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

H.V.A. Caetano[A,B], *M.P. Milazzotto*[A,B], *M.D. Goissis*[B], *C. Yamada*[B], *M.G. Marques*[B], *M.E.O.A. Assumpcao*[B], *and J.A. Visintin*[B]

[A]Biotechnology Department; [B]Department of Animal Reproduction, University of Sao Paulo, Sao Paulo, Brazil. Email: helocae@yahoo.com.br

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

L. Nel-Themaat[A,B], *M.C. Gomez*[B,A], *G. Wirtu*[B,C], *A. Cole*[B], *K.R. Bondioli*[A], *B.L. Dresser*[B,D], *R.A. Godke*[A], *and C.E. Pope*[B]

[A]Department of Animal Sciences, Louisiana State University Agricultural Center, Baton Rouge, LA 70803, USA; [B]Audubon Center for Research of Endangered Species, New Orleans, LA 70178, USA; [C]Department of Veterinary Clinical Sciences, Louisiana State University School of Veterinary Medicine, Baton Rouge, LA 70803, USA; [D]Department of Biological Sciences, University of New Orleans, New Orleans, LA 70148, USA. Email: lnel1@lsu.edu

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

prevented contact between cells and the substrate, and/or damaged somatic cell membranes. Using the complete semen sample requires the sacrifice of valuable live spermatozoa without the certainty of obtaining a somatic cell population. Therefore, the purpose of the present study was to develop a method for isolating somatic cells from semen before culture. Separation by discontinuous Percoll gradient centrifugation was performed. Ejaculates obtained by electro-ejaculation from two Louisiana Native rams and rectal massage of one eland bull were washed, layered on columns of 2.5 mL each of 20% (P-20), 50% (P-50), and 90% (P-90) Percoll in 15-mL conical tubes, and centrifuged for 20 min at 400*g*. Bands were obtained at the three interfaces, as well as a pellet in the P-90 fraction. Each Percoll layer plus the interface band immediately below it was collected by aspiration. Cells from each fraction were re-suspended in serum-supplemented Minimum Essential Medium Alpha containing 500 IU/mL of penicillin, 0.5 mg/mL of streptomycin and 250 g/mL of gentamycin, and were plated on collagen-1 coated dishes. Trypan blue staining was used to determine cell viability in each fraction. After 24 h, dishes were washed and the culture continued until cells attached and proliferated. For both species, the surface fraction yielded primarily dead ELC that were morphologically flat and angular in shape. In the P-20 band, more viable cells were isolated compared with other fractions, although dead cells were also present. In the P-50 and P-90 fractions, few ELC were observed compared with the surface and P-20 fractions. A negligible number of sperm was observed in the surface and P-20 fractions. In contrast, mostly dead sperm were found at the P-50 and P-90 interface band, whereas live spermatozoa were detected in the P-90 pellet. Epithelial-like cells isolated from semen of both species attached (A, initial 24 h of culture), divided (D, within 3 days after plating), and proliferated (P, after 1 week of culture) (see table). Ram ELC derived from the surface and from the P-20 and P-50 layers attached, but proliferation was detected only with cells collected in the P-20 fraction. In contrast, eland cells collected from the surface and P-20 layers readily attached and proliferated. In summary, a technique has been developed for the isolation of somatic cells from semen using a three-layer Percoll gradient. This method also allows the isolation of motile spermatozoa, which are available for further use.

Species (no. animals)	*n*	Cell stage	Surface (%)	P-20 (%)	P-50 (%)	P-90 (%)
Sheep (2)	14	A	1 (50)[a]	6 (43)[b]	2 (14)[ab]	0[a]
		D*	1 (100)	4 (67)	0	0
		P**	0	3 (50)	0	0
Eland (1)	2	A	1 (50)[a]	2 (100)[a]	0[a]	0[a]
		D*	1 (100)	2 (100)	0	0
		P**	1 (100)	2 (100)	0	0
Total (3)	16	A	2 (13)[a]	8 (50)[b]	2 (13)[a]	0[a]
		D*	2 (13)	6 (75)	0	0
		P**	1 (6)	5 (63)	0	0

n = replicates within species. *Ratio of attached samples that divided; **ratio of attached samples that proliferated.
[a,b] Values with different superscripts within rows are significantly different (Fisher's exact test, $P < 0.05$).

329 A NOVEL SUSPENSION CULTURE SYSTEM FOR BOVINE OVIDUCT EPITHELIAL CELLS

R. Rottmayer[A], *S.E. Ulbrich*[B], *S. Koelle*[C], *K. Prelle*[D], *H.H.D. Meyer*[B], *F. Sinowatz*[C], *E. Wolf*[A,E], *and S. Hiendleder*[A]

[A]Institute of Molecular Animal Breeding and Biotechnology, Ludwig-Maximilian University Munich, 81377 Munich, Germany; [B]Physiology Weihenstephan, Technical University Munich, 85350 Freising, Germany; [C]Institute of Veterinary Anatomy II, Ludwig-Maximilian University Munich, 80539 Munich, Germany; [D]CRBA Gynecology & Andrology, Schering AG, Berlin, Germany; [E]Bavarian Research Center for Biology of Reproduction (BFZF), 85764 Oberschleissheim, Germany. Email: r.rottmayer@gen.vetmed.uni-muenchen.de

Bovine oviduct epithelial cells (BOEC) are difficult to culture without dedifferentiation. Frequently observed changes in morphology during culture suggest that gene expression patterns are affected as well. We explored a novel short-term culture system for BOEC – suitable for co-culture experiments with embryos – and evaluated the cells with respect to morphological criteria, candidate gene expression, and hormone responsiveness. Simmental heifers were slaughtered on Day 3.5 after standing heat and BOEC were obtained by squeezing along the ampulla with forceps. The cell sheets were separated mechanically by repeated passages through syringes and pipetting, and recovered by sedimentation. Cells from the ipsi- and contralateral oviduct were cultured separately at a density of 10^6 cells per well in 24-well plates with 800 µL TCM-199 supplemented with 2% OCS (estrous cow serum, as used in embryo culture) or CS 3.5 (cow serum, Day 3.5 after standing heat, adequate to the cycle stage in which cells were obtained) and 0.25 mg/mL gentamicin. For cell counting, an aliquot was further disaggregated by passing 15 times through a 30-gauge needle to achieve a single-cell suspension. Culture took place at 38°C in a humidified atmosphere of 5% CO_2 in air. Cells were examined by light microscopy at seeding and after 6, 12, 24, and 48 h. Cell aggregates showed a worm-like structure, displaying numerous vigorously beating cilia on their surfaces throughout the culture period. Trypan blue staining indicated that cells contained in aggregates were viable while single cells stained predominantly positive (non-viable). The purity of the epithelial cell culture was >95%, as determined by immunohistochemistry using antibodies against vimentin and cytokeratin. For electron microscopic investigations, BOEC were sampled at seeding and after 24 h in culture. Cultured BOEC showed a morphology highly similar to that of BOEC *in vivo*. Both secretory cells with numerous secretory granules and mitochondria, and ciliated cells with long, well developed and actively moving kinocilia were visible. RT-PCR data for candidate genes (*ERα*, *ERβ*, *HMGCR*, *PHGPx*, *PR*) obtained from BOEC samples at seeding and after 6, 12, 24, and 48 h in culture showed that gene expression was stable for the majority of transcripts after 6 h in culture. There was no significant difference between cells cultured with OCS or CS 3.5 and no difference between cells obtained from the ipsi- or contralateral oviduct. Estradiol-17β (E2, 10 pg/mL) or progesterone (P4, 10 ng/mL) stimulation showed that the cultured BOEC are able to respond to hormonal signals in a manner similar to their reaction *in vivo* (Ulbrich *et al*. 2003 J. Steroid. Biochem. Mol. Biol. 84, 279–89). Progesterone receptor mRNA was up-regulated by E2 and estrogen receptor β mRNA was up-regulated by P4. The culture system for bovine oviduct epithelial cells thus provides an adequate tool to investigate mechanisms of the embryo-maternal communication in cattle.

This work was supported by the Deutsche Forschungsgemeinschaft (Research Unit "Mechanisms of Embryo-Maternal Communication"; FOR 478/1).