The maturational stage of oocytes at the time of insemination has been suggested as influencing the sex ratio of resulting embryos (Dominko and Oocyte Maturation

### 274 NUCLEAR MATURATION KINETICS AND IN VITRO EMBRYO DEVELOPMENT OF BOVINE OOCYTES TREATED WITH BUTYROLACTONE I COMBINED OR NOT WITH ROSCOVITINE

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Cyclin-dependent kinase inhibitors (CDKIs) have been used for prematuration culture the aim at improving oocyte competence. However, CDKIs seem to accelerate nuclear maturation (Hashimoto et al., 2002 Biol. Reprod. 66, 1696–1701). The aim of the present work was to compare the effect of butyrolactone I (BLI) alone or combined with roscovitine (ROS) at low dose (Ponderato et al., 2001 Mol. Reprod. Dev. 60, 579–585) on nuclear maturation kinetics and embryo development. To assess maturation kinetics (Experiment 1), oocytes were cultured in 100 μM BLI (B) or 6.25 μM BLI + 12.5 μM ROS (BR) in TCM-199 for 24 h. After prematuration, oocytes were submitted to in vitro maturation (IVM in TCM-199 + 0.5 μg mL⁻¹ FSH, 50 μg mL⁻¹ LH, 10% FCS) for another 24 h. Oocytes were fixed every 3 h (40–50 oocytes/time point/group in 4 replicates) to assess nuclear status. In Experiment 2, oocytes were submitted to prematuration, but the inhibitors were diluted in TCM-199 or DMEM. IVM lasted 21 h in DMEM (same hormone supplementation as in TCM-199 + 5% FCS and 50 ng mL⁻¹ EGF). After IVM, all groups (140–150 oocytes/group in 7 replicates) were in vitro fertilized. Oocytes and sperm (2 × 10⁶ sperm cells mL⁻¹) were co-cultured for 18 h. Embryos were cultured in CR2aa in co-culture with granulosa cells for 8 days. All cultures were in microdrops under oil, at 38.5 °C under 5% CO₂ in air. In both experiments, control oocytes (C) were submitted only to IVM. Data were analyzed by GLM and GENMOD procedures (SAS program; SAS Institute, Inc., Cary, NC, USA), for Experiments 1 (4 replicates) and 2 (7 replicates), respectively. Cell numbers were analyzed by ANOVA and Tukey test. In Experiment 1, at 0 h, C and B oocytes were all (100%) at germinal vesicle stage (GV). BR had less GV oocytes (89% ± 1%, P < 0.05), indicating that BR was less effective in maintaining meiotic block for 24 h. After 3 h IVM, B and BR had less oocytes in GV (85 ± 2% and 80 ± 1%, respectively; P > 0.05) than C (100%, P < 0.05), suggesting an acceleration of oocyte maturation. At 12 h, however, most oocytes were at intermediate stages (metaphase I to telophase I) in all groups (78 ± 1–83 ± 2%, P > 0.05). After 21 and 24h, all groups had similar metaphase II (MII) rates (77 ± 1–89 ± 1% for 21 h and 85 ± 2–96 ± 8% for 24 h; P > 0.05). These results suggest that after 12 h, meiosis acceleration was less evident and oocytes proceeded nuclear maturation at similar rates. In Experiment 2, cleavage (79 ± 3–84 ± 3%, P > 0.05) and Day 7 blastocyst rates (26 ± 4–37 ± 4%, P > 0.05) were similar for all groups. After 8 days in culture, all groups presented similar blastocyst rates (35 ± 4–40 ± 4%, P > 0.05), except for the group prematured with BR in DMEM, which presented lower blastocyst rates (32.3 ± 4%) only when compared with C (40 ± 4%, P < 0.05). Hatching rates were similar (10 ± 3–16 ± 3%, P > 0.05) as were total cell numbers (141 ± 5–170 ± 10). In conclusion: (a) BR is less effective in maintaining meiosis block; (b) B and BR accelerate the first half of meiosis progression in about 3 h; and (c) BR used in DMEM during prematuration may negatively affect developmental rates.

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### 275 THE EFFECTS OF MATURATION CULTURE PERIOD ON THE SEX RATIO OF BOVINE IVF EMBRYOS

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The maturational stage of oocytes at the time of insemination has been suggested as influencing the sex ratio of resulting embryos (Dominko and First, 1997 Theriogenology 47, 1041–1050). However, there are very few reports concerning the relation between the maturation culture period of oocytes and the sex ratio of resulting embryos. The objective of this study was to investigate the effects of maturation culture period of oocytes on the sex ratio of bovine IVF blastocysts, using a novel technique of loop-mediated isothermal amplification (LAMP). Cumulus-oocyte complexes (COCs) were collected from ovaries of slaughtered cows. The COCs were cultured for various times (16, 22, 28 and 34 h) in maturation medium (TCM 199 supplemented with 5% fetal cow serum (FCS), 0.02 mg mL⁻¹ of follicle-stimulating hormone and 50 μg mL⁻¹ of gentamicin). After maturation culture for each period, the oocytes were inseminated with frozen-thawed spermatozoa (4 × 10⁶ spermatozoa mL⁻¹). After incubation with spermatozoa for 5 h, oocytes were transferred into culture medium (TCM199 supplemented with 5% FCS, and 5 μg mL⁻¹ of insulin) and cultured for 7 days at 38.5 °C in a humidified atmosphere of 5% CO₂ in air. The zona pellucida of blastocysts collected on Day 7 after insemination (Day 0) was removed by brief exposure to 0.2% pronase, and the sex determination of embryos was conducted by the LAMP method, using a bovine embryo sexing kit (Eiken Chemical Co., Ltd., Tochigi, Japan). The rates of blastocyst formation were analyzed by ANOVA, and the sex ratios of embryos were compared by chi-square analysis. As shown in Table 1, the rate of blastocyst formation after insemination was significantly higher (P < 0.05) in the oocytes matured for 22 h than in the oocytes matured for 16, 28, and 34 h. The proportion of male blastocysts derived from oocytes matured for 34 h was significantly higher (P < 0.05) than from oocytes matured for 16 and 22 h. Moreover, the proportions of male blastocysts increased with delaying insemination. These results indicate that skewing of the sex ratio of IVF blastocysts is significantly influenced by the maturation culture period of oocytes.
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276 THE EFFECT OF CUMULUS CELLS DURING MATURATION ON THE RISE IN THE CONCENTRATION OF INTRACELLULAR Ca²⁺ ([Ca²⁺]i) OF PORCINE OOCYTES INDUCED BY INOSITOL 1,4,5-TRIPHOSPHATE

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Increase of inositol 1,4,5-triphosphate (IP3) in the cytoplasm of mammalian oocytes is said to be responsible for [Ca²⁺]i oscillation observed in the oocytes immediately after sperm penetration, and the [Ca²⁺]i oscillation is known to be essential for the development of embryos. On the other hand, cumulus cells have been reported to play an important role in cytoplasmic maturation of oocytes and affecting the embryonic development. To obtain more information about the role of cumulus cells in cytoplasmic maturation, the effects of cumulus cells during maturation on the rise in [Ca²⁺]i and on the rate of activation of porcine mature oocytes induced by IP3 injection were investigated. The immature porcine oocytes were divided into three groups: COCs (intact cumulus-oocyte complexes), DOs (oocytes denuded of their cumulus cells), Co-culture (DOs attached to separated cumulus cells). These groups of immature oocytes were cultured in NCSU23 46 h for maturation. To examine the function of cumulus cells, two groups of immature oocytes were also prepared: DOs + pyruvate (DOs put into NCSU23 with pyruvate) and COCs-glucose-free (COCs put into NCSU23 without glucose). The mature oocytes from each group were loaded with Ca²⁺ indicator fluorescent dye Fura2-AM, and then were irradiated by 340 nm and 360 nm ultraviolet immediately after the injection of IP3. The intensities of emission light caused by the irradiation of 340 nm and 360 nm ultraviolet were recorded as E340 and E360. Since coupling of Ca²⁺ and the dye intensifies E340, but does not change E360, the level of [Ca²⁺]i was shown as R (ratio = E340/E360) in this study. Activation rate was calculated by counting the number of the oocytes that formed pronuclei by injection of IP3. ANOVA and Student’s t-test were used in this study. Transient rise in [Ca²⁺]i was observed in the mature oocytes from every group. The peak R of the rise in [Ca²⁺]i of the mature oocytes derived from COCs, DOs, and Co-culture and induced by IP3 were 7.2, 4.0, and 6.9, respectively. The R of DOs was significantly lower than those of the others (P < 0.05). Also, the activation rate of the mature oocytes from DOs was significantly lower than those from COCs and Co-culture (31, 66, and 66%). The mature oocytes from DOs + pyruvate showed the same level of peak R compared with those from COCs (7.4 and 6.3), but COCs-glucose-free showed a slight but significantly lower peak R compared with the mature oocytes from COCs (6.0 and 7.4, P < 0.05). In conclusion, cumulus cells appeared to support the rise in [Ca²⁺]i of porcine oocytes induced by IP3 during maturation and the following activation. Moreover, a function of cumulus cells supposedly produces pyruvate by metabolizing glucose and provides it to oocytes during maturation for promoting the cytoplasmic maturation.

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277 NUCLEAR STAGE AND p34cdc2 EXPRESSION IN DIFFERENT SIZES OF PREPUBERTAL GOAT OOCYTES

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Developmental competence of prepubertal (1- to 2-month old) goat oocytes is compromised, probably because of an incomplete cytoplasmic maturation. Oocyte selection for IVM-IVF is based on morphological criteria. The main regulator of oocyte nuclear maturation is maturation promoting factor (MPF), and it could also be involved in cytoplasmic maturation. The present study tried to determined p34cdc2 (the catalytic subunit of MPF) expression in oocytes of different sizes before and after IVM. Prepubertal goat oocytes were matured in a conventional IVM medium (TCM199 with serum, hormones, and cysteamine) for 27 h. At collection time, a sample of oocytes was classified into 4 groups according to their diameter (<110 μm; 110–125 μm; 125–135 μm; and >135 μm), and nuclear stage was evaluated. After IVM, oocytes were classified by diameter (as described before) and stained to analyze nuclear stage. Before and after IVM, a sample of 10 oocytes of each diameter group was frozen at −80°C. These oocytes were used to perform the detection of p34cdc2-RNA by RT-PCR. Briefly, RNA of 10 oocytes was extracted with TRIReagent (Sigma-Aldrich, Madrid, Spain), and used to perform RT using the Thermoscript kit (Invitrogen, Barcelona, Spain). The cDNA corresponding to two oocytes was amplified by PCR, and each amplification band was measured by densitometry using the Quantity One PC program. Rabbit globin
mRNA was used as an extrinsic control of the whole process. Nuclear stage data were analyzed by Fisher’s exact test, and RT-PCR data were analyzed by one-way ANOVA test. At collection time, a high percentage of prepubertal goat oocytes had resumed meiosis. After IVM, the percentage of MII-oocytes was higher in larger-sized oocytes. At collection time, significantly higher p34cdc2-RNA expression was found in 125–135 µm oocytes. At collection time, a high percentage of prepubertal goat oocytes had resumed meiosis. After IVM, the percentage of oocytes of different diameters showed no difference in p34cdc2-RNA expression.

In contrast, an increase of p34cdc2-RNA was found in 110–125 µm oocytes. The nuclear stage in the smallest oocytes show their reduced ability to resume meiosis. IVM-oocytes of different diameters showed no difference in p34cdc2-RNA expression.

In the bitch, mechanisms responsible for the meiotic resumption and progression of the oocytes are not known. In order to better understand cellular signals involved in canine oocyte meiosis, the present study was performed to investigate the ability of bitch oocytes to resume meiosis in vitro (1) after culture with okadaic acid (OA), and (2) after cell fusion with bovine MII oocytes. For this purpose oocytes were collected from ovaries of bitches undergoing ovariecotomy. Ovaries were sliced repeatedly to release oocytes; only cumulus-oocytes complexes with two or more dense layer of cumulus cells, darkly granulated cytoplasm, and undergoing ovariectomy. Oocytes (+) were pre-incubated for different times (1, 3, 20 h) in TCM 199 + 20% FCS for 72 h as control. At the end of culture, oocytes were stained with glycerol-Hoechst 33342 and evaluated. Results indicated that incubation with 2 µM OA for 1, 3, and 20 h determined a significantly higher (P < 0.001) meiotic resumption (GVBD) of canine oocytes compared to that in the control group, but the percentage of oocytes reaching MI and MII did not increase. Similar results were obtained after culture with 0.5 µM OA for 3 and 20 h. However, meiotic progression to MI and MII was significantly improved (P < 0.05) after incubation with 0.5 µM OA for 1 h. In the second experiment canine oocytes at GV stage were fused with MII bovine oocytes matured in vitro. This experiment was designed to test whether the high activity of MPF of MII bovine oocytes was able to determine modification of GV of canine oocytes. The zonae pellucidae from both GV and MII oocytes were removed using 0.1% pronase. Pairs of oocytes (n = 37) were agglutinated in medium containing 250 mg/mL phythoemoagglutinin placed between two electrodes in 0.5% glucose fusion medium, fused with a single pulse of direct current (1.25 KV/cm for 80 ms) and cultured for 2–3 h in TCM 199 + 10% FCS. After culture fused partners (n = 36) were stained with glycerol-Hoechst 33342 and evaluated. Results indicated that the fusion of MII bovine oocytes to GV reporter canine oocytes failed to induce nuclear membrane disassembly, chromatin condensation, or modification of canine nuclei in all of the fused combinations. These data suggest that OA induces meiotic resumption of canine oocytes. However, the cell fusion results seem to indicate that increased levels of MPF are not able to determine cell cycle progression. Furthermore, incubation times and concentration of okadaic acid could be refined to optimize a system for meiotic maturation of bitch oocytes.

| Table 1. Meiotic progression of bitch oocytes after incubation with okadaic acid |
|-----------------------------|---------|---------|---------|---------|---------|---------|
| Oocytes (n) | GV (%) | GVBD (%) | MI (%) | MII (%) | DEG |
| Control | 57 | 29 (50.9) | 14 (24.6) | 0 | 4 (7.0) | 10 (17.5) |
| OA | 2 µM | 1 h | 54 | 3 (5.6) | 46 (85.2) | 0 | 4 (7.4) | 1 (1.8) |
| | | 3 h | 45 | 3 (6.7) | 36 (80.0) | 0 | 4 (8.9) | 2 (4.4) |
| | | 20 h | 73 | 3 (4.1) | 60 (82.2) | 0 | 6 (8.2) | 4 (5.5) |
| | 0.5 µM | 1 h | 39 | 2 (5.1) | 20 (51.3) | 5 (12.8) | 12 (30.8) | 0 |
| | | 3 h | 43 | 2 (4.6) | 34 (79.1) | 1 (2.3) | 4 (9.3) | 2 (4.7) |
| | | 20 h | 69 | 5 (7.2) | 58 (84.1) | 0 | 6 (8.7) | 0 |

* vs. † P < 0.001; ‡ vs. § P < 0.05; χ² test. DEG = degenerate embryos.

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279 PLASMA MEMBRANE ELECTRICAL PROPERTIES AND INTRACELLULAR CALCIUM STORES IN IMMATURE AND IN VITRO-MATURED ADULT AND JUVENILE SHEEP OOCYTES


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The low developmental efficiency recorded in juvenile oocytes represents, besides its technological relevance, an opportunity for increasing the knowledge of mechanisms regulating developmental competence in the oocytes. To analyze the biological reasons that make an adult oocyte different from a juvenile one, we monitored membrane electrical properties, i.e., resting potential, steady-state conductance and calcium currents, and calcium stores in these two oocyte types both at immature (GV) stage and after in vitro maturation (MII). Ovaries of cycling ewes and 40-day-old lambs were collected at abattoir and transported at 30°C for 24 h. Zona pellucida of immature and in vitro-matured oocytes was removed after incubation for 1–1.5 min in 0.5% (w/v) protease solution. Zona-free oocytes were placed in Ham F10 at 38.5°C and voltage clamped by standard techniques (Tosti et al. 2002 Reproduction 124, 835–846). After obtaining a giga-seal, the patch was ruptured. The permeability of the plasma membrane was verified by applying depolarizing and hyperpolarizing voltage steps of 10 mV and 500 ms before and at the peak current to generate the voltage-dependent currents. The voltage clamp was set at −80 and −30 mV to differentiate the Ca²⁺ current components, i.e., L-type Ca²⁺ channels. For intracellular calcium determinations, oocytes were placed in Ham F10 and injected with the 0.5 mM calcium green dextran (Mr 10,000). Ca²⁺ stores were evoked by the addition of 5 μM Ca²⁺ ionophore, monitored using a computer-controlled photo-multiplier system, and measured as relative fluorescence units (RFU) by normalizing fluorescence against baseline fluorescence. In lamb and ewe, differences in electrical features and calcium dynamics between GV (n = 36 and 17) and MII (n = 42 and 32) oocytes were tested by ANOVA and expressed as mean ± SEM. Resting potential was higher at MII than GV stages (−15.2 ± 0.9 vs. −12.1 ± 1.1 mV, respectively; P < 0.02) but it did not differ between animal age. GV stage and ewe showed either a higher steady-state conductance (25.4 ± 0.2 vs. 21.7 ± 0.2 mS and 15.4 ± 0.2 mS, respectively; P < 0.01) or L-type Ca²⁺ channels (9.7 ± 1.4 vs. 2.7 ± 1.3 pA and 9.2 ± 1.5 vs. 3.2 ± 1.1 pA, respectively; P < 0.01). No differences were found between resting potential peaks yielded after Ca²⁺ ionophore exposure but a higher ion activation current was found in lamb oocytes (489 ± 56 vs. 300 ± 73 pA; P < 0.05). Ca²⁺ stores did not differ between animal age but they were larger at MII than at GV stage (0.70 ± 0.07 vs. 0.44 ± 0.07 RFU; P < 0.01). These results supply further information on both reproductive biology in ovine species and the physiology of oocytes collected from juvenile and adult individuals.

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280 EFFECTS OF ß-ENDORPHIN AND NALOXONE ON INTRACELLULAR CALCIUM LEVELS IN CUMULUS CELLS OF EQUINE OOCYTES

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Changes in intracellular calcium levels in the cumulus oocyte complex (COC) have a crucial role in oocyte maturation. In previous studies we demonstrated that the µ-opioid receptor is expressed in the bovine COC and participates in the signaling associated with oocyte maturation, by inducing an intracellular calcium increase (Dell’Aquila ME et al. 2002 Mol. Reprod. Dev. 63, 210–222). In this work we evaluated modifications of intracellular calcium induced by ß-endorphin (ß-end) or Naloxone (Nx) in cumulus cells of equine oocytes in relation to the time of the year and cumulus morphology at retrieval. Cumulus cells, isolated by mechanical treatment from compact (Cp, n = 120) or expanded (Exp, n = 120) COCs, recovered from the ovaries of slaughtered mares (follicles <20 mm in diameter) during anestrus, breeding season, spring transition, and autumnal transition, were cultured for 24 h and loaded with 5 μM Fura2-AM for microspectrofluorometric measurements of cytoplasmic ionized calcium (Dell’Aquila et al., 2002). The changes in ß-end (30 μM)- or Nx (1 mM and 10 μM)-induced calcium concentration were calculated in single cells (n = 194) and are expressed as Δ fluorescence (Fmaximal effect – Fbaseline) before and after 1-min perfusion with the drugs. The use of 1 mM Nx induced a significant increase of intracellular calcium levels in cumulus cells of oocytes recovered in all periods of the year in both Cp and Exp (P < 0.01). The addition of 10 μM Nx or 30 μM ß-end significantly increased intracellular calcium only in cumulus cells from oocytes recovered in anestrus (P < 0.05). These results confirm previous observations, carried out on bovine oocytes, in which Nx behaved as a µ-receptor agonist when used at high concentration (Dell’Aquila et al. 2002). The effects of ß-end and Nx may be explained in terms of a binding of the two substances at the µ-receptor with consequent intracellular calcium increases due to extracellular calcium entry or depletion of intracellular stores. These findings could be related to differential expression and/or activation status of the µ-opioid receptor in COCs retrieved in different seasons. These substances can be used to modulate intracellular calcium in the equine COCs, and consequent effects on the stimulation/inhibition of oocyte maturation in this species need to be further investigated.

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Mitogen-activated protein kinase (MAPK) is involved in many signal processes within eukaryotic organisms. Its active form is phosphorylated. For meiotic resumption in oocytes the MAPK cascade plays a central role, because it participates in the transfer of the extracellular gonadotropin signal into the nucleus. In pigs it could be shown that for a gonadotropin-induced germinal vesicle breakdown (GVBD), an activation of MAPK in oocytes is not essential, but in the surrounding cumulus cells the MAPK has to be phosphorylated (Ohashi et al. 2003 Biol. Reprod. 68, 664–669). Because cumulus cells are very important for signal transfer, the present investigation dealt with the relevance of porcine cumulus cells and the phosphorylation of MAPK for the resumption of meiosis. Oocytes of slaughtered pigs were collected and cultured (medium: TCM 199, insulin, t-glutamine, gentamycin, 20% (v/v) FCS, and with or without 2.5 µg/mL FSH and 5.0 µg/mL LH). The proteins of isolated cumulus cells and oocytes were separated by gel electrophoresis (cumulus cells of 10 cumulus-oocyte complexes and 40 oocytes per lane, respectively) followed by an immunoblot with antibodies against MAPK and p90rsk (ERK 1 (sc-94) and Rsk-1 (sc-231), respectively; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Alternatively the nuclear maturation was determined by orceine staining. The following results were achieved: The phosphorylation of MAPK in cumulus cells began very early during the in vitro maturation period. This was demonstrated already after 0.5 h unlike in oocytes where phosphorylation of MAPK does not occur until 18 h. The phosphorylation in cumulus cells occurs both in the presence and in the absence of FSH/LH, but without FSH/LH almost no GVBD occurs (after 26 h IVM: 86.9% GV oocytes, n = 59). The phosphorylation in the absence of gonadotropins could be caused by components of FCS, but with an exchange against polyvinylpyrrolidone (0.3%), the phosphorylation without FSH/LH still existed. The specificity was examined with the MAPK kinase inhibitor U0126. A concentration of 10 µM U0126 prevented GVBD and phosphorylation of MAPK in oocytes. However, in cumulus cells the phosphorylation of MAPK was reduced only minimally. In the presence of 50 µM U0126, a distinct decrease was observed during the first hours of maturation. But after 26 h phosphorylated MAPK appeared in cumulus cells despite the high concentration of U0126. The p90rsk is an important substrate of MAPK, which is phosphorylated by activated MAPK in oocytes. In our investigations we could detect only unphosphorylated forms of p90rsk in the cumulus cells. It seems that there are different ways for phosphorylation of MAPK to occur in cumulus cells, but they do not have the same consequences. The phosphorylation of MAPK in cumulus cells is necessary for a gonadotropin induced meiotic resumption, but phosphorylation does not always lead to GVBD. Furthermore, the p90rsk appears not to have the same importance as a substrate of MAPK in cumulus cells as in oocytes.

282 EFFECT OF THE MATURATION TIME ON THE OUTPUT OF THE INTRACYTOPLASMIC SPERM INJECTION (ICSI) OF PIG OOCYTES PRECULTURED WITH ROSCOVITINE

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This study was conducted to evaluate the effect of different maturation times on the output of intracytoplasmic sperm injection (ICSI) with pig oocytes precultured with roscovitine, a specific inhibitor of MPF kinase activity (Meijer and Raymond 2003 Acc. Chem. Res. 36, 417–425). Cumulus-oocyte complexes (COCs) were collected from nonartetric follicles from slaughtered gilts. Just after recovery, oocytes (n = 456) were cultured in the presence of 50 µM roscovitine for 28–30 h, which maintains them at the GV stage until use without affecting further development (Coy et al. 2004 Human Reprod. 19, 41–42). Then, oocytes were matured in a conventional maturation system (Coy et al. 2002 Reproduction 124, 279–288) for 36, 40, or 44 h. Matured oocytes were fertilized by ICSI. Insemination was performed as described by Martín (2000 Biol. Reprod. 63, 109–112) but in D-PBS medium with 10% FCS. Injected oocytes were transferred to TALP medium (Rath et al. 1999 J. Anim. Sci. 77, 3346–3352) for 24 h, after which they were fixed and Hoechst-stained to check for fertilization. Results (data were analyzed by ANOVA and Tukey test) showed similar activation rates (oocytes with female pronucleus) for 36, 40, and 44 h (83.6%, 81.7%, and 90.7%, respectively). Differences were observed in male pronuclear formation (80.2%, 67.6%, and 57.8%) and in putative embryos (zygotes showing two pronuclei or two cell stage 24 hpi: 74.3%, 62.9%, and 55.1%) for 36, 40, and 44 h, respectively (P < 0.05), where the 36-h group showed the best result. Data show that pre-treatment of pig oocytes with roscovitine and further maturation for 36 h can improve the output of ICSI.

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283 DEVELOPMENT OF CAPRINE EMBRYOS PRODUCED BY ICSI AND IVF AND CULTURED IN DIFFERENT MEDIA

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Embryo development of IVM-IVF prepubertal goat oocytes is low. The ICSI techniques may allow fertilization of all IVM oocytes. The objective of this study was to assess the efficacy of ICSI compared with IVF, for embryonic development of prepubertal goat oocytes. Prepubertal (1- to 2-month old) goat oocytes were recovered from a local slaughterhouse and matured in a conventional IVM medium (TCM199 with serum, hormones and cysteamine) for 27 h. Spermatozoa from fresh ejaculates were selected by swim-up and capacitated with heparin. IVM oocytes were divided into three groups: (1) oocytes fertilized using conventional IVF methodology, (2) ICSI oocytes activated by sequential treatment with 5 mM ionomicyn for
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In conclusion, ICSI improved the percentage of embryos developing beyond the 8-cell stage. However, this difference could be due to the additional effect of the chemical activation. Embryo development was also improved by using G1.3/G2.3 embryo culture medium rather than TCM-199 with granulosa cells. The ICSI procedure could be a useful tool to study more accurately the oocyte competence for embryo development.

Maturation of mammalian oocytes is a very important process for subsequent embryo development after fertilization. Prolonged maturation time by meiotic inhibitors could be an effective method for improvement in the meiotic and developmental competence of mammalian oocytes. Roscovitine, a cyclin dependent kinase inhibitor, is known to specifically inhibit M-phase promoting factor (MPF) kinase activity and prevent the resumption of meiosis. The aim of this study was to examine the effect of roscovitine on the maturation and subsequent development of porcine oocytes. Ovaries were collected from slaughtered prepubertal gilts and COCs were aspirated from 2- to 5-mm antral follicles. In control, porcine cumulus oocyte complexes (COCs) were cultured in the maturation medium (TCM-199 supplemented with 0.3% BSA, 1 µg/mL FSH, 1 µg/mL LH, and 10 ng/mL EGF) for 44 h. In the experimental group, COCs were cultured in the inhibition medium (TCM-199 supplemented with 0.3% BSA and roscovitine) for 24 h, and then further cultured in the maturation medium for 44 h. Matured oocytes from both groups were activated by electrical pulse (1.2 kV/cm for 24 h, and then further cultured in the maturation medium for 44 h. Matured oocytes from both groups were activated by electrical pulse (1.2 kV/cm for 24 h and then further cultured in the maturation medium for 44 h). The results suggest that roscovitine was sufficient to prevent meiotic resumption in 79.2% (76/96, 5 replicates) of the porcine oocytes after 24 h of culture when compared to 0 (15.4%, 15/99, 5 replicates) of the oocytes treated with roscovitine. The second experiment was carried out to examine the kinetics of maturation of roscovitine-treated porcine oocytes. The concentration of roscovitine used was 50 µM. A total of 75.8% (50/66, 3 replicates) of roscovitine-treated oocytes reached metaphase II stage compared with 70.8% (46/65) of control. The third experiment was performed to compare embryo development between control and treated group after parthenogenetic activation. No differences (P > 0.05) were found between the control and the treated group in cleavage rate (77.2%, 132/171 vs. 68.0%, 115/169), blastocyst rate (26.9%, 46/171 vs. 17.8%, 30/169), and total cell number per blastocyst (4 replicates). The results suggest that roscovitine can be used to prolong maturation time of porcine oocytes without reducing meiotic maturation but also without significantly decreasing their subsequent developmental competence. Further studies are necessary to improve the developmental competence of porcine oocytes treated with roscovitine.

### Table 1. Embryo development of oocytes after IVF, ICSI, and parthenogenetic activation and culture in G1.3/G2.3 or co-culture in TCM199 with granulosa cells

<table>
<thead>
<tr>
<th></th>
<th>IVF</th>
<th>ICSI</th>
<th>Parthenogenetic</th>
<th>IVF</th>
<th>ICSI</th>
<th>Parthenogenetic</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total embryos</strong></td>
<td>215</td>
<td>70</td>
<td>157</td>
<td>208</td>
<td>63</td>
<td>139</td>
</tr>
<tr>
<td>No. of oocytes</td>
<td>215</td>
<td>70</td>
<td>157</td>
<td>208</td>
<td>63</td>
<td>139</td>
</tr>
<tr>
<td>2–7-cell stage</td>
<td>146</td>
<td>57</td>
<td>86a</td>
<td>121</td>
<td>39bc</td>
<td>56d</td>
</tr>
<tr>
<td>8–16-cell stage</td>
<td>132b</td>
<td>38a</td>
<td>83a</td>
<td>106c</td>
<td>32bc</td>
<td>56c</td>
</tr>
<tr>
<td>Morulae</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Morula-cell number</td>
<td>18</td>
<td>19–50</td>
<td>0</td>
<td>17–25</td>
<td>46</td>
<td>0</td>
</tr>
</tbody>
</table>

a-d Values with different superscripts within each row differ significantly (P < 0.05).

This study was supported by MCYT (Spain), Grant AGL 2000-0353.

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**284 KINETICS OF OOCYTE MATURATION AND SUBSEQUENT DEVELOPMENT OF PARTHENOGENETIC PORCINE EMBRYOS AFTER MEIOTIC INHIBITION WITH ROSCOVITINE**


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285 SUPPRESSION OF MPF AND MAPK ACTIVITIES BY DIBUTYRYL CAMP DURING FIRST MEIOTIC MATURATION IMPROVES SUBSEQUENT DEVELOPMENT OF PORCINE OOCYTES


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Maintaining the germinal vesicle (GV) stage in growing oocytes is essential for developmental competence of the eggs. In pig oocytes, MPF and MAPK activities are low during the GV stage, and their activities increase with progression of meiosis I. In general, cAMP that exists at high levels in GV oocytes inhibits germinal vesicle break down (GVBBD). After gonadotropin stimulation, the amount of cAMP in oocyte cytoplasm is decreased gradually for meiotic resumption. This study was conducted to examine the effect of dibutyryl cAMP (dbcAMP) on nuclear maturation, fertilization, and early embryo development of porcine oocytes. Oocytes were cultured in NCSU23 medium with or without dbcAMP for 22 h, and then cultured in fresh maturation medium for an additional 22 h prior to fertilization. The activities of MPF and MAPK were evaluated by Western blot analysis using specific antibodies such as anti-cdc2 and anti-ERK1/2 in maturing pig oocytes at 22 h and 44 h.

In vitro fertilization was performed with fresh ejaculated spermatozoa in the modified TRIS-buffered medium, and fertilized embryos were cultured in NCSU23 medium. When treated with dbcAMP for 22 h, most oocytes (204/224, 91.9%) were arrested in GV stage by blocking meiotic resumption. The activities of constituent proteins (cdc2 and ERK1/2) of MPF and MAPK were also suppressed in dbcAMP-treated oocytes. After completion of IVM, dbcAMP-treated oocytes showed a higher proportion of the metaphase II stage than non-treated ones (156/171, 91.3% vs. 121/167, 72.8%; P < 0.05). Furthermore, incubation of 44 h matured oocytes with dbcAMP for 22 h increased the MPF and MAPK activities. In the dbcAMP-treated group, penetration rate was increased (126/145, 80.0%) and polyspermy rate was reduced (26/126, 22.4%) as compared to the nontreated group (97/140, 69.3% and 46/97, 47.4%, respectively; P < 0.05). Furthermore, blastocyst formation of dbcAMP-treated eggs was also improved compared to the nontreated group (47/126, 37.0% vs. 28/146, 17.2%; P < 0.05). Our results suggest that the synchronization of meiotic resumption by dbcAMP may support meiotic maturation and in vitro development of pig eggs.

286 THE EFFECT OF IN VITRO MATURATION MEDIUM ON CRYOSURVIVAL, CELL NUMBERS AND APOPTOTIC INDEXES OF BOVINE EMBRYOS


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The success of in vitro maturation (IVM) has a significant impact on the oocytes ability to develop to blastocyst stage. The quality of the produced blastocysts can be evaluated using staining techniques. The aims of this study were (a) to compare the effect of different IVM media on embryo production rates, and (b) to utilize differential (DF) and TUNEL staining to evaluate the quality and cryosurvival of the produced blastocysts. Abattoir-derived oocytes were randomly divided into the IVM groups: (1) M199 IVM (n = 2305): TC-M199 with glutamax-I (GIBCO, Paisley, UK), 0.25 mM Na-pyruvate, 100 IU mL⁻¹ penicillin, and 100 μg mL⁻¹ streptomycin; (2) FBS IVM (n = 2484): M199 IVM medium with hormones (10 μg mL⁻¹ LH, 2 μg mL⁻¹ FSH, and 100 μg mL⁻¹ β-estradiol), and 10% FBS (GIBCO, New Zealand); and (3) FAFBSA IVM (n = 2411): as group (2), but FBS was replaced with 4 mg mL⁻¹ fatty acid free albumin. Fertilized oocytes were denuded and cultured in modified SOFaaeci +4 mg mL⁻¹ FAFBSA in 5% O₂ (Holm P et al. 1999 Theriogenology 52, 683–700). Fresh, Grade I Day 7 blastocysts were stained with TUNEL (n = 114) or with DF (n = 149). In addition, 184 Grade I Day 7 blastocysts were frozen in AG Freeze (AB Technology, Pullman, WA, USA), thawed, and cultured for 24 h. The re-expansion rates (%) after thawing were 86.5, 90.6, and 73.3 for the FBS, FAFBSA, and M199 IVM groups, respectively. Freezing reduced the ICM proportions and elevated the apoptotic indexes (P < 0.0001): 74.0% and 15.0% for the FAFBSA and M199 IVM groups (P < 0.0001): 74.0% and 15.0% for the FAFBSA and M199 IVM groups (P < 0.0001); 74.0% and 15.0% for the FAFBSA and M199 IVM groups (P < 0.0001); 74.0% and 15.0% for the FAFBSA and M199 IVM groups (P < 0.0001); and 76.1% and 8.8% for the M199, respectively. The re-expansion rates (%) after thawing were 86.5, 90.6, and 73.3 for the FBS, FAFBSA, and M199 IVM groups, respectively. Freezing reduced the ICM proportions and elevated the apoptotic indexes (P < 0.0001). The rate of ICM reduction after freezing was not influenced by the IVM medium. There was a significant interaction between the apoptotic index and the IVM group (P = 0.04). The increase of the apoptotic index was smallest in FAFBSA IVM and greatest in M199 IVM. The results indicate that exclusion of serum from IVM medium results in lower embryo cleavage and development rates. Freezing reduced ICM and increased apoptotic index of Day 7 embryos in every IVM group studied. FAFBSA IVM seemed to produce embryos of better quality as evidenced by the smallest increase in the apoptotic index after freezing.

287 INHIBITION OF THE PENTOSE PHOSPHATE PATHWAY RESULTS IN MEIOTIC ARREST IN PORCINE OOCYTES THAT CAN BE OVERCOME BY THE ADDITION OF PATHWAY COFACTORS AND END PRODUCTS

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Glucose metabolism is an indicator of oocyte developmental competence, and is also correlated with meiotic maturation. In vitro maturation of porcine oocytes with the pentose phosphate pathway (PPP) inhibitor diphosphopyridine nucleotide (DPN) blocks meiotic progression to metaphase II. The
objectives of this study were (1) to examine the reversibility of meiotic arrest induced by DPI; and (2) to overcome metabolically induced meiotic arrest by the addition of PPP end products and cofactors downstream of DPI inhibition. Oocytes were matured for 40 h in standard (defined) maturation media containing 0, 25, 50, or 100 nM DPI. At that time half the oocytes in each treatment (TRT) were fixed, and half were moved into standard maturation medium with no DPI for an additional 40 h, at which time all remaining oocytes were fixed. Two oocytes were matured for 40 h in one of 11 media: standard (defined) maturation medium (STND), standard with 50 nM DPI (DPI), standard with 50 nM DPI and 0.25, 2.5, or 5 mM phosphoribosyl diphosphate (PRPP), nicotinamide adenine dinucleotide phosphate (NADP), or ribose-5-phosphate (R5P). Additionally, 10 mM R5P and 12.5 mM PRPP were examined. All oocytes were fixed. Oocytes were assigned a meiotic score: germinal vesicle (GV) = 1, GV breakdown (GVBD) = 2, condensed chromatin (CC) = 3, metaphase I (MI) = 4, anaphase (A) = 5, telophase (T) = 6, and metaphase II (MII) = 7. Immature oocytes were classified as those at GV or GVBD stages, and mature oocytes as those at A, T, or MII stages. Data were analyzed by ANOVA and are presented as mean ± SEM. After 40 h of arrest (n = 79–87/TRT), increasing concentrations of DPI significantly increased the % of immature oocytes (0, 7.2 ± 2.9; 25, 26.4 ± 4.8; 50, 53.2 ± 5.7; 100, 75.9 ± 4.8) and decreased the % of mature oocytes (0, 73.5 ± 4.9; 25, 52.9 ± 5.4; 50, 20.3 ± 4.6; 100, 0). After an additional 40 hours in standard maturation medium (n = 89–93/TRT), there was no difference in the % of immature oocytes between treatments (0, 7.5 ± 2.8; 25, 14.4 ± 3.7; 50, 13.0 ± 3.5; 100, 9.0 ± 3.0) although the % of mature oocytes significantly decreased with increasing DPI concentration (0, 90.3 ± 3.1; 25, 68.9 ± 4.9; 50, 35.9 ± 5.0; 100, 10.1 ± 3.2). Data from experiment 2 are presented below. Meiotic maturation is significantly inhibited by DPI in a dose-dependent manner. Ability of the oocyte to reach MII following 40 h of arrest is also concentration-dependent, although all treatments resulted in GVBD following removal from DPI. Metabolic arrest can be overcome, resulting in numbers of mature oocytes equal to standard controls, by NADP and PRPP but only moderately by R5P. These data demonstrate that glucose metabolism via the PPP is a critical control mechanism of meiotic maturation in porcine oocytes.

### Table 1. Effect of PPP cofactors and end products on overcoming metabolically induced meiotic arrest in porcine oocytes

<table>
<thead>
<tr>
<th>TRT</th>
<th>mM</th>
<th>n</th>
<th>% Immature</th>
<th>% Mature</th>
<th>Meiotic score</th>
</tr>
</thead>
<tbody>
<tr>
<td>STND</td>
<td>93</td>
<td>7.5 ± 2.7</td>
<td>80.6 ± 4.1</td>
<td>6.1 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>DPI</td>
<td>102</td>
<td>35.3 ± 4.8</td>
<td>25.5 ± 4.3</td>
<td>3.6 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>NADP</td>
<td>0.25</td>
<td>91</td>
<td>53.8 ± 5.3</td>
<td>4.4 ± 2.2</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>2.5</td>
<td>107</td>
<td>19.6 ± 3.9</td>
<td>50.5 ± 4.9</td>
<td>4.9 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>PRPP</td>
<td>0.25</td>
<td>41</td>
<td>61.0 ± 7.6</td>
<td>9.8 ± 4.3</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>2.5</td>
<td>44</td>
<td>59.1 ± 7.5</td>
<td>9.1 ± 4.4</td>
<td>2.5 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>R5P</td>
<td>0.25</td>
<td>38</td>
<td>55.3 ± 8.2</td>
<td>2.6 ± 2.6</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>2.5</td>
<td>77</td>
<td>44.2 ± 5.7</td>
<td>22.1 ± 4.8</td>
<td>3.3 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>94</td>
<td>27.7 ± 4.6</td>
<td>35.1 ± 4.9</td>
<td>4.0 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>63</td>
<td>17.5 ± 4.8</td>
<td>55.6 ± 6.3</td>
<td>5.1 ± 0.3</td>
<td></td>
</tr>
</tbody>
</table>

Concentrations within chemical treatments are compared to each other, to STND and to DPI, within column; different superscripts are different, P < 0.05.

### 288 RELATIONSHIP BETWEEN CHROMATIN ORGANIZATION AND OOCYTE-CUMULUS CELL COMMUNICATION IN GERMINAL VESICLE STAGE BOVINE OOCYTES

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Chromatin configuration in the germinal vesicle (GV) undergoes dynamic changes during oocyte growth, and the progressive chromatin condensation has been related to the acquisition of embryonic developmental potential. However, little is known about the mechanisms that regulate chromatin remodeling. In immature mouse oocytes, chromatin condensation and redistribution around the nucleolus are associated with transcriptional repression in both in vivo-derived and in vitro-cultured oocytes in the presence of an intact cumulus oophorus (de la Fuente et al. 2001 Dev. Biol. 229, 224). It is widely accepted that oocyte communication with the somatic cell compartment is essential for both oocyte growth and acquisition of meiotic competence (Eppig et al. 1997 Hum. Reprod. 12, 127). In particular, cumulus cells play an active role in modulating the levels of transcription in the nucleoplasm and in perinuclear domains as well as in chromatin configuration of GV stage oocytes. In cattle, a heterogeneous population of cumulus-oocyte complexes (COCs) has been found after isolation from the follicle, and this is characterized by a different functional degree of gap junction-mediated communication (Luciano et al. 2004 Biol. Reprod. 70, 465). This study was aimed at investigating the possible correlation between the chromatin configuration of immature bovine oocytes and the status of communication between the oocyte and cumulus cells, and oocyte developmental competence. In the first experiment, 138 COCs, isolated from follicles 2–6 mm in diameter, were injected with a 3% solution of Lucifer Yellow to assess the communication status between oocytes and cumulus cells. Successively, COCs were freed of cells, and denuded oocytes (DOs) were stained with Hoechst 33342 to determine the chromatin configuration. In a second experiment, 330 COCs were denuded and stained with Hoechst 33342 in order to assess chromatin configuration and then matured in vitro according to their GV stage. After IVM, DOs were fertilized, and presumptive zygotes were cultured for 7 days at which time blastocyst rate was assessed. Data were analyzed by ANOVA and Fisher’s
The arylhydrocarbon receptor (AhR), a ligand activated transcription factor, has been extensively characterized from a toxicological point of view due to its ability to mediate the adverse effects of a variety of halogenated aromatic hydrocarbons. Recent reports on AhR knockout mice suggest that the AhR may play a role in ovarian physiology. We have previously demonstrated that AhR activity (as indicated by the up-regulation of the target gene cytochrome p450 1A1: CYP1A1) is stimulated during bovine oocyte IVM in the absence of exogenous ligands. Furthermore, exposure to specific AhR antagonists, besides down-regulating the expression of CYP1A1, significantly impairs the ability of the oocyte to complete maturation until the metaphase II stage (Pocar et al. 2004 Endocrinology 145, 1594–1601). The aim of the present study was to further investigate the mechanisms underlying the AhR activation during IVM. Several reports point to a critical role of phosphorylation in the regulation of the AhR-complex. Furthermore, the mitogen-activated protein kinase (MAPK, extracellular regulated kinase (ERK 1 and 2)) cascade has been shown to play a crucial role in regulating meiotic cell cycles during bovine oocyte maturation. A total of 572 bovine cumulus-oocyte complexes were used to investigate the potential role of the MAPK in modulating the activity of the AhR during IVM. The effect of the broad-spectrum serine/threonine kinase inhibitor, 6-dimethylaminopurine (6-DMAP), on the induction of CYP1A1 during oocyte maturation was investigated. As expected, exposure to 6-DMAP induced meiotic arrest (at the stage of germinal vesicle/germinatal vesicle breakdown) and down-regulated the expression level of phosphorylated ERK 1 and 2. Interestingly, a significant down-regulation of the target genes CYP1A1 and CYP1B1 (9.5% and 26.8% of control, respectively) and
an up-regulation of the AhR (199.4% of control) were observed at the mRNA level. This phenomenon was partially reversible after a period of further 24 h of culture in the absence of 6-DMAP. In this condition, besides a recovery of oocyte maturation and phosphorylation status of ERK 1 and 2 to levels comparable to control, a significant up-regulation of CYP1A1 mRNA was observed (68.3% of control). Finally, to confirm the role of serine/threonine kinases in modulating the activity of AhR during resumption of meiosis, we exposed the oocytes to cycloheximide, a protein synthesis inhibitor, also known to arrest oocyte maturation. Furthermore, although cycloheximide exposure induced meiotic arrest, no significant differences in the expression levels of AhR or its target genes compared to control were observed. Each experiment was replicated at least three times. Data were assessed using ANOVA followed by Duncan's multiple range test. The criterion for significance was set at P < 0.05. In conclusion, our results strongly suggest that 6-DMAP-sensitive kinase(s) is (are) involved in the regulation of AhR during bovine oocyte maturation. Further analyses are necessary to understand the biological significance of these observations.

### 29.1 EFFECT OF DIFFERENT TRANSPORT TEMPERATURES (+4°C, +32°C) ON IN VITRO MATURATION OF OOCYTES COLLECTED FROM CATTLE AND SHEEP OVARY


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At present, blastocyst rates in embryos obtained from in vitro maturation of oocytes, and their fertilization and culture, is still not at the desired level. One of the most important problems encountered in in vitro culture studies is seen in the maturation period of oocytes until they reach the fertilizable level. Transport time of the ovaries and, in particular, temperature of the transport medium used are among the factors affecting complete maturation. The aim of this study was to determine the effects of different transport temperatures (4°C, 32°C) of sheep and cattle ovaries on the in vitro maturation of oocytes. Two experimental groups were formed in the study. Sheep and cattle ovaries were put into saline solution at 32°C. The ovaries were transported at the same temperature (Group I) or at 4°C following a 10-min incubation at room temperature (Group II). In 2–4 h to the laboratory (n = 6). For each group, oocytes were collected from ovaries using the dissection method and selected oocytes were matured in their own group in 700 µL TCM-199 (supplemented with pyruvate, LH, FCS) for 23 h at a gas atmosphere of 5% CO₂, 5% O₂, 90% N₂ and at 38.8°C. At the end of maturation, oocytes were cleansed from their cumulus oophorus cells and fixed in acetic acid-ethyl alcohol (1:3) for 48 h. The developmental stages until MII of oocytes stained with aceto-orcein were then examined under the phase contrast microscope. The chi-square test was used for statistical analysis (Table 1). While oocytes obtained from sheep ovaries transported at +32°C reached the MII stage at a faster rate compared to those at +4°C (P < 0.001), no statistically significant difference was observed between the maturation to the MII stage of oocytes obtained from cattle ovaries transported at both +4°C and +32°C. As a result of this study, while it was established that cattle ovaries could be transported at both +4°C and +32°C and that there was no difference in oocyte maturation, a medium temperature of +4°C was determined to be unsuitable for transporting sheep ovaries.

### Table 1. Stages of development in sheep and cattle oocytes after 23 h of culture

<table>
<thead>
<tr>
<th>Species</th>
<th>Transport temp.</th>
<th>Oocytes used</th>
<th>No. of GV (%)</th>
<th>No. of GVBD (%)</th>
<th>No. of MI (%)</th>
<th>No. of AI-TI (%)</th>
<th>No. of MII (%)</th>
<th>No. of UDNM (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep</td>
<td>+4°C</td>
<td>144/175</td>
<td>32b (22.2%)</td>
<td>14b (9.7%)</td>
<td>44b (30.6%)</td>
<td>3b (2.1%)</td>
<td>22b (15.3%)</td>
<td>29b (20.1%)</td>
</tr>
<tr>
<td>Sheep</td>
<td>+32°C</td>
<td>133/158</td>
<td>11b (8.3%)</td>
<td>3b (2.3%)</td>
<td>51b (38.3%)</td>
<td>5b (3.8%)</td>
<td>44b (33.1%)</td>
<td>19b (14.3%)</td>
</tr>
<tr>
<td>Cattle</td>
<td>+4°C</td>
<td>104/129</td>
<td>7 (6.7%)</td>
<td>6 (5.8%)</td>
<td>18 (17.3%)</td>
<td>5 (4.8%)</td>
<td>48 (46.8%)</td>
<td>20 (19.2%)</td>
</tr>
<tr>
<td>Cattle</td>
<td>+32°C</td>
<td>101/126</td>
<td>4 (4.0%)</td>
<td>4 (4.0%)</td>
<td>20 (19.8%)</td>
<td>5 (5.0%)</td>
<td>56 (55.4%)</td>
<td>12 (11.9%)</td>
</tr>
</tbody>
</table>

a,b Rates with different letters in the same column are statistically significant in sheep (P < 0.001).

This work was supported by Istanbul University.

### 29.2 MATURATION IN A STRAW IS EFFECTIVE ON THE DEVELOPMENT OF BOVINE OOCYTES IN VITRO


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In vitro embryo development is strongly influenced by oocyte maturation environments. Maturation of bovine oocytes is processed in a culture dish. However, the development rate to the transferable blastocyst stage was 10 to 30%. This experiment was to examine the effect of the size of straw
This study was supported by grant of GA CR No. 524/02/0674.

A major factor affecting oocyte viability during in vitro maturation (IVM) is oxidative stress. Oxidative modifications could be responsible for oocyte-defective in vitro maturation and consequently compromise subsequent fertilization and embryonic development. Low-molecular-weight thiol compounds such as cysteamine, added during in vitro culture of bovine, porcine, and ovine oocytes, increase intracellular glutathione (GSH) synthesis, which prevents oxidative damages and consequently improves in vitro maturation and embryo development. The present study was aimed at investigating whether equine oocyte maturation efficiency and embryonic developmental capability following ICSI benefit from the addition of cysteamine during in vitro maturation (IVM). Cummulus oocyte complexes (COCs) were collected from slaughtered ovaries and cultured for 30 h at 38.5°C in 5% CO2 in air with maximum humidity. Data from three replicates were analyzed by chi-square test. In Experiment 1, we examined the effect of the instrument of maturation (dish or 0.25-mL and 0.5-mL straws) on embryo development. There were no difference in the cleavage (2-cell) among treatment groups. However, the development rate to the 8-cell and blastocyst stage was significantly higher in the 0.5-mL straw (38.5 and 17.0%) than in the 0.25 mL-straw (26.6 and 7.4%, all respectively). In Experiment 2, the KC oocytes were matured in 0.5-mL straws based on the results of Experiment 1, and we examined the effect of the conditions such as circulation and exchange of maturation medium at 9 h after the start of IVM on embryo development. The development rates to the 2-cell, 8-cell, and blastocyst stage were significantly higher in the circulation group (83.3, 58.0 and 31.3%) than in the control (72.0, 44.7 and 19.3%) and exchange groups (71.3, 40.0, and 18.0%, all respectively). The results of this study suggest that the maturation of KNC oocytes in 0.5-mL straws accompanied by circulation of medium at 9 h is effective in the development to the blastocyst stage.

293 TWO-STEP MATURATION OF BOVINE OOCYTES WITHOUT CDK INHIBITORS: AN ALTERNATIVE TO AFFECT THEIR SUBSEQUENT DEVELOPMENTAL COMPETENCE


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The acquisition of meiotic and developmental competence seems to correlate not only with the size of follicles and oocytes but also with the morphology and transcriptional activity of the oocyte nuclei and nucleoli. To secure or increase the fertilization and the developmental competence of bovine oocytes, we have developed a two-step culture system using the specific cyclin dependent kinase inhibitors (Butyrolactone I, Bohemine). However, these drugs have several side effects during the prolonged time of culture. To avoid this disadvantage, we have used in the present experiments modified culture conditions simulating the intrafollicular block of meiosis. In the first step of culture, bovine oocytes isolated from small, medium, and large follicles (2–3, 3–4, and 4–6 mm in diameter, respectively) were kept under conditions that secured for at least 48 h the intact germinal vesicle stage (GV) in more than 90% of oocytes. The second step represented the subsequent 20–22 h in conditions stimulating resumption of meiosis. The effectiveness of this model depended mainly on medium composition: reduced NaHCO3, substitution of serum with serum albumin, addition of antioxidants (curcumin), increased viscosity of a medium by agar (0.3%), and reduction of oxygen concentration (within 6–9%). The reduction of the proportion between the number of cumulus-oocyte complexes (COC) and the amount of medium (within 6–7 mL per COC) should amplify the GVBD-inhibiting effect of oocyte-surrounding granulosa cells. The COC were situated in clots of 6–7 COC per clot. The effectiveness and reversibility of GVBD inhibition depends also on the duration of COC isolation. The full reversibility of GVBD inhibition was controlled morphologically and also by measuring histone H1 and MAP kinase activities. The two-step versus one-step (24 h) maturation technique was evaluated by the percentage of total and hatched Day 9 blastocysts. When compared with one-step maturation, the two-step culture showed a slightly increased proportion of total and hatched blastocysts developed from the smallest follicular category (13.9 vs. 7.1% and 9.2 vs. 3.3% for total and hatched blastocysts, respectively). No significant difference was noticed between between one- and two-step culture when oocytes from large healthy follicles were used. However, the two-step maturation of oocytes from regressing follicles substantially reduced the blastocyst yield (9.7 vs. 39.1% and 4.9 vs. 26.7% for total and hatched blastocysts, respectively).

This study was supported by grant of GA CR No. 524/02/0674.

294 EFFECT OF CYSTEAMINE ADMINISTRATION DURING EQUINE OOCYTE MATURATION ON GLUTATHIONE CONTENT, NUCLEAR MATURATION, AND DEVELOPMENTAL CAPABILITY AFTER INTRACYTOPLASMIC SPERM INJECTION


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In the recent years, assisted reproduction methods have produced only limited success in equine species in comparison with other domestic mammals. A major factor affecting oocyte viability during in vitro culture is oxidative stress. Oxidative modifications could be responsible for oocyte-defective in vitro maturation and consequently compromise subsequent fertilization and embryonic development. Low-molecular-weight thiol compounds such as cysteamine, added during in vitro culture of bovine, porcine, and ovine oocytes, increase intracellular glutathione (GSH) synthesis, which prevents oxidative damages and consequently improves in vitro maturation and embryo development. The present study was aimed at investigating whether equine oocyte maturation efficiency and embryonic developmental capability following ICSI benefit from the addition of cysteamine during in vitro maturation (IVM). Cummulus oocyte complexes (COCs) were collected from slaughtered ovaries and cultured for 30 h at 38.5°C in 500 μL of control medium (TCM199 + 0.4% BSA + 0.1 IU/mL rhFSH + 50 ng/mL EGF) either supplemented with 100 μM cysteamine or not. After culture, nuclear stage was assessed by Hoechst 33342 staining after cellum cum cellum removal, and MI oocytes were analyzed for GSH content (Baker MA et al. 1990 Anal. Biochem. 190, 360–365). Groups of COCs matured under the same conditions were denuded with hyaluronidase and only oocytes with a visible polar body were fertilized by ICSI. The number of embryos that reached the 2–4 cell stage was assessed by nuclear staining with propidium
Development of two-photon laser scanning microscopy (TPLSM) has made it possible to conduct several recordings over time of early stage embryos without compromising viability. To use TPLSM to study structures within the oocyte it is necessary to remove at least part of the cumulus cells to prevent emitted light from being blocked. Aspiration of cumulus oocyte complexes (COC) through a denudation pipette creates a “window” through which the emitted light can escape and be recorded. To allow repeated recordings of the same location within an object it is important to prevent emitted light from being blocked. Aspiration of cumulus oocyte complexes (COC) through a denudation pipette creates a “window” through which the emitted light can escape and be recorded. To allow repeated recordings of the same location within an object it is important to

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Maturation (%)</th>
<th>GSHi (μmol/oocyte)</th>
<th>&gt;2 cells at 72 h (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>70.7 (41/58)</td>
<td>7.8 (15)</td>
<td>68.4 (21)</td>
</tr>
<tr>
<td>Cysteamine</td>
<td>70.1 (40/57)</td>
<td>9.9 (18)</td>
<td>71.6 (23)</td>
</tr>
</tbody>
</table>

Development of two-photon laser scanning microscopy (TPLSM) has made it possible to conduct several recordings over time of early stage embryos without compromising viability. To use TPLSM to study structures within the oocyte it is necessary to remove at least part of the cumulus cells to prevent emitted light from being blocked. Aspiration of cumulus oocyte complexes (COC) through a denudation pipette creates a “window” through which the emitted light can escape and be recorded. To allow repeated recordings of the same location within an object it is important to

This work was supported by a 2003 UniMi Grant.

295 COMPARISON OF TWO METHODS TO AVOID MOVEMENT OF BOVINE OOCYTES DURING IN VITRO MATURATION

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Development of two-photon laser scanning microscopy (TPLSM) has made it possible to conduct several recordings over time of early stage embryos without compromising viability. To use TPLSM to study structures within the oocyte it is necessary to remove at least part of the cumulus cells to prevent emitted light from being blocked. Aspiration of cumulus oocyte complexes (COC) through a denudation pipette creates a “window” through which the emitted light can escape and be recorded. To allow repeated recordings of the same location within an object it is important to prevent emitted light from being blocked. Aspiration of cumulus oocyte complexes (COC) through a denudation pipette creates a “window” through which the emitted light can escape and be recorded. To allow repeated recordings of the same location within an object it is important to

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296 LEUKEMIA INHIBITORY FACTOR INFLUENCES SHEEP OOCYTE PARTHENOGENETIC DEVELOPMENT DURING THE TRANSITION FROM GERMINAL VESICLE TO EARLY PRONUCLEAR STAGE

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Leukemia inhibitory factor (LIF) is an indispensable cytokine for female fertility. The influence of LIF on embryo development and particularly implantation has been recently confirmed; however, the effect of this cytokine on the oocyte has not been studied. The presence of LIF in human follicular fluid implies its possible role in the acquisition of oocyte competence. Furthermore, the up-regulation of LIF by steroid hormones in sheep makes entirely feasible the hypothesis that ovulatory estradiol peak plays a role in the preparation of female gamete for fertilization. With

This work was supported by a 2003 UniMi Grant.
this in mind, we studied the effect of LIF during in vitro development of sheep oocytes mimicking the physiological expression of LIF induced by the ovulatory peak of estradiol in mice. GV stage oocytes matured and chemically activated in the presence of LIF and anti-LIF antibody were cultured to the blastocyst stage in our standard media. To eliminate the effect of the putative presence of LIF in heat inactivated fetal calf serum used for oocyte maturation, aliquots of LIF were treated at 56°C for 30 min and added to the maturation medium. The proportion of embryos that reached the blastocyst stage in vitro was significantly higher (P < 0.001) for oocytes matured and activated with LIF (36/93; 39%) than for the group incubated with antibody against LIF (6/68; 9%). The significant effect of anti-LIF antibody (P < 0.001) was also observed when compared with blastocysts developed from the control group of oocytes matured without LIF addition (31/106; 29%). Although the beneficial influence of LIF treatment on embryo development demonstrated with those preliminary data was not confirmed statistically, due to low number of oocytes involved, the proportion of embryos reaching the blastocyst stage in vitro was about 10% higher for those incubated with LIF than for either those cultured without the cytokine or those, matured in the presence of heat-treated LIF (15/55; 27%); however, the rate of blastocyst development appeared very similar to that of the control group. This study revealed for the first time a role of LIF in determining oocyte competence. Further investigation to determine how LIF achieves its effects on the oocyte are ongoing in our laboratory.

This work was supported by FIRB RBNE01HPMX, COFIN 2002074357, COFIN 2003073943 002, and British Council 2004.

297 A CASE REPORT: THE OUTCOME OF IVP MAY BE RELATED TO THE BATCH OF TCM-199 IN IVM


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Chemicals used in bovine IVP may be inhibitory to embryo development (see, e.g., Van Soom A et al. 1994 Theriogenology 41, 325). The present study continued earlier experiments to optimize serum-free IVM protocol for bovine oocytes (Rätty M. 2004 Reprod. Fert. Dev. 16, 281). The embryo development rate was much lower in the new experiments than in our previous studies. Thorough testing indicated that the batch of TCM-199 medium used in maturation was the reason for the lower embryo development results. In total, 14,589 abattoir-derived bovine oocytes in 23 batches were matured for 24 h in TCM-199 with glutamax-I (GIBCO, Paisley, UK), 0.25 mM Na-pyruvate, 100 IU mL⁻¹ penicillin, 100 µg mL⁻¹ streptomycin, 10 µg mL⁻¹ LH, 2 µg mL⁻¹ FSH, and 1 µg mL⁻¹ β-estradiol supplemented as follows: (1) 10% FBS (GIBCO, New Zealand); (2) 4 mg mL⁻¹ fatty acid-free albumin (FAFBSA) (Sigma-Aldrich, Helsinki, Finland); (3) 4 mg mL⁻¹ polyvinylpyrrolidone (PVP) (Sigma) + growth factors (GF; 100 ng mL⁻¹ IGF-I + 100 ng mL⁻¹ EGF); or (4) as (3) but without FSH, LH, and β-estradiol (PVPGFwoH). Fertilized oocytes were denuded and cultured in modified SOFaaci + 4 mg mL⁻¹ FAFBSA in 5% O₂. (Holm P. et al. 1999 Theriogenology 52, 683–700). Two TCM-199 batches were used in separate IVM runs, batch 1 (Lot#3075638; 10 runs) and batch 2 (Lot#3081334; 13 runs). The statistical analyses were based on generalized linear mixed models. The estimated probabilities for embryo cleavage and development are shown in Table 1. The use of TCM-199 batch 1 resulted in significantly lower embryo development rates than the use of TCM-199 batch 2 in every IVM group studied. PVP IVM groups were the most sensitive for the TCM-199 batch and resulted in reduced embryo cleavage as well as strikingly low Day 7 embryo development. Our results indicate that TCM-199 batch 1 disturbed embryo development. In defined IVM groups (PVP groups), the maturation inhibiting role of TCM-199 batch 1 was seen already at the cleavage stage. It is possible that FBS and FAFBSA may have protected the oocytes to some extent against inhibitory effects of TCM-199. There is evidence for BSA acting as a chelating agent (see, e.g., Flood L.P. and Shirley B. 1991 Mol. Reprod. Dev. 30, 226–231).

Table 1. Estimated probability for embryo cleavage at 38–42 hpi and Day 7 embryo development after IVM in two different batches of TCM-199

<table>
<thead>
<tr>
<th>IVM treatment</th>
<th>TCM batch</th>
<th>No. oocytes</th>
<th>Estimated probability for Cleavage</th>
<th>Day 7 embryo development</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBS (#1)</td>
<td>1</td>
<td>1247</td>
<td>0.83</td>
<td>0.19a</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2773</td>
<td>0.81</td>
<td>0.31b</td>
</tr>
<tr>
<td>FAFBSA (#2)</td>
<td>1</td>
<td>1222</td>
<td>0.80</td>
<td>0.14c</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2354</td>
<td>0.78</td>
<td>0.23c</td>
</tr>
<tr>
<td>PVPGFwoH (#4)</td>
<td>1</td>
<td>1152</td>
<td>0.65c</td>
<td>0.02c</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2343</td>
<td>0.83d</td>
<td>0.15d</td>
</tr>
<tr>
<td>PVPGF (#3)</td>
<td>1</td>
<td>1240</td>
<td>0.48c</td>
<td>0.01c</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2258</td>
<td>0.79d</td>
<td>0.11c</td>
</tr>
</tbody>
</table>

Different values within IVM group in the same column differ (a-b P < 0.01, c-d P < 0.0001).

298 DISRUPTION OF NUCLEAR MATURATION, APOPTOSIS AND CYTOSKELETAL CHANGES IN BOVINE OOCYTES EXPOSED TO HEAT SHOCK

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Heat shock (HS) can cause apoptosis and induce changes in cytoskeletal elements. A series of experiments were performed to determine whether physiologically relevant HS disrupts the progression of oocytes through meiosis, fertilization, and zygote formation, and causes corresponding...
changes in the cytoskeleton and apoptosis. Cumulus-oocyte complexes (COCs) were cultured at 38.5 (38C), 40 (40C), or 41°C (41C) for the first 12h of maturation. Incubation during the last 10h of maturation and 18h post insemination (hpi) was at 38.5°C and 5% (v/v) CO\textsubscript{2} for both treatments. The CATMOD procedure of SAS (SAS Institute, Inc., Cary, NC, USA) was used to analyze the distribution of oocytes into various classes of nuclear maturation and the proportion of apoptotic oocytes. In Exp. 1, matured oocytes were fixed in 4% (w/v) paraformaldehyde, and either stained with Hoechst 33342 or labeled with TUNEL kit (Roche Diagnostics, Indianapolis, IN, USA). Pronuclei were classified as being either condensed or at metaphase I (MI), metaphase II (MII), anaphase I, or telophase I. HS affected (P < 0.001) the distribution of oocytes into stages of meiosis. The majority of 38C oocytes reached MII while 41C oocytes were mostly at MI. Both 40C and 41C increased the percentage of oocytes having TUNEL-positive nuclei (P < 0.001). In Exp. 2, matured oocytes were fixed and stained with Hoechst and markers for either filamentous actin (phalloidin) or microtubules (anti-bovine-a-tubulin labeled with Zenon). Microfilament localization was affected by stage of nuclear maturation and by HS. Actin microfilaments were more prominent in the cytoplasm of heat-shocked oocytes than for 38C oocytes. In addition, the intense ring of actin present under the plasma membrane was reduced for 41C oocytes and the transzonal actin processes present in 38C oocytes were absent in 41C oocytes. A subset of heat-shocked oocytes possessed misshapen MI spindles with disorganized microtubules and unaligned chromosomes. In Exp. 3, addition of 50 nM sphingosine 1-phosphate (S1P) to maturation medium blocked the effect of HS on progression through meiosis and apoptosis. There was a temperature × S1P interaction (P < 0.001) of distribution of oocytes into nuclear classes because S1P increased the proportion of 41C oocytes that were at MI. S1P also blocked the increase in proportion of TUNEL positive oocytes (temperature × treatment, P < 0.005). In Exp. 4, examination of the chromosomal organization for putative zygotes (18 hpi) revealed that HS affected (P < 0.001) their distribution into nuclear classes. The percentage of putative zygotes with a normal diploid pattern was 57% vs. 20% for 38C and 41C oocytes, respectively. In conclusion, HS during the first 12h of maturation disrupts nuclear maturation, induces apoptosis, alters the cytoskeleton, and reduces subsequent fertilization. These alterations are likely to be involved in the mechanism underlying heat shock induced disruption of oocyte competence and can be reduced by S1P.

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299 IN VITRO DEVELOPMENT OF IMMATURE PORCINE OOCYTES FERTILIZED IN VITRO TO THE BLASTOCYST STAGE

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\textsuperscript{A}Developmental Biology Department, National Institute of Agrobiological Sciences, Ibaraki, 305-8602, Japan; \textsuperscript{B}Genetic Diversity Department, National Institute of Agrobiological Sciences, Ibaraki, 305-8602, Japan; \textsuperscript{C}Animal Reproduction Unit, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan; \textsuperscript{D}Prime Tech Ltd., Hangzhou, Zhejiang, China; \textsuperscript{E}Institute of Animal Breeding, University of West Hungary, Masonmagyarovar, 9200, Hungary; \textsuperscript{F}Present address: Department of Biology, University of Pennsylvania, Philadelphia, PA 19104, USA; \textsuperscript{G}Present address: Department of Research Planning and Coordination, National Institute of Livestock and Grassland Science, Tsukuba, Ibaraki, Japan. Email: somek76@freemail.hu

In vitro fertilization (IVF) and embryonic development of mature and meiotically arrested porcine oocytes were compared in this study. After in vitro maturation (IVM) for 48 h of cumulus-oocyte complexes, 75.4% (n = 442) of them extruded a visible polar body (PB). Most oocytes with a polar body (PB+ group) were found to be at metaphase II (M-II) stage (91.4%). Most oocytes without a visible polar body (PB− group, n = 144) appeared to be arrested at the germinal vesicle (GV) (41.6%) and first meiotic metaphase (M-I) (34.0%) stages. After IVF of oocytes (the day of IVF = Day 0), there was no significant difference between PB+ and PB− groups in rates of sperm penetration, monospermy, and oocyte activation after the penetration. Embryonic development was assessed by staining with 1% orcein. On Day 2, although there was no difference between the embryo cleavage in PB+ (n = 447) and PB− (n = 217) groups (47.0% and 35.9%, respectively), PB+ embryos had more cells than the PB− embryos (3.37 and 2.81 cells, respectively) (P < 0.05; ANOVA). On Day 4, the cleavage rate of PB+ embryos was higher than that of PB− embryos (45.4% and 24.3%, respectively), and PB+ embryos had more cells than the PB− embryos (8.26 and 6.0 cells, respectively) (P < 0.05; ANOVA). On Day 6, a significantly higher number of PB+ embryos developed to the blastocyst stage than that of the PB− embryos (34.6% and 20.7%, respectively) (P < 0.05). However, by subtracting the GV oocytes from the PB− group, there was no difference in blastocyst rates between the M-I arrested and M-II oocytes (35.3% and 34.6%, respectively). The number of blastomer nuclei in embryos obtained from the PB+ group (52.0) was significantly higher than that of the PB− group (29.1); however, the proportion of inner cell mass and trophoderm cells in PB+ and PB− blastocysts did not differ significantly (1.19 and 1.22, respectively) (P < 0.05). Chromosome analysis revealed that PB+ blastocysts had significantly more diploid blastomeres (69.7%) than PB− blastocysts (44.0%), whereas PB− blastocysts had significantly more triploid cells (34.0%) compared with PB+ oocytes (8.4%) (P < 0.05; \chi\textsuperscript{2} test). These results indicate that porcine oocytes arrested at the M-I stage undergo cytoplasmic maturation during culture and have the same ability to develop to blastocysts after IVF as M-II oocytes but with a lower cell number; the latter might be caused by the slower embryonic development.

300 GLUCOSAMINE SUPPLEMENTATION DURING IN VITRO MATURATION LEADS TO PERTURBED DEVELOPMENTAL CAPACITY OF BOVINE CUMULUS OOCYTE COMPLEXES

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Glucose is a primary energy substrate required for successful in vitro oocyte maturation (IVM). However, most maturation media contain more glucose than that seen in follicular fluid (2.3 mM vs. 5.6 mM in TCM199). Glucosamine (Glc) as an alternative substrate for extracellular matrix
during cumulus expansion reduced glucose uptake by bovine cumulus oocyte complexes (COCs, Sutton-McDowall et al. 2004 Reproduction 128, 313–319). As this could enable a reduction in glucose concentrations to physiological levels in IVM medium of COCs, the aim of this study was to investigate the influence of Glc supplementation on oocyte developmental capacity. Bovine COCs were matured in synthetic follicular fluid medium (SFFM, a defined medium based on the composition of follicular fluid, plus 5.6 mM glucose, FSH, hCG and BSA, Sutton-McDowall et al. 2004 Reprod. Fertil. Dev. 16 sup, 204) ± 5 mM Glc. After 24 h, either nuclear maturation (rep = 8, n = 160) or blastocyst development 8 days post-fertilization (rep = 5, n = 400) was determined. Data was arc sine transformed and analyzed by ANOVA, followed by Tukey’s test. While the presence of Glc did not affect the completion of nuclear maturation and early cleavage, +Glc led to severely perturbed blastocyst development (−Glc, 32.5 ± 1.9% vs. +Glc, 4.7 ± 3.9%, P < 0.001). Glc supplementation in somatic cells is well-known to down-regulate the phosphatidylinositol-3-kinase (PI3K) signaling pathway, reducing protein synthesis and other cell survival mechanisms. Therefore, oocyte protein synthesis (measured by [2,3,4,5,6-³H]phenylalanine incorporation, rep = 5, n = 200) and embryo development (rep = 6, n = 720) following IVM in SFFM ± Glc ± EGF (a PI3K pathway stimulator) was determined. Glc supplementation led to a 40% decrease in protein synthesis compared to −Glc, while the combination of +Glc + EGF significantly increased protein synthesis by 60%. However, IVM + EGF + Glc did not improve blastocyst rates (main effect: −Glc 41.6 ± 6.6% vs. +Glc, 6.6 ± 1.7%, P < 0.001). Additionally, COCs were also cultured in SFFM ± 50 μM LY294002 (a specific PI3K inhibitor) and nuclear maturation (rep = 5, n = 200) or blastocyst development 8 days post-fertilization (rep = 4, n = 200) was determined. Despite the presence of LY294002 leading to 43% less COCs completing nuclear maturation (P < 0.001), blastocyst development was not affected (mean = 38.8 ± 3.2%). These results demonstrate that Glc supplementation during IVM has no effect on nuclear maturation or early development but is detrimental to oocyte developmental capacity by severely perturbing blastocyst development. However, the diminished developmental capacity appears to be independent of the well-characterized Glc down-regulation of the PI3K signaling pathway.

This work was supported by the Australian Research Council (SPIRT, C00107702) and Cook Australia Pty Ltd.

301 EFFECT OF ENERGY SUBSTRATES ON METABOLISM, NUCLEAR MATURATION, AND DEVELOPMENT OF GILT AND SOW OOCYTES DURING IN VITRO MATURATION

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Metabolic mechanisms control both nuclear and cytoplasmic maturation in oocytes. Elevated glucose metabolism is typically associated with improved developmental competence. The objective of this study was to compare nuclear maturation, oocyte metabolism, and subsequent embryonic development following the use of different energy substrates during in vitro maturation (IVM) and to determine the specific role of each substrate. Cumulus-oocyte complexes (20–50/treatment (Trt)/replicate) were placed into maturation medium for 42 h in 7% CO2 in air at 38°C. Maturation treatments included a negative control (NC; 0.01 mM pyruvate and 6 mM lactate), addition of 1:100 dilution of fatty acids (FA; Gibco, Grand Island, NY, USA), 1 × NEAA/0.5 × EAA/1 mM glutamine (AA), or 2 mM glucose (GLU) individually; and a positive control (PC; addition of all three substrates). For each of six replicates, metabolism of 10 denuded oocytes/treatment was measured in hanging drops containing labeled glucose (0.0125 mM 5-³H glucose, glycolysis; 0.482 mM 1-¹C glucose, pentose phosphate pathway, PPP). Oocytes were then fixed and stained for determination of meiotic stage. Remaining oocytes were fertilized and cultured in vitro. Cleavage and blastocyst development were recorded at 30–40 and 144 h post-insemination, respectively. The Purdue Porcine Media system was used throughout (PPM; Herrick et al. 2003 Reprod. Fertil. Dev. 15, 249–254). All data were subjected to analysis of variance. Oocyte metabolism and embryonic development are presented in Table 1. Except for FA, energy substrate influenced the percentage of oocytes reaching metaphase II (NC, 1.37 ± 0.06; PC, 54.29 ± 4.10, P < 0.001) or blastocyst development 8 days post-fertilization (rep = 4, n = 200) was determined. Despite the presence of LY294002 leading to 43% less COCs completing nuclear maturation (P < 0.001), blastocyst development was not affected (mean = 38.8 ± 3.2%). These results demonstrate that Glc supplementation during IVM has no effect on nuclear maturation or early development but is detrimental to oocyte developmental capacity by severely perturbing blastocyst development. However, the diminished developmental capacity appears to be independent of the well-characterized Glc down-regulation of the PI3K signaling pathway.

Table 1. Metabolism and development of oocytes after IVM

<table>
<thead>
<tr>
<th>Age</th>
<th>Trt</th>
<th>n</th>
<th>Glycolysis1</th>
<th>PPP1</th>
<th>% Cleaved</th>
<th>% Blast2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gilt</td>
<td>NC</td>
<td>17</td>
<td>1.0 ± 0.4a</td>
<td>0.6 ± 0.11b,c</td>
<td>97</td>
<td>0a</td>
</tr>
<tr>
<td></td>
<td>FA</td>
<td>22</td>
<td>0.9 ± 0.4a</td>
<td>0.5 ± 0.2b,c</td>
<td>93</td>
<td>5.6 ± 2.5d,e</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>25</td>
<td>3.3 ± 0.6a</td>
<td>0.7 ± 0.1b,y</td>
<td>100</td>
<td>34.9 ± 3.7b,z</td>
</tr>
<tr>
<td></td>
<td>GLU</td>
<td>21</td>
<td>0.6 ± 0.1a</td>
<td>0.3 ± 0.1a,y</td>
<td>100</td>
<td>21.9 ± 4.9b,c</td>
</tr>
<tr>
<td>Sow</td>
<td>PC</td>
<td>27</td>
<td>3.3 ± 0.4a</td>
<td>0.8 ± 0.1a,b</td>
<td>97</td>
<td>44.1 ± 10.1b,y</td>
</tr>
<tr>
<td></td>
<td>NC</td>
<td>5</td>
<td>0.1 ± 0.1a</td>
<td>0.1 ± 0.1a</td>
<td>58</td>
<td>0a</td>
</tr>
<tr>
<td></td>
<td>FA</td>
<td>10</td>
<td>0.2 ± 0.1a</td>
<td>0.2 ± 0.1a</td>
<td>62</td>
<td>0a</td>
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<tr>
<td></td>
<td>AA</td>
<td>14</td>
<td>1.1 ± 0.3b</td>
<td>0.3 ± 0.1a,b</td>
<td>62</td>
<td>10.5 ± 5.0b,c</td>
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<tr>
<td></td>
<td>GLU</td>
<td>26</td>
<td>1.2 ± 0.3b</td>
<td>0.4 ± 0.1a,b</td>
<td>58</td>
<td>15.8 ± 4.6b,c</td>
</tr>
<tr>
<td></td>
<td>PC</td>
<td>27</td>
<td>3.3 ± 0.4a</td>
<td>0.8 ± 0.1b</td>
<td>62</td>
<td>24.9 ± 7.6b</td>
</tr>
</tbody>
</table>

1 pmol/oocyte/3 h; 2 blastocyst/cleaved embryos.
Different superscripts within columns and ages are different (a,b,c x P < 0.05) or signify a trend (b,c ± P < 0.09).
302 PROTEIN SUPPLEMENTATION TO IVM MEDIUM IN RELATION TO THE INCIDENCE OF APOPTOSIS IN BOVINE OOCYTES MATURED IN VITRO

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Mammalian embryos derived from in vitro fertilization display lower developmental competence and quality when compared to their in vivo counterparts. The composition of culture media significantly contributes to this phenomenon. Media supplemented with FBS or the serum derivate BSA are described as biochemically undefined. Those macromolecules were shown to exert a wide range of effects on cultured embryos, dependent on batch-to-batch variability. Therefore, replacement of these protein sources with a synthetic macromolecule such as polyvinyl pyrrolidone (PVP) or polyvinyl alcohol (PVA) provides a possibility to use a chemically defined culture medium (Ali et al. 2002 Biol. Reprod. 66, 901–905). Apoptosis as programmed cell death naturally occurs in mammalian oocytes and embryos; however, its incidence is significantly higher in vitro. The aim of this study was to investigate whether protein supplementation (FBS, fatty acid-free (faf)-BSA, PVP40) of IVM medium affects the incidence of apoptotic oocytes. In the present study, the IVM system previously described by Makarevich et al. (2002 Biol. Reprod. 66, 386–392) was used. Briefly, follicular oocytes aspirated from slaughterhouse ovaries were matured in vitro in one of three maturation media supplemented with FBS (10%), faf-BSA (6 mg mL\(^{-1}\)) or PVP40 (4 mg mL\(^{-1}\)). The terminal TUNEL assay kit was used to detect the DNA fragmentation in apoptotic cells (DeadEnd\textsuperscript{TM} Fluorometric TUNEL system, Promega, Madison, WI, USA). The data were analyzed by chi-square test of independence. Altogether, 630 oocytes collected during 12 IVM experiments were subjected to the Tunel test, and 563 (89.4%) of them were successfully investigated: 426 after maturation in vitro and 137 follicular, non-matured. The remaining 67 cells were lost during manipulation. The rate of Tunel-positive cells differed (\(P < 0.001\)) between matured (11.8%) and follicular oocytes (1.5%). Protein supplementation of IVM media did not significantly affect the rate of apoptotic oocyte occurrence, which was 9% in the faf-BSA group, 11.5% in the FBS group, and 15% in the PVP group. No differences were observed in the rate of Tunel-positive cells between oocytes at MII and MI stages. In conclusion, protein supplementation of IVM medium used in the present study did not affect the incidence of apoptotic oocytes after maturation in vitro.

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303 EFFECT OF SERUM SUPPLEMENTATION AND ESTRUS CYCLE STAGE ON IN VITRO NUCLEAR MATURATION OF CANINE OOCYTES

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The present study investigated the effects of the estrus cycle stage and serum supplementation on nuclear maturation of canine oocytes. Ovaries were collected from a private clinic after ovariohysterectomy and classified into follicular, luteal, or anestrus stages through a combination of ovarian morphology and vaginal cytology. A total of 2214 oocytes from 196 ovaries (903 oocytes from 96 anestrus ovaries, 609 oocytes from 36 follicular ovaries, and 702 oocytes from 64 luteal ovaries) were used for experiments. The oocyte retrieval per ovary was 10, 19, and 12 for anestrus, follicular, and luteal-phase ovaries, respectively. In Exp. 1, immature oocytes were cultured for 72 h in TCM-199 alone or TCM-199 supplemented with 10% canine anestrus (CAS), estrus (CES), or diestrus (CDS) serum or fetal bovine serum (FBS). In Exp. 2, immature oocytes were cultured for 72 h in TCM-199 supplemented with 0, 5, 10, or 20% CES. After staining with Hoechst 33342, chromatin stage and position as well as spindle formation were evaluated to determine the stage of meiosis: germinal vesicle (GV) stage, germinal vesicle breakdown (GVBD), metaphase I (MI) stage, metaphase II (MII) stage. The experiments with anestrus and luteal-phase oocytes were repeated eight times and follicular-phase oocytes were repeated six times. Data were subjected to analysis of variance (ANOVA) and protected least significant difference (LSD) test to determine differences among experimental groups by using the Statistical Analysis System (SAS, SAS Institute, Inc., Cary, NC, USA) program. Statistical significance was determined where \(P\) value was less than 0.05. In Exp. 1, the in vitro maturation of oocytes up to MII stage was higher when oocytes were collected from ovaries in follicular phase. The maturation rate up to MII stage was 0.0 to 1.7%, 1.3 to 10.2%, and 1.0 to 3.2% for the oocytes collected from the anestrus, follicular, and luteal-phase ovaries, respectively, depending on the culture media used. In basic TCM media only, 0.0, 1.3, and 2.3% oocytes reached the MII stage for anestrus, follicular, and luteal-phase oocytes, respectively. A significantly higher rate of maturation was obtained when oocytes collected from follicular phase were cultured in TCM-199 supplemented with 10% CES (10.2%), compared to 10% CAS (4.0%), CDS (2.7%), FBS (1.3%), or the control (1.3%). In Exp. 2, supplementing with 10% CES induced the highest (\(P < 0.05\)) maturation rate to the MII stage in oocytes collected from follicular-stage ovaries (11.5%) compared to supplementing with 0% (1.0%), 5% (1.3%), or 20% CES (5.1%). Supplementing with CES (5, 10, or 20%) did not have a significant effect on nuclear maturation of canine oocytes collected from anestrus or luteal-stage ovaries. In conclusion, supplementing in vitro maturation medium with 10% CES increased nuclear maturation of canine oocytes, and canine oocytes collected from follicular-stage ovaries are the most suitable to complete nuclear maturation in vitro.

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