of oocyte growth for *in vitro* fertilization (IVF) in several farm animals. The aim of this study was to evaluate the effectiveness of BCB staining on the selection of developmentally competent oocytes, collected from cows and calves by OPU, before *in vitro* maturation by assessing the embryonic development to the blastocyst stage after IVM/IVF. OPU was performed once weekly for a period of 7 weeks on Japanese Black prepubertal calves (9 months old, $n = 4$) and adult cows ($n = 4$) without gonadotropin stimulation. Calf and cow oocytes with homogeneous cytoplasm were exposed to 26 μM BCB for 90 min and classified according to their cytoplasmic coloration: blue coloration (BCB+) or colorless (BCB−). Classified oocytes were then matured for 20 h in TCM-199 supplemented with 5% calf serum (CS). Matured oocytes were inseminated with Percoll-separated spermatozoa (3×10^6/mL) for 6 h in BO solution supplemented with 5 mM hypotaurine and 2 U mL^{-1} heparin. Presumptive zygotes were cultured in CR1aa supplemented with 5% CS for 8 days. Data were analyzed by Student's *t*-test. The mean (± SEM) percentage of oocytes classified as BCB+ in calves was significantly lower than that in cows (34.4 ± 2.9% and 69.2 ± 2.1%, respectively; $P < 0.01$). In cows, BCB+ oocytes showed significantly higher cleavage and blastocyst formation percentages (72.5% and 42.4%, respectively) than those of BCB− oocytes (47.0% and 13.0%, respectively). In contrast, in calves there were no significant differences in cleavage and blastocyst formation percentages between BCB+ oocytes (56.9% and 25.3%, respectively) and BCB− oocytes (65.4% and 22.4%, respectively). The mean (± SEM) numbers of usable oocytes and blastocysts obtained per calf (19.0 ± 1.5 and 4.5 ± 0.6, respectively) were similar to those obtained per cow (16.4 ± 1.1 and 5.2 ± 0.6, respectively). No significant difference was observed in the numbers between calves and cows. These results indicate that the selection of developmentally competent oocytes before IVM/IVF, using the BCB staining, was effective for cow oocytes but not for calf oocytes, and that blastocysts could be produced by OPU-IVF of oocytes from 9-month-old prepubertal calves at an efficiency equivalent to that achieved from adult cows.

317 ANTI-HYALURONIDASE ACTION OF ELLAGIC ACID EFFECTIVELY PREVENTS POLYSPERMY THROUGH SUPPRESSION OF THE ACROSOME REACTION INDUCED BY THE SPERM–ZONA INTERACTION DURING PORCINE *IN VITRO* FERTILIZATION

I. Tokeshi[A], *H. Tatemoto*[A], *N. Muto*[B], *T. Yoshimoto*[A], *S. Nakamura*[C], *and T. Nakada*[A]

[A]Faculty of Agriculture, University of the Ryukyus, Nishihara, Okinawa 903-0213, Japan;
[B]Faculty of Life and Environmental Sciences, Prefectural University of Hiroshima, Shobara, Hiroshima 727-0023, Japan;
[C]Okinawa Prefectural Livestock Research Center, Nakijin, Okinawa 905-0426, Japan

We previously reported that the anti-hyaluronidase agents oligosaccharide and tannic acid (TA) were efficient probes for promoting the normal fertilization process in terms of an effective decrease in the incidence of polyspermy, not only in cumulus-enclosed but also in denuded oocytes in pigs. It was unclear, however, why the polyspermic penetration into the zona pellucida (ZP) was directly prevented by the anti-hyaluronidase action. The present study was conducted to examine the effects of 3 tannin relatives [TA, gallic acid (GA), and ellagic acid (EA)] on IVF parameters and the acrosome reaction induced by the sperm–ZP interaction. The anti-hyaluronidase and radical-scavenging activities of tannin relatives were measured by the colorimetric and the DPPH methods, respectively. Porcine cumulus–oocyte complexes (COCs) were cultured for 44 h in 0.1 mL of TCM-199 supplemented with 0.6 mM cysteine, 40 mU mL^{-1} of FSH, 20 mU mL^{-1} of LH, and 10% porcine follicular fluid. After *in vitro* maturation (IVM), the COCs were freed from their cumulus cells and inseminated by frozen–thawed ejaculated sperm in modified Tris-buffered medium (IVF medium) containing 0 (control) or 5 μg mL^{-1} of tannin relatives. After 2 h of co-incubation, the oocytes were gently pipetted to remove loosely bound sperm and stained with Hoechst 33342 to count the number of ZP-bound sperm, or stained with fluorescein isothiocyanate (FITC)-PNA, PI, and 4′,6-diamidino-2-phenylindole to evaluate the acrosomal status. At 10 h post-insemination, IVF parameters were examined by lacmoid staining. The data were analyzed by ANOVA and the Tukey-Kramer test. None of the tannin relatives caused a protective proteolytic modification of the ZP matrix or a reduction of the acrosomal proteolytic activity or the number of ZP-bound sperm. There was no difference in the sperm penetration rate even in the presence of tannin relatives (73–82%). However, the incidence of polyspermy was remarkably prevented by TA (32%; 31/98) and EA (21%; 20/94) compared with the control (58%; 58/100; $P < 0.05$), resulting from their strong anti-hyaluronidase actions, whereas GA without the anti-hyaluronidase action had no effect on the prevention of polyspermy (51%; 43/84). The rate of acrosome reaction induced by the sperm–ZP interaction was decreased by TA (15%; 123/833) and EA (16%; 110/708) compared with the control (25%; 238/939; $P < 0.05$), and a similar result was found in sperm binding to the pretreated ZP with 500 U of hyaluronidase for 2 h (18%; 351/1959). Interestingly, when sperm were incubated in IVF medium with 10 μg mL^{-1} of progesterone 0.5 h to induce a compulsory acrosome reaction instead of the ZP, EA never disturbed the acrosome reaction (23%; 98/424) as control (23%; 2/437), although this reaction was blocked by TA (13%; 57/427) and GA (13%; 50/375), which possessed higher levels of radical-scavenging activity than EA ($P < 0.05$). These results indicate that the anti-hyaluronidase action of TA and EA effectively prevented polyspermy during porcine IVF as a consequence of suppression of the acrosome reaction functionally induced by the sperm–ZP interaction requiring the hyaluronidase intervention.

318 EFFECT OF MATERNAL HEAT STRESS ON OOCYTE QUALITY AND *IN VITRO* COMPETENCE IN *BOS INDICUS* CATTLE

J. R. S. Torres Júnior[A], *M. F. A. Pires*[B], *W. F. Sá*[B], *A. M. Ferreira*[B], *J. H. M. Viana*[B], *L. S. A. Camargo*[B], *A. A. Ramos*[B], *I. M. Folhadella*[B], *J. Polisseni*[B], *C. Freitas*[B], *C. A. A. Clemente*[B], *M. F. Sá Filho*[A], *C. M. Martins*[A], *G. A. Bó*[C], *and P. S. Baruselli*[A]

[A]Animal Reproduction Department, FMVZ/USP, São Paulo, Brazil;
[B]Dairy Cattle Research Center, EMBRAPA, Juiz de Fora, Brazil;
[C]Animal Reproduction Institute, Córdoba, Argentina

High temperatures can be harmful to the competence of cumulus–oocyte complexes and to embryo development (Al-Katanani *et al.* 2002 J. Dairy Sci. 85, 390–396). The aim of this study was to evaluate the effect of maternal heat stress on *in vitro* embryo yield. Ten multiparous nonlactating Gir

(*Bos indicus*) cows were kept in tie stalls for an adaptive period of 28 days [pre-heat-stress period (PRE-HS)/Days −28 to −1]. Cows were subjected to 2 OPU (ovum pickup) sessions (Days −14 and −7). In the heat-stress period (HS; Days 0 to 28), cows were divided into control (C: $n = 5$) and heat-stressed (HS: $n = 5$) groups. During this period, OPU sessions were performed once a week from Days 0 to 28. The C group remained in a thermo-neutral environment, and the HS group was kept in a climatic chamber with controlled temperature and humidity (38°C and 80% during the day and 30°C and 80% during the night). In the post-heat-stress period (POST-HS; Days 28 to 147), all cows returned to thermo-neutral conditions. Then 17 OPU sessions were performed once a week from Days 35 to 147. In all periods, blood samples were collected weekly for progesterone (P4) analysis, and ovarian follicles were counted, measured, and aspirated. The COCs were evaluated and selected for the IVF procedure. Data were analyzed by ANOVA (PROC MIXED of SAS) and a chi-squared test. The luteal phase was defined as the period between 2 samples with P4 below 1.0 ng mL^{-1}. A handling accident caused the exclusion of an HS cow after the sixth session. The C and HS groups were subjected to 125 and 107 OPU sessions, respectively. Means ± SEM for the C vs. HS groups, in the PRE-HS, HS, and POST-HS periods, respectively, were visualized follicles: 25.5 ± 2.5 vs. 28.5 ± 2.8, 24.2 ± 1.1 vs. 24.0 ± 1.9, and 15.3 ± 0.6 vs. 15.8 ± 0.8; largest follicle diameter: 12.1 ± 1.5 vs. 11.1 ± 1.7, 13.3 ± 0.8 vs. 13.0 ± 0.6, and 11.4 ± 0.4^{b} vs. 14.0 ± 0.4^{a}; $P < 0.05$; 2nd largest follicle diameter: 6.2 ± 1.3 vs. 6.0 ± 1.2, 5.9 ± 0.6 vs. 7.1 ± 0.8, and 6.3 ± 0.3^{b} vs. 8.7 ± 0.5^{a}; recovered COCs: 11.2 ± 2.8 vs. 14.3 ± 2.5, 9.6 ± 1.0 vs. 11.0 ± 1.3, and 8.6 ± 0.7 vs. 7.9 ± 0.6; COCs selected for IVF: 69/112 (61.6%)^{b} vs. 108/143 (75.5%)^{a}, 164/241 (68%) vs. 172/265 (64.9%), and 426/712 (59.8%) vs. 305/535 (75.0%); cleavage: 44/59 (74.5%) vs. 87/105 (82.9%), 72/101 (71.3%) vs. 74/121 (61.2%), and 226/317 (71.3%) vs. 159/230 (69.1%); embryos per cow/OPU: 2.1 ± 1.1^{y} vs. 4.1 ± 1.0^{x}, 0.4 ± 0.3 vs. 0.5 ± 0.3, and 0.9 ± 0.2^{x} vs. 0.4 ± 0.1^{y}; $P < 0.1$; and blastocyst yield: 16/59 (27.1%) vs. 33/105 (31.5%), 11/31 (35.5%) vs. 13/52 (25.0%), and 76/279 (27.2%)^{a} vs. 25/188 (13.3%)^{b}. In conclusion, maternal heat stress increased the percentage of short estrous cycles, decreased the P4 concentrations, and decreased the number of embryos produced by *Bos indicus* cows, mainly from 28 to 147 days post-heat-stress, showing long-term deleterious effects on blastocyst development.

319 PROTEIN GENE PRODUCT 9.5 REGULATES SPERM PENETRATION DURING PORCINE FERTILIZATION *IN VITRO*

Y. J. Yi[A,B], *G. Manandhar*[B], *M. Sutovsky*[B], *C. S. Park*[A], *and P. Sutovsky*[B,C]

[A]Research Center for Transgenic Cloned Pigs, Chungnam National University, Daejeon, Korea;
[B]Division of Animal Sciences, University of Missouri-Columbia, Columbia, MO 65201, USA;
[C]Department of Obstetrics and Gynecology, University of Missouri-Columbia, Columbia, MO 65201, USA

The protein gene product 9.5 (PGP9.5) belongs to a family of ubiquitin C-terminal hydrolases (UCHs), which regenerate monoubiquitin from ubiquitin–protein complexes or polyubiquitin chains by cleaving the amide linkage next to the C-terminal glycine of ubiquitin. Identified in the acrosome of boar spermatozoa, we hypothesized that PGP9.5 might regulate sperm–zona pellucida interactions during porcine IVF. The cumulus–oocyte complexes isolated from slaughterhouse ovaries were cultured in TCM-199 media for 44 h at 38.5°C, 5% CO_2 in air. After completion of *in vitro* maturation (IVM), cumulus cells were removed by 0.1% hyaluronidase, and metaphase II (MII) oocytes were used for IVF. In Experiment 1, oocytes were co-incubated with different sperm concentrations (1×10^6, 5×10^5, and 1×10^5 sperm mL^{-1}) in TBM medium with or without anti-PGP9.5 antibody (1 : 50 dilution). In Experiment 2, oocytes were inseminated with 1×10^6 sperm mL^{-1} in TBM medium containing different concentrations of extracted oviductal fluids (0, 0.1, 0.5, 1, 2, and 3 μg mL^{-1}) for 6 h. After IVF, oocytes were transferred into NCSU23 medium containing 0.4% BSA for further culture. The fertilization rates were evaluated by DAPI staining at 13 to 19 h. Data were analyzed by ANOVA and Duncan's multiple range test using the SAS program. Polyspermy was increased by the addition of anti-PGP9.5 antibody to the IVF medium (56.5–60.2% at polyspermy). This PGP9.5-antibody-induced polyspermy increase was sustained even with decreasing sperm concentrations. The polyspermy rates were reduced by the addition of isolated porcine oviductal fluid to IVF medium (50.4, 44.8, 28.0, 31.1, 1.6, and 0.0% at oviductal fluid concentrations of 0, 0.1, 0.5, 1, 2, and 3 μg mL^{-1}, respectively). Biochemical analysis by Western blotting detected the appropriate 24-kDa PGP9.5 band in porcine oviductal fluid used for these experiments. Enzymatic UCH activity comparable to activity of recombinant UCH-L3 was detected in sperm extract, whole spermatozoa, and isolated oviductal fluid by fluorometric assay using fluorogenic UCH-substrate ubiquitin-AMC. This UCH activity was not reduced by the general protease inhibitor phenyl methyl sylfonyl fluoride, but it was reduced in a statistically significant manner ($P < 0.05$) by the specific UCH-inhibitor ubiquitin aldehyde. In conclusion, the polyspermy increased with different concentrations of sperm in the anti-PGP9.5 antibody, and PGP9.5 was detected in oviductal fluids, suggesting that PGP9.5 is involved in the sperm–zona pellucida interaction during porcine fertilization.

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320 EFFECT OF OXYGEN TENSION AND FOLLICLE CELLS DURING *IN VITRO* CULTURE OF PORCINE OOCYTES IN FOLLICULAR FLUID ON THEIR MATURATION AND FERTILIZATION

P. Yongnan[A,B], *F. Daiichiro*[C], *S. Shoichiro*[C], *O. Akira*[C], *S. Eime*[B], *and T. Nagai*[A]

[A]National Institute of Livestock and Grassland Science, Tsukuba, Japan;
[B]Tohoku University, Sendai, Japan;
[C]National Institute of Agrobiological Sciences, Tsukuba, Japan

If we could use porcine follicular fluid (pFF) as a solo *in vitro* maturation (IVM) medium, the preparation of complicated medium wouldn't be necessary. In this study, we investigated the effects on nuclear maturation and subsequent IVF of oxygen tension and follicle cells (FC) during IVM

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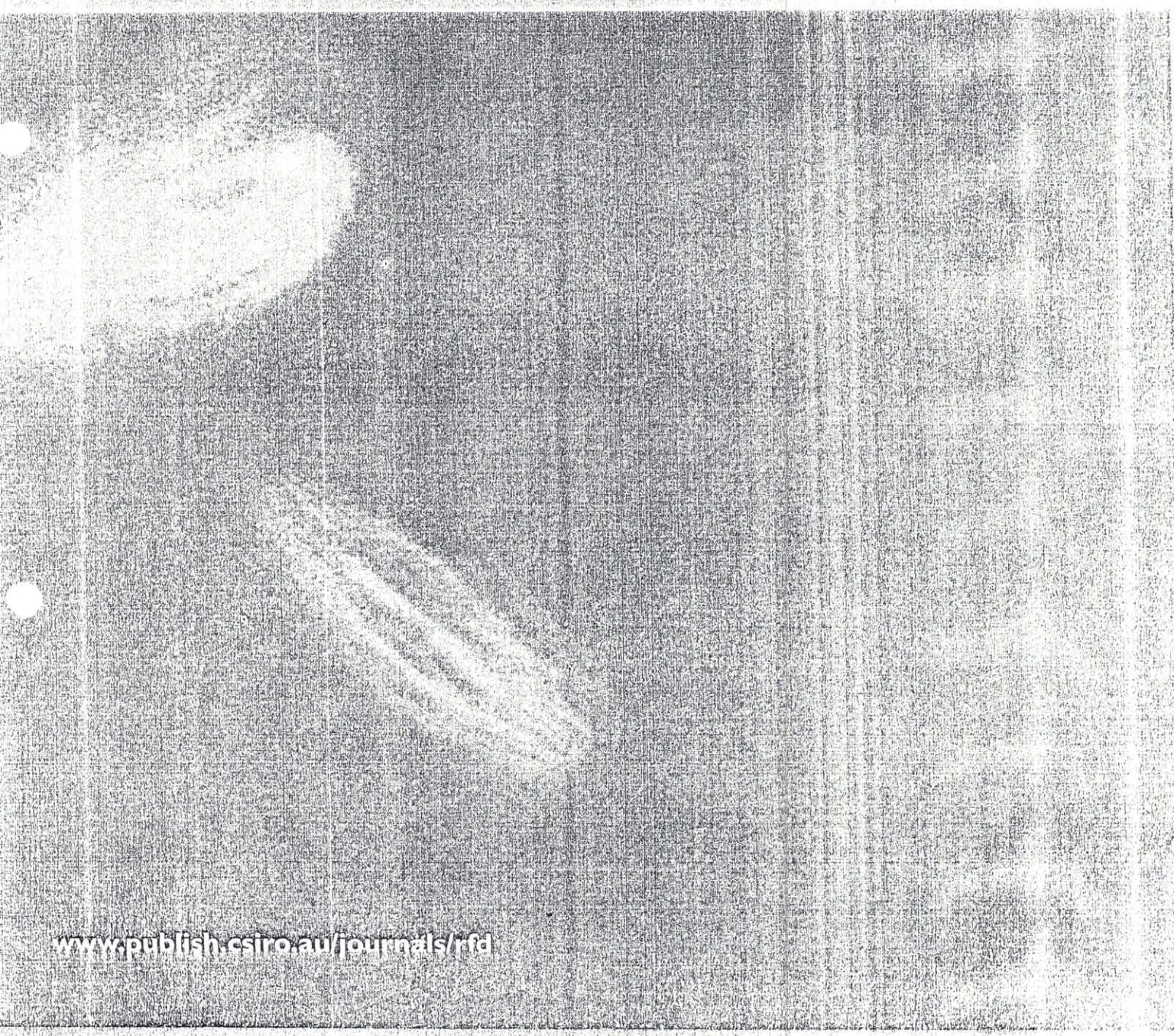

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