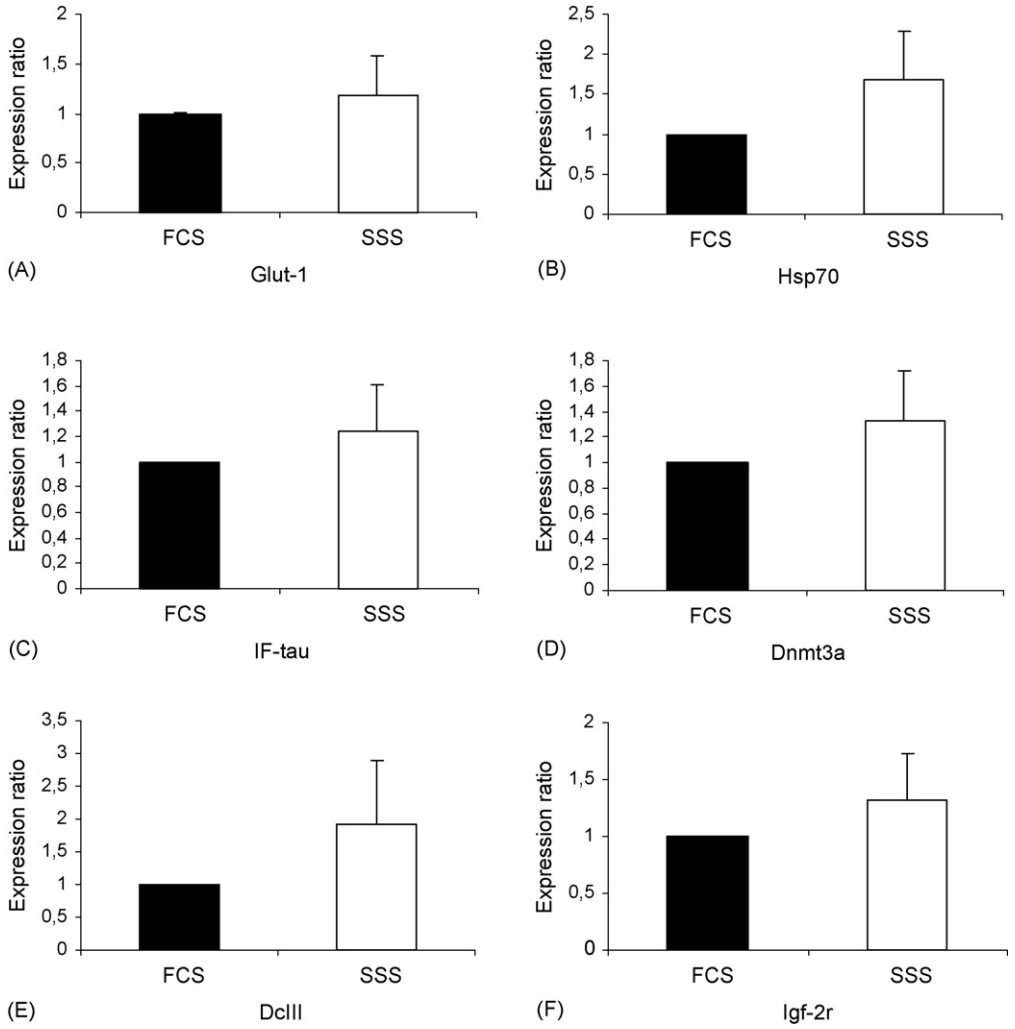

Fig. 3. Relative abundance of various developmentally important gene transcripts in bovine blastocysts obtained from FCS Group (solid bars) and SSS Group (open bars). Ratio of mRNA for Glut-1, Hsp70, IF-τ, Dnmt3a, DcIII and Igf-2r genes are demonstrated in (A–F), respectively.

and the mean cell numbers of FCS and SSS Groups (Table 4). However, these results may not necessarily indicate the equal developmental potential. Developmental potential was tested further using the TUNEL assay to determine apoptotic cell percentages for both groups. Blastocysts from the SSS Group had a significantly greater percentage of TUNEL-positive cells than the FCS Group (Table 5).

Dramatic programming of mRNA occurs during embryonic genome activation (EGA), an essential event starting as early as 1-cell zygotic stage in the bovine and increasing gradually as embryonic development advances (Memili and First, 1999). Characteristics of EGA include utilization or degradation of maternal RNA and stage specific activation of embryonic genes during early embryogenesis. It is this programming of mRNA that sets the stage for later development. It

was shown that in vitro conditions have a profound effect on the patterns of some gene transcripts in bovine and mouse embryos (Ho et al., 1994, 1995; Lazzari et al., 2002; Niemann and Wrenzycki, 2000; Rinaudo and Schultz, 2004; Rizos et al., 2002; Wrenzycki et al., 2001a; Sagirkaya et al., 2006). The present mRNA patterns in Experiment I showed that different in vitro maturation conditions affect the relative abundance of the gene transcripts and indicate that in vitro maturation is a critical factor causing differences in mRNA content. On the contrary, Knijn et al. (2002) reported that there was no significant difference among a panel of six gene transcripts of blastocysts derived from oocytes matured differently, but cultured identically.

Differences in the amount of mRNA for the Glut-1 gene between the Control Group and Group II also reflects the differences in energy metabolism between embryos matured in different conditions (Fig. 2A). It was reported that environmental stressors such as free oxygen radicals and increased temperature induce gene expression prior to major activation of gene transcription in bovine embryos (Edwards et al., 1997; Edwards and Hansen, 1996, 1997). In the present study, expression of Hsp70 gene in Group II was significantly different from Group I. In Group II, Hsp70 mRNA was less in the presence of SSS and EGF (Fig. 2B). Results of the present study suggested that the use of serum substitute supplement in culture media could create more sensitivity in response to heat shock since Hsp70 has a role in stress adaptation (Wrenzycki et al., 1999). It was previously reported that IF-τ mRNA was present in blastocysts, but not in morulae (Hernandez-Ledezma et al., 1993; Wrenzycki et al., 1999). IF-τ is exclusively secreted by the trophectodermal cells of blastocysts and primary factor responsible for maternal recognition of pregnancy in cattle (Roberts et al., 1992). It is commonly believed that a higher amount of IF-τ mRNA is an indicator of poor quality blastocysts (Wrenzycki et al., 2001b). In the present study, expression of the IF-τ gene was significantly less in Groups I and II when compared to Control Group (Fig. 2C). Although relative implantation was not studied, results of the present study indicated that the use of serum substitute supplement in culture media could decrease the implantation rate. Methylation of DNA in mammals is essential for development and it is involved in X chromosome inactivation, cell differentiation and imprinting, and usually results in gene silencing (Kaneda et al., 2004). Interestingly, in Experiment I, Dnmt3a mRNA was significantly less in Groups I and II when compared to Control Group, and there was also a significant reduction in Group II when compared to Group I (Fig. 2D). Presence of synthetic serum with or without EGF in culture media might have some effects in global gene methylation which may result in different transcription patterns in further development.

DcIII plays a role in the compaction and cavitation during blastocyst formation. In the present study, expression of DcIII gene in Groups I and II was significantly different from that in the Control Group (Fig. 2E). This might decrease blastocyst rate and cause developmental block at morulae stage. It has been postulated that insulin-like growth factors play a role as a survival factor in preimplantation bovine embryos (Yaseen et al., 2001). The mRNA of Igf-2r in Group II significantly differed from the amount in both the Control Group and Group I (Fig. 2F). This result indicates that the presence of EGF down-regulates Igf-2r gene expression, which might result in, decreased blastocyst rate. However, in the present study, something unknown balanced this negative effect.

In Experiment II, surprisingly the relative abundance of mRNA between the FCS and SSS Groups did not differ significantly (Fig. 3). Successful oocyte maturation affects the subsequent fertilization and embryonic development. The maturation status of oocyte cytoplasm plays a major role in reprogramming of gene expression at the onset of embryonic development. Successful EGA during embryogenesis is a prerequisite for further development, and thus oocyte maturation impacts both pre- and post-implantation development.

In conclusion, SSS and EGF in maturation medium instead of FCS support oocyte maturation and embryo development in vitro. Linking morphological and developmental indicators of oocyte and embryo viability with the expression and function of specific genes can provide valuable insights into mechanisms contributing to embryonic mortality. However, perturbations of global transcriptome in different culture conditions should be analyzed using DNA microarrays. Furthermore, correlations between amount of mRNA and developmental potential of in vitro derived embryos should be further studied by methods such as embryo transfer to recipient cows.

Acknowledgments

Authors would like to thank Brad Haley for help in obtaining bovine ovaries and Kindra Kelly-Quagliana for laboratory set up and preparations of the manuscript.

This project was supported by The Life Sciences and Biotechnology Institute, Mississippi State University (Assignment number: J-10829).

References

Abe, H., Yamashita, S., Itoh, T., Satoh, T., Hoshi, H., 1999. Ultrastructure of bovine embryos developed from in vitro-matured and -fertilized oocytes: comparative morphological evaluation of embryos cultured either in serum-free medium or in serum-supplemented medium. Mol. Reprod. Dev. 53, 325–335.

Chanson, A., Nocera, D., Senn, A., De Grandi, P., Germond, M., 2001. Development of a well-defined medium for the in vitro maturation of immature bovine cumulus–oocyte complexes. J. Assist. Reprod. Genet. 18, 97–105.

Dominko, T., First, N.L., 1997. Timing of meiotic progression in bovine oocytes and its effect on early embryo development. Mol. Reprod. Dev. 47, 456–467.

Edwards, J.L., Ealy, A.D., Monterroso, V.H., Hansen, P.J., 1997. Ontogeny of temperature-regulated heat shock protein 70 synthesis in preimplantation bovine embryos. Mol. Reprod. Dev. 48, 25–33.

Edwards, J.L., Hansen, P.J., 1996. Elevated temperature increases heat shock protein 70 synthesis in bovine two-cell embryos and compromises function of maturing oocytes. Biol. Reprod. 55, 341–346.

Edwards, J.L., Hansen, P.J., 1997. Differential responses of bovine oocytes and preimplantation embryos to heat shock. Mol. Reprod. Dev. 46, 138–145.

Fedorcsak, P., Storeng, R., 2003. Effects of leptin and leukemia inhibitory factor on preimplantation development and STAT3 signaling of mouse embryos in vitro. Biol. Reprod. 69, 1531–1538.

Gordon, I., 1994. Laboratory Production of Cattle Embryos. CAB International, Wallington, pp. 30–142.

Hernandez-Ledezma, J.J., Mathialagan, N., Villanueva, C., Sikes, J.D., Roberts, R.M., 1993. Expression of bovine trophoblast interferons by in vitro-derived blastocysts is correlated with their morphological quality and stage of development. Mol. Reprod. Dev. 36, 1–6.

Ho, Y., Doherty, A.S., Schultz, R.M., 1994. Mouse preimplantation embryo development in vitro: effect of sodium concentration in culture media on RNA synthesis and accumulation and gene expression. Mol. Reprod. Dev. 38, 131–141.

Ho, Y., Wigglesworth, K., Eppig, J.J., Schultz, R.M., 1995. Preimplantation development of mouse embryos in KSOM: augmentation by amino acids and analysis of gene expression. Mol. Reprod. Dev. 41, 232–238.

Holm, P., Booth, P.J., Callesen, H., 2002. Kinetics of early in vitro development of bovine in vivo- and in vitro-derived zygotes produced and/or cultured in chemically defined or serum-containing media. Reproduction 123, 553–565.

Kane, M.T., 1987. Culture media and culture of early embryos. Theriogenology 27, 49–57.

Kaneda, M., Okano, M., Hata, K., Sado, T., Tsujimoto, N., Li, E., Sasaki, H., 2004. Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. Nature 429, 900–903.

Knijn, H.M., Wrenzycki, C., Hendriksen, P.J., Vos, P.L., Herrmann, D., van der Weijden, G.C., Niemann, H., Dieleman, S.J., 2002. Effects of oocyte maturation regimen on the relative abundance of gene transcripts in bovine blastocysts derived in vitro or in vivo. Reproduction 124, 365–375.

Lazzari, G., Wrenzycki, C., Herrmann, D., Duchi, R., Kruip, T., Niemann, H., Galli, C., 2002. Cellular and molecular deviations in bovine in vitro-produced embryos are related to the large offspring syndrome. Biol. Reprod. 67, 767–775.

Leibfried, M.L., Bavister, B.D., 1982. Effects of epinephrine and hypotaurine on in-vitro fertilization in the golden hamster. J. Reprod. Fertil. 66, 87–93.

Lonergan, P., Carolan, C., Van Langendonckt, A., Donnay, I., Khatir, H., Mermillod, P., 1996. Role of epidermal growth factor in bovine oocyte maturation and preimplantation embryo development in vitro. Biol. Reprod. 54, 1420–1429.

Lonergan, P., O'Kearney-Flynn, M., Boland, M.P., 1999. Effect of protein supplementation and presence of an antioxidant on the development of bovine zygotes in synthetic oviduct fluid medium under high or low oxygen tension. Theriogenology 51, 1565–1576.

Mastromonaco, G.F., Semple, E., Robert, C., Rho, G.J., Betts, D.H., King, W.A., 2004. Different culture media requirements of IVF and nuclear transfer bovine embryos. Reprod. Domest. Anim. 39, 462–467.

Memili, E., First, N.L., 1999. Control of gene expression at the onset of bovine embryonic development. Biol. Reprod. 61, 1198–1207.

Mtango, N.R., Varisanga, M.D., Dong, Y.J., Rajamahendran, R., Suzuki, T., 2003. Growth factors and growth hormone enhance in vitro embryo production and post-thaw survival of vitrified bovine blastocysts. Theriogenology 59, 1393–1402.

Niemann, H., Wrenzycki, C., 2000. Alterations of expression of developmentally important genes in preimplantation bovine embryos by in vitro culture conditions: implications for subsequent development. Theriogenology 53, 21–34.

Oyamada, T., Iwayama, H., Fukui, Y., 2004. Additional effect of epidermal growth factor during in vitro maturation for individual bovine oocytes using a chemically defined medium. Zygote 12, 143–150.

Parrish, J.J., Krogenaes, A., Susko-Parrish, J.L., 1995. Effect of bovine sperm separation by either swim-up or Percoll method on success of in vitro fertilization and early embryonic development. Theriogenology 44, 859–870.

Parrish, J.J., Susko-Parrish, J.L., Leibfried-Rutledge, M.L., Critser, E.S., Eyestone, W.H., First, N.L., 1986. Bovine in vitro fertilization with frozen–thawed semen. Theriogenology 25, 591–600.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucl. Acids Res. 29, e45.

Pfaffl, M.W., Horgan, G.W., Dempfle, L., 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucl. Acids Res. 30, e36.

Rinaudo, P., Schultz, R.M., 2004. Effects of embryo culture on global pattern of gene expression in preimplantation mouse embryos. Reproduction 128, 301–311.

Rizos, D., Lonergan, P., Boland, M.P., Arroyo-Garcia, R., Pintado, B., de la Fuente, J., Gutierrez-Adan, A., 2002. Analysis of differential messenger RNA expression between bovine blastocysts produced in different culture systems: implications for blastocyst quality. Biol. Reprod. 66, 589–595.

Roberts, R.M., Cross, J.C., Leaman, D.W., 1992. Interferons as hormones of pregnancy. Endocr. Rev. 13, 432–452.

Russell, J.B., Knezevich, K.M., Fabian, K.F., Dickson, J.A., 1997. Unstimulated immature oocyte retrieval: early versus midfollicular endometrial priming. Fertil. Steril. 67, 616–620.

Sagirkaya, H., Yagmur, M., Nur, Z., Soylu, M.K., 2004. Replacement of fetal calf serum with synthetic serum substitute in the in vitro maturation medium: effects on maturation, fertilization and subsequent development of cattle oocytes in vitro. Turk. J. Vet. Anim. Sci. 28, 779–784.

Sagirkaya, H., Misirlioglu, M., Kaya, A., Parrish, J.J., First, N.L., Memili, E., 2006. Developmental and molecular correlates of bovine preimplantation embryos. Reproduction 131, 895–904.

Sakaguchi, M., Dominko, T., Leibfried-Rutledge, M.L., Nagai, T., First, N.L., 2000. A combination of EGF and IGF-I accelerates the progression of meiosis in bovine follicular oocytes in vitro and fetal calf serum neutralizes the acceleration effect. Theriogenology 54, 1327–1342.

Thompson, J.G., Allen, N.W., McGowan, L.T., Bell, A.C., Lambert, M.G., Tervit, H.R., 1998. Effect of delayed supplementation of fetal calf serum to culture medium on bovine embryo development in vitro and following transfer. Theriogenology 49, 1239–1249.

Vigneron, C., Perrau, C., Dupont, J., Uzbekova, S., Prigent, C., Mermillod, P., 2004. Several signaling pathways are involved in the control of cattle oocyte maturation. Mol. Reprod. Dev. 69, 466–474.

Wrenzycki, C., Herrmann, D., Carnwath, J.W., Niemann, H., 1999. Alterations in the relative abundance of gene transcripts in preimplantation bovine embryos cultured in medium supplemented with either serum or PVA. Mol. Reprod. Dev. 53, 8–18.

Wrenzycki, C., Herrmann, D., Keskintepe, L., Martins Jr., A., Sirisathien, S., Brackett, B., Niemann, H., 2001a. Effects of culture system and protein supplementation on mRNA expression in pre-implantation bovine embryos. Hum. Reprod. 16, 893–901.

Wrenzycki, C., Herrmann, D., Lucas-Hahn, A., Lemme, E., Korsawe, K., Niemann, H., 2004. Gene expression patterns in in vitro-produced and somatic nuclear transfer-derived preimplantation bovine embryos: relationship to the large offspring syndrome? Anim. Reprod. Sci. 82-83, 593–603.

Wrenzycki, C., Wells, D., Herrmann, D., Miller, A., Oliver, J., Tervit, R., Niemann, H., 2001b. Nuclear transfer protocol affects messenger RNA expression patterns in cloned bovine blastocysts. Biol. Reprod. 65, 309–317.

Yaseen, M.A., Wrenzycki, C., Herrmann, D., Carnwath, J.W., Niemann, H., 2001. Changes in the relative abundance of mRNA transcripts for insulin-like growth factor (IGF-I and IGF-II) ligands and their receptors (IGF-IR/IGF-IIR) in preimplantation bovine embryos derived from different in vitro systems. Reproduction 122, 601–610.

Zeuner, A., Muller, K., Reguszynski, K., Jewgenow, K., 2003. Apoptosis within bovine follicular cells and its effect on oocyte development during in vitro maturation. Theriogenology 59, 1421–1433.