GOT (91.73 ± 3.59 μ/L) and GPT (16.09 ± 3.23 μ/L) activities were recorded in high yielder and the lowest mean values of GOP (72.58 ± 4.79 μ/L) and GPT (13.05 ± 1.99 μ/L) activities were observed in low yielder cows. Based on the findings of this study, it was concluded that although the occurrence of moderate fatty liver was 41.67%, this could be associated with the infertility condition of crossbred dairy cows. It is quite possible that cows showing mild liver damage were in recovery stage after severe or moderate liver damage, as the postpartum period in cross bred cows under study varied from 1.5 to 8 months.

Embryo Culture

129 RESPIRATION ACTIVITY OF BOVINE EMBRYOS CULTURED IN SERUM-FREE AND SERUM-CONTAINING MEDIA

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Oxygen consumption is a ubiquitous parameter which can provide valuable information about metabolic mechanisms and embryo quality. Recently, we succeeded in non-invasively and quantitatively determining oxygen consumption of individual bovine embryos by the scanning electrochemical microscopy (SECM). The aim of this study was to assess by SECM the oxygen consumption of individual bovine embryos at different developmental stages cultured in serum-free and serum-supplemented media. Bovine oocytes were matured in IVMD101 medium [Research Institute for the Functional Peptides (IFP), Shimojo, Yamagata, Japan] and inseminated in BO-based medium. For serum-free culture, inseminated ooocytes were cultured to the blastocyst stage in IVD101 medium in an atmosphere of a low oxygen condition (5% CO_2/5% O_2/90% N_2) at 38.5°C. For serum-supplemented culture, inseminated oocytes were cultured in HPM199 medium (IFP) supplemented with 5% calf serum (HPM199 + CS) in the presence of bovine cumulus/granulosa cells in a humidified atmosphere of 5% CO_2 in air. Oxygen consumption by individual bovine embryos was non-invasively quantified by the SECM measuring system. Some embryos were prepared for transmission electron microscopy. The oxygen consumption rates are presented in the table. Oxygen consumption rates (F) of the single embryos were low from 2-cell to 8-cell stages (0.45–0.52 × 10^{-14} mol s^{-1}). In serum-free culture, an increase in oxygen consumption rate was found at the morula (1.03 × 10^{-14} mol s^{-1}) stage, and blastocysts showed an even higher oxygen consumption rate (1.86 × 10^{-14} mol s^{-1}). On the other hand, the oxygen consumption of morulae and blastocysts produced in serum-supplemented medium was lower than that of embryos cultured in serum-free medium. Electron microscopic study demonstrated that many of the mitochondria of morulae and blastocycts cultured in HPM199 + CS medium were an immature form, indicating a correlation between respiration activity and development of mitochondria. These results suggest that the culture conditions affect the respiration activity of bovine embryos. The SECM procedures may have a wide application for judging embryo quality and culture conditions for embryos.

Table 1. Oxygen consumption rates (F × 10^{-14} mol s^{-1}) of the bovine embryos at various developmental stages

Embryonic stage	IVD101	HPM199 + CS
2 cell	0.46 ± 0.05 (17)	0.52 ± 0.04 (6)
4 cell	0.45 ± 0.03 (17)	0.47 ± 0.04 (6)
8 cell	0.46 ± 0.02 (10)	0.52 ± 0.04 (10)
Morula	1.03 ± 0.05 (27)	0.70 ± 0.05 (12)*
Blastocyst	1.86 ± 0.07 (21)	1.33 ± 0.10 (12)*

* Significance of differences compared with IVD101 ($P < 0.05$).
The numbers in parentheses represent the numbers of embryos examined.

130 THE EFFECT OF ALTERED ENERGY SUBSTRATE CONCENTRATIONS ON THE DEVELOPMENT OF DIPLOID PARTHENOGENETIC PORCINE EMBRYOS CREATED FROM OOCYTES FROM GILTS AND SOWS

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There is building evidence that altering the concentration of energy substrates in the culture medium to more closely approximate the concentrations thought to be present in the reproductive tract improves porcine embryo development. The aim of this experiment was to examine the development of porcine parthenogenetic embryos in such a modified version of NCSU23. The embryos were created from both sow- and gilt-derived oocytes to see whether the source of oocytes influences how the embryos respond. Ovaries from slaughtered sows or prepubertal gilts were collected, follicles (3–6 mm) were aspirated and oocytes surrounded by at least three layers of compact cumulus cells were collected and matured in TCM199 containing cysteamine, insulin, FSH, EGF, and 10% sow follicular fluid, for approximately 40 h. Cumulus cells were removed and good quality mature oocytes

with a visible polar body were electrically activated (approximately 44 h after the start of maturation) by 2 DC pulses (1.5 $kVcm^{-1}$, 60 μs) applied 1 s apart, cultured in NCSU23 + 7.5 μg/mL cytochalasin B for 3 h and then placed into the treatment droplets. All long-term culture was in 50-μL droplets under mineral oil in an atmosphere of 5%CO_2, 5%O_2, 90%N_2 at 38.5°C for a total of 6 days (144 h). The treatments were: NCSU23 (N23); a modified NCSU23 containing 0.6 mM glucose, 0.2 mM pyruvate, and 5.7 mM lactate (PLG); or the modified NCSU23 for the first 48 h and then NCSU23 for the remaining 4 days (dPLG). All treatments were transferred into fresh droplets of the appropriate medium at 48 h. Heat inactivated fetal bovine serum (10%) was added early on Day 5. On Day 6, the embryos were assessed for morphological development and those embryos judged to be good blastocysts were stained for cell count. Experiments using sow or gilt oocytes were conducted and analyzed separately. Morphological data were analyzsed by χ^2 and cell number data by ANOVA with the LSD test used to determine differences between treatments. There were no statistical differences in the percentage of activated sow or gilt oocytes developing to blastocysts (sow: N23 51.3%, PLG 48.9%, dPLG 55.1%; and gilt: N23 25.6%, PLG 38.2%, dPLG 38.6%). The blastocysts produced in the dPLG treatment contained significantly more cells than those produced in the N23 treatment for both sow- (mean ± SEM; N23 47.1 ± 2.1; dPLG 53.8 ± 1.7; $P < 0.001$) and gilt (N23 39.3 ± 3.4; dPLG 50.5 ± 3.0; $P < 0.05$)-derived embryos. The number of cells per blastocyst produced in the PLG treatment did not differ significantly from the other treatments (sow: 49.9 ± 2.0; gilt: 43.4 ± 2.8). These data demonstrate that culturing for the first 48 h with the modified energy substrate concentrations improved the cell number of the resulting blastocysts but did not affect the proportion developing into blastocysts by day 6 of culture. This effect appeared to be consistent whether sow- or gilt-derived parthenogenetic embryos were used.

131 MODIFICATION OF AMINO ACID CONCENTRATIONS IN MEDIUM BY PIG EMBRYOS FROM THE ZYGOTE TO THE BLASTOCYST STAGE

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Pre-implantation embryos can produce and consume amino acids in a manner dependent upon stage of embryonic development (Partridge and Leese 1996 Reprod. Fert. Dev. 8, 945) that may also be predictive of subsequent viability (Houghton *et al*. 2002 Hum. Reprod. 17, 999). To examine these relationships in the pig, the appearance or depletion of 18 amino acids from a presumptive near-physiological mixture was determined by HPLC in porcine *in vitro*-produced embryos from the zygote to the blastocyst stage. Cumulus oocyte complexes derived from slaughterhouse prepubertal pig ovaries were matured for 40 h in modified TCM-199 before being fertilized (Day 0) with frozen thawed semen in *tris*-based medium. After 6 h, presumptive zygotes were denuded and cultured in groups of 20 in NCSU medium modified to contain a physiological mixture of 18 amino acids including 0.1 mM glutamine (NCSUaa). Groups of 2–10 embryos (dependent on stage) were removed on Day 0 (1 cell), Day 1 (2- and 4-cell), Day 4 (compact morula), and Day 6 (blastocyst) and placed in 4 μL NCSUaa for 24 h. After incubation, the embryos were removed and the medium analyzed by HPLC. Each stage was replicated 3–9 times. Since amino acid profiles of 2- and 4-cell embryos were not different, data were combined. Overall, arginine (1.19 ± 0.33), glutamine (0.78 ± 0.34) and threonine (0.05 ± 0.04) were significantly ($P < 0.01$) depleted from the medium whereas alanine (0.21 ± 0.1), glycine (0.20 ± 0.06), asparagine (0.13 ± 0.5), lysine (0.1 ± 0.03), isoleucine (0.08 ± 0.01), valine (0.05 ± 0.01), leucine (0.04 ± 0.02), phenylalanine (0.03 ± 0.01), and histidine (0.02 ± 0.04) significantly ($P < 0.05$) accumulated (mean of the 4 sampling timepoints; all values pmol/embryo/h ± SEM). The difference between amino acid accumulation and depletion (balance) was approximately equivalent between Day 0 and the morula stage although turnover (sum of depletion and accumulation) steadily decreased during this period from 3.1 on Day 0 to 1.35 pmol/embryo/h at the morula stage. However, at the blastocyst stage, turnover and balance increased to 6.32 and 2.42 pmol/embryo/h, respectively, i.e. net appearance occurred. Notable changes in amino acid profile during development included decreases in accumulation of asparagine, glutamate, and glycine in the medium and the depletion of glutamine over Days 0, 1, and 4, followed by reversal of these trends by Day 6. These data suggest that pig embryos can alter the accumulation and depletion rates of amino acids in a manner that is dependent on the specific amino acid and the stage of embryonic development.

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132 BLASTOCYST DEVELOPMENT OF EQUINE OOCYTES WITH LOW MEIOTIC COMPETENCE HELD IN ROSCOVITINE BEFORE *IN VITRO* MATURATION

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At the time of recovery, immature equine oocytes may be separated into those with either expanded cumuli (Ex) or compact cumuli (Cp). The Cp oocytes originate from viable follicles but are largely juvenile, with low meiotic competence (20 to 30% maturation to MII), and possibly reduced developmental competence. We previously found that in Cp oocytes recovered immediately after slaughter, suppression of meiosis with roscovitine for 24 h before maturation increased embryo development at 4 days after intracytoplasmic sperm injection (ICSI; Franz *et al*. 2003 Reproduction 125, 693–700). The present study was conducted to evaluate the effect of roscovitine suppression on nuclear maturation and blastocyst formation of Cp oocytes recovered after transport of ovaries from the abattoir (i.e. recovered 5–9 h after slaughter). Compact oocytes recovered from transported ovaries were cultured in M199 with 10% FBS containing 66 μM roscovitine with or without an oil cover. After 16–18 or 24 h, oocytes were fixed to examine the chromatin configuration. Treatment for 16–18 h without oil resulted in the lowest rate of meiotic resumption (0%); thus this treatment was utilized in further studies. Resumption in other treatments ranged from 3 to 6%. Following roscovitine suppression, oocytes were cultured for 30 h in M199 with 10% FBS and 5 $\mu U\,mL^{-1}$ FSH for maturation; control oocytes were cultured for 30 h in the same medium immediately after recovery. Mature oocytes were subjected to ICSI, then cultured in DMEM/F-12 with 10% FBS with or without co-culture with equine oviductal epithelial cells under mineral oil in 5% CO_2 in air at 38.2°C, and then evaluated at 7.5 days. Progression to MII (82/376, 22%) after maturation of roscovitine-treated oocytes was similar to that for control oocytes (74/395, 19%). There was no significant difference in cleavage rates after ICSI

(72–78%) among treatments. Development to blastocyst was highest in roscovitine-treated oocytes in DMEM/F-12 with co-culture (11/30, 37%); this was significantly higher than that of non-treated oocytes in DMEM/F-12 alone (5/36, 14%), but similar to that of non-treated/DMEM/F-12/co-culture (10/37, 27%) and roscovitine/DMEM/F-12 alone (8/39, 21%). These data indicate that roscovitine induces a fully reversible meiotic suppression in Cp equine oocytes recovered 5–9 h after slaughter, and that this suppression does not harm subsequent developmental competence. This treatment may be used to manipulate the time of onset of maturation of equine oocytes for ease of subsequent procedures. Co-culture with oviductal epithelial cells tended to increase blastocyst rate ($P = 0.1$, Fisher's exact test) in contrast to our previous findings with embryos from Ex oocytes (Choi *et al*. 2004 Biol. Reprod. 70, 1231–1238). Further work is needed to determine whether this is related to differences in intrinsic developmental competence between oocyte types.

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133 INSULIN, TRANSFERRIN AND SELENIUM WITH OR WITHOUT BSA IN A SERUM-FREE CULTURE SYSTEM FOR BOVINE EMBRYO, AND ITS SUITABILITY FOR EMBRYOS CULTURED IN SMALL GROUPS

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Serum in embryo culture medium may be a potential cause of abnormal accumulation of lipid droplets, which is correlated to a higher sensitivity to cryopreservation. Moreover, serum may introduce pathogens. With the aim of developing a serum-free culture medium, we first (Experiment 1) investigated the effect of adding ITS (5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL selenium) as a serum substitute in SOF medium on embryos cultured in large groups (20 embryos per culture drop of 20 μL) and we then (Experiment 2) analyzed the effect of adding BSA. In this second experiment, our serum-free culture media were also tested on embryos cultured in small numbers (5 embryos per drop of 20 μL) in order to mimic ovum pickup (OPU) conditions. Embryos were obtained from slaughterhouse oocytes, matured *in vitro* for 24 h in a serum-free enriched 199 medium (Donnay *et al*. 2004 Reprod. Fertil. Dev. 16, 274) containing ITS, and fertilized for 18 h. In experiment 1, embryos were cultured in SOF (Holm *et al*. 1999 Theriogenology 52, 683–700) supplemented with 0.1 mg/mL polyvinylpyrrolidane (PVP) without (SOF) or with ITS (SOF-ITS), or with 5% FCS (SOF-FCS). Cavitation occurred earlier in presence of serum (table). Adding ITS to SOF increased blastocyst rates at Day 7 and Day 8 post-insemination (p.i.) and also the hatching rate. In experiment 2, embryos were cultured in SOF-FCS, SOF-ITS, or SOF-ITS supplemented with 4 mg/mL fatty acid free BSA (SOF-ITS-BSA). Within each condition, no differences were observed for blastocyst and hatching rates between embryos cultured in large or in small groups. Adding BSA to SOF-ITS increased blastocyst rate at Day 6 p.i. and also the hatching rate. At Days 7 and 8 p.i., blastocyst rates were higher in SOF-FCS than in SOF-ITS and tended to be higher than in SOF-ITS-BSA, especially for embryos cultured in small groups. Cell numbers of the resulting embryos were unaffected. These results indicate that: (1) ITS as supplement to SOF medium promotes embryo development *in vitro*. (2) BSA as protein supplement to SOF-ITS medium accelerates blastulation and improves hatching rate. (3) SOF-ITS and SOF-ITS-BSA are two serum-free culture media that can sustain development of embryos, also when cultured in small number, even though SOF-FCS tended to afford better rates of development. Further studies will include evaluation of other quality parameters including resistance to cryopreservation.

	Groups	Treatments		% blastocysts			
			n	Day 6	Day 7	Day 8	Hatched/D8
Experiment 1	Large	SOF	241	6^{a}	22^{a}	32^{a}	17^{a}
		SOF-ITS	239	7^{a}	34^{b}	43^{b}	$26^{a,b}$
		SOF-FCS	239	24^{b}	41^{b}	42^{b}	37^{b}
Experiment 2	Large	SOF-ITS	200	10^{e}	31^{e}	37^{e}	20^{e}
		SOF-ITS-BSA	202	18^{f}	$33^{e,f}$	$39^{e,f}$	40^{f}
		SOF-FCS	200	18^{f}	42^{f}	47^{f}	25^{e}
	Small	SOF-ITS	200	7^{x}	27^{x}	35^{x}	19^{x}
		SOF-ITS-BSA	200	16^{y}	28^{x}	41^{x}	39^{y}
		SOF-FCS	200	26^{z}	49^{y}	52^{y}	$30^{x,y}$

Data with different superscripts within a column and a group are significantly different (chi-square, $P < 0.05$).

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134 EVALUATION OF APOPTOSIS IN BOVINE EMBRYOS BY FLUORESCENT LABELING OF CASPASES-3 AND -7

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Reports indicate that apoptosis is more prevalent in *in vitro*-produced (IVP) embryos than in *in vivo*-derived (IVD) embryos. These studies most often utilize the TUNEL procedure to label fragmented DNA. DNA fragmentation, however, is not unique to apoptosis as cells undergoing necrosis also

exhibit substantial DNA fragmentation. Also, improper tissue handling has been reported to induce enough DNA fragmentation for TUNEL-positive staining of nuclei. Thus, the objective of this study was to evaluate the presence of caspases-3 and -7 as indicators of apoptosis in bovine fresh IVD embryos (F-IVD; $n = 32$), frozen-thawed IVD embryos (FT-IVD; $n = 32$) and IVP embryos cultured in CR1aa ($n = 64$). Embryos (quality grades, QG = 1 to 4) were evaluated for apoptotic cells by using a fluorescence-based assay for the detection of active caspaces (Caspatag®, Caspase 3,7 In Situ Assay Kit, Chemicon International, Inc., Temecula, CA, USA). To validate this assay, positive controls (PC) were prepared by incubating IVP-embryos in cyclohexamide for 3 to 4 h prior to staining. After staining, embryos were placed on slides and the number of apoptotic cells/embryo were counted. Embryos in which individual fluorescent points were not apparent were categorized as having profuse staining patterns (PSP) and were assigned an arbitrary apoptotic cell value of 20. Data were analyzed using PROC GLM of SAS with a post hoc LSD test. More than 65% of all embryos displayed at least 1 fluorescent cell, regardless of treatment. PC embryos consistently exhibited PSP. Overall, culture conditions did not markedly affect the incidence of apoptosis. FT-IVD embryos had significantly more ($P < 0.05$) apoptotic cells (8.2 ± 2.9) than F-IVD (0.9 ± 0.3). However, when embryos exhibiting PSP were excluded, the statistical difference was no longer present (1.5 ± 0.9 vs. 0.9 ± 0.3, respectively). It is unclear why some embryos exhibit specific staining patterns while others exhibit PSP, but we hypothesize that PSP embryos are likely degenerate. Interestingly, different degrees of fluorescence were often present within an embryo. Often, small pinpoint areas of fluorescence were detected while other times the fluorescence appeared to consume the entire cytoplasm of the cell. The capase protein has been localized in the cytoplasm of the cell, so the different degrees of fluorescence may represent different degrees of apoptosis. There was no major effect of culture environment on apoptosis when embryos were categorized by QG. Furthermore, within a treatment, there was no significant difference in apoptosis across embryo QG. To our knowledge, this is the first report of apoptosis evaluated in relation to embryo QG. Based on these findings, apoptosis does not appear to be heavily involved in the reduced developmental potential of IVP bovine embryos.

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135 REGULATION OF GLUCOSE METABOLISM TO DECREASE LIPID CONTENT OF *IN VITRO*-PRODUCED BOVINE EMBRYOS

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Our objective was to improve normality of embryos produced *in vitro* with regulators of carbohydrate metabolism at doses optimized in earlier experiments. Eight- to 16-cell embryos were produced *in vitro* in the G1/G2 system (chemically defined sequential medium with recombinant human serum albumin), and then cultured 3 days in G2 containing metabolic regulators as follows: phenazine ethosulfate (PES), 0.3 μM; NaN_3, 27 μM; 2,4-dinitrophenol (DNP), 30 μM; and control. The following responses were analyzed by ANOVA in 2 to 4 replicates of 8–12 embryos each: glucose uptake and metabolism (uptake measured by microfluorometry of medium after incubating an embryo 3 h; metabolism measured as ^3H_2O released after incubating an embryo 3 h in medium containing 5-^{3}H glucose), % of glucose metabolized via the pentose phosphate pathway (PPP rate), lactate production, glycolysis (% of lactate produced from glucose taken up on a molar basis), lipid accumulation (number of >2 μM Sudan Black B positive granules/10^3 μm^2), % live Day 14 embryos recovered from embryos transferred to recipients at Day 7, and average surface area of embryos collected. *In vivo*-derived embryos were included as a second control for lipid evaluation. PES-treated embryos had higher glucose metabolism ($P < 0.05$) and lower glucose uptake ($P < 0.01$) than embryos in NaN_3 and tended to have a higher PPP rate ($P < 0.11$) than controls; however, glycolysis was higher for PES than other treatments ($P < 0.01$) (Table 1). Lipid accumulation of embryos from PES was markedly lower than any other *in vitro* treatments ($P < 0.01$), but higher than *in vivo* embryos (3.31 ± 2.78 lipid granules) ($P < 0.01$). NaN_3- and DNP-treated embryos both accumulated lipid similar to *in vitro* controls. No treatment differences were found in developmental competence when Day 7 embryos were transferred to recipients and recovered 1 week later (43 to 54% live embryos recovered), nor were there any significant differences ($P > 0.1$) in surface area. Embryos exposed to PES at the compaction and post-compaction stages accumulated much less lipid than controls or embryos exposed to other metabolic regulators, making this a very promising treatment. PES oxidizes NADPH; the molecular mechanism of PES appears to involve increased flux of glucose through the PPP while decreasing availability of NADPH for fatty acid synthesis.

Table 1. Response of embryos to metabolic regulators

Response	PES	NaN_3	DNP	Control
Glucose uptake[1]	13 ± 6.9^{a}	39 ± 4.2^{b}	34 ± 4.7^{ab}	24 ± 4.8^{ab}
Glucose metabolism[1]	18 ± 1.7^{c}	11 ± 1.7^{d}	17 ± 1.7^{cd}	15 ± 1.7^{cd}
PPP rate (%)	51 ± 8^{e}	26 ± 8^{ef}	28 ± 8^{ef}	22 ± 8^{f}
Lactate production[1]	52 ± 12^{gh}	59 ± 7^{g}	36 ± 8^{gh}	33 ± 8^{h}
Glycolysis (%)	187 ± 21^{a}	88 ± 13^{b}	94 ± 14^{b}	93 ± 14^{b}
Lipid granules[2]	15 ± 2.6^{a}	28 ± 2.7^{b}	25 ± 2.8^{b}	25 ± 3.0^{b}

[1]Picomol/embryo/h; [2]No. >2 μm Sudan Black positive granules/10^3 μm^2.
[ab]($P < 0.01$); [cd]($P < 0.05$); [ef]($P < 0.11$); [gh]($P < 0.08$): Statistical significance within rows (LS mean d ± SE).

136 EVIDENCE OF A DIRECT EFFECT OF P_4 ON IVF-DERIVED BOVINE 8-CELL EMBRYOS

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There has been much debate over a direct role for progesterone (P_4) in early bovine embryo development. While previous attempts to supplement bovine embryos *in vitro* with P_4 produced results that vary and are often contradictory, this may be a response of administering P_4 at inappropriate times. Therefore, the objective of these experiments was to determine if P_4 could exert a direct effect on developing IVF-derived bovine embryos when administered at an appropriate time of embryo development. In Exp. I, IVF-derived bovine 8-cell embryos were randomly allotted to treatments: (1) control, CR1aa medium ($n = 168$); (2) vehicle, CR1aa + ETOH (0.01%) ($n = 170$); and (3) P4, CR1aa + ETOH + P_4 (20 ng/mL in 50-μL droplet) ($n = 173$). In Exp. II, IVF-derived bovine 8-cell embryos were randomly allotted to treatments: (1) control, CR1aa medium ($n = 160$); (2) vehicle, CR1aa + DMSO (0.01%) ($n = 180$); and (3) P4, CR1aa + DMSO (0.01%) + P_4 (20 ng/mL in 50-μL droplet) ($n = 170$). All embryos were evaluated on Days 6 to 9 post-insemination and rates calculated from 8-cell embryos. In Exp. I, ETOH tended to have a detrimental effect with significantly fewer ($P < 0.05$) embryos (53%) developing to the blastocyst stage on Day 7 compared with the control (62%) and P_4 (71%) groups. At Day 7, significantly more embryos cultured in P_4 (71%) developed to the blastocyst stage compared with the control group (62%). P_4 treatment significantly increased the number of Grade 1 blastocysts (25%) on Day 7 compared with vehicle (15%) and control (17%) groups. At the end of culture, there were also significantly more Day 9 hatched blastocysts in the P_4 group (33%) compared with vehicle (22%) and control (21%) groups. Supplementing P_4 in the culture medium increased the rate of development, resulting in significantly more blastocysts (8%) on Day 6 and hatched blastocysts (21%) on Day 8 compared with vehicle (3% and 12%) and control (0% and 8%) groups, respectively. In Exp. II, there were no significant differences between treatment groups for Day 7 blastocysts (control 54%, DMSO 61%, P_4 57%) and Day 9 hatched blastocysts (control 46%, DMSO 51%, P_4 46%). However, there were significantly more Grade 1 blastocysts in the P4 group (22% and 36%) on Days 6 and 8 compared with vehicle (11% and 23%) and control (13% and 23%) groups, respectively. The lack of improvement in Day 7 blastocysts and Day 9 hatched blastocysts rates leads to further uncertainty in understanding the P_4 vehicle interactions. In conclusion, the results of these two experiments indicate that P_4 can exert a direct effect on the developing IVF-derived bovine embryo; however, due to P_4 vehicle interactions; other inert vehicles need to be explored to further evaluate the direct effects of P_4 on the developing bovine embryo.

137 EFFECT OF FLUNIXIN MEGLUMINE IN CO-CULTURE MEDIUM ON THE DEVELOPMENT OF *IN VITRO* MATURED AND FERTILIZED BOVINE EMBRYOS

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PG concentration is often increased during uterine manipulation with embryo transfer. Embryo viability is affected by the increase in the $PGF_{2\alpha}$ concentration accompanying manipulation of the uterus during embryo transfer. Schrick et al. (2001 Theriogenology 55, 370 abst) observed that treatment with flunixin meglumine, an inhibitor of prostaglandin, increased pregnancy rates depending on the stage and quality of embryos transferred. On the other hand, prostaglandin was secreted by a cumulus cell monolayer in an *in vitro* culture of bovine oocytes. The present study aimed to assess the effects of flunixin meglumine in culture medium on the development of *in vitro*-matured and fertilized bovine embryos. COCs were collected from ovaries of slaughtered cows by aspiration. The COCs were matured for 20 h in TCM-199 supplemented with 5% fetal bovine serum (FBS) and antibiotics at 38.5°C under an atmosphere of 2% CO_2 in air. Matured COCs were inseminated with 1.0×10^7 sperm mL^{-1} in BO medium (Brackett and Oliphant 1975 Biol. Reprod. 12, 260–274) containing 5 mM theophillin and 5 μg mL^{-1} heparin for 5 h. All of the inseminated oocytes were introduced into the maturation medium that had been kept with the cumulus cells in the CO_2 incubator. At 48 h after insemination, all embryos over the 4-cell stage were cultured in TCM-199 plus 5% FBS supplemented with each of five concentrations of flunixin meglumine (0, 0.0025, 0.005, 0.01, and 0.025%) with a cumulus cell monolayer. Development to the blastocyst stage and quality were examined at Days 7 to 8 (Day 0 = day of insemination) using a microscope. The experiment was replicated four times. Data were analyzed by the chi-square test. The total blastocyst rates from the over-4-cell embryos were 61.2 (52/89), 53.7 (44/89), 65.6 (59/90), 57.3 (51/89), and 33.7% (31/92) for 0, 0.0025, 0.005, 0.01, and 0.025%, flunixin meglumine, respectively. The total blastocyst rate with the flunixin meglumine concentration of 0.025% was significantly lower than those with the other concentrations ($P < 0.05$). The proportion of grade 1 blastocysts with the flunixin meglumine concentration of 0.005% was significantly higher than that with the 0, 0.0025, and 0.025% concentrations (27.8 vs 11.2, 14.6, and 5.4%; $P < 0.05$). Our present results show that the addition of 0.005% flunixin meglumine to the co-culture medium is positively associated with blastocyst quality in bovine embryos.

138 THE EFFECT OF SYNTHETIC HYALURONAN, BSA AND SERUM ON *IN VITRO* DEVELOPMENT AND GENE TRANSCRIPTION OF BOVINE BLASTOCYSTS

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The objective of this study was to examine the effect of synthetic hyaluronan (s-HA), BSA and fetal calf serum (FCS) on bovine embryo *in vitro* development, ultrastructure, and mRNA transcription of four developmentally important genes: apoptosis (BAX), oxidative stress (SOX), growth

factor (IGF-II), and cell-to-cell adhesion (Ecad). A total of 1406 presumptive zygotes (7 replicates) were cultured initially in two Groups: 1, SOFaa + 4% BSA only, and 2, SOFaa + 4% BSA and 10% FCS. On Day 4 (96 h after insemination) of culture, the number of zygotes that developed to the <8-cell-stage were recorded, and 2.5 mg/mL of s-HA (MAP-5; Bioniche Inc, Belleville, ON, Canada) was added to half of the embryos from each group; the other half received an equivalent volume of corresponding SOF medium without s-HA. Embryos were cultured in 50-μL drops (25 zygotes per drop) under paraffin oil at 39°C and 5% CO_2 in humidified air. Cleavage rates were recorded on Day 2 and the number of blastocysts on Days 7, 8, and 9. At least five blastocysts from each replicate and from each treatment were frozen for evaluation of gene expression patterns. Poly(A) mRNA was prepared from 4–5 groups of pools of 10 embryos. The quantification of all gene transcripts was performed by real time quantitative RT-PCR in three replicates. The fine structure of blastocysts was studied using transmission electron microscopy. Embryo developmental stages and blastocyst formation were analyzed by chi-square analysis, and data on mRNA expression were analyzed by one-way repeated-measures ANOVA. No differences in cleavage rates were observed between groups. There was no difference between the BSA group with or without s-HA in the percentages of embryos developed to the blastocyst stage at Days 7, 8, and 9 (38.3 and 38.1%, respectively). However, significantly ($P < 0.05$) less blastocysts developed in medium supplemented with BSA + FCS (18.3%) or with BSA + FCS + s-HA (27.4%). Synthetic HA added to the medium containing BSA significantly ($P < 0.05$) increased the level of expression of EGF-II and decreased ($P < 0.05$) the level of expression of BAX, SOX, and Ecad. On the other hand, presence of FCS significantly ($P < 0.05$) increased the level of SOX and decreased the level of IGF-II ($P < 0.05$), and the addition of s-HA to SOF containing FCS showed no effect on the level of transcription of any analyzed genes. The general fine structure of embryos cultured with s-HA regardless of protein supplement was conspicuously improved in comparison with the respective controls. It can be concluded that, within our culture system, addition of s-HA on Day 4 of culture to the SOFaa medium supplemented with BSA but not in combination with FCS showed a positive effect on embryo development and molecular composition of the embryos.

139 BOVINE AMNIOTIC FLUID FOR THE CULTURE OF TWO-CELL MURINE EMBRYOS

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The objective of the experiment was to evaluate bovine amniotic fluid as an alternative medium for culture of two-cell murine embryos. Variability in embryo development rate and high purchase prices of medium supplements such as fetal bovine serum have prompted the search for a serum alternative. Amniotic fluid was collected postmortem from first-trimester bovine fetuses, pooled, heat inactivated at 56°C for 30 min and stored at −20°C until used. Two-cell mouse embryos were collected from (Balb/C × C57BL6) F_1 females superovulated with 10 IU PMSG and 10 IU hCG. The experiment was performed to evaluate the effect of commercial fetal bovine serum (F4135, Sigma, South Africa) supplementation to amniotic fluid. Treatments consisted of (1) bovine amniotic fluid, (2) bovine amniotic fluid supplemented with 10% fetal bovine serum, (3) M16 (M7292, Sigma) supplemented with 10% fetal bovine serum, and (4) Medium 199 (M4530, Sigma) supplemented with 10% fetal bovine serum. The latter two media were controls. Twenty-four hours before use the culture media were supplemented with 1% antibiotic antimycotic solution (A5955, Sigma). Media were equilibrated for 24 hours at 37°C and 5% CO_2 before use. Embryos were cultured in 50-μL droplets with oil overlay at 37°C in 5% CO_2. A minimum of 5 and maximum of 10 embryos per droplet were allowed. Six replications per treatment were done giving a total of 292 embryos in treatment (1), 318 in treatment (2), 304 in treatment (3), and 303 in treatment (4). The embryos were monitored under an inverted microscope (Olympus model IX70) at 24-h intervals for 72 h for blastocyst formation. The differences between embryo growth in the different culture media were assessed by one-way analysis of variance. All culture media supported the development of mouse embryos to the hatched blastocyst stage. A higher ($P < 0.05$) number of embryos hatched in M16 (64.6%) and Medium 199 (55.0%) supplemented with 10% fetal bovine serum than in frozen bovine amniotic fluid (12.2%) and frozen bovine amniotic fluid supplemented with 10% fetal bovine serum (17.8%). M16 was superior to all other treatments in supporting embryo development up to the morula stage. More than twice the number of embryos (94.9%) reached the morula stage in M16 than in frozen bovine amniotic fluid with (37.4%) or without (29.1%) serum supplementation. Bovine amniotic fluid obtained from postmortem first trimester fetuses supported the development of two-cell mouse embryos; however, embryo development in frozen fetal fluid was lower ($P < 0.05$) than that obtained in the control media. Fetal bovine serum, when added to amniotic fluid, did not increase the development rate. It seems likely that freezing the amniotic fluid had an adverse effect on *in vitro* embryo development.

140 EXPRESSION OF LEPTIN LIGAND AND RECEPTOR AND EFFECT OF EXOGENOUS LEPTIN SUPPLEMENTATION ON *IN VITRO* DEVELOPMENT OF PORCINE *IN VITRO* FERTILIZED AND SOMATIC CELL NUCLEAR TRANSFER EMBRYOS

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The present study investigated the expression of ligand and receptor for leptin, and the effect of leptin supplementation on preimplantation development of porcine *in vitro*-fertilized (IVF) and somatic cell nuclear transfer (SCNT) embryos. The IVF embryos were produced using frozen boar semen and SCNT embryos were obtained by nuclear transfer of fetal fibroblasts into enucleated oocytes. In Exp. 1, *in vitro*-matured porcine oocytes and embryos in 2-, 4-, and 8-cell as well as morula and blastocyst stages derived from IVF or SCNT were immunostained for leptin ligand and receptor with their specific antibodies. The expression of leptin ligand and receptor proteins was detected in oocytes and all stages of IVF and SCNT embryos. The IVF (Exp. 2; $n = 635$, 630, 633, 635, respectively) or SCNT oocytes (Exp. 3; $n = 256$, 258, 251, 258, respectively) were cultured in modified North Carolina State University (mNCSU)-23 medium supplemented with various concentrations (1, 10, 100, or 1000 ng/mL) of leptin. For the control

group, IVF ($n = 635$) or SCNT embryos ($n = 249$) were cultured without leptin supplementation (0 ng/mL leptin). Embryo development and cell number in blatoscysts after differential staining according to a modified staining procedure (Thouas *et al*. 2000 Reprod. Biomed. Online 3, 25–29) were evaluated. The IVF or SCNT embryos were randomly distributed, and experiments were replicated at least 11 times. The differences in embryo development among experimental groups were analyzed using one-way ANOVA after arcsine transformation (without arcsine transformation for cell number of blastocysts) to maintain homogeneity of variance. Post hoc analyses to identify between-group differences were performed using the LSD test. In SCNT embryos, the cleavage rate was not different in leptin-treated groups (73.8, 77.5, 75.7, or 78.7%, respectively) compared to the control (76.3%). The rate of blastocyst formation (at 166 h after the day of injection of donor cells) in SCNT embryos was significantly increased ($P < 0.05$) in 1000 ng/mL leptin-supplemented group (20.2%) compared with the control (12.9%) and 1 ng/mL leptin-supplemented (12.5%) groups. Supplementing mNCSU-23 with 1000 ng/mL leptin also significantly increased ($P < 0.05$) the number of total cells (54.6) and trophectoderm (TE) cells (39.1) in SCNT blastocysts ($n = >25$) compared with the control [45.1 (total cells) and 31.6 (TE cells)] and 10 ng/mL leptin-supplemented group [44.4 (total cells) and 31.7 (TE cells)]. In IVF embryos, leptin supplementation did not affect pre-implantation embryo development and cell number in blastocysts. In conclusion, the present study demonstrated the expression of leptin ligand and receptor proteins in porcine *in vitro* matured oocytes, IVF and SCNT embryos, and the embryotropic role of leptin in the SCNT embryo development.

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141 BENEFICIAL EFFECT OF ETHYLENEDIAMINETETRAACETIC ACID COMBINED WITH HEMOGLOBIN ON PRE-IMPLANTATION DEVELOPMENT OF PORCINE *IN VITRO* PRODUCED EMBRYOS

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Developing a porcine embryo culture system is important for increasing the rates of implantation and pregnancy of somatic cell nuclear transfer (SCNT) embryos. Ethylenediaminetetraacetic acid (EDTA) was shown to inhibit glycolytic activity of cleavage stage embryos, thereby preventing the premature stimulation of glycolysis and enhancing development. However, EDTA should not be used for later-stage embryos as the inhibition of glycolysis reduces energy production at the blastocyst stage and significantly inhibits inner cell mass development. On the other hand, addition of a nitric oxide (NO) scavenger, hemoglobin (Hb), to the culture medium is known to promote embryo development to the blastocyst stage. This study was conducted to evaluate the beneficial effect of EDTA combined with Hb on pre-implantation development of porcine embryos *in vitro*. Porcine embryos produced by *in vitro* maturation and fertilization were cultured for 6 days in North Carolina State University (NCSU)-23 medium supplemented with EDTA or/and Hb. All data were subjected to one-way ANOVA and protected least significant difference (LSD) test using the general linear models (GLM) procedure of the statistical analysis system (SAS Institute, Inc., Cary, NC, USA) program to determine differences among experimental groups. Statistical significance was determined when the P value was less than 0.05. In Exp. 1, culturing porcine zygotes with 100 mM EDTA ($n = 537$) significantly increased cleavage rates (85.3%) at 48 h post-insemination compared to supplementing with 0, 1, or 10 mM EDTA (78.9, 79.7, or 78.2%, respectively). However, EDTA at these concentrations did not promote blastocyst formation compared to the control. In addition, no difference was observed in total cell numbers in blastocysts among the experimental groups (41.8, 42.6, 45.8, 44.5, respectively). In Exp. 2, *in vitro*-fertilized oocytes were cultured with 0, 1, or 10 mg/mL Hb. Culturing with Hb did not promote porcine embryo development, but significantly increased the total cell number of blastocysts obtained from 1 mg/mL Hb supplementation ($n = 566$) compared to that of the control (56.8 vs. 41.6). In Exp. 3, culturing embryos ($n = 548$) with 100 mM EDTA + 1 mg/mL Hb significantly improved rates of cleavage (84.0% vs. 75.2%) and blastocyst formation (19.2% vs. 12.7%), and the total number of cells in blastocysts compared to those of the control (58.4 vs. 42.3). In conclusion, our results demonstrated that EDTA or Hb have different roles in supporting *in vitro* pre-implantation development of porcine embryos; EDTA mainly stimulated early cleavage up to the 2- to 4-cell stage, and Hb promoted the total cell number of blastocysts. However, combined supplementation with these two chemicals improved cleavage, blastocyst formation, and total cell number in blastocysts.

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142 *IN VITRO* DEVELOPMENT OF BOVINE EMBRYOS CULTURED IN KSOM, CR1aa, OR KSOM/CR1aa

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In vitro embryo culture is an important step of *in vitro* production of bovine embryos. It has been shown that IVF-derived bovine embryos cultured in KSOM or CR1aa have high development rates. In our laboratory, we have observed that 8-cell embryos are morphologically superior when embryos are cultured in KSOM whereas blastocysts are morphologically superior when embryos are cultured in CR1aa. Based on these observations, we hypothesized that development of IVF-derived bovine embryos can be improved by sequential use of these media (KSOM and CR1aa). The aim of this experiment was to compare the *in vitro* development of bovine embryos cultured in KSOM, CR1aa or KSOM/CR1aa supplemented with BSA at Day 0 and BSA and FBS at Day 3. In order to accomplish the sequential culture, fertilized oocytes where cultured in KSOM to the 8-cell stage and then transferred to CR1aa for further development. Oocytes were purchased from Bomed (Madison, WI, USA), and after 22 hours of maturation

were fertilized with frozen-thawed semen for 5 hours at 39°C in 5% CO_2. After fertilization, the presumptive zygotes were denuded from cumulus cells by votexing and were randomly allotted to one of 3 treatments: (1) cultured only in KSOM ($n = 110$), (2) cultured only in CR1aa ($n = 102$), and (3) cultured in KSOM in the first 3 days and then in CR1aa from Day 3 to Day 9 ($n = 110$). The embryo culture was carried out in 50-μL droplets of medium that were placed in an airtight modular incubator filled with 5% CO_2, 5% O_2 and 90% N_2. The embryos were evaluated on Days 6 to 9 post insemination. All embryo developmental rates were calculated from presumptive zygotes. The Day 6 morula rates were 52%, 40%, and 47% for KSOM, CR1aa, and KSOM/CR1aa, respectively. The Day 7 blastocyst rates for KSOM (40%), CR1aa (25%), and KSOM/CR1aa (30%) were not significantly different; however, Day 9 hatched blastocyst rates were significantly higher ($P < 0.05$) for KSOM (22%) compared to CR1aa (9%) but not different from KSOM/CR1aa (14%). Regarding embryo quality, Day 7 transferable embryos rates (Grade 1 and Grade 2) were 35%, 25%, and 30%, respectively for KSOM, CR1aa, and KSOM/CR1aa; however, no significant difference was observed. These results indicate that IVF-derived bovine embryos can develop in KSOM, CR1aa, or KSOM/CR1aa with no significant difference among morula, blastocyst and hatched blastocyst rates. However, the combination of KSOM and CR1aa during *in vitro* culture did not decrease the morula and blastocyst rates.

143 *IN VITRO* DEVELOPMENT OF OVINE OOCYTES FROM EWES OF CONTRASTING VITAMIN B12 STATUS

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Suboptimal circulating concentrations of vitamin B12 are commonly found in cattle and sheep grazing cobalt-deficient pastures. Vitamin B12 is a co-factor for enzymes involved in energy metabolism (methylmalonyl CoA mutase) and DNA synthesis/methylation (methionine synthase), and vitamin B12 status may therefore impact on cell division and gene expression in early embryos. The aim of this study was to determine the effect of vitamin B12 status on the *in vitro* development of ovine oocytes to the blastocyst stage. Mature Scottish Blackface ewes from cobalt-deficient farms were housed for ~ four months and fed a cobalt-deficient diet (0.06 mg cobalt kg DM^{-1}). At housing, 55 of the ewes were given an intra-ruminal slow-release cobalt bolus to compensate for the dietary deficit, and 52 remained untreated. The ovaries of all ewes were recovered at slaughter within the natural breeding season. Oocytes were aspirated and those with evenly granulated cytoplasm and >3 layers of cumulus cells were pooled according to ewe cobalt treatment, matured, fertilized, and cultured *in vitro* (~20 oocytes per 50-μL drop under mineral oil). Oocytes were matured for 24 h in M199 + 10% fetal calf serum at 38.5°C in a humidified atmosphere of 5% CO_2 in air prior to co-incubation for 18 h with frozen-thawed semen from a single ejaculate (1×10^6 live sperm mL^{-1}). Presumptive zygotes were cultured for 7 Days in synthetic oviduct fluid + 0.4% fatty acid-free BSA (5% CO_2, 5% O_2, 90% N). Blastocysts formed at the end of the culture period were fixed and stained (Hoechst 33258) to count cell numbers. Data were analyzed by ANOVA and chi-square. For cobalt-supplemented and non-supplemented ewes, circulating concentrations of vitamin B12 at the time of slaughter were 1244 ± 52.5 and 372 ± 27.9 pmol L^{-1} ($P < 0.001$), respectively. Numbers of small (<5 mm) follicles per ewe were 17.6 ± 1.22 and 17.1 ± 1.31, and large (>5 mm) follicles per ewe were 1.8 ± 0.16 and 1.6 ± 0.18, respectively (NS). Cobalt-supplemented ewes yielded a lower proportion of matured oocytes that cleaved but an increased proportion of cleaved oocytes that formed blastocysts by Day 6 of the culture period (Table 1). The proportion of grade 1 and 2 blastocysts was also increased but cobalt treatment did not affect blastocyst cell numbers. In conclusion, results suggest that cleaved eggs derived from ewes of adequate, compared to suboptimal vitamin B12 status have improved developmental competence *in vitro*.

Table 1. Effect of cobalt/vitamin B12 status on the *in vitro* development of ovine oocytes

No. (proportion of) ova	Cobalt-supplemented	Non-supplemented	Significance
Selected (selected/aspirated)	379 (0.67)	415 (0.63)	NS
Matured (matured/selected)	367 (0.97)	399 (0.96)	NS
Cleaved (cleaved/matured)	321 (0.87)	369 (0.92)	$P < 0.05$
Blastocysts Day 6 (Day 6/cleaved)	183 (0.57)	177 (0.48)	$P < 0.05$
Blastocysts Day 6 + 7 (Day 6 + 7/cleaved)	228 (0.71)	240 (0.65)	NS
Blastocysts Grade 1 & 2 (g1 & 2/Day 6 + 7)	147 (0.65)	124 (0.52)	$P < 0.01$
Mean ± SEM cell numbers (d6 + 7)	102 ± 2.9	93 ± 2.8	NS

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144 ROLE OF GnRH ON MOUSE PRE-IMPLANTATION EMBRYONIC DEVELOPMENT *IN VITRO*

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The interaction between GnRH and its receptor on gonadotropes within the anterior pituitary gland represents a key point for regulation of the reproduction. In addition, GnRH can act in multiple extrapituitary tissues via autocrine/paracrine mechanisms. Protein for GnRH and mRNA for

both GnRH and its receptor have been detected in human uterine endometrium and oviduct as well as in embryos at the morula/blastocyst stage in the mouse and human. Therefore, we hypothesized that GnRH may have a critical role in the development of pre-implantation embryos. To address this question, we examined the effect of a GnRH agonist and antagonist on the development of mouse embryos *in vitro*. For these studies, 1-cell embryos were randomly allocated to culture in KSOM containing the appropriate treatment for 144 h at 37°C in a 5% CO_2 in air environment. The medium was changed every 12 h and embryos were scored daily for development. The data were compared using a χ^2 test. First, we wanted to determine if a GnRH agonist, histrelin, could enhance embryonic development. Embryos were cultured with ($n = 35$) or without ($n = 36$) 10 μM histrelin. The addition of histrelin did not increase morula or blastocyst formation *v*. control. Second, we cultured embryos in the presence of different concentrations (0, 0.001, 0.01, 0.1, 1, and 10 μM) of the GnRH antagonist, SB-75 (cetrorelix; $n = 22$/treatment) in order to determine its effect on embryonic development. The 10 μM SB-75 treatment blocked embryo development beyond the compact morula stage ($P < 0.001$). To determine if this was a receptor mediated effect, we attempted to rescue development of SB-75 treated embryos with a histrelin challenge. Our treatments consisted of control ($n = 30$), 10 μM histrelin ($n = 27$), 10 μM SB-75 ($n = 29$), and 10 μM SB-75 in combination with either 1 μM ($n = 27$) or 10 μM ($n = 25$) histrelin. Both levels of histrelin partially rescued the inhibition of blastocyst formation by SB-75 ($P < 0.01$). Next, we were interested in examining the signaling cascade activated following binding of GnRH to its receptor in pre-implantation embryos. Toward this end, we treated embryos with inhibitors of either PKC or PKA. First, embryos were cultured in the presence of 0 ($n = 33$), 0.1 ($n = 35$), 1 ($n = 35$), or 10 ($n = 35$) μM GF109203X (GFX), a PKC inhibitor. Similar to the results obtained with SB-75, treatment with 10 μM GFX significantly reduced development to the compact morula stage and completely blocked blastocyst formation. Second, we treated embryos ($n = 15$ to 17/treatment) with different concentrations (0, 0.01, 0.1, 0.5, or 1 mM) of the PKA inhibitor, SQ22536. In contrast to treatment with GFX, rates of blastocyst formation were decreased only by 35% ($P < 0.05$) at the highest concentration of SQ22536. The percentage of embryos developing to the hatched blastocyst stage was decreased in a dose-dependent manner following SQ22536 treatment ($P < 0.05$); however, this effect was not consistent with SB-75 inhibition of blastocyst formation. We suggest that GnRH has an important autocrine effect on early embryonic development, potentially signaling via PKC.

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145 COMPARISON OF DEVELOPMENT AND QUALITY OF PORCINE EMBRYOS CULTURED IN DIFFERENT OXYGEN CONCENTRATIONS

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In spite of valuable uses of pig IVP embryos, including IVF and cloned embryos for production of valuable offspring that provide recombinant protein and xenotransplantation, several problems limit the success. In cattle embryo culture, lowering the oxygen concentration resulted in increased development, quality, and cell number, and reduced apoptosis incidence. The high O_2 concentration in *in vitro* culture caused overproduction of reactive oxygen specias (ROS), causing damage to the cell membrane or DNA. The present study, therefore, evaluated the developmental ability of porcine embryos by culturing in either low or high oxygen concentration on the rates of cleavage, development, cell number, and apoptosis. These values were then compared to parthenote embryos, which were activated with electric stimulation by 2 DC pulses of 2.0 kV/cm, 30 μs in 0.3 M mannitol containing 100 μM $CaCl_2$ and 100 μM $MgCl_2$ and then transferred into NCSU23 medium supplemented with 7.5 μg/mL cytochalasin B for 6 h. Cumulus-oocyte complexes from slaughterhouse ovaries were matured in TCM 199 medium supplemented with 10 ng/mL EGF, 0.5 μg/mL FSH, 0.5 μg/mL LH, 0.57 mM cysteine, and 0.91 mM Na pyruvate for 24 h and further cultured in the same medium without FSH and LH for 20 h at 38.5°C, 5% CO_2 in air. IVF was carried out in mTBM for 5 h with 1×10^5 sperm/mL by following the previously reported protocols (2004 Methods Mol. Biol. 253, 227–234) with minor modifications. Zygotes were allocated two O_2 concentrations; zygotes were cultured in NCSU23 medium supplemented with 0.17 mM Na pyruvate, 2.73 mM Na lactate, and 0.4% BSA for 54 h and subsequently cultured in the same medium supplemented with 5.55 mM glucose for 90 h at 38.5°C, 5% CO_2, 5% O_2 and 90% N_2 (Treatment 1) or 5% CO_2, 20% O_2 in air (Treatment 2). Statistical analysis was performed with one-way ANOVA by SPSS 10.0 ($P < 0.05$). Most oocytes (>82%) cleaved and the rates did not differ between groups. However, the rates of blastocyst development from oocytes used as parthenote controls (5% O_2, 40.0 ± 13.0 (70/162); 20% O_2, 34.5 ± 11.2 (56/161)) were significantly ($P < 0.05$) higher than those in IVF embryos (5% O_2, 27.9 ± 8.0 (70/247); 20% O_2, 27.1 ± 7.2 (68/249)). Similarly cell number and apoptosis index by TUNEL staining of blastocysts in parthenote (5% O_2, 42 and 15%; 20% O_2, 39 and 20%) were significantly ($P < 0.05$) higher than those in IVF (5% O_2, 32 and 13%; 20% O_2, 27 and 14%, all respectively). In conclusion, no differences between low and high oxygen concentrations in culture of porcine IVF and parthenote embryos were observed on the rates of cleavages and development into blastocyst cell number and apoptosis incidence. Further research should be carried out to develop a reliable IVP system.

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146 THE EFFECT OF CULTURE TEMPERATURE ON THE CLEAVAGE, DEVELOPMENT, AND GENE TRANSCRIPTION PATTERNS OF BOVINE EMBRYOS

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Bovine oocytes matured and fertilized at 39°C had significantly higher rates of fertilization than at 37°C (Lenz *et al*. 1983 Biol. Reprod. 29, 173–179). However, embryo culture temperature that may affect molecular composition and metabolism of embryo membranes was not investigated. The objective of this study was to determine the effect of two culture temperatures, 37 and 39°C on the cleavage, *in vitro* development, and

gene transcription patterns in bovine embryos. A total of 794 oocytes (5 replicates) were matured in TCM-199 medium containing 10% FCS and 10 ng/mL epidermal growth factor and inseminated with 1×10^6/mL spermatozoa in groups of 50 in 250 μL in Fert-Talp supplemented with 25 mM bicarbonate, 22 mM sodium lactate, 1 mM sodium pyruvate, 6 mg/mL BSA-FAF, and 10 μg/mL heparin at 39°C. After 24 h of oocyte-sperm co-incubation, presumptive zygotes were divided into two groups and cultured at 37 or 39°C under paraffin oil and 5% CO_2 in humidified air. Embryos were cultured in SOFaa medium supplemented with 4% BSA in 50-μL drops (25 zygotes per drop). Cleavage rates were recorded on Day 2 and zygote development on Days 4, 7, 8, and 9. At least 5 blastocysts from each replicate from each treatment were frozen for evaluation of gene expression patterns. Poly(A) mRNA was prepared from 6 groups of pools of 5–7 embryos. A total of 11 developmentally important mRNA transcripts were examined. The quantification of all gene transcripts was performed by real time quantitative RT-PCR in three replicates. Embryo development was analyzed by chi-square analysis and data on mRNA expression by one-way repeated-measures ANOVA. There was no effect of culture temperature on embryo cleavage (65.2 and 72.0% for 37 or 39°C, respectively) but at Day 4 significantly ($P < 0.05$) more embryos developed to <8 cells at 39°C (32.5%) than at 37°C (25.9%). There was no difference in the total number of blastocysts produced in either temperature (22.4% at 37°C and 22.1% at 39°C), and significantly ($P < 0.05$) more zygotes that were at <8-cell stage at Day 4 progressed to the blastocyst stage at 37°C (86.4%) than at 39°C (66.6%). Transcript levels for genes related to response to stress (SOX, IFNτ) and glucose metabolism (Glut-1, G6pd) were higher ($P < 0.05$) in the blastocysts cultured at 39°C, and levels of Glut-5 (glucose metabolism) and Oct-4 (factor related to pluripotency) were higher ($P < 0.05$) in the blastocysts cultured at 37°C. No difference was found in mRNA transcription of genes related to apoptosis (BAX), response to heat (Hsp70), compaction (Ecad, Na/K, DcII), and cell connection (Cx43) at either temperature tested. It can be concluded that culture temperature may affect embryo molecular composition and the kinetics of embryo development.

147 EFFECT OF CULTURE SYSTEM ON THE DEVELOPMENT OF *IN VITRO*-FERTILIZED OR DNA-INJECTED BOVINE EMBRYOS

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DNA microinjection has become the most widely applied method for gene transfer in mammals. However, the production of the transgenic bovine is relatively inefficient with the pronuclear microinjection technology. This experiment was designed to compare the two different *in vitro* production systems the serum-containing system (IVM, IVF, and IVC; TCM199, TALP, and CR1aa) and the Serum-free system (IVM, IVF, and IVC; IVMD101, IVF100 and, IVMD101), on the development of *in vitro*-fertilized embryos (Experiment 1) and DNA-microinjected embryos (Experiment 2). Korean Native Cow (KNC) ovaries were obtained from slaughterhouse and cumulus oocyte complexes (COCs) were aspirated from 2- to 8-mm follicles. In the serum-containing system, groups of 15 COCs were matured in TCM199 supplemented with 10% fetal calf serum (FBS), 1 μg/mL FSH, 10 μg/mL LH, and 1 μg/mL estradiol-17β for 18 h. *In vitro* matured oocytes were fertilized using frozen-thawed percoll separated spermatozoa (Day 0) in fer-TALP medium for 20 h. Presumptive zygotes were cultured in CR1aa medium supplemented with 0.3% BSA (before Day 3) or 10% FBS (after Day 3). In the serum-free system, groups of 15 COCs were matured in IVMD101 medium, fertilized in IVF100 medium and cultured in IVMD101 medium (Hochi *et al*. 2003 Theriogenology 59, 675–685). The DNA used for microinjection was a green fluorescent protein. The zygotes were centrifuged in TALP medium at 15,000*g* for 7 min, and then were microinjected into the pronucleus. All cultures were maintained in an incubator at 39°C, 5%CO_2 in air with maximum humidity. Data from three replicates were analyzed by chi-square test. In Experiment 1, there were no differences in the cleavage rates between treatments (71.8% v. 65.3%, respectively). The development rate to the 8-cell stage was significantly higher in the serum-free system than in the serum-containing system ($P < 0.05$; 47.2% v. 24.7%, respectively). However, the development rates to the blastocyst stage were not different (20.4% v. 16.0%, respectively). In Experiment 2, the development rates to the cleavage, 8-cell, and blastocyst stages were significantly higher in the serum-free system than in the serum-containing system ($P < 0.05$; 47.2, 25.0, and 5.6% vs. 16.5, 3.5, and 0%, respectively). The results of this study suggest that the serum-free system was not effective on the development of *in vitro*-fertilized embryos, but it was effective on the development of DNA-microinjected embryos.

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148 AN EFFECT OF MELATONIN ON DEVELOPMENT OF BOVINE EMBRYOS CULTURED *IN VITRO* UNDER OPTIMAL OR ENHANCED OXYGEN TENSIONS

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Many different systems of free radical scavengers have been investigated during the last few years for *in vitro* culture of mammalian embryos. Melatonin is a potent reactive oxygen species scavenger and has been tested in the promotion of mouse embryo development *in vitro* (Ishizuka *et al*. 2000 J. Pin. Res. 28, 48–51). An effect of melatonin on bovine embryo development *in vitro* is described here. Slaughterhouse-derived oocytes were subjected to standard *in vitro* maturation and fertilization procedures. Presumptive zygotes randomly allocated to experimental groups were cultured for 3 days (Day 1–Day 3) in CR1aaLA medium (Papis *et al*. 2000 Theriogenology 54, 651–658) supplemented with two different concentrations of melatonin (10^{-6} M or 10^{-4} M; Sigma, St. Louis, MO, USA) or without melatonin (control). Culture was performed under two different gas atmospheres containing 4% CO_2 and either normal (7%) or enhanced (20%) oxygen concentration (2 × 3 factorial analysis). At the end of Day 3, embryos from each treatment group, developed to at least the 4-cell stage, were collected and cultured without melatonin until Day 10 at optimum 4% CO_2 and 7% O_2 atmosphere. The numbers of blastocysts at Day 8 and hatching/hatched blastocysts at Day 10 were recorded. Five replicates of

each treatment were performed. Blastocyst formation rates of presumptive zygotes and of Day 3, 4-cell embryos were calculated for each group. Differences between groups were analyzed using chi-square and/or Fisher's exact tests where appropriate. $P < 0.05$ was considered statistically significant. Out of 100, 100, and 101 presumptive zygotes cultured for the first 3 days in 7% oxygen with 10^{-4} M, 10^{-6} M, or no melatonin, 31 (31%), 40 (40%), and 44 (43.5%) developed to blastocyst stage and 25 (25%), 33 (33%), and 36 (36%) to hatching/hatched blastocyst stage, respectively. On the other hand, out of 102, 102, and 100 zygotes cultured in the same concentrations of melatonin, but under 20% of oxygen, an opposite tendency was observed, as 42 (41%), 25 (24.5%), and 32 (32%) blastocysts and 26 (25.5%), 21 (20.6%), and 25 (25%) hatching/hatched blastocysts developed, respectively. No statistical significance was reached here. However, out of 4-cell embryos put into *in vitro* culture after initial treatments in different melatonin concentrations, a decreased ratio of blastocyst formation was observed in the 10^{-4} M melatonin group (31/65, 47.7%) compared to that of the control (44/65, 67.7%; $P = 0.0327$) when the lower oxygen concentration was applied. However, a beneficial effect of melatonin was observed in the presence of 20% oxygen. Out of 61 embryos, 42 (68.9%) developed to the blastocyst stage after treatment in 10^{-4} M melatonin concentrations, vs. 32/63 (50.8%; $P = 0.0458$) blastocysts developed in control group. In conclusion, a beneficial or a harmful effect of melatonin on bovine embryo *in vitro* development was observed depending on the oxygen concentration during the treatment. Results presented seem to confirm a potent free radicals scavenging activity of melatonin in a bovine embryo culture system.

149 INTRAUTERINE CULTURE OF *IN VITRO* PRODUCED BOVINE EMBRYOS AND RECOVERY OF THE EMBRYOS AT DAYS 12–14

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For stem cell production and detailed morphological analysis 12–14-day-old bovine embryos are suitable. However, it has been proven to be difficult to extend the *in vitro* culture period beyond Days 8–9, and it was the aim of the present experiment to examine whether it might be possible to culture 6–7-day-old *in vitro*-produced (IVP) embryos for a period of 5–7 days in the uterine horns of heifers. The IVP embryos were produced by standard procedures. Briefly, IVM took place in DMEM medium supplemented with 5% serum, EGF, and eCG/hCG, and IVF was carried out in TALP medium under 5% CO_2 in humidified air and at 38.5°C. IVC took place in SOFaaci supplemented with 10% serum under 5% CO_2, 5% O_2 and 90% N_2 at 38.5°C .The embryos were cultured *in vitro* to Days 6–7 post insemination, when morulas and blastocysts of excellent quality were placed in HEPES-buffered TCM199 with 10% serum, loaded in numbers of 10–30 into 0.25 mL straws, and then transported to the place of transfer in a portable incubator at 38.5°C. The embryos were transferred nonsurgically to the mid or distal part of the uterine horns of 28 dairy heifers which were heat synchronized with injections of cloprostenol (Estrumat Vet, Schering-Plough, Farum, Denmark) to a cycle stage of embryo age +1 day. In 16 heifers, embryos were transferred into both sides and for the remaining ones only into the horn ipsilateral to the ovary bearing the corpus luteum. After 5–7 days, the heifers were flushed nonsurgically by standard method, using a flushing catheter of large caliber (Minitab® 18 G) and slow infusion and evacuation of the fluid. The differences in recovering rate among horns were identified by Fisher's Exact test. Data are given as LS means ± SEM values and statistical differences assigned at the $P < 0.05$ level. In 6 of the 28 heifers no embryos were obtained; in these 6 cases, the quality of the transferred embryos, the transfer procedure, the heifers, and the flushing procedures did not differ in any obvious way from those of the successful flushings, which numbered 22 (79%). The mean embryo recovery rate was 40 ± 3% with a variation from 7% to 93%. There was a minor but not statistically significant difference between the overall recovery rate of embryos from the ipsi- versus contralateral horn, respectively (44 ± 5% vs. 38 ± 6%). In only 4 of the 16 heifers where transfer occurred to both horns was the recovery rate higher in contralateral side, compared to 9 heifers where the highest recovery rate was seen in the ipsilateral side. The oldest elongated embryos were in one occasion damaged and in another tangled, making it difficult to isolate the individual embryo; apart from that, all of the embryos seemed of excellent quality making it possible to isolate the embryonic discs. It can be concluded that it is possible to culture *in vitro* produced Day 6–7 bovine blastocysts in the uterus of synchronized heifers and to achieve an acceptable recovery of Day 12–14 embryos.

150 ENVIRONMENT OF THE EARLY EMBRYO AND ITS EFFECT ON DEVELOPMENT AND POSTNATAL LIFE

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We have investigated the impact of mouse early embryo *in vitro* culture environment on (a) short-term blastocyst development and (b) long-term postnatal growth and physiology after embryo transfer. *In vitro*-developed blastocysts, cultured from the 2-cell stage, had reduced inner cell mass (ICM) and trophectoderm (TE) cell numbers when compared to *in vivo*-derived blastocysts at 96 h post-hCG ($n = 13$–39, $P < 0.05$). Despite the retardation in blastocyst development, the ICM:TE ratio was equivalent in both treatment groups. Using embryo transfer techniques, we compared the postnatal development of embryos cultured *in vitro* from the 2-cell to the blastocyst stage (termed "*in vitro*" mice) with offspring generated from blastocysts developed *in vivo*, but which also underwent embryo transfer (termed "*in vivo*" mice). These two treatment groups were in turn compared with mice derived from naturally mated mothers, which had their mean litter size at birth adjusted to a size comparable with that of the *in vitro* and *in vivo* mice (a mean of 6 animals) and which had not been transferred. All data were analyzed using a multilevel random effects regression model which took into account between-mother and within-mother variation in litter size for parameters measured from individual animals. No significant differences in birth weight were observed between *in vitro* and *in vivo* offspring. However, *in vitro* offspring were significantly lighter than *in vivo* offspring in a gender-dependent manner at 2 weeks of age (males, $P = 0.009$) and at 6 and 11 weeks of age (females, $P = 0.037$ and

0.035, respectively). In addition, at 4 weeks of age, the *in vivo* males became significantly lighter when compared to the naturally mated males ($P = 0.034$). At 8 weeks of age, the *in vivo* females had a significantly elevated systolic blood pressure when compared to the *in vitro* females ($P = 0.003$); however, at 21 weeks of age, both *in vitro* males and females had a significantly elevated blood pressure when compared to *in vivo* offspring ($P < 0.003$). At 8, 15, and 21 weeks of age, offspring derived from transferred embryos developed with significantly elevated systolic blood pressure when compared to non-embryo transfer offspring ($P < 0.05$). No significant differences in serum angiotensin-converting enzyme activity (a potent regulator of systolic blood pressure) was observed between the treatment groups. Significantly altered liver:body weight ratios were observed between the *in vitro* and *in vivo* males, and between the *in vitro* and the naturally mated (6) females ($P < 0.038$). All of the above data are independent of litter size. These data support the hypothesis that early embryo environment can influence postnatal growth and cardiovascular physiology.

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151 EFFECTS OF HEXOSES SUPPLEMENTED IN THE MATURATION AND PRE IMPLANTATION MEDIUM ON THE *IN VITRO* DEVELOPMENT OF PORCINE OOCYTES

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The present study was conducted to determine the effects of hexoses on *in vitro* maturation, fertilization, and development of porcine oocytes. In the first experiment, porcine oocytes were matured in modified North Carolina State University (NCSU)-37 medium supplemented with 0.6 mM cysteine, 1 mM dibutyryl cyclic AMP (dbc AMP), 10 IU/mL equine chorionic gonadotrophin, 10 IU/mL human chorionic gonadotrophin, 50 μ/mL gentamycin (Sigma-Aldrich, Tokyo, Japan), 10% (v/v) porcine follicular fluid, and various hexoses (glucose, fructose, and galactose) at various concentrations of 0 (control), 2.5, 5.5, and 10 mM. They were subsequently cultured in the maturation medium without hormones and dbcAMP for an additional 22 h. Fertilization was performed according to Kikuchi *et al*. (2002 Biol. Reprod. 66, 1033–1041); 15 oocytes were co-incubated with 1 million frozen thawed sperm/mL in fertilization medium for 5 h. Supplementation of either glucose (2.5 or 5.5 mM) or fructose (5.5 mM) in the maturation medium significantly increased the percentages of maturation to metaphase II (68.5%, 79.4%, and 70.2%, respectively) and monospermic fertilization of oocytes (55.0%, 64.5%, and 58.9%, respectively), as compared with control group (metaphase II: 52.8%; monospermic: 42.7%; $P < 0.05$). Supplementation of galactose had no effect on the meiotic maturation and monospermic fertilization of oocytes. In the second experiment, presumptive zygotes were cultured in modified NCSU-37 supplemented with 4 mg/mL BSA, 0.17 mM sodium pyruvate, 2.73 mM sodium lactate, and 50 μg/mL gentamycin. The cleaved embryos were collected at Day 3 after *in vitro* fertilization and then cultured for a further 4 days in modified NCSU-37 medium supplemented with 5.5 mM of glucose, fructose, or galactose. The percentages of blastocyst formation, calculated from cleaved embryos, were significantly higher in the glucose and fructose groups (20.4% and 18.4%, respectively) than in the galactose group (12.9%) and the non-supplemented control group (9.2%). Although fructose supplementation did not accelerate blastocyst formation, it did significantly increase the mean cell number of blastocysts (48.0 ± 2.9 vs. 38.6 ± 1.8) and reduced the index of DNA-fragmented nuclei in the blastocysts stained by the TUNEL method (7.6 ± 0.9 vs. 11.8 ± 0.9), as compared with glucose supplementation. In the present study, although all hexoses were used through the glycolysis pathway, supplementation of galactose in the maturation and embryo culture medium had no beneficial effect on development. In contrast, both glucose and fructose enhanced development with an optimal concentration of 5.5 mM. Replacement of glucose with fructose did not accelerate blastocyst formation but did enhance embryo quality in terms of increasing cell number and decreasing the number of fragmented nuclei.

Embryo Manipulation

152 *IN VITRO* AND *IN VIVO* DEVELOPMENT OF BOVINE IVP EMBRYOS FOLLOWING SINGLE-CELL BIOPSY ON DAY 4

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Maximum advantage can be gained from gene discovery programs, by screening embryos carrying the desired genes(s) prior to immediate transfer. This requires an efficient and reliable genotyping system and a method for biopsy preparation that does not compromise subsequent embryo or fetal development. The present study examined the effect of removing a single-cell from the developing 8–16 cell embryo on its subsequent ability to continue development to at least the late morula stage *in vitro* and then survive following triple transfer to recipients. Abattoir-sourced ovaries were obtained and subjected to IVP as previously described (van Wagtendonk-De Leeuw AM *et al*. 2004 Reprod. Fert. Dev. 16, 214 abst). Briefly, oocytes were matured in TCM199 +10% FCS, 10 μg/mL FSH, 10 μg/mL LH, 1 μg/mL estradiol, and 100 μM cysteamine under 5% CO_2 in air at 38.5°C for 24 h. Percoll-separated sperm (1×10^6/mL) were then co-incubated with the matured oocytes (Day 0) for 24 h with the presumptive zygotes further cultured in mSOF medium under 5%CO_2, 7% O_2, 88% N_2. On Day 4 embryos with a minimum of 8 cells were selected and held at 38.5°C in HEPES-buffered SOF (HSOF) until biopsy at ambient temperature. Embryo biopsy was performed in HSOF medium + 5 μg/mL cytochalasin B. A single cell was removed using a 30 μm biopsy pipette. Both biopsied and control embryos were then further cultured in mSOF in individual