

IVF/IVP

237 INCREASE OF BLASTOCYST DEVELOPMENTAL RATE *IN VITRO* BY SELECTION OF DEVELOPMENTALLY COMPETENT COW OOCYTES BEFORE IVM USING A STAINING TEST

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A large proportion of bovine oocytes fail to develop to blastocyst stage following maturation, fertilization, and culture *in vitro*. While suboptimal culture conditions undoubtedly contribute to this poor development, it is recognized that immature oocytes, especially from cows with reduced reproductive performance or which are slaughtered on the end of their use, are heterogeneous in quality and developmental competence (Gordon 2003). The aim of the present study was to increase the efficiency of blastocyst production from cows after IVM/IVF by oocyte selection before maturation. Immature oocytes are known to synthesize a variety of proteins (Wassarman PM 1988, *Annu. Rev. Biochem.* 57, 415–442), among them, glucose-6-phosphate dehydrogenase (G6PDH). This enzyme is active in the growing oocyte, but has decreased activity in oocytes that have finished their growth phase. Brilliant cresyl blue (BCB) has been used to measure G6PDH activity. The BCB test is based on the capability of the G6PDH to convert the BCB stain from blue to colorless (Erisson *et al.* 1993 *Theriogenology* 39, 214). The ovaries were obtained from a slaughterhouse and transported to the laboratory; cumulus-oocyte complexes (COCs) were recovered by slicing the surface of the ovary. Only oocytes with a compact cumulus investment were used. Oocytes were placed into three groups: (1) control – placed immediately into culture; (2) holding control – COCs kept in PBS containing 0.4% BSA for 90 min at 38.5°C before placement into culture; and (3) treatment – incubation with brilliant cresyl blue for 90 min at 38.5°C before culture. Treated oocytes were then divided into BCB– (colorless cytoplasm, increased G6PDH) and BCB+ (colored cytoplasm, low G6PDH) on their ability to metabolize the stain. Activity of G6PDH was determined via measurement of NADP reduction in control, BCB–, and BCB+ groups; activity was significantly increased in BCB– COCs in comparison to the control and BCB+ COCs. After IVM, oocytes were fertilized *in vitro*. Embryos were cultured to Day 8. The rate of maturation to metaphase II was significantly higher for control and BCB+ oocytes (77.1 and 72.5%, respectively) than for BCB– oocytes (58.1%). The BCB+ oocytes yielded a significantly higher proportion of blastocysts (34.1%) than either control group (18.3 and 19.2%); and both controls and BCB+ oocytes had significantly higher blastocyst development than did BCB– oocytes (3.9%). The number of nuclei in the blastocysts was comparable in BCB+ and both control groups (105.5 ± 5.8 and 117.5 ± 8.5, 101.8 ± 6.2, respectively). Blastocysts in the BCB– group had a significantly lower cell number (61.0 ± 2.6) than did controls. The results show that the staining of COCs from cows before IVM may be useful in increasing the efficiency of blastocyst production during standard IVF procedures. In addition, classification of G6PDH activity on the basis of BCB staining may be used to effectively select cow oocytes with further developmental competence. To our knowledge, this is the first study to evaluate the association between G6PDH activity in oocytes and further blastocyst development in cows.

238 ASSESSMENT OF SPERM MOTILITY, VIABILITY, AND FERTILIZATION RATE OF TWO GROUPS OF BULLS SHOWING A DIFFERENT ABILITY TO PROMOTE EMBRYO DEVELOPMENT *IN VITRO*

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Low developmental rates after IVF with some AI bulls are one of the most frequent problems encountered with *in vitro* production of bovine embryos. In this study, two groups of high and low *in vitro* fertility bulls (3 bulls per group) were selected based upon blastocyst rates. We first evaluated sperm motion characteristics using computer-assisted sperm analysis (CASA) before and after Percoll separation. The following parameters were compared between bulls: proportion of motile sperm (% MOT), curvilinear velocity (VCL), and straightness (STR), which allowed us to evaluate the proportion of progressive semen (VCL > 80 µm/s and STR > 60%). Concerning % MOT, differences between bulls were observed before Percoll separation (42 to 59% – ANOVA2: $P = 0.017$). Following separation, the values of % MOT were globally increased (ANOVA3: $P < 0.001$) and no further difference was observed between bulls (68 to 76%). The same pattern was obtained for the proportion of progressive spermatozoa (before: 43 to 55% vs. after: 48 to 57% – ANOVA3: $P = 0.005$). For viability measurements, two DNA-binding fluorochromes, propidium iodide and Hoechst 33342, were used, which respectively stain dead sperm nuclei in red and living sperm nuclei in blue. This viability evaluation was carried out after 0, 2, 6, and 18 h of incubation in TALP medium supplemented with heparin (10 µg/mL), under the same conditions as IVF but without oocytes. In the two groups of bulls, a sharp decrease in the proportion of living sperm was already observed at 2 h (only 50 to 20% of living sperm), while, at 18 h, 17 to 9% of sperm were still intact. Two bulls from the high *in vitro* fertility group showed a percentage of living sperm significantly more important at 6 and 18 h than the other 4 bulls (chi square – $P < 0.05$). In a third experiment, we assessed the fertilization (2 pronuclei) and the polyspermy (>2 pronuclei) rates after staining the zygotes with Hoechst at 18 hpi. The high fertility group showed higher fertilization rates than the low fertility one (75 to 80% vs. 59 to 67% – ANOVA2: $P < 0.008$). No significant difference was noticed between bulls concerning polyspermy. The present results confirm the efficiency of Percoll separation in enhancing some of the motility parameters such as the proportion of motile and progressive sperm. Moreover, this gradient allows us to abolish differences between bulls for those motility parameters. However, the viability and the rate of fertilization still varied between bulls after Percoll separation. In our study, only fertilization rates seemed related to the blastocyst rates. Further investigations are under way to better understand the sperm characteristics of these two groups.

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239 CAPACITATION OF BUFFALO SPERMATOZOA *IN VITRO*

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The efficiency of *in vitro* embryo production (IVEP) in buffalo is hampered by the poor cleavage rate. The quality of the frozen semen may affect fertilization efficiency, due to damages of the male gamete that occur following cryopreservation. However, it is not possible to rule out that the process of capacitation, required by spermatozoa to acquire the fertilizing ability, is impaired in the IVF system. Although several agents have been proven to induce sperm capacitation *in vitro*, heparin is still the most efficient method in most of the domestic species. The aim of the study was to evaluate the efficiency of buffalo estrus serum (BES) and follicular fluid (FF) to induce buffalo sperm capacitation *in vitro*, indirectly assessed by estimating the capability of spermatozoa to acrosome react. Frozen semen from a bull previously tested for IVF, thawed at 37°C for 40 s in water, was treated by swim-up in order to select only the motile population. Spermatozoa ($n = 1546$) were assessed immediately after swim-up separation, to evaluate the incidence of acrosomal loss in nontreated cells (time 0). The remaining spermatozoa were incubated in the presence of 0.01 mM heparin (control; $n = 3531$), 20% BES ($n = 2442$) and 20% FF ($n = 1419$), the latter recovered from a pool of dominant follicles, for 1, 2, and 3 hours. Sperm was then exposed for 15 min to $60 \mu\text{g mL}^{-1}$ of lysophosphatidylcholine, an agent known to induce acrosome reaction only on capacitated spermatozoa. Trypan blue was used to differentiate live from dead spermatozoa, and the dried smears were then fixed in 37% formaldehyde and stained with Giemsa for acrosome evaluation by microscopic examination. The proportion of acrosome-reacted spermatozoa in each group was used to assess the efficiency of capacitation under different incubation conditions. Differences between groups were analyzed by χ^2 . No dead spermatozoa were found in all groups. Acrosomal loss was observed in only 15.3% of the sperm population at time 0; it may be accounted for either by damages preceding cell death or by freezing-induced capacitation. No differences were found between incubation times within each treatment group. Interestingly, sperm treatment with both BES and FF resulted in a significantly higher incidence of acrosome reaction compared with heparin (84.3, 94.5 vs. 50.1%, respectively; $P < 0.001$), the capacitating agent currently used in the IVF system, and, in particular, FF showed the highest percentage of acrosome reaction at all incubation times, even when compared with BES ($P < 0.01$). It is likely that factors derived by BES and FF, present in the oviduct environment around fertilization, play a critical role in processing the male gamete *in vivo*. These preliminary results show the possibility of significantly improving the efficiency of sperm capacitation *in vitro* in buffalo species with BES and FF and strongly suggest investigating the effects of these factors also on the fertilizing capability of buffalo spermatozoa.

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240 GESTATION LENGTH AND BIRTH WEIGHT OF *IN VITRO* PRODUCED EMBRYOS FROM ZEBU DAIRY CATTLEL.S.A. Camargo^A, J.H.M. Viana^A, A.A. Ramos^A, W.F. de Sa^A, A.M. Ferreira^A, J.F. Fonseca^B, and V.R. Vale Filho^C^AEmbrapa Dairy Cattle, Federal University of Juiz de Fora, Minas Gerais 36038-330, Brazil;^BEmbrapa Goats, Sobral 62011-970, Brazil; ^CFederal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil. Email: camargo3112@hotmail.com

Gir cattle are a popular zebu dairy breed in tropical and subtropical regions because of their tolerance of heat stress and resistance to tick-borne disease. The use of *in vitro* embryo production (IVP) technology may help accelerate genetic improvement of this breed. However, in general, IVP systems have been implicated in the production of large offspring and a greater proportion of male calves. Natural breeding results in newborn Gir calves weighing around 25 kg despite the fact that dams may weigh over 500 kg. It is unknown whether *in vitro*-produced Gir embryos also result in large offspring. The aim of this work was to evaluate the effect of *in vitro* embryo production on gestation length, birth weight, and sex ratio in Gir cattle. COCs were harvested by oocyte pickup from mature non-lactating Gir cows and *in vitro*-matured in TCM 199 medium (Gibco, Sao Paulo, Brazil) with 10% inactivated estrous cow serum for 24 h under 5% CO₂ at 38.5°C in air. Gir spermatozoa were obtained through the swim-up method and co-incubated with oocytes in Fert-TALP media (Parrish JJ *et al.* 1988 Biol. Reprod. 38, 1171–1180) with 10 $\mu\text{g/mL}$ heparin (Sigma, Sao Paulo, Brazil) and 6 mg/mL fatty acid-free bovine albumin (Sigma) for 18 h in 5% CO₂ at 38.5°C in air. Presumptive zygotes were co-cultured with their own cumulus cells in CR2aa medium (Wilkinson RF *et al.* 1996 Theriogenology 45, 41–49) with 10% fetal calf serum in humid atmosphere of 5% CO₂ at 38.5°C in air. Fresh Day 7 blastocysts were transferred to synchronized *B. indicus* \times *B. taurus* crossbred recipients. Data of gestation length, birth weight, and gender ratio from 26 IVP calves (IVP group) were recorded and compared to data obtained from Gir calves produced by artificial insemination or natural mating ($n = 24$; control group) using ANOVA or chi-square analysis. There was no difference ($P > 0.05$) in gestation length between pregnancies of the IVP and control groups (mean \pm SEM, 285.4 ± 1.5 vs. 284.4 ± 1.1 days, respectively). IVP and control calves showed similar ($P > 0.05$) weight at calving (29.6 ± 0.9 vs. 26.9 ± 1.2 kg for IVP and control male calves, and 27.0 ± 2.5 vs. 25.2 ± 0.5 kg for IVP and control female calves, respectively). The percentage of male calves was greater ($P < 0.05$) in the IVP group than in the control group (76.9% vs. 43.4%, respectively). IVP calves did not show abnormalities associated with Large Offspring Syndrome, such as breathing difficulty and perinatal death. These data suggest that *in vitro* production may affect the development of Gir embryos, biasing the sex ratio in a manner similar to previously reported for *in vitro*-produced embryos from *Bos taurus* breeds.

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241 EFFECT OF MEIOTIC ARREST BY ROSCOVITINE AND SUBSEQUENT IVM TIME ON DEVELOPMENTAL COMPETENCE OF IMMATURE BOVINE OOCYTES

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Maintaining immature bovine oocytes at the germinal vesicle stage by inhibiting M-phase promoting factor (MPF) activity is a reversible process when using roscovitine, and this can improve cytoplasmic maturation *in vitro*. However, optimum meiotic arrest times and subsequent IVM times have not been determined, so we evaluated the developmental competence of oocytes in relation to these times. Two by two factorial treatments consisting of 2 arrest times (8 h, 16 h) and 2 subsequent IVM times (16 h, 22 h) plus a control were replicated 6 times in this study. Semen from two bulls was used three times. Chemically defined media (CDM) were used throughout (Olson and Seidel 2000 *J. Anim. Sci.* 78, 152–157). Slaughterhouse-derived oocytes were arrested in meiosis in 1 mL of CDM-M without any hormones, but containing 50 μ M roscovitine and 0.5% fatty acid-free (FAF)-BSA under 5% CO₂ in air at 38.5°C. After 8 or 16 h of meiotic arrest, oocytes were washed and matured in 1 mL of CDM-M containing 0.5% FAF-BSA, 2 mM glucose, 50 ng/mL EGF, 15 ng/mL NIDDK-oFSH-20, 1 μ g/mL USDA-LH-B-5, 1 μ g/mL E2, and 0.1 mM cysteamine for 16 or 22 h under 5% CO₂ in air at 38.5°C. Oocytes for the control group were matured in 1 mL of the CDM-M with hormones for 22 h. Ten oocytes from each group were fixed after IVM, stained with orcein, and evaluated for maturation to MII. For fertilization, motile sperm recovered from frozen-thawed semen were co-incubated for 18–20 h with ~20 oocytes/group at a final sperm concentration of 0.5×10^6 sperm/mL in F-CDM. Presumptive zygotes were cultured in 0.5 mL of CDM-1 for 2.5 days and then in CDM-2 for 5.5 days in 5% CO₂, 5% O₂, 90% N₂ in a humidified incubator at 39°C. Cleavage rates were evaluated after culture in CDM-1. Blastocyst rate, blastocyst stage (5 = early, 6 = full, 6.5 = expanding, 7 = expanded, 7.5 = hatching, 8 = hatched), and embryo quality (1 = excellent, 2 = good, 3 = fair, 4 = poor) were evaluated after CDM-2. Data were subjected to ANOVA; the arc sin transformation was used for percentage data, and least-squares means are presented. There were no significant differences in % cleavage (Cle), cell stage, or blastocyst quality among treatments ($P > 0.1$). However, meiotic arrest of oocytes for 16 h and subsequent IVM for 16 h improved embryo development to blastocysts compared to other roscovitine treatments (Table 1, $P < 0.05$). A bull effect for % blastocysts was observed, 19.9% and 25.2% for bulls 1 and 2, respectively ($P < 0.08$). Blastocyst production was improved by shortening oocyte maturation time from 22 to 16 h, when meiotic progression was previously inhibited for 16 h with roscovitine.

Table 1. Effect of meiotic arrest and IVM times on oocyte maturation and embryo development

Treatment (h) (Roscovitine/IVM)	No. oocytes	MII (%)	Cle (%)	Bl (%)	Stage	Grade
8/16	151	58 ^a	84	14.7 ^a	6.5	1.6
8/22	162	89 ^b	87	20.0 ^a	6.6	1.7
16/16	161	67 ^{ab}	88	30.7 ^b	6.8	1.5
16/22	170	81 ^b	84	18.7 ^a	6.8	1.6
0/22	156	71 ^{ab}	84	28.7 ^b	6.5	1.6

^{a,b} Means within columns without common superscripts differ ($P < 0.05$).

242 DEVELOPMENT OF BAC FISH PROBES FOR GENETIC ANALYSIS OF NON-HUMAN PRIMATE GAMETES AND EMBRYONIC STEM CELLS

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Several factors relating to reproductive health and gamete competence must be considered when implementing programs for propagation of genetically valuable primates via natural breeding or assisted reproductive technologies. These relevant factors include ascertaining gamete chromosome normality of individuals. Our early attempts at characterizing aneuploidy levels of gametes within a model primate species, the rhesus macaque, utilizing Y-sis centromeric fluorescent *in situ* hybridization (FISH) probes, failed to produce a signal. Commercially available probe sets for interphase cytogenetics rely partly on repetitive DNA probes. Therefore, we have employed either pools of human BACs or clones selected from a rhesus macaque BAC library for interphase cytogenetics. Bacterial artificial chromosome (BAC) FISH probes specific to regions of human DNA that are homologous to macaque DNA were produced, in collaboration with researchers at the University of California-Davis. The most common aneuploidies implicated in human birth defects or pregnancy failures are those involving the sex chromosomes and chromosomes HSA (human) 13, 16, 18, and 21 (Munne 2003 *Placenta* 24, 70–76). Hence, we have initially used human BAC FISH probes on the equivalent chromosome homologs in *Macaca mulatta* (MMU): X, Y, MMU 17 (HSA13), MMU 20 (HSA16), and MMU18 (HSA18). The homolog of HSA 21 is part of a larger chromosome in the macaque (MMU3) that also contains HSA 7 (based on nomenclature by Cambefort *et al.* 1976 *Ann. Genet.* 19, 5–9). Since aneuploidy has been linked to chromosome size and MMU3 is large, this macaque chromosome may be involved in aneuploidies. In addition, we determined the suitability of commercially available “chromosome paints” that target repetitive sequences and are widely used in determining

karyotypes in human fertility clinics. Initial work was performed on cultured macaque blood cells exposed to Colcemid. Our preliminary data clearly demonstrate the feasibility of using probes that are commonly used in the analysis of human karyotypes to examine chromosomal anomalies in macaque gametes and embryos. Additional probes for other chromosomes are currently under investigation. The development of a large panel of available probes will provide a rapid and accurate method to assess aneuploidy/mosaicism levels in genetically valuable primate spermatozoa, *in vitro*-matured oocytes and blastomeres of IVP embryos. Furthermore, these probes will be useful to determine the karyotypic normalcy of cultured non-human primate embryonic stem cells.

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243 PREGNANCY RATES IN THE FIELD AFTER THE TRANSFER OF BOVINE IVP EMBRYOS VITRIFIED BY THE CRYOLOGIC VITRIFICATION METHOD

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Although large numbers of IVP embryos can be produced from donor cattle in a short period of time, commercial acceptance of the technology depends on the ability to cryopreserve these embryos and achieve a 50% pregnancy rate in large-scale embryo transfer programs. Many studies have reported low pregnancy rates of about 20% after the transfer of cryopreserved IVP embryos. We have developed the user friendly CryoLogic Vitrification Method (CVM) that vitrifies embryos on a solid surface at -196°C and warms them rapidly in a one-step procedure prior to transfer (Lindemans *et al.* 2004 *Reprod. Fertil. Dev.* 16, 174). We present an overview of the pregnancy rates in the field after vitrification of bovine IVP embryos by the CVM. The *bos taurus* IVP embryos in southern Australia (bT) and the *bos indicus*-based IVP embryos in northern Australia (bI) were produced by our standard TVR and IVP methodology (Fry *et al.*, 2003 *Theriogenology* 59, 446). Pregnancy was determined by rectal palpation between Day 40 and Day 90 and differences between treatments were analyzed by chi-square. The development of the CVM has enabled the successful cryopreservation of bovine IVP embryos. In the laboratory the typical survival (90% re-expansion) and development (80% hatching) of IVP embryos post-vitrification is high and, as demonstrated here, the pregnancy rates after transfer are approaching commercially acceptable levels. However, further research is required to identify factors that may influence success under full field conditions, for both the IVP and the vitrification technologies.

Table 1. Pregnancy rates for fresh IVP, CVM-vitrified IVP, and traditionally flushed embryos cryopreserved in glycerol

Embryos	Fresh IVP (%)	CVM vitrified IVP (%)	Traditional glycerol (%)
bT calf	28/52 = 54	21/49 = 43	
bT heifer	31/69 = 45	38/86 = 44	
bT calf		11/18 = 61	
bI heifer		17/44 = 39	3/9 = 33
		21/44 = 48	
		152/439 = 35	71/133 = 53 (P < 0.01)
		10/62 = 16	
bI calf/cow		28/60 = 47	8/11 = 72
		10/63 = 16	
	4/11 = 22	11/50 = 22	
bI cow	17/30 = 57		
bI cow	23/50 = 46	16/50 = 32	
bI cow	27/54 = 50	10/46 = 22 (P < 0.01)	
	5/15 = 33		

244 EFFECT OF β -MERCAPTOETHANOL IN A TRANSIENT-IVF SYSTEM ON SPERM PENETRATION INTO PORCINE IVM OOCYTES

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This study was carried out to determine the effect of β -mercaptoethanol (bME; at 50 μM) during a brief co-culture and the additional culture of inseminated oocytes on sperm penetration of porcine IVM oocytes. Cumulus-oocyte complexes (COCs) collected from 3–6-mm follicles were cultured in modified NCSU-37 supplemented with bME, eCG, hCG, and dibutyryl cAMP for 20 h and then in the same medium without eCG, hCG, and dibutyryl cAMP for additional 24 h. After IVM, the oocytes were denuded with 0.1% hyaluronidase, randomly divided into three groups (as described below) and fertilized *in vitro*. Treatment 1 (T1, control): Oocytes were co-cultured with fresh spermatozoa for 10 min in modified M199

medium (mM199) supplemented with 5 mM caffeine (+caff) in the absence of bME (bME-), then washed gently with caffeine-free mM199, and cultured for further 9 h in mM199; T2: co-culture for 10 min in mM199+caff bME- and additional culture in mM199 in the presence of bME (bME+); and T3: co-culture for 10 min in mM199+caff bME+ and additional culture in mM199 bME+. At 9 h after insemination, oocytes were fixed and stained for the evaluation of sperm penetration. Results from four replicates were analyzed by ANOVA and Duncan's multiple range test. Sperm penetration rate was lower in T3 (79.8% in 92 oocytes) compared with T1 (95.3% in 84 oocytes) and T2 (93.3% in 93 oocytes). Percentage of eggs with male and female pronuclei in penetrated oocytes was higher in T1 (89.6%) than in T2 (56.4%) and T3 (57.2%). Monospermy rate was lower in T1 (48.9%) than in T2 (59.5%) and T3 (64.3%). These results demonstrated that, although the presence of β -mercaptoethanol during co-culture and the additional culture reduced the fertilization rate, the presence during the additional culture period affected the process of fertilization and increased the incidence of monospermy at 9 h after insemination. In conclusion, the progression of sperm penetration may be controlled by utilization of β -mercaptoethanol in porcine IVF system.

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245 OOCYTE RECOVERY BY OVUM PICK UP AND EMBRYO PRODUCTION IN MURRAH AND NILI-RAVI BUFFALOES (*BUBALUS BUBALIS*) IMPORTED IN CHINA

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In this preliminary study, *in vitro* embryo production and cryopreservation in two river type buffaloes (Murrah and Nili-Ravi) imported into China have been carried out. The objective of the study was enhancement of the genetic merit and productive performances of imported river buffaloes in conjunction with the utilization of local swamp buffaloes. In order to improve milk and meat production in China local swamp buffaloes ($2n = 48$), which are the predominant subspecies, have been crossbred with imported river buffaloes (Murrah and Nili-Ravi: $2n = 50$). At present, several hundred thousand crossbred heads have been produced, and although both males and females can reproduce with $2n = 49$ crossbred buffaloes, their reproductive performances are significantly reduced when compared to $2n = 50$ buffaloes. As an alternative approach, a program of embryo production in river buffaloes and transfer into both river and swamp buffaloes has been implemented at the Guangxi Buffalo Research Institute, in Nanning, P.R. China. Some preliminary results are presented: from a start-up experiment, a total of 46 river buffaloes were subjected to 2 to 3 ovum pickup sessions at 4-day intervals. A total of 750 antral follicles were punctured and 495 (66%) cumulus-oocyte complexes (COCs) were retrieved. Only COCs characterized by at least one layer of granulosa cells ($n = 451$; 91.1%) were considered for *in vitro* maturation (IVM). COCs were matured in TCM 199 + 10% FCS, 0.5 μ g/mL FSH, 5 μ g/mL LH, and 1 μ g/mL estradiol in the presence of cysteamine (50 μ M) at 39°C under 5% CO₂ in humidified air for 24 h. Of the initial 451 COCs matured, only 277 could be considered for *in vitro* fertilization (IVF). IVF was performed at 39°C under CO₂ in humidified air in TALP medium supplemented with 0.2 mM penicillamine, 0.1 mM hypotaurine, and 0.01 mM heparin. Frozen/thawed sperm from a tested bull was treated by swim-up procedure and used at a final concentration of 20 million/mL. Following 20 to 22 h of co-incubation, presumptive zygotes were cultured in SOF medium, supplemented with essential and non-essential amino acids and 8 mg/mL BSA, in a gas atmosphere of 5% CO₂, 7% O₂, and 88% N₂. A total of 41 (14.8%) blastocysts were produced, of which 33 were vitrified and 8 transferred immediately into available swamp and river buffalo recipients. Two calves were born (25%) from the transfer of fresh embryos into one river and one swamp buffalo. *In vitro* embryo production in the buffalo species is still characterized by a high degree of variable results. However, these preliminary results reinforce the need to implement newly developed reproductive technologies not only for speeding up genetic gain of already productive species, but also for the utilization of local breeds characterized by reduced productive performance.

246 FOLLICULAR DEVELOPMENT AND OOCYTE QUALITY AFTER OVUM PICKUP IN DONOR COWS

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The present study was designed to assess the renewal of follicular development and oocyte quality after ovum pickup (OPU) in Holstein dry cows. Cows were kept under the same feeding and environmental conditions. In Experiment 1, follicle aspiration (more than 2 mm) by OPU using a 7.5 MHz linear transducer with needle (cova needle, Misawa Medical, Tokyo, Japan) connected to an ultrasound scanner (SSD-1200, ALOKA, Tokyo, Japan) was performed in four cows. After OPU ovaries were observed from Day 4 (Day 0 = the day of OPU) to Day 11 to assess the number of follicles that developed. In Experiment 2, two sessions of OPU ($n = 11$) were performed with a 7-day interval between to assess the quality of developing follicles and oocytes. Oocytes were evaluated by their cumulus cell morphology, cytoplasmic color, and density. To assess the developmental competence of oocytes, collected cumulus-oocyte complexes (COCs) were cultured for 20 h in TCM-199 supplemented with 5% calf serum (CS) in a microdroplet (volume was adjusted to 5 μ L/oocyte) at 38.5°C under atmosphere of 5% CO₂ 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.* (2002 J. Vet. Med. Sci. 64(10), 887–891). The putative zygotes were then cultured in CR1aa supplemented with 5% CS under the same conditions as maturation culture for nine days. Embryo development was assessed by the cleavage rate on Day 2 and the blastocyst formation rate on Days 7 to 9 (the day of insemination = Day 0). Data were analyzed by ANOVA or Student's *t*-test. In Experiment 1, the mean number of developing follicles (larger than 2 mm in diameter) were increased from Day 4 to Day 11 (Day 4: 19.8 ± 10.0 , Day 7: 32.5 ± 9.5 ; Day 11: 39.5 ± 10.7 (mean \pm SD), respectively, $P < 0.05$). In Experiment 2, the mean number of developing follicles and collected oocytes on the day of OPU were significantly ($P < 0.05$) different between the first and second sessions (54.2 ± 12.4 and 40.8 ± 12.7 , 45.7 ± 20.2 and 27.7 ± 8.7 , respectively). The percentage

of Grade 1 and 2 oocytes for the first session was significantly lower ($P < 0.05$) than those for the second session (59.1 ± 8.4 and 69.0 ± 11.8), and no significant differences were found within cleavage and blastocyst rates. The mean numbers of blastocysts obtained per session were 14.2 ± 8.9 and 9.7 ± 6.3 in the first and second sessions, respectively. These results indicate that populations of follicles were increased till Day 11 after OPU, and proportion of normal oocytes were increased in the renewal follicles.

247 USING THE ACIDIC ORGANIC BUFFER MES TO CLEAN IVP BOVINE EMBRYOS FROM FOOT AND MOUTH DISEASE VIRUS

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The risk of transmitting foot and mouth disease virus (FMDV) by *in vivo*-produced embryos is extremely small. *In vitro* produced (IVP) bovine embryos carry a greater risk of transmitting FMDV. IVP Day 7 embryos, exposed to FMDV cannot be freed of the virus by washing (Marquant-LeGuienne *et al.* 1998 *Theriogenology* 50, 109–116). Similarly, IVP embryos exposed to FMDV during IVF cannot be freed of FMDV by washing (Jooste *et al.* 2003 *Theriogenology* 59, 443). The aim of this study was to test if bovine IVP embryos, exposed to FMDV during IVF and IVF, can be rendered free of infective FMDV by treating them with 2-(Morpholino)ethanesulfonic acid (MES) at a pH of 5.5. MES is an organic buffer with pKa 6.1 (MES, Sigma, South Africa, M2933). Four groups of 300 and two groups of 320 oocytes were obtained from ovaries from a local abattoir. Oocytes were matured, fertilized and cultured as described earlier (Jooste *et al.* 2003 *Theriogenology* 59, 443). Virus was added at a concentration of 2×10^6 TCID₅₀ in all treatments. Treatment with MES was between 30 and 60 s in all cases. After exposure to MES, oocytes were washed 5 times in IVF, IVF, or IVC medium. In all groups, virus detection was attempted by PCR and on pig kidney cell monolayers. Group 1 was not exposed to MES and not spiked with FMDV (control). In Group 2, FMDV was added during IVF and IVF. FMDV detection was attempted after the denuded presumptive zygotes were exposed to MES. Group 3 was treated as group 2, except that presumptive zygotes in this group were cultured up to Day 7 before virus detection was attempted. In Group 4, COCs were treated with MES after IVF and viral detection attempted. Group 5 was treated as group 4, but COCs were subjected to IVF. FMDV was added again during IVF. Denuded presumptive zygotes were again treated with MES and viral detection was attempted. Group 6 was treated as group 5, except that virus detection was not attempted after denuding, but presumptive zygotes were cultured up to Day 7 before virus detection was attempted. No FMDV could be demonstrated in the control group. All denuded oocytes/embryos that were treated with MES for 30–60 s were negative for the presence of infective FMDV. FMDV could be detected in all COCs. Treatment with MES is ineffective in rendering COCs free from infective FMDV. Treatment with MES did not negatively affect the blastocyst rate (chi-square test, $P < 0.05$) in this trial. Blastocyst rates were 8.3% (28/300), 10.3% (31/300), and 9.3% (28/300) for Group 1 (control), Group 3, and Group 6, respectively; the low blastocyst rate was ascribed to the production of embryos in a non-designated IVF laboratory. It thus appears that FMDV is protected by the cumulus cells. It is not clear if the virus infects the cumulus cells per se or if it is protected in the intercellular spaces. It also appears that FMDV does not penetrate the zona pellucida and if the surface of the zona pellucida is exposed to an acidic environment, FMDV can be inactivated.

248 EMBRYO DEVELOPMENT IN MICRODROPS AND MICROCHANNELS: COMPARISON OF NCSU23 SEQUENTIAL AND NON-SEQUENTIAL CULTURE MEDIUM

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The *in vitro* culture systems used to produce pig embryos generally result in few embryos developing to the blastocyst stage. The use of pyruvate (pyr) and lactate (lac) during the culture of zygotes to the 8-cell stage followed by glucose (glu) supplementation replacing pyr and lac appears to be beneficial for embryo development in the pig. The aim of this study was to compare the embryo development rates from pig oocytes fertilized with and without cumulus cells in 100- μ L microdrops (MD) and cultured in 100- μ L MD or microchannels (MC), using NCSU23 containing 8 mg/mL of BSA and supplemented with (1) glu or (2) pyr/lac or (3) pyr/lac for the first three days and then with just glu for the remainder of culture period (pyr/lac-glu). Sow oocytes were matured in TCM199 supplemented with gonadotropins for the first 22 h, and for an additional 22 h without hormones. After 44 h of maturation, oocytes were placed in MD of modified tris-buffered medium to be fertilized using 3×10^5 sperm/mL. Oocytes were divided into two groups for fertilization: with and without cumulus cells. Following 6 h of fertilization, all inseminated oocytes were washed, divided into groups of 15, allotted to the three culture media treatment groups as described above, and incubated in either MD or MC. With the exception of one treatment there were no significant differences in development rates among embryos cultured in MD or MC, hence data were pooled from these two culture devices. Only oocytes fertilized without cumulus cells and cultured in pyr/lac in MC appeared to have lower rates of blastocyst formation (11.67%) than those cultured in MD (26.67%) in the same culture medium. When the six treatments were compared, oocytes fertilized with cumulus cells and cultured in glu had significantly higher ($P < 0.05$) blastocyst rates and hatching rates compared with the other treatments, with the exception of those fertilized without cumulus cells and cultured in pyr/lac-glu. There were no significant differences among other treatments in Day 7 blastocyst or in Day 9 hatching rates. In conclusion, both culture devices can be used to reach similar blastocyst rates with different treatments. In this experiment, the removal of cumulus cells before fertilization appeared to enhance embryo development *in vitro* when sequential media are used. On the other hand, the presence of cumulus cells before fertilization seems to enhance embryo development when non-sequential glu medium is used.

Table 1. Embryo development rates on Day 9 for three different culture treatments

Fertilization treatment	Culture treatment	Total oocytes	Blastocysts (%)	Hatched blastocyst (%)
With cumulus	Glu	135	29.6 ^a	8.9 ^a
Without cumulus	Glu	150	16.7 ^b	2.7 ^b
With cumulus	Pyr/lac	120	19.7 ^b	3.3 ^b
Without cumulus	Pyr/lac	150	20.7 ^b	5.3 ^b
With cumulus	Pyr/lac-Glu	120	17.5 ^b	3.3 ^b
Without cumulus	Pyr/lac-Glu	135	21.5 ^b	5.9 ^b

^{a,b} Means with different superscripts in the same column represent significant differences.

249 EFFECT OF REFREEZING BULL SEMEN ON IVF SUCCESS RATE

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Cryopreserved semen from valuable sires may be available in limited quantities in some situations. A large percentage of the spermatozoa in a thawed straw is potentially wasted since a relatively small number of spermatozoa are required for most assisted reproduction techniques. The goal of this study was to determine the effect of dilution and refreezing of bull semen on fertilization and blastocyst development rates following *in vitro* fertilization. The hypothesis was that frozen bull semen that was thawed, diluted, and refrozen could be used successfully for IVF. Oocytes were harvested from cow ovaries collected from a local abattoir and matured *in vitro* for 24 hours. Ova were subsequently assigned to one of four *in vitro* fertilization treatment groups. Group 1 ova ($n = 158$) were fertilized with bull semen frozen at a concentration of 20×10^6 spermatozoa per 0.25 mL straw. Group 2 ova ($n = 157$) were fertilized with semen frozen at an initial concentration of 2×10^6 spermatozoa. Group 3 ova ($n = 157$) were fertilized with semen that had been thawed and refrozen at a concentration of 20×10^6 spermatozoa. Group 4 ova ($n = 150$) were fertilized with semen that had initially been frozen at a concentration of 20×10^6 spermatozoa and then thawed, diluted to a concentration of 2×10^6 spermatozoa, and refrozen. IVF was performed in a medium volume of 100 μ L using 1×10^6 spermatozoa/mL. Cleavage and blastocyst rates were determined 2 days and 7 days, respectively after IVF. Cleavage rates following IVF was highest with semen frozen at 20×10^6 spermatozoa (89.9%), intermediate with semen frozen at 2×10^6 spermatozoa or refrozen at 20×10^6 spermatozoa (71.3% and 73.9%, respectively), and lowest with semen refrozen at 2×10^6 spermatozoa (38.7%) ($P < 0.05$). Blastocyst development rate was not significantly different ($P > 0.05$) among treatment groups. This study confirmed the hypothesis that refrozen bovine semen can be used successfully for *in vitro* fertilization. Although the overall IVF efficiency was lower using diluted refrozen semen, multiple IVF procedures could theoretically be performed over time from one initial straw. Consequently, if a limited amount of frozen semen is available, thawing of a single straw followed by dilution, re-allocation into multiple straws, and refreezing should be considered to facilitate the more efficient use of semen in future assisted reproduction endeavors.

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250 DEHYDRATED COCONUT WATER FOR *IN VITRO* SPERM CAPACITATION IN SWINE

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Recently, progress has been achieved in reproduction biotechnology with coconut water as semen extender for domestic animals and in bovine oocyte *in vitro* maturation and embryo culture. The aim of this study was to evaluate the dehydrated coconut water (ACP250) as semen extender and its effect on *in vitro* sperm capacitation in swine. Eighteen ejaculates were collected from three crossbred boars of Landrace and Duroc. Semen samples were extended in ACP250 solution or Beltsville Thawing Solution (BTS) and submitted to sperm capacitation after extension (EXT) or after cooling (COO) at 16°C for 24 h. The semen was centrifuged at 400g for 8 min. After centrifugation, the samples were standardized at 2×10^7 spermatozoa/mL, subjected to sperm capacitation in TALP medium with 5 mM of caffeine, and incubated at 38.5°C and 5% of CO₂ in high humidity for three h. Four treatments were tested, T1 (ACP250 + EXT), T2 (ACP250 + COO), T3 (BTS + EXT), and T4 (BTS + COO). Sperm capacitation was evaluated by Coomassie Blue G stain, as follows: capacitated spermatozoa were stained in tenuous blue and not capacitated in intense blue. The Coomassie Blue G staining technique was described by Larson and Miller (1999 Mol. Reprod. Dev. 52, 445–449), who demonstrated that sperm from a variety of mammalian species can be stained by this technique, and an adequate observation of the acrosomal status was possible as well. This procedure was described as simple, fast, inexpensive and reliable, and it requires no fluorescence or DIC optics. For statistical analysis, SAS (System for Windows; SAS Institute, Inc., Cary, NC, USA) was utilized ($P < 0.05$). Comparing the sperm capacitation rates in relation to fresh vs. cooled semen, there were significant differences between T1 (22.3%) and T2 (43.7%) and between T3 (23.4%) and T4 (48.1%) ($P < 0.05$). In relation to BTS or ACP250 extenders, there were no significant differences between T1 and T3 and between T2 and T4 ($P > 0.05$). In conclusion, the ACP250 solution is an option for *in vitro* sperm capacitation in swine, specially with chilled extended semen.

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251 CAMELID EMBRYO DEVELOPMENT *IN VITRO*: EFFECT OF PROTEIN SUPPLEMENT IN MATURATION MEDIUM AND SUBSEQUENT CULTURE IN TWO DIFFERENT MEDIA ON FERTILIZATION AND DEVELOPMENT

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Successful IVM/IVF can be used to produce large number of embryos cheaply for transfer and for manipulations. The technology has not previously been reported for the dromedary. The objectives of this study were to determine the effect of protein supplement [BSA vs. heat inactivated estrous camel serum (OCS)] in the maturation medium and subsequent culture of *in vitro*-fertilized zygotes in TCM199 or G1 and G2 medium (Vitrolife, Gothenburg, Sweden) on the rate of cleavage and development of embryos to blastocysts. Cumulus-oocyte complexes (COC) were collected by puncturing the follicles excised from the slaughterhouse ovaries (Nowshari and Wernery, A.E.T.E. 19th Scientific Meeting, September 12–13th 2003, Rostock, Germany). For IVM, TCM199 supplemented with 0.33 mM pyruvate, 10 µg/mL FSH and LH, and 1 µg/mL oestradiol (maturation medium) was used. The maturation medium was supplemented with either 5 mg/mL BSA or 10% OCS. After 30 to 32 h culture, COC were fertilized with epididymal spermatozoa which was stored at 4°C in TRIS-tes-egg yolk diluent for 1 to 8 days and consisted of not less than 50% motile spermatozoa on the day of use. The spermatozoa were swim up in fertilization medium (TALP, Parrish *et al.* 1985 Theriogenology 24, 537). Oocytes and spermatozoa ($2-4 \times 10^6$) were incubated in the fertilization medium for 14–16 h at 38°C under 5% CO₂ in air. Intact oocytes were removed from the fertilization medium and washed three times in the respective culture medium. Oocytes from two of the maturation treatments were divided into two subgroups and cultured in either medium TCM199 supplemented with 0.33 mM pyruvate and 10% OCS or medium G1 plus 10% OCS at 38°C under 5% CO₂, 5% O₂, 90% N₂. Zygotes in medium G1 were transferred to medium G2 on Day 3. Zygotes were examined for cleavage on Day 2 and further development on Day 8. The results are presented in Table 1. The results indicate that supplementation of maturation medium with BSA or OCS does not affect the rate of cleavage and development of embryos, however, culture of zygotes in sequential medium (G1-G2) affects the cleavage rate ($P < 0.01$) but not the further development of *in vitro*-produced dromedary embryos. Further studies are needed to improve the success of IVF and development during culture in this species.

Table 1.

Maturation medium supplement	Culture medium	No. inseminated	No. cleaved* (%)	No. morulae (%)	No. blastocysts (%)
OCS	TCM	206	30 ^b (15)	10 (33)	5 (17)
OCS	G1–G2	206	65 ^a (32)	28 (43)	7 (11)
BSA	TCM	207	32 ^a (15)	12 (37)	2 (6)
BSA	G1–G2	220	66 ^b (30)	28 (42)	7 (11)

* Cleavage based on initial cleavage on Day 2 of culture.

Percent morula and blastocyst development is based on number cleaved initially.

Values with different superscripts differ ($P < 0.01$, chi-square analysis).

252 COMPARISON OF TWO SPERM SEPARATION PRODUCTS FOR USE IN BOVINE IVF

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In commercial bovine *in vitro* fertilization (IVF) companies, there is a continuous need to improve results. Efforts to maximize *in vitro* embryo production have included modifications in the use of sperm separation gradients. The development of commercially available sperm centrifugation gradients represents a new possibility of increasing the number of viable sperm that can be obtained from low concentration (fresh or frozen, sexed or unsexed) semen samples in order to improve the efficiency of the IVF system to make embryo production as efficient as possible. The objective of this study was to compare two different separation gradients, as follows: Group 1: Percoll (Sigma, St. Louis, MO, USA), in 45% and 90% gradients; Group 2: EquiPure (Nidacon, Gathenborg, Sweden), in top and bottom layers. Before and after separation, sperm were evaluated at 200× magnification for total motility, and then stained to assess viability at 400× with fast-green/eosin stain (Sigma). Sperm separation was performed using frozen/thawed semen from one bull. Semen was separated by centrifugation at 200g for 30 min in both density gradients. Results obtained from Groups 1 and 2 were compared by chi-square test. Sperm separation with Percoll yielded lower numbers of sperm (average sperm concentration after separation of 92×10^6 , vs. 159×10^6 sperm/mL for EquiPure; $P < 0.05$) but resulted in higher motility (60% vs. 39%, respectively; $P < 0.05$) of separated sperm. Rates of live sperm cells were not significantly different between groups (69.5% vs. 70%, respectively; $P > 0.1$). These results indicate that the commercial separation medium EquiPure may be associated with higher sperm concentration levels but with lowered sperm motility when compared to Percoll for bovine sperm separation. However, EquiPure provided similar percentages of live sperm when compared to Percoll, which is currently used in our laboratory.

253 COMPARISON OF THREE DIFFERENT IVF PROTOCOLS FOR BOVINE OOCYTES

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Recently, the development of commercially available defined media and sperm centrifugation gradients has offered new possibilities for increasing the efficiency of commercial *in vitro* fertilization (IVF) systems. The objective of this study was to compare three different IVF protocols using two different separation gradients, two fertilization media, and two embryo culture media, as follows: Group 1. sperm separation (SS): Percoll (Sigma, St. Louis, MO, USA), fertilization medium (FM): TALP-Fert (TFM), embryo culture media (ECM): G1/G2 (version 3, Vitrolife, Englewood, CO, USA). Group 2. SS: Percoll, FM: Bovine vitro Fert (Cook, Brisbane, Australia), ECM: Bovine vitro Blast/Bovine vitro Cleave (Cook); and Group 3. SS: EquiPure (Nidacon, Spectrum Technologies, Healdsburg, CA, USA), FM: TFM, ECM: G1/G2. Oocytes were obtained from slaughterhouse ovaries and matured *in vitro* (Looney *et al.* 1994 Theriogenology 41, 67). IVF was conducted using frozen/thawed semen from one bull. Semen was separated by centrifugation at 700g for 30 min in the given density gradients; Percoll was used in a 45% to 90% gradient. Sperm viability after separation was assessed by fast-green/eosin stain (Sigma). IVF was carried out in 0.5 mL of the given fertilization medium supplemented with PHE1 and heparin (10 µg/mL), in humidified 5% CO₂ in air atmosphere at 38.7°C. Final sperm concentration in the IVF wells was 1 × 10⁶/mL. In Experiment 1, a total of 368 oocytes (2 replicates) were fixed and stained (Hoechst 33342, Sigma) 24 h post-IVF to assess sperm penetration (Group 1, n = 128, Group 2, n = 108, Group 3, n = 132). In Experiment 2, a total of 400 embryos (2 replicates) were cultured in 0.5 mL of the given culture medium under mineral oil in a 5% O₂, 5% CO₂, 90% N₂ atmosphere at 38.7°C with high humidity for 112 h before fixation and staining. Embryos in Groups 1 (n = 129) and 3 (n = 139) and Group 2 (n = 132) were changed to G2 and Cleave media, respectively, at 84 h. Sperm separation with Percoll yielded lower numbers of sperm (average sperm concentration after separation of 218 vs. 383 × 10⁶ for EquiPure; P < 0.05), but resulted in higher total motility (60% vs. 41%, respectively; P < 0.05) and higher viability (93% vs. 70%, respectively; P < 0.05) of separated sperm. In Experiment 1, rates of normal fertilization were significantly lower for Group 3 (58%) than for Groups 1 and 2 (74% and 77%, respectively, P < 0.05). In Experiment 2, rates of development to <8, 9 to 16, and >16 cells at 112 h were not significantly different among groups (43, 48, and 46% for Group 1; 22, 18, and 31% for Group 2; and 35, 34, and 23% for Group 3, respectively; P > 0.1). These results indicate that the commercial separation medium, EquiPure, may be associated with lowered sperm motility, viability, and fertilization rates when compared to a standard medium (Percoll) for bovine sperm separation. Commercial fertilization and embryo culture media (Bovine vitro Fert, Cleave, and Blast) provided equivalent embryo development to that currently in use by our laboratory (TFM, G1/G2).

254 EFFECT OF FSH TREATMENT ON NUMBER OF BOVINE OOCYTES RETRIEVED BY NON-ULTRASOUND GUIDED TRANSVAGINAL OVUM PICK-UP AND THEIR DEVELOPMENTAL COMPETENCE

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This study was conducted to investigate the efficacy of FSH stimulation on the number of bovine oocytes retrieved by non-ultrasound-guided transvaginal ovum pickup and their developmental competence. In Experiment 1, to study the effect of FSH treatment on meiotic maturation, oocyte donors were divided into two groups (n = 5/group). Group I received no FSH treatment and aspiration was performed twice weekly, whereas Group II received 200 mg FSH (Folltropin-V) treatment twice daily for 3 days prior to aspiration, which was performed once every two weeks. Both groups were investigated for 8 weeks, after which crossover treatment continued for another 8 weeks. The retrieved oocytes were cultured for 24 h *in vitro*, and the chromosomal stages were evaluated by staining with 1% aceto-orcein. Experiment 2 was designed to determine the effect of FSH treatment on developmental competence of bovine oocytes. Oocytes were obtained from FSH-treated and untreated control cows (n = 3/group), and they were matured, fertilized, and cultured *in vitro*. The results showed that Group II females had a higher number of retrieved oocytes per cow per session than Group I females (7.05 ± 3.88 vs. 2.06 ± 0.99, respectively; P < 0.05). The FSH stimulation led to an increase in the proportion of matured oocytes (69.6% vs. 63.3%) and also increased the percentage of cleavage stage embryos (49.4% vs. 32.2%) compared with untreated control cows (P < 0.05). However, there was no difference between the two groups in percentage of morula and blastocyst formation (11.1%, 8.6% and 10.2%, 6.8%, respectively). In conclusion, the combination of non-ultrasound-guided transvaginal ovum pickup with FSH stimulation increased the number of retrieved oocytes per cow per session and tended to increase *in vitro* embryo production.

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255 PRONUCLEUS FORMATION IN RAT ZONA-FREE OOCYTES CO-CULTURED WITH HOMOLOGOUS POST-THAW SPERMATOZOA

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The aim of the present study was to develop an IVF system with frozen/thawed rat spermatozoa. We examined the effect of cooling rate to 5.0°C on post-thaw sperm motility and membrane integrity, and also investigated the ability of post-thaw spermatozoa to form pronuclei. Under room temperature, epididymal spermatozoa of Wistar rats were collected in 2.0 mL of egg yolk medium containing 8.0% (w/v) lactose monohydrate and

0.7% (v/v) Equex Stem. Samples were loaded into 0.25-mL straws and cooled to 5.0°C in the chamber of a programmed freezer. For cryopreservation, the samples were exposed to liquid nitrogen (LN) vapor for 10 min and then plunged into LN. Straws were thawed in a 37.0°C water bath for 10 s. Ovulated oocytes were collected and the zona pellucidae were removed with 0.1% pronase. One-hundred μ L of thawed samples were put into a droplet of 400 μ L R1ECM and pre-incubated for 1 h. R1ECM solution was added to the droplet to adjust to $0.5\text{--}1.5 \times 10^6$ sperm mL^{-1} . The zona-free oocytes were then transferred into the droplet and co-cultured for 10 h. Oocytes were observed for pronuclei formation by means of an inverted phase contrast microscope. In Experiment I, the influence of sperm cooling rate to 5.0°C on sperm motility and membrane integrity was evaluated. Portions of samples were cooled at 54.0°C/min, 0.9°C/min, 0.5°C/min, and 0.3°C/min. The remainders were then frozen. The non-cooled samples were designated as controls. In Experiment II, we examined whether post-thaw spermatozoa have the ability to form pronuclei *in vitro* or not. All percentage data were arc-sine transformed and then analyzed by the Student's *t*-test. In Experiment I, the membrane integrity between the spermatozoa cooled at 0.5°C/min and the non-cooled spermatozoa was not different (38.1% vs. 37.2%; $P > 0.05$), but the integrity of these was higher than in spermatozoa cooled directly at 54.0°C/min (38.1% vs. 25.3%; $P < 0.05$). After culture for 1 h, the motility of spermatozoa cooled at 0.5°C/min was higher than that of those cooled at 54.0°C/min (61.3% vs. 53.3%; $P < 0.05$). At 2 h post-thaw the motility of spermatozoa cooled at 0.5°C/min was higher than that of spermatozoa cooled at 54.0°C/min and at 0.9°C/min (11.0% vs. 4.5%, 4.9%; $P < 0.05$). The membrane integrity of post-thaw spermatozoa cooled at 0.5°C/min was also higher compared to that of spermatozoa cooled at 54.0°C/min (22.5% vs. 8.4%; $P < 0.01$). In Experiment II, 28 (26.2%) of 107 oocytes had pronuclei when the post-thaw spermatozoa cooled at 0.5°C/min were used. The results indicated that the frozen/thawed spermatozoa cooled to 5.0°C at 0.5°C/min showed higher sperm motility and membrane integrity, and that spermatozoa can form pronuclei in homologous zona-free oocytes *in vitro*. Although in the rat sperm damage occurred during cooling to 5.0°C, and sperm motility and membrane integrity were also decreased by the cold shock, it is possible to decrease the damage by cooling slowly to 5.0°C at 0.5°C/min.

256 COMMERCIAL-SCALE *IN VITRO* PRODUCTION AND TRANSFER OF JAPANESE BLACK EMBRYOS

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In vitro fertilized (IVF) Japanese Black embryos have been preferably transferred to Holstein recipients for beef cattle production in Japan. We have been commercially producing IVF embryos, aiming at increased productivity and hereditary improvement of Japanese Black cattle. However, the use of IVF embryos has been implicated in reduced pregnancy rates as well as in increased risk of abortion and oversized newborns. In this report, we present our cumulative data that summarize the outcome of IVF embryo transfer. The IVF embryos were produced as previously described by Hamano and Kuwayama (1993 *Theriogenology* 39, 703) using semen collected from five stud bulls. Fertilized oocytes were developed to the blastocyst stage by co-culture with cumulus cells. Developed blastocysts were transported to farms all over Japan in two conditions: (1) non-frozen via air or ground shipping using cell transporters, or (2) cryopreserved in glycerol for direct transfer. Both methods were used at approximately the same rate. We transferred 90% of these embryos to Holstein heifers and 10% to hybrids. Although the average pregnancy rate at the farms was initially low (31%), it has exceeded 40% in recent years. After pregnancy diagnosis, abortion occurred in 9% of these cases. We compared 109 pregnancies from *in vivo* embryos with 824 from IVF embryos obtained during the same period: the average gestation period was 281.4 and 283.6 days, respectively, the latter being longer by 2.2 days. The average birth weight from IVF was 33.8 kg, 1.3 kg heavier than that of *in vivo*-derived calves (32.5 kg). In addition, oversized newborns (>50 kg) were absent with *in vivo* embryos, whereas it occurred in 3.1% of the IVF calves with an average gestation periods of 291.3 days. These gestation period were significantly longer ($P < 0.05$) than for the normal sized newborn groups. Our study suggests that the IVF technology can be effectively used to increase beef production in Japan and that oversized newborns are associated with delayed parturition. Due to the improved meat productivity obtained with calves derived from these embryos, our sales further increased with 9014, 9334, and 11,932 embryos sold in 2001, 2002 and 2003, respectively.

257 EFFECTS OF GLUTAMINE AND HYPOTAURINE ON OXIDATIVE STRESS OF PORCINE EMBRYOS CULTURED *IN VITRO*

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We previously developed an *in vitro* production system for porcine embryos and reported that the addition of glutamine and hypotaurine during *in vitro* culture improved blastocyst yield and the total number of cells in the blastocysts. Glutamine and hypotaurine might reduce oxidative stress, allowing the development of embryos cultured *in vitro*, because glutamine reportedly protects embryos against oxidative stress by helping to maintain intracellular levels of cysteine, a precursor of glutathione (GSH), and hypotaurine is a potent antioxidant. In the present study we evaluated the effects of the presence of glutamine and hypotaurine from Day 2 (Day 0 = the day of *in vitro* fertilization) to Day 3 on oxidative stress during *in vitro* development of porcine embryos. Porcine cumulus-oocytes complexes from prepubertal gilts were matured and fertilized *in vitro* using frozen-thawed ejaculated boar semen (Yoshioka *et al.* 2003 *Biol. Reprod.* 69, 2092–2099). Presumptive zygotes were cultured in porcine zygote medium (PZM)-5 (Suzuki *et al.*, 2002) containing 2 mM of glutamine and 5 mM of hypotaurine as a basal culture medium until Day 2. The cleaved embryos were then transferred into one of four media prepared as follows: (1) containing no glutamine or hypotaurine (G–H–), (2) containing glutamine (G+H–), (3) containing hypotaurine (G–H+), (4) containing glutamine and hypotaurine (G+H+) (= PZM-5), and cultured for 24 h. After culture, the total number of cells, intracellular GSH content, and level of hydrogen peroxide (H_2O_2), which is a reactive oxygen species, in the cleaved embryos were examined. Some cleaved embryos were cultured in PZM-5 from Day 3 until Day 5 and the percentage of embryos that developed into blastocysts and the total number of cells in the blastocysts were investigated. Intracellular GSH content and H_2O_2 level on Day 3 were determined by a dithionitrobenzoic acid-glutathione disulfide (DTNB-GSSG) reductase recycling assay and dichlorohydrofluorescein diacetate

(DCHFDA)-based assay, respectively. Data were statistically analyzed by ANOVA and Fisher's PLSD test. The total number of cells (4.3 to 4.4 cells) and intracellular GSH content (2.3 to 2.9 pmol/embryo) in the cleaved embryos on Day 3 did not differ among treatments. On Day 3, the intracellular H₂O₂ level of the cleaved embryos cultured in G+H+ decreased by 49% compared with those cultured in G-H- (100%) ($P < 0.05$). On Day 5, the percentage of embryos that developed into blastocysts in G+H+ (52%, 47/90) and G+H- (41%, 36/88) was significantly higher than in G-H- (11%, 11/90) and G-H+ (21%, 19/89) ($P < 0.05$). The total number of cells in the Day 5 blastocysts from G+H+ (34.5 cells) was significantly ($P < 0.05$) greater than in those from G-H- (25.8 cells). These results suggest that the presence of glutamine and hypotaurine in PZM-5 from Day 2 to Day 3 improves the subsequent development of porcine embryos into blastocysts by reducing intracellular H₂O₂ levels.

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258 APOPTOSIS IN FAST- AND SLOW-CLEAVING BOVINE EMBRYOS *IN VITRO*

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Fast-cleaving embryos have significantly higher chances of reaching advanced developmental stages, and hatched blastocysts developed from fast-cleaving embryos have higher total cell and inner cell mass cell numbers. Few data are available on the prevalence of apoptosis in blastocysts resulting from fast-cleaving bovine embryos. Therefore, it was the aim of this study to search for a correlation between this early quality marker (timing of first cleavage) and a later quality marker (apoptosis in blastocyst stage). A total of 836 immature bovine oocytes were matured and fertilized *in vitro*. Presumed zygotes were denuded 24 h after fertilization and cultured in 50 μ L droplets of modified SOF medium without serum at 39.0°C in 5% CO₂, 5% O₂, and 90% N₂. At 30 h, 36 h and 48 h post-fertilization (PF), zygotes that had developed to the 2-cell stage or beyond were placed in new droplets and cultured in separate groups. In each of the 3 replicates a control group of 100 presumed zygotes was cultured in SOF medium without manipulation. After 24 h of culture, the SOF medium was supplemented with fetal calf serum (FCS) up to 10%. Blastocysts were evaluated at Days 7 and 8, and fixed in 4% paraformaldehyde. After TUNEL staining, total cell number and TUNEL-positive cells were counted for each group. An univariate analysis of variance was used with % apoptotic cells as the dependent variable, timing of first cleavage as the fixed factor, and replicate as the random factor (mixed model ANOVA). The mean cumulative blastocyst yields in the control group were 20.9% and 27.0% at Days 7 and 8, respectively, which were not different from the total yields from manipulated embryos (Table 1). The cumulative cleavage rates for manipulated embryos were 44.9, 65.1, and 85.7% at 30, 36, and 48 h PF, respectively. The blastocyst yields at Days 7 and 8 PF were declining for embryos which cleaved later, with a distinct drop ($P < 0.05$) between the 36-h and 48-h groups. The percentage of apoptotic cells in Day 7 blastocysts was significantly lower in the 30-h compared with the 36-h and 48-h groups ($P < 0.05$). Within the 30-h and 36-h groups, Day 8 blastocysts had significantly more apoptotic cells than Day 7 blastocysts ($P < 0.05$), but no differences could be detected between groups at day 8. In conclusion, this experiment has confirmed the increased chances of early-cleaving embryos to reach the blastocyst stage. The higher percentages of apoptosis in late cleaving embryos at Day 7 and in all blastocysts at day 8 as determined by TUNEL staining could be an indication of lower blastocyst quality.

Table 1. Blastocyst yield (number and percentage) and percentage of apoptotic cells (APC) at Days 7 and 8 PF

Group	Blastocyst Day 7 PF	Blastocyst Day 8 PF	% APC Day 7 PF (mean \pm SD)	% APC Day 8 PF (mean \pm SD)
30 h PF	89 ^a (32.0%)	112 ^a (40.3%)	9.8 ^a \pm 7.1	20.6 \pm 8.6
36 h PF	32 ^a (24.4%)	49 ^a (37.4%)	11.5 ^a \pm 6.9	19.0 \pm 11.4
48 h PF	6 ^b (6.6%)	6 ^b (6.6%)	14.2 ^b \pm 8.4	–
Total	127 (20.3%)	167 (26.7%)	10.4 \pm 7.1	20.1 \pm 9.6

^{ab} Within a column, values with a different superscript differ significantly ($P < 0.05$).

259 INVOLVEMENT OF Ca²⁺/CALMODULIN-DEPENDENT PROTEIN KINASE II IN THE SPONTANEOUS ACTIVATION IN RAT OOCYTES

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Immediately after recovery from the oviduct, rat metaphase-arrested oocytes are very sensitive to spontaneous activation (SA), which is characterized by resumption of meiotic division followed by the cytoplasmic scattering of chromosomes. Although it has been shown that proteasome inhibitor is effective in stopping SA, it is likely that an upstream event is controlling SA. Constitutively active Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) triggers cyclin B destruction and release from MII arrest. To investigate the kinetics and mechanism of rat spontaneous oocyte activation, we (1) compared SA status after different periods of aging in oviducts and examined the effects of hyaluronidase treatment, (2) compared SA between Ca²⁺-free and Ca²⁺-containing conditions, (3) examined the pattern of SA after using CaMKII inhibitor and proteasome inhibitor, and (4) analyzed the activity of CaMKII at different times after oocyte collection. Oocytes were collected from three 4-wk-old PMSG-primed Sprague

Dawley rats. ANOVA Tukey-Kramer HSD was used for statistical analysis using a P value of 0.05. Each experiment contained at least four replicates with at least 100 oocytes. Experiment 1: Oocytes were collected at different times post-hCG injection (14, 18, 22 h) and cumulus cells were removed before or after 2 h of *in vitro* culture with hyaluronidase. There was no difference in SA due to oocyte aging in oviducts or hyaluronidase treatment. Experiment 2: Oocytes were cultured in Ca^{2+} -containing and Ca^{2+} -free KSOM for 2 h after collection at different times (14, 18, 22 h) post hCG injection. Although there were no differences among Ca^{2+} -containing KSOM culture groups (91%, 82%, and 90%, respectively), Ca^{2+} -free KSOM significantly decreased SA at 14, 18, and 22 h post-hCG (19%, 43%, and 51%, respectively). Experiment 3: Oocytes were treated with different doses of inhibitor of CaMKII, myr-AIP (10, 20, 50, 100 μM), and proteasome inhibitor MG132 (10, 20, 50, 100 μM) after oocyte collection. Fifty and 100 μM myr-AIP induced significantly lower SA (36%, and 17%) than 0, 10, 20 μM (91%, 65%, and 53%, respectively). Moreover, higher concentrations of MG132 (50 and 100 μM) induced significantly lower SA rates (6% and 5%) than 0, 10, 20 μM (91%, 41%, and 22%, respectively). We also investigated the effect of short exposures to myr-AIP and MG132 during the first 2 min post-recovery. The myr-AIP group produced significantly lower SA compared to control and MG132-treated groups. Experiment 4: Oocytes collected at different times (0, 10, 20, 30, 60 and 90 min) were used for CaMKII activity assay. CaMKII activity increased at 20 min and remained high for 30 min followed by decreased activity by 60 min. In conclusion, CaMKII seems to be one of the upstream signals that causes rat oocytes to spontaneously activate after recovery.

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260 REPLACEMENT OF PVA WITH FETAL BOVINE SERUM IMPROVES FORMATION AND HATCHING OF PORCINE BLASTOCYSTS PRODUCED *IN VITRO*

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Porcine embryos, derived from *in vitro* maturation and fertilization, were used to investigate the effects of timing of serum inclusion and PVA replacement in the medium for *in vitro* culture (IVC) on rates of blastocyst formation and hatching. In Experiment 1, presumptive zygotes at 20 h post-insemination (hpi) or cleaved embryos obtained by culture in porcine zygote medium (PZM-5) containing 3 mg mL⁻¹ polyvinyl alcohol (PVA) at 48 or 96 hpi were further cultured in either PZM-5 containing PVA or PZM-5 where PVA was replaced by 1%, 5%, or 10% fetal bovine serum (FBS) until Day 6 (Day 0 = the day of *in vitro* insemination). Supplementation with 1% to 10% FBS at 20 and 48 hpi reduced ($P < 0.05$; by ANOVA and Fisher's PLSD test) blastocyst rates on Days 5 (0% to 1%) and 6 (3% to 6%) compared with PVA supplementation (4% and 22%, respectively). However, addition of 10% FBS at 96 hpi increased ($P < 0.05$) blastocyst rates (30%) on Day 5 compared with PVA (11%) and 1% FBS (15%); there was no significant difference among treatments in rates of blastocyst formation on Day 6 (24% to 40%). The total number of blastomeres in Day 6 blastocysts did not differ among treatments at any timing of serum supplementation (26.5 to 48.3 cells). In Experiment 2, presumptive zygotes were cultured from 20 to 96 hpi in PVA medium, and the cleaved embryos were later transferred into PZM-5 containing PVA, or 1%, 5%, or 10% FBS for another 4 days. Hatching rates of embryos on Days 7 and 8 were significantly higher ($P < 0.05$) in PZM-5 where PVA was replaced with 10% FBS (15% and 20%, respectively) than those in PZM-5 containing PVA (1% and 5%, respectively). Moreover, the total cell number in hatching/hatched blastocysts on Day 8 were significantly greater ($P < 0.05$) in medium containing 10% FBS (135.1 cells) than that in PVA medium (77.0 cells). In Experiment 3, at 130 hpi, blastocysts derived from IVC with PZM-5 containing PVA were transferred into PZM-5 containing PVA, 3 mg mL⁻¹ bovine serum albumin (BSA) or 10% FBS for another 2 days. Hatching rates of blastocysts on Days 6, 7 and 8 were significantly higher ($P < 0.05$) in PZM-5 where PVA was replaced with 10% FBS (12%, 56%, and 64%, respectively) than those in PZM-5 containing PVA (0%, 12%, and 20%, respectively) and BSA (0%, 12%, and 20%, respectively). Moreover, the total cell number in hatching/hatched blastocysts on Day 8 were significantly greater ($P < 0.05$) in medium containing 10% FBS (138.7 cells) than that in PVA (71.7 cells) and BSA medium (70.7 cells). The results indicate that the timing of serum inclusion in the culture medium markedly affects porcine embryo development *in vitro* and that replacement of PVA with FBS in PZM-5 at 96 hpi or later improves the subsequent development of embryos to the hatching/hatched blastocyst stage.

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Male Physiology

261 FERTILITY CONTROL BY GnRH ANALOGUES IN DOGS

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GnRH plays a pivotal role in reproduction by stimulating the release of gonadotrophins. Chemical substitutions in the GnRH molecule lead to analogues possessing antagonist or agonist activity (Paramo RM *et al.* 1993 J. Reprod. Fertil. Suppl. 47, 387–397). The highly potent agonist analogue, Buserelin, with up to 20 times of potency, by increasing binding affinity, desensitizing competitive receptors, and resisting metabolic degradation, shuts down rather than stimulates reproductive function (Bertschinger HJ *et al.* 2001 J. Reprod. Fertil. Suppl. 57, 275–283). In man, Buserelin is employed in several gonadal hormone-dependent diseases and for prostatic cancers. We suppress gonadal function in male dogs using Buserelin. Eight intact male German sheep dogs 20 months old were divided into two groups; A, 4 subjects treated for pharmacological castration