ABSTRACTS FOR POSTER PRESENTATION

IVF/IVP

258 ULTRASTRUCTURAL STUDY OF STALLION SPERM FOLLOWING THAWING, CAPACITATING AND IVF PROCEDURES: EFFECT OF THE PRESENCE OF CUMULUS-ENCLOSED OOCYTES

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The use of IVF in horses has a limited efficiency, reflecting low oocyte developmental competence and inadequate sperm capacitation procedures. In a preliminary study, using carboxyfluorescein diacetate/propidium iodide staining, we determined that the freezing-thawing procedure left only 56.6 ± 3.4 % of the sperm cells with an intact membrane. The following incubation in TALP-IVF induced membrane damage at high rates with only 9.58 ± 1.8 % of them intact after 18 h. However, the presence of at least four cumulus-enclosed oocytes (CEO) in the medium significantly increased the number of membrane-intact spermatozoa at the end of incubation (53.87 ± 1.99%). This indicated that the sperm thawing and capacitating procedures can damage the cell membrane but the presence of four or more CEO in TALP-IVF could prevent further damages. The aim of the study was to investigate in detail the membrane damages and to analyze the differences induced by the presence of CEO. Spermatozoa were thawed in water at 37°C, and centrifuged for 30 minutes at 600*g* in a 45–90% Percoll gradient made with modified Tyrode's medium. The sperm pellet was washed once in the same medium and diluted to a final concentration of 1×10^6 spermatozoa/ml TALP supplemented with 0.6% (w/v) BSA fatty acid free and 12 μg mL^{-1} heparin (TALP-IVF). Sperm cells were incubated with 0 or 4 in vitro-matured CEO. Sperm cells were examined after thawing, 0, 2 and 18 h from the beginning of incubation in TALP-IVF. Each experiment was replicated at least 3 times. Both scanning and transmission electron microscopy were performed on sperm samples fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, using standard procedures. Specimens for scanning electron microscopy were examined under a field emission gun JEOL JSM 6301 microscope. For transmission electron microscopy the samples were examined with a JEOL JEM 100 SX. A minimum of 25 cells were analyzed for each group. Immediately after thawing, damaged spermatozoa showed, on the surface of their heads, small vesicles correlated to a progressive process of vacuolisation and degeneration of membrane integrity. The same lesions were visible at all the successive time points taken into account. Moreover, a loss of the acrosome integrity with acrosomal swelling and a decrease of content homogeneity were observed particularly in the spermatozoa cultured for 18 h without CEO. When CEO were present in the IVF medium lesions were visible in a lower percentage of spermatozoa but the type of lesions did not differ from those observed in their absence. These observations confirmed our previous data and gave more details on the lesions that occur during the IVF procedures in the horse. Supported by MURST COFIN grant n. 2001078849.

259 PRODUCTION OF TROPICAL DAIRY CALVES BY EMBRYO TRANSFER USING LOCAL LAISIND (*BOS INDICUS*) RECIPIENTS AND INTERCONTINENTAL FRESH IVF SHIPPED EMBRYOS

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Increasing the diary population and milk production is a goal of many tropical developing countries. We report in this paper an attempt to develop a system of intercontinental shipping for transfer of fresh crossbred *Bos taurus* × *Bos indicus* IVF embryos into local Laisind (*Bos indicus*) recipients as a way to produce tropical dairy calves with highly improved milk productivity. The production of embryos was done at BOMED, Inc (Madison, WI, USA). Oocytes collected from ovaries of Holstein (*Bos taurus*) at slaughter and semen from milking Gir (*Bos indicus*) were used for IVP. Cleaved embryos were selected for air shipping in portable incubators at Day 4 (Group 1), Day 3 (Group 2) or Day 2 (Group 3) after IVF. The duration of shipping varied from 60 to 65 h. Embryo transfer was done in Vietnam. Laisind cows (Yellow cattle × Red Sindhi) with body weight more then 280 kg and normal reproductive activity were selected for treatment of estrous synchronization with double 11-day interval injection of PG2α (Intervet, Boxmeer, The Netherlands) and single injection of eCG (SABC Vietnam) two days before the second injection of PG2α. Timing of injections was calculated according to the IVF schedule. Embryos collected from portable incubators were transferred to a CO_2 incubator for further culture at 39°C. Two experiments were carried out: (1) transfer of embryos without sexing; (2) transfer of embryos after biopsy and sex determination by PCR. In experiment 2, compact morulae or morula-blastocysts were selected for sex determination. Four to five blastomeres were aspirated from each embryo using a cutting pipette and an aspiration pipette of 30-μm diameter. PCR was done as previously described (Uoc *et al.*, 1999 J. Biology). After biopsy, embryos were kept in culture for one day to observe the development in vitro. Embryos developed to morula-blastocyst or hatching blastocyst at Day 7 or Day 8 after IVF were transferred nonsurgically to recipients with estrus detected in the period from 0 to 12 h before or after the starting IVF. Pregnancy was confirmed by rectal palpation 3 months after embryo transfer. The average rate of embryos developed into morula-blastocyst was more then 50% (Table 1) and there were no significant differences among different shipping groups. For experiment 2, more then 87 % of embryos biopsied developed in vitro to expanding and hatching embryos. The average rate of female embryos was 56.3%. The pregnancy rate at 3 months was more then 44% ($n = 188$). The first group of calves was born without unusual birthing problems. In conclusion, the system of embryo transfer using intercontinental shipping of fresh IVF embryos and local *Bos indicus* recipients can be applied for production of dairy calves. Supported by grant from the AIRE-Development agency.

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Table 1. Development in vitro of IVP embryos

Experiment	Exp-1. without biopsy & sexing No. embryos observed	No. morulae at Day 8 (%)	No. blastocysts at Day 8 (%)	Exp. 2 with biopsy & sexing No. embryos treated	No. developed after cuture (%)	% of female embryos
Day 4 embryos	242	38 (15.7)	99 (41.2)	89	74 (83.2)	57.6
Day 3 Embryos	393	98 (24.9)	126 (32.3)	69	59 (85.5)	62.9
Day 2 Embryos	323	56 (17.3)	142 (44.2)	64	61 (95.3)	51.56
Average	958	192 (20.0)	367 (38.3)	222	194 (87.4)	56.3

260 PRELIMINARY RESULTS OF IN VITRO FERTILIZATION FOR THE ENDANGERED MURCIANO-LEVANTINA BOVINE BREED

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The Murciano-levantina cattle from the Spanish Southeast belong to a bovine endangered breed under the special protection of FAO (WWL-DAD:3)*. Their characteristics of rusticity, longevity, docility and disease resistance make the recovery of the breed interesting. The objective of the present work was to determine the efficiency of in vitro bovine embryo production using oocytes collected from postmortem ovaries fertilized with frozen-thawed sperm obtained from either commercial or Murciano-levantina bulls. Cumulus-oocyte complexes were matured for 24 h in TCM199 with 10% FBS, 2 mM L-glutamine, 0.2 mM sodium pyruvate, 0.3 $\mu g\,mL^{-1}$ LH and 5 $\mu g\,mL^{-1}$ FSH. Oocytes presumed matured were fertilized with Percoll-prepared sperm (750 000/mL) in IVF-TALP, as described by Parrish *et al.*, (1988 Biol. Reprod. 38, 1171–1180). Zygotes (at 18 h) or two-cell-stage embryos (at 48 h) were stained with Hoechst 33342 (10 $\mu g\,mL^{-1}$; 20 min) and assessed by means of epifluorescence microscopy. The experiment was replicated on 4 occasions. The results of penetration (PEN-%), average number of spermatozoa inside the oocyte (S/O), monospermy (MON-%), putative embryos (zygotes) with two pronuclei (2 PN-%) and percentages of cleavage at 48 hours were recorded (Table 1). The results show that early embryos can be obtained with no differences in monospermy and cleavage rates regardless whether the sperm source was commercial or Murciano-levantina ($P \geq 0.05$).

*Lista Mundial de Vigilancia para la Diversidad de los Animales Domésticos (1997). http://www.fao.org/docrep/V8300S/V8300S00.htm.

Table 1. In vitro fertilization results with one commercial and one Murciano-levantina bull

	n[a]	PEN[a]	S/O[a]	MON[a]	2 PN[a]	n[b]	1 CELL[b]	2–4 CELLS[b]	>4 CELLS[b]
Commercial	79	62.02 ± 5.49	1.38 ± 0.11	77.55 ± 6.02	61.22 ± 7.03	92	55.43 ± 5.21	34.78 ± 4.99	9.78 ± 3.11
Murciano-levantina	76	60.53 ± 5.64	1.48 ± 0.16	76.09 ± 6.35	73.91 ± 6.54	95	53.68 ± 5.14	29.47 ± 4.70	16.8 ± 3.85
P		0.849	0.657	0.868	0.191		0.811	0.440	0.158

[a]At 18 h. [b]At 48 h.

261 DIFFERENT TRANSVAGINAL OVUM PICK-UP STRATEGIES TO OPTIMIZE THE OOCYTE RETRIEVAL AND EMBRYO PRODUCTION OVER A FIXED PERIOD OF TIME

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The objective of this study was to compare the efficacy of different oocyte retrieval schemes over a period of 10 weeks. Fifteen multiparous Angus cows were randomly assigned ($n = 3$/group) to the following groups: 1) OPU once/week (7-day interval), 2) OPU twice/week (3- and 4-day interval, alternately), 3) Dominant follicle removal (DFR) + OPU once/week. DFR followed by OPU 72 h later, 4) DFR + FSH + OPU once/week. DFR followed 36 h later by FSH (Folltropin, Bioniche, Belleville, Ontario, Canada) (120 mg s.c. and 80 mg i.m. administered simultaneously) followed by OPU 48 h later, 5) FSH + OPU twice/week. FSH followed by OPU_1 30 h later and OPU_2 96 h after OPU_1. The interval between OPU_2 and next FSH was approximately 42 h. The follicles were aspirated using an Aloka ultrasound scanner (Wallingford, CT, USA) and a 5-MHz probe. The COCs were selected based on morphology and matured in TCM-199, supplemented with 10% FCS, 0.01 units mL^{-1} bFSH, 0.01 units mL^{-1} bLH and antibiotics. Fertilization (Day 0) was carried out with TALP-FERT medium containing capacitation factors. Frozen semen from the same bull was used (1×10^6 mL^{-1}) throughout. After 18 h the presumptive zygotes were cultured in SOF with 5% FCS (Holm P *et al.*, 1999 Theriogenology 52, 683–700). The embryos were evaluated based on IETS guidelines (Grades 1 and 2 selected). The data were analyzed by chi-square test and ANOVA. In all parameters, the DFR followed by FSH and subsequent OPU once/week protocol gave the best results on a per-cow-per-week as well as total (3 cows over 10 weeks) basis. Though OPU was done only once/week, this group produced more total oocytes (303) than groups where OPU was done twice/week, either with FSH (286) or without FSH (229) and also produced more total embryos on Day 8 (71 blastocysts, 23.4% of oocytes

cultured) than the latter two groups (64, 22.4% and 49, 21.4%, respectively). Among the nonstimulated groups, the OPU twice/week group had more total oocytes (236) than groups with OPU once/week, either without DFR (137) or with preceding DFR (160). However, a preceding DFR seemed to have a positive effect on oocyte quality as this group had a better embryo development rate (26.9%), producing more total embryos (42). In comparison, OPU twice/week produced total 49 embryos (21.4%) and OPU once/week produced 26 (19.4%). In conclusion, DFR coupled with single-shot FSH administration can be used effectively over a period of at least 10 consecutive weeks and can increase ($P < 0.05$) the oocyte yield by two-fold and embryo production following IVF by two and half-fold, as compared to routine OPU-IVF done once a week.

Table 1. Per cow per week performance

Treatment groups	Follicles aspirated	Oocytes retrieved	Oocytes cultured	Blastocysts Day 7	Blastocysts Days (7 + 8)	Embryos hatched
OPU 1/w	7.8 ± 2.4^{a}	4.6 ± 1.9^{a}	4.5 ± 1.9^{a}	0.6 ± 0.8^{a}	0.9 ± 0.9^{a}	0.2 ± 0.4^{a}
OPU 2/w*	13.0 ± 3.5^{b}	7.9 ± 2.9^{b}	7.6 ± 2.8^{b}	1.3 ± 1.1^{bc}	1.6 ± 1.3^{bc}	0.5 ± 0.9^{ab}
DFR-OPU 1/w	7.3 ± 2.4^{a}	5.3 ± 2.2^{a}	5.2 ± 2.1^{a}	1.2 ± 1.3^{ab}	1.4 ± 1.6^{ab}	0.5 ± 0.8^{ac}
DFR-FSH-OPU 1/w	16.0 ± 5.0^{c}	10.6 ± 4.5^{c}	10.1 ± 4.4^{c}	2.1 ± 1.2^{d}	2.4 ± 1.4^{d}	0.7 ± 0.9^{bc}
FSH-OPU 2/w*	15.1 ± 4.4^{c}	9.8 ± 3.9^{c}	9.5 ± 3.8^{c}	1.8 ± 1.6^{cd}	2.1 ± 1.9^{cd}	0.7 ± 0.8^{bc}

Values not having a common superscript in the same column differ ($P < 0.05$). *2 sessions/week.

262 IN VITRO FERTILIZATION IN MICROFLUIDIC CHANNELS ENHANCES MONOSPERMIC PENETRATION OF SWINE OOCYTES

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In traditional porcine IVF systems, there is a high incidence of polyspermy, a pathological condition which results in aberrant embryonic development. Efforts to improve the in vitro embryo production process in pigs have included modifying the culture medium, the number of spermatozoa inseminated, and the quantity of medium used. Recently, the development of microscale embryo culture devices (microchannels) has opened new avenues for manipulation of the IVF system to improve the efficiency and overall production of porcine embryos by more closely mimicking the function of the oviduct. The volume of medium in the local vicinity of the embryo is smaller (0.125 μL) compared to the typical 5–50 μL microdrops. Additionally, it is believed that the delivery of sperm cells in the microchannel simulates the flow pattern of spermatozoa past the oocytes similar to that in the oviduct. This study was designed to compare the incidence of polyspermy of pig oocytes fertilized in PDMS-glass microchannels (MC) to conventional microdrop methods (controls). Oocytes were obtained by aspiration of ovarian antral follicles. Fifty oocytes were placed into 500 μL of TCM199 medium supplemented with LH, FSH, EGF, cysteine, PVA, and antibiotics. Extended semen was washed with mTBM and re-suspended to 6×10^5 sperm/mL. The sperm suspension was placed in humidified 5% CO_2 in air atmosphere at 39°C for 1 h to allow for capacitation. Concurrently, pre-warmed microchannels were filled with 200 μL of mTBM and allowed to equilibrate for 1 h at 39°C. Cumulus cells were removed from the oocytes using 0.1 mg mL^{-1} hyaluronidase in mTBM. At 44 h of maturation, 15 oocytes were placed into a pre-equilibrated 50 μL drop of mTBM covered with warm paraffin oil in a petri dish, and placed into the microchannel. Capacitated sperm cells were then added to the oocytes to give a final concentration of 3×10^5 sperm/mL; the mixture was incubated for 6 h. Presumptive zygotes were cultured in 100 μL drops of NCSU-23 covered with oil at 39°C in a humidified 5% CO_2 in air for 12 h. At this time, the zygotes were fixed in 1 : 3 (v/v) glacial acetic acid in absolute ethanol for 48–72 h. Aceto-orcein staining data revealed a higher incidence of monospermic penetration and a lower number of spermatozoa per oocyte in the microchannels as compared to the controls (Table 1). Data from six replicates were arranged in a randomized block design and analyzed by the generalized linear model in SAS. These data support the idea that the microchannel environment reduces the incidence of polyspermy during IVF of porcine oocytes ($P < 0.05$) while maintaining comparable penetration and male pronuclear formation rates. Furthermore, it is possible that the number of sperm present near the oocytes during fertilization is decreased using the microchannel. In conclusion, microfluidic technology has shown the potential to improve in vitro fertilization in swine by an increasing monospermic penetration of oocytes.

Table 1. Effect of IVF system on fertilization parameters

IVF System	Oocyte number	Maturation (% of total)	Penetration (% of matured)	Monospermic (% of penetrated)	MPF (% of penetrated)	No. Sperm/ oocyte
Control	138	88.59 ± 1.1	95.71 ± 0.8	20.56 ± 1.6^{a}	77.84 ± 1.7	6.65 ± 0.4^{a}
MC	128	80.22 ± 1.4	86.60 ± 1.6	57.99 ± 1.7^{b}	70.76 ± 2.1	1.77 ± 0.1^{b}

[a,b]different superscripts within columns represent significant differences ($P < 0.05$). Values are mean ± SEM. MC: microchannel; MPF: male pronuclear formation.

263 BINDING OF STALLION SPERM TO EQUINE AND BOVINE ZONAE PELLUCIDAE: EFFECT OF MILK, MILK PROTEINS AND GLUCOSE

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The ability of sperm to bind to zonae pellucidae (ZP) has been correlated with fertilizing capacity of sperm in several species. Limited numbers of equine oocytes are available to perform such assays. Therefore, use of heterologous ZP to perform gamete binding tests with stallion sperm would be useful. We have found that addition of 10% of skim milk-based extender with glucose [EZ-Mixin®, Animal Reproduction Systems, Chino, USA; (EZ)] to TALP significantly increased the number of stallion sperm bound to bovine ZP. Objectives of the present experiments were to determine: (1) if stallion sperm bind in similar numbers to equine and bovine ZP, and (2) the effects of skim milk, milk proteins and glucose on sperm binding to ZP. Denuded bovine (immature) and equine (mature) oocytes were stored at 5°C in salt solution (1.5 M $MgCl_2$, 40 mM HEPES, 0.1% PVP). In Experiment I, 4 ejaculates from 2 stallions were centrifuged at 300*g* for 6 min, and sperm pellets were resuspended in 1 mL of TALP or EZ. Sperm were stained with Hoechst 33342, centrifuged, and resuspended to 2×10^6 sperm mL^{-1}. Oocytes were placed into droplets of 45 μL of TALP (7 to 10 oocytes/trt/ejac). Extended sperm (5 μL) were added to oocytes, resulting in 2×10^5 sperm mL^{-1}, and the mixutre was incubated for 2 h at 38.5°C. Oocytes then were pipetted in TALP to remove loosely attached sperm and observed with fluorescence microscopy; mean numbers of sperm bound to bovine and equine ZP for TALP were 29 ± 1.9 and 36 ± 2.6 ($P > 0.1$) and for EZ, 149 ± 5 and 152 ± 6.3 ($P > 0.1$), respectively. More sperm bound to ZP with EZ than to ZP with TALP ($P < 0.001$). Experiment II used 4 ejaculates from 4 stallions. After initial centrifugation, sperm were resuspended in 1 mL of each of six extenders: TALP, EZ, TALP containing 89.5 mM glucose (TG), TALP containing 163.5 mM glucose (THG), TALP containing 2.4 mg mL^{-1} of skim-milk powder (TSM), and INRA 96® (IMV Technologies, L'Aigle, France) that contains 27 mg mL^{-1} of native phosphocaseinate. Hoechst 33342-stained sperm and bovine oocytes were processed as described for Experiment I. Treatments containing milk proteins resulted in more sperm binding ($P < 0.01$) than those without milk proteins (Table 1). In conclusion, use of bovine oocytes led to similar results for equine and bovine oocytes; therefore, bovine oocytes can be used for binding assays with stallion sperm. High concentrations of glucose increased numbers of sperm bound to ZP; however, presence of milk or milk proteins was more effective in enhancing binding of sperm to ZP. INRA96 contains relatively low glucose (67 mM) and one milk protein. Therefore, we hypothesize that native phosphocaseinate may cause increased sperm binding to ZP.

Table 1. Mean sperm bound per ZP ± SEM ($n = 38$–40/group)

Treatment	TALP	TG	THG	TSM	EZ	INRA 96
Sperm/ZP	53 ± 3.9^a	55 ± 3.6^a	81 ± 4.3^b	169 ± 5.3^c	160 ± 5.5^c	151 ± 5.2^c

[abc]Means with different superscripts differ significantly, ($P < 0.01$); ANOVA of log transformed data.

264 IN VITRO FERTILIZATION OF *MACACA NEMESTRINA* OOCYTES WITH FRESH AND FROZEN-THAWED EPIDIDYMAL SPERM

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In an effort to develop methods to maximize the use of reproductive tissues in our own tissue distribution program (WaNPRC TDP) and to serve as platforms for application of ART to endangered non-human primate (NHP) species, we have examined the effects of collection and cryostorage on epididymal sperm (ES) collected from *Macaca nemestrina* (Mn). Fresh ES was collected by needle aspiration from the cauda epididymis and prepared by centrifugation in mHTF (Irvine Scientific, Santa Ana, CA, USA) + BSA (3 mg mL^{-1}) at 700*g* for 10 min. The resultant pellet was resuspended in fresh mHTF + BSA and held at RT until required. ES subjected to freeze-thaw was collected from the cauda epididymis of testes provided by the WaNPRC TDP by slicing and swim-out into mHTF + BSA. ES was frozen in mTTE medium + 5% glycerol (Sankai T *et al.*, 1994 J. Reprod. Fertil. 101, 273). Frozen ES straws were removed from liquid nitrogen and thawed in air at RT for 10 min. Thawed ES was centrifuged through an 80% PureSperm gradient (Spectrum Technologies, Healdsburg, CA, USA) at 700 g for 15 min. The resultant pellet was retrieved and washed in mHTF + BSA at 600 g for 5 min. For capacitation sperm were incubated in pre-equilibrated HTF + BSA containing 1.0 mM caffeine and 0.1 mM dbcAMP (Sigma, St. Louis, MO, USA) for 5–10 min in a humidified atmosphere of 6% CO_2 in air. In vitro-matured Mn oocytes collected from unstimulated ovaries were exposed to fresh or frozen-thawed ES whereas in vivo-matured Mn oocytes collected from superovulated ovaries were exposed to fresh ES in 50-μL Fertilized oocytes were cultured for 24–48 h and assessed for cleavage on Day 3 post-insemination. Data were analyzed by single-factor ANOVA or *t*-test where appropriate and are expressed as mean ± SD. Fresh Mn ES ($n = 4$) exhibited very low motility at collection ($38 \pm 10\%$) but recovered motility following processing ($58 \pm 20\%$). Frozen-thawed ES ($n = 4$) also exhibited low motility post-thaw ($53 \pm 17\%$) and recovered after processing ($66 \pm 28\%$). The percentage of acrosome-intact sperm was not significantly different ($P > 0.05$) for fresh ES ($58.9 \pm 8.6\%$) compared to frozen-thawed ES ($70.6 \pm 21.9\%$). Fresh and frozen-thawed ES fertilized in vitro-matured Mn oocytes at similar rates (fresh $68 \pm 10.3\%$, $n = 170$ v. frozen-thawed $71.2 \pm 6.7\%$, $n = 90$; $P > 0.05$). Cleavage rates of fertilized IVM oocytes were not significantly different (fresh $79 \pm 7.2\%$ v. frozen-thawed $79.8 \pm 5.0\%$; $P > 0.05$). Fresh ES was also able to fertilize in vivo-matured Mn oocytes collected from superovulated ovaries ($95.1 \pm 2.5\%$, $n = 98$) and fertilized oocytes went on to cleave at a high rate ($96.1 \pm 2.6\%$). These results suggest that fresh and frozen-thawed ES may be useful for applied ART in endangered species. This work was supported by NIH grant #RR00166 and the WaNPRC TDP.

265 EFFECT OF SPECIFIC GRAVITY OF CULTURE MEDIUM ON IN VITRO EMBRYO DEVELOPMENT IN BUFFALO

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In buffalo the success rate of transferable quality embryo production through in vitro procedure is very low as compared to cattle. Sub optimal culture conditions and physical conditions such as specific gravity of the culture medium may lead to a reduced rate of transferable buffalo embryo production from the oocytes matured and fertilized in vitro (Palta & Chauhan,1998 Reprod. Fertil. Dev. 10, 379–391). This experiment was therefore conducted to find out the role of specific gravity of the IVC medium on the development rate of the buffalo embryos in vitro. Follicles of slaughter house ovaries were aspirated and the collected oocytes with cumulus-oocytes complexes (COCs) were cultured in TCM-199 medium supplemented with 10% fetal calf serum, 10% buffalo follicular fluid and 0.5 $\mu g\,mL^{-1}$ FSH in 5% CO_2 incubator at 38.5°C. The matured oocytes were then inseminated with frozen-thawed buffalo semen suspended in BO medium. After 42 h of post-inseminations the cleavage rates were evaluated. The 2–4 cell-cleaved eggs (Day 2 of post-insemination) were randomly divided and cultured for eight days in vitro in 1) modified synthetic fluid (mSOF) + 0.8 % BSA (control), 2) mSOF + 0.8 % BSA + gelatin (1 $mg\,mL^{-1}$) 3) mSOF + 0.8% BSA + 1 $mg\,mL^{-1}$ gelatin + 10 $ng\,mL^{-1}$ epidermal growth factor (EGF). Supplementation of gelatin increased the specific gravity of the mSOF medium from 0.9658 ± 0.009 to 1.0331 ± 0.013 without any change in pH (7.4). The development of embryos to the 8–16 cell-stage on day 4 of in vitro culture were significantly higher ($P < 0.05$) in mSOF + 0.8% BSA + 1 $mg\,mL^{-1}$ gelatin (81.8%; 27/33) than that in mSOF + 0.8% BSA (75.7%; 28/37) and mSOF + 0.8% BSA + 10 ng/mL EGF (68.7%; 22/32). When these embryos were further cultured for another four days (Day 8), the development of transferable quality embryos (morula/blastocyst) was 42.4% (14/33), 48.7% (18/37) and 46.9% (15/32), respectively. Supplementation of gelatin increased the cleavage of eggs up to the 8–16 cell-stage embryo, but did not significantly enhance the rate of development to the morula/blastocyst stage in comparison to control and EGF-supplemented group. However, the percentage of transferable quality embryos was slightly lower in the gelatin-added group but not statistically significant than other groups. The study concluded that increase in specific gravity of the in vitro culture medium enhanced initial cleavage rate but did not have any role in transferable embryo production in buffalo.

266 IN VITRO-DERIVED EMBRYO PRODUCTION WITH SEXED AND UNSEXED SEMEN FROM DIFFERENT BULLS

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The production of pre-sex-selected calves by in vitro fertilization (IVF), using sexed semen, does show some benefits due to the small quantity of sperms needed for the process as compared to other reproductive technologies. The objective of this study was to determine differences among bulls and sperm concentrations in embryo development with sexed and unsexed semen. Follicles ranging from 2 to 6 mm in diameter were aspirated from slaughterhouse ovaries. COC were selected and matured in groups of maximum of 30 in 1.8 mL of TCM-199, supplemented with 10% fetal calf serum, 0.01 $U\,mL^{-1}$ bFSH, 0.01 $U\,mL^{-1}$ bLH and 10 $\mu L\,mL^{-1}$ penicillin-streptomycin for 24 h at 38.5°C. Fertilization (Day 0) was carried out in micro-drops (50 μL) with TALP-FERT medium containing PHE (3 $\mu g\,mL^{-1}$ penicillamine, 11 $\mu g\,mL^{-1}$ hypotaurine and 0.18 $\mu g\,mL^{-1}$ epinephrine), 10 $\mu L\,mL^{-1}$ non-essential amino acid and 2 $\mu g\,mL^{-1}$ heparin. Frozen/thawed sexed (female) and non-sexed sperms from five bulls were selected in a discontinuous percoll gradient. Sperm concentration was 1×10^6 for non-sexed semen and 1×10^6 or 2×10^6 for sexed semen. After 18–20 h, presumptive zygotes were denuded and cultured in groups of 10 in 50-μL micro-drops of SOF citrate with 5% FCS (Holm P *et al.*, 1999 Theriogenology 52, 683–700) under paraffin oil in a 5% O_2, 5% CO_2, 90% N_2 atmosphere with high humidity. On Day 7, blastocysts (BL) were morphologically evaluated and recorded. Results are shown in Table 1. Data was compared by chi-square analysis. Sexed frozen bovine sperm can be used successfully in IVF systems. More research needs to be done to optimize and standardize bovine in vitro fertilization with sexed semen.

Table 1. Results of comparisons between bulls, sperm concentrations, cleavage and embryo development

Bulls	Sperm Concentration	Semen	Cleavage (%)	Development to BL stage on Day 7 (%)
Holstein #1	1×10^6	Sexed	65/225 (29%)[a]	8/225 (3.5%)[a]
Holstein #1	2×10^6	Sexed	62/196 (31.6%)[a]	11/196 (5.6%)[a]
Holstein #1	1×10^6	Unsexed	366/474 (77%)[b]	127/474 (26.8%)[b]
Holstein #2	1×10^6	Sexed	78/321 (24%)[*]	16/321 (5%)[*]
Holstein #2	2×10^6	Sexed	116/215 (54%)[**]	30/215 (14%)[**]
Holstein #2	1×10^6	Unsexed	336/422 (79.6%)[***]	125/422 (29.6)[***]
Holstein #3	2×10^6	Sexed	–	26/118 (22%)
Holstein #3	1×10^6	Unsexed	–	17/108 (15.7%)
Jersey #1	2×10^6	Sexed	–	25/115 (21.7%)
Jersey #1	1×10^6	Unsexed	–	22/65 (33.8%)
Jersey #2	2×10^6	Sexed	–	27/148 (18%)
Jersey #2	1×10^6	Unsexed	–	17/142 (12%)

Value with different superscripts in the same column differ ($P < 0.05$).

267 BODY WEIGHT GAIN VARIATION, HORMONAL AND METABOLIC STATUS AND IN VITRO EMBRYO PRODUCTION IN SUPEROVULATED DAIRY HEIFERS

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This study aimed to investigate the hormonal and metabolic status of heifers subjected to short-term variation of energy intake and associated growth rate; oocytes were collected by ovum pick-up (OPU) for in vitro embryo production. Experimental scheme, diets and OPU protocol have been described previously (Freret *et al.*, 2003 Theriogenology, 59, 445 abst). Briefly, oocytes from 16 Prim′Holstein heifers (14 ± 1 months old, 340 ± 25 kg) were collected by OPU every two weeks after superovulation treatment (total dose of 250 μg FSH (Stimufol®, Merial, France) divided into 5 i.m. injections 12 hours apart, at decreasing doses). They received individually for 6 weeks (Period 1 = OPU 1 to 4) a diet aimed at a 1000 g day^{-1} body weight gain (BWG). Heifers were then allocated to 2 groups (overfeeding or dietary restriction), for 8 weeks after OPU 4 (Period 2 = OPU 5 to 8). COCs were collected 12 h after the last FSH injection for IVF and IVC. Blood sample analyses were performed once a week to determine glucose, insulin, IGF1, non esterified fatty acids (NEFA), β-hydroxybutyrate (βOH) and urea concentrations, and at the time of follicular puncture for estradiol assay. Effects of period, group of growth rate and their interaction where analyzed using the mixed procedure of SAS (female effect as random) and least-squares means were subsequently compared with Scheffe's test. Three groups of growth rate were determined according to results observed during period 2 (Table 1). A period effect was observed for glucose, insuline and estradiol ($P < 0.05$). But Scheffe's test showed a significant variation between periods only in the '600 g day^{-1}' group, with more estradiol and less glucose in period 2 which was associated with more blastocysts and grade 1 embryos (Freret *et al.*, 2003 Theriogenology, 59, 445 abst), and in the '1400 g day^{-1}' group with more insulin in period 2 (associated with more follicles <8 mm 2 days before FSH treatment). In period 2, βOH concentration was significantly higher in the '1400 g day^{-1}' group when compared to the others (Table 1). For urea, NEFA and IGF1 concentrations, no difference between groups or periods was observed. These results illustrate the role of glucose and insulin as mediators of nutritional effects on reproduction in growing animals. These results suggest that nutritional requirements aimed at optimizing follicular growth and embryonic development may be different.

Table 1.

	Group 600 g day^{-1} ($n = 4$)		Group 1000 g day^{-1} ($n = 8$)		Group 1400 g day^{-1} ($n = 4$)	
	Period 1	Period 2	Period 1	Period 2	Period 1	Period 2
BWG (g day^{-1})	940 ± 145	615 ± 100^{a}	980 ± 120	1035 ± 70	890 ± 200	1380 ± 100^{c}
Follicles <8 mm 2 days before FSH	27.2 ± 4.4	23.4 ± 4.4	22.2 ± 3.1	25.3 ± 3.1	19.1 ± 4.4^{a}	28.8 ± 4.4^{b}
Estradiol at OPU (pg mL^{-1})	4.6 ± 2.14^{a}	12.01 ± 2.14^{c}	8.27 ± 1.51	10.1 ± 1.51	10 ± 2.14	11.68 ± 2.14
Glucose (mmol L^{-1})	5.01 ± 0.13^{a}	4.67 ± 0.13^{b}	4.91 ± 0.09	4.76 ± 0.09	5.08 ± 0.13	5.02 ± 0.13
Insulin (pmol L^{-1})	128.32 ± 19.6	156.12 ± 20.39	136.17 ± 13.86	147.56 ± 14.12	138.4 ± 19.6^{a}	207.4 ± 19.6^{c}
βOH (mmol L^{-1})	0.35 ± 0.14	0.35 ± 0.14^{c}	0.73 ± 0.1	0.69 ± 0.1^{b}	1.12 ± 0.14	1.27 ± 0.14^{a}

Least-squares means ± SEM, [a v. b]: $P < 0.05$; [a v. c]: $P < 0.01$.

268 PREGNANCIES FROM FROZEN IVF CATTLE EMBRYOS USING SEX-SORTED AND UNSORTED SPERM

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Previously we demonstrated that sex-sorted sperm could produce IVF embryos from juvenile and adult cattle at rates similar to those for unsorted sperm (Fry *et al.*, 2003 Theriogenology 52, 198). In this study we investigated the pregnancy rates of recipient cattle following the transfer of frozen/thawed IVF embryos generated from young heifers using sex-sorted and unsorted sperm. COCs collected from FSH-stimulated Senepol or Beefex heifers by TVR were matured, fertilized with either sex-sorted or unsorted Senepol sperm and cultured for 6 days under our standard laboratory conditions (Fry *et al.*, 2003 Theriogenology 59, 446, Earl *et al.*, 1997 Theriogenology 47, 255). Embryos reaching the blastocyst or expanded blastocyst stage of development were frozen by the CL-V method of vitrification. Briefly, embryos were equilibrated for 5–10 min in HEPES-199 media containing 20% FCS (HM), placed in HM containing 10% EG, 10% DMSO for approximately 2 minutes and then in HM containing 20% EG, 20% DMSO for between 20–60 sec (Vatja *et al.*, 1997 Cryoletters 18, 191). Vitrification was achieved by collecting between 5–10 IVF embryos in a 3-μL droplet and securing this droplet to a coded CL-V holder. The droplet was vitrified using the CL-V kit (Lindemans *et al.*, 2004 Theriogenology in press) and then sealed in a precooled "straw" for storage in liquid nitrogen. To thaw, the "straw" with specimen was removed from storage; the specimen droplet was withdrawn from the "straw" and placed directly into HM containg 0.2 M sucrose (SM). After approximately 5–10 min each embryo was assessed, loaded into a tomcat catheter in SM and transferred surgically into a recipeint cow within 10–15 min of thaw. Of 129 Brahman and Brahman cross cows receiving 2 injections of 125 μg cloprostenol 11 days apart, 60 exhibited oestrus 2–4 days after the second

injection and 53 were deemed suitable for embryo transfer. Pregnancy was determined by ultrasound on Day 40. No difference in pregnancy rate was found between treatment groups ($P > 0.05$; Table 1). The low submission rate (60/129) and pregnancy rate for the in vivo control group indicate that the fertility of the recipient cows may have been compromised by the drought conditions predominating in Central Queensland. Notwithstanding, the CL-V method for the vitrification of IVF embryos produced by either sex-sorted or unsorted sperm gave similar and very promising pregnancy results of around 40%. This provides new opportunities for the rapid banking of large numbers of sexed IVF embryos generated from elite cattle by TVR for user friendly embryo transfer programs.

Table 1. Pregnancies from IVF embryos derived from sex-sorted and unsorted sperm and frozen by the CL-V method of vitrification, or from in vivo embryos frozen in glycerol

Embryos	Sex-sorted IVF	Unsorted IVF	In vivo
Grade 1	3/6	8/17	3/8
Grade 2	2/7	1/6	0/0
Grade 3	3/7	0/1	0/1
TOTAL	8/20 (40%)	9/24 (38%)	3/9 (33%)

269 IN VITRO FERTILITY OF BOAR SPERMATOZOA PRESERVED AT 10°C FOR 22 DAYS

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Fertility of boar spermatozoa as determined following artificial insemination seems to be maintained during liquid preservation at 10–15°C for several days, although prolonged liquid preservations reduce the pregnancy rate rapidly. However, it is not clear if spermatozoa can penetrate into oocytes in an IVF system even after a prolonged liquid preservation. Oxidative stress could also be one of the possible detrimental factors in liquid preservation of spermatozoa. In the present study, fertility of liquid-preserved spermatozoa was examined using an IVM-IVF system. Whether cysteine can improve the fertility was also determined. Spermatozoa (from four Berkshires) was resuspended at 1×10^8 cells mL^{-1} in Modena solution containing 15% (v/v) boar seminal plasma and 0 or 5 mM cysteine after washing 3 times. Sperm suspensions (1 mL) were then preserved at 10°C for 22 days following a program for cooling down (to 15°C for 4 h, keeping at 15°C for 12 h and then to 10°C for 6 h). At Days 1, 8, 15 and 22 after the start of preservation, spermatozoa (5×10^5 cells mL^{-1}) were co-cultured with IVM oocytes in an IVM/IVF system (Funahashi *et al.*, 1997 Biol Reprod 57, 49–53). Viability and functional status of spermatozoa were also examined at Days 8 and 15 of preservation by using LIVE/DEAD sperm viability kit and CTC fluorescence assay. Data (mean ± SEM) from 4–6 replicates were analyzed by ANOVA and Fisher's protected LSD test. When spermatozoa that had been preserved without cysteine (Cys−) were used, penetration rates were not different ($P > 0.05$) from those with cysteine (Cys+) at Day 8 of preservation (91.4 ± 3.4% in Cys− and 99.3 ± 0.7% in Cys+), but lower ($P < 0.02$) at Days 15 and 22 (72.6 ± 13.6% and 33.8 ± 8.4% in Cys−; 94.8 ± 2.1% and 71.1 ± 10.8% in Cys+, respectively). Both viability and proportion of uncapacitated live cells were higher ($P < 0.05$) in Cys+ than Cys− at Days 8 and 15. These results demonstrate that boar spermatozoa can penetrate into oocytes in vitro even after a liquid preservation at 10°C for 22 days and that cysteine can improve the viability and penetrability in vitro of spermatozoa during liquid preservation. Supported by the Ito Foundation.

270 IN VITRO FERTILIZATION OF BUFFALO (*BUBALUS BUBALIS*) OOCYTES: EFFECTS OF MEDIA AND SPERM MOTILITY INDUCING AGENTS

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The identification of an optimal in vitro fertilization system is critical in order to improve the in vitro embryo production efficiency in buffalo species. The aim of this work was to evaluate the effects of fertilization media and sperm motility inducing factors (SMIF) on cleavage and blastocyst rates in buffalo species. Cumulus-oocytes complexes ($n = 516$), recovered from slaughtered animals, were matured in vitro in TCM 199 + 10 % FCS, 0.5 μg mL^{-1} FSH, 5 μg mL^{-1} LH, 1 μg mL^{-1} 17β-estradiol and 50 μM cysteamine, at 38.5°C under 5% CO_2 in humidified air for 24 hours. The mature oocytes were randomly assigned to four groups for fertilization. In particular, IVF was carried out at 38.5°C under 5% CO_2 in humidified air in either Tyrode's modified medium or Brackett Oliphant medium, in the presence of 0.01 mM heparin; each medium was supplemented with either a mixture of 0.2 mM penicillamine and 0.1 mM hypotaurine or 5 mM caffeine. Frozen-thawed sperm from a tested bull was treated by the swim-up procedure and used at a final concentration of 20^6 mL^{-1}. After 20–22 h presumptive zygotes were cultured in SOF medium, supplemented with essential and non-essential amino acids and BSA, in a gas atmosphere of 5% CO_2, 7% O_2 , and 88% N_2, up to the blastocyst stage. Cleavage rates and blastocyst yields were analyzed by a full factorial model 2 × 2 with medium and SMIF effects (SPSS 11.0). The analysis used permits the identification of statistical differences between treatments irrespective of an interaction (Searle SR. 1971. Linear model. Ed. John Wiley & Sons; XXI:533). The comparison of the two media, irrespective of the SMIF used, did not show any difference in cleavage rate (43.7% v. 39.3%, respectively, in TALP and BO). On the contrary higher cleavage rates were recorded with hypotaurine-penicillamine v. caffeine (47.7% v. 35.3%; $P < 0.05$), regardless of the medium employed. However, a significant interaction between media and SMIF was found; in fact the addition of hypotaurine and

penicillamine significantly improved cleavage rate compared with caffeine in TALP medium (59.6% v. 27.9%; $P < 0.05$) whereas no differences were observed in BO (35.9% v. 42.7%, respectively). With regard to blastocyst yield a significant effect of medium was also found, with the highest embryo production in TALP v. BO (13.9% v. 6.8%; $P < 0.05$). Blastocyst rate was improved in the presence of hypotaurine-penicillamine v. caffeine (13.6% v. 7.2%; $P < 0.05$). Furthermore there was a significant interaction between medium and SMIF, with the highest embryo yields in the presence of hypotaurine-penicillamine v. caffeine in TALP (20.7% v. 7.1%, respectively; $P < 0.05$) but not in BO (6.4% v. 7.2%, respectively). The differences we found disappeared when the embryo yield was calculated in relation to the cleaved eggs, with the exception of a lower efficiency of BO v. TALP (15.9% v. 31.1%; $P = 0.057$), suggesting an influence of BO also on post-fertilization development.

271 INFLUENCE OF ARGININE–GLYCINE–ASPARTIC ACID (RGD) IN BOVINE SPERM-EGG BINDING, AND FERTILIZATION IN VITRO

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Osteopontin (OPN), a secretory RGD-containing phosphoprotein, has been identified in cow oviductal ephitelium and fluid, but its role in fertilization is unclear. RGD peptide is capable of blocking fertilization, inducing intracellular Ca^{2+} transients, and initiating parthenogenetic development when present during bovine fertilization in vitro.This study was conducted to determine whether in vitro sperm binding to the zona pellucida (ZP) and fertilization of bovine oocytes were affected by treating the sperm or oocytes with RGD (arginine–glycine–aspartic acid, a sequence recognized by integrins) or non-RGD-containing peptides. In vitro matured oocytes were incubated (39°C, 5% CO_2 in air) for 2 hours in fertilization medium with: (1) no peptides; (2) 50 $\mu g\,mL^{-1}$ RGD (Calbiochem®, San Diego, CA, USA); (3) 1000 $\mu g\,mL^{-1}$; (4) 50 $\mu g\,mL^{-1}$ non-RGD (Calbiochem®); (5) 1000 $\mu g\,mL^{-1}$ non-RGD. The bovine sperm from two differents bulle was collected by artificial vagina, pooled, washed twice with MTM at 500 g for 10 min and incubated (39°C, 5% CO_2 in air) for two h at 5×10^7 concentration in MTM with: (6) no peptides; (7) 50 $\mu g\,mL^{-1}$ RGD; (8) 1000 $\mu g\,mL^{-1}$; (9) 50 $\mu g\,mL^{-1}$ non-RGD; (10) 1000 $\mu g\,mL^{-1}$ non-RGD. Following incubation, treated and untreated oocytes were washed and inseminated with 1×10^5 treated or untreated fresh spermatozoa per 10 oocytes; after the sperm were recovered from a Percoll gradient (45%/90%). After 18–20 h, oocytes were removed from co-culture, and washed in TL-HEPES. Oocytes used to assess sperm binding were stained with Hoescht 33342, and the number of sperm bound per ZP counted. The remaining oocytes were fixed in acid alcohol, stained with 1% acetato-orcein and observed for the presence of pronuclei. For the five replicates, 100–120 oocytes were used for fertilization and 150–170 oocytes were used for sperm-egg binding assays. Data were analized by SAS. Treatment of sperm or oocytes with the RGD peptide significantly decreased ($P < 0.05$) fertilization compared to the non-treated controls or those treated with non-RGD peptides: (1) 80% ± 3.0; (2) 42% ± 3.0; (3) 30.2% ± 3.0; (4) 78.5% ± 3.0; (5) 79.1% ± 3.0; (6) 78.9% ± 3.0; (7) 41.3% ± 3.0; (8) 29.1% ± 3.0; (9) 79.2% ± 3.0; (10) 80.2% ± 3.0. More sperm bound to the ZP of untreated or non-RGD-treated oocytes or sperm than those incubated with the RGD peptide: (1) 71.2 ± 4.1; (2) 33.2 ± 4.2; (3) 24.2 ± 4.1; (4) 69.5 ± 4.1; (5) 70.2 ± 4.2; (6) 71.9 ± 4.2; (7) 29.8 ± 4.2; (8) 19.8 ± 4.2; (9) 68.9 ± 4.2; (10) 70.6 ± 4.2. These studies demonstrated that incubation of bovine oocytes or spermatozoa with a RGD peptide inhibits sperm-egg binding and fertilization in vitro. These findings support the notion that the role of osteopontin in bovine fertilization may involve inteaction with integrins via its RGD sequence.

272 OVUM RECOVERY AND BLASTOCYST DEVELOPMENT FOLLOWING INTRACYTOPLASMIC SPERM INJECTION IN CHIMPANZEES

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In the present study, we report on oocyte collection, intracytoplasmic sperm injection and early embryogenesis in chimpanzees. Eight adult female chimpanzees, 11–27 years of age, received a single s.c. injection of 3.75 mg GnRH (Leuplin, Takeda Co. Ltd., Osaka, Japan) 1 to 3 days after the beginning of menstruation. Daily i.m. injections of hMG (Humegon, Nippon Organon K.K., Tokyo, Japan) were initiated the following day. The dose of hMG was altered from 75 to 300 IU according to serum estradiol levels. When at least one follicle of 17 mm or more in diameter was observed, 10 000 IU of hCG (Pregnyl, Nippon Organon K.K.) were administered by i.m injection. Oocytes were recovered by ultrasound-guided transvaginal follicular aspiration 30.5 to 35.5 h after hCG injection. Mature oocytes were denuded of cumulus cells by treatment with 0.1% hyaluronidase, and injected with a frozen-thawed or fresh spermatozoan using a Piezo-driven micromanipulator. Zygotes were cultured in Quinn's Advantage Fertilization Medium (Cooper Surgical, Inc., Trumbull, CT, USA) with 10 serum protein substitute (SPS) at 37°C in a 5% CO_2 atmosphere until the pronucleus stage. The medium was replaced by Quinn's Advantage Cleavage Medium with 10 SPS from the pronuclear to 8-cell stage, and Quinn's Advantage Blastcyst Medium with 10 SPS, thereafter. Mild ovarian hyperstimulation syndrome (OHSS) occurred in one female chimpanzee with estradiol levels of 7520 $pg\,mL^{-1}$. No oocytes were collected from 2 chimpanzees in which large follicles were observed. Thirty-five mature oocytes, one immature oocyte and 6 degenerate/fragmented oocytes were retrieved from 6 chimpanzees, including the one with OHSS. Among 35 mature oocytes injected with spermatozoa, 26 oocytes (74%) produced two pronuclei; 23 zygotes (66%) cleaved to the 2-cell stage, 22 (63%) to the 4-cell stage, 14 (40%) to the 8-cell stage, and 9 (26%) to the morula stage. Seven zygotes (20%) developed to the blastocyst stage by 120 h. There were no differences in fertilization rate or early embryogenesis between frozen and fresh spermatozoa. Results indicate that techniques used for human-assisted reproduction may be applicable to the chimpanzee to help preserve this endangered species.

273 EFFECT OF EPIDERMAL GROWTH FACTOR (EGF) DURING OOCYTE MATURATION ON IN VITRO PRODUCTION OF BOVINE EMBRYOS

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Medium components during in vitro maturation (IVM) can significantly influence oocyte maturation and subsequent embryo development in vitro (Rose TA and Bavister BD 1992 Mol. Reprod. Dev. 31, 72–77; Harper K and Brackett B 1993 Biol. Reprod. 48, 409–416). The aim of this experiment was to evaluate the effect of EGF during IVM on further development of bovine embryos in vitro. Bovine ovaries were obtained at a slaughterhouse. Cumulus-oocyte complexes (COC) were aspirated from follicles 2–5 mm in diameter. COC were incubated for 24 h in either of 3 maturation media: T1 ($n = 72$): modified TCM-199; T2 ($n = 45$): modified TCM-199 supplemented with 10 ng mL^{-1} of EGF; or T3 ($n = 46$): modified TCM-199 supplemented with 10% fetal bovine serum (FBS). After 24 h of IVM, COC were inseminated with 2×10^6 motile spermatozoa/ml. After 18 h of gamete coincubation, presumptive zygotes were denuded and placed in culture in SOF rich in glutamine (g-SOf) for 72 h, at which time, cleavage rate (%) wass assesed (embryos with >4 cells). Subsequently, cleaved embryos were incubated for an additional 72 h in c-SOF (SOF rich in citrate and glucose). Finally, embryos were cultured in modified TCM-199 for 24–48 h, at which time blastocyst formation rate (%) was evaluated. Cleavage rates were similar between T2 and T3 but significantly greater than in T1 ($P > 0.05$; see Table 1). Addition of EGF during IVM (T2; 11/45, 24.4%) did not yield more blastocysts compared to the other two treatments (6/57, 10.5% and 10/29, 34.5%, T1 and T3, respectively). Nonetheless, T3 (with serum) had a greater yield of blastocysts compared to T1 ($P > 0.01$). Results in this study show that the addition of EGF to chemically defined media results in similar cleavage rates and blastocyst yields to those obtained when using serum during IVM. Key words: in vitro maturation, EGF, cleavage, bovine, embryo.

Table 1. Effect of EGF and serum during IVM on cleavage rate of bovine oocytes

Treatments	COC *n*	Cleavage rate *n* (%)
T1	72	18 (25.0[a])
T2	45	19 (42.2[b])
T3	46	21 (45.7[b])

[a,b]Values with different superscripts within the same column differ significantly ($P < 0.05$).

274 SIMILARITY OF EMBRYOS PRODUCED BY OVUM PICK-UP AND IN VITRO FERTILIZATION IN IDENTICAL TWIN CATTLE

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The present study was designed to assess the similarity of follicular development, oocyte quality, and their subsequent development on ovum pick-up (OPU)-IVF in identical twin cattle. Four pairs of identical twin Japanese black cows (A, B pairs at 5 years old and C, D pairs at 3 years old) were kept under the same feeding and environmental conditions. OPU was performed for these cows once a week for seven continuous weeks. OPU was done by using a 7.5-MHz linear transducer with needle (17 G, 530-mm length) connected to an ultrasound scanner (SSD-1200, ALOKA, Tokyo, Japan). Oocytes were evaluated by their cumulus cell morphology, cytoplasmic color, and density. To assess the development, collected COCs were cultured for 20 h in TCM-199 supplemented with 5% calf serum (CS) in a microdroplet (volume was adjusted to 5 μL/oocyte) at 38.5°C under atmosphere of 5% CO_2 in air. After maturation, the COCs were inseminated with frozen-thawed semen collected from the same ejaculation of a single bull. The fertilization was performed with BO solution as described by Imai *et al* (J. Vet. Med. Sci., 2002, 64(10), 887–891). The zygotes were then cultured in CR1aa supplemented with 5% CS under the same condition of maturation for nine days. Embryo development was assessed by the cleavage rate on Day 2 and the blastocyst production rate on Days 7 to 9 (insemination day = Day 0). Blastocysts were classified according to the IETS creteria. Data were analyzed by ANOVA. A total 56 sessions of OPU were performed in this study. The overall mean number of developing follicles (larger than 2 mm in diameter), collected oocytes, and produced blastocysts were 30.3 ± 9.2, 20.1 ± 9.2 and 6.3 ± 3.8 (mean ± SD) per session, respectively. The mean number of developing follicles on the day of OPU were significantly different between B and D pairs (38.6 ± 7.5 and 21.9 ± 6.5, $P < 0.01$); however, no significant difference was found within each twin. In oocyte quality, C and D pairs were significantly higher grade than the A pair. The percentages of cleaved oocytes and embryos developed to the blastocyst stage (34 ± 16, 27 ± 10, 41 ± 17 and 39 ± 24) showed no differences among 4 pairs and within each twin. However, the percentage of Grade 1 blastocyst of B pair was significantly lower ($P < 0.01$) than that of other pairs, and C pair was significantly higher ($P < 0.05$) than that of A and D pairs (67 ± 25, 41 ± 22, 93 ± 10 and 71 ± 25; A, B, C and D pairs, respectively). There was no significant difference within twins. These results show little statistical variation between cows of the same genetic background in the production of embryos in vitro.

275 FOLLICULAR ATRESIA IN SMALL, NON-FSH-DEPENDENT BOVINE FOLLICLES IS ASSOCIATED WITH INCREASED DEVELOPMENTAL POTENTIAL OF OOCYTES FOLLOWING IVP

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Oocytes from small, non-FSH-dependent follicles are associated with reduced developmental competence following in vitro embryo production (IVP) compared to oocytes from larger follicles. It has been suggested that, for small follicles, oocytes derived from atretic follicles are more developmentally competent than those from healthy follicles (Blondin P and Sirard MA, 1995 Mol. Reprod. Dev. 41, 54–62). Little is known of the characteristics of small follicles that support developmentally competent oocytes. Here we examine the development to blastocyst stage of oocytes collected from histologically-assessed bovine 2–5 mm follicles. Ovaries were obtained at a local abattoir; 4 follicles were dissected from each ovary and oocytes were recovered. A section of each follicle wall was taken and fixed in 2.5% glutaraldehyde for histological assessment of the follicle and characterization of the morphology of the follicular basal lamina by electron microscopy (Irving-Rodgers HF and Rodgers RJ, 2000 J. Reprod. Fert. 118, 221–228). Oocytes recovered from follicles underwent IVP utilizing a novel single IVP system. Oocytes were matured for 24 h (10 μL per COC) in TCM199, supplemented with FSH, hCG, FCS, cysteamine and pyruvate. Mature oocytes were inseminated with 1×10^6 sperm mL^{-1} for an additional 24 h using Bovine Fertilization Medium (10 μL per COC; Cook, Australia). Following insemination, putative zygotes were stripped of remaining cells and placed within individual micro-wells prepared in 1% agar in Bovine Early Cleavage Medium, Cook, Australia. The agar (350 μL) was prepared within wells of a 4-well plate and small plugs of agar were removed to form micro-wells. The agar was over-laid with 450 μL of Early Cleavage Medium and 250 μL mineral oil, and equilibrated overnight before putative zygotes were placed individually within micro-wells. Culture was performed under 7% O_2, 6% CO_2, and 87% N_2 at 39°C. On Day 5 following insemination, fetal calf serum (final concentration 10% v/v) was added to facilitate blastocyst development. Blastocyst formation was assessed on Day 8. A total of 211 oocytes were cultured and 69% were from healthy follicles; 67 oocytes (32%) had developed to the blastocyst stage by Day 8. Forty-three percent of oocytes recovered from atretic follicles (28/65) had developed to the blastocyst stage by Day 8, as compared to only 27% (39/146) oocytes recovered from healthy follicles, this difference was significant ($P < 0.05$, chi-square analysis). Seventy-eight percent (14/18) of oocytes from healthy follicles with additional follicular basal lamina material (Irging-Rodgers HF and Rodgers RJ, 2000 J. Reprod. Fert. 118, 221–228) failed to develop, whereas only 44% (4/9) of oocytes from healthy follicles with a normal basal lamina failed to develop ($P > 0.08$). The present study finds a direct association between the follicle morphology and oocyte maturational potential within non-FSH dependent follicles, revealing that high levels of development (>40%) can be obtained from atretic follicles. Furthermore, differences between healthy follicles may also contribute to developmental variation.

276 INFLUENCE OF SPERM TREATMENTS ON BLASTOCYST DEVELOPMENT IN VITRO AND CELL NUMBER IN CATTLE

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Experiments were designed to examine the effects of developmental rate and cell numbers in embryos produced by in vitro fertilization (IVF) using sperm from 2 bulls (sperm A and B purchased from commercial sale) isolated by three methods. Cumulus-oocyte-complexes collected from ovaries harvested from a local slaughter house were matured in 50 μL droplets of serum-free M199 medium supplemented with 1 μg mL^{-1} estradiol-17β, 10 μg mL^{-1} LH and FSH under silicone oil at 39°C in a humidified atmosphere of 5% CO_2 in air. After 24 h of culture, oocytes were fertilized with the sperm treated by three different methods of isolation; percoll gradient, swim-up and glass wool filtration; a final concentration of 2×10^6 cells mL^{-1} was used. At 16 h after fertilization, presumptive zygotes were co-cultured in serum-free M199 with BOEC for up to 192 h post-insemination. At 48 h and 120 h post-insemination, the cultures were fed with 25 μL of serum-free M199. The embryos were compared for their rates of cleavage at 48 h post-insemination, development to the blastocyst stage, and hatching, and also the cell number at 192 h post-insemination. Differences between treatments were analyzed using one-way ANOVA after arc-sine transformation of the proportional data of cleavage, development into blastocyst stage and hatching. Comparisons of means among treatments were performed using Tukey-Kramer multiple comparisons test. The results are summarized below. The rates of cleavage in embryos produced by IVF using sperm from 2 bulls isolated by percoll, swim-up and glass wool were not significantly different. The blastocyst development and hatching rates between sperm treatment were not significant within bull sperm A and within sperm B. However, although the hatching rate in percoll treatment of bull sperm A was higher than in bull sperm B, the difference was not statistically significant. The mean cell numbers in percoll treatment of bull sperm A (176.5 ± 7.1) were significantly higher ($P < 0.001$) than the others. In bull sperm B the cell numbers in percoll treatment were higher than the other two treatments, but the differences were not statistically significant. In conclusion, these results support the concept that sperm preparation using percoll has beneficial effects on blastocyst cell number.

Table 1. Developmental rates of in vitro embryos using sperm from 2 bulls isolated by three methods with 4 replicates

Sperm source	Treatment	No. of oocytes	No. cleaved at 48 h (%)	No. of blastocysts	No. hatching (%)	Cell number at 192 h (mean ± SEM)
Sperm A	Percoll	230	202 (87.8)	67 (29.1)[a]	28 (12.2)[a]	176.5 ± 7.1^{a}
	Swim-up	228	192 (84.2)	57 (25.0)[a,b]	23 (10.1)[a]	140.4 ± 4.7^{b}
	Glass wool	233	194 (83.3)	53 (22.7)[a]	14 (6.1)[a,b]	131.6 ± 3.6^{b}
Sperm B	Percoll	228	194 (85.1)	40 (17.5)[a,b]	13 (5.7)[a,b]	$143.6 \pm 13.5^{a,b}$
	Swim-up	229	172 (75.1)	38 (16.6)[b]	9 (3.9)[b]	$147.3 \pm 7.0^{a,b}$
	Glass wool	230	165 (71.7)	53 (15.2)[b]	16 (7.0)[a,b]	133.4 ± 8.3^{b}

[a,b]Values with different superscripts within a column differ significantly ($P < 0.05$).

277 ENERGY REQUIREMENT DURING DEVELOPMENT TO THE BLASTOCYST STAGE OF PORCINE EMBRYOS PRODUCED IN VITRO

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A successful in vitro production (IVP) of porcine blastocysts, which enables piglet production after transfer to recipients, was reported (Kikuchi *et al.*, 2002 Biol. Reprod. 66, 1033–1041). Generally, in the IVP system, both glucose and glutamine as energy sources were included in vitro culture (IVC) medium from Day 2 (Day 0 = the day of in vitro fertilization) until Day 6. However, the exact requirement of these substances for the development to the blastocyst stage of IVP embryos has not yet been clarified. The objective of the present study was to evaluate whether these two substances are necessary for embryonic development to the blastocyst stage in culture during the period. Porcine cumulus-oocyte complexes were matured for 46 h and fertilized in vitro as reported by Kikuchi *et al.* (see above). After removal of cumulus cells and spermatozoa, the oocytes were cultured subsequently in NCSU-37 supplemented with pyruvate and lactate (IVC-PyrLac) for 2 days. Then they were cultured until Day 6 in other IVC medium prepared as follows (1–6); Basic IVC medium (BM) was a modified NCSU-37 consisting of 108.7 mM NaCl, 4.8 mM KCl, 1.7 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25.1 mM $NaHCO_3$ and 4 mg mL^{-1} fatty acid-free BSA. Then one or more of the following energy sources were supplemented to BM; (1) 12 mM sorbitol (SigmaUltra), 5.55 mM glucose (Wako special grade) and 1.0 mM glutamine (Sigma) (NCSU-37/Gln+), (2) 19.2 mM sorbitol and 1.0 mM glutamine (IVC-Sorbitol/Gln+); (3) 19.2 mM mannitol (SigmaUltra) and 1.0 mM glutamine (IVC-Mannitol/Gln+), (4) 12 mM sorbitol and 5.55 mM glucose (NCSU-37/Gln−); 5) 19.2 mM sorbitol (IVC-Sorbitol/Gln−); and 6) 19.2 mM mannitol (IVC-Mannitol/Gln−). The osmolarity of these media was adjusted to 283–285 osmol g^{-1}. All embryos were fixed as whole mounts, stained and evaluated. The rate of blastocysts in NCSU-37/Gln+ (26.8%) was significantly higher ($P < 0.05$; by analysis of variance and Duncan's multiple range test) than those in IVC-Sorbitol/Gln+, IVC-Mannitol/Gln+ and NCSU-37/Gln− (19.0%, 17.0% and 15.5%, respectively). A remarkable decrease in the rates in IVC-Sorbitol/Gln− and IVC-Mannitol/Gln− ($P < 0.05$; 1.4% and 2.0%, respectively) was observed. The cell numbers of NCSU-37/Gln+, IVC-Sorbitol/Gln+, IVC-Mannitol/Gln+ and NCSU-37/Gln− (55.5, 52.0, 49.6 and 58.7, respectively) had a tendency to be higher than those of IVC-Sorbitol/Gln− and IVC-Mannitol/Gln− (38.0 and 35.2, respectively). These results confirm that the supplementation of maturation medium with at least one energy source (glucose or glutamine) promotes embryonic development in vitro to the blastocyst stage, that the combination of both sources improves the chance of the embryonic survival, and that porcine embryos do not utilize sorbitol or mannitol as an energy source. The importance of glucose and glutamine is suggested for the development to the blastocyst stage of porcine IVP embryos.

278 EFFECT OF GLYCOSAMINOGLYCAN SUPPLEMENT ON PORCINE PREIMPLANTATION EMBRYO DEVELOPMENT AND EXPRESSION OF RECEPTORS FOR GLYCOSAMINOGLYCANS IN PORCINE EMBRYOS

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The present study investigated the effect of glycosaminoglycan (GAG) supplement on developmental competence of porcine in vitro fertilized (IVF) embryos and GAG receptor expression in the porcine embryos. In vitro-matured oocytes were inseminated with frozen-thawed boar semen and cultured in North Carolina State University (NCSU)-23 medium in the absence or presence of various GAGs (hayaluronic acid, heparin or both). Developmental competence was evaluated by monitoring the numbers of 2-cell embryos and blastocysts at Days 2 and 7, respectively. Differential staining was performed in blastocysts at Day 7. All data were analyzed by ANOVA using a Generalized Linear Model (SAS). Inseminated oocytes were cultured in NCSU-23 supplemented with different concentrations (0, 0.1, 0.5 or 1.0 mg mL^{-1}) of hyaluronic acid (in Experiment 1) or heparin (in Experiment 2). Supplementing NCSU-23 with 0.5 mg mL^{-1} hyaluronic acid significantly increased ($P < 0.05$) the total number of cells (55.9) and the number of trophectoderm (TE) cells (41.7) compared with the other culture groups (44.7, 45.0 and 31.3, 31.8, respectively). The rate of blastocyst formation was significantly increased ($P < 0.05$) in the 1.0 mg mL^{-1} heparin-supplemented group (21.8%) compared with that in the control culture group (16.4%). In experiment 3, inseminated oocytes were cultured in NCSU-23 supplemented with 0, 0.1 mg mL^{-1} heparin, 0.5 mg mL^{-1} hyaluronic acid, or 0.1 mg mL^{-1} heparin + 0.5 mg mL^{-1} hyaluronic acid. The rate of blastocyst formation was significantly increased ($P < 0.05$) in the 1.0 mg mL^{-1} heparin group (21.5%) and the 1.0 mg mL^{-1} heparin + 0.5 mg mL^{-1} hyaluronic acid group (22.8%) compared with that of control culture group (16.6%). In experiment 4, total RNA was prepared from oocytes, 2-, 4-, and 8-cell stages, morulae, and blastocysts and reverse-transcribed using the First-strand cDNA Synthesis kit (Amersham Biosciences, Piscataway, NJ, USA). The cDNA was subjected to polymerase chain reaction with primers specific for hyaluronic acid receptor (CD44) and heparin binding protein (HIP). The expression of CD44 gene was detected in oocytes, and in 2- and 4-cell stages; HIP gene was detected in all preimplanatation-stage embryos. In conclusion, the present study demonstrated the embryotrophic role of GAG and expression of GAGs receptors in porcine IVF embryos. This study was supported by the Advanced Backbone IT Technology Development (IMT 2000-C1-1).

279 VASCULAR MORPHOMETRY OF BOVINE PLACENTOMES IN LATE GESTATION FROM EMBRYOS PRODUCED IN VIVO OR IN VITRO

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The role of the vascular supply in the development of placentas from embryos produced in vitro is poorly understood. The objective of this study was to determine the effects of in vitro embryo production on morphometry of blood vessels within fetal (cotyledonary) and maternal (caruncular)

components of the placentome during late gestation. In vivo-produced embryos were recovered from superovulated Holstein cows on Day 7 after estrus. For in vitro embryo production, oocytes were aspirated from the ovaries of Holstein cows, matured in vitro, and then fertilized. Presumptive zygotes with their cumulus cells were transferred into M-199 with 10% estrus cow serum and cultured for 168 h post-insemination. Semen from the same Holstein sire was used for the production of in vivo and in vitro embryos. Single blastocysts from each production system were transferred into the uteri of heifers. On Day 222 of gestation, fetuses and placentas were recovered in utero (in vivo, $n = 12$; in vitro, $n = 12$). Placentomes were collected, fixed and sectioned. Fetal and maternal blood vessels were identified within placentome sections using immunocytochemistry for vascular endothelial growth factor (VEGF) protein. A total of 4.8×10^5 μm^2 of tissue were examined from each placentome. Stereological methods were used to determine the volume densities of fetal and maternal blood vessels. Data were analyzed by GLM procedures. Fetuses were heavier ($P = 0.03$) in the in vitro group (20.7 ± 1.0 kg, LS mean $\pm$ SEM) compared to the in vivo group (17.3 ± 1.0 kg). Placentas were also heavier ($P = 0.06$) for the in vitro group (2.5 ± 0.2 kg) compared to the in vivo group (2.0 ± 0.2 kg). Placental efficiency, calculated as fetal weight/placental weight, was similar between the two treatment groups (9.0 ± 0.5 and 8.9 ± 0.5 for in vivo and in vitro, respectively). Fetal vascular volume density in placentomes was not different between the two treatment groups ($5.4 \pm 0.3\%$ and $5.4 \pm 0.3\%$ for in vivo and in vitro, respectively). In contrast, maternal vascular volume density was greater ($P = 0.02$) for placentomes in the in vitro group ($5.9 \pm 0.3\%$) compared to in vivo controls ($4.9 \pm 0.3\%$). In summary, compared to placentomes from embryos produced in vivo, placentomes from embryos produced in vitro had similar volume density of fetal vessels, but had significantly increased volume density of maternal vessels. Supported by the State of North Carolina.

280 LAMBS BORN AFTER IN VITRO EMBRYO PRODUCTION FROM PREPUBERTAL LAMB OOCYTES AND FROZEN-THAWED UNSORTED AND SEX-SORTED SPERMATOZOA

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Developments in sperm sexing technology have resulted in the birth of a number of offspring after IVF of oocytes from adult animals (Johnson LA, 2000 An. Reprod. Sci. 60–61, 93–107). The aim of this study was to combine sperm sexing technology with juvenile breeding. Merino lambs, 2–3 weeks ($n = 43$) were hormone stimulated (Morton KM *et al.*, 2003 Proc. Soc. Reprod. Fert., P18), and COCs were matured in TCM-199 (Sigma) with 10 $\mu g\,mL^{-1}$ p-FSH (Folltropin-V; Bioniche Animal Health Australasia), 10 $\mu g\,mL^{-1}$ pLH (Bioniche), and 20% sheep serum (v/v) in a humidified 6% CO_2, 5% O_2, 89% N_2 atmosphere for 22 h. Semen collected from Merino rams was diluted and frozen as pellets (Unsorted), or stained with H33342, separated into X and Y sperm using a SX MoFlo (Cytomation Inc., Fort Collins, CO, USA), and frozen as pellets (Sorted). Sperm were prepared for IVF by swim-up under 0.5 mL of SOF with 2% sheep serum (v/v; SOF+) for 45 min (Unsorted), or diluted in 0.5 mL of Sydney IVF Sperm Buffer (Cook IVF, Brisbane, Australia) and centrifuged at 650*g* for 3 min (Sorted). After IVM, oocytes were transferred to SOF+, and cultured with 0.5×10^6 mL^{-1} (Unsorted) or 1.0×10^6 mL^{-1} (Sorted) motile sperm for 18 h. Presumptive zygotes were transferred to Sydney IVF cleavage and blastocyst medium (Cook IVF) for 3 and 5 days, respectively. Oocyte maturation and fertilization were assessed by orcein staining 18 h post-insemination (hpi). Two Day-7 blastocysts were transferred to each recipient ewe ($n = 9$; 3 per group) and pregnancies diagnosed by ultrasound on Day 57 of gestation. Data were analyzed by chi-square test. Oocyte maturation was 83.9% (73/87), and monospermic fertilization did not differ for Unsorted (22/32; 68.7%), X- (6/14; 42.8%), and Y-sperm groups (15/27; 55.6%). Polyspermic fertilization was 9.4% (3/32) and 7.4% (2/27) for the Unsorted and Y groups. Cleavage was reduced with X- and Y-sperm compared with Unsorted, but blastocyst formation (from cleaved oocytes) did not differ (Table 1). There were three (100%), zero (0%), and one (33.3%) pregnancies from Unsorted, X- and Y-embryos, respectively, all of which survived to birth, demonstrating that juvenile breeding can be successfully combined with sperm sexing.

Table 1. Cleavage and blastocyst formation after IVF with Unsorted, X- or Y-sperm. Values in parenthesis are percentages

Group	No. oocytes	No. oocytes cleaving (%)		Blastocyst formation (%)		
		24 hpi	48 hpi	Day 6	Day 7	Day 8
Unsorted	632	217 (34.3)[a]	332 (52.4)[a]	62 (18.7)[a]	115 (34.6)	132 (39.8)
X	556	113 (20.3)[b]	206 (37.1)[b]	19 (9.2)[b]	56 (27.2)	68 (33.0)
Y	551	88 (16.0)[b]	171 (31.0)[b]	31 (18.1)[a,b]	68 (39.8)	76 (44.4)

[a,b] Values within columns with different supersripts differ significantly.

281 REDUCTION OF POLYSPERMY IN PORCINE IN VITRO FERTILIZATION BY MODIFIED SWIM-UP METHOD

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In vitro production (IVP) of porcine embryos facilitates research related to biotechnology and biomedicine. Even though many attempts have been made to optimize the IVP of porcine embryos, the outcome is still unsatisfactory compared to other species, such as mouse and cattle. The

high incidence of polyspermic fertilization is one of the major causes lowering the overall efficiency of porcine IVF. The common procedure for fertilization in vitro involves the co-culture of both gametes in the medium drop, which increases sperm concentration and incidence of polyspermy. Therefore, the present study was carried out to increase the efficiency of porcine IVF by reducing polyspermy using a modified swim-up method. This method modifies conventional swim-up washing by placing oocytes directly at the time of washing. Porcine oocytes were aspirated from ovaries and matured. Sperm pellet was prepared in the tube and mature oocytes were placed on a cell strainer with 70-μm pore size (Falcon 2350) at the top of the tube. After fertilization, the oocytes were fixed and stained for examination. Also, the developmental potential of fertilized embryos was measured to evaluate for the feasibility of this method. While penetration rates were similar in both methods (86.67 ± 2.36% to 83.33 ± 1.36%), there was a significant reduction of polyspermy in the modified swim-up method (17.50 ± 1.60%) compared to the control (44.1 ± 3.70%) ($P < 0.05$). Subsequent culture showed higher rate of blastocyst formation in the modified swim-up method (20.44 ± 0.99%) than in the control (15.73 ± 3.26%) ($P < 0.05$), even though the difference was not significant. These results suggest that, by controlling the number of spermatozoa reaching the oocytes, porcine oocytes might be protected from polyspermy in vitro. Also, the developmental potential of the fertilized embryos using this method could be improved by increasing the pool of spermatozoa with better quality. Further optimization of the procedure is required to impliment this method in routine porcine IVF.

282 MATURATION MEDIUM EXCHANGE IS EFFECTIVE ON BOVINE EMBRYO DEVELOPMENT IN VITRO

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In vitro embryo development is strongly influenced by IVM conditions. Increased duration of IVM may cause aging of the oocytes, which has a harmful effect on the embryo development. Oocyte maturation depends upon the synthesis of several proteins that may play important roles in the cytoplasmic maturation. These experiments were conducted to determine the effect of IVM duration(18-h or 24-h) and medium exchange (at 18 h) on embryo development, and to investigate the protein quantities in IVM medium. Korean Native Cow (KNC) ovaries 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 50-μL drops of TCM-199 supplemented with 10% fetal calf serum (FBS), 1 μg mL^{-1} MFSH, 10 μg mL LH and 1 μg mL^{-1} Estradiol-17β for 18 h or 24 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 types of cultures were carried out in an incubator at 39°C, 5% CO_2 in air. The total protein quantity in IVM medium at 18 h or 24 h were compared by 2-dimensional gel electrophoresis using a 10–15% polyacrylamide gradient gels. Data from three replicates were analyzed by chi-square test. The proportions of oocytes reaching the blastocyst stage was significantly higher in 18 h IVM group than 24 h IVM group (Table 1). However, there was no difference detected in blastocyst rate between 18 h IVM group and 18 h medium exchange group. Total protein quantity was reduced between 18 h and 24 h in IVM medium. There were 299 protein spots identified in IVM medium; there was an increase at 10 spots in the IVM medium analyzed at 18 h and a decrease of 20 spots at 24 h. This study suggests that duration of IVM affects subsequent embryo development. The total protein quantity was decreased between 18 h and 24 h in IVM medium. These proteins may be absorbed into the oocytes and reduce development to the blastocyst stage. However, this may be overcome by IVM medium exchange.

Table 1. Effects of duration of IVM and medium exchange on embryo development of KNC oocytes

Total maturation time	Medium exchanging	No. of examined oocytes	>2-cell	8-cell	Blastocyst
18 h	No	121	94 (77.4 ± 0.7)[a]	59 (48.8 ± 2.5)[a]	31 (26.0 ± 1.1)[a]
24 h	No	118	80 (67.7 ± 8.6)[a,b]	27 (22.9 ± 3.2)[b]	16 (14.4 ± 4.6)[b]
24 h	Yes (18 h)	113	74 (65.6 ± 5.6)[b]	36 (31.9 ± 12.8)[b]	27 (23.0 ± 3.9)[a,b]

[a,b]Values in the same column with different superscripts are significantly different ($P < 0.05$), Mean ± SEM.

283 ASSESSMENT OF CALVES PRODUCED BY IVP IN A SEMI-DEFINED MEDIUM

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Heavy birth weight, increased calving difficulty, heart function defects, increased perinatal mortality and organ immaturity have been reported for calves produced from IVP embryos compared to those produced from MOET or AI (van Wagtendonk AM *et al.*, 2000 Theriogenology 53, 575–597; Jacobsen H *et al.*, 2002 Anim Reprod Sci 70, 1–11). In this study we examined birth weight (BWT), and blood chemistry at 1 day of age, gestation length and heart function at 7 days, and response to an ACTH challenge at 21 days of calves derived from IVP in a 'semi-defined' IVC system (Thompson JG *et al*., 2000 J. Reprod. Fertil. 118, 47–55) and of contemporary MOET or AI calves. Holstein Friesian (HF) 2- and 3-year-old recipients carrying single HF calves (101 × IVP and 21 × MOET) were monitored in this study. Within 1 day of birth the calves were weighed and a blood sample taken for analysis. At 7d, ultrasound measurement of the left ventricle diastolic diameter (LVEDd) and % ejection fraction (EF%) was determined. Each calf was then transported to a rearing unit. At 3 weeks of age, 30 IVP and 30 control AI calves of the same age were

injected i.v. with Synacthen (synthetic ACTH, Ciba Corporation, 0.1 $\mu g\,kg^{-1}$ body weight). Blood samples were collected at −30, 0, 30, 60 and 90 min (0 min = time of injection) for cortisol measurements. There was no difference in BWT for MOET or IVP calves (40.9 ± 4.7 v. 35.6 ± 4.8 kg, respectively). Moreover, gestation lengths (279 days v. 281 days) and calving assistance scores (1.3 v. 1.6) did not differ. Calf mortality at birth was higher for IVP calves (16%) than for MOET calves (5%). All but 7 surviving calves (6 × IVP and 1 × MOET) had high GGT levels at 1 day. Blood chemistry revealed no differences between the calf types, all measures being within normal ranges. For all calves, heart function analysis revealed no abnormalities with mean LVEDd = 4.1 ± 0.6 cm and mean EF% = 78.5 ± 8.4%. All calves exhibited elevated cortisol following ACTH challenge. There was no difference between control and IVP calves for mean cortisol concentration at any time point (0 min, 13.8 ± 5.2; 30 min, 46.6 ± 9.8; 60 min, 42.8 ± 9.9; 90 min, 28.1 ± 8.9 ng mL^{-1}). These data suggest that, unlike calves produced in less defined culture systems, calves produced by IVP in a semi-defined culture system have birth weight and gestation lengths similar to those of MOET calves. Moreover, no abnormalities in organ (heart, adrenal) function were detected. However, of concern was the high number of unexplained deaths for IVP calves. This may be due to an overall lack of vigour in IVP calves that, in an unsupervised calving, results in calf death. More vigilence at calving may be needed to ensure calf survival. The authors thank Juliet Jensen, Waikato Hospital, for ultrasound measurements and David Stewart, Morrinsville Veterinary Services, for calf care. This study was funded by Vialactia Biosciences and FRST.

284 THE EFFECT OF FEEDING PROPYLENE GLYCOL TO DAIRY COWS DURING THE EARLY POSTPARTUM PERIOD ON SERUM INSULIN CONCENTRATION AND THE RELATIONSHIP WITH OOCYTE DEVELOPMENTAL COMPETENCE

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High yielding dairy cows are typically in negative energy balance postpartum (pp). It has been shown that initiation of the first pp ovulation and, therefore, the resumption of normal oestrous cycles is delayed in high genetic merit dairy cows and is associated with lower circulating insulin concentration (Gutierrez *et al.*, 1999 J. Reprod. Fertil. 24, 32 abst). Evidence shows that propylene glycol (PG) rapidly elevates systemic concentrations of insulin (Bremmer *et al.*, 2000 J. Dairy Sci. 83, 2239–2251). The aim of this study was to determine the effects of PG feeding to dairy cows in the early pp period, on serum insulin and ovarian function, and on oocyte developmental competence after in vitro maturation, fertilization, and culture. Thirteen Holstein-Friesian cows were assigned to PG ($n = 6$) or control ($n = 7$) groups. Each treated cow received 500 mL of PG and each control was given 500 mL of water daily from Day 5 pp until day of AI. Blood samples for insulin were collected at 0, 30, 60 and 90 min post-drenching on Days 5, 15 and 25 pp. All cows were fed 3 kg concentrates at milking (twice daily) and had ad libitum access to a 50 : 50 maize silage : grass silage forage from the time of last blood collection. Oocytes were collected by ovum pick-up (OPU) in four sessions (following treatment with pFSH) beginning on Day 25–35 pp. The recovered oocytes were graded (Grade 1 to 4) in terms of their surrounding cumulus cells and the appearance of the cytoplasm. Grade 1–2 oocytes were matured in vitro, then fertilized using frozen-thawed bull semen, and subsequently cultured up to Day 8 in synthetic oviduct fluid. All data were analyzed using SAS version 6.12 and split-plot designs, following square root or arc sine transformation, if appropriate. PG significantly increased ($P < 0.001$) serum insulin concentration (0 min: 1.55 ± 0.19; 30 min: 4.48 ± 0.82; 60 min: 4.74 ± 0.72; 90 min: 4.10 ± 0.56) compared to the control group (0 min: 1.91 ± 0.28; 30 min 1.96 ± 0.27; 60 min: 2.37 ± 0.44; 90 min: 2.04 ± 0.26). The follicle size distribution was similar between treated and control cows for categories 2–4 mm (4.0 ± 0.47; 4.3 ± 0.70), 8–10 mm (3.2 ± 0.47; 2.5 ± 0.39), and >10 mm (0.42 ± 0.12; 0.67 ± 0.17). However, there were significantly more follicles in the 5–7 mm category (6.2 ± 0.82 v. 3.3 ± 0.43; $P < 0.05$) for treated cows. The number of follicles punctured (13.8 ± 1.02; 10.7 ± 1.04), the number of oocytes recovered (4.5 ± 0.53; 3.5 ± 0.61), and the number of Grade 1–2 oocytes (2.8 ± 0.35; 1.8 ± 0.35) were not different between treated and control cows. Although cleavage rate (68.3 v. 58.9%) and blastocyst yield (25.3 v. 14.4%) were higher for treated cows, the differences were not significant. In conclusion, these results indicate that feeding cows with PG during the early pp period increased the circulating insulin concentration. However, the developmental competence of the recovered oocytes did not differ between the groups.

285 BIRTH OF PIGLETS AFTER NON-SURGICAL TRANSFER OF PORCINE EMBRYOS CULTURED IN PZM-4 WITH ALTERED CONCENTRATIONS OF AMINO ACIDS

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We previously developed an in vitro production (IVP) system for porcine embryos and obtained piglets after surgical transfer of blastocysts cultured in Porcine Zygote Medium (PZM)-4. However, the developmental competence of pig IVP embryos to the blastocyst stage is still low and further improvement of IVC medium is needed. In the present study, we evaluated the effects of the addition of glutamine (Gln), hypotaurine (HT), taurine (Tau), BME-essential (EA) and MEM-nonessential (NA) amino acids solutions to PZM-4, and the replacement of polyvinyl alcohol (PVA) with BSA on embryo development to blastocysts. Moreover, the developmental competence of IVP blastocysts after nonsurgical embryo transfer (NS-ET), using a flexible catheter (FC) for deep intrauterine insemination, was investigated. Porcine COC from prepubertal gilts were matured and fertilized in vitro, using frozen-thawed ejaculated boar semen. Presumptive zygotes were cultured in PZM-4, as a basal culture medium, until Day 5 after IVF. Data from six replicates were analyzed by ANOVA. Addition of 0.25 to 4 mM Gln to PZM-4 (containing 5 mM HT) significantly increased the percentage of embryos that developed to blastocysts (15 to 31%), with addition of 2 mM Gln significantly increasing the total cell numbers in blastocysts (43 ± 17 cells) compared with no addition (3% and 20 ± 4 cells, respectively). Addition of 1.25 to 10 mM HT to HT-free PZM-4 supplemented with 2 mM Gln (named PZM-5) significantly increased the percentage of embryos that developed to blastocysts (22 to 28%) compared with control (no HT; 4%). In the culture with HT-free PZM-5, addition of 5 mM Tau significantly increased blastocyst yield (17%) compared with

control (4%). However, Tau addition in the presence of 5 mM HT had no effect on development to the blastocyst stage. In combinations of EA and NA added to PZM-5, a single dose of EA significantly increased the percentage of embryos that developed to blastocysts (27%) compared with no dose (19%) or with a double dose of EA (20%), while a double dose of NA significantly increased the total cell numbers in blastocysts (43 ± 16 cells) compared with no NA (37 ± 6 cells). Replacement of PVA with BSA in PZM-5 had no effect on embryo development to the blastocyst stage. Crossbred sows were used as recipients for NS-ET, and had their estrous cycle synchronized by a described previously method (Yoshioka *et al.*, 2002 Biol. Reprod. 66, 112–119). Five days after hCG injection, a FC was introduced via the cervix into the uterine horn of recipients without sedation. Day-5 blastocysts cultured in PZM-5 were then transferred together with 5 ml of TALP-Hepes (45 to 50 blastocysts/recipient). Of 6 recipients, one sow became pregnant and farrowed 7 piglets. Our results indicate that the addition of amino acids to PZM-4 can improve porcine embryo development to the blastocyst stage, and that blastocysts cultured in a chemically defined medium, PZM-5, can develop to full-term following NS-ET.

286 SPERM–OOCYTE INTERACTION DURING IN VITRO FERTILIZATION IN THE HORSE

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In vitro fertilization (IVF) has proven to be a surprisingly unsuccessful way of producing horse embryos. The aim of this study was to investigate the interaction between sperm and the cumulus oocyte complex (COC) during IVF. In experiment 1, three IVF conditions were tested: (A) COCs recovered from slaughtered mares were categorized with respect to cumulus morphology (C: compact, $n = 86$, or E: expanded, $n = 55$) and matured in TCM199 containing 0.01 IU/mL porcine FSH and equine LH (IVM); after IVM, the oocytes were denuded and those with a visible polar body were incubated with sperm (IVF) in the presence or absence of 150 ng/mL progesterone (P4) to induce the acrosome reaction (AR); (B) IVM oocytes from C-COCS were denuded ($n = 52$) or not ($n = 67$) before IVF in the presence of P4; (C) in vivo-matured oocytes ($n = 15$) recovered by transvaginal ultrasound-guided aspiration from preovulatory follicles 32 h after the donor mare was treated with hCG, were fertilized in vitro in the presence of P4. In all cases, IVF was performed with frozen-thawed, Percoll-selected sperm from a single stallion, at a final concentration of 1×10^6spermatozoa/ml in fertil-TALP for 20 h (Parrish *et al.*, 1988 Biol. Reprod. 38, 1171–1180). In experiment 2, the possibility that semen cryopreservation or stallion critically influenced IVF was examined by incubating denuded IVM oocytes with fresh or frozen/thawed sperm from the same (fresh; $n = 17$ for both C- and E-COCs and frozen-thawed; $n = 12$ and 21 for C and E-COCs, respectively) or one other stallion (Fresh; $n = 12$ and 19 and frozen-thawed; $n = 12$ and 19 for C and E-COCs, respectively), in the presence of P4 for 20 h. In both experiments, the resulting sperm-oocyte complexes were fixed, permeabilized and labelled with fluorescein-conjugated peanut agglutinin (EY Laboratores, San Mateo, CA, USA) and ethidium homodimer (Molecular Probes, Eugene, OR, USA) to stain the acrosomal membrane and DNA, respectively, so that membrane status and position of the sperm within the oocyte investments could be detected by confocal laser scanning microscopy. The total number of sperm bound per oocyte was compared between treatments using one-way ANOVA with pair-wise multiple comparison (Bonferroni *t*-test). Despite binding to the zona pellucida (ZP), neither fresh nor frozen/thawed sperm from either stallion acrosome-reacted or penetrated any oocytes, irrespective of cumulus morphology at the onset of IVM, denudation prior to IVF or the presence of P4. However, more sperm bound to the ZP of cumulus-denuded IVM oocytes (65 ± 32 and 62 ± 28 [mean ± sd] for C and E-COCs, respectively), than cumulus-intact IVM (5 ± 4) or in vivo-matured oocytes (23 ± 17: $P < 0.001$). None of the other factors investigated affected bound sperm numbers. In all cases, ZP-bound sperm failed to AR in the classical fashion, and all oocytes remained arrested at the MII stage. In summary, fertilization failed because sperm did not acrosome-react after binding to the ZP. It is concluded that failure to adequately activate stallion sperm is an important obstacle to successful IVF in horses.

287 IN VITRO PRODUCTION OF HOLSTEIN EMBRYOS USING SEX SORTED SEMEN

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Our objective was to explore the synergy between sexed semen and in vitro embryo production and assess benefits of these technologies on commercial farms. Ovaries were collected from high genetic merit Holstein cull cows via colpotomy or at the time of slaughter. Oocytes were aspirated from the ovaries, fertilized 20–24 h later, and matured to the morula or blastocyst stage. Embryos were transferred into recipient Holstein cows and heifers on the same farms. Seven Wisconsin herds participated, and 365 embryos were produced from 104 donor cows. Only 272 of these embryos were transferred due to limited availability of recipients. Sexed semen from three Holstein sires was used. On average, 3.5 ± 0.37 transferable embryos were produced per donor, including 1.4 ± 0.18 grade 1 embryos and 1.5 ± 0.20 grade 2 embryos. Individual farms averaged from 1.6 to 5.8 transferable embryos per donor. Laboratory data also revealed interesting results. On average 43.7 ± 4.0 oocytes were collected per donor, and the number of usable oocytes (33.9 ± 3.4), and percent embryos cleaved (52.1 ± 1.9), were significant predicators of the number of blastocysts developed. We divided the usable oocytes and embryos cleaved per donor into quartiles. The fourth quartile for embryos cleaved was significantly greater ($P < 0.05$) than the lower three quartiles, and the usable oocyte quartiles all significantly differed from each other. Semen freeze date was also a significant predicator of the number of blastocysts developed, suggesting significant variation in the quality of sorted semen per ejaculate. To preliminarily test the effect of sorting on the percentage of embryos developing to blastocyst stage, oocytes were recovered from ovaries collected at a slaughterhouse and fertilized using non-sorted semen or sex-sorted semen from the same sires. Oocytes ($n = 3312$) fertilized using non-sorted semen tended ($P = 0.06$) to produce more embryos developing to blastocyst stage than oocytes ($n = 1577$) fertilized using sex-sorted semen ($20.1 \pm 2.9\%$ v. $12.2 \pm 2.3\%$, respectively). Preliminary pregnancy results show strong farm and sire effects. Overall conception rate was 36% for heifer recipients and 18milking cow recipients. These results suggest that low cost in vitro embryo production may have promise as an early system for utilizing sexed semen in dairy cattle breeding programs.

288 EFFECTS OF OVIDUCTAL EPITHELIAL AND CUMULUS CELLS ON FERTILIZATION AND EMBRYO DEVELOPMENT OF PIG OOCYTES IN VITRO

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This study was carried out to investigate the effect of adding porcine oviductal epithelial cells (POEC) and also the presence of cumulus cells during in vitro fertilization on fertilization rate and subsequent embryo development of pig oocytes matured in vitro. Cumulus-oocyte complexes (COCs) aspirated from 2- to 6-mm follicles were matured in TCM 199 supplemented with cysteine, EGF, eCG, hCG, and PVA for 20–22 h, and cultured in the same medium without hormone for an additional 20–22 h. Oviducts attached to ovaries without CL were used to obtain epithelial cells. After removal of the fimbria and utero-tubal junction, oviducts were flushed with MEM supplemented with 10% FBS, and POEC clumps were cultured in the same medium for 48 h. After the completion of maturation, COCs were randomly divided into four groups; cumulus-denuded (D), cumulus-denuded with POEC (DP), cumulus-enclosed (E), and cumulus-enclosed with POEC (EP). Eight to 10 POEC clumps were co-cultured with sperm and oocytes in a 100-μL fertilization drop. Oocytes were fertilized with frozen-thawed spermatozoa for 6 h in modified Tris-buffered medium containing caffeine and BSA. Presumptive zygotes were cultured in NCSU23. Oocytes were fixed and stained for the evaluation of penetration at 12 h after IVF ($n = 549$ oocytes), and cleavage rate and blastocyst formation were evaluated at 48 and 144 h after IVF ($n = 1531$ oocytes), respectively. Results were analyzed by Duncan's multiple range test using GLM procedure in SAS. Although the sperm penetration rate in group E was lowest among all groups ($P < 0.05$), the monospermic fertilization rate was not significantly different among treatment groups (68.6–81.9%). Although the cleavage rate and percentage blastocyst in group E were significantly lower than other groups (38.1 v. 53.6, 52.0 and 44.6%, and 15.0 v. 21.2, 23.4 and 18.5% in group D, DP, and EP, respectively), blastocyst cell number was not significantly different among treatment groups (24.9–27.3) These results suggested that the presence of cumulus cells alone during fertilization interferes with sperm penetration, cleavage, and blastocyst formation and that POEC may improve both cleavage and blastocyst formation rate.

289 EFFECT OF FERTILIZATION TIME OF PIG OOCYTES MATURED IN-VITRO BY BOAR SPERM STORED AT 4°C

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The use of boar sperm stored at 4°C may be a useful tool for in vitro production of pig embryos. Therefore, this study was undertaken to investigate the effect of fertilization time of pig oocytes matured in-vitro by boar sperm. The sperm-rich fraction (30–60 mL) was slowly cooled to room temperature (20–23°C) by 2 h after collection. Semen was transferred into 15-mL tubes, centrifuged at room temperature for 10 min at 800*g*, and the supernatant solution was poured off. The concentrated sperm was resuspended with 5 mL of the LEN (11.0 g lactose hydrate, 20 mL egg yolk, 0.05 g N-acetyl-D-glucosamine and 100 mL distilled water) diluent to provide 1.0×10^9 sperm mL^{-1} at room temperature. The resuspended semen was cooled in a refrigerator to 4°C. The medium used for oocyte maturation was TCM-199 supplemented with 26.19 mM sodium bicarbonate, 0.9 mM sodium pyruvate, 10 μg mL^{-1} insulin, 2 μg mL^{-1} vitamin B_{12}, 25 mM HEPES, 10 μg mL^{-1} bovine apotransferrin, 150 μM cysteamine, 10 IU mL^{-1} PMSG, 10 IU mL^{-1} hCG, 10 ng mL^{-1} EGF, 0.4% BSA, 75 μg mL^{-1} sodium penicillin G, 50 μg mL^{-1} streptomycin sulfate and 10% pFF. After about 22 h of maturation, oocytes were cultured without cysteamine and hormones for 22 h at 38.5°C, 5% CO_2 in air. Oocytes were inseminated with boar sperm stored at 4°C for 2 days after collection. Oocytes were coincubated for 1, 3, 6 and 9 h in 500 μL TBM fertilization media with 1×10^6 mL^{-1} sperm concentration. Thereafter, oocytes were transferred into 500 μL NCSU-23 culture medium containing 0.4% BSA for further culture of 6, 48 and 144 h, fixed and stained for the evaluation of fertilization parameters and developmental ability. Data were analysed by ANOVA and Duncan's multiple range test using the SAS program. The rates of sperm penetration and male pronuclear formation were higher in the fertilization times of 6 and 9 h than in those of 1 and 3 h. The percentage of polyspermic oocytes was highest in fertilization time of 9 h compared with other incubation times. The rates of cleaved oocytes were higher in the fertilization times of 6 and 9 h (85.0 and 84.6%) compared with those of 1 and 3 h (61.1 and 76.8%). The percentage of blastocyst formation from the cleaved oocytes was highest in the fertilization time of 6 h (33.6%) than in that of 1, 3 and 9 h (11.4, 23.0 and 29.6%). Mean cell numbers per blastocyst were 32.9 ± 3.3, 27.6 ± 2.7, 26.3 ± 2.2 and 24.4 ± 1.8 in the fertilization times of 6, 9, 3 and 1 h, respectively. In conclusion, we found out that boar sperm stored at 4°C could be used for in vitro fertilization of pig oocytes matured in vitro. Also, we recommend the coincubation time of 6 h in 500 μL TBM fertilization medium with 1×10^6 mL^{-1} sperm concentration for in vitro fertilization of pig oocytes matured in vitro.

290 EFFECTS OF HYALURONAN ON IN VITRO DEVELOPMENT OF PORCINE EMBRYOS CULTURED IN A CHEMICALLY DEFINED MEDIUM

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Hyaluronan (HA), a glycosaminoglycan present in follicular and oviductal fluids, has been related to sperm capacitation, fertilization and embryo development. We have previously developed an in vitro-production (IVP) system of porcine embryos, where porcine blastocysts can be produced by IVF and IVC in chemically defined media and can develop to full-term by transfer to recipients. The application of a chemically defined medium to IVP in pigs allows the analysis of the physical action of substances on the development of pre-implantation embryos. In the present study, the effects

of HA on the development of porcine embryos in a chemically defined medium were investigated. Porcine presumptive zygotes were produced by IVM and IVF of COC from pre-pubertal gilts and frozen-thawed ejaculated boar semen. The zygotes were cultured in Porcine Zygote Medium (PZM)-5 containing different concentrations of HA (0 [control], 1, 2, 5, 10, 20 and 50 $\mu g\,mL^{-1}$) until 6 days after IVF, and representative specimens were fixed for cell counting and transmission electron microscopy. Data of percentages and cell numbers were statistically analyzed by one-way ANOVA and Fisher's PLSD test. The percentage of embryos that developed to the blastocyst stage (15.8% [23/144] to 19.5% [27/139]) did not differ among treatments. However, addition of 5 or 10 $\mu g\,mL^{-1}$ HA increased ($P < 0.05$) the total number of cells in blastocysts (56.1 and 58.3 cells [$n = 22$ and 23], respectively) compared to control (no HA, 42.0 cells [$n = 23$]). To evaluate proliferation rates of inner cell mass (ICM) and trophectoderm (TE), embryos were cultured in PZM-5 for various periods of exposure to 10 $\mu g\,mL^{-1}$ HA. The numbers of ICM and TE cells in Day-6 blastocysts cultured in the presence of exogenous HA from Day 0 to Day 3 (18.3 and 34.4 cells, respectively [$n = 38$]) or Day 6 (17.9 and 35.9 cells, respectively [$n = 36$]) were significantly ($P < 0.05$) higher than those cultured without HA through the culture period (13.5 and 24.2 cells, respectively [$n = 26$]). In the presence of HA from Day 3 to 6, only the number of TE cells (37.1 cells [$n = 33$]) increased ($P < 0.05$), compared to PZM-5 alone. Differences in ultrastructure were noticed among blastocysts cultured with or without 10 $mg\,mL^{-1}$ HA. Blastocysts cultured with HA had mainly mature mitochondria while many mitochondria appeared morphologically immature in the blastocysts cultured without HA. Lipid droplets in the blastocysts cultured with HA seemed to be more homogeneous in comparison with those in the blastocysts cultured in PZM-5 alone. Further differences were seen in the numbers of lysosome-like structures, which were greater in blastocysts cultured with HA. This study demonstrates that exogenous HA improves cell proliferation and normality of ICM and TE in porcine embryos cultured in a chemically defined medium, depending on the exposure periods to HA. (Supported by MAFF, Japan and STINT, Sweden.)