Developmental Biology

99 COMPARISON OF IN VITRO DEVELOPMENT FOLLOWING CRYOPRESERVATION OF MEISHAN AND WHITE CROSS SWINE EMBRYOS

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Development of improved protocols for cryopreservation of zona pellucida-intact porcine embryos could greatly impact the swine industry. Our aim was to investigate in vitro development following cryopreservation of embryos from Chinese Meishan (M) and occidental white cross (WC) breeds using a modified protocol described previously (Mitsui K et al. 2003 Theriogenology 60, 253–260). First-parity M sows (n = 11) and WC gilts (n = 13) were observed for estrus every 12 h and inseminated at 12 and 24 h after estrous onset within breed using semen from 2 different boars. Females were sacrificed between Days 4.5 and 6 after estrus and embryos were collected using Beltsville embryo culture medium (BECM). Compact morula (CM) or blastocyst stage embryos from each female within breed were randomly allocated either directly into the culture system to serve as controls (68 M and 48 WC embryos) or to undergo cryopreservation. A total of 101 M and 78 WC embryos were cryopreserved using the following protocol: (1) 5 min in BECM + 10% ethylene glycol (EG); (2) 5 min in BECM + 10% EG + 0.27 M sucrose + 1% polyethylene glycol (PEG); and (3) 30 to 45 s in BECM + 40% EG + 0.36 M sucrose + 2% PEG. In the last solution, 5 to 10 embryos in a 5- to 10-μL microdrop attached to a fine glass pipette were exposed to the vapor phase of liquid nitrogen (LN2) for 15 s and then plunged into LN2. The pipette tip was broken and the tip and associated frozen microdrop were placed inside an LN2-submerged 2-mL cryotube containing a hole in the lid for 1 h. Next, embryos were thawed using a 4-step (5 min each) procedure: (1) BECM + 5% EG + 0.57 M sucrose; (2) BECM + 2.5% EG + 0.29 M sucrose; (3) BECM + 0.3 M sucrose; and (4) BECM alone. All procedures were performed with solutions maintained at 37°C. Cryopreserved and control embryos were cultured in 50 μL drops of modified Whitten’s medium + 1.5% BSA under oil at 37°C in a 5% CO2 in air environment and scored daily for development. For embryos undergoing cryopreservation, retrieval rates from cryovials were 92% and 96% for M and WC, respectively. The percentage of embryos surviving 24 h after cryopreservation without lysis or degeneration was higher for M (72%) than for WC (44%; P < 0.001; χ²-test). However, in vitro development of embryos that survived cryopreservation was not different between M and WC at the expanded (64%) or hatched (22%) blastocyst stages. Developmental rates were significantly higher for control embryos than for frozen embryos from both breeds. Rates of expanded blastocyst formation did not differ between M and WC control embryos (98% and 95%, respectively), but more M embryos developed to the hatched blastocyst stage (22% for M vs. 9% for WC; P < 0.05). Our results suggest that M embryos have a higher capacity to survive the vitrification process than WC embryos.

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Developmental Biology

100 DIET AND FATTY ACID COMPOSITION OF BOVINE PLASMA, GRANULOSA CELLS, AND CUMULUS–OOCYTE COMPLEXES

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The effects of altering dietary carbohydrates and lipids to oocyte donors during superovulation and ovum pickup (OPU) on in vitro embryo production was reported previously (Adamia et al. 2004 Reprod. Dev. Fert. 16, 193–194). Here we report the effects of these dietary treatments on the fatty acid (FA) composition of plasma, granulosa cells (GCs), and cumulus–oocyte complexes (COCs) from the 32 heifers used in that study. Blood samples were collected by jugular venipuncture. GCs and COCs were harvested from each heifer by OPU as described previously but were pooled between pairs of heifers within treatment to provide adequate material for FA analysis. Both GCs and COCs were washed twice in PBS supplemented with 0.3% (w/v) BSA (FA-free) before being transferred into 2:1 (v/v) chloroform:methanol solution for FA extraction. FA composition was determined using gas chromatography as described previously (Reis et al. 2002, Theriogenology 57, 507). Data were analyzed by ANOVA. Total plasma FA content averaged 1.12 μg/mL and was unaffected by body condition score (BCS). Low BCS heifers had more saturated (54.0 ± 1.76 vs. 49.2 ± 1.74%) and monounsaturated (22.4 ± 1.08 vs. 18.2 ± 0.69%) FA, but less polyunsaturated FA (PUFA) (23.7 ± 1.75 vs. 32.8 ± 2.21%) in plasma than moderate BCS heifers (P < 0.01). Animals fed high relative to low fiber diets had greater plasma FA (1.3 ± 0.15 vs. 1.0 ± 0.12 μg/mL)
This work was supported by Defra and The Perry Foundation.

The use of a single dose of GnRH antagonists during the progestagen treatment prior to superovulatory treatment protocols in sheep increases the developmental competence of oocytes collected by ovum pick (OPU) from GnRH-antagonist treated sheep during an ovarian by perstimulation protocol. Adult Sarda sheep (Ovis aries) increased plasma FA (1.6 ± 0.07 vs. 0.6 ± 0.04 µg/mL) and PUFA (30.0 ± 1.92 vs. 26.4 ± 0.30%) (P < 0.05), but reduced plasma saturated FA (48.9 ± 1.12 vs. 54.2 ± 2.14%; P < 0.01) in the animals fed high compared to low fiber diets, respectively. In contrast to their effects in plasma, BCS and diet had little effect on FA composition of GCs and COCs. Although low BCS and dietary lipids both increased FA in COCs (78.3 ± 2.01 vs. 69.2 ± 2.80, P < 0.01; and 77.1 ± 2.96 vs. 70.5 ± 2.26 ng/COC; P < 0.05, respectively), neither factor significantly altered the FA composition of COCs. Across treatments, the FA composition of GCs and COCs differed markedly from that of plasma (Table 1), confirming the presence of a selective uptake mechanism in the follicle that can moderate dietary induced fluctuations in FA supply from peripheral circulation.

### Table 1. Concentrations of fatty acids in plasma, GCs, and COCs expressed as mean percentages (w/w) of total fatty acids

<table>
<thead>
<tr>
<th></th>
<th>Plasma</th>
<th>GCs</th>
<th>COCs</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated FA</td>
<td>51.6 ± 1.35</td>
<td>56.2 ± 2.10</td>
<td>87.3 ± 1.00</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Monounsaturated FA</td>
<td>20.3 ± 0.82</td>
<td>32.7 ± 0.68</td>
<td>11.1 ± 0.66</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PUFA</td>
<td>28.2 ± 1.80</td>
<td>11.1 ± 2.23</td>
<td>2.1 ± 0.35</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>n-3 PUFA</td>
<td>1.8 ± 0.10</td>
<td>0.9 ± 0.13</td>
<td>0.5 ± 0.15</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>n-6/n-3 ratio</td>
<td>16.6 ± 1.06</td>
<td>10.9 ± 1.94</td>
<td>3.8 ± 0.71</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

This work was supported by the Spanish MEC (projects SC 00-051-C3.1 and HI2002-0004) and the Italian MIUR (cofin).
102 ANALYSIS OF EARLY EMBRYONIC TRANSCRIPTION IN THE BOVINE EMBRYO USING A DEDICATED cDNA LIBRARY

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Early embryonic development is initially dependent on mRNAs that have been transcribed during oocyte growth (maternal transcripts). Newly formed zygotic transcripts then become required during what is called the maternal-to-zygotic transition. In cattle, this transition initiates at the 8-cell stage and ends before the morula stage. Because of its decisive role in the further development of the embryo we are interested in characterizing the regulative functions of those cattle zygotic transcripts that are differentially expressed at the end of this transition. For that reason a subtracted cDNA library corresponding to the first zygotic transcripts was established at the early compacted morula stage by subtracting abundant transcripts from an early compacted morula stage library using suppressive subtractive hybridization (SSH; Clontech, LePont de Claix, France). Morula derived cDNAs were used as Tester and 4-cell stage cDNAs as Driver materials. Cattle embryos were obtained from slaughterhouse-derived ovas using standard in vitro maturation and fertilization techniques. Since, in cattle, early cleaving (2-cell-stage) zygotes are more likely to develop to the blastocyst stage than their later-cleaving counterparts, all embryos used to establish the cDNA library were selected from zygotes that were already at the 2-cell stage 32 h post-in vitro fertilization. Total RNA was extracted from batches of 140 (morula) and 200 (4-cell-stage) embryos and the amount of Poly\textsuperscript{A} + RNAs was estimated according to Duranthon and Renard (in \textit{Biological and Pathology of the Oocyte}, Trounson and Gosden eds, Cambridge Univ. Press, 2003, p. 96). Double-stranded cDNAs were synthesized with the SMART cDNA amplification kit (Clontech) before SSH was undertaken. Upon RNA extraction, exogenous transcripts obtained from \textit{Arabidopsis thaliana} (Stratagene, La Jolla, CA, USA) were added either to the Tester only (at three concentrations: \(10^{-3}, 5 \times 10^{-3}, 5 \times 10^{-2}\)) or to both the Tester and the Driver materials (at two concentrations: \(5 \times 10^{-3}, 5 \times 10^{-2}\)). These transcripts allowed us to report on the efficiency of our subtraction procedure and on the quality of the bacterial library in terms of tester-specific transcript enrichment. We found the library to be enriched in specific transcripts (Stratagene, La Jolla, CA, USA) combined with ALEXA 488 conjugated secondary antibodies (Cambridge Bioscience, Oxford, UK) was used to determine the distribution of PKCs and junctional proteins in intact blastocysts and fully and partially isolated ICMs after immunosurgery.

These conditions are thus beneficial for the isolation of rare zygotic transcripts present at an initial concentration of only \(10^{-3}\). Normalization of the library, as determined from the proportion of exogenous transcripts after bacterial transformation, was effective for those added initially at \(10^{-3}\) or moderate \((5 \times 10^{-3})\) concentrations but not for abundant ones \((5 \times 10^{-2})\). These conditions are thus beneficial for the isolation of rare zygotic transcripts present at an initial concentration of only \(10^{-3}\) of the messengers. Ongoing study using various different screening of this cattle library with morula- and 4-cell-stage probes will now allow us to identify zygotic transcripts specifically expressed at the onset of genome activation and not present in the pool of maternal transcripts up to the 4-cell stage.

This work was supported by an INRA CIRAD grant (BioDiva) to LCB.

103 GAP JUNCTIONAL INTERCELLULAR COMMUNICATION IS DISPENSIBLE DURING REGULATION OF TIGHT JUNCTION MEMBRANE ASSEMBLY BY CELL CONTACT PATTERN AND PKC SIGNALING

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Contact symmetries are involved in regulating cell lineage segregation during blastocyst biogenesis when tight junction (TJ) membrane assembly is restricted to the epithelial trophectoderm (TE). Manipulation of cell contact patterns by immunosurgical isolation of inner cell masses (ICMs) providing a contact-free cell surface serves as a switch to induce TE differentiation upon \textit{in vitro} culture. In this model, protein kinase C (PKC)-mediated signaling up-regulates TJ membrane assembly. Whether signaling via gap junctional intercellular communication (GJIC) affects these processes is controversial. The current study investigates the interrelationship between changes in cell contact pattern, PKC signaling, and GJIC on mediated signaling up-regulates TJ membrane assembly. This modulation may affect GJIC. Taken together, our data suggest that cell contact pattern regulates TJ assembly via PKC signaling pathways and may also affect GJIC. GJIC appeared dispensable during cavitation, TJ assembly, and PKC signaling. A better understanding of the interrelationships between different signaling mechanisms may help to improve embryo culture methods and viability.

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104 EFFECT OF FROZEN MEDIA ON IGFT EXPRESSION OF BOVINE EMBRYOS CULTURED ENTIRELY IN VITRO UNTIL DAY 14


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Freezing and stocking ready to use IVP medium, including hormones and fetal serum, is a practical alternative to rationalize work and reduce costs in in vitro embryo production. In our laboratory routine, embryo culture in frozen or fresh medium (produced weekly) has shown equal Day 7 blastocyst rates. Although morphological aspects were also similar, it is known that culture environment may alter gene expression patterns and embryo development on later stages. Thus, a preliminary study of mRNA expression of IGFT in Day 14 embryos produced in both culture conditions (frozen and fresh medium) was performed. For that, IVM (TCM 199 with hormones, antibiotics, t-glutamine and 10% fetal bovine serum) and IVC medium (SOFaaci) were split into 2-mL aliquots into Eppendorf tubes and frozen at −80°C temperature four weeks prior to use. The thawing was performed in a 4–5°C refrigerator overnight and the medium was stabilized in the incubator at least 4 h prior to use. Abattoir-derived oocytes were collected and randomly distributed into two culture groups: T1 (fresh IVM, fresh IVC) and T2 (frozen IVM, frozen IVC). On Day 7, blastocysts classified as Grades 1 and 2 (n = 12) were selected and continued in vitro culture in the Post Hatching Development system; PHD system (Brandão et al. 2004 Reprod. Fertil. Dev. 16, 123–124) under 38.5°C, 5% CO2 in air. On Day 14, elongated embryos from T1 and T2 were removed from culture and analyzed by RT-PCR. Total RNA of each group was prepared from two D14 embryos of two distinct replicates using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol with modifications. The reverse transcription (RT) was done using the EZ-First Strand cDNA Synthesis Kit (Biological Industries, Israel). The ß-actin gene was used as a constitutive control and PCR reactions for the two genes were carried out in triplicate using a PTC-100 MJ Research thermocycler. PCR products were electrophoresed on a 1.5% agarose gel. The two genes were detected in both samples; the relative expression of IGFT was higher in embryos cultivated in fresh medium (control). The consequences of IGFT levels in embryo development are still unknown, but highlight the late effects of culture conditions on embryonic gene expression. The perspective use of the PHD system might be an alternative embryo development for monitoring until further stages. In conclusion, IGFT appears to be a candidate marker gene for embryo development and, although frozen ready-to-use medium is a practical strategy, further studies on molecular trends are necessary to confirm its use.

105 EXPRESSION OF PLURIPOTENCY-DETERMINING FACTORS OCT-4 AND NANOG IN PRE-IMPLANTATION GOAT EMBRYOS


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The objective of this study was to determine the expression patterns of the pluripotency-determining factors, Oct-4 and Nanog, in pre-implantation goat embryos. The Pou/octamer-binding domain transcription factor Oct-4 and the homeobox transcription factor Nanog have been shown to play key roles in the maintenance of pluripotency in the inner cell mass (ICM) of pre-implantation mouse embryos and in embryonic stem cells. As Oct-4 protein has been observed in human, monkey, bovine, and porcine pre-implantation embryos, its role in embryonic development and differentiation may be conserved across these species. The patterns of mRNA expression for Oct-4 and Nanog have not been reported for ruminant embryos. In this study, total RNA was extracted from 10 in vivo-derived goat embryos at each stage (8-cell, morula, and blastocyst) using an Absolutely RNA Nanoprep Kit (Stratagene, La Jolla, CA, USA). The first-strand cDNAs were synthesized using Superscript III (Invitrogen, Carlsbad, CA, USA) and cDNAs were amplified with PfuUltra hotstart PCR master mix (Stratagene). Oct-4 primers were designed based on bovine Oct-4 open-reading sequence, while Nanog primers were designed based on the human Nanog open-reading sequence. Expression screening by PCR was performed. Oct-4 mRNA expression was detected at the 8-cell, morula and blastocyst stages. Sequencing of the 1.1-kb PCR product with Oct-4 primers revealed 87% homology to human cDNA sequence and 96% homology to the bovine sequence. Protein localization of Oct-4 as observed by immunocytochemistry was diffuse at the morula stage, but moved to a more nuclear location at the blastocyst stage. Oct-4 protein and mRNA expression were detected in both the ICM and trophectoderm of expanded blastocysts. This pattern of protein expression is similar to that reported by others in the pig and cow. As caprine, bovine, and porcine embryos all show extensive proliferation and elongation of the trophectoderm, continued expression of Oct-4 protein in the trophectoderm may be necessary to prevent premature differentiation of the trophectoderm. Nanog mRNA was detected at the morula and blastocyst stages. Nanog mRNA was detected in the ICM but not the trophectoderm of expanded goat blastocysts, a pattern that follows the expression observed in mice. Sequencing of the 698 bp PCR product obtained by RT-PCR from goat blastocysts confirmed that the mRNA detected was Nanog. Sequence alignment (ClustalW) showed that the cDNA sequence identities were 96% between goat and human and 70% between goat and mouse. The amino acid identities were 93% between goat and human and 52% between goat and mouse. To our knowledge this is the first report of detection of Nanog in domestic animals. These results are supportive of the premise that core components involved in the control of pluripotency are analogous across vertebrate species.
106 GENOMIC IMPRINTING OF IGF2R IN TISSUES OF BOVINE FETUSES GENERATED BY ARTIFICIAL INSEMINATION OR IN VITRO FERTILIZATION


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The insulin-like growth factor 2 receptor gene (IGF2R) is involved in fetal growth regulation. A study in sheep associated fetal overgrowth after in vitro embryo culture with abnormal DNA methylation and expression of IGF2R (Young et al. 2001 Nat. Genet. 27, 153–154). This suggested that abnormal IGF2R imprinting is a major cause of fetal overgrowth. To test this hypothesis in bovine fetuses, we developed a microsatellite marker for IGF2R from cdNA sequence data and screened 45 Day-80 fetuses generated in vitro, by artificial insemination (AI), or in vitro fertilization (IVF) procedures, for parent-of-origin-specific gene expression. A total of 17 fetuses were heterozygous, but available parental DNA samples showed that only 12 (8 AL 4 IVF) allowed unambiguous discrimination of parental alleles. Parent-of-origin-specific allelic expression patterns indicated that bovine IGF2R was expressed predominantly from the maternal allele and thus imprinted in fetal heart, kidney, liver, lung, muscle, and cotyledon tissue. However, the relative amount of expression from the paternal allele was tissue-specific and ranged from 6.4 ± 0.8% in skeletal muscle up to 27.4 ± 0.9% in cotyledon (SPSS or 11.5, ANOVA, P < 0.001). Tissues that originated from the same germ layer showed similar allelic expression ratios whereas significantly different expression ratios (P < 0.05) were observed between tissues originating from different germ layers. Contrary to expectations from sheep data, there was no evidence for gross abnormalities in IGF2R imprinting in tissues from overgrown (n = 2) or normal sized (n = 2) IVF fetuses. However, relative paternal expression levels in several tissues showed significant relationships (P < 0.05–0.001) with growth parameters and pointed to subtle changes in paternal IGF2R expression in overgrown IVF fetuses.

We thank W. Scholz and M. Weppe rt for excellent technical assistance.

107 INITIAL RESULTS FROM MALE GERM CELL TRANSFER BETWEEN CATTLE BREEDS


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Male germ cell transfer has produced offspring in mice (Brinster & Zimmermann 1994 PNAS 91, 11 298–11 302). Recently the first livestock animal, a goat, was produced (Honaramooz et al. 2003 Mol. Reprod. Dev. 64 422–64 428.), and early results in cattle are promising (Izadyar et al. 2003 Reproduction 126, 765–774; Oatley et al. 2002 J. Anim. Sci. 80, 1925–1931). We have assessed the outcome of male germ cell transfer between breeds of cattle and the efficacy of two vital dyes as markers of donor cells following transfer. Testis cells from three Bos taurus (Angus) bull calves were used as donor cells to transfer into six Bos indicus cross (predominantly Brahman bloodline) bull calves. Each of the calves was prepubertal and aged between 5 and 7 months. The calves were castrated; then a single-cell suspension of testis cells was prepared enzymatically using collagenase, DNAase, and trypsin. Prior to transfer into the recipient calves, the testis cell suspensions were dyed with one of two long-term vital dyes (PKH26 or CFDA). Approximately 300 million cells were injected into the rete of each testis under ultrasonographic guidance. In four of the six recipients, CFDA was injected into one testis and PKH26 into the other. These four recipients were castrated at 2, 4, 6, and 8 weeks after transfer. The other two recipients received either CFDA or PKH26 into both testes and were castrated at 8 weeks after transfer. Following castration, PKH positive donor cells were found in freshly isolated tubules of each of the five recipients that received PKH-dyed cells, while no CFDA-positive donor cells were conclusively identified in any of the recipients. In the freshly isolated tubules, clumps of PKH-positive donor cells were observed, which indicated either cell division or substantial local colonization of certain areas of the tubules. Frozen sections were used to further localize the PKH positive donor cells. Positive cells were located on the seminiferous tubule basement membrane, which indicates these cells had successfully migrated from the tubule lumen and were likely to be spermatagonia. There was variation in the amount of fluorescence for individual cells, which indicated either cell division or variable uptake of the stain during the staining procedure. We were disappointed to find no conclusive evidence of CFDA stained cells as we encountered high background fluorescence from the majority of testis cells. Although this fluorescence was quenched within 10 s, we were unable to find positive cells with any certainty. We have concluded that PKH26 was more suitable for labeling donor testis cells and that donor cells can be identified for at least 2 months following transfer. Each of the recipients that received PKH26 stained cells retained these cells in the tubule epithelium, which suggests that transfer between different animals, and indeed between breeds, can be achieved. Further studies will aim to demonstrate that donor cells are able to undergo spermatogenesis in the recipient animals.

108 THE ROLE OF NITRIC OXIDE SYNTHASE IN IN VITRO DEVELOPMENT OF BOVINE OOCYTES AND PRE-IMPLANTATION EMBRYOS


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Nitric oxide (NO) is a free radical that serves as a key-signal molecule in various physiological processes including reproduction. Four isoforms of nitric oxide synthase (NOS) have been characterized: endothelial (eNOS), inducible (iNOS), neuronal (nNOS), and mitochondrial (mtNOS). The
The aim of the present study was to examine transient expression of transgene injected into nuclei of rat 2-cell stage embryos. We also investigated the localization of the protein in pre-implantation embryos. Oocytes and embryos were grown in the media with NOS inhibitor added at a level of 0 mM (control), 1 mM, and 10 mM to either maturation or culture medium. Each experiment was conducted in four replicates each containing 100 oocytes for IVP. Cleavage and blastocyst rate were recorded at Days 2 and 7, respectively. Data were analyzed using the General Linear Model in SAS version 8.02 (SAS Institute, Inc., Cary, NC, USA) with the main factors being the level of 1-NAME and the point of application. Pairwise comparisons were done using the Tukey test. Protein localization in bovine oocytes and embryos was performed by immunocytochemistry using eNOS- and iNOS-specific antibodies. Embryos were fixed in 3.7% paraformaldehyde, permeabilized in 0.1% Triton-X100, and washed three times in PBS supplemented with BSA. They were incubated with eNOS and iNOS primary antibody (1:200 dilutions) and washed before incubation with secondary antibody conjugated to FITC. After washing they were mounted on glass slides and examined under a confocal laser scanning microscope (Carl Zeiss Jena, Carl Zeiss AG, Oberkochen, Germany). In the controls the primary antibodies were omitted. As shown in the table below, the presence of 1-NAME in the maturation medium significantly reduced the cleavage and blastocyst rate independent of the dosage applied. However the presence of 1-NAME in the culture medium had an influence only on the blastocyst rate. The immunocytochemical staining results showed that both eNOS and iNOS are expressed in the cytoplasm of the MII oocytes, and during the pre-implantation stage the fluorescence signal was observed in nuclei and cytoplasm. However, the nuclear signal was much weaker. In conclusion, the present study is the first to determine the role of NO in bovine oocyte maturation and preimplantation embryo development. These results indicate that nitric oxide may play an important role as a diffusible regulator of bovine oocyte maturation and preimplantation embryo development.

### Table 1. Effect of 1-NAME addition in maturation or culture medium on embryo development

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Maturation medium</th>
<th>Culture medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cleavage rate (%)</td>
<td>Blastocyst rate (%)</td>
</tr>
<tr>
<td>0 mM</td>
<td>83.7 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.5 ± 2.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1 mM</td>
<td>68.9 ± 6.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.9 ± 2.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>10 mM</td>
<td>69.4 ± 2.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.2 ± 1.3&lt;sup&gt;l&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values with different superscript within columns are significantly different (P < 0.05).

### 109 A BRIDGE OF SPERM TAIL BETWEEN BLASTOMERES ENHANCED PROTEIN MIGRATION IN THE RAT TWO-CELL STAGE EMBRYOS

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The aim of the present study was to examine transient expression of transgene injected into nuclei of rat 2-cell stage embryos. We also investigated the relationship between expression in both blastomeres and tail position of penetrated spermatozoa in rat 2-cell stage embryos. Rat 2-cell stage embryos were recovered from superovulated Wistar females mated with same strain mature males at 48 h after hCG injection. DNA fragments, as the transgene containing the EGFP (enhanced green fluorescent protein) gene controlled under the CMV-IE promoter, were microinjected into one nucleus of 2-cell stage embryos. After microinjection, embryos were cultured in KRB at 37.0°C in a 5% CO2 and 95% humidified air until observation. First, transient EGFP expression in 151 injected embryos was observed using a fluorescence microscope at 6 h intervals until 48 h after injection. At 6 h after microinjection fluorescent embryos were detected, and the proportion of fluorescent embryos increased over time. The rate reached maximum (84%, 52/62) at 24 h after microinjection, and several fluorescent patterns of fluorescent blastomeres in the embryos were observed. There were blastomeres with the same or different fluorescence levels and a single fluorescent blastomere. Second, to assess tail position of the penetrated sperm in the fluorescent embryos, 75 whole mount specimens were observed by inverted phase-contrast microscopy at 24 h after the injection. Also, parthenogenetic 2-cell stage embryos that never contained sperm tail were microinjected with the transgene and observed in the same manner. To obtain parthenogenetic 2-cell embryos, 80 ovulated ova were collected from non-mated females, and incubated with 2 mM 6-DMP for 4 h. The ova were additionally cultured for 20 h in KRB at 37.0°C in a 5% CO2 and 95% humidified air. In embryos with both blastomeres fluorescent (94%, 33/35), the sperm tail existed in both blastomeres like a bridge between blastomeres. In contrast, in one embryo with a single fluorescent blastomere (4%, 1/24), the sperm tail existed in both blastomeres, and in other embryos with a single fluorescent blastomere (75%, 18/24), the sperm tail was positioned in the one blastomere. On the other hand, in 63 parthenogenetic rat 2-cell embryos in which there was no sperm tail, most embryos (86%, 54/63) had a single fluorescent blastomere at 24 h after microinjection. The results indicated that the sperm tail position in the 2-cell embryos makes the protein migration variable. In conclusion, when the CMV-IE/EGFP gene was microinjected into nuclei of rat 2-cell embryos, 86% (54/63) of a sperm tail in both blastomeres may influence EGFP distribution.

### 110 NUCLEAR LAMIN A/C EXPRESSION IN BOVINE IVP EMBRYOS

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Lamin A/C expression is generally associated with terminally differentiated cell types; however, numerous conflicting reports in the literature demonstrate the presence of lamin A/C in pluripotent cells of pre-implantation embryos. This study characterized lamin A/C expression in bovine
pre-implantation embryos using two monoclonal lamin A/C antibodies: anti-A/C IgM (A/C1) and anti-A/C IgG (A/C2) (Santa Cruz, California). Bovine embryos were produced as previously described (Foulad-Nashta et al. 1998 Biol. Rep. 59, 255–262) and collected at various stages for immunofluorescence staining. Embryos were fixed in 100% methanol at −20°C for 20 min and then blocked for 1 h (4% goat serum in PBS) at RT. Samples were then incubated overnight at 4°C with mouse lamin A/C antibodies or with blocking solution as a control. Following the primary incubation, embryos were washed extensively in 1% BSA in PBS and then incubated with rabbit anti-mouse immunoglobulins (1:20) (DAKO, Denmark) for 1 h at RT. Unbound secondary antibody was removed with washing in 1% BSA in PBS, and embryos were counter-stained with 4′,6-diamidino-2-phenylindole (2 μg/ml). Bovine fetal fibroblasts (BFF1) and human embryonic teratocarcinoma cells (EC1 and EC2) were processed identically to the embryos and used as positive and negative controls, respectively (Stewart and Burke 1987 Cell 51, 383–392). Images were viewed using epifluorescence (Leica DMR, Germany) and confocal microscopy (Leica TCS). BFF1 cells reacted with both lamin A/C1 and A/C2 antibodies. EC1 and EC2 stained positively for A/C2 whereas A/C1 was negative in both. All germinal vesicle (GV)-stage oocytes stained strongly for A/C2; however, for A/C1 only 67.5% were positive, and staining intensity was variable. Metaphase II oocytes stained negatively for both antibodies. One-cell zygotes exhibited a variable staining pattern similar to that of GV-stage oocytes. In contrast, all embryos from the 2-cell to blastocyst stage were negative for A/C1 but positive for A/C2. Our observations in embryos and EC cells indicate that the mouse anti-lamin A/C1 is specifically binding to lamin A/C whereas A/C2 is cross-reacting with other nuclear envelope proteins, possibly lamin B1/B2. The cross-reactivity of A/C2 has led to contradicting results in previous reports on lamin A/C expression in pre-implantation embryos. Our results with A/C1 show that lamin A/C is present in GV oocytes and 1-cell zygotes, suggesting that lamin A is important for pronuclear formation after fertilization. These results suggest that active remodelling of the nuclear envelope occurs during the early stages of bovine embryo development.

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112 THE PRESENCE OF LAMIN A/C ANTIGENS IN PORCINE EMBRYOS

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Nuclear laminas are components of the nuclear lamina, and their primary role is to support the nuclear envelope and provide anchorage sites for the chromatin. While type B laminas are expressed in all cells, type A laminas (including laminas A and C) are developmentally regulated and expressed in differentiated cells only. There are conflicting results about the presence of lamin A/C in early mammalian embryos. Lamin A/C was found to localize in the nuclear envelope of bovine, pig, and mouse embryos, while recently it has been reported that early mouse and bovine embryos lacked lamin A/C antigens. It has also been suggested that the existence of lamin A/C in the pronuclei of mouse and bovine nuclear transfer embryos indicated faulty reprogramming. The aim of this study was to investigate the presence of lamin A/C in porcine embryos of different origins (in vivo, parthenogenetic, and nuclear transfer). Embryos of various developmental stages were collected from inseminated gilts. For the production of parthenogenetic embryos, mature oocytes were electroporated and cultured for up to seven days. Fibroblast cells served as differentiated controls; progenitor cells from the olfactory bulb of a porcine fetus were used as undifferentiated controls. Lamin A/C was visualized by immunocytochemistry. Olfactory bulb progenitor cells lacked lamin A/C (0 out of 50 cells showed staining) while all fibroblast nuclei (n = 50) reacted positively with
Developmental Biology

113 THE DISTRIBUTION OF THE LEPTIN PROTEIN WITHIN BOVINE OOCYTES AND PRE-IMPLANTATION EMBRYOS MATURED AND FERTILIZED IN VITRO

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It has recently been documented that leptin regulates processes linked to reproduction including preimplantation development, embryonic implantation (trophoblast invasion), and fetal growth. Transcripts for the leptin gene (\textit{LEP}) and the leptin receptor gene (\textit{LEPR}) have been identified in ovary, testis, placenta, endometrium, ovarian follicles, and oocytes, and also in rat, mouse, and bovine pre-implantation embryos. Moreover, the leptin protein was detected in mouse and human oocytes and embryos, and its localization was polarized. The distribution of regulatory proteins within oocytes and pre-implantation embryos is critical for early mammalian development, such as determination of the animal pole and the establishment of the trophoblast and the inner cell mass cells (ICM). So far there is no published evidence concerning this phenomenon in bovine oocytes and embryos. Therefore, the aim of this work was to analyze the leptin protein distribution within bovine oocytes and preimplantation embryos matured and fertilized (\textit{in vitro}). The material for this work consisted of oocytes collected from slaughterhouse ovaries and sperm collected from AI bulls. \textit{In vitro} oocyte maturation and fertilization were carried out according to the method described by Makarevich and Markkula (2002 Biol. Reprod. 66, 386–392). The preliminary experiment of leptin protein localization by immunofluorescent staining included immature and matured oocytes and blastocysts. Oocytes and embryos were fixed in PBS containing 4% paraformaldehyde and reacted with affinity-purified polyclonal rabbit primary antibody directed against leptin (0.1 mg/mL; Ob Y20, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); and then exposed to secondary goat-anti-rabbit antibody (1.0 mg/mL; Santa Cruz Biotechnology Inc.)-fluorescein isothiocyanate (FITC) conjugate. Finally, chromatin was visualized by propidium iodide staining (0.5 μg/mL). Slides were examined under a conventional fluorescence microscope (Nikon) and confocal microscope (Zeiss). The preliminary results demonstrate that the distribution of leptin differed between immature and mature oocytes: it was spherical in immature oocytes (a rim beneath the oolemma) whereas it became evenly distributed after maturation. In blastocysts, leptin signals were present in both the trophoblast cells and in the ICM cells. This is in contrast with studies on mouse embryos which showed the presence of the LEP protein in the trophoblast only. Future experiments will include studies of embryos at the 2-cell, 4-cell, 8–16-cell, and morula stages. The present study for the first time shows the pattern of leptin protein distribution within bovine oocytes and preattachment embryos.

114 PORCINE EMBRYO FRAGMENTATION, DEVELOPMENT AND APOPTOSIS: A CONFOCAL MICROSCOPY STUDY

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The relationship between embryonic fragmentation, embryonic arrest, and apoptosis has been the subject of some controversy (Hardy K 1999 Rev. Reprod. 4, 125–134). In order to investigate possible links, \textit{in vivo}-produced, \textit{in vitro}-cultured porcine embryos (n = 132) were scored for developmental stage and fragmentation at 7 days post insemination (dpi) and processed for propidium iodide and annexin V labelling. After fixation, embryos were processed for terminal deoxynucleotidyl transferase mediated dUTP nick end labelling (TUNEL). Using confocal microscopy, a cell was categorized apoptotic if (i) it had a fragmented or condensed nucleus, (ii) the cell membrane was annexin V-positive, and (iii) the nucleus was TUNEL labelled. An apoptotic cell ratio (ACR) was determined as the percentage of apoptotic cells per embryo. Differences in the % of fragmented and apoptotic embryos and correlations were analyzed using chi-square. Logistic regression was used to compare the average fragmentation % and the ACR. Sixty-one embryos (46%) arrested during the culture period, with 8 embryos arresting before or at the 4-cell stage. Significantly more arrested embryos were fragmented compared to embryos that were blastocysts at 7 dpi. Also, the average fragmentation percentage was significantly higher for arrested embryos compared to blastocysts. The correlation detected between developmental arrest and fragmentation was 0.60 (P < 0.05). None of the embryos without fragmentation had cells categorized as apoptotic, whereas 30 out of 55 embryos with fragmentation possessed apoptotic cells, which led to a correlation of 0.87 (P < 0.01) between fragmentation and apoptosis. The percentage of embryos with apoptotic cells was significantly higher for embryos arrested during the 5-cell to the morula stage compared to embryos that arrested before or at the 4-cell stage and embryos with blastocyst development at 7 dpi. The average ACR of embryos arrested during the 5-cell to the morula stage was significantly higher compared to the average ACR of blastocysts at 7 dpi. The correlation detected between the developmental arrest, during the 5-cell to the morula stage period and apoptosis was 0.57 (P < 0.01). Taken together, significant correlations between fragmentation, developmental arrest and apoptosis were detected. However, the association between embryonic arrest and apoptosis could be established only for embryos arrested after embryonic genome activation.
This work was supported by Wakayama Prefecture Collaboration of Regional Entities for the Advancement of Technological Excellence of the JST, and by a Grant-in-Aid for the 21st Century COE Program of the Japan MEXT.

115 CYTOLOGICAL ANALYSIS OF HEPATIC GENE EXPRESSION AND IMMUNOLOGICAL RESPONSE OF MHC ANTIGENS IN MOUSE AMNIOTIC EPITHELIAL CELLS

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Human amniotic epithelial cells (hAECs) have been reported to have unique properties. They express almost no class I and class II MHC antigens and lack response to interferon-\(\gamma\) (IFN-\(\gamma\)) which mediates the expression of those MHC molecules. Moreover, hAECs express some genes characteristic of hepatic cells. Therefore, hAECs seem to have multipotency and are expected to substitute for hepatic tissues in part. We aimed to develop the experimental model for investigating AECs in mice (mAECs). In this study, we examined the induction of MHC molecules by IFN-\(\gamma\) and the hepatic gene expression in mAECs. Murine amniotic membranes were collected from C57BL/6J females at 17.5 days of gestation. They were digested by 0.03% hyaluronidase followed by 0.2% collagenase treatment. Dissociated mAECs were cultured on dishes in DMEM supplemented with 10% FBS at 37\(\degree\)C under 5%CO\(_2\) in air. Embryonic fibroblasts (EFs) collected from C57BL/6J fetuses at 13.5 dpc were cultured in the same condition as mAECs. In Experiment I, the effect of IFN-\(\gamma\) on induction of MHC molecules in mAECs was examined. mAECs and EFs cultured in the presence or absence of IFN-\(\gamma\) at 1 \times 10\(^{3}\) \(\mu\)l\(^{-1}\) for 72 h were recovered and incubated with FITC-conjugated antibodies against mouse H-2 MHC class I or I-A/I-E MHC class II antigens. The cells were analyzed by flow cytometry. In Experiment II, the expression of the genes in mAECs was examined by RT-PCR. mRNA was purified from adult liver, EFs, fresh mAECs, and mAECs cultured for 5 days. As the genes characteristic for hepatic cells, HNF-3\(\alpha\), HNF-3\(\beta\), HNF-3\(\gamma\), HNF-4, transthyretin (TTR), albumin, \(\alpha\)-fetoprotein (AFP), glucose-6-phosphatase (G6P), and asialoglycoprotein receptor-1 (Asgr1) were examined. In Experiment I, cell-surface expression of class I and class II MHC antigens in response to IFN-\(\gamma\) was observed weakly in mAECs as compared to EFs, suggesting different property in hAECs which lack the expression of those antigens. In Experiment II, RT-PCR analysis showed that all of the genes except G6P were expressed in fresh mAECs. However, the expression of transcription factors such as HNF-3\(\alpha\), HNF-3\(\beta\), HNF-4, and TTR, serum proteins such as albumin and AFP, and Asgr1 decreased after in vitro culture, contrary to the case of hAECs in which, for example, albumin appeared after cultivation. In conclusion, it was evident that mAECs have quite different properties, both in the inducitivity of MHC molecules and the expression of hepatic genes, from hAECs.

This work was supported by Wakayama Prefecture Collaboration of Regional Entities for the Advancement of Technological Excellence of the JST, and by a Grant-in-Aid for the 21st Century COE Program of the Japan MEXT.

116 ACTIVE METHYLATION AND ACETYLATION OF HISTONE H3-K9 IN MOUSE EMBRYO WITH DIFFERENT PROPORTIONS OF MATERNAL AND PATERNAL GENOME

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Epigenetic modification of parental genomes plays a prominent role in regulating genome expression in the early development of embryos. In general, histone H3 of the paternal genome is demethylated at lysine 9 (H3-K9) during the first and second mitotic divisions in fertilized embryos, while the maternal genome is methylated. We investigated the effects of maternal genomes (Mgen) and paternal genomes (Pgen) on H3-K9 methylation and acetylation during the early development of murine embryos. Histone H3-K9 methylation and acetylation were detected by anti-trimethyl histone H3-K9 and anti-triacetyl histone H3-K9 antibodies. The following embryos were used in this study: (1) intracytoplasmic sperm injection (ICSI) embryo (about 25% Mgen, about 75% Pgen); (2) parthenogenetic diploid embryos (100% Mgen, 0% Pgen); (3) somatic nuclear transfer embryos (50% Mgen, 50% Pgen); (4) ICSI embryos with fragmentation (about 25% Mgen, about 75% Pgen). Each experiment was repeated five times to obtain more than 120 embryos per group. Our results show that: (1) in the ICSI embryo, histone H3 methylation occurs in Mgen but not in Pgen at the first and second mitotic divisions; (2) in the parthenogenetic embryo, histone H3 methylation occurs in both nuclei at the first and second mitotic divisions; (3) in the somatic nuclear transfer embryo, histone H3 is methylated in all of the nuclei at the first and second mitotic divisions; (4) in the androgenetic embryo, methylated H3-K9 is detected weakly in the heterochromatin enclosed around the nucleolus of the one-cell embryo, and methylated in the entire nuclei of the two-cell embryo; and (5) in the haploidized somatic and sperm embryo, the pattern of histone H3-L9 methylation resembles that of the ICSI embryo. While histone H3-K9 acetylation occurs in both paternal and maternal genomes during interphase, even when the nuclear membrane is completely degraded and the chromosome is condensed, it disappears rapidly when the chromosome enters the real metaphase, and reappears at the early stage of pronuclear formation in all types of embryo. These results suggest that the absence of maternal genomes results in histone H3-K9
methylation in the paternal genomes during the first and second mitotic divisions of embryos in mice. In addition, histone H3-K9 acetylation is independent of the presence or absence of maternal or paternal genomes during pre-implantation development in mice.

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117 CYTOPLASMIC FACTORS INFLUENCE DEVELOPMENTAL POTENTIAL OF SAMP1/Yit MOUSE EMBRYOS

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Nuclear transplantation is an efficient means to investigate nucleo-cytoplasmic interactions of mammalian embryos during early development. A recent study has shown that the developmental potential of embryos is affected by the type of cytoplasm. The SAMP1/Yit mouse, an inbred strain that develops spontaneous chronic ileitis resembling Crohn’s disease (Matsumoto S 1999 Bioscience Microflora 18, 1–9), has poor reproductive performance, and the developmental ability of embryos is low (unpublished data). Therefore we need to enhance productivity of the SAMP1/Yit mouse. Recently it was reported that cytoplasm of F1 mouse egg supported the development of embryos which have low developmental ability (Muggleton-Harris A et al. 1982 Nature 299, 460–462). In the present study, we examined the influences of the nucleus and cytoplasm on the development of reconstructed embryos in vitro and in vivo, using reciprocal nuclear transplantation between SAMP1/Yit and B6P1F1 (C57BL/6J × SAMP1/Yit) mouse embryos. We evaluated the developmental ability of reconstructed embryos by the development rate into blastocysts in vitro and by the rate of offspring after transfer of blastocysts to recipient mice. Pronuclear transplantation was carried out as reported previously (McGrath J and Solter D 1983 Science 220, 1300–1302). Briefly, karyoplasts from one-cell SAMP1/Yit embryos were introduced into enucleated B6P1F1 zygotes (SAMP1/B6P1F1) and fused by addition of inactivated HVJ (2700 U L\(^{-1}\)). The other group of reconstructed embryos (B6P1F1/SAMP1) was manipulated similarly. After fusion, reconstructed embryos were cultured in drops of KSOM medium for 120 h at 37°C in 5% CO\(_2\) in humidified air. Some reconstructed and control (unmanipulated) embryos that developed to the blastocyst stage were transferred to the uteri of recipient mice. Data were compared using chi-square test; differences were considered significant at \(P < 0.01\). The development rate of [SAMP1/B6P1F1] embryos to the blastocyst stage was significantly (\(P < 0.01\)) higher (75.0%) than that of SAMP1/Yit controls (39.1%). The rate of offspring in [SAMP1/B6P1F1] was also significantly (\(P < 0.01\)) higher (47.5%) than that of SAMP1/Yit controls (22.1%). On the other hand, [B6P1F1/SAMP1] embryos showed low developmental potential compared to B6P1F1 control embryos. These results indicate that the source of the cytoplasm strongly influences the development of reconstructed embryos containing SAMP1/Yit karyoplasts.

Table 1.

<table>
<thead>
<tr>
<th>Group</th>
<th>Karyoplast</th>
<th>Cytoplasm</th>
<th>No. of blastocysts developed (%)</th>
<th>No. of offsprings transferred (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (unmanipulated)</td>
<td>SAMP1</td>
<td>SAMP1</td>
<td>77/197 (39.1)</td>
<td>15/68 (22.1)</td>
</tr>
<tr>
<td>Control (unmanipulated)</td>
<td>B6P1F1</td>
<td>B6P1F1</td>
<td>118/138 (85.5)</td>
<td>57/87 (65.5)</td>
</tr>
<tr>
<td>Reconstructed</td>
<td>SAMP1</td>
<td>B6P1F1</td>
<td>69/92 (75.0)*</td>
<td>29/61 (47.5)*</td>
</tr>
<tr>
<td>Reconstructed</td>
<td>B6P1F1</td>
<td>SAMP1</td>
<td>56/87 (64.4)</td>
<td>20/56 (35.7)</td>
</tr>
</tbody>
</table>

\*Significantly different from corresponding column of control (SAMP1) at \(P < 0.01\).

118 TRANSFER OF IRON FROM MOTHER TO FETUS IN WATER BUFFALO: ERYTROPHAGOCYTOSIS AND UTEROFERRIN


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The transplacental transport of iron by uteroferrin (Uf), and the hemorphagous areas in the water buffalo placenta were analyzed to clarify the mechanism of blood extravasation in the maternal-fetal interface with consequent transfer of iron to the fetus through the trophoblastic erythrophagocytosis (Murai and Yamauchi 1986 Nippon Juigaki Zasshi 48, 75–88) and in the endometrial glands (Bazer et al. 1991 Exp. Hematol. 19: 910–919). In the water buffalo placenta this mechanism remains unclear; uteroferrin is very important in this process because it is both an iron transporter and a progesterone-induced hematopoietic growth factor. Our objective was to characterize these hemorphagous areas and the endometrium of the water
This work was funded by FAPESP.

A chimaera is an organism composed of cells derived from two (or more) zygotes. Spontaneously originated diploid-triploid (2n-3n) chimaeric embryos were created by aggregation of diploid embryos with triploid embryos at 4–8 cell stage. In chimaeras created according to this procedure, the triploid component was agouti and produced the 1B1B isoform of glucose phosphate isomerase (GPI) and the diploid component was albino and produced the GPI-1A1A isoform. Electrophoresis of GPI was performed in order to determine the contribution of both populations of cells in tissues of embryos and individuals. Over a thousand oocytes were subjected to triploidization. A total number of 201 diploid-triploid chimaeric embryos were created by aggregation of diploid embryos with triploid embryos at 4–8 cell stage. 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Early Pregnancy/Pregnancy Recognition

Reproduction, Fertility and Development

121 HEAT SHOCK TO PIG OOCYTES DOES NOT INDUCE APOPTOSIS BUT REDUCES EMBRYO DEVELOPMENT

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Oocytes are susceptible to heat shock (HS) during the maturation process. It has been demonstrated that HS induces apoptosis and/or the expression of HS protein 70 (hsp 70) in in vitro-produced oocytes and embryos. The objectives of this study were to analyze the effects of HS on the development and apoptosis of pig oocytes and embryos. Porcine ovaries were collected from a local slaughterhouse and the cumulus-oocyte complexes (COCs) were aspirated from follicles 3–6 mm in diameter and subjected to standard in vitro maturation procedures at 39°C for 42 h. The in vitro matured oocytes were then randomly allocated to different HS treatments at 41.5°C for 0 (control, C0h), 1 (HS1h), 2 (HS2h), or 4 h (HS4h). An additional control group of oocytes was cultured for 4 h without HS (C4h). Data were analyzed by chi-square test. In Experiment 1, anti-hsp 70 (SPA-810AP, Stressgen, San Diego, CA, USA) and Western blotting were used to examine the expression of hsp 70. Results indicated that no significant difference of hsp 70 expression in metaphase II porcine oocytes occurred between controls and HS groups (P > 0.05, 7 replicates). In Experiment 2, apoptosis of metaphase II oocytes after HS was identified by annexin V-FITC (Sigma, St. Louis, MO, USA) staining and TUNEL assay (Roche, Indianapolis, IN, USA). No significant apoptotic signal was detected in the HS groups compared to the controls. The intensity of annexin V staining was not affected by HS, but it increased with the time of culture (P < 0.05, n = 24–37). In Experiment 3, the apoptotic rate and developmental competence of the HS-oocytes were evaluated by TUNEL assay (n = 123–137, 4 replicates). Parthenogenetic activation (n = 123–137) was performed by an electric pulse (2.2 kV/cm−1) combined with 6-dimethylaminopurine treatment (6-DMP, 2.5 μM, 4 h, Sigma). The cleavage rates in HS2h (43 ± 29%) and HS4h (35 ± 28%) decreased (P < 0.05) compared to those in C0h (62 ± 12%) and C4h (66 ± 8%). In addition, the blastocyst formation rates and total cell numbers reduced (P < 0.05) after 2 h (11 ± 10%, 20 ± 16) and 4 h (11 ± 8%, 19 ± 8) of HS treatments compared to those in C0h (23 ± 14%, 32 ± 22) and C4h (21 ± 11%, 27 ± 17), all respectively. The numbers of blastocysts with TUNEL-positive signals were not significantly different between the HS and control groups, but the signals increased (P < 0.05) before the 8-cell stage in HS groups (22–24%) compared to the C0h and C4h controls (16 and 11%), respectively. These results indicate that reduction in developmental competence of in vitro-matured pig oocytes after heat shock is not closely correlated to the expression of hsp 70 in the oocytes and to the apoptotic cell numbers in the blastocyst. Whether detection of apoptosis by TUNEL or annexin V-FITC in oocytes is a good indicator requires further investigation.

Early Pregnancy/Pregnancy Recognition

122 USE OF A DAY-14 EMBRYONIC ARRAY TO STUDY THE ELONGATION PHASE OF THE BOVINE EMBRYO

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In cattle, more than 30% of embryonic losses observed after artificial insemination (AI) have an early origin, coincident with a marked elongation of the trophoblast which occurs before implantation, between the 13th and 19th days of pregnancy. During this exponential growth phase, physiological interactions essential for pregnancy are established between the embryo and the uterus. Our work focuses on the identification of transcripts that regulate this key developmental period in several domestic species. For that, we generated a nylon membrane that contained 1920 gridded inserts originating from a Day-14 bovine embryo cDNA library (dBEST ID.15979; Hue et al., in preparation). Gene expression profiles in trophoblasts of increasing sizes were compared using ovoid (10–18-mm), tubular (50–60-mm), and early filamentous (140–150-mm) stages as control probes. Trophoblasts were collected and immediately snap-frozen. RNA extractions were performed using RNauplus (QuantumAppligene, Illkirch 67402, France). Due to the scarce amount of mRNA per embryo, amplified material was used to hybridize the array. For that, antisense-RNA (aRNA) and cDNA were generated starting from 1 μg of total RNA, as described by the MessageAmp aRNA kit instructions (Ambion,ustin, TX 78744, USA) and according to Revel et al. (1995 Zygote 3, 241–250). Five hundred nanograms of aRNA or cDNA were random-primed and labelled with 32P-alpha-dATP [aRNA, according to the procedure of Decraene et al. 1999 BioTechniques 27, 962–966; cDNA using the Atlas SMART Probe Amplification kit, (Clontech, Oxyme, Saint Quentin Yvelines 78053, France)]. For each protocol, two probes were generated independently and each of these probes was hybridized to four identical membranes according to Clontech instructions. These were then exposed to phosphorscreens and scanned after 7 days. Quantifications were done using ImaGene 5.1 (BioDiscovery, El Segundo, CA 90245, USA) and statistically analyzed.