ABSTRACTS FOR POSTER PRESENTATION

Developmental Biology

125 MORPHOLOGICAL CHARACTERIZATION OF DAY 14 AND DAY 21 IVP AND CLONED BOVINE EMBRYO DEVELOPMENT

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The establishment of viable embryonic development following in vitro production (IVP) or nuclear transfer by SUZI (subzonal injection) and handmade cloning (HMC) methods is pivotal for practical implementation of embryo technology. The development of cloned embryos is currently impeded by the high rate of pre- and postnatal losses. This project is aimed at developing screening methods using both ultra-structural and molecular markers of development of post-hatching/pre-implantation stage embryos to enable selection of embryos with greater developmental competence. Initially twenty embryos derived by IVP or nuclear transfer (SUZI or HMC) were transferred to six recipient cows on Day 7 ($n = 120$). Embryos were collected on Days 14 and 21 by flushing the uterine tracts of slaughtered cows. Embryos were fixed in 4% paraformaldehyde and processed for paraffin embedding. Serial sections were stained with haematoxylin and eosin and evaluated by light microscopy. On each day of collection, certain characteristics were expected to be observed according to normal in vivo development (Maddox-Hyttel *et al.*, 2003, Reproduction 125, 607–623). On Day 14, IVP embryos ($n = 6$) were ovoid to filamentous in shape. Three embryos had extensive foldings of trophoblast in the process of forming an amniotic cavity. NT-SUZI embryos ($n = 4$) had a mainly round uneven shape. No NT-HMC embryos were recovered. On Day 21, IVP embryos ($n = 4$) more closely resembled in vivo embryos than any other group and formed either a neural groove or a neural groove with somites. NT-SUZI embryos ($n = 6$) were mainly filamentous, with one embryo showing complete development of the neural tube and 5 somite pairs. NT-HMC ($n = 17$) development ranged from ovoid to multi-protrusions of the yolk sac. One embryo displayed the formation of a neural tube. Embryos, irrespective of production method, which had an abnormal morphology, presented only an incomplete hypoblast and severe degeneration of the epiblast. Those embryos that were morphologically comparable to in vivo embryos also displayed developmental stages which ranged from presentation of a primitive streak and formation of a neural tube to having a neural tube, differentiation of mesoderm and somites. At present, the low number of embryos recovered as well as their abnormal development clearly reflect the low success rate to term of live calves. It is possible that no Day 14 NT-HMC embryos were recovered due to collection efficiency not being optimal. It is apparent that IVP and cloned embryos have different developmental time lines as compared to that of in vivo embryos.

126 MOUSE BLASTOCYST FORMATION IS REGULATED BY SPECIFIC PKC ISOFORMS THAT AFFECT LOCALIZATION OF Na/K-ATPase

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During blastocyst biogenesis, establishment of a trans-trophectodermal ion gradient by the Na/K-ATPase ion transporter family is essential for fluid accumulation within the blastocoelic cavity. In the mouse, the $\alpha 1\beta 1$ isozyme combination confined to the basolateral membrane within the trophectoderm is considered mainly responsible. Enzyme subunits, e.g. $\alpha 1$, can become inactive by internalization upon phosphorylation potentially mediated by protein kinase C (PKC). At least 11 PKC isotypes with variable biological functions dependent upon cellular contexts have been

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identified so far. The present study examined the role of specific PKC isoforms during cavitation in relation to potential influence on Na/K-ATPase localization using cell-permeable PKC isoform-specific activating or inhibiting peptides. At 20 h post-compaction, in vitro-generated (T6-BSA) late morulae (MF1) were incubated for up to 4 hrs in 500 μL DMEM with 10% FCS and 0, 0.1, 0.5 or 1 μM PKCδ agonist or antagonist or PKCζ antagonist alone or with 0.1 μM PKCδ and ζ antagonist combined. Either peptides (obtained from Dr. Daria Mochly-Rosen, Stanford University, Standford, CA, USA) were renewed after 2 h or embryos were washed and placed into peptide-free medium (release). Cavitation was recorded hourly before fixation in ice-cold methanol for 10 min. Embryos were double-labelled with antibodies against a-catenin for visualization of the cell membrane (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and the α1 subunit of the Na/K-ATPase (Upstate Biotechnology, Wolverton Mill South, UK) combined with ALEXA 488 or 568 conjugated respective secondary antibodies (Molecular Probes, Eugene, OR, USA). Protein localization was analyzed (Confocal Assistant software) after laser confocal microscopy (BioRad MRC 600, Hemel Hampstead, Hertfordshire, UK) at 60× magnification under oil. Cavitation was significantly ($P < 0.05$; ANOVA) delayed in a partially dose-dependent and reversible manner by PKCδ and/or ζ antagonists compared to controls ($n = 60$–73 per treatment; cavitation after e.g. 3 h: 7.4–21% in control, 1.2–2.9% in PKCδ or ζ antagonist alone or combined v. 12.5–18.5% in PKCδ agonist or 5.5–7.5% peptide release). Toxicity was excluded as carrier peptides alone had no effect on cavitation, and 70–90% formed blastocysts after overnight culture with peptides. In addition, inhibition of PKCζ or activation PKCδ caused internalization of the α1 subunit in 85–87.5% of embryos as compared to 22.2% within the control embryos ($P < 0.05$, ANOVA; $n = 17$–43 per treatment). This reversal of membrane insertion of one functional enzyme subunit indicative of decreased enzyme function occurred similarly within cavitated and non-cavitated embryos. Taken together, our data suggest that PKCδ and ζ participate in regulating cavitation by affecting different mechanisms. While decreased PKCζ activity delays cavitation and reduces Na/K-ATPase function, the various targets of PKCδ need further examination. A better understanding of underlying mechanisms involved in regulating the complex process of cavitation could help to improve embryo viability. Funding by the Wellcome Trust and MRC is gratefully acknowledged.

127 APOPTOSIS IN BOVINE BLASTOCYSTS FROM FIVE DIFFERENT PRODUCTION SYSTEMS

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Regulation of apoptosis may be affected by factors during preimplantation development, and this is possibly related to embryo developmental potential. Here we investigate differences in the incidence of apoptotic nuclei in Day 7 bovine blastocysts produced by two different in vivo and three different in vitro methods. In vivo embryos were produced either by a regular superovulation procedure (reg group; $n = 29$; Laurincik *et al*., 2003, Mol. Reprod. Dev. 65, 73–85), or by postponement of the LH surge (pp group; $n = 35$; van de Leemput *et al*., 2001, Therio. 55, 573–592). In vitro embryos were derived from systems using either co-culture (cc group; $n = 30$, Avery and Greve 2000, Mol. Reprod. Dev. 55, 438–445), or culture in synthetic oviduct fluid (SOF) with (S + group; $n = 35$) or without serum (S− group; $n = 38$; Holm *et al*., 1999, Theriogenology, 52, 683–700). Embryos were collected at approx. 168 h post ovulation/insemination and subjected to chromatin staining and detection of DNA degradation by TUNEL reaction. The total number of nuclei, number of nuclei displaying apoptotic morphology (+M), number of nuclei displaying TUNEL reaction (+T), and number of nuclei displaying both markers simultaneously (M&T) were scored according to J.O. Gjørret *et al*. (2003 Biol. Reprod. 69. in press). Only M&T nuclei were regarded as apoptotic, and +M, +T, and apoptotic (M&T) indices (%) were calculated for the trophoblast (*tb*), inner cell mass (*i*) and the total blastocysts (*t*) in each group. Significant differences were observed for all parameters when all groups were compared (ANOVA, P ranging from 0.024 to < 0.0001). Highest number of total nuclei were observed in the S + group, whereas the lowest indices were observed in the pp group, which had significant lower indices in the *i* and *t* than in the reg., S+ and S− groups ($P < 0.05$; Tukey's post test for ANOVA). Highest indices were generally observed in the S− group. The results demonstrate that not only embryo cell numbers but also incidences of apoptotic markers are affected by the mode of production. However, in Day 7 bovine blastocysts high cell number is not consistent with a low incidence of apoptosis. Even though cell numbers appeared comparable in the two in vivo groups, their incidences of apoptosis were different, and the reg group displayed indices comparable to the in vitro groups, highlighting the importance of ovulation protocols when in vivo embryos are used as reference material in general.

Table 1.

Group		Cell *n*	+M	+T	M&T
pp	*tb*	62.1 ± 6.0	2.3 ± 0.5	2.4 ± 0.6	1.8 ± 0.5
	i	106.5 ± 5.8	6.2 ± 0.7	4.8 ± 0.7	4.2 ± 0.6
	t	168.5 ± 10.6	4.8 ± 0.6	4.0 ± 0.6	3.4 ± 0.5
reg	*tb*	47.1 ± 3.8	4.1 ± 1.0	3.9 ± 1.0	3.1 ± 0.8
	i	106.2 ± 8.3	9.0 ± 0.9	8.8 ± 1.5	6.9 ± 0.8
	t	153.3 ± 9.2	7.7 ± 0.8	7.2 ± 1.1	5.9 ± 0.7
cc	*tb*	72.6 ± 5.4	3.8 ± 0.5	3.2 ± 0.5	2.7 ± 0.4
	i	107 ± 7.1	11.2 ± 1.2	7.3 ± 1.0	6.4 ± 1.0
	t	179.6 ± 10.9	8.1 ± 0.7	5.4 ± 0.6	4.7 ± 0.6
S+	*tb*	85.3 ± 4.4	3.4 ± 0.7	3.2 ± 0.6	2.5 ± 0.5
	i	122.1 ± 5.0	10.6 ± 1.0	9.1 ± 0.8	8.3 ± 0.8
	t	207.4 ± 7.8	7.5 ± 0.7	6.6 ± 0.6	5.9 ± 0.6
S−	*tb*	60.7 ± 4.0	6.6 ± 1.0	5.9 ± 1.0	5.1 ± 0.9
	i	94.1 ± 4.7	10.7 ± 0.9	9.1 ± 0.8	8.0 ± 0.7
	t	154.7 ± 7.0	8.8 ± 0.8	7.6 ± 0.7	6.8 ± 0.7

Values are mean ± SEM.

128 CHANGES WITHIN OXYGEN ENVIRONMENT DURING IVF AND IVC IMPROVE BOVINE EMBRYONIC DEVELOPMENT IN VITRO

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Oxygen concentration during both IVF and IVC affects embryonic development. A low O_2 atmosphere during IVC has been reported to be beneficial for embryos in culture without somatic cells. Similarly, a reduction in spermatozoa (sp) concentration can influence the oxygenation grade of fertilization medium, and O_2 requirements can vary according to the embryonic stage of development. This experiment investigated whether prolonged contact with sperm cells interacts with effects of oxygen tension during the first days of culture. Slaughterhouse bovine COCs matured with Vero-cells in TCM199 with FCS and EGF were co-incubated (CI) with swim-up separated sp. COCs with attached sp were either removed after 2 h and placed in IVF medium without sp up to completion of 18 h (sp-restricted CI), or COCs and sp were left together for 18 h (sp-prolonged CI). Zygotes were cultured in serum-free, B2 medium conditioned with Vero cells (Marquant-Le Guienne *et al.*, 1999 Theriogenology 51, 386), modified as described by Gomez and Diez (2000 Anim. Reprod. Sci. 58, 23–37). According to IVF procedure (sp-restricted or sp-prolonged), embryo culture was made either in 20% O_2 from Day 0 up to Day 3 and in 5% O_2 later on, or in 5% O_2 from Day 0 up to Day 8, in a 2×2 factorial design. IVF medium was monitored for Na^+, K^+, Ca^{2+}, pH, O_2, CO_2 , HCO_3^- and lactate (i-STAT analyzer; 5 replicates -R-) at 0 h, 2 h and 18 h without cells (controls), at 2 h in CI and at 18 h both in sp-restricted and sp-prolonged CI. Embryo development was analyzed by CATMOD for effects, and all data were processed by GLM and Duncan test and expressed as LSM ± SE. The presence of cells decreased pO_2 ($P < 0.01$) in IVF medium at 2 h of CI (154 ± 2.3 without cells v. 144 ± 2.3 with cells). There were differences in pO_2 at 18 h ($P < 0.05$) between sp-prolonged (140.8 ± 2.3) and sp-restricted CI (148.6 ± 2.3). At 18 h in CI there was K^+ depletion ($P < 0.05$), while Na^+, Ca^{2+}, pH and pCO_2 remained invariable throughout. Lactate production increased ($P < 0.05$) in CI at 2 h and, together with HCO_3- ($P < 0.01$), at 18 h in sp-prolonged CI. Increasing O_2 up to 20% during the first 3 days of culture improved total and medium to late (M + L; early blastocysts excluded) blastocyst rates upon sp-prolonged CI, which is comparable to sp-restricted CI under both % O_2 conditions during embryo culture. Under 5% CO_2 throughout culture, higher expansion rates occurred in sp-restricted CI, suggesting that this system could improve embryonic viability. Metabolic effects can be inferred from differences in pO_2 , lactate and HCO_3^- , and research will explore the role of oxygen free radicals in these experimental conditions. Grant support: Eureka 2573; CDTI, PROFIT, and FICYT.

Sp + COCs CI	O_2 in culture	N	R	Day 8 Blastocysts (%)		
				Total	M + L	Expanded
18 h	20% > 5%	134	4	34.9 ± 3.7^{c}	23.7 ± 3.3^{c}	17.4 ± 2.4
18 h	5% > 5%	156	5	23.5 ± 3.2^{d}	14.9 ± 2.9^{d}	11.9 ± 2.1^{a}
2 h	20% > 5%	161	5	27.6 ± 3.2	21.3 ± 2.9	16.8 ± 2.1
2 h	5% > 5%	154	5	26.6 ± 3.2	23.7 ± 2.9	19.1 ± 2.1^{b}

Unlike superscripts differ significantly [a,b] ($P < 0.05$; [c,d] $P < 0.08$). N: fertilized oocytes. R: no. of replicates.

129 IN UTERO TRANSPLANTATION OF HUMAN HEMATOPOIETIC STEM CELLS (HSC) INTO FETAL GOATS OFFERS A NEW APPROACH TO STUDIES OF HSC EXPANSION AND DEVELOPMENT

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In utero transplantation of xenogeneic stem cells into animals (such as mouse, sheep, goats) is a new approach to the studies of the engraftmen, expansion and development of foreign cells in vivo. In addition, such an approach is able to offer a model to clinical potential for correcting or replacing damaged tissues in a variety of disorders, including many that are inherent as well as acquired. Recently, we generated a human/goat hematopoietic stem cell (HSC) xenogeneic model to investigate the engraftment, expansion and development of human hematopoietic cells at an in vivo level. Human HSCs (lin-cells) were isolated and purified from fresh cord blood, and then injected intraperitoneally into pre-immune fetal goats at 55–65 gestation days (term: 145 days). A total of 50 fetal goats were injected. The growth and development of the recipient goats were monitored by B-type ultrasound scan. Ten goats suffered miscarriage due to GVH response; 39 recipients survived a complete gestation and were born alive. All these live-born goats were healthy and grew very well. They could produce offspring normally. In order to determine whether human cells were present longterm in the circulatory blood of the recipient goats, FACS, real-time PCR, RT-PCR, and Southern-blot hybridization as well as FISH were used to analyze the genotype and phenotype up to 11 months after birth. The results showed that hematopoietic chimerism (on average, $>10^8$ human cells per goat) was documented in 90% (35/39) of live-born goats that had been originally transplanted with 10^5 human HSCs. The regenerated human cells included myeloid, B-lymphoid and erythroid lineages as well as more primitive cells as shown by specific positive staining for human CD14, CD20, glycophorin A (GPA) and CD34, and the detection of human-specific DNA sequences and human GPA and CD34 transcripts in the blood of the transplanted goats. No human T or NK-lineage cells were detected. These studies demonstrate the feasibility of using in utero-transplanted fetal goats as a new approach for evaluating the longterm engraftment potential, expansion and development of human hematopoietic cells in vivo.

130 INSULIN-LIKE GROWTH FACTOR-1 AND INTERLEUKIN-11 AS POSSIBLE SURVIVAL FACTORS FOR THE BOVINE PREIMPLANTATION EMBRYO EXPOSED TO STRESS

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Both IGF-1 and interleukin-11 (IL-11) are survival factors that modify response to cell injury. Moreover, IGF-1 promotes preimplantation development (Mol. Reprod. Dev. 62, 489) and IL-11 has been reported to reduce effects of heat shock on bovine embryos (Theriogenology 59, 343). For this study, it was hypothesized that IGF-1 and IL-11 improve survival of bovine embryos exposed to lethal stimuli. Embryos were produced in vitro and cultured in KSOM medium. Treatment effects were analyzed using least squares ANOVA with the GLM procedure of SAS (SAS Inst., Inc., Cary, NC, USA). In Exp. 1, 100 ng mL^{-1} IGF-1 increased ($P < 0.01$) the percent of oocytes that became blastocyst at 8 days post-insemination (dpi) (19.0% for control v. 24.5% for IGF-1; SEM = 1.3%; 7 replicates; 105 embryos/treatment). For Exp. 2, embryos were cultured ± 100 ng mL^{-1} IGF-1. At 5 dpi, embryos ≥16 cells were cultured at either 38.5°C for 24 h or 41°C for 9 h and then 38.5°C for 15 h followed by TUNEL analysis (7 replicates; 86–100 embryos/treatment). At 38.5°C, IGF-1 did not affect total cell number (60.5 v. 64.4 for control and IGF-1, respectively; SEM = 2.3) or percent of blastomeres undergoing apoptosis as determined by TUNEL (5.9% v. 5.7%; SEM = 0.6%). Heat shock reduced total cell number ($P < 0.05$) and increased the percent of cells that were TUNEL-positive ($P < 0.001$). For heat-shocked embryos, total cell number was 46.0 for control v. 59.8 for IGF-1 (SEM = 2.3) and percent of TUNEL-positive blastomeres was 11.6% for control v. 5.9% for IGF-1 (SEM = 0.6%). Effects of heat shock were less for IGF-1-treated embryos (temperature × IGF-1, $P = 0.07$ for cell number and $P < 0.01$ for TUNEL). For Exp. 3 (4 replicates; 111–136 embryos/treatment), embryos were cultured ± 100 ng mL^{-1} IGF-1 beginning at the 1-cell stage in control medium (KSOM), ethanol (1.0%; v/v) or gossypol (10 μg mL^{-1} in 1% ethanol). Percent of blastocysts at 8 dpi was affected by treatment ($P < 0.01$) and IGF-1 ($P < 0.04$). Without IGF-1, least-squares means were 24.6%, 13.6% and 0.9% for control, ethanol, and gossypol, respectively (SEM = 1.7%). With IGF-1, least-squares means were 29.3 ± 3.4%, 26.5 ± 1.6%, and 8.7% ± 1.7% for control, ethanol, and gossypol (control v. ethanol, NS; control v. gossypol, $P < 0.01$). Thus, IGF-1 blocked the effect of ethanol on development. For Exp. 4, putative zygotes were cultured ± 10 ng mL^{-1} human IL-11 (4 replicates; 201–214 zygotes/treatment). At 3 dpi, embryos remained at 38.5°C or were cultured at 41°C for 9 h and then returned to 38.5°C. Heat shock reduced ($P < 0.01$) the percent of putative zygotes and cleaved embryos that became blastocysts at 8 dpi but IL-11 had no effect at either temperature (percent zygotes to blastocysts = 25.3% and 25.6% for control and IL-11 at 38.5°C and 14.0% and 11.8% for control and IL-11 at 41°C; SEM = 3.4%). In conclusion, IGF-1 blocked induction of apoptosis caused by heat shock and the reduction in development caused by ethanol. Thus, IGF-1 may play an important role in early development by acting as a survival factor. There was no evidence that IL-11 conferred thermoprotection to bovine embryos. (Support: USDA NRICGP 2002-35203-12664, USDA IFAFS #2001-52101-11318, and USDA TSTAR 2001-34135-11150.)

131 DIFFERENTIAL DNA METHYLATION CHANGES OF THE REPETITIVE SEQUENCES DURING PREIMPLANTATION DEVELOPMENT IN THE MOUSE

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DNA demethylation as an epigenetic change is a unique event genome-wide, occurring at preimplantation and germ cell stages during mouse development. The paternal genome after fertilization is demethylated first, referred to active demethylation, followed by demethylation of the maternal genome during preimplantation development in the mouse. To examine methylation changes in the early mouse embryo, methylation states of various genomic regions such as intracisternal A-particle (IAP), early retrotransposon (Etn) and centromeric satellite sequences were determined by means of a bisulfite sequencing method. For methylation analysis, genomic DNA was first isolated from each developmental stage of embryo (about 300 cells in total), respectively, and exposed to 1.9 M sodium bisulfite overnight. Targeted DNA sequences were amplified from bisulfite-treated genomic DNAs by PCR, cloned into pGEM T-easy vector and sequenced. Results indicated that IAP sequences maintained high levels of methylation until the morula stage and were demethylated in blastocysts. In contrast to the IAP sequences, methylation states of Etn elements were remarkably erased after fertilization, completely demethylated at the 8-cell stage and then remethylated at the morula stage. Centromeric satellite sequences showed low methylation states throughout all preimplantation stages of embryos, indicating that the satellite sequences are substantially demethylated in both paternal and maternal genomes. The results suggest that differential epifenetic changes among the repetitive sequences may be responsible for peculiar chromatin structure of respective genomic loci and/or may regulate gene expression during preimplantation development in the mouse.

132 INCIDENCE OF OXIDATIVE STRESS-INDUCED APOPTOSIS IN OVINE EMBRYOS IS ALLEVIATED IN VITRO BY TROLOX, A WATER-SOLUBLE VITAMIN E ANALOGUE

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The ability of ovine embryos to develop in vitro can be curtailed severely by culture conditions that predispose, or medium constituents such as docosahexaenoic acid (DHA; C22: 6n − 3) that are vulnerable to oxidative stress (Reis *et al.*, 2003 Reprod. Abs. Ser. 30, 48). This study examined the consequences of culture in such adverse circumstances in terms of resultant blastocyst cell metabolism and survival, and investigated whether presence of a water-soluble vitamin E analogue (Trolox; Sigma, St. Louis, MO, USA) from the zygote stage onward could safeguard ovine blastocyst integrity. Day 6 blastocysts were produced in Synthetic Oviductal Fluid (10 replicates) supplemented with 0.4% w/v fatty acid-free BSA in the

absence (SBSA) or presence of DHA (SBSAD) or in the same media with 200 μM Trolox (SBSAT, SBSADT). For determination of the functional competence of their mitochondria, individual blastocysts (5 replicates) were incubated for 3 h in the presence of [2-^{14}C]pyruvic acid (American Radiolabeled Chemicals, St. Louis, MO, USA; Sp. act. 5.5 mCi mmol^{-1}) using a 'hanging drop' technique, and then fixed for cell counts (Hoechst 33342). In addition, the incidence of apoptosis in corresponding blastocysts (5 replicates) was determined by TUNEL assay (Apoptag Red kit, Serologicals Corporation, Norcross, GA, USA). Cell counts and pyruvate metabolism data (log transformed) were tested using 2-way ANOVA. Blastocyst apoptotic cell indices were analyzed by Generalized Linear Model (binomial distribution; Genstat 6, Version 6.2). Neither DHA nor Trolox significantly affected metabolism of [2-^{14}C]pyruvic acid on a $^{14}CO_2$ per cell (pmol/3 h) basis (Table 1) in Day 6 blastocysts, but there was an interaction (DHA × Trolox, $P < 0.001$). Total cell numbers were lowered ($P = 0.053$) in blastocysts produced in the presence of DHA, and Trolox did not significantly affect this parameter. However, inclusion of Trolox reduced apoptotic indices (mean ± SEM; $P < 0.001$) which, for blastocysts from SBSA, SBSAD, SBSAT and SBSADT, were 12 ± 0.8 ($n = 43$), 13 ± 1.1 (20), 9 ± 0.7 (64) and 9 ± 0.8% (36), respectively. The data indicate that Trolox supplementation of DHA-containing media sustained mitochondrial metabolism in Day 6 ovine blastocysts. Moreover, independently of DHA status, Trolox lowered the incidence of apoptosis, presumably by protecting vital but vulnerable cellular constituents. Funded by SEERAD; AR supported by Portuguese Ministry of Science and Technology.

Table 1. Mean (± SEM) pyruvate metabolism indices (pmol/3 h) and cell numbers for Day 6 blastocysts

	No. of blastocysts	Blastocyst cell numbers	$^{14}CO_2$/embryo (pmol/3 h)	$^{14}CO_2$/cell (pmol/3 h)
SBSA	21	87.8 ± 6.7	25.7 ± 1.7	0.31 ± 0.02
SBSAD	16	70.1 ± 4.6	15.5 ± 2.3	0.20 ± 0.02
SBSAT	23	89.7 ± 5.3	20.6 ± 1.0	0.24 ± 0.01
SBSADT	22	83.6 ± 5.7	21.5 ± 1.2	0.27 ± 0.02
Probability	DHA	0.053	0.005	NS
	Trolox	NS	NS	NS
	Interaction	NS	< 0.001	0.001

133 SUBOPTIMAL OOCYTE ACTIVATION CAUSES METHYLATION CHANGE IN TWO IMPRINTED GENES IN THE MOUSE

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In mammals, the dynamic of Ca^{2+} oscillations induced by fertilization causes the remodeling of the parental chromosomes and activates the developmental processes. The objective of the present study was to evaluate whether the modulation of the regime of Ca^{2+} oscillations during the process of egg activation is capable of inducing specific chromatin modification. The sensitivity of chromatin modification during oocyte activation was evaluated by comparison of the methylation profile of Igf2r, the maternal allele of which is methylated, and H19, the maternal allele of which is unmethylated. Freshly ovulated mouse oocytes were parthenogenetically activated by a series of repetitive Ca^{2+} ion influxes induced by electropermeabilization of the plasma membrane in a microfluidic processor. The first treatment (T1) consisted of 24 electrical pulses (1.45 kV cm^{-1}) given every 8 min for 3 h in the presence of 100 μM of Ca^{2+}. The treated oocytes were almost all activated, 98% (328/338), and formed pronuclei 3 or 4 h after the first pulse. The second treatment (T2) was made up of 16 electrical pulses of lower amplitude (1.12 kV cm^{-1}), given every 8 min for 2 h under the same conditions. The rate of egg activation dropped to 29%, (86/298), and the time course of pronucleus formation was completed 7 h after the first pulse. The remaining oocytes, 71% (212/298), extruded the second polar body, but their chromatin did not undergo full decondensation and then did not proceed further to pronucleus formation. The methylation profiles of the two imprinted genes were analyzed by using the bisulfite sequencing method. Oocytes that formed pronuclei after being subjected to either the T1 or the T2 treatment displayed methylation profiles of the two alleles similar to those of the non-treated oocytes. The Igf2r-DMR was found fully methylated on all 13 copies examined, and the H19 DMR was found mostly unmethylated (13 out of 19 copies; 6 were partially methylated). Thus, when parthenogenetic activation results in formation of a pronucleus the methylation profile of these two genes is maintained. In contrast, those from oocytes that responded by PB extrusion showed the following: fully unmethylated Igf2r-DMR copies (9/9) and partially methylated H19-DMR copies (4/11). These results reveal that rapid de novo methylating and demethylating activity are present when cells are subjected to partial activation. Thus, when the biological response of the oocyte is incomplete, that is to say, when the chromatin remains partially decondensed after suboptimal activation, both active demethylation and de novo methylation can be remarkably dynamic. While the molecular mechanism needs to be clarified, these results reveal that modulation of the process of egg activation might give new opportunities to better understand the epigenetic chromatin changes caused by fertilization.

134 INCOMPLETE HISTONE ACETYLATION OF SOMATIC CHROMATIN IN BOVINE OOCYTES

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Histone acetylation as an important regulatory mechanism of chromatin structure preceeding zygotic gene expression in early embryo development. After fertilization, transcriptional activation of the embryo begins during the S/G2 phase of the first cell cycle. However, the precise mechanism

underlying activation of zygotic transcription remains to be understood, especially in bovine nuclear transfer (NT) embryos. It is known that acetylation of histone H4 lysine 5 (H4K5) represents hyperacetylation state, which is correlated with gene expression. In this study, the acetylation of H4K5 was observed during pronuclear formation by using immunofluorescence analysis with anti-AcH4K5. Our data were analyzed by the general linear models (GLM) procedure of the SAS. In IVF embryos, acetylation of H4K5 occurred on the paternal chromatin at 8 h after fertilization but did not occur on the maternal chromatin until 10 h after fertilization. Reconstructed oocytes with deactylated somatic cell nuclei began to show signs of acetylation on chromatin at 3 h after fusion. When acetylation intensity was calculated using an image analyzer, IVF embryos presented a higher acetylation signal than NT embryos ($P < 0.05$). To induce hyperacetylation in NT embryos, somatic cells were exposed to trichostatin A (TSA, 1 μM for 60 h), a specific inhibitor of histone deacetylase (HDAC), prior to NT. Acetylated signals of H4K5 increased significantly in TSA-treated cells as compared with non-treated cells ($P < 0.05$). The reconstructed embryos with TSA-treated cells showed a higher fluorescence intensity than the oocytes with non-treated cells ($P < 0.05$), but weak signals compared to IVF embryos. Thus, the results demonstrated low histone acetylation level of somatic cell nuclei after NT during the zygotic progress. Our findings suggest that developmental failures of NT embryos may be due to incomplete chromatin remodeling of somatic cell nuclei during early embryonic development.

135 DNA METHYLATION PROFILES IN THE PREIMPLANTATION PORCINE EMBRYOS

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DNA methylation at CpG dinucleotides is an important epigenetic regulation process, which is associated with gene expression without any change in DNA sequence. During early development of the mouse embryo, dynamic changes in DNA methylation of the genome occur. After fertilization, active demethylation occurs on the paternal genome followed by passive demethylation until morula stage and then de novo methylation at the blastocyst stage. This study was designed to investigate changes in DNA methylation of in vivo- and in vitro-fertilized (IVF) porcine embryos. DNA methylation states were observed in preimplantation porcine embryos by using an immunofluorescence method after staining with an antibody against 5-methylcytosine. In contrast to the data from mouse embryos, active demethylation of the genome from the paternal pronucleus was not observed in the porcine embryos. Also, no passive demethylation was detected in in vivo- and IVF-derived embryos until the morula stage. Moreover, differential de novo methylation was not shown on the genome of the inner cell mass. Whole genomes of inner cell mass and trophectoderm cells were fully methylated. Our results demonstrate that DNA methylation of porcine embryos is different from that of mouse embryos during preimplantation development, suggesting that the machinery to regulate DNA methylation may be species-specific in mammals.