

ABSTRACTS FOR POSTER PRESENTATION

Student Competition Finalists

1 LONG TERM HEALTH AND BEHAVIOR OF ICSI PRODUCED MICE

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Intracytoplasmic sperm injection (ICSI) is a relatively new treatment for human male-related infertility (1992) and for the production of transgenic animals (1995). However, ICSI bypasses many natural biological processes such as sperm maturation, interaction within the female genital tract, sperm capacitation, interaction with oocyte vestments, and sperm membrane fusion with the oocyte. With the widespread use of this technology, its potential adverse outcomes need to be ascertained. It is theoretically possible that ICSI may cause specific problems through injury to the sperm or egg or injection of damaged or defective sperm. Here, we determined if ICSI has a long-term effect on mouse growth, behavior, and health. Female CD1 mice were superovulated and oocytes were injected with frozen-thawed spermatozoa (without cryoprotector or chelating agent) obtained from CD1 mice epididymes (Moreira *et al.* 2004 Biol. Reprod. 71, in press). Embryos were cultured 24 h in KSOM, and 2-cell embryos were transferred into CD1 females. Fifty-six mice (36 males and 20 females) produced by ICSI and 41 control mice (18 males and 23 females) obtained from *in vivo*-fertilized mice were analyzed. On week 20, animals were submitted to the following behavior tests: locomotor activity (open field), exploratory/anxiety behavior (elevated plus maze, open field), and spatial memory (free-choice exploration paradigm in Y maze). Comparison between groups was made using analysis of variance followed by least significant difference post hoc test. Postnatal weight gain of female mice produced by ICSI was heavier than for their control counterparts from 10 weeks on ($P < 0.01$). Males produced by ICSI showed more anxiety and lower locomotion in the p-maze and the Y-maze tests ($P < 0.05$), but no significant differences were found in the open-field test. Also, no differences were found in spatial memory or in the habituation pattern. Anatomopathological analysis of animals at 16 months of age showed some large organs (heart, lung, and liver; $P < 0.01$) and an increase in pathologies (15% of animals produced by ICSI presented some solid tumors in lung, dermis of back, or neck). Loss of imprinting is one of the most common epigenetic changes associated with the development of a wide variety of tumours. An association between some imprinting disorders, rare tumors, and ICSI has recently been reported in humans (Wittermer *et al.* 2004 Med. Sci. 20, 352). We are now analyzing the epigenetic modifications that may be induced by our ICSI protocol and whether the sperm DNA fragmentation that may take place during sperm freezing before the ICSI procedure might not only affect postnatal development, growth, and physiology, but also increase the risk of tumors in adult animals. Our data suggest that our ICSI method produces mice with sex-dimorphic alterations in aberrant growth and anxiety, as well as with a higher probability of developing a solid tumor.

2 CORRELATION BETWEEN OXYGEN RESPIRATION RATES AND MORPHOLOGY, SEX, DIAMETER AND DEVELOPMENTAL STAGE OF SINGLE BOVINE IVP-EMBRYOS

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A simple, non-invasive, rapid and sensitive oxygen microsensor system was developed to investigate correlations between oxygen respiration rates of individual bovine embryos and their morphology, sex, diameter and developmental stage. Bovine IVP-embryos ($n = 78$; Holm *et al.* Theriogenology 52, 683–700) were analysed around the 8-cell stage (Day 3; $n = 18$) and at various blastocyst stages (Day 7; $n = 60$). Each embryo was morphologically evaluated, its outer diameter measured and was then loaded into a glass tube (i.d. 0.68 mm, length 3 mm). After 1 h, oxygen concentration gradient generated by the embryo's respiration were measured over app. 8 min with an oxygen microelectrode (www.unisense.com). Five embryos were measured in one round together with an empty tube as control. The procedure was repeated twice for each embryo with app. 1 h interval. Individual respiration rates in nL O₂/embryo/h (nL/h) were calculated from these gradients. The measurements were performed at 38.5°C under constant flow of humidified 5% CO₂ in air (app. 19% O₂). After this, 64 embryos (14 Day 3; 50 Day 7) were lysed for sex diagnosis by PCR. Values are given as mean \pm SD. The sensitivity of the oxygen measurement system was high (controls: 0.034 ± 0.035 nL/h, $n = 15$) and its repeatability from 1st to 2nd measurement was $99.7 \pm 9.8\%$ ($n = 71$). The average embryo respiration rate was 0.39 ± 0.05 nL/h on Day 3 ($n = 18$) and 1.31 ± 0.52 nL/h on Day 7 ($n = 60$). For Day 7 embryos, the respiration rates varied according to their morphological quality, being 1.87 ± 0.46^a ($n = 18$), 1.17 ± 0.32^b ($n = 23$), $0.95 \pm 0.27^{b,c}$ ($n = 14$) and 0.72 ± 0.24^c ($n = 4$) nL/h for quality 1, 2, 3, and 4 embryos, respectively (Proc Mixed,^{a,b,c}: $P < 0.05$; values with different superscripts differ significantly). The sex ratio (male:female) was 9:5 (Day 3) and 32:18 (Day 7), and on Day 7 this ratio varied between qualities: 11:2, 12:8, 8:4, and 1:3 for quality 1, 2, 3, and 4, respectively. The average respiration rate on day 3 was the same for males and females, as it was on day 7 (1.22 ± 0.43 nL/h (females) and 1.31 ± 0.58 nL/h (males), $P > 0.05$). There was a correlation between embryo diameter and respiration rate ($r^2 = 0.65$, $n = 74$), which was even stronger for Day 7 male embryos ($r^2 = 0.72$, $n = 32$). In conclusion, a highly reliable, repeatable and sensitive system was established for measuring respiration rates in single bovine embryos, even at early developmental stages. The respiration rate was lower on day 3 compared to Day 7 embryos, and it was correlated with the morphological embryo quality on Day 7. Oxygen consumption could be a valuable supplementary indicator of embryo viability, especially in difficult evaluations (e.g. quality 2 and 3 after IVP). It remains to be demonstrated if such measurements can also reveal quality differences already at Day 3, which would be of interest in, e.g. the human field.

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3 X-LINKED GENE EXPRESSION IN BOVINE PRE-IMPLANTATION EMBRYOS OBTAINED *IN VIVO* AND *IN VITRO* AS A MEASURE OF IMPACT OF EMBRYO PRODUCTION TECHNOLOGIES

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The X chromosome provides an ideal system to study the impact of assisted reproduction technologies on gene expression because it holds a wide range of diversely functional genes and also the epigenetic features of X-inactivation are inherently susceptible to *in vitro* culture-mediated alterations. Using quantitative real-time RT-PCR we studied the expression of a panel of X-linked genes (*ANT3*, *GAB3*, *RPS4*, *MECP2*, *XIAP*, *XIST*) in pooled pre-attachment bovine embryos produced *in vivo* and *in vitro*. We collected pools of ten embryos *in vivo* (de la Fuente R. *et al.* 1999 Biol. Reprod. 60, 769–775) at the morula ($n = 3$ pools) and blastocyst ($n = 3$ pools) stages. Pools of ten matured oocytes ($n = 10$ pools), 2–4 cell ($n = 10$ pools), 8–16 cell ($n = 10$ pools), morula ($n = 7$ pools) and blastocyst ($n = 7$ pools) stage embryos were produced in LSOF (Robert *et al.* Biol. Reprod. 67, 1465–1472) and TCM-199/bovine oviductal epithelial cells (BOEC) co-culture (de la Fuente *et al.* 1999 Biol. Reprod. 60, 769–775). Total RNA was extracted with the Absolutely RNA[®] Microprep kit (Stratagene, La Jolla, CA, USA) and reverse transcribed using Oligo-dT_{12–18} primers and Superscript II RT (Invitrogen, Burlington, Ontario, Canada). Specific primers were designed for each gene and PCR products were used to build standard curves for absolute quantification in a Light Cycler[™] instrument with the Light Cycler FastStart DNA Master Mix SYBRGreenI kit (Roche Diagnostics, Laval, Quebec, Canada). The data were analyzed with SAS (SAS Institute Inc., Cary, NC, USA) using a factorial ANOVA design and a log transformation. Significance level was set at $\alpha = 0.05$. There were no significant differences in transcript levels between IVF systems in mature oocytes, morulae, or blastocysts. Significant differences were observed for some of the genes tested at the 2–4-cell (*XIAP*) and at the 8–16-cell stages (*ANT3*, *GAB3*, *XIAP*) but not others. There were significant differences between IVF (both BOEC and LSOF) and *in vivo* embryos at the morula and blastocyst stages, with IVF embryos showing an average of 8 (*ANT3*), 10 (*XIAP*), 127 (*MECP2*), and 593 (*XIST*) times more transcripts than their *in vivo* counterparts. *GAB3* was detected in only a few samples prior to the blastocyst stage where it was consistently detected in IVF but not *in vivo* embryos. It was concluded that there is an effect of the IVF system on gene transcription just before and during the period of activation of the embryonic genome during the 4th cell cycle, as well as marked differences between IVF and *in vivo* produced embryos that are evident in the differential expression of X-linked genes. This system provides a good tool to monitor the effects of embryo production conditions and help in their improvement.

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4 EFFECT OF THE TIMING OF ARTIFICIAL INSEMINATION ON THE NUMBER OF SPERMATOZOA DISCOVERED IN THE UTERINE CRYPTS OF THE BITCH

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Canine spermatozoa may be stored for several days within the genital tract of the bitch since natural matings 8 to 9 days before ovulation may result in litters. Several studies have suggested that the sperm reservoir in the dog is located in the uterine crypts and the uterotubal junction (UTJ). In the present study, we investigated the effect of the timing of artificial insemination (AI) in relation to ovulation on the sperm distribution in the genital tract of the bitch. Ten beagle dogs were inseminated intravaginally with 500×10^6 spermatozoa. Based on progesterone concentration, three dogs were inseminated 1–2 days before ovulation, four dogs during ovulation, and three dogs 2–3 days after ovulation. Ovariohysterectomy was performed 24 h after AI. The genital tract was divided into eight segments (i.e. corpus uteri; caudal, middle, and cranial parts of the uterine horn; UTJ; isthmus; ampulla; and infundibulum) which were processed for histology. From each segment, 30 histological sections were evaluated. For the UTJ and the different segments of the oviduct, the total number of spermatozoa was determined. For the different parts of the uterus, on each of these 30 sections, 100 uterine crypts of comparable size were evaluated for the presence of spermatozoa. The crypts were divided into crypts without spermatozoa, crypts with 1 sperm cell, crypts with 2 to 5 spermatozoa, and crypts with either more than 5 spermatozoa or in which the spermatozoa were clustered. The data were analyzed using univariate analysis of variance. Histology revealed that the spermatozoa were located mainly in the uterine crypts and at the UTJ, while very few spermatozoa were detected in the different parts of the oviduct. Insemination during ovulation resulted in higher percentages of crypts with spermatozoa in the different parts of the uterus ($P < 0.05$). Moreover, for the ovulatory group, 54.7% of the uterine crypts with spermatozoa contained more than 5 spermatozoa (or clusters) compared to 19.9% and 28.2% for the pre- and post-ovulatory groups, respectively ($P < 0.05$). In the pre-ovulatory group, 59.6% of the uterine crypts with spermatozoa contained only 1 sperm cell whereas in the post-ovulatory group, frequently 1 (34.0%) or 2 to 5 spermatozoa (37.9%) were found per crypt. In conclusion, sperm transport in the genital tract of the bitch is affected by the time of AI in relation to ovulation. Insemination during the ovulation period resulted in higher percentages of uterine crypts with spermatozoa, and most of these crypts contained 5 or more spermatozoa. Further research should determine whether the number of sperm binding sites expressed on the epithelium of the canine uterine crypts is influenced by the ovulation event.

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5 NEUTRAL SEGREGATION OF DONOR CELL MITOCHONDRIA IN FETAL AND ADULT TISSUES OF SOMATIC CELL CLONES IN CATTLE

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Until now, animal cloning has been extremely inefficient: only 1–2% of nuclear transfer (NT) clones survive to birth. Some of these anomalies may be related to an incompatibility between nuclear and mitochondrial genes (Cummins JM 2001 Hum. Reprod. Update 7, 217–228). Controversy exists as to the levels of donor cell mitochondrial DNA (mtDNA) inheritance in somatic clones (heteroplasmy). Whereas some researchers found very low quantities (0.1–4%) (Steinborn R *et al.* 2000 Nat. Genet. 25, 255–257), others found levels of heteroplasmy ranging from 6 to 40% (Takeda *et al.* Mol. Reprod. Dev. 64, 429–437). Since it remains unclear whether mtDNA segregation is neutral or selective, the purpose of this study was to analyze the transmission of the mtDNA from donor somatic cells in fetal and adult clones using a particular mtDNA marker (mtDNA *Bos taurus* with one mutation in the D-loop of 40 base pairs plus than the wild type). Fibroblasts from a fetus of 60 days were used as donor cells. The fetus was produced by artificial insemination of a Holstein (*Bos taurus*) heifer carrying an mtDNA mutation with semen from a Zebu (*Bos indicus*) bull. Oocytes derived from slaughterhouse ovaries of Holstein cows carrying wild-type mtDNA were used as recipient cells. The presence of the mutated mtDNA from the donor cell (heteroplasmy) was analyzed in a male cloned fetus of 60 days and in three adult male clones at 18 months of age. Heteroplasmy was detected in 7 tissues in the foetus: muscle, skin, stomach, testicle, thymus, tongue, and umbilical cord. Three tissues were analyzed from the adult clones: semen, skin, and white blood cells. Heteroplasmy was detected in all the tissues by nested PCR amplification of the D-loop and analyzed by ANOVA and Tukey-Kramer multiple comparison test. The mean (%) of the mutated mtDNA of the donor cell in the seven tissues of the 60-day-old fetus was 1.14 ± 0.34 (SEM). There was no differences in the means of heteroplasmy (%) between the tissues of the fetus ($P > 0.05$). The mean level of heteroplasmy in the three adult clones analyzed (clones A, B, and C) was 1.41 ± 0.18 (SEM). Analysis of heteroplasmy between the tissues of each clone showed no differences ($P > 0.05$) with the exception of clone B, where semen was different ($P < 0.05$) from white blood cells. There were significant differences ($P < 0.05$) between some clones (taking together all the results of all tissues of each clone). The heteroplasmy in clone B (%) (2.59 ± 0.18 SEM) was different ($P < 0.05$) from that of both clone A (1.04 ± 0.18) and clone C (1.46 ± 0.18). There was no difference between the heteroplasmy (%) of clone A and that of clone C ($P > 0.05$). These results show that the tissues of the fetus and the adult clones were heteroplasmic at similar levels, suggesting neutral segregation of the donor cell mtDNA during development and tissue differentiation.

6 VITRIFICATION OF BOVINE EMBRYOS WITHOUT ANIMAL-DERIVED PRODUCTS

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Media free of animal products would facilitate import and export requirements for embryos. Our goal was to test replacement of bovine serum albumin (BSA) (2.5 µg/mL) with sodium hyaluronate (SH) (4.2 µg/mL, 12.5 µg/mL, or 37.5 µg/mL) as the macromolecule in a very simple system in which embryos were vitrified in 0.25-mL straws suitable for direct embryo transfer. Day 7 blastocysts ($n = 384$) were produced *in vitro* with semen of 3 bulls, 2 replicates each. For vitrification, embryos were placed into chemically defined, HEPES-buffered medium (HCDM-2) and then transferred to V1 (5 M ethylene glycol, 0.5 M galactose in HCDM-2 plus BSA or SH) for 3 min. Next, embryos were placed in a 6-µL drop of V2 (7 M ethylene glycol, 0.5 M galactose, and 18% w/v Ficoll 70 in HCDM-2 plus BSA or SH) for 45 s. During equilibration, dilution medium (0.5 M galactose in HCDM-2 plus BSA or SH) was aspirated into 0.25 mL straws, followed by the 6-µL drop of V2 plus embryos, and a final short column of dilution medium. When 45 s had elapsed, the heat-sealed end of straw was dipped into liquid nitrogen to cover the embryo and then plunged slowly. Straws were thawed in air for 10 s and then in 37°C water for 20 s. Straws were then shaken like a clinical thermometer 4 times to mix columns, and held in 37°C water for 10 min before expelling embryos to be rinsed and cultured in CDM-2 + 5% FCS. Survival (as determined by expansion of blastocysts), quality score (1 = excellent, 2 = fair, 3 = poor), inner cell mass quality (ICM) (1 = large and compact, 2 = clearly visible, 3 = not discernable), and blastocyst stage (5 = early, 6 = full, 7 = expanded, 8 = hatching, 9 = hatched) were evaluated 24 h post-thaw and analyzed by ANOVA. Due to poor embryo recovery from straws in replicates 1–3, 0.1% polyvinyl alcohol (PVA) was added to vitrification solutions in replicates 4–6. Survival was calculated as a percentage of noncryopreserved controls in the same replicate (control: survival 93%, quality 1.52, ICM 1.43, stage 7.47). The lowest dose of SH replaced BSA efficaciously with this vitrification procedure (Table 1). Addition of PVA greatly improved all responses (main effects): recovery 99% vs. 69%; survival 78% vs. 53%; quality 2.07 vs. 2.43; ICM 2.10 vs. 2.47; stage 5.98 vs. 4.94; all $P < 0.001$. Successful vitrification of embryos in solutions containing 4.2 µg/mL SH plus PVA in place of BSA in 0.25 mL straws, from which embryos can be transferred directly, further increases the appeal of vitrification as an alternative to conventional cryopreservation.

PVA	Macromolecule	Subclass means				
		Survival	Quality	ICM	Stage	Recovery
+	2.5 µg/mL BSA	85%	1.81	2.01	6.64	98%
+	4.2 µg/mL SH	91% ^a	1.96	1.93	6.10	98%
+	12.5 µg/mL SH	65%	2.30 ^b	2.25	5.37	100%
+	37.5 µg/mL SH	71%	2.22	2.21	5.82	98%
–	2.5 µg/mL BSA	61%	2.23	2.27	5.25	94%
–	4.2 µg/mL SH	66% ^a	2.29	2.27	5.54	63% ^b
–	12.5 µg/mL SH	45%	2.50	2.54	4.40	63% ^b
–	37.5 µg/mL SH	40%	2.70 ^b	2.82 ^b	4.58	54% ^b

^a Different (main effect) from other SH doses ($P < 0.05$), Tukey's hsd.

^b Different from respective BSA controls ($P < 0.05$), Dunnett's test.