322 DIFFERENTIAL DEVELOPMENT OF RABBIT EMBRYOS FOLLOWING MICROINSEMINATION USING SPERM AND SPERMATIDS

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Microinsemination is a technique that delivers male germ cells directly into the ooplasm. The efficiency of fertilization and subsequent embryo development after microinsemination varies with species and the male germ cells used. This study examined the developmental ability of rabbit embryos *in vitro* and *in vivo* following microinsemination using haploid male germ cells at different stages. First, we injected rabbit spermatozoa, elongated spermatids, and round spermatids into mouse oocytes to assess their oocyte-activating capacity. Mouse oocytes are a good experimental model for assessing the oocyte-activating capacity of male germ cells from different species. The majority of mouse oocytes were activated irrespective of the stage of rabbit male germ cells injected (77, 61, and 73% for spermatozoa, elongated spermatids, and round spermatids, respectively). By contrast, these male germ cells activated homologous rabbit oocytes at rates of 100, 59, and 29%, respectively. After 120 h in culture, 69, 55, and 13% of these activated rabbit oocytes (pronuclear eggs) developed into blastocysts, respectively. The rate of embryo development into blastocysts following round spermatid injection was significantly improved when oocytes were activated by an electric pulse shortly before microinsemination. The total number of cells was counted in embryos that reached the morula/blastocyst stages in culture using nuclear-staining with propidium iodide. The average cell number of embryos derived from elongated (89 ± 41; mean ± SD) or round spermatid (98 ± 34) injection was significantly lower than that of control embryos (*in vivo* fertilization) (211 ± 44) ($P < 0.01$). After 24 h in culture, some four- to eight-cell-stage embryos were transferred into the oviducts of pseudopregnant females. Normal pups were born from embryos involving sperm (4 offspring/16 transfers; 25%) and elongated spermatid (3/26; 12%) injection, but none from those involving round spermatid injection (0/68). These findings indicate that rabbit male germ cells acquire the ability to activate oocytes and to support subsequent embryo development as they undergo spermiogenesis. Immaturity of the nuclear genome or difficulty in coordinating the behavior of the male and female chromosomes might compromise embryo development.

Superovulation

323 THE QUALITY OF PREOVULATORY FOLLICLES DURING FINAL MATURATION IN COWS STIMULATED WITH oFSH AND A DEFINED LH SURGE

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Multiple preovulatory follicles developing upon superovulation (SO) are heterogeneous in quality, which may be the consequence of follicular development deviating from that in untreated cyclic cows. Therefore, we investigated follicle performance in terms of estradiol (E), progesterone (P), and testosterone (T) concentrations in the fluid of stimulated preovulatory follicles (FF), in particular at onset of final maturation as initiated by the LH surge. Pre-synchronized HF cows ($n = 25$) were treated with oFSH (Ovagen; ICP, Auckland, New Zealand) for SO, and FF were collected 2 h pre-LH surge ($n = 9$ cows), and 6 h ($n = 8$) and 22 h C($n = 8$) post-LH. At Day 9 (estrus = Day 0), a norgestomet ear implant (Crestar; Intervet International BV, Boxmeer, The Netherlands) was inserted, and SO treatment was started at Day 10 using oFSH i.m. twice daily in decreasing doses during 4 days (total dose 17 mL). Prostaglandin (22.5 mg PG; Prosolvin, Intervet) was administered i.m. concomitant with the 5th dose of FSH. Ear implants were removed 50 h after PG and then GnRH (0.021 mg Receptal; Intervet) was administered i.m. inducing the LH surge 2 h later. Ovaries were collected by laparotomy at the time of GnRH, and 8 and 24 h later and all follicles sized >10 and <16 mm were aspirated to collect FF (pre-LH, $n = 79$; 6 h post-LH, $n = 78$; and 22 h post LH, $n = 78$ follicles). For comparison, E and P in FF from pre-LH groups that had been collected previously in 2 other experiments of our group were studied: (1) 86 FF collected by ultrasound-guided aspiration of follicles >8 mm from 23 cows at 30 h after PG, that is, preceding the LH surge, following treatment with 3000 IU eCG i.m. (Folligon; Intervet) on Day 10 and 15 mg PG on Day 12; (2) 12 FF of the dominant follicle from 12 untreated cyclic cows after ovariectomy 48 to 62 h after onset of luteolysis, that is, shortly before the natural LH surge. The concentrations (ng/mL FF) of E, P, and T were estimated by our validated RIAs. Data (mean ± SEM) were analyzed by ANOVA. The levels of E, P and T of the oFSH group were pre-LH: 399 ± 35, 49 ± 6 and 13 ± 2; 6 h post-LH: 194 ± 11, 202 ± 12, and 14 ± 1; and 22 h post-LH: 35 ± 2, 200 ± 23, and 7 ± 1, respectively. Although the change in E and P levels between the different time points after LH is in agreement with that reported for untreated cyclic cows (Dieleman *et al*. 1983 J. Endocrinol. 97, 31–42), the concentrations were lower. However, the most striking finding was the significantly lower E in pre-LH FF after oFSH compared to that after eCG (1302 ± 82) or of cyclic cows (1942 ± 200). The P levels in FF of the respective pre-LH groups were not significantly different. The much lower E level after oFSH is possibly due to the low or even absent LH bioactivity for oFSH in comparison to eCG. It could also indicate a lower developmental potential of oocytes following oFSH. However, it is known that SO with oFSH results in regular yields of transferable embryos similar to that after eCG. In view of the high variability of the E level in FF, it is concluded that selection of follicles for high E levels is a prerequisite when investigating oocyte development and maturation.

324 ADJUSTING SPERM CONCENTRATION USED TO INSEMINATE SUPERSTIMULATED BEEF COWS, IN ORDER TO AVOID DECLINE IN EMBRYO PRODUCTION

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The quantity and quality of semen may affect conception rate after artificial insemination (AI). The concentration of motile spermatozoa, after thawing, varies according to the sire and lot of frozen semen used. The minimum amount of spermatozoa required in a semen straw was determined to obtain pregnancy after a single AI of a non-superovulated animal. However, superstimulation of embryo donors results in the availability of many more oocytes (an average 10–20) for fertilization than in a non-superovulated female (1 oocyte). The purpose of the present work was to verify if by adjusting the concentration of motile spermatozoa, in straws with low sperm concentration, the percentage of viable embryos is comparable to those obtained using straws with high sperm concentration after thawing. Nelore cows (*Bos taurus indicus*) were superstimulated with a protocol termed P36 (Barros CM *et al*. 2003 Theriogenology 59, 524 abst), in which the ovulation is induced by exogenous LH (12.5 mg, Lutropin®, Vetrepharm, London, Ontario, Canada), administered 36 h after $PGF_{2\alpha}$. One sample of each lot of semen was analyzed by CASA (computer-assisted semen analysis), and motile sperm concentration, after thawing, was adjusted to a minimum of $25–30 \times 10^6$ spermatozoa, which is approximately 3 to 4 times higher than the sperm concentration used for a regular AI. Fixed-time AI (FTAI) was performed 12, 24, and sometimes 36 h after exogenous LH. The number of semen straws necessary to obtain at least 25×10^6 spermatozoa varied from 2 to 6 (Groups 2 to 6, respectively). Since at least two semen straws were used per animal, there is no Group 1. The number of FTAI was adjusted according to the number of straws used, i.e., 2 straws (FTAI 12 and 24 h after LH), and 3 or more straws (12, 24, and 36 h after LH). Mean total structures (oocytes, viable embryos and degenerate embryos), mean viable embryos per flushing, and viability rate (percentage of viable embryos/total structures) were, respectively: 12.2, 8.9, and 73.6% (Group 2, $n = 19$ flushings); 13.5, 9.6, and 70.9% (Group 3, $n = 101$); 13.3, 9.4, and 70.9% (Group 4, $n = 22$); 5.5, 4.0, and 72.7% (Group 5, $n = 4$); and 24.0, 13.0, and 54.2% (Group 6, $n = 1$). When the results from Groups 4, 5, and 6 were pooled, total structures, viable embryos, and viability rate were: 12.5, 8.7, and 69.8% ($n = 27$). The statistical analysis was performed using the ratio of viable embryos/total structures for each flushing, transformed in square root followed by arc sin. Data from groups 4, 5 and 6 were pooled before comparing to the other groups by ANOVA. In order to facilitate the comprehension of the results, data were presented as viability rate instead of means of arc sin. There was no difference when comparing pooled data from Groups 4, 5, and 6 with the other groups (2 or 3; $P = 0.87$; ANOVA). It is concluded that by adjusting the concentration of motile spermatozoa in straws with low sperm concentration (Groups 4, 5, and 6), the viability rates are comparable to those obtained using semen with high sperm concentration (Group 2 or 3).

Nogueira has a fellowship from FAPESP (São Paulo).

325 INSULIN-LIKE GROWTH FACTOR-I CONCENTRATIONS IN OVIDUCT FLUID OF SUPEROVULATED EWES DURING THE PERI-OVULAR PERIOD

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This study examined the concentrations of insulin-like growth factor-1 (IGF-1) in oviduct fluid during the peri-ovular period as a reference for the establishment of optimal *in vitro* culture conditions for sheep embryos. Six mature ewes (4–5 years, 58–67 kg) of comparable body condition were fed a standard diet for two weeks before the start of fluid collection. Ewes were superovulated using conventional treatment involving a progestagen, FSH, and GnRH treatment. Oviducts were catheterized four days (which is sufficient time to recover from surgery) before collection of oviductal fluid, which started one day (Day 1) before the time of ovulation (Day 0) and continued until five days later (Day 5). Oviductal fluid was acidified by diluting into 0.8 M acetic acid/0.2 M trimethylamine, pH 2.8, mixed, and incubated to dissociate IGFs from IGF-binding proteins (IGFBPs). Following incubation, acidified fluid was centrifuged at 10,000*g* through a 0.1-mm Micro-spin centrifuge filter; the filtrate transferred to glass high-performance liquid chromatography (HPLC) vials. IGFs and IGFBPs were separated from one another by high-performance size-exclusion liquid chromatography using a Protein-Pak 125 column (Waters Corporation, Milford, MA, USA) and 0.2 M acetic acid, 0.05 M trimethylamine, pH 2.8, at a flow rate of 1 mL/min. Oviductal fluid IGF-I was collected in a single 2-mL fraction directly from the HPLC and its concentration measured by an IGF-I-specific enzyme-linked immunosorbent assay (Diagnostic Systems Laboratories, Inc. Webster, TX, USA). The data were analyzed by analysis of variance. The non-superovulation group had significantly higher concentrations of oviductal IGF-I compared with the superovulation group. In the superovulated group, there was, however, a significant effect of day on the oviductal fluid IGF-I concentration ($P < 0.01$) such that the concentrations of IGF-I first increased for three days and then decreased for the remaining four days. In the non-superovulation group, there was no significant two-way interaction between ovulation and day. It can be concluded that the levels of IGF-I increase over time and then decrease.

Authors express thanks to the help of Jenn Skye and Hemish Turretfield Research Station SA.

326 OPTIMIZING FREQUENCY OF FSH APPLICATION FOR SUPEROVULATORY TREATMENT IN CATTLE

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The aim of the study was to examine the influence of the application frequency of Pluset® (Calier, Spain) and Folltropin® (Vetrepharm, London, Ontario, Canada) on the results of embryo recovery in Simmental cattle. Moreover, pulsatility of LH concentrations in plasma should be studied

before and after application of Pluset. FSH was given in a treatment of eight or four injections on four consecutive days starting between Days 9 and 13 of the estrous cycle. Luteolysis was induced by application of 500 μg Estrumate® (Essex Tierarznei, Munich, Germany), applied 72 and 84 h after the first FSH injection. Embryo recovery was performed non-surgically on Day 7 of the following estrous cycle. Heifers received either a total amount of 500 IU Pluset according to the International Standard for human urine FSH and LH (P1: 8 injections, $n = 20$; P2: 4 injections, $n = 54$) or 200 mg Folltropin according to NIH bFSH-B1 (F1: 8 injections, $n = 117$; F2: 4 injections, $n = 60$). Cows received either 550 IU Pluset (P3: 8 injections, $n = 54$; P4: 4 injections, $n = 183$) or 260 mg Folltropin (F3: 8 injections, $n = 36$; F4: 4 injections, $n = 159$). Altogether 8 heifers were bled throughout 8 h every 10 min on Days 9 and 12 (before and during stimulation with FSH). The samples were analyzed for LH by ECLIA (intra-assay coefficient of variation (VK) 6.4%, inter-assay VK 8.9%). The evaluation of the data was carried out with the procedure 'GLM' of the statistics software package SAS (SAS Institute, Inc., Cary, NC, USA) and PULSAR analysis. As a post hoc test Student's *t*-test was used. Significance was set at $P = 0.05$. Results of embryo recovery are shown in the table. Number of LH pulses in 8 h (LSM ± SE) was 2.3 ± 0.4 on Day 9 and 0.6 ± 0.4 on Day 12 ($P < 0.05$). We conclude that four injections of both FSH products can lead to results comparable with those reached with eight injections in a 12 hours interval. Therefore, the effort for superovulatory treatments can be reduced. However, it has to be considered that the portion of transferable embryos was reduced, partly significantly, after four FSH injections. This result was independent of the drug used.

Table 1. Results of embryo recovery

Group	Ova/embryos (*n*) (LSM/SE)	*P* value	Portion of transferable embryos (%) (LSM)	*P* value
P1	12.9/1.9	0.09	70.3	0.08
P2	9.2/1.1		54.7	
P3	18.4/1.4	0.4	69.5	0.002
P4	17.0/08		55.0	
F1	14.1/0.9	0.11	79.0	0.03
F2	16.5/1.2		69.7	
F3	19.2/1.6	0.08	61.2	0.56
F4	16.2/0.7		57.8	

Tissue Culture

327 CHARACTERIZATION OF FETAL AND ADULT FIBROBLASTS FROM NELORE BOVINE FOR NUCLEAR TRANSFER

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Cloning by nuclear transfer is a technology that has provided major advances in reproductive sciences. It is an efficient method: (1) to produce transgenic embryos, fetuses, and animals; (2) to multiply genetically superior adult animals; and (3) to generate proteins, tissues, and organs for xenotransplants of human interest. Basic studies on nuclear transfer have contributed to understanding how genomic activation and cell cycle synchrony affect nuclear reprogramming and cloning efficiencies. The aim of this study was to establish a stable lineage of Nelore adult and fetal fibroblasts for embryo reconstruction. Cultures were characterized by morphological aspects, and by immunocytochemical and ultrastructural analysis. Cultures of fetal and adult fibroblasts at passage 2 stained positively for cytokeratin, a typical protein from the intermediate filament of epithelial cells, in some cells but staining was not found in subsequent passages. A dense and organized network occurred in adult and fetal fibroblast cultures presenting positive immunostainig for vimentin in pass 5, 10, and 15 subcultures, but did not stain for cytokeratin (negative control). The results demonstrated that fibroblasts continued expressing vimentin and maintained their morphological aspects in culture. The ultrastructural analysis showed the presence of organelles involved in protein synthesis including dilated rough endoplasmatic reticulum, Golgi cisternae, and polyribosomes. Elongate mitochondria were distributed around the Golgi complex, suggesting high metabolic activity. These results demonstrated that adult and fetal fibroblasts continued to develop biological activities and maintained specific characteristics under these culture conditions. In conclusion, adults and fetal fibroblasts can be successfully used as donor cells for nuclear transfer.

This work was supported by FAPESP 01/11931-8 and 01/13944-0.

328 SOMATIC CELL ISOLATION FROM SEMEN BY PERCOLL GRADIENTS

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We previously isolated epithelial-like cells (ELC) from sheep (*Ovis aries*) and eland (*Taurotragus oryx*) ejaculates (Nel-Themaat *et al*. 2004 Reprod. Fertil. Dev. 16, 152). Success rates were low, and the presence of live sperm during initial culture may have altered medium properties, physically