ABSTRACTS FOR POSTER PRESENTATION

Embryo Culture

143 DIETARY CARBOHYDRATES AND LIPIDS AFFECT IN VITRO EMBRYO PRODUCTION FOLLOWING OPU IN HEIFERS

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The superstimulation protocol of Blondin *et al.* (2002; Biol. Reprod. 66, 38–43) produces cumulus-oocyte complexes (COCs) of high developmental competence for IVP. Using a similar protocol we assessed the affects of alterations in oocyte donor carbohydrate and lipid metabolism during ovarian stimulation on the production and viability of blastocysts in vitro. A 2 × 2 factorial experiment offered two diets: Fiber (F) and Starch (S) alone (0) or with 6% w/w (6) protected lipid (calcium soaps of fatty acids). Thirty-two heifers ranked by body condition score (scale: 1 = thin, 5 = obese) were allocated within score to one of the 4 treatments: F0, F6, S0 and S6. COCs were collected 5 days after estrus by OPU for lipid analysis. Ovarian stimulation (4 doses of FSH (9 mg NIADDK oFSH) given 12 h apart) commenced 2 days later. COCs were collected 40 h after the last FSH injection. GnRH (0.012 mg Buserelin) was administered i.v. 6 h prior to OPU. A second period of ovarian stimulation and OPU then followed. Following IVM/IVF, zygotes were cultured in SOF with 0.3% w/v fatty acid-free BSA under oil (38.8°C, 5% CO_2, 5% O_2, 90% N) until Day 8 of development, when blastocysts were subjected to total cell counts and TUNEL analysis. Data were analyzed by ANOVA. Neither follicles aspirated (25.9 ± 1.87) nor oocytes recovered (12.1 ± 0.92) differed between treatments. Total fatty acids in plasma were greater ($P < 0.001$) for the F than for the S diets and increased with the inclusion of protected lipid (0.75, 1.82, 0.50 and 1.39 $\mu g\,mL^{-1}$ for F0, F6, S0 and S6, respectively; SED = 0.076). The dietary lipid-induced increase in plasma fatty acids was reflected in an increase ($P < 0.05$) in total fatty acids within the oocyte (70.4, 74.7, 69.9 and 78.4 ng/oocyte; SED = 3.41). Retrospective analysis by body condition indicated that S diets reduced ($P = 0.006$) blastocyst yields in thin heifers and reduced ($P = 0.02$) cleavage rates in fat heifers (Table 1). Blastocyst yields were lower ($P = 0.1$) for fat heifers on the F0 diet. Total cell numbers

 10.1071/RDv16n1Ab155

were greater for thin heifers on S0 than F0 diet. TUNEL-positive cells averaged 4.2 ± 0.48% and did not differ between treatments. In conclusion, modification of oocyte donor carbohydrate and lipid metabolism prior to OPU can influence IVP outcome in a complex manner dependent on body composition. Supported by Defra and The Perry Foundation.

Table 1. In vitro blastocyst yields and total cell numbers

Carbohydrate Protected Lipid (%)	Fiber		Starch		SED	*P*
	0	6	0	6		
(a) Thin heifers (2.5 ± 0.06 units)						
Cleavage rate (%)	72.3	83.3	75.0	81.0	9.8	NS
Blastocysts of cleaved (%)	45.6	43.9	30.9	14.7	8.0	0.006
Total cell count (*n*)	111.3	131.7	154.2	123.2	13.4	0.02
(b) Fat heifers (3.5 ± 0.08 units)						
Cleavage rate (%)	71.8	86.1	66.9	64.1	7.0	0.02
Blastocysts of cleaved (%)	11.4	31.1	35.0	33.9	11.0	0.1
Total cell count (*n*)	105.1	134.5	119.4	104.7	18.9	NS

144 ARE BLASTOCYST RATES A RELIABLE INDICATOR OF THE QUALITY OF AN IVP SYSTEM?

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Normally blastocyst rates are used to document the efficiency of an IVP system, because routine transfer of all embryos is not a realistic approach. Even though pregnancies are established, there will only be a weak correlation to a given IVP system because the embryos for transfer have been highly selected. The aim of this study was to analyze the in vitro development of bovine IVM/IVF oocytes after culture in SOFaa medium with or without the presence of bovine oviduct cells (BOEC) under 5% or 20% O_2 in 5% CO_2 and 38.5°C in order to select the optimal IVC system under the given circumstances. The study was based on six replicates and 2373 inseminated oocytes retrieved from abattoir ovaries, and the quality markers were Day 8 blastocyst rates (BL per inseminated oocytes), morphology, kinetics, and cell count. From the relative proportion of BL, XB, and H, an average developmental stage (kinetics) could be assigned. Ranking was based on BL rate, rates of A and B graded BL, and the average developmental stage. Established standard procedures were used for IVM (23 h in DMEM with 5% serum and eCG/hCG), and IVF (23 h in TALP with heparin), and the inseminated oocytes were randomly allocated into four IVC groups (5% O_2, 5% O_2/BOEC; 20% O_2, and 20% O_2/BOEC) to be cultured in groups of 25 in 0.1 mL oil-covered droplets of SOFaa with 5% serum (Holm P *et al.* 1999 Theriogenology 52, 683–700). The morphology was graded as A: compact and distinct inner cell mass, regular morphology of trophoblast cells, development corresponding to the expected; B: smaller or less distinct inner cell mass, a few degenerated trophoblast cells or slight fragmentation, development corresponding to the expected; C: dispersed or no inner cell mass, degenerated trophoblast cells or much fragmentation, developmental arrest. For cell counts the zona and cytoplasm from the individual blastocysts were lysed in 0.01 M HCL and 0.1% Tween 20, leaving the isolated nuclei to be fixed in 3:1 methanol:acetic acid on a slide (Viuff D *et al.* 2002 Biol. Reprod. 63, 1143–1148). The kinetics were assessed as hatched per total BL at Day 8 (Fisher's exact test, $P < 0.01$). The BL rates were significantly lower in the 20% O_2 group (23% v. 31%, 32%, 33% in the other groups, respectively), while the hatching rate was significantly higher in the 5% O_2 group (35% v. 12%, 10%, 18%). The frequency of A-quality blastocysts was significantly higher in the 5% O_2 and 20% O_2/BOEC groups (46%, 41%) than in the 20% O_2 and 5% O_2/BOEC groups (27%, 22%). The B-quality frequency did not differ between the four groups (41%, 40% v. 48%, 45%), whereas the C-quality inversely reflected the A-quality (13%, 19% v. 25%, 33%). There were no differences in the cell counts between the same quality grades in the four systems. An A-grade expanded BL had 134 ± 50 cells (mean ± SD), a B-grade 94 ± 45; a hatched A-grade BL had 168 ± 48 cells, a B-grade 143 ± 54. This study shows that regardless of differences in average developmental stages (kinetics) and morphology, similar blastocyst rates can be obtained. Using these criteria our four IVC groups would be ranked as (1) 5% O_2, (2) 20% O_2/BOEC, (3) 5% O_2/BOEC (4) 20% O_2. In conclusion, when evaluating the suitability of an IVP system, morphology and kinetics should be considered as well as blastocyst rates.

145 COMBINED GLUCOSE AND FRUCTOSE SUPPLEMENTATION IN PROTEIN-FREE KSOM IMPROVES PREIMPLANTATION DEVELOPMENT OF BOVINE TRANSGENIC CLONED EMBRYOS

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The present study investigated the effect of fructose supplementation in protein-free potassium simplex optimization medium (KSOM) on the preimplantation development of bovine transgenic cloned embryos. An expression plasmid containing bovine mutant PrP gene and enhanced green fluorescent protein (eGFP) as a marker gene was constructed and transfected into bovine fetal fibroblasts using FuGene6 (Roche, Indianapolis, IN, USA) as a lipid carrier. Transfected cells were cultured for 5 to 6 days to achieve chromosomal integration of the gene and then used for nuclear

transfer. The somatic cell nuclear transfer was carried out by transferring a GFP-expressing donor cell into the perivitelline space of an enucleated oocyte. After electrical fusion and chemical activation, 525 (11 replicates) fused embryos were cultured in KSOM supplemented with 0.01% (w/v) PVA for 192 h at 39°C under 5% CO_2, 5% O_2 and 90% N_2 gas atmosphere. The embryos were randomly allocated for 4 culture groups; KSOM supplemented with 1) 0.2 mM glucose, 2) 1.5 mM fructose, 3) 0.2 mM glucose + 1.5 mM fructose and 4) vehicle (without glucose and fructose). We used 0.2 mM glucose as per formulation of KSOM (Biggers JD *et al.* 2000 Biol. Reprod. 63, 281–293) and 1.5 mM fructose due to its benefical effect on embryo development (Kwun J *et al.* 2003 Mol. Reprod. Dev. 65, 167–174). As a control experiment, 1043 (17 replicates) in vitro fertilized (IVF) embryos were cultured in the same culture system. The data were analyzed by PROC-GLM using SAS program. In IVF embryos, no significant differences in rates of cleavage (71.7 to 75.5%), morulae (34.1 to 37.1%) and blastocysts formation (21.0 to 24.5%) among the culture groups were observed. In contrast, significantly ($P < 0.05$) higher rate in blastocysts formation (19.2%) was obtained when transgenic cloned embryos were cultured in KSOM supplemented with 0.2 mM glucose + 1.5 mM fructose than with 0.2 mM glucose (10.0%). However, the differences in blastocysts formation rates among other culture groups (13.2% for 1.5 mM fructose and 11.9% for vehicle) were not significant. Moreover, the rates of cleavage (76.2 to 79.1%), morulae (19.3 to 23.8%) and GFP expression in blastocysts (68.0 to 78.6%) did not differ significantly among the culture groups for transgenic cloned embryos. This study demonstrated that the requirement of energy substrates in culture medium for bovine transgenic cloned and IVF embryos might be different. Now, we are conducting experiments to confirm whether the beneficial effect on transgenic cloned embryo development is due to combined effect of glucose and fructose or due to total concentration of available energy. This study was supported by Biogreen 21-1000520030100000.

146 COMPARISON OF EMBRYO CULTURE MEDIA FOR BLASTOCYST DEVELOPMENT AFTER INTRACYTOPLASMIC SPERM INJECTION OF IN VITRO-MATURED EQUINE OOCYTES

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Research on in vitro culture of equine embryos has been scant, due to failure of equine in vitro fertilization to be repeatably successful. We have recently obtained high fertilization rates of equine oocytes via intracytoplasmic sperm injection (ICSI) using a piezo drill (Choi *et al.*, 2002 Reproduction 123, 455–465). Culture of presumptive zygotes in G1.2/2.2 medium resulted in 63% cleavage and an average of 15 cells at 4 d, but only 2 to 9% blastocyst development at 7 days (Choi *et al.*, 2003 Theriogenology 59, 1219–1229). In the present study, we evaluated the effect of two different culture media, G1.3/G2.3 v. DMEM/F-12, with or without FBS, on blastocyst development after ICSI. Oocytes were collected from slaughterhouse-derived ovaries by follicular scraping and were matured in vitro for 24 h in M199 with 10% FBS and 5 $\mu U\,mL^{-1}$ FSH. After culture, oocytes having a polar body (198/305; 65%) were fertilized by ICSI with frozen-thawed equine sperm using a piezo drill. Presumptive zygotes were cultured in 1 of 4 media: G1.3/G2.3 (which includes 0.8% BSA) with or without 10% FBS, or in DMEM/F-12 with 0.5% BSA, with or without 10% FBS. Culture was performed in microdroplets at 5 μL/zygote under oil at 38.2°C in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 for 7.5 days. In G1.3/2.3 treatments, G1.3 media were completely refreshed at 48 h, zygotes were transferred to G2.3 (with or without FBS as per the first stage) at 96 h, and were completely refreshed with the same media at 144 h. In DMEM/F-12 treatments, media were completely refreshed every other day. Three to 5 replicates were performed in each treatment, and data were analyzed by chi-square test. There were no significant differences in cleavage rates (59–64%) among treatments. The rate of development to blastocyst, per oocyte injected, in G1.3/G2.3/BSA (1/49, 2%) was significantly lower ($P < 0.05$) than that for the other three treatments: G1.3/2.3/BSA/FBS (9/49, 18%), DMEM/F-12/BSA (9/50, 18%), or DMEM/F-12/BSA/FBS (10/50, 20%). There was no significant difference in blastocyst development among the latter three treatments. These findings indicate that G1.3/2.3 media with BSA only do not adequately support growth of equine embryos. Development of up to 20% of injected oocytes to the blastocyst stage in G media supplemented with FBS, in DMEM/F-12/BSA or in DMEM/F-12/BSA/FBS represents the highest in vitro equine blastocyst rate in medium alone (i.e. without co-culture) yet reported. The success of DMEM/F-12 as an embryo culture medium may provide a relatively simple basis for equine in vitro culture programs. To determine whether this medium was able to support further developmental competence, we cultured equine embryos resulting from nuclear transfer of in vitro-matured oocytes in DMEM/F-12 + 10% FBS (without BSA). We transferred 4 resulting blastocysts to recipient mares by transcervical transfer; one pregnancy is ongoing at 230 d gestation at the time of this writing. This work was supported by the Link Equine Research Endowment Fund, Texas A&M University.

147 ION COMPOSITION OF CULTURE MEDIUM INFLUENCES MITOCHONDRIAL DISTRIBUTION AND BLASTOCYST DEVELOPMENT OF PREIMPLANTATION PORCINE EMBRYOS

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Elevated intracellular calcium (Ca^{2+}) concentrations impair hamster embryo metabolism and viability (Lane M and Bavister B 1998 Biol. Reprod. 59, 1000–1007). Extracellular magnesium (Mg^{2+}) regulates intracellular Ca^{2+} by controlling its uptake and release. In the present study, we examined the effects of altering Ca^{2+} and Mg^{2+} ion concentrations in Purdue Porcine Medium (PPM1) on porcine embryo mitochondrial distribution, metabolic (glycolytic and Krebs cycle) activity, and in vitro developmental potential. Cumulus-oocyte complexes collected from abattoir ovaries were matured for 40–42 h, inseminated with 5×10^5 sperm mL^{-1} for 5 h, and initially cultured in 1 : 0.4 or 2 : 1 ratio of Ca^{2+} to Mg^{2+} (concentrations in mM) at 38.7°C, in 6% CO_2, 10% O_2, balance N_2. At 22–26, 46–50, and 70–74 h post-insemination, 2-, 4-, and 8-cell embryos, respectively, were removed from culture to evaluate mitochondrial distribution (confocal microscopy after tetramethylrhodamine methyl ester staining) and glycolytic and Krebs cycle activity (5-[^{3}H]-glucose and 2-[C^{14}]-pyruvate, respectively). Remaining embryos were further cultured to determine developmental competence (2:1, $n = 548$; 1 : 0.4, $n = 560$). Cleavage was assessed on Day 3 (2 : 1, $n = 552$; 1 : 0.4, $n = 560$) of culture. All data were analyzed using GLM ANOVA, except mitochondrial distribution data which were analyzed using GLIMMIX. A majority ($P < 0.05$) of 2-cell (65%, 13/20) and 4-cell (67%, 22/33) embryos cultured in 2:1 displayed a homogeneous mitochondrial distribution. More (70%, 21/30; $P < 0.05$) 8-cell embryos

cultured in 2:1 had a perinuclear mitochondrial distribution. When cultured in 1:0.4, a majority (61%, 14/23; $P < 0.05$) of 2-cell embryos displayed a cortical mitochondrial distribution, whereas most ($P < 0.05$) 4-cell (66%, 19/29) and 8-cell embryos (69%, 18/26) displayed a homogeneous distribution. Glycolytic and Krebs cycle activities were similar ($P > 0.05$) between treatments and across all cell stages examined. Treatment had no effect ($P > 0.05$) on cleavage or blastocyst total cell number. Unlike hamster embryos, culturing pig embryos in a higher Ca^{2+} concentration resulted in more embryos developing to the blastocyst stage. Culture medium containing 2 mM Ca^{2+} and 1 mM Mg^{2+} best supports in vitro blastocyst development, possibly by supporting a more correct mitochondrial distribution. These results are not mediated via changes in glycolytic or Krebs cycle activity, thus suggesting that another cellular mechanism plays a key role in developmental competence in early pig embryos.

Table 1. Effects of Ca^{2+} : Mg^{2+} on porcine embryonic development and metabolic activity (mean ± SEM).

Ca^{2+} : Mg^{2+} (mM)	Cleavage day 3 (%)	Blastocyst day 6 (%)	Embryo cell stage (*n*)	Glycolysis*	Krebs cycle*
2 : 1	72.5 ± 1.8[a]	20.9 ± 2.0[a]	2 (27)	5.1 ± 0.4[a]	1.0 ± 0.3[a]
			4 (20)	5.1 ± 0.4[a]	0.9 ± 0.1[a]
			8 (40)	5.8 ± 0.7[a]	0.9 ± 0.1[a]
1 : 0.4	77.3 ± 2.0[a]	13.6 ± 1.6[b]	2 (25)	5.8 ± 0.4[a]	1.0 ± 0.1[a]
			4 (26)	4.7 ± 0.5[a]	1.0 ± 0.2[a]
			8 (18)	5.4 ± 0.6[a]	0.6 ± 0.1[a]

[a,b] Different superscripts indicate significant differences within a column ($P < 0.05$). *pmol/embryo/3 h

148 EPIDERMAL GROWTH FACTOR INDUCES BCL-XL GENE EXPRESSION AND REDUCES APOPTOSIS IN PORCINE PARTHENOTES DEVELOPING IN VITRO

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Epidermal growth factor (EGF) induces well-documented mitogenic and differentiating effects on murine and bovine preimplantation embryos. However, the effects of EGF on apoptosis and apoptosis-related gene expression in porcine embryos developing in vitro have not been evaluated. The objective of this study was to determine the effects of exogenous EGF in the presence and absence of BSA on the preimplantation development of porcine diploid parthenotes. In addition, we measured cell number, apoptosis, and expression of apoptotic-related genes of the blastocysts that developed in these culture conditions. In vitro-matured oocytes were parthenogenetically activated by square electrical direct current pulses. After 3 h of culture in North Carolina State University (NCSU) 23 medium with 0.4% BSA containing 7.5 mg mL^{-1} cytochalasin B, embryos were washed and cultured in NCSU 23 medium with 0.4% BSA for 48 h. The general linear models (GLM) procedure in the Statistical Analysis System was used to analyze developmental rates. A paired Student's *t*-test was used to compare relative gene expression. Presumptive diploid 4-cell parthenote embryos were randomly cultured in the same medium containing 0 or 10 ng mL^{-1} EGF in the presence and absence of 0.4% BSA. More 4-cell embryos developed into blastocysts at Day 7 when BSA was present at 0.1% (54.8% ± 4.9) and 0.4% (55.1% ± 3.1) than when BSA was absent (43.0% ± 3.5, $P < 0.05$). The addition of 10 ng mL^{-1} EGF into the medium did not significantly increase the developmental rate (40.2 ± 2.2 v. 35.1 ± 2.1), but EGF in the presence of 0.1 and 0.4% BSA significantly increased the cell numbers per blastocyst (51.3 ± 2.8 v. 37.8 ± 2.5 in 0.1% BSA; 51.8 ± 2.0 v. 42.7 ± 2.3 in 0.4% BSA, $P < 0.01$ for both comparisons). Furthermore, EGF treatment in the absence of BSA did not inhibit apoptosis in the blastocysts (6.8% ± 0.9 v. 8.8% ± 1.0), while addition of EGF in the presence of 0.4% BSA significantly reduced the degree of apoptosis in the blastocysts (4.0% ± 0.9 v. 9.3% ± 1.0, $P < 0.01$). To investigate whether EGF modulates mRNA expression of apoptosis-related genes, mRNA was prepared from single blastocysts and each preparation was subjected to RT-PCR for Bcl-xL and Bak transcripts. EGF enhanced the relative abundance of Bcl-xL expression in the presence of BSA ($P < 0.01$). The relative abundance of Bak expression was not altered by EGF treatment in either the presence or the absence of BSA. These results suggest that EGF and BSA synergistically enhance Bcl-xL gene expression, which may result in a net increase in cell number in porcine presumptive diploid parthenotes developing in vitro.

149 GLUCOSE METABOLISM OF IN VITRO AND IN VIVO PRODUCED BOVINE EMBRYOS

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It has been documented that higher glucose metabolism of bovine blastocysts is correlated with higher pregnancy rates following embryo transfer. The aim of this study was to determine the effect of switching embryos between in vivo and in vitro conditions on glucose metabolism. Four types of embryos were produced: Vivo-vivo: embryos were developed entirely in vivo (7.5 days); embryos were collected from superovulated cows at Day 5 following estrus, transferred to a recipient and collected again 2.5 days later to make an appropriate control. Vivo-vitro: embryos developed 5 days in vivo and then were cultured in vitro for 2.5 days. Vitro-vivo: embryos were produced in vitro using slaughterhouse oocytes and frozen semen, cultured until Day 5, and then transferred to recipients and recovered 2.5 days later. Vitro-vitro: embryos were produced entirely in vitro up to Day 7.5. Embryos were cultured in a chemically defined, sequential system (G1/G2), using recombinant human albumin as a protein source, supplemented with hyaluronan and citrate. At Day 7.5, glucose metabolism was measured by determining the amount of $3H_2O$ released when individual embryos

($n = 111$ for all groups) were placed in a 3-μL hanging drop containing ^{3}H-glucose in a closed chamber for 3 h. Embryos were graded 1 (good quality) or 2 (fair or poor). Data were analyzed by ANOVA using a 4×2 factorial design with factors group (vivo-vivo, vivo-vitro, vitro-vivo, vitro-vitro) and quality (1, 2). To validate the vivo-vivo group, 15 embryos produced in vivo (without collection and re-transfer) were analyzed for glucose metabolism, resulting in similar values. Grade 1 embryos metabolized more glucose ($P < 0.01$) than Grade 2 embryos (16.6 ± 1.2 v. 10.7 ± 2.5 pmol/embryo/h) The vivo-vivo and vitro-vitro embryos metabolized more glucose ($P < 0.05$) than the vivo-vitro and vitro-vivo embryos (15.4 ± 1.7 and 19.3 ± 2.0 v. 9.0 ± 4.5 and 10.7 ± 1.6 pmol/embryo/h, respectively). There was an interaction ($P < 0.05$) due to higher glucose metabolism of Grade 1 over Grade 2 embryos in the vitro-vivo and vitro-vitro groups, but no difference between grades for the vivo-vivo and vivo-vitro groups. We conclude that assessment for visual quality was meaningful, and that changing development conditions (vivo to vitro or vitro to vivo) appears to have a detrimental effect on glucose metabolic capabilities of bovine blasocysts lasting up to 2.5 days, and possibly on their developmental competence. Significantly, bovine blastocysts cultured in sequential media G1/G2 had equivalent glucose metabolism to those embryos developed completely in vivo.

150 PREGNANCY AND PARTURITION FOLLOWING TRANSFER OF BOVINE EMBRYOS CULTURED IN TWO MEDIA UNDER TWO OXYGEN CONCENTRATIONS

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Developmental aberrations following transfer of in vitro-produced bovine embryos can result in early gestational losses and offspring abnormalities. An ongoing study tests the hypothesis that such aberrations occur with equal frequency among commonly employed culture systems. In year 1, embryos were produced using oocytes from abattoir-derived ovaries (breed unspecified) and a proven Angus bull selected for low birth weight. IVC treatments were 2×2 factorial for medium (KSOMaa or SOFaa) and oxygen concentration (5% or 20%). Angus recipients ($n = 61$; 32 cows, 29 heifers) were randomly allotted to treatments for Day 7 transfers. Pregnancy was diagnosed with ultrasound several times during gestation (Table 1). At parturition calf weight, shoulder height, chest circumference, crown-rump length, and humeral and femoral length data were collected. Statistical analyses (Statistical Analysis System, Cary, NC) were logistic regression with a binomial distribution for pregnancy rate, and the general linear models procedure for calf measurements; included were fixed effects of medium, oxygen, and their interaction, with additional fixed effects of dam parity and calf sex where appropriate. No significant effects of medium or oxygen were found for pregnancy rate or calf measurements other than birth weight. Mean birth weight was higher in the KSOM, 20% oxygen treatment (Table 1), and medium-oxygen interaction for calf weight was also significant ($P < 0.01$). In year 2 embryos were produced using the same Angus bull and Angus oocytes. Angus recipients ($n = 38$; 32 cows, 6 heifers) were randomly allotted to treatments. Fetal crown-rump lengths were measured by ultrasound weekly from Days 33 to 54 and were analyzed as repeated measures using the mixed procedure. Pregnancy outcome and LS means for crown-rump lengths are included in Table 1. Though insufficient recipient numbers preclude determination of statistical significance, of interest is the relatively small fetal size in early gestation and large birth weights in the KSOM, 20% oxygen treatment. This treatment also contained a Day 33 pregnancy, subsequently lost by Day 40, in which the fetus was too small to obtain an accurate measurement. Fetal growth will continue to be monitored throughout gestation. Data will be collected at parturition as in year 1, and pooled analyses will be done.

Table 1.

IVC	Year 1						Year 2		
	n transfers	*n* pregnant		*n* at term (%)	Mean birth weight, lbs.	Birth weight range, lbs.	Mean crown-rump length, mm (*n* pregnant)		
		Days 35–56	Days 71–92				*n* transfers	Day 33	Day 54
KSOM, 5% O_2	16	7	7	6 (37.5)	82.8[a]	75–87	10	11.8 (5)	47.1 (4)
KSOM, 20% O_2	16	9	9	7 (43.8)*	106.8[b]	86–124	9	10.7 (5)	46.0 (4)
SOF, 5% O_2	14	7	7	6 (42.9)	93.1[ab]	83–105	10	13.9 (2)	48.8 (2)
SOF, 20% O_2	15	5	4	3 (20)	83.7[a]	79–97	9	14.1 (4)	47.8 (3)

[ab] Values with different superscripts are significantly different ($P < 0.01$). *Two calves died from suffocation due to severe dystocia.

151 A SIMPLE AND FAST METHOD FOR CONCURRENT DIFFERENTIAL STAINING AND TUNEL LABELLING OF BOVINE BLASTOCYSTS

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Currently techniques of TUNEL labelling for detection of apoptosis and differential staining for counting the ratio of inner cell mass (ICM) to trophoectoderm (TE) cells are used separately for assessment of embryo quality in different species. The majority of these techniques are antibody-based, and time-consuming, frequently giving inconsistent results. Here we report on the development of a simple and fast method for simultaneous

differential staining and TUNEL labelling of bovine embryos. Cleaved embryos produced from in vitro-matured and fertilized oocytes were cultured to the blastocyst stage in synthetic oviductal fluid culture medium (SOF) supplemented with 4 mg mL^{-1} BSA and 5% FCS. Embryos were partially permeabilized in 0.5% Triton X-100 solution containing 2 μM mL^{-1} TOTO-3 dye (Molecular Probes, Eugene, OR, USA) for 30 s. TOTO-3 is a cell-impermeant nucleic acid dye; thus only permeabilized cells are stained red. The embryos were then quickly washed in PBS containing 3 mg mL^{-1} PVA, fixed for 15 min at RT in 4% paraformaldehyde containing 10 μg mL^{-1} Hoescht, and TUNEL-labelled using a Cell Death Kit (Roche Applied Science, East Sussex, UI) for 30 min at 37°C in a humid chamber. The embryos were then treated with RNase A (50 U mL^{-1}) for 30 min at 37°C, washed and mounted in a small drop of glycerol on a glass slide. RQ1-DNase (3 U mL^{-1})-treated embryos were used as a positive control. After three-dimensional reconstruction using a Leica TCS SP2 confocal microscope, we determined the number of ICM (blue), TE (red) and apoptotic nuclei (green). Only peripheral cells of the blastocysts were labelled red, indicating that TE cells were permeabilized by the short exposure to the detergent Triton. ICM cells were consistently stained blue by the cell permeant dye Hoechst. Apoptotic nuclei were found in both types of cells. More consistent differential staining was observed in hatched blastocysts ($n = 30$) than in zona-enclosed blastocysts ($n = 35$); also, more apoptotic nuclei were observed. No differences were found in the consistency of the technique for embryos grown with or without FCS. When compared to dual staining without Tunel, no differences in cell number (74 ± 22) , and ICM/TE ratio (0.28 ± 0.06) were detected, indicating that the Tunel procedure does not affect the labeling of the DNA. Preliminary observations also indicate that this method can be successfully applied to porcine and ovine embryos. This technique has the advantage of being fast and can be applied for assessment of embryo quality. It can also be used to determine the time and origin of ICM and TE differentiation while monitoring the degree of apoptosis in different culture systems and in different species. This work was in part supported by Department of Environment Food and Rural Affairs (Defra) UK.

152 EFFECT OF REPLACEMENT OF PYRUVATE/LACTATE IN CULTURE MEDIUM WITH GLUCOSE ON PREIMPLANTATION DEVELOPMENT OF PORCINE EMBRYOS IN VITRO

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Kikuchi *et al.* (2002 Biol. Reprod. 66, 1033–1041) reported that replacement of pyruvate and lactate with glucose, as energy substrates, at 48 h of culture in IVC medium enhanced the quality of IVP porcine blastocysts. However, the exact time during early cleavage stages when the utilization of glucose as an energy source is optimal has not yet been determined. The purpose of this study was to examine the effects of glucose supplementation at different times of culture on the developmental competence of IVP porcine embryos. Porcine cumulus-oocytes complexes were matured in modified NCSU-37 solution and fertilized in vitro according to Kikuchi *et al*. All cultures were performed at 38.5°C, 5% O_2, 5% CO_2, and 90% N_2. In experiment 1, after being fertilized (Day 0), putative zygotes (1158 in 6 trials) were cultured in NCSU-37 supplemented with 0.4% BSA, 0.17 mM sodium pyruvate, and 2.73 mM sodium lactate (IVC-pyr/lac). Embryos (30–50 in each group) were then transferred into NCSU-37 supplemented with 0.4% BSA and 5.55 mM D-glucose (IVC-glu) at 24, 48, 72, 96, or 118 h of culture. As control groups, putative zygotes (391) were cultured in IVC-pyr/lac or IVC-glu for the whole culture period. In experiment 2, after being fertilized, putative zygotes (543 in 4 trials, 30–50 in each group) were cultured in IVC-pyr/lac, and then were transferred into IVC-glu at 48 h, 53 h, 58 h, or 63 h of culture, because glycolytic activity of in vitro-derived porcine embryos was reported to increase around the 8-cell stage, and some embryos develop to that stage before 72 h of culture in experiment 1. All embryos were cultured for 6 days, and then development to the blastocyst stage and number of cells per blastocyst were assessed. When IVF embryos were cultured in IVC pyr/lac for 24 h or 48 h and subsequently in IVC-glu until day 6 in experiment 1, the rates of blastocyst formation were significantly higher ($P < 0.05$, ANOVA test) than those of embryos cultured in IVC-pyr/lac for the whole culture period (24.4% and 23.0% v. 14.5%, respectively). However, when IVC pyr/lac was replaced with IVC-glu, there were no significant differences between the energy source replacement groups and the glucose-only group in terms of the proportions of cleavage, development to the blastocyst stage and mean cell number per blastocyst ($P > 0.05$, ANOVA test) (15.2%–24.4%, and 16.8%, respectively). Replacement of pyruvate and lactate with glucose at 58 h of culture in experiment 2 significantly enhanced the rate of blastocyst formation ($P < 0.05$, ANOVA test) but not the mean cell number compared with zygotes in which the replacement was done at 48, 53, and 63 h of culture (31.3% v. 20.6%, 20.8%, and 21.1%, respectively) ($P < 0.05$, ANOVA test). In conclusion, replacement of pyruvate and lactate with glucose as energy substrates was optimal at 58 h of culture for the in vitro development of pig embryos to the blastocyst stage.

153 EXPRESSION OF TGF-BETA I AND TYPE I AND TYPE II OF TGF-BETA RECEPTORS IN BOVINE EMBRYOS

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Although the effects of TGFβ1, as an important factor in the mice embryo development have been reported, little information relevant to this subject is known in the bovine embryo. The objectives of this study were to investigate the presence and expression patterns of TGFβ1 and TGFβ1 receptors, types I and II, in unfertilized oocytes and fertilized bovine embryos in normal and NT embryo development. We postulated that TGFβ1 may have a beneficial effect on the preimplantation embryo and show different expression patterns at different stages of bovine embryo development. Immature bovine oocytes were aspirated from follicles of ovaries obtained from a local abattoir and they were cultured for up to 24 h and fertilized in vitro. Reverse transcription-polymerase chain reaction (RT-PCR) and immunocytochemistry were used to investigate the presence of TGFβ1 and type I and type II of TGFβ1 receptors (the essential components of the TGFβ1 signaling pathway) in unfertilized oocytes and preimplantation embryos. Also, mRNA and protein expression patterns of TGFβ1 and their receptors at various stages of embryos were examined. It was found that both receptors, as well as TGFβ1, were present in the unfertilized bovine oocytes, indicating that TGFβ1 is a maternally expressed protein. Although

the type I TGFβ1 receptor was present at the morulae and blastocyst stages, the type II TGFβ1 receptor was not present at both stages. It was also confirmed that the expression level of TGFβ1 was high at the 8-cell stage, and mRNA and protein expression patterns of TGFβ1 and their receptors were not coincident. Interestingly, TGFβ1 protein was not detected at blastocyst stage of embryos, whereas the mRNA expression level was high at this stage. The results of this experiment indicate that TGFβ1 protein may be needed by embryos after the blastocyst stage and may be expressed in hatched embryos for implantation. These findings support the hypothesis that there may be an interaction between the TGFβ1 and TGFβ1 receptors in the unfertilized oocytes and preimplantation embryos, and that TGFβ1 signaling may be important for the development of the oocytes and the preimplantation embryos.

154 THE EFFECT OF FIBROBLAST GROWTH FACTOR-4 ON THE DEVELOPMENT OF NUCLEAR TRANSFER BOVINE EMBRYOS

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Fibroblast growth factor-4 (FGF-4) has been shown to be preferentially produced in the inner cell mass (ICM) of mouse blastocysts, to stimulate the proliferation of mouse trophectoderm (TE) cells and to repress their transformation and differentiation into giant trophoblasts. Recent studies have shown that nuclear transfer (NT) bovine embryos have aberrant allocations of ICM and TE cells (Koo *et al.*, 2002 Biol. Report. 67, 487–492) and aberrant expression of FGF-4 gene (Daniels *et al.*, 2000 Biol. Reprod. 63, 1034–1040). In this study, we examined whether recombinant human FGF-4 (rhFGF-4) stimulates development of nuclear transfer bovine embryos. As donor cells for NT, bovine ear skin fibroblast cells of passage 5 to 8 were used. Oocytes were enucleated after in vitro maturation in TCM 199 supplemented with 10% FBS, 1 $\mu g\,mL^{-1}$ FSH and 1 $\mu g\,mL^{-1}$ estradiol-17β for 20 h. Enucleated oocytes were fused with donor cells by a DC pulse of 25V/150 μm for 10 μs in Zimmerman cell fusion medium. For activation, fused oocytes were exposed to 10 μM Ca-Ionophore for 5 min, followed by 2 mM 6-dimethylaminopurine for 3 h. NT embryos were subsequently cultured in CR2 medium (containing 0.5% BSA) without or with rhFGF-4 (1, 10 and 100 $ng\,mL^{-1}$) at 39.0°C in 5% O_2, 5% CO_2 and 90% N_2. After 7 days of culture, blastocyst formation was observed. Apoptotic cells in blastocysts were detected by a terminal deoxynucleotidyl transferase-mediated d-UTP nick end-labeling (TUNEL) assay and total cell number was examined by propidium iodide (PI) counterstaining. Data were analyzed by chi-square test and Student's *t*-test. Supplementation of serum-free medium with rhFGF-4 increased the proportion of embryos developing to the blastocyst stage (18.4, 29.4 and 23.5% for 0, 1 and 10 $ng\,mL^{-1}$ FGF-4, respectively) and total cell number of blastocysts (66.3 ± 11.4, 75.9 ± 25.5 and 74.4 ± 22.4 for 0, 1 and 10 $ng\,mL^{-1}$ FGF-4). Particularly, 100 $ng\,mL^{-1}$ FGF-4 significantly ($P < 0.05$) increased the proportion of blastocysts (40.4%) and total cell number of blastocysts (86.7 ± 26.5) when compared with TCM 199 medium alone. FGF-4 also decreased the mean proportion of apoptotic cells in blastocysts (10.6 ± 7.8, 7.4 ± 5.3, 8.6 ± 5.3 and 7.5 ± 4.1% for 0, 1, 10 and 100 $ng\,mL^{-1}$ FGF-4). Our results suggest that FGF-4 may play a role in the early development of NT bovine embryos and might be a useful molecule for increasing development of NT bovine embryos in serum-free culture systems.

155 OXYGEN MEASUREMENTS AS INDICATORS OF CULTURE CONDITIONS AND VIABILITY OF CATTLE EMBRYOS

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Experiments were performed to study oxygen and temperature conditions experienced by embryos during routine in vitro production (IVP) (Exp. 1) and to evaluate oxygen consumption of single embryos as a viability indicator (Exp. 2). In both experiments, bovine IVP embryos were used (Holm et al. 1999, Theriogenology 52: 683–700), and a combined oxygen-temperature microsensor (tip diameter approx. 50 μm) was used to attain oxygen partial pressure gradients and temperature profiles. In Exp. 1, 4-well dishes with in vitro culture (IVC) medium (400-μL or 50-μL droplets) covered with 400 μL oil were taken from the incubator (5% CO_2 , 5% O_2 , 90% N) and measured under atmospheric air conditions at room temperature (24–25°C). The profiles were determined over 2 min, starting 2 min after the dishes were taken out of the incubator. The 400 μL medium was measured from the top layer and gradually 3/4 down into the well (seven replicates). The oxygen partial pressure decreased from 21.0 ± 0.66% O_2 at the top layer to 15.0 ± 1.6% O_2 at the bottom layer, while the average temperature increased from 27.2 ± 3.0°C to 31.7 ± 0.7°C. In the 50-μL droplets, the profiles were obtained in the middle of the droplet (five replicates). The oxygen partial pressure was 17.1 ± 2.44% O_2 and the temperature 31.0 ± 1.11°C. Consequently, routine handling of culture dishes outside the incubator seems to have a strong influence on both temperature and oxygen conditions. In Exp. 2, Day 3 and Day 7 embryos were evaluated morphologically before being loaded consecutively into one of the holes in a measuring block developed for this purpose. The block was previously covered with 40 mL IVC medium before being incubated for 3 days under 5% CO_2 , 95% N (38.5°C, 100% humidity). The oxygen consumption of each embryo was measured from 75 ± 5 min after removal from the incubator. Each measurement lasted approx. 5 min, was repeated three times for each embryo, and was performed under atmospheric conditions on a warming stage held at 37 ± 1°C. The average oxygen consumption by Day 3 embryos was 0.25 ± 0.14 nL/embryo/h ($n = 20$), and no clear relation between respiration rate and embryo morphology could be demonstrated. For Day 7 embryos, the average oxygen consumption was 0.90 ± 0.56 nL/embryo/h ($n = 22$), and this varied according to their morphological quality (1.17 ± 0.70 ($n = 7$), 0.98 ± 0.49 ($n = 6$) and 0.46 ± 0.38 ($n = 9$) nL/embryo/h for good, fair and poor quality embryos, respectively). In conclusion, this study illustrated the changes in O_2 partial pressure that embryos can temporarily be exposed to under routine handling, even for rapid procedures such as cleavage inspection. The results also show that respiration rate is lower in Day 3 than in Day 7 embryos. Furthermore, oxygen consumption values of Day 7 embryos seem to be in agreement with the morphological embryo quality, supporting the idea that oxygen consumption can be a valuable parameter for the evaluation of embryo viability.

156 INFLUENCE OF SERUM AND BSA ON BLASTOCYST DEVELOPMENT AND HATCHING IN IVF AND NUCLEAR TRANSFER BOVINE EMBRYOS

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Important differences exist between in vivo- and in vitro-produced bovine embryos. Studies have shown that various components in culture media affect embryo development, with serum producing some of the more detrimental effects. Efforts to develop a serum-free culture system have included looking at the effects of BSA, polyvinyl pyrrolidone and polyvinyl alcohol on embryo development. In this study, we compare serum and BSA during oocyte maturation and embryo culture of IVF and nuclear transfer (NT) embryos. *Experiment A:* Oocytes were aspirated from follicles and matured in either collection medium (Hams F-10 + 2% steer serum (SS); F-10) or in follicular fluid alone (FF). They were subjected to IVM-IVF-IVC as follows: 20–22 h maturation in synthetic oviductal fluid +8 mg mL^{-1} fatty acid-free BSA (SOF + BSA-FAF) supplemented with hormones, 18 h co-incubation with sperm in IVF-TALP, and culture for 9 days in SOF + BSA-FAF. *Experiment B:* Oocytes were randomly distributed for IVM-IVF-IVC into the following treatment groups: (i) IVM and IVC in SOF + 2% SS (SER), (ii) IVM in SOF + 2% SS and IVC in SOF + BSA-FAF (SER-FAF), (iii) IVM and IVC in SOF + BSA-FAF (FAF), and (iv) IVM and IVC in SOF + BSA-FrV (FrV). *Experiment C:* Oocytes were matured for 18 h in either SOF + 2% SS (SER) or SOF + BSA-FAF (FAF). Couplets were constructed with adult skin fibroblasts, exposed to a single pulse of 1.5 kV cm^{-1} for 40 s and activated using ionomycin and cycloheximide. Embryos were cultured in SOF + BSA-FAF. Three replicates with 100–120 oocytes per treatment group were carried out. *Results:* Cleavage rates were similar among all treatments in experiments A and B. No differences were observed between oocytes collected in F-10 or FF indicating that short-term exposure to serum does not have long-term effects on embryo development. Although a higher number of blastocysts was observed in the SER group on Day 6 (3.2% v. <0.5%; $P < 0.05$), no differences were seen in blastocyst development among the IVF treatment groups from Day 8 onwards (SER: 29.7%, SER-FAF: 21.1%, FAF: 20.4%, FrV: 19.9%). However, hatching rates on Days 8 and 9 were significantly greater ($P < 0.05$) in groups with serum, with the exception of FAF on Day 9 (SER: 31.1%, 57.2%; SER-FAF: 29.4%, 50.6%; FAF: 23.1%, 46.4%; FrV: 18.5%, 34.2%). In the NT treatment groups, the presence of serum during IVM resulted in a higher proportion of MII oocytes at 18 h, better oocyte quality for manipulation, and increased blastocyst development and hatching rates (SER: 31.4%, 18.2%; FAF: 21.7%, 4.8%). These results indicate that both serum and fatty acid-free BSA provide comparable embryo development during IVF. However, development in serum occurs at an accelerated rate as indicated by the shorter nuclear maturation times and blastocyst development on Day 6, which has been associated with adverse outcomes. Despite this, serum may provide the oocyte with factors that are important for membrane flexibility and repair, enabling greater survival after manipulation. Funding from NSERC and OMAFRA. Sperm provided by Gencor.

157 A WATER-SOLUBLE VITAMIN E ANALOGUE (TROLOX) IMPROVES OVINE EMBRYO DEVELOPMENT DURING SERUM-FREE CULTURE IN THE PRESENCE OF DOCOSAHEXAENOIC ACID (C22:6n−3)

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Vitamin E (α-tocopherol) supplementation of culture media can safeguard embryo development in vitro but, as it is fat-soluble, usually serum must be present. This study investigated whether Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid; Sigma, St. Louis, MO, USA), a water-soluble vitamin E analogue reported to scavenge peroxyl radicals in a manner similar to that of its parent compound, could provide antioxidant protection for ovine embryos in serum-free culture conditions in the presence or absence of supplementary docosahexaenoic acid (DHA, C22:6n−3), a polyunsaturated fatty acid found in vulnerable membranes. Abattoir-derived oocytes were matured and fertilized in vitro (IVF = Day 0). On Day 1, cleaved eggs were assigned to culture (5% CO_2, 5% O_2, 90% N_2; 38.5°C) to the blastocyst stage in synthetic oviductal fluid plus amino acids (SOF, $n = 15$ replicates, mean ± SEM = 231 ± 6 per replicate) supplemented with 0.4% w/v fatty acid-free BSA in the absence (SBSA) or presence of (SBSAD) DHA or the same media with 200 μM Trolox (SBSAT, SBSADT). Respective DHA concentrations (μg/mL; mean ± SEM) in these media were 0.0, 0.5 ± 0.12, 0.0 and 0.7 ± 0.27. Blastocyst lipids were separated into polar and neutral fractions (solid-phase extraction, aminopropyl silica columns), and fatty acid composition of each fraction was determined by gas chromatography (splitless method). Using a 2-way factorial design, blastocyst yields (Generalized Linear Model; binomial distribution) and fatty acid profiles (ANOVA) were analyzed. Days 6 + 7 blastocyst yields were 37 ± 1.5%, 12 ± 0.7%, 56 ± 2.6% and 34 ± 2.1% (mean ± SEM) for SBSA, SBSAD, SBSAT and SBSADT, respectively (+DHA effect, $P < 0.001$; + Trolox effect, $P < 0.001$; Interaction, $P < 0.001$). Corresponding cell counts (86 ± 4, $n = 64$; 72 ± 4, $n = 19$; 89 ± 3, $n = 103$; 79 ± 3, $n = 62$) were affected only by presence of DHA ($P < 0.01$). Fatty acid content in neutral lipids (ng/blastocyst) was 64 ± 3, 73 ± 2, 66 ± 5 and 66 ± 5 and, in polar lipids, 47 ± 3, 56 ± 3, 46 ± 3 and 51 ± 7, for SBSA, SBSAD, SBSAT and SBSADT respectively (NS). These data indicate that addition of Trolox to DHA-supplemented culture medium improved production of blastocysts. Presence of DHA, however, was not associated with alterations in neutral and polar lipid content or fatty acid composition of the blastocysts. The fact that Trolox enhanced blastocyst yields in the absence of supplementary DHA indicates that conventional media may benefit from provision of this antioxidant. Funded by SEERAD; AR supported by Portuguese Ministry of Science and Technology.

Table 1. Fatty acid complement in neutral and polar lipids (ng/embryo) from blastocysts produced in vitro

Data presented are mean ± s.e.m. values per test sample ($n = 2$ to 4 samples)

	SBSA	SBSAD	SBSAT	SBSADT
Neutral lipids				
Saturates	57 ± 3.4	73 ± 2.2	58 ± 3.5	58 ± 5.0
Monounsaturates	4.5 ± 1.7	0	5.9 ± 1.8	4.8 ± 1.4
Polyunsaturates	2.0 ± 0.3	0	2.4 ± 0.2	2.9 ± 1.7
Polar lipids				
Saturates	43 ± 1.5	54 ± 6.0	39 ± 0.6	47 ± 6.2
Monounsaturates	3.7 ± 1.3	2.3 ± 2.3	5.9 ± 2.9	3.2 ± 0.7
Polyunsaturates	0.6 ± 0.6	0.4 ± 0.4	0.8 ± 0.4	0.8 ± 0.3

Within rows, differences are not significant.

158 APOPTOSIS IN IN VITRO PRODUCED BOVINE EMBRYOS ACCORDING TO DEVELOPMENTAL KINETICS

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Apoptosis has been previously reported in embryos during late pre-implantation development. Fast-developing embryos are known to present higher developmental competence. The aim of the present work was to evaluate the quality of in vitro-produced bovine embryos with fast (8-cells at 48 hours post-insemination (hpi) and slow (8-cells at 90 hpi) cleavage and study the correlation of this phenotype with programmed cell death occurrences. Embryos were produced from immature oocytes obtained from slaughtered cow ovaries, after maturation and fertilization, presumed zygotes were cultured in CR2 medium with 10% FCS, together with granulosa cells under 5% CO_2 atmosphere. The number of nuclei in the inner cell mass and trophectoderm (ICM/TE), as well as the number of nuclei with fragmented DNA, were estimated by applying differential staining and TUNEL, respectively; data were analyzed by ANOVA (JMP—SAS Institute). To test the expression of apoptosis regulating genes, a pool of fifty 8-cell embryos from each group (fast and slow) were collected. After RNA extraction and reverse transcriptase reaction, cDNA was amplified with Bax and Bcl2 primers, individually. Results indicated, as expected, higher quality in fast-cleaving embryos, estimated by the number of ICM nuclei (20.8 ± 1.4 and 15.6 ± 2.1—$P \leq 0.05$); however, the number of TE didn't show significant differences (54.9 ± 2.4 and 53.2 ± 3.8); the same was observed for total cell number (75.7 ± 2.8 and 68.8 ± 4.4). The frequency of blastocyst TUNEL-positive nuclei as an estimate of total cell number was significantly larger in the slow group when compared to the rapid development group (19.0 ± 2.5% and 8.5 ± 1.4%, respectively, $P \leq 0.05$). The greater proportion of morphologic abnormal nuclei in both groups was located in the ICM, and may explain the lower number of ICM nuclei in slow developing embryos. Hence, embryos of slow development show TUNEL-positive blastomeres at the 8-cell stage, but no fragmented nuclei were observed in embryos at 48 hpi. Bax and Bcl2 cDNA amplification showed that both mRNAs were constitutively present at the 8-cell stage in both groups. It can be concluded that in vitro-produced bovine blastocysts, with slow development to the 8-cell stage, present lower quality compared with fast development homologues, estimated by mean number of ICM nuclei, as well as nuclei fragmentation in blastomeres (TUNEL-positive). There is a difference in fragmented nuclei proportion between both groups at the 8-cell stage, but this result may be biased by the numbers of hours in culture. It was possible to demonstrate the presence of mRNA for pro (Bax) and anti-apoptotic (Bcl2) genes in slow- and fast-developing embryos at the 8-cell stage, and the future determination of the ratio between these two transcripts may allow the evaluation of the participation of pre-transcriptional regulation of these genes on the induction of DNA fragmentation. Financial support: Grant 99/12351-3 FAPESP São Paulo, Brazil.

159 EFFECT OF GRANULOCYTE-MACROPHAGE COLONY STIMULATING FACTOR (GM-CSF) AND SERUM, ALONE OR IN COMBINATION, ON INTERFERON-TAU (IFNT) PRODUCTION BY OVINE EMBRYOS

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The objectives were to establish whether GM-CSF, which may stimulate IFNT production by blastocysts at implantation, influences IFNT production by ovine embryos in culture and whether GM-CSF can explain stimulation of IFNT production by ovine serum (Rooke *et al.*, 2003 Reprod. Abstr. Series 30 : 56). Oocytes, aspirated from sheep ovaries obtained from a local abattoir on 4 separate days, were matured and fertilized by standard procedures. On Day 1, cleaved zygotes (approximately 10 per 0.05-mL^{-1} drop; total, 175 per treatment) were cultured in synthetic oviductal fluid containing either 0.3% bovine serum albumin and amino acids (SOFA) or 10% sheep serum (SOFS) in the presence (+) or absence (−) of 5 ng mL^{-1} ovine GM-CSF. Medium was changed every 2 days. On Days 6 and 7, blastocysts were removed from their group drops and assigned in a balanced manner individually to 0.05-mL^{-1} drops of one of the 4 media for a further 24 h. Embryo grade, diameter and cell number were recorded. IFNT concentrations in conditioned media samples were determined by ELISA. The presence of serum accelerated blastocyst development (cross-tabulation; chi-square analysis; $P = 0.039$; Table 1). Both serum and GM-CSF in group culture media increased (2 × 2 factorial ANOVA, $P < 0.001$) IFNT concentrations (ng mL^{-1}; SOFA−, 0.5; SOFA+, 1.7; SOFS−, 2.7; SOFS+, 4.2; SED, 0.47) with no interaction between serum and GM-CSF. After 24 h individual culture of Day 6 blastocysts, IFNT concentrations (2 × 2 factorial ANOVA; Table 1) were influenced mainly by

the origin of the blastocyst (group treatment covariate, serum, $P < 0.001$; GM-CSF, $P < 0.001$) rather than by individual blastocyst culture media (GM-CSF, NS; serum, $P < 0.001$). The pattern of results was similar for Day 7 blastocysts. Incubation of zygotes in the presence of GM-CSF had no effect on embryo grade or diameter but blastocysts (both day 6 and 7) incubated individually for 24 h in the presence of GM-CSF had more cells (128 v. 119; SED, 3.52; $P = 0.001$) than blastocysts incubated concurrently without GM-CSF. However, the pattern of IFNT production after correction for cell number was unchanged. In conclusion, culture of zygotes in medium containing GM-CSF or serum, alone or in combination, from Days 1 to 7 increased IFNT production, and IFNT production by individually cultured blastocysts depended on their origin. SAC receives financial support from the Scottish Executive Environment and Rural Affairs Department.

Table 1. Blastocyst yields (% of zygotes) on Days 6 and 7 and IFNT concentrations (ng mL^{-1}) in media after individual culture of Day 6 blastocysts

	Original (group) media				SED
	SOFA−	SOFA+	SOFS−	SOFS+	
Blastocyst yields					
Day 6 (%)	52 (30)	51 (29)	56 (32)	69 (39)	
Day 7 (%)	47 (27)	33 (19)	36 (21)	26 (15)	
IFNT production (ng mL^{-1})					
24 h SOFA−	1.8	4.6	8.3	10.7	0.72
24 h SOFA+	2.0	5.5	8.7	10.9	
24 h SOFS−	2.1	5.2	12.5	11.0	
24 h SOFS+	2.1	5.3	10.4	12.3	

160 EFFECTS OF EXTRACELLULAR ION CONCENTRATIONS ON IN VITRO DEVELOPMENT OF DOMESTIC CAT IVF EMBRYOS

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Domestic cat embryos typically are cultured in media formulated for somatic cells or embryos from rodents or livestock species. Under these conditions, blastocyst development has been inconsistent and delayed relative to embryos grown in vivo, and embryo viability following transfer has been low. Our goal is to systematically define the culture requirements of the feline embryo to improve embryo development and viability. The objective of this study was to determine the ionic (NaCl, KCl, KH_2PO_4, and $CaCl_2 : MgSO_4$) preferences of domestic cat IVF embryos. Anestral female cats were injected (i.m.) with 150 IU eCG followed 84 h later by 100 IU hCG. Oocytes were recovered via laparoscopic follicular aspiration approximately 24 h post-hCG injection (Day 0). Semen was collected from one of two males by means of an artificial vagina and washed once in HEPES-buffered IVF medium. Mature cumulus-oocyte complexes were co-incubated with 2.5–5 $\times 10^5$ motile sperm mL^{-1} in IVF medium (100 mM NaCl, 4.0 mM KCl, 1.0 mM KH_2 PO_4, 2.0 mM $CaCl_2$, 1.0 mM $MgSO_4$-7H_2O, 25.0 mM $NaHCO_3$, 3.0 mM glucose, 0.1 mM pyruvate, 6.0 mM L-lactate, 1.0 mM glutamine, 0.1 mM taurine, 1 × MEM nonessential amino acids, 50 μg mL^{-1} gentamicin, and 4.0 mg mL^{-1} BSA) for 19 to 22 h in 6% CO_2 in air (38.7°C). Cumulus cells were removed and embryos cultured (8–11 embryos/50 μL drop; 6% CO_2, 5% O_2, 89% N_2, 38.7°C) in media containing 100.0 or 120.0 mM NaCl, 4.0 or 8.0 mM KCl, 0.25 or 1.0 mM KH_2PO_4, and 1.0 mM : 2.0 mM or 2.0 mM : 1.0 mM $CaCl_2 : MgSO_4$ (2 × 2 × 2 × 2 factorial design). The remaining components of the culture medium were identical to the IVF medium (but w/o gentamicin). Development to the blastocyst stage by Day 6, metabolism (glycolysis and pyruvate) of each blastocyst, and final cell number (Hoechst 33342 staining) of all embryos were evaluated. Final cell number of cleaved embryos and development to the blastocyst stage were analyzed using analysis of variance in the GLIMMIX macro of SAS. A total of 236 oocytes were inseminated, yielding 128 cleaved embryos (54%), including 6 blastocysts (4.7% of cleaved embryos). Cell number was not ($P > 0.05$) affected by NaCl, KCl, or KH_2PO_4 concentrations, but tended ($P = 0.057$) to be higher after culture in 2.0 mM : 1.0 mM $CaCl_2 : MgSO_4$. Treatments did not significantly affect ($P > 0.05$) development to the blastocyst stage, but numerically more blastocysts were produced in 100.0 mM NaCl (4/6), 8.0 mM KCl (5/6), or 1.0 mM KH_2PO_4 (5/6). Both $CaCl_2 : MgSO_4$ ratios resulted in 3 blastocysts. Blastocysts contained 61.08 ± 5.1 (mean ± SEM, $n = 6$) cells and actively metabolized glucose (glycolysis, 3.7 ± 0.8 pmol/embryo/3h or 0.06 ± 0.01 pmol/cell/3h) and pyruvate (0.75 ± 0.27 pmol/embryo/3h or 0.013 ± 0.005 pmol/cell/3h). These results suggest that the ionic composition of culture media influences the in vitro development of cat IVF embryos. (Supported by NIH grant RR15388.)

161 EFFECT OF BOVINE PITUITARY EXTRACT ON HATCHING AND POST-HATCHING DEVELOPMENT OF IVP BOVINE EMBRYOS

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The effect of bovine pituitary extract (BPE; BD Biosciences, Inc., San Jose, CA, USA) on development of in vitro-produced (IVP) bovine blastocysts after Day 7 was studied. IVP embryos were produced from in vitro-matured COCs processed from local slaughterhouse ovaries or

obtained from Bomed, Inc., Madison, WI, USA. The first 7 days of embryo culture following in vitro fertilization were in G1/G2 medium in $5\%O_2 + 5\%CO_2 + 90\%N_2$. Embryos that had reached the compacted morula or early blastocyst stage on Day 7 (29% of total) were identified and randomly assigned to four media treatments ($n = 75$/treatment, $r = 7$). DMEM/199 medium (D2) or G2, each supplemented by the addition of BSA, insulin, transferrin, and selenium (ITS), were used with or without BPE at 30 $\mu g\,mL^{-1}$. Extended embryo culture was continued until Day 11. Further development was assessed by counting hatched blastocysts and measuring the size (cross-sectional index) and the number of cells per blastocyst. Size diameters of blastocysts ranged from 200 μm to 400 μm across treatments. Cell counts were performed by staining the fixed blastocysts with Hoechst 33342 and counting the nuclei of squashed blastocysts with a fluorescent microscope. The percentage of hatched blastocysts was significantly greater ($P < 0.05$) in G2 with BPE ($63.7 \pm 6.8\%$) than in G2 alone ($29.6 \pm 7.3\%$). D2 with BPE ($59.3 \pm 6.8\%$) was not significantly different from D2 alone ($56.8 \pm 7.9\%$). Area index gave similar results; G2 + BPE (267 ± 168) was significantly higher ($P < 0.05$) than G2 alone (76 ± 28), and D2 + BPE (192 ± 139) and D2 alone (155 ± 106) were not different. Cell number gave similar results ($P < 0.05$); G2 + BPE (303 ± 164), G2 alone (123 ± 47), D2 + BPE (254 ± 166) and D2 alone (233 ± 120). A second experiment randomly assigned 7 day embryos (33% of total) to extended culture in G2 or D2 medium with or without irradiated STO feeder cells and under either 5% O_2 or 20% O_2 ($n = 49$/treatment, $r = 5$). All treatments contained BPE and a layer of agarose. Hatched blastocysts were counted on Day 11 and percentages were not different ($P < 0.05$) for both media without STO in low O_2 ($G2 = 75 \pm 12$ %; $D2 = 67 \pm 8\%$) and in air ($G2 = 82 \pm 19\%$; $D2 = 83 \pm 11\%$). Hatching was not different ($P < 0.05$) with STO in both media in air ($G2 = 73 \pm 10\%$; $D2 = 68 \pm 15\%$), but was significantly reduced ($P < 0.05$) when STO was combined with low oxygen ($G2 = 28 \pm 14\%$; $D2 = 4 \pm 6\%$). Further culture to 18 days was completed, but many of the embryos collapsed by Day 12 and thereafter grew as lobed bodies and occasionally attached to each other. By 18 days a few embryos continued uncomplicated growth, however, and were composed of as many as 6900 cells. These results indicate that promotion of in vitro bovine blastocyst development in a minimal medium such as G2 requires BPE while a more complex medium, D2, supports further development without BPE. STO feeder cells in combination with low oxygen culture was inhibitory to blastocyst hatching and survival.

162 INSULIN IMPROVES FREQUENCY OF CLEAVAGE AND IN VITRO DEVELOPMENT OF BOVINE EMBRYOS IN DEFINED MEDIUM

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Pre-elongation stage bovine embryos contain insulin receptor, ligands and receptors of IGF-I and IGF-II, and possibly EGF ligand. Cumulus cells and blastocysts also express EGF receptor; however, insulin is not produced (Watson AJ *et al.* 1992 Mol. Reprod. Dev. 31, 87–95; Tetens F. *et al.* 2000 Anat. Embryol. 201, 349–55; Yaseen MA *et al.* 2001 Reproduction 122, 601-10). Reported effects of external growth factors during bovine IVC are conflicting. The present study evaluated effects of EGF and insulin on the development of in vitro-produced embryos in a chemically defined IVC medium. IVM medium was TCM199 plus fetal bovine serum, LH and estradiol. IVF was done in Tyrodes solution containing BSA, lactate, pyruvate, heparin, penicillamine-hypotaurine-epinephrine and nonessential amino acids. At 18 h post-insemination, ova were vortexed, washed and placed in IVC treatments. Modified KSOM (Yang BK *et al.* 1995 J. Reprod. Dev. 41, 213–18) with 1X MEM nonessential and 1X BME amino acids was the base IVC medium. Incubations were done at 39°C in a humidified atmosphere of 5% CO_2 in air (IVM, IVF) or 5% CO_2, 10% O_2 and 85% N_2 (IVC). Effects of three EGF (0, 50, 100 $ng\,mL^{-1}$; Experiment 1) or five insulin (0, 5, 10, 15, 20 $\mu g\,mL^{-1}$; Experiment 2) doses were tested, in five replicates, by supplementation during the entire IVC period (up to Day 9; Day 0 = day of insemination). Embryos were placed in fresh medium on Day 4 post-insemination. Data were analyzed using one-way ANOVA and Tukey's test. In Experiment 1 ($n = 646$ oocytes), EGF treatment did not affect percentages of Day 2 cleavage (83 ± 3, 82 ± 3, and $81 \pm 2\%$, respectively; mean ± SEM.) and Day 9 blastocysts (34 ± 4, 33 ± 4 and $33 \pm 5\%$, respectively) or cell number per blastocyst after Hoechst staining (95 ± 5, 88 ± 4 and 89 ± 5, respectively). In Experiment 2 ($n = 687$ oocytes), insulin improved the frequency of cleavage and percentage of expanding, hatching and total blastocysts. It also increased blastocyst cell number (see Table 1). Lack of effect of EGF on bovine IVC is in contrast to its widely reported beneficial effects in mice. In many bovine IVC studies, the beneficial effects of external insulin were seen at or after the morula stage. The present study demonstrates that insulin can influence embryonic development as early as the initial cleavage stage and before embryonic genome activation. Reseach was funded in part by J. Bennett Johnston Science Foundation.

Table 1. Effects of insulin ($\mu g\,mL^{-1}$) on development to >2-cell (Day 2), expanded, hatched and total blastocysts (Day 9: % ± SEM) and on blastocyst cell number (Day 9: mean ± SEM)

Insulin	Oocytes, *n*	> 2-cell	Expanded+	Hatched	Total	Cell number
0	133	79 ± 2^{a}	24 ± 2^{a}	8 ± 2^{a}	31 ± 2^{a}	92 ± 8^{a}
5	166	85 ± 2^{ab}	35 ± 5^{b}	22 ± 5^{b}	43 ± 8^{ab}	122 ± 12^{ab}
10	137	89 ± 0^{b}	38 ± 7^{b}	19 ± 5^{b}	48 ± 7^{b}	165 ± 15^{b}
15	123	91 ± 3^{b}	43 ± 5^{b}	24 ± 2^{b}	45 ± 4^{ab}	161 ± 17^{b}
20	128	86 ± 4^{ab}	34 ± 4^{b}	19 ± 5^{b}	42 ± 5^{ab}	163 ± 13^{b}

[ab] Intracolumn values bearing different letters differ significantly ($P < 0.05$).

163 IMPROVEMENT OF IN VITRO PRODUCTION OF PIG EMBRYOS BY SYNCHRONISATION OF OOCYTE MEIOTIC MATURATION WITH CYCLOHEXIMIDE

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It is suggested that the relatively high rates of polyspermic fertilization and poor development of pig embryos produced in vitro are caused by asynchronous oocyte maturation. We have recently shown that pre-treatment of pig oocytes with cycloheximide (CHX) is an efficient way of synchronizing their meiotic maturation in vitro. However, it is not known whether this procedure affects fertilization or further development. The present study examined the effects of CHX-synchronised meiotic maturation on subsequent embryo development and the response to FSH. Pig ovaries were collected from a local abattoir. Cumulus-oocyte complexes (COCs) were aspirated from 3–5 mm diameter follicles with a translucent appearance and extensive vascularization. COCs were first pre-incubated in defined maturation medium (DM; M199 with Earle's salts, 25 mM HEPES and sodium bicarbonate, 3 mM L-glutamine, 0.1% (w/v) BSA, 0.57 mM cysteine, 10 ng mL^{-1} EGF, 0.2 μg mL^{-1} pLH, 100 iu mL^{-1} penicillin and 0.1 mg mL^{-1} streptomycin) or in DM supplemented with 50 ng mL^{-1} pFSH (DMF) and 5 μg mL^{-1} CHX for 12 h. COCs were then further cultured in the same DM without CHX for 24–30 h or in DMF for 36 h. For controls, COCs were cultured conventionally in DM for 42 h or DMF for 48 h. After removal of cumulus cells, all cultured oocytes were inseminated with ejaculated sperm at a final concentration of 300 000 mL^{-1} for 6 h. The IVF medium was modified Tris-buffered medium containing 0.1% BSA, 20 μM adenosine and 0.2 mM reduced glutathione. Putative embryos were cultured in NCSU23 without glucose but supplemented with 4.5 mM Na lactate and 0.33 mM Na pyruvate for 2 days. Cleaved embryos were further cultured in normal NCSU23 for 4 days. IVM and IVF were performed in 5% CO_2 in air and IVC in 5% CO_2, 5% O_2, 90% N_2, all at 39°C and 95% RH. Three replicates with DM, with or without CHX, and one with DMF, with or without CHX, were performed with 30–50 oocytes in each replicate. Statistical comparisons were by *t*-test. The result with DM showed that the rate for normal cleavage at 2 days after insemination of CHX-treated oocytes (40.6 ± 3.8%) was similar to that of controls (40.4 ± 3.5%). However, the proportion developing to healthy blastocysts at Day 6 was significantly higher in the CHX-treated group (16.9 ± 1.2%) than in controls (9.6 ± 1.3%; $P < 0.05$). A significantly higher number of Day 2-cleaved embryos from CHX-treated oocytes developed to the day 6 blastocyst stage compared with controls (44.7 ± 5.0% and 22.3 ± 2.4%, respectively; $P < 0.05$). Supplementation of the basic maturation medium with pFSH increased the rate of cleavage in both CHX-treated oocytes (73.2%) and controls (76.9%) and increased the proportions developing to healthy blastocysts at Day 6 (CHX-treated: 39.0%; control: 11.5%). We conclude that oocytes pre-treated with CHX retain their developmental competence and that meiotic synchronization with CHX improves the efficiency of in vitro production of pig embryos. (Supported by BBSRC 42/S18810.)