ABSTRACTS FOR POSTER PRESENTATION

193 CHARACTERIZATION OF PORCINE TROPHECTODERM-DERIVED CELLS IN THE PRESENCE OF hBMP4 IN THE ABSENCE OF A FEEDER LAYER

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A porcine trophoblastic cell line could provide a powerful model for understanding trophoblast cell biology as well as placental gene expression and proteomics in vitro. In this experiment, we derived porcine trophoblastic cells from trophectoderm tissue and assessed their growth on three different extracellular matrix substrates and in three different concentrations of human recombinant bone morphogenetic protein 4 (hBMP4). Human BMP4 has been shown to induce differentiation of human embryonic stem cells into trophoblast lineages. Elongated embryos were flushed using DPBS supplemented with 1% fetal calf serum and penicillin-streptomycin (1X) from the hysterectomized uteri of superovulated and bred prepuberal gilts 15 days post-insemination. The embryonic disc was visualized with a dissecting microscope. The trophectoderm tissue was cut 2–3 mm away from the embryonic disc with a scalpel and the trophectoderm tissue was manually dissected into cell aggregates. These aggregates were plated on collagen type IV, Matrigel, and human extracellular matrix (laminin, collagen type IV and heparan sulfate proteoglycan derived from human placenta) in culture medium (DMEM with 15% FCS, 0.1 mM 2-mercaptoethanol, 4 ng mL^{-1} basic FGF4 and 1X P/S) in the presence or absence of hBMP4 at 0, 10, or 20 ng mL^{-1}. Cell outgrowth was observed within 24 hours of culture. After three days of culture, various cell types (based on size and morphology) were present. Among cultures of predominant large cells were colonies of smaller cells with epithelial-type morphology that had a prominent nucleus and a high nuclear-to-cytoplasmic ratio. The epithelial-type cells grew in tight colonies with definite borders and contained cytoplasmic structures resembling lipid-containing vesicles. These colonies initially appeared on all matrices across all hBMP4 concentrations. After seven days in culture the colonies developed distinct differences across groups. Cell growth on collagen was comprised of tight colonies having definite borders among large cells. Colonies on collagen were larger and more pronounced in both the hBMP4-supplemented groups than when cultured without hBMP4. The Matrigel coated plates contained large sheets of epithelial-type cell growth instead of compact colonies. This type of growth characteristic was present in all hBMP4 treatments on Matrigel. In contrast, few cells survived and propagated on human extracellular matrix. Only small colonies having the desired morphology were among the large cells on human extracellular matrix when cultured in medium containing 10 ng mL^{-1} hBMP4. Cells were passaged and only cells growing on Matrigel could be further cultured. These data suggest that both the cell substrate and hBMP4 affect initial trophoblast outgrowths. Further analysis including immunocytochemistry and RT-PCR is currently being performed to better characterize these cells.

Epidemiology/Diseases

194 THE EFFECT OF OVIDUCTAL INCUBATION OF OOCYTES AND EMBRYOS ON ADHERENCE OF BOVINE VIRAL DIARRHEA VIRUS

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The zona pellucida (ZP) plays a major role as a protective shell against infection of embryonic cells and as a carrier of infectious agents in the spread of livestock diseases through embryo transfer practices. It has been demonstrated that pathogenic agents are more likely to adhere to the surface of ZP of IVF embryos than to that of in vivo fertilized embryos. It has been suggested that divergent conditions for the production of these two types of embryos may lead to changes in their morphology and to differences in the interaction of the ZP with pathogens. The objective of this study was to investigate whether or not experimentally induced changes in hardening ZP (HZP) affect adherence of BVDV to the ZP of oocytes and embryos produced in vitro. To induce HZP, the oocytes or IVF embryos in groups of 30–50 were incubated in the ligated, ampullar part of the oviduct at 38°C in 5%CO_2, 5%O_2 and 90% nitrogen for 5 h. Following incubation, a proportion of oocytes/embryos was exposed to 1% pronase to determine HZP (the time of ZP lysis), while the remaining oocytes and embryos were incubated with 10^6 $TCID_{50}$/mL of either noncytopathic (NY-1) or cytopathic (NADL) strain of BVDV at 38°C for 3 h. Subsequently, oocytes and embryos were washed according to the method recommended by IETS and then tested for the presence of BVDV (virus isolation and PCR tests). At the end of experiments the oviductal tissues were tested by PCR and proven free of BVDV. For immature and matured oocytes and embryos not exposed to the oviduct, the ZP dissolution times were 3.6 ± 0.24 (mean $\pm$ SEM, $n = 20$), 3.8 ± 0.24, and 4.0 ± 1.24 min, respectively (Chi-square test; $P > 0.05$). Corresponding times for those incubated in the oviduct were 393 ± 47, 431.0 ± 50, and 467 ± 61 min, respectively ($P > 0.05$). There was no difference between the number of virus-positive oocytes and embryos ($n = 965$ in 193 samples) following experimental exposure to BVDV regardless of whether or not they were previously incubated in the oviduct ($P > 0.05$). Lower, but not significant, differences in percentages of samples associated with the infectious cytopathic strain of BVDV as compared to a noncytopathic strain were detected ($P > 0.05$). It was concluded that the modification in proteolytic resistance properties of ZP during in vitro oviductal incubation did not influence the adherence of BVDV to ZP of oocytes or IVF embryos. Further studies are warranted to determine why IVF embryos are more prone to the adherence of pathogenic agents than are in vivo fertilized embryos.

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195 REMOVAL OF EQUINE ARTERITIS VIRUS FROM STALLION SEMEN

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Infection of breeding horses with equine arteritis virus (EAV) can result in abortion in up to 50% of mares (Del Piero F 2000 Vet. Pathol. 37, 287–296). Viral transmission occurs in body fluids, including semen (Golnik W *et al.* 1986 Zentralblatt für Veterinarmedizin 33, 413–417), with infected males potentially shedding virus indefinitely. Previously, the only means of preventing EAV transmission via semen was to remove identified shedders from the breeding pool. Recent medical studies have shown that viral infectivity can be removed from the semen of HIV or hepatitis C patients by a sequential method of sperm preparation: i.e. centrifugation on a discontinuous density gradient, followed by swim-up, (e.g. Bujan *et al*. 2002 Fertil. Steril. 78, 1321–1323; Levy *et al*. 2002 Hum. Rep. 17, 2650–2653). Human sperm prepared by this method have been used in over 1000 assisted reproduction attempts without sero-conversion of mothers or children (Lyerly A *et al.* 2001 Fertil. Steril. 75, 843–858). The current study investigates whether a sequential preparation technique of centrifugation on an EquiPure density gradient followed by a swim-up into a sperm maintenance medium can remove EAV from stallion ejaculates. Aliquots (1 mL) of stallion semen, extended in Kenny's medium, were spiked with known quantities of EAV at three levels corresponding to 1.0, 10 and 100 TCID50/mL^{-1}. The latter was considered to be representative of levels seen in natural infection (Timoney PJ *et al.* 1987 J. Reprod. Fertil. (Suppl. 35), 95–102). Aliquots of spiked semen were prepared by centrifugation on EquiPure gradients. After centrifuging the resulting sperm pellets in EquiSperm Wash, the sperm were subjected to a swim-up treatment (all sperm preparation material from NidaCon, Gothenburg, Sweden). Aliquots of the sperm preparations, the unspiked extended semen, and spiked extended semen were stored at −70°C for viral assay by nested PCR (Belak S *et al.* 1994 Proc. 7th Int. Conf. Equine Inf. Dis. pp 33–38). The sensitivity of this assay is less than 1 PFU mL^{-1} of virus in seminal plasma, as validated by Belak *et al.* Using the PCR technique, a region from the nucleocapsid gene of EAV is amplified, resulting in a 170-base-pair product. Details of the primer sequences used are as follows: first TCGATGGCGTCAAGACGATCAC and GGTTCCTGGGTGGCTAATAACTACTTCAAC; second CGCAACCCACTCAGGCTATTATTG and GGTAGGAACCCAACTGACGGTG. The untreated spiked samples were all positive for EAV, whereas the sperm preparations from the spiked semen, after density gradient and swim-up, were negative for EAV. A negative control (water) and the unspiked extended ejaculate were also negative. These preliminary results indicate that the sequential technique of centrifugation on an EquiPure density gradient followed by a swim-up is potentially a useful and simple tool for the removal of EAV from the semen of shedding stallions. Further experiments will investigate whether the virus can be removed from naturally infected ejaculates. We are grateful to Prof. Twink Allen and Miss Clare Tiplady of the Equine Fertility Unit, Newmarket, UK, for providing samples of stallion semen. This study is partially funded by Eureka (E-2967).

196 PREVENTION AND TREATMENT OF BOVINE VIRAL DIARRHEA VIRUS INFECTIONS IN FETAL FIBROBLAST CELLS

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Unnoticed infections with bovine viral diarrhea virus (BVDV) can occur in cultured cells used for somatic cell nuclear transfer. Aromatic cationic molecules have exhibited inhibitory activity against in vitro replication of BVDV. The purpose of this research was to evaluate the ability of aromatic cationic compounds to prevent or treat noncytopathic BVDV infections of fetal fibroblast cells. Aromatic compounds tested were 2-(4-[2-imidazolinyl]phenyl)-5-(4-methoxyphenyl)furan (DB606); 2-(2-benzimidazolyl)-5-[4-(2-imidazolino) phenyl]furan dihydrochloride (DB772); and 2-(1-methyl-2-benzimidazolyl)-5-[4'-(2-imidazolino)-2'-methylphenyl]furan dihydrochloride (DB824). To evaluate prevention of BVDV infections, 10 cell lines in the absence or presence of 7 dilutions of each of the 3 compounds were inoculated with BVDV. The concentrations of BVDV in medium and cell lysates were determined by serial dilution and virus isolation. Samples were obtained 72 hours post-inoculation. Bovine viral diarrhea virus in cell culture medium and cell lysate samples was evaluated by comparison to equivalent samples from control cultures in which no compound was added (percent of control = cell culture infective doses (50%; $CCID_{50}$) of BVDV in compound sample/$CCID_{50}$ of BVDV in control sample lacking compound). The viral inhibitory concentrations (99%) of compounds were calculated with JMP software by least-squares regression techniques. Cumulatively, the 99% endpoints for inhibition of viral replication in fetal fibroblast cell lines for the 3 compounds were 0.1 μM, 0.007 μM and 0.028 μM, respectively. To evaluate therapeutic treatment of established BVDV infections, the concentration of BVDV in medium and cell lysates of 2 fetal fibroblast cell lines were evaluated. The cell lines were previously determined to be infected with a genotype 1a strain of BVDV. Samples were obtained during 4 sequential passages in the absence or presence of 0.04 μM and 4 μM concentrations of DB772 or DB824. Presence of BVDV was determined by reverse transcription nested polymerase chain reaction and virus isolation. While BVDV persisted in cultures supplemented with no aromatic compound or 0.04 μM, both DB772 and DB824 effectively cured BVDV infections after 1 passage in 4 μM, and cells remained viable. Results indicate that BVDV infections can be effectively prevented or treated in fetal fibroblast cultures. Further research is needed to determine if exposed cells are competent for production of normal embryos via nuclear transfer.

197 SUSCEPTIBILITY OF PORCINE MORULAE AND BLASTOCYST STAGE EMBRYOS TO PSEUDORABIES VIRUS AND PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS

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Porcine preimplantation embryos are refractory to infection with pseudorabies virus (PRV) and porcine reproductive and respiratory syndrome virus (PRRSV) during the 2–4 to 16-cell stage as described by Bolin *et al.* (1981 Am. J. Vet. Res. 42: 1711–1712) and Prieto *et al.* (1996 Theriogenology

46: 687–693), respectively. Research on the effects of PRV and PRRSV on embryonic cells of morulae, blastocysts and hatched blastocysts is limited. Therefore, the objectives of the present study were (i) to assess the effects of PRV and PRRSV exposure on further embryonic development, and (ii) to determine whether PRV and PRRSV are able to replicate in embryonic cells of porcine morulae and blastocysts. In vivo produced ZP-intact and ZP-free morulae (6 days post-insemination), early blastocysts (7 days post-insemination), and hatched blastocysts (8 days post-insemination) derived from 22 superovulated sows were exposed to 10^5 $TCID_{50}$ PRV (strain 89v87, second passage in swine testicle cells) or to 10^5 $TCID_{50}$ PRRSV (Lelystad virus strain, 13th passage in swine alveolar macrophages) for 1 h at 39°C. Control embryos were incubated under the same circumstances without viruses. Each group of morulae and blastocysts consisted of approximately 20 embryos. Embryonic development was assessed every 12 h and differences in rates of development were analyzed using Chi-square analysis or Fisher's exact test. At 48 h post-incubation, embryos were collected and examined for viral antigen by indirect immunofluorescence. Further embryo development of ZP-intact and ZP-free morulae and blastocysts was not affected by exposure to PRV or PRRSV compared to controls ($P < 0.05$). Moreover, using indirect immunofluorescence, no PRV or PRRSV antigen-positive cells were detected. Exposure of hatched blastocysts to PRV inhibited further embryo development as 100% ($n = 5$) of the embryos degenerated 24 h after viral exposure. This was significantly different ($P < 0.05$) from the controls and the PRRSV-incubated hatched blastocysts that did not experience any negative influence on embryo development. Based on these results it can be concluded that embryonic cells are not susceptible to a PRRSV infection up to the hatched blastocyst stage. Embryonic cells of morulae and blastocysts are refractory to PRV, but the virus has a detrimental effect on further embryo development of hatched blastocysts. More experiments are necessary to confirm these results and to investigate whether, or at which preimplantation stage, embryos are susceptible to a PRRSV infection.

198 BOVINE VIRAL DIARRHEA VIRUS (BVDV) IN CELL LINES USED FOR SOMATIC CELL CLONING

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Most isolates of BVDV cause unapparent infections in cultured cells. Fetuses, postnatal animals or fetal bovine serum are possible sources of the virus for cultivated cells used as karyoplasts in cloning. Routine screening by veterinary diagnostic laboratories of 39 fetal fibroblast cell lines used in cloning research had revealed that 15 (39%) were positive for BVDV by various assays including RT-nPCR. As some were valuable transgenic cell lines, a rigorous protocol for evaluation of each line was undertaken to confirm infection with BVDV. A cryopreserved vial of each line was thawed, medium discarded and cells incubated (38.5°C in 5% CO_2 and air) through 2 passages (6–10 days) in α-MEM supplemented with 10% equine serum. At the end of the second passage, cells were separated from medium, washed and assayed for presence of BVDV using virus isolation in 2 sequential passages in Madin Darby Bovine Kidney Cells and RT-nPCR. Available lots of fetal bovine serum and medium that had been used to culture the cells also were tested for BVDV. When the virus was detected, the RT-nPCR products were sequenced and compared. Also, an attempt was made to evaluate the earliest available cryopreserved passage of any positive cell lines. Results indicated that just 5 of 39 of the original cells tested (13%) were positive. Since cryopreserved earlier passages of 4 of the cell lines were available, they were assayed with the result that 2 of the 4 were not infected at the earliest passage. Further, BVDV was isolated from one lot of fetal bovine serum that was used to culture one of the cell lines. Sequence analysis verified that only 2 of these 4 cell lines were infected with the same isolate of BVDV, and one isolate was identical to the virus found in the fetal bovine serum used in medium to culture it. The discrepancy between our viral detection and that of the diagnostic laboratories is explained in part by the presumed test protocols. All BVDV-positive cells, as reported by the diagnostic laboratories, were positive by RT-nPCR. We presume that they did not separate medium from cells before assays. Thus, any noninfectious viral RNA that was in the medium (e.g. as would be expected in many lots of irradiated serum) would have been reported positive. The only possible sources for BVDV in these cell lines were the fetuses from which they originated or fetal bovine serum used in medium. Sequence analysis confirmed that serum was the source of viral infection in one line. The likely source of virus for 2 other lines was serum, since they were not infected at earlier passages. The 2 remaining cell lines were positive at the earliest available passages, so the fetuses from which cells were harvested could not be discounted as the source of BVDV. This report highlights the risks of introducing BVDV in embryo technologies and the difficulties that can be encountered in attempting accurate diagnosis of the presence of infectious virus.