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 (2×10^6 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% CO_2 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 24 h, 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 (4×10^6 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% CO_2 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 apparently influenced by the maturation culture period of oocytes.

Table 1. Effect of maturation culture period on the development of bovine oocytes after IVF and the sex ratio of blastocysts

Maturation culture period (h)	No. of oocytes examined	Mean ± SEM of blastocysts	No. of blastocysts examined	Mean of male blastocysts
16	278	13.5 ± 1.8^{a}	22	31.8^{a}
22	219	22.7 ± 2.3^{b}	22	$40.9^{a,b}$
28	274	15.2 ± 1.4^{a}	22	$68.2^{b,c}$
34	435	11.7 ± 1.2^{a}	22	72.7^{c}

[a–c] Values with different superscripts in the same column are significantly different ($P < 0.05$).

276 THE EFFECT OF CUMULUS CELLS DURING MATURATION ON THE RISE IN THE CONCENTRATION OF INTRACELLULAR Ca^{2+} ($[Ca^{2+}]i$) OF PORCINE OOCYTES INDUCED BY INOSITOL 1,4,5-TRISPHOSPHATE

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Increase of inositol 1,4,5-triphosphate (IP3) in the cytoplasm of mammalian oocytes is said to be responsible for $[Ca^{2+}]i$ oscillation observed in the oocytes immediately after sperm penetration, and the $[Ca^{2+}]i$ 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 $[Ca^{2+}]i$ 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 Ca^{2+} indicator fluorescent dye Fura2-AM, and then were irradiated by 340 nm and 360 nm of 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 Ca^{2+} and the dye intensifies E340, but does not change E360, the level of $[Ca^{2+}]i$ 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 $[Ca^{2+}]i$ was observed in the mature oocytes from every group. The peak R of the rise in $[Ca^{2+}]i$ 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 $[Ca^{2+}]i$ 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. After IVM, no differences were found among oocyte groups. During maturation, a decrease of p34cdc2-RNA was found in 125–135 μm oocytes. 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.

Table 1. p34cdc2-RNA expression of prepubertal goat oocytes according to nuclear stage and oocyte sizes

Size (μ)	Oocytes at collection time				Oocytes after IVM			
	Nuclear stage			p34cdc2-RNA (mean ± SD)	Nuclear stage			p32cdc2-RNA (mean ± SD)
	N°	GV	MII		N°	GV	MII	
≤110	75	62[a]	0	0.26 ± 0.10[a]	138	117[a]	5[a]	0.43 ± 0.42
110–125	105	51b	0	0.24 ± 0.20[a,α]	474	66[b]	200[b]	1.58 ± 0.19[β]
125–135	250	34[c]	4	2.40 ± 0.71[b,α]	414	10[c]	267[c]	1.19 ± 0.49[β]
≥135	198	3[d]	4	0.36 ± 0.38[a]	70	0[c]	50[c]	1.08 ± 0.62

[a,b,c] Values within each column and [α,β] within each row differ significantly ($P < 0.05$).

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278 MEIOTIC RESUMPTION *IN VITRO* OF CANINE OOCYTES: COMPARATIVE METHODS

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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 ovariectomy. Ovaries were sliced repeatedly to release oocytes; only cumulus-oocytes complexes with two or more dense layer of cumulus cells, darkly granulated cytoplasm, and >110 μm in diameter were selected for experiments. In the first experiment, oocytes were pre-incubated for different times (1, 3, 20 h) in TCM 199 + 20% FCS + 0.5 μM or 2 μM OA and thereafter cultured for 48 h in the same medium without OA. A group of oocytes was matured in TCM 199 + 20% FCS for 72 h as control. At the end of culture, oocytes were stained with glycerol-Hoechst 33342 to evaluate meiotic stage. 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 phytohemoagglutinin 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)[a]	0	4 (7.0)[c]	10 (17.5)
OA	2 μM	1 h	54	3 (5.6)	46 (85.2)[b]	0	4 (7.4)[c]	1 (1.8)
		3 h	45	3 (6.7)	36 (80.0)[b]	0	4 (8.9)[c]	2 (4.4)
		20 h	73	3 (4.1)	60 (82.2)[b]	0	6 (8.2)[c]	4 (5.5)
	0.5 μM	1 h	39	2 (5.1)	20 (51.3)[b]	5 (12.8)	12 (30.8)[c]	0
		3 h	43	2 (4.6)	34 (79.1)[b]	1 (2.3)	4 (9.3)[c]	2 (4.7)
		20 h	69	5 (7.2)	58 (84.1)[b]	0	6 (8.7)[d]	0

[a] vs. [b] $P < 0.001$; [c] vs. [d] $P < 0.05$; χ^2 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. Cumulus-oocyte complexes (COC) were recovered by mincing. *In vitro* maturation was carried out in TCM199 supplemented with 10% fetal calf serum, 10 IU/mL of LH, 0.1 IU/mL of FSH, and 1 mg/mN of 17β-estradiol at 39.0°C in 5% CO_2 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 Ca^{2+} current components, i.e. L-type Ca^{2+} channels. For intracellular calcium determinations, oocytes were placed in Ham F10 and injected with the 0.5 mM calcium green dextran (Mr 10,000). Ca^{2+} stores were evoked by the addition of 5 μM Ca^{2+} 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. 11.7 ± 0.2 nS and 21.7 ± 0.2 vs. 15.4 ± 0.2 nS, respectively; $P < 0.01$) or L-type Ca^{2+} 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 Ca^{2+} ionophore exposure but a higher ion activation current was found in lamb oocytes (489 ± 56 vs. 300 ± 73 pA; $P < 0.05$). Ca^{2+} 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 β-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 β-endorphin (β-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 β-end (30 μM)- or Nx (1mM and 10 μM)-induced calcium concentration were calculated in single cells ($n = 194$) and are expressed as Δ fluorescence ($F_{maximal\ effect} - F_{baseline}$) 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 β-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 β-end and Nx may be explained in terms of a binding of the two subtances 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 espression 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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281 MITOGEN-ACTIVATED PROTEIN KINASE IN PORCINE CUMULUS CELLS

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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, 604–609). 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, L-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 nonatretic 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. Injection was performed as described by Martin (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 embryonc development of prepubertal goat oocytes. Prepubertal (1- to 2-month old) goat oocyte 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 ionomycin for

5 min and 2 mM 6-DMAP for 4 h; and (3) parthenogenetic oocyte group used as a control for chemical activation. Presumptive zygotes and activated oocytes were cultured in TALP fertilization medium for 24 h. After fertilization, all groups were divided into two groups and cultured for 192 h in: (a) sequential media G1.3/G2.3; or b) TCM199 with granulosa cells. The number of cell nuclei in embryos was assessed by Hoescht staining. Each experiment was repeated seven times, and results were analyzed using chi-square or Fisher's test. The percentage of total embryos obtained after IVF, ICSI, and chemical activation was higher ($P < 0.05$) for ICSI than for IVF in G1.3/G2.3 (81.4 and 67.9%, respectively) but not in co-culture (61.9 and 58.2%, respectively). The percentage of total embryos was higher ($P < 0.05$) in G1.3/G2.3 than in co-culture for both IVF oocytes (67.9 and 58.2%, respectively) and ICSI oocytes (81.4 and 61.9%, respectively). The highest percentage of 8- to 16-cell embryos (28.1%) was obtained in the ICSI group cultured in G1.3/G2.3 medium. We did not find significant differences among the experimental groups in the percentage of morulae. 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.

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

	G1.3/G2.3			Co-culture		
	IVF	ICSI	Parthenogenetic	IVF	ICSI	Parthenogenetic
No. of oocytes	215	70	147	208	63	139
Total embryos	146^{b}	57^{a}	86^{bc}	121^{c}	39^{bc}	58^{d}
2–7-cell stage	132^{ab}	38^{c}	83^{a}	106^{b}	32^{bc}	55^{ab}
8–16-cell stage	13^{bc}	16^{a}	3^{c}	13^{bc}	6^{ab}	3^{bc}
Morulae	1	3	0	2	1	0
Morula-cell number	18	19–50	0	17–25	46	0

$^{a–d}$ Values with different superscripts within each row differ significantly ($P < 0.05$).

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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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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 30 μs), and then cultured in PZM-3 medium for 6 days. Apoptotic cells in blastocysts were detected by TUNEL assay and total cell number was examined by propidium iodide (PI) counterstaining. Data were analyzed by chi-square and Student's *t*-test. The first experiment was conducted to determine the effect of roscovitine (0, 12.5, 25, 50, and 100 μM) on meiotic inhibition of GV oocytes. This effect was dose-dependent, and a concentration of 50 μM 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%, 15/97), 12.5 (32.1%, 36/112), 25 (57.4%, 54/94), and 100 μM (77.8%, 77/99). 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 (33.7 ± 12.4 vs. 35.1 ± 12.6) and apoptotic (2.2 ± 2.4 vs. 2.2 ± 1.2) 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.

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 (GVBD). After gonadotrophin 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 by 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 ($n = 2305$): TCM-199 with glutamax-I (GIBCO, Paisley, UK), 0.25 mM Na-pyruvate, 100 IU mL^{-1} penicillin, and 100 $\mu g\,mL^{-1}$ streptomycin; (2) FBS IVM ($n = 2484$): M199 IVM medium with hormones (10 $\mu g\,mL^{-1}$ LH, 2 $\mu g\,mL^{-1}$ FSH, and 100 $\mu g\,mL^{-1}$ 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 SOFaaci + 4 mg mL^{-1} FAFBSA in 5% O_2 (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-expanded embryos were stained with TUNEL ($n = 96$) or with DF ($n = 88$). Modified TUNEL protocol (Upstate, Lake Placid, NY, USA; Makarevich A and Markkula M 2002 Biol. Reprod. 66, 386–392) and DF staining protocol (Thouas G.A. *et al.* 2001 Reprod Biomed Online 3, 25–29) were used. The results of embryo cleavage and D7 embryo development are based on logistic regression models, and the results of proportion of ICM and apoptotic index on general linear mixed models.

After FBS IVM, 83.6% of the fertilized oocytes were estimated to cleave and 25.5% to develop to the blastocyst stage by Day 7. The estimations for embryo cleavage and Day 7 development rates were significantly lower in FAFBSA IVM and M199 IVM groups ($P < 0.0001$): 74.0% and 15.0% for the FAFBSA, 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 ($P < 0.001$). 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 diphenyleneiodonium (DPI) 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 phosphoribose 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

TRT	mM	n	% Immature	% Mature	Meiotic score
STND		93	$7.5 \pm 2.7^{a,l,v}$	$80.6 \pm 4.1^{a,l,w}$	6.1 ± 0.2^{a}
DPI		102	$35.3 \pm 4.8^{a,b,j,y,z}$	$25.5 \pm 4.3^{b,j,y,z}$	3.6 ± 0.2^{b}
NADP	0.25	91	53.8 ± 5.3^{b}	4.4 ± 2.2^{b}	2.5 ± 0.2^{c}
	2.5	107	19.6 ± 3.9^{a}	$50.5 \pm 4.9^{a,b}$	4.9 ± 0.2^{d}
	5	107	13.1 ± 3.3^{a}	66.4 ± 4.6^{a}	$5.6 \pm 0.2^{a,d}$
PRPP	0.25	41	61.0 ± 7.7^{k}	$9.8 \pm 4.7^{j,k}$	2.5 ± 0.3^{c}
	2.5	44	59.1 ± 7.5^{k}	$9.1 \pm 4.4^{j,k}$	2.5 ± 0.3^{c}
	5	37	62.2 ± 8.1^{k}	0^{k}	2.1 ± 0.2^{c}
	12.5	19	$5.3 \pm 5.3^{i,j}$	68.4 ± 11.0^{i}	5.4 ± 0.4^{a}
R5P	0.25	38	55.3 ± 8.2^{w}	2.6 ± 2.6^{y}	2.2 ± 0.2^{c}
	2.5	77	$44.2 \pm 5.7^{w,y}$	$22.1 \pm 4.8^{y,z}$	$3.3 \pm 0.3^{b,c}$
	5	94	$27.7 \pm 4.6^{x,z}$	$35.1 \pm 4.9^{x,z}$	4.0 ± 0.2^{b}
	10	63	$17.5 \pm 4.8^{v,x}$	55.6 ± 6.3^{x}	5.1 ± 0.3^{a}

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

PLSD test. Three stages of GV oocytes were identified: GVI, with filamentous chromatin distributed in the nucleoplasm; GVII, with chromatin condensed into thick clumps; and GVIII, with chromatin condensed into a single clump. The GVIII stage showed a lower proportion of functional open communication than the GVI and GVII groups (8.5 vs. 45.7 and 46.1, respectively, $P < 0.05$). However, when compared with each other, the GVI stage oocytes showed lower embryonic developmental competence (12.9 in GVI vs. 22.1 and 24.2 in GVII and GVIII, respectively, $P < 0.05$). Our findings indicate that the status of communication between oocytes and cumulus cells could be related to the chromatin organization in immature bovine oocytes. A direct correlation between the communications grade, the modulation of oocyte transcriptional activity, and the acquisition of oocyte developmental competence remain to be confirmed.

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289 EFFECTS OF THE STORAGE OF BOVINE OVARIES ON THE NUCLEAR MATURATION AND DEVELOPMENT OF *IN VITRO* PRODUCED EMBRYOS

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The objectives of this study were to investigate effects of storage of bovine ovaries on the maturation of oocytes and to determine the optimal maturation time for oocytes obtained from the stored ovaries. Ovaries were obtained at a local abattoir and transported in physiological saline to the laboratory (18°C, 3 h; storage group). As a control, oocytes were collected from ovaries without storage. Other ovaries were kept in a plastic bag without solution (Bag-group) or with saline (Saline-group). These ovaries were preserved at 20°C for 18 h. Then cumulus-oocyte complexes were collected and maturated in TCM-199 + 5% CS. In Experiment 1, to investigate effects of the storage methods of bovine ovaries on the timing of germinal vesicle breakdown (GVBD) and the progression to MII in oocytes obtained from ovaries, oocytes were fixed every 2 h (0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 h) from the start of *in vitro* maturation, and then stained for examination of their nuclear stage. In Experiment 2, to investigate effects of length of *in vitro* maturation (18, 20, 22, 24 h) of oocytes (18-h, 20-h, 22-h and 24-h group, respectively) obtained from the ovaries stored in a saline for 18 h at 20°C on the subsequent *in vitro* development after IVF and IVC. Following insemination, the presumptive zygotes were cultured in CR1aa + 5% CS for 6 days to assess the development of embryos on Day 2 (Day 0 = the day of IVF) for rates of cleavage and on Day 6 for rates of embryo development to morulae (M), compacted morulae (CM), and blastocyst (BL) stages. The data of nuclear stage were analyzed by ANOVA after transformation to arcsine, and the rates of embryo development were analyzed by chi-square. There were two peaks of GVBD in the storage group, one occurred at 2 h of maturation culture, the other at 4–8 h of culture as control. There were between-treatment differences in the timing of increase in the rates of oocytes to reach MII. After 12 h of culture 21.2 ± 1.1% of oocytes in the Saline-group and 11.6 ± 4.6% of oocytes in the Bag-group reached MII, but no oocytes in the control group reached MII ($P < 0.05$). Furthermore, the rate of oocytes in the Saline-group matured to MII at 20 h of culture was lower than that of the control group (Bag-group: 67.9 ± 7.3%; Saline-group: 61.2 ± 14.5%; control: 82.9 ± 5.3%) ($P < 0.05$). The rates of embryos that cleaved after IVF of IVM oocytes in the 18-h group (90.2 ± 7.0%) was higher than those of the other groups (20-h group: 81.3 ± 8.2%, 22-h group: 80.5 ± 13.2%, 24-h group: 75.8 ± 6.0%) ($P < 0.05$). The rate of embryos developed to M, CM, and BL stages in the 18-h group (48.4 ± 6.7%) was the highest among the treatments, and significantly higher than that of the 24-h group (36.2 ± 6.7%) ($P < 0.05$). These results indicated that the timing of undergoing GVBD and reaching MII of oocytes obtained from the stored ovaries was earlier than that of oocytes obtained from the non-preserved ovaries, and the optimal maturation time for oocytes obtained from stored ovaries was 18 h.

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290 MODULATION OF ARYLHYDROCARBON RECEPTOR ACTIVITY DURING *IN VITRO* MATURATION OF BOVINE OOCYTES

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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/germinal 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 Duncans multiple rance 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.

291 EFFECT OF DIFFERENT TRANSPORT TEMPERATURES (+4°C, +32°C) ON *IN VITRO* MATURATION OF OOCYTES COLLECTED FROM CATTLE AND SHEEP OVARIES

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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% CO_2, 5% O_2, 90% N_2 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 +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

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

[a,b] Rates with different letters in the same column are statistically significant in sheep ($P < 0.001$).
* The differences between the groups are not significant in cattle.
No. of: number of oocytes reaching indicated stage;
GV: Germinal vesicle; GVBD: Germinal vesicule break down;
MI: Metaphase I; AI-TI: Anaphase I-Telephase I; MII: Metaphase II;
UDNM: Undefined material.

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292 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

and the medium exchange on the development of Korean Native Cow (KNC) oocytes. Ovaries of KNC were obtained from a local slaughterhouse and cumulus oocyte complexes (COCs) were aspirated from 2- to 8-mm follicles. Groups of 15 COCs were matured in TCM-199 supplemented with 10% fetal calf serum (FCS), 1 μg/mL FSH, 10 μg/mL LH, and 1 μg/mL estradiol-17β for 18 h. *In vitro*-matured oocytes were fertilized using frozen-thawed percoll-separated spermatozoa (Day 0) in fer-TALP medium for 20 h and cultured in CR1aa medium supplemented with 0.3% BSA (before Day 3) or 10% FBS (after Day 3). All cultures were maintained in an incubator at 39°C, 5% CO_2 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 KNC 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 $NaHCO_3$, 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).

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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). Cumulus 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 cumulus cell removal, and MII 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

iodide after 72 h of culture in SOF supplemented with 5% calf serum at 38.5°C in a modified atmosphere (5% CO_2, 5% O_2, and 90% N_2). Our data indicated that oocytes cultured in the presence of cysteamine had a nuclear maturation rate similar to those cultured in control medium (Table 1). Intraoocyte GSH content increased during IVM, and the addition of cysteamine induced a significant GSH accumulation in matured oocytes. After ICSI, a similar proportion of zygotes in each group developed beyond the two-cell stage after 72 h of culture. The results of this study demonstrate that the addition of cysteamine to the IVM medium increases GSH content in equine oocytes. However, this affects neither the maturation rate nor the capability to reach the early embryonic development after ICSI. We hypothesize that factor(s) other than GSH content are responsible for the limited *in vitro* developmental capability of equine oocyte.

Table 1. Effect of cysteamine administration on maturation rate, oocyte GSH content (pmol/oocyte), and early embryonic development after ICSI

Treatment	Maturation (*n*)	GSHi (*n*)	>2 cells at 72 h (*n*)
Control	70.7 (41/58)	7.8 (15)[a]	68.4 (21)
Cysteamine	70.1 (40/57)	9.9 (18)[b]	71.6 (23)

[a,b] Values with different superscripts differ significantly; $P < 0.05$ (non-parametric Kruskal-Wallis test).

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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 avoid movement of the object. Gelatine (Gel) and poly-L-lysine (PLL) have previously been used to promote adhesion of cells in culture. The aim of our study was to develop a method to avoid movement during IVM of partially denuded COCs without compromising oocyte viability. Previous experiments in our lab showed that partial denudation of COC had no effect on embryo development (unpublished). Bovine COCs were obtained from abattoir ovaries. In the control group COCs were placed in non-treated dishes. In the experimental groups, they were placed in Gel- or PLL-coated dishes, either intact or partially denuded, where the length of cumulus cell "tails" was shortened to around 200 μm on each side of the oocyte. The coated dishes were prepared 24 h prior to IVM with 200 μL of 0.1% Gel (Sigma, Copenhagen, Denmark, G2500) or 200 μL 0.01% PLL (Sigma, P-4832). Partial denudation of COCs was performed with a 127–129 μm diameter denudation pipette. Standard procedures were used for IVM (23 h in DMEM with 5% serum and eCG/hCG), IVF (23 h in TALP), and IVC (SOF with 10% serum); IVM and IVF were incubated at 38.5°C in 5% CO_2 in air, and IVC at 5% CO_2 in 5% O_2. The study was based on a total of 1151 oocytes and 3 replicates. Day 8 blastocyst (BL) rates, BL kinetics, and morphology were used as endpoints to assess oocyte maturation. Kinetics/morphology were graded by a scoring system: hatched/excellent 3, expanded/good 2, non-expanded/poor 1. COCs placed in Gel- or PLL-coated dishes did not move during handling of the dishes. The BL rates in the Gel group were 37%, 25%, and 17%, and in the PLL group 24%, 21%, and 12%, for the control, intact, and partially denuded COCs, respectively. In the Gel group the BL rates showed a decreasing trend ($P < 0.0036$), whereas in only the PLL group the BL rates from the partially denuded COC differed from the control and the intact COCs ($P < 0.008$). No significant differences were seen between blastocyst kinetics (Gel/PLL 1.9/1.9, 1.8/1.9, 1.6/1.7) or morphology (Gel/PLL 2.2/2.4, 2.0/2.5, 2.2/2.1) in the control, intact or partially denuded groups. Fisher's exact test used. We conclude that it is possible to avoid movement of COCs during IVM without compromising oocyte maturation in dishes coated with Gel or PLL, if the cumulus layer is intact. The BL rates are compromised if COCs are partially denuded and the "cumulus tails" shortened before IVM in Gel or PLL coated dishes, whereas kinetics and morphology are unaffected.

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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 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.

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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 (Räty 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 $\mu g\,mL^{-1}$ streptomycin, 10 $\mu g\,mL^{-1}$ LH, 2 $\mu g\,mL^{-1}$ FSH, and 1 $\mu g\,mL^{-1}$ 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% O_2. (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

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

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 12 h of maturation. Incubation during the last 10 h of maturation and 18 h post insemination (hpi) was at 38.5°C and 5% (v/v) CO_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-α-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 ($P < 0.001$) on distribution of oocytes into nuclear classes because S1P increased the proportion of 41C oocytes that were at MII. S1P also blocked the increase in percentage 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 12 h of maturation disrupts nuclear maturation, induces apoptosis, alters the cytoskeleteon, 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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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 trophectoderm cells in PB+ and PB− blastocysts did not differ significantly (1:1.9 and 1:2.2, 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%)($P < 0.05$; χ^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 arcsine 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) signalling pathway, reducing protein synthesis and other cell survival mechanisms. Therefore, oocyte protein synthesis (measured by [2,3,4,5,6-^{3}H] 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 signalling pathway.

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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% CO_2 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-^{3}H glucose, glycolysis; 0.482 mM 1-^{14}C 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 ± 0.01; FA, 1.35 ± 0.01; AA, 33.33 ± 0.06; GLU, 25.81 ± 0.06; PC, 54.29 ± 0.06) but age of oocyte donor did not. Blastocyst metabolism and cell number were not affected by treatment. In general, sows were more responsive to treatment effects. These data demonstrate that exogenous fatty acids do not play a role in porcine oocyte maturation. Amino acids appear to promote meiosis and glycolysis, but do not support oocyte developmental potential. Elevated metabolism in this treatment may be due to a recovery effect when glucose-starved oocytes were placed into glucose containing metabolism medium. Glucose appears to be important for meiosis and cytoplasmic maturation leading to developmental competence with minimal effect on oocyte metabolism. The success of the positive control suggests that a combination of glucose and amino acids is beneficial to maturation and embryonic development of porcine oocytes.

Table 1. Metabolism and development of oocytes after IVM

Age	Trt	*n*	Glycolysis[1]	PPP[1]	*n*	% Cleaved	% Blast[2]
Gilt	NC	17	1.0 ± 0.4^{x}	$0.6 \pm 0.1^{x,y,z}$	97	0^{a}	0^{x}
	FA	22	0.9 ± 0.4^{x}	$0.5 \pm 0.2^{x,z}$	93	$5.6 \pm 2.5^{a,c}$	0^{x}
	AA	25	3.3 ± 0.6^{y}	$0.7 \pm 0.1^{x,y}$	100	34.9 ± 3.7^{b}	0^{x}
	GLU	21	0.6 ± 0.1^{x}	0.3 ± 0.1^{z}	100	$21.9 \pm 4.9^{b,c}$	0^{x}
	PC	27	3.3 ± 0.4^{y}	0.8 ± 0.1^{y}	97	44.1 ± 10.1^{b}	6.2 ± 4.1^{y}
Sow	NC	5	0.1 ± 0.1^{a}	0.1 ± 0.1^{a}	58	0^{a}	0^{x}
	FA	10	0.2 ± 0.1^{a}	0.2 ± 0.1^{a}	62	0^{a}	0^{x}
	AA	14	1.1 ± 0.3^{b}	0.3 ± 0.1^{a}	62	$10.5 \pm 5.0^{a,b}$	7.1 ± 7.1^{x}
	GLU	26	1.2 ± 0.3^{b}	0.4 ± 0.1^{a}	58	$15.8 \pm 4.6^{a,b}$	$33.3 \pm 17.8^{x,y}$
	PC	27	3.3 ± 0.4^{c}	0.8 ± 0.1^{b}	62	24.9 ± 7.6^{b}	42.9 ± 20.2^{y}

[1] pmol/oocyte/3 h; [2] blastocyst/cleaved embryos.

Different superscripts within columns and ages are different (^{a,b,c} $P < 0.05$) or signify a trend (^{x,y,z} $P < 0.09$).

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^{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 state 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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