# ABSTRACTS FOR POSTER PRESENTATION



*Oocyte Maturation*

## 307 CHARACTERIZATION OF PROTEIN PHOSPHORYLATIONS IN THE COURSE OF MEIOTIC MATURATION OF BOVINE OOCYTES

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The importance of protein phosphorylations during meiotic maturation (transition from prophase I to metaphase II) of oocytes is documented by the fact that the inhibition of the M-phase kinases, cdc2k or MAPK, arrests the oocytes in the GV stage. A detailed knowledge of the targets of these kinases during this stage of development is still missing. Therefore, we have analyzed the proteome of bovine oocytes by high resolution 2D-gel electrophoresis to detect differences in the expression and phosphorylation state of proteins in the course of in vitro maturation (IVM). Bovine oocytes were matured for different times in TCM 199 containing 3% BSA and 300 oocytes each in GV stage (0-h maturation), in GVBD/M I (10-h maturation) or in M II stage (240 h maturation) were separated on the gels. The proteins were visualized by staining them with silver or with the fluorescence dye Sypro Ruby, and phosphorylated proteins were detected by Western Blotting with Ser-, Thr-, or Tyr-phosphorylation specific antibodies or by staining with the phosphoprotein specific fluorescence dye Pro-Q Diamond. Gels made from oocytes at the above mentioned maturation stages were compared by a computerized gel-overlay software program (2D Decodon, Greitswald, Germany). The overall protein synthesis was statistically analysed by ANOVA (SigmaStat, Ekrath, Germany), pairwise multiple comparison procedure. Only distinct spots with a difference greater than 30% in their optical densities were considered to be differently expressed or phosphorylated. The results showed a three-fold increase in the rate of overall protein synthesis (p 0.05) during GVBD. Newly synthesized proteins were detected mainly in the higher molecular weight (MW) range (60–80 kDa), and protein degradations were found mainly in the lower MW range (20–40 kDa) after GVBD. Preliminary data obtained by analyzing the phosphorylation pattern showed that obviously no phosphorylated proteins could be detected in the GV-stage oocytes. Phosphorylation of different proteins was observed at the time of GVBD after 6 to 10 h IVM, concomitantly with the activation of cdc2k and MAPK. A maximum of phosphorylated proteins was observed in metaphase II. The first results obtained by performing peptide mass fingerprinting using MALDI-Tof showed that members of the family of heat-shock proteins, ribosomal proteins and putative zinc finger proteins (transcription regulators) were differently expressed or phosphorylated during IVM. This work was supported by the DFG, To 178/1-1, 2 and by the Eibl-Stiftung.

## 308 MITOCHONDRIA RELOCATION, MICROTUBULE ASSEMBLING AND PARTHENOGENETIC DEVELOPMENTAL COMPETENCE OF PIG OOCYTES

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Developmental competence of in vitro-produced porcine embryos appears to be limited by specific maternally inherited cytoplasmic factors. We previously reported a relationship between mitochondria distribution during IVM, energy status, and oocyte developmental ability after parthenogenetic activation. The aim of the present study was to investigate the timing of mitochondria relocation during meiosis and the possible relationship with cytoskeleton organization in high and low competence oocytes. To this purpose, homogeneous groups of oocytes were matured in vitro (IVM) with 25% or 0% porcine follicular fluid (pff) to obtain different cytoplasmic competence (high and low, respectively) but similar nuclear maturation (Brevini *et al*., 2003, Theriogenology, 59, 440). After maturation, oocytes were parthenogenetically activated and cultured as previously described by Grupen *et al*., 2002 (Mol. Reprod. Dev., 62, 387–396). Active mitochondria were stained with MitoTracker® Orange CMTM Ros (Molecular Probes, Leiden, The Netherlands) at GV, MI and MII meiotic stages. At the same time microtubule organization was determined by immuno-cytochemistry using an antibody raised against α-tubulin (Sigma, St. Louis, MO, USA). Meiotic stages were assessed with DAPI. Specimens were examined with a Leica TCS-NT confocal microscope through an equatorial optical section. Nuclear maturation rate was comparable in the two groups at the end of IVM (46 h). Mitochondria relocation from the periphery to the center of the oocyte was evident as early as 20 h IVM in the 25% pff group (high competence), while 0% pff oocytes (low competence) did not show any mitochondria relocation at this time point. In agreement with the literature, α-tubulin was not detectable in GV oocytes, while at the end of IVM, α-tubulin was associated with the DNA, forming the meiotic spindle both in high and low competence oocytes. However, oocytes in the 25% pff group displayed a cytoplasmic microtubular organization that co-localized with mitochondria at 20 to 28 h IVM. Conversely, α-tubulin was not detected in the cytoplasm of 0% pff oocytes at the same time points and 71% of these oocytes did not undergo any mitochondria relocation at all by the end of IVM. Altogether the present results show that mitochondria relocation takes place at a well-defined time during IVM and is temporally associated with the formation of the microtubule mesh in the oocyte cytoplasm. Low-developmental-competence oocytes display an altered mitochondria distribution and microtubule arrangement or seem to lack the temporal coupling of the two phenomena. We speculate that a tightly linked timing of mitochondria relocation and cytoskeleton microtubule formation in the cytoplasm of the oocyte during IVM may represent a key cytoplasmic factor regulating pig embryo parthenogenetic development.

## 309 CHROMOSOME CONDENSATION IS CORRELATED WITH HISTONE H3 PHOSPHORYLATION WITHOUT CDC2 KINASE AND MAP KINASE ACTIVITIES IN PIG OOCYTES

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Chromosome condensation is the first step of oocyte maturation. When the oocytes resume meiosis, chromosomes start to condense and Cdc2 kinase becomes activated. However, recent findings show that the chromosome condensation does not always correlate with Cdc2 kinase activity in pig

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oocytes. The objectives of this study were to examine (1) the correlation between chromosome condensation and histone H3 phosphorylation at serine 10 (Ser10) during meiotic maturation of pig oocytes, and (2) the effects of protein phosphatase 1/2A (PP1/PP2A) inhibitors on the chromosome condensation and the involvement of Cdc2 kinase, MAP kinase and histone H3 kinase in this process. Oocyte-cumulus-granulosa cell complexes (OCGCs) were collected from follicles of 4–6 mm in diameter. OCGCs were cultured in modified TCM 199 for different periods of time to obtain oocytes at the germinal vesicle (GV, 0 h), diakinesis (18 h), metaphase I (24–27 h), anaphase I to telophase I (30–33 h), and metaphase II (42 h) stages. To examine the effects of PP1/PP2A inhibitors on the chromosome condensation, oocyte-cumulus-complexes (OCCs) were cultured in modified TCM 199 with either 2.5 μM okadaic acid (OA) or 50 nM calyculin A (CL-A) for 0.5, 1, 2, 3, 4 and 6 h. To inhibit the MAP kinase activity in the oocytes treated with the PP1/PP2A inhibitor, OCCs were cultured in medium containing CL-A and the MEK inhibitor, U0126 (0.1 mM). Morphology of the chromosome and nuclear membrane, and phosphorylation of histone H3 were examined by the immunofluorescent microscopy. In each group 30 oocytes were examined for OA or CL-A and 60 oocytes for CL-A + U0126 treatments. Activities of Cdc2 kinase, MAP kinase and histone H3 kinase were also examined. Phosphorylation of histone H3 (Ser10) was not detected in the oocytes at the GV stage. The phosphorylation was first detected in the clump of condensed chromosomes at the diakinesis stage of prophase I and maintained until metaphase II. The kinase assay also showed that histone H3 kinase activity was low in GV oocytes, increased at the diakinesis stage, and then maintained high activity until metaphase II. PP1/PP2A inhibitors induced rapid chromosome condensation in pig oocytes. Histone H3 phosphorylation (Ser10) became detectable together with the chromosome condensation in the treated oocytes after 2 h. After 6 h, oocytes had highly condensed chromosomes with phosphorylated histone H3 (81% in CL-A- and 71% in OA-treated oocytes). Both histone H3 kinase and MAP kinase were activated in the treated oocytes, although Cdc2 kinase was not activated. In the oocytes treated with CL-A and U0126, neither Cdc2 kinase nor MAP kinase were activated, although histone H3 kinase was still activated and chromosomes condensed. These results suggest that phosphorylation of histone H3 (Ser10) occurs in condensed chromosomes during maturation in pig oocytes. Futhermore, the chromosome condensation is correlated with histone H3 kinase activity, but not with Cdc2 kinase and MAP kinase activities.

## 310 ENRICHING A DEFINED MATURATION MEDIUM IMPROVES SUBSEQUENT EMBRYONIC DEVELOPMENT OF BOVINE OOCYTES CULTURED IN SMALL AND LARGE GROUPS

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When performing Ovum Pick Up (OPU) in unstimulated animals, the number of oocytes collected per donor is often reduced. Culturing oocytes and embryos in small groups impairs embryonic development by comparison with embryos cultured in large groups: less blastocysts are obtained and their appearance is delayed. The aim of the study was to evaluate the effect of an enriched define maturation medium on further embryonic development of oocytes and embryos cultured in large and small groups during IVM, IVF and IVC. Bovine cumulus-oocyte complexes (COC) were collected from abattoir ovaries, selected on morphological criteria, and then allocated to two maturation media: TCM 199 + 10 ng mL$^{-1}$ mEGF or the same medium enriched with 5 μg mL$^{-1}$ insulin, 5 μg mL$^{-1}$ transferrin, 5 ng mL$^{-1}$ selenium, 19 ng mL$^{-1}$ IGF-1, 2.2 ng mL$^{-1}$ FGF, 90 μg mL$^{-1}$ L-cystein, 28 μM *myo*-inositol, 100 μM β-mercaptoethanol, 75 μg mL$^{-1}$ ascorbic acid, 720 μg mL$^{-1}$ glycine, 0.1 mg mL$^{-1}$ glutamine, 5 UI mL$^{-1}$ hCG and 10 UI mL$^{-1}$ eCG. For both media, COC were cultured either in groups of 18 to 20 (large) or in groups of 4 to 5 (small) in 500 μL of medium in 4-well plates. After 24 h maturation at 39°C and in 5% $CO_2$ in air, the COC were fertilized and then cultured in modified SOF medium with 5% FCS at 39°C and in 5% $CO_2$, 5% $O_2$ and 90% $N_2$. No selection was performed after the maturation step and the oocytes and embryos were kept in small or large groups throughout the experiment. Blastocyst development was evaluated at Day 7 and 8 post insemination. Results are shown in Table 1. As expected, a significant decrease was observed in blastocyst rates when oocytes and embryos were cultured in small groups. Enriching the maturation medium led to an important increase in blastocyst rates regardless of the number of oocytes cultured together, but the increase was greater when culture was performed in small groups from the maturation step (56% increase at Day 8 v. 31% increase for embryos cultured in large groups). The enriched medium also accelerated the appearance of the blastocysts in embryos cultured in small groups (74% of the blastocysts appeared on Day 7 instead of 50% in the control medium) which could indicate an improvement in blastocyst quality. The rate of hatching was not significantly increased. In conclusion, enriching the maturation allowed an increase in the developmental competence of abattoir oocytes matured in small and large groups. Although further experiments are needed, this could be of particular interest to improve embryonic development from OPU oocytes.

**Table 1. Effect of an enriched defined maturation medium on blastocyst development from oocytes cultured in small or large groups**

| Maturation medium | Size of the group | *n* | Day-7 blastocysts (%) | Day-8 blastocysts (%) | Hatched Day-8 blastocysts (%) |
|---|---|---|---|---|---|
| Control | Large | 308 | 22.1[a] | 27.3[a] | 20 |
| Control | Small | 78 | 6.4[b] | 12.8[b] | 10 |
| Enriched | Large | 322 | 34.5[c] | 39.4[c] | 35 |
| Enriched | Small | 79 | 21.5[a] | 29.1[a,c] | 26 |

[a,b,c]Values with different letters are significantly different within the same column (ANOVA2, followed by Scheffe's test, $P < 0.05$). Results of 4 replicates.

## 311 EFFECT OF OOCYTE MATURATION MEDIA ON THE SPEED OF MEIOTIC PROGRESSION AND BLASTOCYST DEVELOPMENT OF BOVINE EMBRYOS

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Environment is crucial for in vitro development of gametes and embryos. The recent progression of culture media towards defined conditions brought to surface the impact of different medium supplements on oocyte and embryo development. In this work we evaluate the effect of various oocyte culture media on bovine oocyte maturation and subsequent embryo development. Bovine cumulus-oocyte complexes were recovered from slaughterhouse ovaries and matured in vitro in either TCM-199 (Gibco) or SOF (Synthetic Oviduct Fluid) media supplemented with BSA (fatty acid-free) or serum (fetal bovine serum). Oocytes from each treatment group were denuded and fixed at 18, 20, 22, 24, 26 and 28 h post-maturation (p.m.). Oocyte meiotic progression was monitored in each of the groups ($n = 28$–40 oocytes/group) by immunofluorescence microscopy of chromatin. Oocytes matured in SOF showed a slower rate of meiotic progression when compared to the other groups, with the highest percentage of oocytes reaching the MII stage by 28 h p.m. (60.71% SOF-BSA, 71.43% SOF-Serum). The fastest developmental rate was observed in oocytes matured in TCM-serum (77.15% at 24 h p.m.) followed by oocytes matured in TCM-BSA (74.29% at 26 h p.m.). In order to evaluate the effect of nuclear maturation on chromosome segregation, chromosomal organization of MII oocytes was evaluated by immunofluorescence microscopy within each media group ($n = 26$–31 oocytes/group) at 18, 22 and 26 h p.m.. No chromosomal abnormalities were found at 18 h p.m.. Both media supplemented with BSA induced lower frequencies of chromosomal abnormalities (0 to 3.23%) and (3.57 to 7.69%) for SOF and TCM, respectively, when compared to their serum-supplemented counterparts (7.14 to 11.54%) and (10 to 10.71%) for SOF and TCM, respectively at 22 and 26 h p.m.. Remarkably, the maturation medium and its supplements influenced the speed of blastocyst development. For this experiment, oocytes were matured in TCM-BSA, TCM-Serum, SOF-BSA or SOF-serum, fertilized in vitro in a TALP-base media supplemented with BSA and cultured in SOF-BSA. Blastocyst development was assessed at 7, 8 and 9 days of culture. Cleavage rates were similar between the groups (84–90%), whereas development rates to blastocyst stage varied among treatment groups. Maturation in SOF-BSA induced a delay in blastocyst formation that reached its highest percentage only on day 9 of culture (30.8%); moreover, blastocyst development was carried over until Day 12. When oocytes were matured in the presence of serum, the number of blastocysts did not increase after Day 8 of culture (26.6%, TCM-serum). These results provide evidence of a severe impact of oocyte culture media on the nuclear maturation of oocytes and their subsequent embryonic development after IVF. Moreover, the difference in the rate of oocyte maturation and blastocyst formation emphasizes the necessity for reviewing and adapting current protocols to new systems such as SOF-BSA. [Research funded by NSERC and OMAF of Canada.]

## 312 THE EFFECTS OF ECG AND ESTRADIOL ON CANINE OOCYTES DURING IN VITRO MATURATION

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The effects of eCG and estradiol (E2) were evaluated on canine oocytes during in vitro maturation (IVM). Canine ovaries were obtained from routine ovariohysterectomies, and oocytes with one or more intact cumulus layers and a consistently dark and smooth cytoplasm were collected by mincing the ovaries in TL HEPES warmed to 38°C. Oocytes were cultured for 72 h at 38.5°C in 5% $CO_2$ in humidified air in medium containing TCM 199, 2.92 mM calcium lactate, 2.0 mM pyruvic acid, 4.43 mM HEPES, 10% fetal bovine serum, and 1% Pen/Strep solution (12166 GibcoBRL, Grand Island, NY, USA) and with no added hormones (control), 2 ng/mL E2, 10 IU/mL eCG, or both E2 and eCG. At the end of culture, oocytes were removed and denuded by aspiration in a medium containing 50% v/v Trypsin-EDTA 10X solution (T-4174Sigma, St. Louis, MO, USA) and 50% v/v PBS with 3 mg $mL^{-1}$ polyvinyl alcohol. Denuded oocytes were fixed in 3.7% paraformaldehyde for 1 h and then transferred to a 1.9 mM Hoechst 33342 solution in glycerol. Nuclear status was observed under UV light and recorded. Chi-square analysis was performed to determine statistical significance where $P < 0.05$. Oocytes cultured with eCG were observed as having a significantly larger percentage of retained germinal vesicles (GV) at the end of culture (78%) compared to those cultured with E2 (62%) or in the control (66%). Addition of E2 with eCG was observed to increase the GV retention percentage (84%); however, it was not significantly greater than treatment with eCG alone. The eCG- and eCG with E2-treated oocytes also showed cumulus cell mass expansion where the cumulus oocyte complex diameter greatly increased and became 'fluffy' in appearance. The control and E2-supplemented media were the only treatments where MII oocytes were observed (5% and 9%, respectively), and the percentages of meiotic resumption for these treatments (15% and 17%, respectively) were significantly greater than those for eCG-(8%) and eCG with E2 (2%)-treated oocytes. The percentage of oocytes in which there was no observable DNA at the end of culture remained relatively constant between all four treatment groups and was also average compared to most reports of canine IVM in the literature. These data suggest that it is possible eCG promotes cumulus cell expansion and acts as a meiotic inhibitor for canine oocytes in vitro. The addition of E2 appears to augment the inhibitory effect of eCG, but in culture alone did not significantly increase the percentage of oocytes that resumed meiosis compared to the control. The authors would like to acknowledge Genetic Savings and Clone for making this work possible.

**Nuclear status of oocytes after culture**

| Treatment | *n* | GV (%) | GVBD (%) | MI (%) | AI (%) | MII (%) | RM[z] (%) | No DNA |
|---|---|---|---|---|---|---|---|---|
| Control | 183 | 121 (66)[a] | 7 (4) | 12 (6) | 0 (0) | 9 (5)[a] | 28 (15)[a] | 34 (19)[a] |
| E2 | 115 | 71 (62)[a] | 2 (1) | 8 (7) | 0 (0) | 10 (9)[a] | 20 (17) [a] | 24 (21)[a] |
| eCG | 151 | 118 (78)[b] | 1 (1) | 10 (6) | 1 (1) | 0 (0) | 12 (8)[b] | 21 (14)[a] |
| eCG + E2 | 126 | 106 (84)[b] | 1 (1) | 1 (1) | 0 (0) | 0 (0) | 2 (2)[b] | 18 (14)[a] |

[z]Oocytes that resumed meiosis; [a,b]Different superscripts with columns represent significant diffeences ($P < 0.05$).

## 313 IN VITRO MATURATION AND FERTILIZATION OF OOCYTES COLLECTED FROM UNSTIMULATED *MACACA NEMISTRINA* OVARIES

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Reports describing the IVM of *Macaca nemestrina* (Mn) oocytes are limited (Cranfield MR *et al.* 1989 Zoo. Biol. (Supp. 1), 33). The use of gonadotrophins (Gnt) for IVM of non-human primate (NHP) oocytes is common but the concentrations used are often high (8–40 IU $mL^{-1}$) and the species of origin and biological activity of Gnt varies (Schramm RD and Paprocki AM, 2000 Hum. Reprod. 15, 2411). We have compared two different IVM systems with human Gnt on maturation and fertilization of oocytes collected from unstimulated Mn ovaries ($n = 6$–10 animals). Oocytes were subjected to IVM in modified (minus PVA and pantothenic acid, plus 20 amino acids) HECM −10 + 15% FCS (Zheng P *et al.*, 2001 Mol. Reprod. Dev. 58, 348) for a) 36 h in the presence (mHECM +36, $n = 322$) or absence (mHECM −36, $n = 99$) of FSH and LH applied sequentially (FSH 1 IU $mL^{-1}$ 0–24 h; 10 IU $mL^{-1}$ FSH and LH 24–36 h) or b) 24 h in the presence (mHECM +24, $n = 119$) or absence (mHECM −24, $n = 56$) of static concentrations of Gnt (FSH and LH 1 IU $mL^{-1}$ 0–24 h; no Gnt 24–30 h). Oocytes exhibiting first polar body extrusion at 36 and 30 h were recorded as mature (MII) and subjected to IVF in HTF + BSA (3 mg $mL^{-1}$) with Mn sperm pretreated with 1.0 mM caffeine and 0.1 mM dbcAMP. Fertilized oocytes (pronuclei and/or 2nd polar body extrusion) were cultured in sequential culture medium for 48 h, assessed for cleavage and either fixed or frozen. Proportional data (mature/total, fertilized/mature or cleaved/fertilized) were compared by chi-square analysis and are reported as percentages. Oocytes cultured in mHECM+36 and mHECM −36 exhibited similar rates of GVBD (58.7% v. 53.5%) but the percentage of MII oocytes was significantly higher ($P = 0.0244$) in mHECM +36 (41.3%) v. mHECM −36 (28.3%). Fertilization rates were comparable between mHECM+36 (61.5%) and mHECM −36 (60.9%), whereas cleavage rates were significantly higher ($P = 0.0004$) in mHECM +36 (74.6%) v. HECM −36 (21.4%). Oocytes cultured in mHECM +24 and mHECM −24 exhibited similar rates of GVBD (76.5% v. 62.5%) but the proportion of MII oocytes was significantly higher ($P = 0.0159$) in mHECM +24 (55.5%) v. mHECM −24 (35.7%). Fertilization and cleavage rates were comparable between mHECM +24 (58.8% v. 63.3%) and mHECM −24 (50.0% v. 42.8%). A comparison between mHECM +36 and mHECM +24 indicated a significantly lower ($P = 0.0005$) percentage of GV oocytes and a significantly higher ($P = 0.0096$) percentage of MII oocytes in mHECM +24 (23.5% v. 55.5%) compared to mHECM +36 (41.3% v. 41.3%). Fertilization and cleavage rates were not significantly different between mHECM +36 and mHECM +24. Oocyte maturation and fertilization and embryo cleavage were not different for mHECM −36 and mHECM −24 ($P = 0.3138$–$0.8202$). Mn oocytes exhibit high rates of Gnt-independent GVBD (52.5%–53.5%) and maturation (28.3%–35.7%) in vitro, and maturation rates were improved in Gnt supplemented maturation medium. However, reduced exposure to lower concentrations of FSH and increased exposure to lower concentrations of LH was associated with higher rates of oocyte maturation in vitro. The use of lower concentrations of FSH and LH for reduced periods may improve IVM of NHP oocytes. This work was supported by the Tissue Distribution Program of the WaNPRC (NIH grant # R00166).

## 314 EFFECT OF MACROMOLECULE SUPPLEMENTATION DURING IN VITRO MATURATION ON THE DEVELOPMENTAL COMPETENCE OF GOAT OOCYTES

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In vitro maturation of goat oocytes has traditionally involved the use of serum or BSA. However, these products introduce variability and complicate evaluation of the effects of other medium components. The objective of this study was to examine the effects of citrate and hyaluronate in the absence or presence of BSA during IVM on the developmental competence of goat oocytes. Abattoir-derived, cumulus-oocyte complexes (COC) were matured for 20–22 h (6.0% $CO_2$ in air, 38.7°C) in modified SOF medium (1.5 mM glucose, 3.0 mM L-lactate, 0.1 mM pyruvate, 1.0 mM glutamine, 0.1 mM taurine) supplemented with 1 × MEM nonessential amino acids, 0.5 × MEM essential amino acids, 1 × MEM vitamins, 0.1 mM cysteamine, 5 μg $mL^{-1}$ insulin, 5 μg $mL^{-1}$ transferrin, 5 ng $mL^{-1}$ selenium, 50 ng $mL^{-1}$ EGF, 0.01 U $mL^{-1}$ LH and FSH, and 50 μg $mL^{-1}$ gentamicin. Treatments were: (1) 1 mg $mL^{-1}$ PVA (protein-free, defined); (2) 4 mg $mL^{-1}$ BSA (semi-defined); (3) 0.5 mM citrate and 0.5 mg $mL^{-1}$ hylauronate (C + H, defined); and (4) 0.5 mM citrate and 0.5 mg $mL^{-1}$ hylauronate with 4 mg $mL^{-1}$ BSA (C + H + BSA, semi-defined). At the end of IVM, COC were transferred to modified Brackett and Oliphant's medium with 7.7 mM Ca-(l)-lactate and 20% FCS for IVF. Frozen-thawed sperm were processed through a 45%:90% Percoll gradient and added to IVF drops (50 μL) containing COC at a final concentration of 14–15 × $10^6$ sperm $mL^{-1}$. Gametes were coincubated in the presence of heparin (25 μg $mL^{-1}$) for 22–24 h in 7% $CO_2$ in air at 38.7°C. After coincubation, cumulus cells were removed and zygotes were cultured (6% $CO_2$, 5% $O_2$, 89% $N_2$, 38.7°C) in G1 v.3 for 3 days followed by 4 days in G2 v.3. Cleavage was evaluated when embryos were moved to G2, and development to the blastocyst stage was assessed at the end of culture. All blastocysts were fixed and stained with Hoechst 33342 for total cell counts. Analysis of variance was performed using the general linear mixed model macro of SAS. Means are presented ±SEM and probability values $P < 0.05$ were considered significant. The use of BSA did not improve ($P > 0.05$) the developmental potential of goat oocytes (Table 1). Furthermore, a similar proportion ($P > 0.05$) of oocytes developed to the blastocyst and hatching blastocyst stage after maturation under defined conditions compared to oocytes matured with BSA. In conclusion, developmentally competent goat oocytes can be produced by IVM under defined conditions.

**Table 1. Development of goat oocytes following IVM with different macromolecules.**

| Treatment ($n$ = oocytes) | (%) Cleavage | (%) Blastocyst/oocyte | (%) Blastocyst/cleaved | (%) Hatching blastocyst/blastocyst | Blastocyst total cell# |
|---|---|---|---|---|---|
| PVA(178) | 59.2 ± 4.5[a] | 24.7 ± 5.4[a] | 43.4 ± 10.3[a] | 50.6 ± 8.9[a] | 80.1 ± 6.4[a] |
| BSA(180) | 52.8 ± 6.7[a] | 19.6 ± 3.4[a] | 38.3 ± 6.0[a] | 52.0 ± 5.6[a] | 87.9 ± 8.0[a] |
| C + H(170) | 53.2 ± 7.4[a] | 22.4 ± 2.7[a] | 46.5 ± 10.1[a] | 58.2 ± 6.8[a] | 82.4 ± 7.8[a] |
| C + H + BSA(170) | 55.3 ± 4.8[a] | 15.5 ± 1.5[a] | 28.5 ± 2.4[a] | 52.7 ± 7.9[a] | 85.0 ± 7.6[a] |

[a]Similar superscripts indicate no significant treatment effect ($P > 0.05$).

## 315 THE REDUCTION OF MATURATIONAL COMPETENCE BY STREPTOMYCIN DURING IN VITRO MATURATION OF GOAT FOLLICULAR OOCYTES

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Antibiotics are commonly added to mammalian oocyte maturation media, but their effects on oocytes maturation have not been examined thoroughly. Goat follicular oocytes were used to investigate whether penicillin, streptomycin or gentamycin affect maturational competence of oocytes and subsequent parthenogenetic activation potential in vitro. Cumulus-oocyte complexes collected from a local abattoir were matured for 24 h in five treatments, and matured oocytes were cultured for 48 h in five treatments after parthenogenetic activation by treatment with ionomycin, followed by immediate exposure to 6-diethlaminopurine; (1) Control: TCM-199 medium with no antibiotics, (2) TCM-199 with 100 IU/mL$^{-1}$ penicillin (P-4687, Sigma, St. Louis, MO, USA), (3) TCM-199 with 50 μg mL$^{-1}$ streptomycin (S-1277, Sigma), (4) TCM-199 with 50 μg mL$^{-1}$ gentamycin (G-1264, Sigma) and (5) TCM-199 with both 100 IU mL$^{-1}$ penicillin and 50 μg mL$^{-1}$ streptomycin. Maturation rates at 24 h post-in vitro maturation and parthenogenetic cleavage development at 48 h post-activation were evaluated. Data were analyzed by ANOVA and Student's *t*-test. Penicillin and gentamicin treatment groups did not affect maturation rates and percentages of cleavage to 2–4 cell stage at 48 h post-chemical oocyte activation. However, when streptomycin was present in the maturation medium, the percentages of matured oocytes at 24 h post-in vitro maturation of immature goat oocytes were significantly lower than those from the other groups. However, among the five treatments, there was no significant difference in cleavage rates of matured oocytes at 48 h post-activation (Table 1). Therefore, streptomycin did interfere with the maturation of immature goat oocytes, but did not affect the subsequent development of matured goat oocytes. The mechanism by which streptomycin affects the maturation of goat follicular oocytes needs to be investigated further. We conclude that streptomycin in oocyte maturation medium can be detrimental during in vitro maturation of goat follicular oocytes.

**Table 1. Effect of antibiotics on maturational competence of goat follicular oocytes and subsequent parthenogenetic activation potential in vitro**

| Treatment | N[a] | Matured oocytes | | Cleaved | |
|---|---|---|---|---|---|
| | | *n* | % ± SEM[b] | *n* | % ± SEM[c] |
| Control | 84 | 62 | 73.8 ± 7.2[d] | 50 | 79.9 ± 8.4 |
| Penicillin | 88 | 62 | 69.1 ± 11.1[d] | 50 | 83.5 ± 11.8 |
| Streptomycin | 84 | 36 | 42.5 ± 8.3[e] | 30 | 80.4 ± 14.2 |
| Gentamycin | 84 | 60 | 71.3 ± 10.1[d] | 50 | 81.9 ± 10.5 |
| Pen+Strep | 82 | 38 | 45.7 ± 8.5[e] | 30 | 80.2 ± 16.9 |

[a]No. of goat follicular oocytes cultured in 4 replicate experiments. [b]% = (matured oocytes/cultured oocytes) × 100. [c]% = (cleaved/matured oocytes) × 100. [d,e]Different superscripts within column are significantly different, $P < 0.01$.

## 316 EFFECTS OF ESTRADIOL-17β AND PROGESTERONE SUPPLEMENT ON THE RESUMPTION OF MEIOSIS OF CANINE OOCYTES MATURED IN VITRO

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In the bitch, oocytes are ovulated at the germinal vesicle (GV) stage and mature in the isthmus of the oviduct around 3 days after ovulation, it is not known what elements trigger the release of this meiotic arrest. Canine IVM has shown limited success with maturation rates, usually around 20% (MII) (Farstad W, 2000 Anim. Reprod. Sci. 60–61, 375–387). Estrogen and progesterone are suggested to play a significant role in causing oocyte resumption of meiosis and progression to MII stage. The purpose of this study was to investigate the role of estradiol-17β (E2) and progesterone (P4) during in vitro maturation of canine oocytes in serum-free tissue culture medium (TCM)-199. Canine oocytes collected from bitches were categorized into three groups based on estrous stages, follicular, luteal, or anestrus, at routine ovariohystrectomy. Oocytes were cultured in vitro in TCM-199 supplemented with E2, P4 or E2 + P4 according to experimental design at 39°C in 5% $CO_2$ and $O_2$. After 72 h of maturation culture, oocytes were denuded, fixed in a 3.7% paraformaldehyde solution for 10 min, stained with Hoechst 33342 in glycerol, and observed under the UV light. Three groups of oocytes were cultured in TCM-199 supplemented with different concentrations (0, 0.1, 1.0 or 2.0 μg mL$^{-1}$) of E2 (Experiment 1, $n = 898$, replications: 5) or P4 (0, 0.5, 1.0 or 2.0 μg mL$^{-1}$, Experiment 2, $n = 734$, replications: 5). Multiple comparisons were implemented using Generalized Linear Models in the SAS 8.12 program. The rates of oocyte maturation to MII stage were higher ($P < 0.05$) in follicular stage oocytes cultured with 2 μg mL$^{-1}$ E2 (17.9%) compared to other supplement groups (0 to 7.6%). No differences ($P < 0.05$) in rate of MII stage oocytes among P4 supplement groups were observed. In Experiment 3, to investigate the combined effects of E2 and P4 on in vitro maturation, three groups of oocytes were cultured in TCM-199 supplemented with 2 μg mL$^{-1}$ E2 and various concentration of P4 (0, 0.5, 1.0 or 2.0 μg mL$^{-1}$, Experiment 3, $n = 1613$, replications: 5). The rate of oocyte maturation to MII stage (11.5%) was higher ($P < 0.05$) in follicular stage oocytes cultured with 2 μg mL$^{-1}$ E2 + 2.0 μg mL$^{-1}$ P4 supplement compared to other supplement groups (0 to 6.4%). In conclusion, the present study demonstrated

that E2 supplement in the culture medium increased maturation of canine oocyte to MII stage and that supplement of P4 alone did not promote oocyte maturation. However, P4 supplemented with E2 further promoted oocyte maturation in the follicular stage compared to E2 supplement alone, indicating that P4 acts synergistically with E2 on canine oocyte maturation in the presence of E2. From our results, we conclude that canine oocytes are exposed to high levels of P4 during maturation due to the preovulatory luteinization of canine follicles which gives rise to high intrafollicular as well as intratubal P4 concentrations-this is very different from the situation in oocytes from other domestic animal species. This study was supported by Biogreen 21-1000520030100000.

## 317 EMBRYONIC DEVELOPMENT AFTER HOLDING BOVINE OOCYTES IN UNDILUTED FOLLICULAR FLUID FOR A 6-HOUR PREMATURATION PERIOD

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Follicular fluid has been implicated in follicular growth, initiation of steroidogenesis, regulation of granulosa cell function and oocyte maturation. Although individual components of the follicular fluid have been analyzed, it is still unclear how undiluted follicular fluid may affect subsequent in vitro oocyte maturation (IVM), in vitro fertilization (IVF) and embryo development. The objective of this study was to simulate recovery of bovine oocytes as if under field conditions to determine the effect of holding these oocytes in follicular fluid for a 6-h holding period before performing IVF. Ovaries were obtained from a local abattoir and transported to the laboratory at ~22°C in a 0.9% saline and antibiotic solution. At the laboratory, ovaries were rinsed with an ethanol solution and randomly allotted to four treatment groups. Follicles ranging in size from 2 to 9 mm were aspirated using a 20-gauge needle attached to a sterile plastic syringe. Dominant follicles were not included. Oocytes harvested from the ovaries in Treatment 1 (Control) were placed directly into a standard laboratory maturation medium consisting of TCM-199 supplemented with LH, FSH, fetal bovine serum, estradiol and gentamicin for 22 h. Standard laboratory IVF was then performed with frozen-thawed semen from a fertile bull. In Treatment 2, 3 mL of pooled follicular fluid was dispensed into a 6-mL conical centrifuge tube and co-incubated with the harvested oocytes at room temperature (22°C) for 6 h. Oocytes recovered from the ovaries in Treatment 3 were placed into 3 mL of Ringer's lactate solution for a 6-h holding period at 22°C. Oocytes obtained from the ovaries in Treatment 4 were placed into a mixture of 2 mL of Ringer's lactate plus 1 mL of the same pooled follicular fluid and were held for 6 h at 22°C. After being held for a 6-h period, oocytes were recovered from each centrifuge tube and were placed into IVM medium for 22 h and then subjected to standard IVF. Embryo development in CR1aa culture medium was assessed at 72, 168 and 216 h post-insemination in each treatment group. In summary, no significant difference was detected between the standard IVF procedure and oocytes held in follicular fluid 6 h prior to IVF (Treatment 1 v. 2). Also, follicular fluid apparently had a positive effect on the oocytes over that of holding oocytes 6 h in Ringer's lactate alone, as indicted by a significantly greater rate of blastocyst development (Treatment 3 v. 4). In conclusion, it should not be overlooked that bovine oocytes, aspirated under field conditions, could be held up to 6 h in 22°C undiluted bovine follicular fluid until they can be delivered to a full-service IVF laboratory.

| Treatment group | No. of ovaries* | No. of oocytes | No. (%) degenerated [72 h] | No. (%) developed to | | |
|---|---|---|---|---|---|---|
| | | | | Cleavage [72 h] | Blastocyst [168 h] | Hatch [216 h] |
| 1 | 32 | 340 | 20 (6) | 302 (89)[a] | 88 (26)[a] | 58 (17)[a] |
| 2 | 32 | 335 | 25 (7) | 289 (86)[a] | 85 (25)[a] | 58 (17)[a] |
| 3 | 32 | 297 | 101 (34) | 135 (45)[b] | 14 (5)[b] | 6 (2)[b] |
| 4 | 32 | 310 | 60 (19) | 210 (68)[c] | 48 (15)[c] | 26 (8)[c] |

*Total of 8 replicates. [a,b,c]Columns with different superscripts are significantly different ($P < 0.01$) (chi-square analysis).

## 318 EFFECT OF PIG FOLLICLE FLUID AND FETAL CALF SERUM ON PORCINE OOCYTE MATURATION AND SUBSEQUENT DEVELOPMENT AFTER ACTIVATION AND SOMATIC CELL NUCLEAR TRANSFER

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In vitro maturation of porcine oocytes is very important for understanding porcine somatic cell nuclear transfer (SCNT). In order to develop an in vitro maturation system that can provide more high quality oocytes, the effect of porcine follicle fluid (pFF) (gathered from 3–5-mm porcine follicles) and fetal calf serum (FCS: Sigma, St. Louis, MO, USA), as an important additional component of a chemically-defined medium was studied. Cumulus-oocyte complexes (COC) derived from follicles 3–5 mm in diameter were cultured in three different media: a chemically-defined medium (CDM: TCM-199 with 0.1 mg mL$^{-1}$ cysteine, 10 ng mL$^{-1}$ EGF, 0.5 μg mL$^{-1}$ LH and 0.5 μg mL$^{-1}$ FSH); CDM with 10% pFF (CDM + p); and CDM with 10% FCS (CDM + F). After 42–44 h of maturation, oocytes with a clear polar body were classified as matured oocytes. Matured oocytes stimulated by electric pulse (120 v, 30 μs, 2 pulse), or enucleated and fused with fibroblasts to construct SCNT embryos by using the same electrical parameters. All of these parthenogenetic and SCNT embryos were cultured in Porcine Zygote Medium-3. The blastocyst rate was assessed

under a stereomicroscope on Day 6, and the number of nuclei in the blastocysts was counted under a fluorescent microscope after staining with 5 μg mL$^{-1}$ of Hoechst 33342. All data were subjected to a Generalized Linear Model Procedure (PROC-GLM) of Statistical Analysis System (SAS). The maturation rates of porcine oocytes in CDM and CDM + p were 53.2 ± 3.8% (539/1050) and 69.7 ± 3.8% (587/847), respectively; in CDM and CDM+F, 61.1 ± 3.1% (471/776) and 70.2 ± 3.7% (577/844), respectively. Oocytes matured in CDM + p and CDM + F showed a higher ($P < 0.05$) maturation rate than those in CDM. The percentages of parthenogenetic blastocysts of oocytes matured in CDM and CDM + p were 13.9 ± 2.1% (35/250) and 20.2 ± 5.3% (64/300), and the numbers of nuclei in these blastocysts were 25.8 ± 2.3 and 25.8 ± 1.4, respectively. The blastocyst rate from CDM- and CDM + F-matured oocytes were 20.1 ± 2.0% (53/272) and 22.2 ± 4.7%(71/298), and the numbers of nuclei in these blastocysts were 24.7 ± 1.5 and 25.3 ± 1.5, respectively. There were no significant ($P > 0.05$) differences in the percentages of parthenogenetic blastocysts and nuclei numbers between CDM and CDM + p, or CDM and CDM + F. The percentages of blastocysts in SCNT embryos derived from CDM and CDM + p were 8.1 ± 1.5% (14/192) and 12.3 ± 1.9% (24/192), while the nuclei numbers in these blastocysts were 26.6 ± 1.2 and 34.5 ± 2.2, respectively. The percentages of blastocysts after SCNT from oocytes matured in CDM and CDM + F were 24.3 ± 4.9% (35/139) and 27.1 ± 5.5% (45/176), while the numbers of nuclei were 29.8 ± 2.5 and 32.2 ± 1.9, respectively. There were no significant ($P > 0.05$) differences between CDM and CDM + p, or CDM and CDM + F in SCNT embryo blastocyst rate, but the SCNT embryos derived from CDM + p showed a higher ($P < 0.05$) nuclear number. In conclusion, these results indicate that 10% pFF or FCS in CDM can promote a higher maturation rate of porcine oocytes. As recipient cytoplasm for SCNT, oocytes matured in CDM + p can support development of blastocysts that contain more nuclei than those matured in CDM alone. Supported in part by Food for the 21st Century and RR13438.

## 319 IN VITRO MATURATION OF EQUINE OOCYTES IN A COMPLETELY DEFINED MEDIUM SUPPLEMENTED WITH PROGESTERONE

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A completely defined medium for in vitro maturation (IVM) of equine oocytes has not yet been developed, since most of the media used for IVM are supplemented with serum or BSA. Furthermore, in this species there is no report about the influence of progesterone on maturation, although it has already been used as supplement (500 ng mL$^{-1}$) in EMMI (Maclellan LJ *et al*., 2001, Theriogenolgy 55, 310 abst). The aims of this study were to develop a completely defined medium for equine oocyte maturation and to investigate the effect of progesterone on nuclear maturation. Equine oocytes were collected by follicular scraping of abattoir-derived ovaries between April and June. The basal medium for maturation was SOFaa supplemented with pFSH-LH 0.1 IU mL$^{-1}$ (Pluset, Laboratorios Calier, Barcelona, Spain), EGF* 50 ng mL$^{-1}$, ITS (Insulin, Transferrin, Sodium selenite), L-cysteine 1.2 mM, Maturation SOF (MSOF). Compact cumulus-oocyte complexes were selected, washed three times in H-SOF and matured in one of the following media (15–20 oocytes mL$^{-1}$): (1) MSOF + FCS 10% (MSOF-FCS), (2) MSOF + progesterone 100 ng mL$^{-1}$ (MSOF-P4), (3) MSOF. After 24 h of culture in 5% $CO_2$ in air at 38.5°C, the oocytes were denuded by gently pipetting in a 0.25% trypsin solution, washed and stained with Hoechst 33258 (10 μg mL$^{-1}$ in PBS) for 30 min at room temperature. Oocytes were examined under a fluorescent microscope to assess nuclear maturation. Only oocytes with an evident polar body and metaphase II plate (MII) were considered mature. The experiment was done in 6 replicates. Chi Square test was used for statistical analysis (Statistica for Windows – Stat Soft Inc., Tusla, OK, USA). Significance was assessed for $P < 0.05$. The results of this study show that MSOF can be considered a suitable completely defined medium for IVM of equine oocytes. Adding progesterone significantly ($P < 0.05$) increases the nuclear maturation rate at 24 h of culture. It can be speculated that although cumuls cells produce this hormone, supplementation is useful to reach progesterone concentrations similar to those present in follicular fluid (early dominant 63.4 ± 19.3 ng mL$^{-1}$, healthy preovulatory follicle 1094.3 ± 170.9 ng mL$^{-1}$; Gerard N *et al*., 2002, Reproduction 124, 241–248). Further studies are needed to investigate the influence of progesterone on cytoplasmic maturation and to test the effect of different progesterone concentrations and time of maturation in a completely defined system.

*All chemicals were purchased from Sigma, St. Louis, MO, USA, unless otherwise stated.

**Table 1. Maturation of equine oocytes in different media**

| IVM medium | *n*. oocytes | *n*. Met. II (%) |
|---|---|---|
| MSOF-FCS | 95 | 46 (48.4)[b] |
| MSOF-P4 | 93 | 61 (65.6)[a] |
| MSOF | 92 | 46 (50.0)[b] |

[a,b] $P < 0.05$.

## 320 EFFECT OF CYSTEAMINE DURING IN VITRO MATURATION ON FURTHER EMBRYONIC DEVELOPMENT AND POSTTHAW SURVIVAL OF IVP BOVINE EMBRYOS

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The uptake of cysteamine by immature oocytes may facilitate the synthesis of glutathione (GSH) during in vitro maturation, as reported by Matos *et al*. (1995 Mol. Reprod. Dev. 42 432–436). GSH plays an important protective role in relation to reactive oxygen species generated by normal

oxidative metabolism. This study investigated the effects of the presence of cysteamine during in vitro maturation on subsequent in vitro embryonic development and postthaw in vitro survival. Immature Cumulus-Oocyte-Complexes (COCs) were recovered from ovaries 6 to 8 h after slaughter. COCs were matured in vitro for 22 to 24 h in TCM199/FCS/LH/FSH supplemented either with or without cysteamine (0.1 mM), Subsequently, matured oocytes were fertilized with frozen-thawed Percoll-separated semen and further cultured for seven days in SOFaaBSA. Morulae grade 1 (IETS) and blastocysts grades 1 and 2 (IETS) were frozen on Day 7 in 10% Glycerol using a conventional slow freezing procedure (Wagtendonk-de Leeuw *et al*. 1995 Cryobiology; 32 157–167). In vitro survival was measured by rates of blastocyst formation and reexpansion at 24 h and hatching/ed blastocysts at 72 h in SOFaaBSA supplemented with 5% FCS. Results were analyzed by Chi-square analyses. The presence of cysteamine during in vitro maturation significantly affected the embryo production rate (19.4% and 24.0% for control and cysteamine at Day 7, respectively). The higher number of embryos at Day 7 was totally due to an increased number of blastocysts (Table 1); however, the distribution of embryos among the different quality grades was not affected. Addition of cysteamine did not affect the post thaw survival of the frozen/thawed embryos (85% v. 91% reexpansion and 33% v. 34% hatching/ed for control v. cysteamine, respectively). These results show that the presence of cysteamine during in vitro maturation, does affect further in vitro embryonic development, resulting in a higher embryo production rate. Embryo quality, expressed in morphological grades and postthaw survival rates, were not affected. A field trial will be conducted in order to confirm these results with ovum pick up-derived oocytes.

**Table 1. Effect of cysteamine during in vitro maturation on subsequent in vitro embryonic development of IVP bovine embryos (number of replicates: 5)**

| Group | # Oocytes | # Cleavage (%) | # Embryos Day 7 (%) | | Total |
|---|---|---|---|---|---|
| | | | Morulae | Blastocysts | |
| Control | 1056 | 586 (55.5) | 81 (7.7) | 124 (11.7)[a] | 205 (19.4)[a] |
| Cysteamine | 1070 | 634 (59.3) | 79 (7.4) | 178 (16.6)[b] | 257 (24.0)[b] |

[a,b]Values in columns with different superscript are significantly different, $P < 0.05$.

## 321 EFFECTS OF BUTYROLACTONE-I AND CYCLOHEXIMIDE ON GERMINAL VESICLE BREAKDOWN IN BOVINE OOCYTES AND SUBSEQUENT IN VITRO DEVELOPMENT AFTER IVM–IVF–IVC

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The present study aimed to compare the effects of butyrolactone-I (BL-I) and cycloheximide (CHX) on inhibition of germinal vesicle (GV) breakdown (GVDB) in bovine oocytes and subsequent in vitro development after in vitro maturation and fertilization. Furthermore, in experiment 2, we compared the kind of supplemented protein with CHX during inhibition of GVBD of oocytes obtained from ovaries stored for 1 day, and examined time extension of storage of oocytes. In experiment 1, bovine cumulus-oocyte complexes (COCs) collected by the aspiration of 3- to 5-mm follicles of ovaries from at a local abattoir were preincubated for 24 h in TCM-199 supplemented with 100 μM BL-I and 3 mg mL$^{-1}$ BSA or 100 μL mL$^{-1}$ CHX and 5% CS. As a control, fresh COCs were used without preincubation. In experiment 2, the COCs were collected from ovaries stored in phygiological saline for 1 day at 20°C. The collected COCs were preincubated for 24 h in TCM-199 supplemented with 100 μL mL$^{-1}$ CHX and 3 mg mL$^{-1}$ BSA or 5% CS (CHX + BSA, CHX + CS). As a control, fresh COCs collected from ovaries stored in the same condition were used without preincubation. In both experiments, the COCs were maturated and inseminated with frozen-thawed spermatozoa. After preincubation, maturation and fertilization, some oocytes or zygotes were fixed to assess the rates of oocytes at the GV stage, MII or sperm penetration. Following insemination, the presumptive zygotes were cultured in CR1aa (Rosenkrans, C.F. Jr. *et al*., 1993 Biol. Reprod. 49, 459–462) supplemented with 5% CS for 8 days. Embryo development was evaluated for cleavage rates on Day 2, and for blastocyst rates on Days 7 and 8 (IVF = Day 0), respectively. To evaluate embryo quality, the total cell numbers in the blastocysts were counted by means of the air-drying method. Three replicates were carried out for each experiment. Data were analyzed by chi-square test (cleavage and blastocyst rates) and ANOVA (cell numbers). In experiment 1, there were no differences in the rates of the oocytes at the GV stage between BL-I (71.4 ± 10.7%, mean ± SD) and CHX (86.7 ± 10.9%), but the rates of the oocytes at the MII stage for BL-I (59.6 ± 7.4%) tended to be lower than for those in CHX (80.0 ± 14.1%, $P < 0.1$). The rate of MII stage for control was 67.5 ± 18.4%, and there were no differences between control and other treatments. No differences were found in sperm penetration, normal fertilization and polyspermy after in vitro fertilization. The cleavage rate for oocytes in CHX (81.0 ± 1.2%) was significantly higher than for those in BL-I (65.0 ± 13.1%, $P < 0.01$), and there was a tendency for the cleavage rate in BL-I to be lower than that of the control (75.5 ± 4.7%, $P < 0.1$). A significantly lower percentage of embryos cultured in BL-I (19.2 ± 13.8%) developed to the blastocyst stage than those of embryos in the control (32.0 ± 11.2%, $P < 0.05$), but there were no differences in the blastocyst rate between BL-I and CHX (25.9 ± 8.8%). Cell numbers in the blastocysts in BL-I (177.2 ± 15.9, $n = 21$) and CHX (191.2 ± 12.9, $n = 31$) were not significantly different compared to the control (198.4 ± 14.3, $n = 34$). In experiment 2, no significant differences were found in the cleavage rates (CHX+CS, 64.0 ± 18.7%; CHX+BSA, 68.1 ± 10.8% and control, 72.2 ± 8.3%). However, the blastocyst rates in CHX+CS (4.0 ± 7.8%) and CHX+BSA (7.7 ± 9.2%) were significantly lower than the control (20.4 ± 3.7%, $P < 0.05$). These results suggested that CHX can reversibly inhibit the GVBD of bovine oocytes for 24 h without compromising subsequent developmental competence after in vitro maturation, fertilization, and culture. However, COCs collected from stored ovaries for 1 day and preincubated with CHX failed to develop into blastocysts regardless of the kind of supplemented protein.

## 322 MEIOTIC COMPETENCE OF CANINE OOCYTES EMBEDDED IN COLLAGEN GELS

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The low meiotic competence of canine oocytes cultured in vitro is a major obstacle to the in vitro production of canine embryos. The objectives of the present study were to examine meiotic competence of oocytes embedded in collagen gels and to investigate the effects of timed exposure of the oocytes embedded in collagen gels to hormone supplements on the nuclear maturation. Ovaries were collected from 17 bitches at various stages of the estrous cycles by ovariohysterectomy following anesthesia at local veterinary practices. Only non-degenerate COCs were collected and then suspended in TCM-199 supplemented with 5% fetal calf serum. Only oocytes with diameter >110 μm were selected and used for this study. In the first experiment, the effect of embedding in collagen gels of COCs on their meiotic competence was tested. Half of selected oocytes were embedded in 0.3 mL of collagen gels (3 to 4 COCs per gel) as described by Yamamoto *et al.* (Yamamoto K *et al.*, 1999 Theriogenology 52, 81–89). The COCs with or without collagen gels were cultured in a dish containing 2.5 mL of TCM-199 supplemented with 0.1 IU $mL^{-1}$ HMG and 10 IU $mL^{-1}$ hCG (3 to 4 COCs per dish) for 72 h at 38.5°C in a humidified atmosphere of 5% $CO_2$ in air. In the second experiment, the effect of removal of hormonal supplements from maturation medium on nuclear maturation in vitro was examined. At 24 and 48 h after the start of culture, the COCs embedded in collagen gels were cultured in TCM-199 without HMG and hCG for 48 and 24 h, respectively. As a control, the COCs embedded in collagen gels were cultured with hormone supplement for 72 h. After 72 of maturation culture, the oocytes were fixed, stained with Hoechst 33342 and examined for the meiotic stage of the oocytes using a fluorescence microscope. Data were analyzed by ANOVA. The proportion of oocytes that resumed meiosis was significantly higher ($P < 0.05$) in the COCs with collagen gels than in the control COCs without collagen gels (50.6 v. 26.5%). Significantly more oocytes reached metaphase I to metaphase II stage (MI/II) in the collagen gels culture than in the control culture ($P < 0.05$; 27.4 v. 8.3%). The proportion of collagen embedded-oocytes that resumed meiosis was significantly higher ($P < 0.05$) in COCs cultured with hormone supplements for 24 h than in COCs cultured for 48 h (59.1 v. 30.4%) but not different from COCs exposed for 72 h (41.9%). Moreover, there were no significant differences of MI/MII rates (22 to 24%) among the three treatment groups. These observations indicate that embedding of COCs in collagen gels enhances the meiotic competence of canine oocytes, but removal of hormone supplement from maturation medium does not improve the ability of the oocytes to reach MI/MII stage.

## 323 OPTIMIZATION OF SERUM-FREE IVP PROTOCOL: EFFECT OF SERUM-FREE IVM ON BOVINE EMBRYO DEVELOPMENT

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The use of serum in IVP medium may cause abnormalities, e.g. reduced post-thaw survival, in bovine IVP embryos (Abe H *et al.*, 1999 Mol. Reprod. Dev. 53, 325–335). Moreover, serum may be a source of contamination and its composition is highly variable. Several reports have shown that serum-free IVF–IVC does not compromise embryo development (see, e.g. Keskinteipe L *et al.*, 1996 Biol. Reprod. 55, 333–339). Here we studied the effect of serum-free IVM on bovine IVP embryo development, aiming for production of good quality Day 7 embryos for freezing. In total, 11 734 abattoir-derived bovine oocytes in 13 batches were washed with emCare Complete Medium with BSA (1 mg $L^{-1}$) and 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 either with 1) 10% FBS (Gibco, New Zealand), 2) 4 mg $mL^{-1}$ fatty acid-free albumin (FAF-BSA), 3) 4 mg $mL^{-1}$ FAF-BSA + growth factors (GF; 100 ng $mL^{-1}$ IGF-I + 100 ng $mL^{-1}$ EGF), 4) 4 mg $mL^{-1}$ polyvinylpyrrolidone (PVP), 5) 4 mg $mL^{-1}$ PVP+GF and 6) PVP + amino acids (10 μL $mL^{-1}$ MEM + 20 μL $mL^{-1}$ BME). After 20 h fertilization in FERT-TALP + 2 mg $mL^{-1}$ BSA with semen of pre-tested IVF-bull, the oocytes were denuded and cultured in modified SOFaaci + 6 mg $mL^{-1}$ FAF-BSA in 5% $O_2$ (Holm P *et al.*, 1999 Theriogenology 52, 683–700). The statistical analyses are based on logistic regression models with IVP batch and treatment as explanatory variables. The estimated probabilities ($P$) are shown in Table 1. The upper and lower values of 95% confidence intervals varied within $P \pm 0.05$, $P \pm 0.03$, and $P \pm 0.02$ for cleavage, Day 7 embryo development and development of good quality Day 7 embryos, respectively. PVP+GF-IVM supported cleavage equally well as FBS-IVM ($P = 0.49$), whereas the remaining serum-free IVM treatments had lower embryo cleavage rate than FBS-IVM ($P < 0.01$). On Day 7 none of the serum-free IVM treatments supported embryo development and development of good quality embryos as well as FBS-IVM ($P < 0.01$). Addition of GF in FAF-BSA-IVM reduced embryo cleavage, Day7 embryo development and development of good quality embryos compared to that of FAF-BSA alone ($P < 0.03$). PVP-IVM resulted in lower embryo cleavage rate than PVP+GF ($P < 0.005$), whereas according to the two other criteria there were no differences between the treatments ($P > 0.13$). In conclusion, these preliminary results indicate that replacing the FBS as a protein source in IVM needs more optimization.

**Table 1. Estimated probability (proportion) for embryo cleavage at 34–38 hpi (Y1), Day 7 embryo development (Y2) and development of good quality Day 7 embryos (Y3) after serum-containing and serum-free IVM**

| IVM treatment | No. of oocytes | Estimated probability for | | |
|---|---|---|---|---|
| | | Y1 | Y2 | Y3 |
| FBS | 1174 | 0.87 | 0.20 | 0.11 |
| PVPGF | 1184 | 0.85 | 0.14 | 0.07 |
| FAFBSA | 1186 | 0.77 | 0.13 | 0.06 |
| PVP | 1181 | 0.79 | 0.12 | 0.06 |
| PVPAA | 1188 | 0.81 | 0.08 | 0.04 |
| FAFBSAGF | 1203 | 0.72 | 0.09 | 0.04 |

## 324 SPHINGOSINE-1-PHOSPHATE PROTECTS CULTURED BOVINE OOCYTES FROM PHYSIOLOGICALLY RELEVANT THERMAL STRESS

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Sphingosine-1-phosphate (S1P) is a sphingolipid metabolite that can block the sphingomyelin cell-death pathway by suppressing ceramide-induced apoptosis. The present study was performed to test whether S1P protects oocytes from heat shock during in vitro maturation. Cumulus-oocyte complexes obtained by slicing follicles were placed in maturation medium with or without 50 nM S1P and cultured at 38.5°C (CON) or 41°C (41C) for the first 12 h of maturation. Incubation during the last 10 h of maturation (22-h total maturation time), fertilization, and embryonic development were performed at 38.5°C and 5% (v/v) $CO_2$. Blastocyst development was recorded at 8 days post-insemination (dpi) and activity of group II caspases in 8-day blastocysts was determined using a fluoroprobe, PhiPhiLux-G1D2 (OncoImmunin, Gaithersburg, MD, USA). Data were analysed by least-squares ANOVA with the GLM procedure of SAS. Percentage data were subjected to arcsin transformation before analysis. Exposure of oocytes to thermal stress during the first 12 h of maturation reduced cleavage rate ($P < 0.01$) and the number of oocytes developing to the blastocyst stage ($P < 0.04$). There was a temperature x S1P interaction for cleavage rate ($P < 0.03$) because S1P blocked effects of thermal stress on cleavage rate. Without S1P, the percentage of oocytes that cleaved by 3 dpi were $83.6 \pm 2.7\%$ and $65.8 \pm 2.7\%$ for CON and 41C, respectively. In the presence of S1P, percent cleavage was $86.7 \pm 2.7\%$ and $83.9 \pm 2.7\%$ for CON and 41C, respectively. There was a trend ($P = 0.06$) for a temperature x S1P interaction for percent oocytes developing to blastocyst stage because S1P blocked effects of heat shock on development. Without S1P, the percentages of oocytes that developed to the blastocyst stage were $28.7 \pm 3.0\%$ and $15.2 \pm 3.0\%$ for CON and 41C, respectively. In the presence of S1P, percent blastocysts were $24.3 \pm 3.4\%$ and $23.9 \pm 3.0\%$ for CON and 41C, respectively. When development was expressed as percentage of cleaved embryos, however, there were no effects of temperature, S1P, or temperature x S1P on percent development to the blastocyst stage. Blastocyst caspase activity was not affected by temperature or S1P. In summary, exposure to physiologically relevant thermal stress during the first 12 h of maturation has a deleterious effect on oocyte competence and this effect can be reduced by S1P. The fact that heat shock reduced the percentage of oocytes but not the percentage of cleaved embryos that became blastocysts suggests that oocytes that survive effects of heat shock and cleave have normal potential to develop to the blastocyst stage. Moreover, since heat shock did not affect caspase activity, it is likely that blastocysts from heat-shocked oocytes have normal developmental potential, at least as determined by caspase activity. Support: BARD FI-330-2002 and USDA Grants 2002-35203-12664 and 2001-52101-11318.

## 325 RETINOID-DEPENDENT POLY(A) mRNA CONTENTS IN BOVINE OOCYTES PREMATURED AND/OR MATURED IN VITRO

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Retinoic acid (RA) can induce cell differentiation and plays a role in controlling events within the cell cycle, but little is known of RA post-transcriptional modifications in the oocyte. Bovine oocyte and cumulus cells express most of RA receptors, and the presence of 9-*cis*-RA during in vitro prematuration and maturation (IVM) improves oocyte developmental competence (Duque *et al.*, 2002 Hum. Reprod. 17, 2706–2714; Hidalgo *et al.*, 2003 Reproduction 125, 409–416). This work analyzes the mRNA stability in bovine oocytes during in vitro prematuration and/or maturation. Cumulus-oocyte complexes (COCs) were cultured in defined medium with polyvinyl alcohol (DM). Those COCs undergoing prematuration were cultured for 24 h in DM with 25 μM roscovitine. For IVM, COCs were cultured in DM containing pFSH, LH and $E_2$ for 24 h, and some prematured COCs were then allowed to mature. Incubations were made at 39°C in 5% $CO_2$ in air and high humidity. Within experiments, COCs were cultured with 5 nM 9-*cis*-RA, in 1% ethanol (both as a vehicle and as an inhibitor of endogenous RA synthesis), 3% ethanol, 5% ethanol and untreated. Groups of 10 COCs per treatment were cultured, and oocytes detached from cumulus cells were analyzed. Poly(A) mRNA quantification was based on the pyrophosphorylation property of the DNA polymerase (Klenow). ATP production was measured by luminometric assay as a function of numbers of poly(A) tails. Data (4 replicates) were analyzed by ANOVA and Duncan's test ($^{v,x,y,z}P < 0.01$; $^{a,b}P < 0.05$), and poly(A) mRNA (pg oocyte$^{-1}$) was expressed as LSM ± SE. After prematuration, poly(A) mRNA contents differed between 9-*cis*-RA ($125.7 \pm 4.8^{x}$) and untreated ($95.5 \pm 4.8^{y}$) oocytes, as compared to 1% ethanol ($72.2 \pm 4.8^{z}$) and immature ($71.5 \pm 4.8^{z}$) oocytes. After IVM, untreated oocytes ($23.0 \pm 2.2^{v}$) showed the lowest poly(A) mRNA amount, and poly(A) mRNA in 9-*cis*-RA ($36.2 \pm 2.2^{y}$) basically equalled that in 1% ethanol ($35.2 \pm 2.2^{y}$), while 3% ($44.5 \pm 2.2^{yz}$) and 5% ethanol ($52.0 \pm 2.2^{z}$) increased poly(A) mRNA levels. All groups of matured oocytes showed poly(A) mRNA contents lower than in immature ($71.5 \pm 4.8^{x}$). After prematuration + maturation, poly(A) mRNA values were $34.2 \pm 2.2^{v}$ (untreated + untreated), $36.5 \pm 2.2^{v}$ (9-*cis*-RA + untreated), $49.5 \pm 2.2^{xa}$ (untreated + 9-*cis*-RA), $41.0 \pm 2.2^{vxb}$ (9-*cis*-RA + 9-*cis*-RA) and $59.0 \pm 2.2^{y}$ (untreated + 1% ethanol). Levels of poly(A) mRNA from prematured + matured oocytes were again lower than in immature ($71.5 \pm 4.8^{x}$). Our study shows that beneficial effects of RA on the oocyte developmental competence can be represented in part as a gain in the quality of mRNAs stored. Grant support: Spanish Ministry of Science and Technology (AGL-2002-01175).

## 326 FOLLICULAR SIZE, BUT NOT STAGE OF REPRODUCTION OR SEASON, INFLUENCES MEIOTIC MATURATION OF DOMESTIC DOG OOCYTES

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The current in vitro maturation system (IVM) for dog oocytes is inefficient. On the average, only 15% of ovarian oocytes complete nuclear maturation in vitro. For unknown reasons, the ability of oocytes to develop to the metaphase II stage (MII) varies markedly among bitches (Songsasen *et al.*,

2002, Mol. Reprod. Dev. 62, 407–415). The objective of this study was to identify the cause(s) underlying these significant variations in nuclear maturation. Initially, we retrospectively analyzed data obtained during the past 3 years; 1661 oocytes were obtained from 74 bitches where stage of reproduction for the donor was known based on ovarian morphology. Oocytes were cultured in TCM 199 + 0.1% polyvinyl alcohol at 38.5°C in 5% $CO_2$ in humidified air under various experimental conditions. Analysis of variance (ANOVA) was performed to compare differences in meiotic competence of oocytes obtained at various reproductive stages and during different seasons. Stage of reproduction did not influence meiotic abilities of oocytes. Percentages of oocytes obtained during proestrus/estrus ($n = 468$ oocytes), diestrus/metestrus ($n = 333$), anestrus ($n = 331$) or prepuberty (6–8 months of age, $n = 479$) and developing to MII were $17.9 \pm 2.9\%$, (mean ± SEM), $24.0 \pm 6.0\%$, $20.8 \pm 4.7\%$, and $17.8 \pm 5.2\%$, respectively ($P > 0.05$). A similar analysis across seasons (spring, summer, fall, winter) also indicated no influence of time of year on nuclear maturation ($P > 0.05$). Because there is a known strong link between follicular growth and meiotic competence of goat oocytes (De Smedt *et al.*, 1994 J. Exp. Zool. 269, 128–139), we also examined the impact of follicular size on nuclear maturation. The cortex of ovaries from 15 bitches was horizontally dissected (5 mm thickness) so follicles could be observed and divided into three classes: (1) <0.5 mm diameter ($n = 60$); (2) ≥0.5 to <1 mm ($n = 110$); and (3) 1–2 mm ($n = 72$). Follicles were separated according to these size classes; oocytes were recovered and cultured in TCM 199 + 0.25 mM pyruvate, 2 mM glutamine, 25 mM β-mercaptoethanol, 10 ng/mL epidermal growth factor (Basal TCM) supplemented with 0.5 IU/mL equine chorionic gonadotropin for 1 h. Oocytes then were cultured in Basal TCM for 48 h before staining with 1% orcein to assess nuclear status. Follicular size influenced meiotic competence of the oocytes (ANOVA, $P < 0.05$). Mean percentages of MII oocytes were $14.2 \pm 7.2$, $15.6 \pm 4.5$, and $30.9 \pm 8.2$, for oocytes recovered from <0.5-mm, ≥0.5 to <1-mm and 1–2-mm diameter follicles, respectively. This study revealed that stage of reproduction and season have no impact on in vitro nuclear maturation of the dog oocyte. However, the findings demonstrate that dog oocytes acquire meiotic competency during follicular development. Because the source of most dog oocytes for IVM are small follicles, results suggest that oocytes may be incapable of completing nuclear maturation under in vitro conditions that are designed for fully-grown oocytes.

## 327 THE EFFECTS OF GLUCOSE AND GLUCOSAMINE ON CUMULUS EXPANSION AND NUCLEAR MATURATION OF BOVINE CUMULUS-OOCYTE COMPLEXES

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Glucose is the primary energy substrate consumed by bovine COCs during in vitro maturation (IVM), with most accounted for by glycolysis (L-lactate production). However, antral follicular fluid (FF) contains less than half the glucose of standard IVM media (TCM199 = 5.6 mM, FF = 2.3 mM). We have previously demonstrated that from 20 to 24 h of IVM, a significant proportion of the glucose utilized is directed into pathways other than lactate production (Sutton *et al.*, 2003 Reproduction 126, 27–34). We hypothesize that glucose is utilized for cumulus matrix synthesis. The aim of this study was to determine the influence of glucosamine (an intermediate for matrix components) on FSH-stimulated glucose uptake and cumulus expansion. The influence of different glucose concentrations and glucosamine on nuclear maturation was also investigated. Bovine COCs were collected from abattoir-derived ovaries. In Exp. 1, individual COCs ($n = 60$, 3 replicates) were cultured in 10-μL drops of TCM199 (plus pyruvate, hCG and BSA, containing 5.6 mM glucose), ± FSH (0.1 IU $mL^{-1}$) and ± glucosamine (5 mM). After 20 h, COCs were transferred to fresh media and cultured a further 4 h. Cumulus expansion and glucose/L-lactate levels in spent medium from 0–4-h and 20–24-h culture periods were measured. In Experiment 2, COCs ($n = 300$, 6 replicates) were cultured in groups of 10 in 100 μL of Bovine FF medium (a defined medium based on the composition of bovine antral FF, also containing amino acids, FSH, hCG and BSA) ± glucosamine (5 mM) in 2.3 or 5.6 mM glucose, or in conventional TCM199 IVM media (as above). Nuclear maturation was assessed at 24 and 30 h using orcein staining. Treatment differences were determined using two-way ANOVA. The influence of FSH and glucosamine (Exp. 1) on the measured parameters was evident at 20–24 h, with FSH increasing diameter, glucose uptake and L-lactate production ($P < 0.05$). Although glucosamine alone did not influence diameter or glucose/L-lactate concentrations, glucosamine plus FSH led to a decrease in glucose uptake compared to FSH-stimulation alone ($P < 0.05$). The proportion of oocytes at MII (Exp. 2) was significantly lower when COCs were cultured in low glucose (main effect, 24 h: 2.3 mM = 38% v. 5.6 mM = 64%; $P < 0.005$). The presence of glucosamine tended to stimulate meiotic maturation (main effect, 24 h: 0 mM = 45% v. 5 mM = 59%; $P = 0.1$). MII frequency in TCM199 controls at 24 h was 68%. These experiments support the hypothesis that synthesis of cumulus matrix is a major pathway for glucose metabolism, especially in the absence of glucosamine. Furthermore, oocytes matured in media based on a physiological concentration of glucose (2.3 mM), have delayed meiosis compared to oocytes cultured in higher glucose (5.6 mM). Thus, glucose has multiple functions, involving matrix formation and meiosis regulation during bovine IVM. Supplementation of medium with glucosamine appears to partly reduce the dependency of COCs on glucose. Supported by Australian Research Council and COOK Australia.

## 328 MEIOTIC COMPETENCE AND DNA FRAGMENTATION OF PORCINE OOCYTES FROM OVARIES STORED IN VARIOUS TEMPERATURES

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The aim of this study was to investigate the effects of storage of porcine ovaries at different temperatures before oocyte collection on the nuclear maturation and DNA fragmentation of cumulus-oocyte complexes (COCs). Ovaries were collected at a local abattoir and randomly kept in physiological saline at 4°C, 15°C, 25°C and 35°C. Ovaries were stored for 6 hours prior to follicle aspiration. After storage at each temperature (about 80 oocytes each group), COCs were fixed immediately after aspiration and stained by the terminal deoxynucleotidyl transferase (TdT) nick-end labeling (TUNEL) method to examine the DNA fragmentation under fluorescein microscope. To investigate meiotic competence of the oocytes, some COCs of each treatment group (about 100 oocytes each group) were matured in vitro for 45 hours in a modified North Carolina State University (NCSU)-37

solution supplemented with 10% (v/v) porcine follicular fluid, 0.6 mM cysteine, 10 IU $mL^{-1}$ eCG and 10 IU $mL^{-1}$ hCG. After maturation culture, the cumulus cells were removed from COCs and fixed in acetic acid-ethanol (1:3, v/v) for 48–72 h. The fixed oocytes were stained with acetic-orcein (1% orcein in 45% acetic acid) and examined under a phase-contrast microscope. Data were subjected to arc-sin transform before analyzing by ANOVA. The proportions of oocytes with DNA fragmentation increased with increasing storage temperature of ovaries (25.2% in 4°C, 31.8% in 15°C, 37.4% in 25°C and 54.7% in 35°C, respectively). There was no significant difference between the proportions of germinal vesicle breakdown (GVBD) of 25°C and 35°C storage groups (74.7 and 83.6%, respectively), but the proportions of 25°C and 35°C storage groups were significantly higher ($P < 0.05$) than those of 4°C and 15°C storage groups (58.1 and 59.6%, respectively). The proportions of oocytes reaching metaphase II (MII) was significantly higher ($P < 0.05$) in the 25°C storage group than in other groups (48.0% in 25°C v. 0% in 4°C, 0% in 15°C and 40.1% in 35°C). Moreover, none of oocytes in 4°C and 15°C storage groups reached MII. These results indicate that 25°C is the most suitable temperature for long-term storage of ovaries to maintain meiotic competence and prevent DNA fragmentation of porcine oocytes.