SECTION I

PART A UPDATE

****Disease Transmission Through In Vivo Produced Embryos****

**Cattle**

**Bovine viral diarrhea virus**

Bovine diarrhea virus (BVDV) causes a variety of economically important enteric and infertility problems. For that reason, several countries have eradicated the disease and some others have schemes in progress to achieve freedom from it. Although there is a considerable amount of information about the risk of BVDV transmission through contaminated semen used for AI, there is no available evidence to indicate whether the resulting embryos, when used for embryo transfer (ET), can lead to the transmission of BVDV to recipients and offspring. For this experiment, semen from a bull persistently infected with BVDV ($10^5$ TCID$_{50}$/mL of NY strain) was used for insemination (2 times at estrus) of BVDV-seronegative, superovulated heifers ($n=27$). All heifers seroconverted to BVDV within 10 days post-insemination. Embryos and unfertilized oocytes were collected nonsurgically ($n=92$) or postmortem ($n=52$) 7 days post-insemination and were either washed according to IETS recommendations (without trypsin treatment) or left unwashed. In total, out of 144 unfertilized oocytes and embryos collected, 23 (16%) were of ET quality. Most of the embryos were degenerated or unfertilized. On 17 occasions, 1 or 2 washed embryos were transferred to BVDV-seronegative recipients. After ET, all pregnant and nonpregnant recipients remained free of BVDV and antibodies. In total, 6 heifers became pregnant and 5 calves free of BVDV and BVDV antibodies, including 2 sets of twins, have been born to date. Post-insemination, BVDV was detected in 29% (12/41) of unwashed and 10% (4/40) of washed embryos, 100% (4/4) of follicular fluid samples, oviductal epithelial cells, endometrium, and corpora lutea tissues as determined by the virus isolation test. Results herein suggest that BVDV can be transmitted by AI, resulting in the production of some proportion of contaminated embryos. However, it appears that such embryos, when washed according to the IETS guidelines, do not cause BVDV transmission to recipients or their offspring.


The aim of the present study was to evaluate the development and ultrastructure of preimplantation bovine embryos that were exposed to bovine viral diarrhea virus (BVDV) in vitro. The embryos were recovered from superovulated and fertilized Holstein-Friesian donor cows on day 6 of the estrous cycle. Compact morulae were microinjected with 20 pl of BVDV suspension ($10(5.16)$ TCID(50)/ml viral stock diluted
1:4) under the zona pellucida (ZP), then washed in SOF medium and cultured for 24-48 h. Embryos were evaluated for developmental stages and then processed immunocytochemically for the presence of viral particles, using fluorescent anti-BVDV-FITC conjugate. Ultrastructure of cellular organelles was analysed by transmission electron microscopy (TEM). After microinjection of BVDV under the ZP, significantly more (p<0.001) embryos (83.33%) were arrested at the morula stage compared with the intact control (30.33%). Immunocytochemical analysis localized the BVDV-FITC signal inside the microinjected embryos. TEM revealed: (i) the presence of virus-like particles in the dilated endoplasmic reticulum and in cytoplasmic vacuoles of the trophoblast and embryoblast cells; (ii) the loss of microarchitecture; and (iii) abnormal disintegrated nuclei, which lacked reticular structure and the heterochromatin area. In all, the embryo nuclear structure was altered and the microarchitecture of the nucleolus had disappeared when compared with the nuclei from control embryos. Dilatation of the intercellular space and the loss of the intercellular gap junctions were often observed in bovine BVDV-exposed embryos. These findings provide evidence for the adverse effect of BVDV virus on the development of bovine embryos, which is related to irreversible changes in the ultrastructure of cell organelles.


The objective was to determine the average amount of bovine viral diarrhea virus (BVDV) associated with single in vivo-derived and in vitro-produced bovine embryos following recommended processing procedures for embryos. In vivo-derived and in vitro-produced bovine embryos at 7d post-fertilization were exposed (for 2h) to 2 x 10^5-7 cell culture infective dose (CCID(50))/mL of SD-1 (a noncytopathic, Type 1a strain of BVDV), and then washed according to International Embryo Transfer Society (IETS) guidelines prior to testing. Of the 87 in vivo-derived embryos tested, 27% were positive for virus by quantitative polymerase chain reaction (qPCR). The range in amount of virus associated with 99% of the contaminated embryos was <or=6.62+/-1.57 copies/5 microL; 90% of the contaminated embryos had <or=4.64+/-1.57 viral copies/5 microL of embryo-associated virus, using tolerance intervals (P<0.05). The SEM was 0.33 and the mean of averages was 1.12/5 microL. Of the 87 in vitro-produced embryos, 42% were positive for virus. The range in amount of virus associated with 99% of the contaminated embryos was <or=3.44+/-0.89 copies/5 microL; 90% of the contaminated embryos had <or=2.40+/-0.89 viral copies/5 microL of embryo-associated virus using tolerance intervals (P<0.05; S.E.M. was 0.14 and the mean of averages was 0.55/5 microL). Therefore, although many embryos were positive for virus, there were limited numbers of copies, thereby posing doubt regarding their potential for contamination following embryo transfer.

Gard JA, Givens MD, Marley MS, Galik PK, Riddell KP, Stringfellow DA, Zhang Y, Edmondson MA. Bovine viral diarrhea virus (BVDV) associated with single in

Infectious bovine rhinotracephitis (BHV-1)

The aim of our study was to examine whether: (1) the exposure of bovine embryos to the BHV-1 virus in vitro can compromise their further development and alter the ultrastructural morphology of cellular organelles; (2) whether the zona pellucida (ZP) can be a barrier protecting embryos against infection; and (3) whether washing with trypsin after viral exposure can prevent virus penetration inside the embryo and subsequent virus-induced damages. The embryos were recovered from superovulated Holstein-Friesian donor cows on day 6 of the estrous cycle. Only compact morulas or early blastocysts were selected for experiments with virus incubation. We used the embryos either with intact ZP (either with or without trypsin washing) or embryos in which the ZP barrier was avoided by using the microinjection of a BHV-1 suspension under the ZP. ZP-intact embryos (n = 153) were exposed to BHV-1 at 10(6.16) TCID(50)/ml for 60 min, then washed in trypsin according to IETS guidelines and postincubated in synthetic oviduct fluid (SOF) medium for 48 h. Some of the embryos (n = 36) were microinjected with 20 μl of BHV-1 suspension under the ZP, the embryos were washed in SOF medium and cultured for 48 h. Embryo development was evaluated by morphological inspection, the presence of viral particles was determined both immunocytochemically, using fluorescent anti-IBR-FITC conjugate and by transmission electron microscopy (TEM) on the basis of the ultrastructure of the cellular organelles. It was found that BHV-1 exposure impairs embryo development to higher preimplantation stages independent of the presence of the ZP or the trypsin treatment step, as most of the embryos were arrested at the morula stage when compared with the control. Immunofluorescence analysis confirmed the presence of BHV-1 particles in about 75% of embryos that were passed through the trypsin treatment and in all the BHV-1-microinjected embryos. Ultrastructural analysis, using TEM, revealed the presence of virus-like particles inside the BHV-1-exposed embryos, where the trypsin washing step was omitted. Conversely, in trypsin-treated BHV-1-exposed embryos, TEM detected only the envelope-free virus-like particles adhered to pores of the ZP. The embryos that were microinjected with BHV-1 suspension showed the presence of BHV-1 particles, as well as ultrastructural alterations in cell organelles. Taken together these findings may suggest that BHV-1 infection compromises preimplantation development of bovine embryos in vitro and therefore the ZP may not be enough on its own to prevent virus-induced damage, unless it is not accompanied with trypsin washing.

Makarevich AV, Pivko J, Kubovicova E, Chrenek P, Slezakova M, Louda F. Development and viability of bovine preimplantation embryos after the in vitro infection with bovine herpesvirus-1 (BHV-1): immunocytochemical and ultrastructural studies. Zygote. 2007;15(4):307-15. (Note that the pdf of this article was forwarded with the 2009 update to facilitate discussion on Agenda item #5a.)
Although porcine-origin trypsin will effectively remove bovine herpesvirus 1 (BHV-1) associated with in vivo-derived embryos, TrypLE™, a recombinant trypsin-like protease, has not been evaluated. In Experiment 1, 17 groups of 10 in vivo-derived embryos were exposed to BHV-1, treated with TrypLE™ Express or TrypLE™ Select (10× concentration) for varying intervals, and assayed as 2 groups of 5 embryos. TrypLE™ Select treatment for 5 and 10 min (two and seven groups of five embryos, respectively) effectively inactivated BHV-1. In Experiment 2, 22 groups of 10 IVF embryos were treated and assayed. Treatment with TrypLE™ Select for 7 and 10 min (six groups of five embryos each) and with TrypLE™ Select diluted 1:2 for 10 min (seven groups of five embryos) was also effective. In Experiment 3, 17 groups of 10 IVF embryos were further evaluated with TrypLE™ Select undiluted and diluted 1:2 for 10 min. Treatment with the diluted product was effective (18 groups of five embryos), whereas the undiluted product was not completely effective (virus isolated from 2 of 16 groups). In Experiment 4, IVF embryos were treated as described in Experiment 3 and then cultured individually or as groups of five on uterine tubal cells (UTCs) for 48 h; 60% of UTC samples associated with groups of embryos and 35% of UTC associated with individual embryo samples were positive for BHV-1. Therefore, although TrypLE™ Select appeared to have promise for the treatment of in vivo-derived embryos, it cannot be recommended for treatment of in vitro-derived embryos.

Marley MS, Givens MD, Galik PK, Riddell KP, Looney CR, Stringfellow DA. Efficacy of a recombinant trypsin product against bovine herpesvirus 1 associated with in vivo- and in vitro-derived bovine embryos. Theriogenology. 2008;69(6):74-57. (Note that the pdf of this article was forwarded with the 2009 update to facilitate discussion on Agenda item #5a.)
**Bluetongue virus (serotype 8)**

The aim of this study was to describe the effects of BTV infection on foetal mortality in 53 herds located in the east of France and infected by the virus between August and November 2007. In 2008, two visits were performed 50 days apart by local veterinary surgeons and pregnancy was checked by ultrasonography. BT status was found positive in 69.5% of the cows (80% of beef vs 67% of dairy cows). At first visit, ultrasonography was performed in cows which were supposed to be pregnant. At 2nd visit, a positive pregnancy status has been confirmed in 78% of the cows, but this proportion was higher in seronegative cows when compared to seropositive cows (81% vs 76%, p<0.02). Out of 1311 pregnant cows at first visit, only 37 presented a foetal mortality at 2nd pregnancy check (2.8%). The rate of foetal mortality was also related to the BT seropositive status of cows (seronegative: 6/418 = 1.4 vs seropositive 31/893 = 3.5%, p<0.05).


**Neospora caninum**

The aim of this research was to determine the influence of Neospora caninum (NC) infection on reproductive parameters of Holstein dairy cows. The study was performed at 32 farms in the south of Brazil, latitude 27°30′S to 31°S and longitude 51°30′W to 55°30′W. Farms included in this study met rigorous vaccination schedule for infectious bovine rhinotracheitis, bovine viral diarrhea virus, leptospirosis, and brucellosis. Blood samples collected from the dairy cows were submitted to serologic testing by an indirect fluorescent antibody test (IFAT) and were distributed into 2 groups: seropositive (GP; n = 160) and seronegative (GN; n = 558). The dogs (n = 51) of the farms were tested by IFAT. After abortion, fetuses were frozen at -80°C and submitted to PCR detection of NC in tissues. Seropositive cows that aborted were also tested by IFAT. Pregnancies of GP and GN cows were monitored by periodic ultrasound exams. After parturition or abortion all cows in GP and GN groups were submitted to gynecological exams. Cows that developed disease (clinical mastitis, metabolic disease, or laminitis) after parturition or abortion were excluded from the analysis to prevent any distortion of data. Farms were divided into 3 categories according to production technology, specifically, average milk production per cow per lactation: high (>25 kg), medium (15-25 kg), and low (<15 kg). Data was analyzed by ANOVA using SAS® software (SAS Institute, Cary, NC, USA) at a level of 5% significance and Student’s t-test for average reproductive parameters. The alternative hypothesis of the study was that at least 1 reproductive parameter was influenced in GP after abortion compared with GN cows that aborted. The null hypothesis was that there was no influence of NC antibody titer on reproductive parameters. The prevalence of antibody for NC in dairy cows was 24.48%. There was no prevalence difference among production systems (P > 0.05). The prevalence in dogs was
48.6% and did not differ among production systems. Fifty-nine (41.37%) NC-seropositive cows aborted. This differs significantly from the seronegative cow abortion rate (n = 17; 2.6%; P < 0.001). Occurrence of retained fetal membranes (RFM), endometritis, absence of uterine infection after abortion, and days between abortion and first estrus can be observed in Table 1. Abortion occurred between the fifth and eighth month of pregnancy. Occurrence of RFM was more frequent in cows that were seropositive. The period between abortion and first estrus was 79.06 v. 65.8 days for seropositive and seronegative groups, respectively, which differed significantly (P < 0.001). The results suggest that the higher occurrence of RFM after abortion in dairy NC-seropositive cows affects uterine involution, resulting in a delay of ovarian activity.


Neospora caninum, an intracellular protozoon, causes encephalomyelitis in dogs (Bjerkas I et al. 1984 Zentralblat fur Parasitenkunde 70, 271-274). For the past decade, neosporosis has been a main cause of abortion in dairy cattle worldwide (Anderson M et al. 2000 Anim. Reprod. Sci. 60-61, 417-431; Dubey JP 2003 Korean J. Parasitology 41, 1-16). Vertical transmission has been indicated as an important way of spreading neosporosis (Hall CA et al. 2005 Vet. Parasitology 31, 231-41); thus, we investigated whether the protozoon could be transferred by embryo production techniques. Blood samples were collected from 92 dairy cows with history of reproductive failure and abortion within the previous 90 days at 7 dairy farms in Tizayuca, Mexico. For serology evaluation, a commercial indirect ELISA kit (Civtest Bovis Neospora, Laboratories Hipra S.A, Girona, Spain), yielded 46.74% (43/92) positive results, 46.74% (43/92) negative results, and 6.52% (6/92) suspicious to N. caninum infection. Thirteen positive cows were chosen for uterine flush (UF), ovum pickup (OPU), and a blood sample collection. Lymphocytes from blood and cells within the UF and OPU collection fluids were collected after centrifugation and DNA was extracted. All samples were tested for the presence of N. caninum by PCR, using primers and protocols that amplified a 275-bp fragment of the genomic region (5-GGGTGAACCGAGGGAGTTG-3 and 5-CCTCCCAATGCGAACGAAA-3). The N. caninum vaccine (Bovilis® NeoGuard, Intervet, Santiago Tianguistenco, Mexico) was used as a positive control and water as a negative control. Uterine flush could not be obtained from 1 cow. From 13 cows seropositive to N. caninum, only 38% were positive to PCR from blood lymphocytes. In contrast, PCR amplification was obtained from OPU cell sediment in 92.31% (12/13) and in 33.33% (4/12) of UF. Of these 12 OPU- and 4 UF-positive samples, only 5 and 3 of their corresponding blood lymphocytes were positive. Our results using uterine and follicular fluid were contradictory to those published by Moskwa et al. (2008 Vet. Parasitology 158, 370-375) where oocytes and embryos were evaluated. These results indicate that N. caninum is present in the ovary and uterine lumen of the cows, suggesting a possible risk of neospora transmission during oocyte and embryo collection and transfer techniques.
Sheep

Classical scrapie

The objective of the study was to examine whether or not the preimplantation embryo can act as a carrier of classic scrapie infection. The study was carried out on quarantined premises with sheep of highly susceptible scrapie genotypes. Uninfected embryos, collected from New Zealand-derived Suffolk ewes, were surgically transferred into recipient ewes that were also of New Zealand origin. Seventeen negative control lambs were born on the study premises from these embryo transfers. Thirty-nine experimental lambs were from embryos collected from naturally infected donor ewes. The experimental lambs were also born on the study premises after their surgical transfer into recipient ewes of New Zealand origin. These embryos had been collected from donor ewes in a scrapie-infected flock where the ewes were clinically sick with scrapie or developed clinical scrapie after embryo collection. All lambs were confirmed as scrapie susceptible of the ARQ/ARQ genotype. Twenty-eight experimental animals survived to the end point of the study at 5 yr of age with a mean survival of 1579 d. In the negative control group, 12 of 17 sheep survived to 5 yr of age with a mean survival of 1508 d. Postmortem examinations were carried out on all animals derived by embryo transfer, and in none was histologic or immunohistochemical evidence of scrapie found. In contrast, in the originating flock the majority of scrapie cases occurred in ARQ/ARQ genotyped animals where a 56% mortality from scrapie had been recorded in animals of this genotype. Thus, the study provides no evidence for transmission of scrapie and reinforces published evidence that vertical transmission of scrapie may be circumvented by embryo transfer procedures.

Low JC, Chambers J, McKelvey WA, McKendrick IJ, Jeffrey M. Failure to transmit scrapie infection by transferring preimplantation embryos from naturally infected donor sheep. Theriogenology. 2009;72(6):809-16. (Note that the pdf of this article was forwarded with the 2009 update to facilitate discussion on Agenda item #3b.)
Miscellaneous species

Mice

Mouse Minute Virus (MMV; parvovirus)

Murine parvoviruses, including minute virus of mice (MVM), represent major infectious disease problems encountered in contemporary laboratory animal research facilities with embryo transfer (ET), one of the most widely used techniques for rederivation. Using an in vivo approach, the objectives of this study were to assess the risk of MVM transmission during rederivation and to provide data that allow recommendation of preventive measures. Therefore, we determined whether immunosuppressive variant MVMi viral DNA is detectable in reproductive organs, gametes (oocytes and spermatozoa), and embryos collected from experimentally infected mice and whether washing as recommended before ET eliminates MVMi sufficiently from gametes and embryos. Fractions of reproductive organs tested positive from Day 5 to Day 30 postinoculation, demonstrating a risk for a minimum period of 4 wk; the highest incidence of positive organs was found between Day 9 and Day 13 postinoculation. Real-time PCR detected viral DNA to a lesser extent in male than in female reproductive organs. MVMi DNA was detected in oocytes and sperm cells derived after in vivo infection but not in two-cell embryos. In vitro contamination studies revealed that the virus firmly adheres to the zona pellucida after 10 wash steps, indicating that even extensive washing might not eliminate MVMi completely from embryos. According to this systematic in vivo approach, recommended measures to prevent transmission of MVM during rederivation include sufficient washing of embryos, accompanying testing using adequate (PCR) methods, and using embryos rather than in vitro fertilization techniques; furthermore, the exchange of gametes should be considered a risk factor.


Miscellaneous topics

Pathogens in semen

Acute or chronic BVDV infection on bulls is associated with BVDV excretion in the semen that can be transient in case of acute infection or permanent for a persistently infected animal. Under certain circumstances BVDV acute infection on bull can lead to a permanent spermatic infection. During a 6 months follow-up, a total of 2 bulls (0.19 %, out of 1033 seropositive AI bulls) were diagnosed as permanent spermatic infected whereas the BVDV antibodies remained stable.

This study investigated the effects of the vaccination of rams with a serotype 2 bluetongue virus vaccine on the quality of their semen. One group of 23 rams was vaccinated on days 0 and 47, and 23 rams were left unvaccinated. Samples of blood, serum and semen were collected regularly in order to detect the virus genome, and to compare the quality of the semen from the vaccinated and unvaccinated rams. Segment 10 of the genome of the vaccine strain was detected in the blood of the vaccinated animals by reverse transcriptase-PCR (RT-PCR) on days 7, 13 and 19 after the first vaccination, but no virus was isolated from the RT-PCR-positive blood or from any of the semen samples from the vaccinated animals. There was a significant decrease in the concentration and motility of the spermatozoa and an increase in the proportion of abnormal and dead spermatozoa after the first vaccination ; however, after the second vaccination only smaller, non-significant changes were observed. On day 69, the quality of the semen of the vaccinated animals was not significantly different from that of the controls.

**Abstract 15.1**

### Pathogen Transmission through Breeding

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The recent outbreak of Contagious Equine Metritis (CEM) in the U.S. demonstrates that venereal diseases are still an imminent problem for horse breeders. However, when looking at *viral* venereal diseases, numerical data on prevalences, transmission and virus shedding status of individuals, are not always available. For example, sero-prevalences for Equine Viral Arteritis (EVA) vary between 6 and 41% depending on country, season and breed. Furthermore, the European legislation prescribes a rather strict compliance with the sanitary guidelines concerning transport of animals and semen, although only little consideration is given to new purifying techniques, as density gradient centrifugation, and possible infectious risks of embryo transfer in the horse. Recently, new findings have been published on the potential risk of equine herpes virus transmission (*Helba et al. 2007: Theriogenology 67: 1485-1491*) and methods to purify stallion semen from viral and bacterial contaminants have become available (*Morell and Geryartgy 2006: Equine Vet J 38, 224-229*). It might be useful to discuss how stringent regulations can or have to be concerning transcontinental semen and embryo transport. These data have been proven to be useful in the field and might be essential in the discussion.

**Abstract 15.2**

### Risk of Pathogen Transmission through Breeding in Horses

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Veneral transmission: which risks? Some infectious bacterial, viral and protozoal reproductive pathogens have a well-known venereal transmission. The stallion can be an asymptomatic penile carrier of pathogenic bacteria that are transmitted to the mare: *Tetratoga equitans*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. Streptococcus zooepidemicus may also cause significant infertility in mares but its eventual venereal transmission has never been formally proven. The ability of the Equine Arteritis Virus to be transmitted by semen is well established. Equine coital examination caused by Equine Herpes Virus 3 was described from many years. In some countries, the causative agent of dourine, trypanosoma equiperdum, is sometimes found. Equine herpesvirus types 1 and 4 are two major pathogens in the horse, with a large incidence of asymptomatic latent infections. In many mammalian species viruses from the Herpesviridae family are frequently found in and transmitted via semen and embryos. In the past, a few sporadic cases of the isolation of the virus in the semen have been described following the experimental or natural infection of stallions with the EHV-1 virus, but never in healthy stallions. In a recent study (Helba-Fellah et al, 2009), samples of semen from 390 different stallions were tested using specific PCR to detect EHV-1 and EHV-4 DNA. EHV-4 viral DNA was not detected in any of the semen samples. EHV-1 DNA was identified in 51 of the 390 samples (13%). One hundred and eighty two samples came from 6 studs and there was significant difference (p < 0.05) in the proportion of stallions whose semen tested positive for viral DNA from 0 to 55% among the studs. In another study (Helba-Fellah et al, 2009) testis and epididymis tissues sampled from 60 healthy stallions during castration were tested by the same specific PCR. EHV-1 DNA was detected in 2 from 60 stallions’ testes samples (3%), but was not detected in any stallions’ epididymis samples. Those results demonstrate for the first time the presence of the EHV-1 in the testes and semen of naturally infected stallions and suggest that EHV-1 virus may be transmitted via semen at mating or via AI, similar to other herpesviruses in other species.

Further studies are needed to determine in which testicular cells the virus resides and which consequences the genital and semen location of EHV1 has on fertility and viral transmission. Would other viruses be present in semen and could be sexually transmitted? The question has to be asked for example for influenza virus, West-Nile virus... For a few viruses, there is a partial response: Equine Infectious Anemia (EIA) virus has been reported in semen from infected stallions, without any evidence of sexual transmission of disease (Lu & Morressey, 2007). Transmission via embryo transfer: are there risks? European and french sanitary regulations for the inter and intra European countries trade of equine embryos recommend embryos to be washed 10 times over in accordance with the standardized procedure described for ruminant and porcine embryos by IETS guidelines. However, the risks of any infectious transmission by embryo transfer in horses have not been really evaluated. Embryo contamination was reported once by Carvalho et al. (2000), who collected an equine embryo naturally contaminated by EHV-1 from a clinically healthy donor mare. In two studies (Helba et al., 2007, 2008), we demonstrated that after *in vitro* contamination of D6.5, D7.5 and D8 embryos cannot be removed from embryos by the washing procedure (10 washes) recommended by sanitary european regulations and IETS guidelines. In the second of these experiments (Helba et al., 2008), an enzymatic treatment (bath of 0.25% trypsin for 90 s) before the washing protocol has proven to be efficient to decontaminate equine early blastocysts (D6.5) surrounded by zona pellucida as previously reported for bovine embryos infected by BHV-1, but ineffective to do the same on older blastocysts (D8) surrounded only by capsule, as previously reported for porcine embryos infected by PRV. The persistence of EHV-1 after washing makes the embryo a potential means of transmitting the virus, and disease, to recipient mares. Further studies are needed to determine which different interactions exist between EHV-1 and embryos surrounded by ZP or by capsule, to evaluate the effect of virus on embryo viability and to determine whether the transfer of infected embryos could infect recipient mares. Other pathogens would be tested on equine embryos, and eventual sanitary risks of embryo transfer would be more evaluated in horses.

**Abstract 15.3**

### Prevention of Pathogen Transmission through Breeding in Horses

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A method for removing pathogens from semen is urgently required, to protect naïve inseminated animals from viruses which may be shed in the semen before the male has same-converted. Recently, a method was described for removing equine arteritis virus (EAV) from stallion semen to be used for artificial insemination (AI) (*Morell and Geryartgy 2006: Equine Vet J 38, 224-229*). The method, a double semen processing technique consisting of a density gradient centrifugation (DGC) followed by a ‘wet-up’ procedure, removed EAV from both spiked and naturally infected semen samples, using PCR and virus isolation to test for the virus (*Morell and Geryartgy 2006: Equine Vet J 38, 224-229*). Preliminary results of a similar experiment using a simplified technique, Single Layer Centrifugation (SLC) with Androco-F®, plus ‘wet-up’, considerably reduced the virus titer from boar ejaculates spiked with porcine circovirus (*Morerell et al., unpublished data*). Since SLC is less time-consuming than DGC and can be scaled-up to process large volumes of ejaculate, it might be feasible to use it for processing whole animal ejaculates. DGC is also reported to remove bacteria from human semen (*Nicholson et al. 2000: Hum Reprod 15, 656-666*). Therefore, studies using SLC with animal semen are currently underway to investigate the removal of bacterial contamination occurring during semen collection. Routine use of SLC in processing animal semen for AI-doses could thus protect against accidental transmission of viruses from shedding males and reduce the amount of antibodies used in semen doses for AI.
In the horse, the risk of excretion of two major equine pathogens (equine herpesvirus types 1 (EHV-1) and 4 (EHV-4)) in semen is unknown. The objective of our study was to assess the possible risks for the horizontal transmission of equine rhinopneumonitis herpesviruses via the semen and the effect of the viruses on stallion fertility. Samples of stallion semen (n=390) were gathered from several different sources. Examination of the semen involved the detection of viral DNA using specific PCR. The mean fertility of the stallions whose sperm tested positive for viral DNA and the mean fertility of stallions whose sperm did not contain viral DNA, were compared using the Student's t-test. EHV-4 viral DNA was not detected in any of the semen samples. EHV-1 DNA was identified in 51 of the 390 samples, (13%). One hundred and eighty-two samples came from 6 studs and there was significant difference (p<0.05) among the proportion of stallions whose semen tested positive for viral DNA from 0 to 55% between the studs. There was a significant difference (p<0.014) between the fertility of stallions whose semen tested positive for viral DNA and those whose semen was free from viral DNA. The stallions that excreted the EHV-1 virus in their semen appeared to be more fertile than the non-excretors, but this difference was in fact related to the breeding technique since higher proportion of excretors were found among those whose semen was used fresh rather than preserved by cooling or freezing. In conclusion, this study suggests that the EHV-1 virus may be transmitted via the semen at mating or by artificial insemination as demonstrated with other herpes viruses in other species.

Disease transmission through in vitro produced embryos

Cattle

Bovine viral diarrhea virus

The objective was to determine the average amount of bovine viral diarrhea virus (BVDV) associated with single in vivo-derived and in vitro-produced bovine embryos following recommended processing procedures for embryos. In vivo-derived and in vitro-produced bovine embryos at 7d post-fertilization were exposed (for 2h) to 2 x 10^5-7 cell culture infective dose (CCID(50))/mL of SD-1 (a noncytopathic, Type 1a strain of BVDV), and then washed according to International Embryo Transfer Society (IETS) guidelines prior to testing. Of the 87 in vivo-derived embryos tested, 27% were positive for virus by quantitative polymerase chain reaction (qPCR). The range in amount of virus associated with 99% of the contaminated embryos was <or=6.62+/-1.57 copies/5 microL; 90% of the contaminated embryos had <or=4.64+/-1.57 viral copies/5 microL of embryo-associated virus, using tolerance intervals (P<0.05). The SEM was 0.33 and the mean of averages was 1.12/5 microL. Of the 87 in vitro-produced embryos, 42% were positive for virus. The range in amount of virus associated with 99% of the contaminated embryos was <or=3.44+/-0.89 copies/5 microL; 90% of the contaminated embryos had <or=2.40+/-0.89 viral copies/5 microL of embryo-associated virus using tolerance intervals (P<0.05; S.E.M. was 0.14 and the mean of averages was 0.55/5 microL). Therefore, although many embryos were positive for virus, there were limited numbers of copies, thereby posing doubt regarding their potential for contamination following embryo transfer.


The objective of this study was to perform a comprehensive risk assessment on infectious disease transmission in the system of in vitro embryo production via somatic cell nucleus transfer (SCNT) technology using bovine viral diarrhea virus (BVDV) as a model. The risks of BVDV transmission in each step of the SCNT embryo production procedure, from donor cells to preimplantation SCNT embryo culture, were carefully examined using a sensitive real-time polymerase chain reaction assay. The identified primary source of BVDV transmission in SCNT embryo production was donor cell infection, most likely caused by contaminated fetal bovine serum in the culture medium. The risk of disease transmission through contaminated oocytes from an abattoir was relatively low, and it can be greatly minimized by cumulus cell removal and adequate oocyte washing procedures. Of the 200 cumulus-oocyte complexes (COCs) and more than 1500 cumulus cell-free oocyte (CFO) samples collected from multiple sources over a course of 7 months, only 2.5% of the COCs were BVDV positive, and all of the CFOs (100%) were BVDV negative. To evaluate the risk of BVDV introduction during in vitro SCNT
embryo culture, 324 SCNT embryos were produced from 18 different cell lines using oocytes from 26 different batches collected over a course of 9 months. The embryos were cultured in vitro for 7 days and then tested for BVDV. All of the 324 SCNT embryos (100%) were negative, indicating that the embryo culture system is virtually risk-free for BVDV transmission. Based on these results, a standard operational protocol (SOP) for SCNT embryo production was proposed to greatly minimize the risk of BVDV transmission through the SCNT embryo production system. This SOP could be a starting point to produce a SCNT system that is virtually risk-free for disease transmission in general.


The purpose of this study was to determine whether or not embryos derived from in vitro fertilization of oocytes from persistently infected (PI) cattle would contain infectious virus. Three in vitro embryo production treatment groups were assessed: 1) oocytes and uterine tubal cells (UTC) free of bovine viral diarrhoea virus (BVDV) (negative control), 2) oocytes free of BVDV fertilized and cultured in media containing UTC obtained from PI heifers, and 3) oocytes from PI heifers fertilized and cultured in media containing UTC free of BVDV. The developmental media, UTC and embryos (individual or groups of five) were assayed for virus. Virus was not isolated from any samples in treatment group 1. As shown in previous studies, a proportion of embryo samples were positive for BVDV in treatment group 2. In treatment group 3, the virus associated with the oocytes contaminated the developmental media and infected susceptible co-culture cells used during fertilization and culture. In addition, 65% (11/17) of the degenerated ova from treatment group 3 had infectious virus associated with them. While none of the ova developed into transferable embryos, the study did confirm that use of oocytes from PI cows could lead to amplification of BVDV and cross contamination during in vitro embryo production.


Bovine viral diarrhea virus & Bovine herpesvirus-1

The objective was to determine the effect of cryopreservation by conventional slow controlled cooling (0.5 degrees C/min) and by vitrification on the presence of bovine viral diarrhea virus (BVDV) and bovine herpesvirus-1 (BHV-1) infectivity associated with frozen-thawed Day 7 bovine embryos. In this study, Day 7 embryos generated by in vitro fertilization (IVF) were exposed in vitro for 1.5h to BVDV (N=393) and BHV-1 (N=242) and subsequently tested before and after cryopreservation for the presence of infectivity. Exposure of embryos to viral agents resulted in 72% of them infected prior to
cryopreservation. Stepwise exposure of embryos to cryoprotectants, as well as their removal, substantially reduced the proportion of contaminated embryos (46% vs. 72%, \(P<0.05\)). Overall, both freezing methods reduced the percentage of infectious embryos compared with that of embryos similarly exposed to viruses but not cryopreserved (31% vs. 72%, respectively; \(P<0.001\)). The percentage of embryos with infectious viruses was not significantly higher after vitrification than after slow cooling (38% vs. 22%). In addition, after cryopreservation, a higher percentage (\(P<0.002\)) of embryos exposed to BHV-1 (42%) remained infectious than did embryos exposed to BVDV (24%). In conclusion, cryopreservation reduced the proportion of infected embryos but did not render all of them free from infectious pathogens.


Bluetongue virus (serotype 8)

In 2006 and 2007, Bluetongue virus serotype 8 (BTV-8) caused devastating outbreaks in Northern Europe; the outbreaks were controlled in 2008 and 2009 by an international vaccination policy. Remarkably, BTV-8 differs from other serotypes in that it spread transplacentally (De Clercq K et al. 2008 Transboundary and Emerging Diseases 55, 352-359). Apart from the transplacental spreading, a significant increase in the incidence of abortions was reported in Belgium (Meroc E et al. 2009 Transboundary and Emerging Diseases 56, 39-48). The aim of the present study was to investigate the susceptibility of bovine-hatched, in vitro-produced blastocysts to BTV-8. A total of 1390 immature bovine oocytes were matured and fertilized in vitro. Presumed zygotes \((n = 1148)\) were denuded 24 h post-insemination and cultured in 50-μL droplets of modified synthetic oviduct fluid (SOF) medium with 10% fetal calf serum (tested negative for BTV antibodies) at 39.0°C in 5% CO2, 5% O2, and 90% N2. At 7 days post-insemination (dpi), blastocysts were grouped to enhance hatching. For virus incubation, BTV-8 Bel 2006/2 from Veterinary and Agrochemical Research Centre (VAR, Brussels, Belgium) was used. At 8.5 dpi, hatched embryos were placed in 800μL of minimum essential medium (MEM) containing 103.8 50% tissue culture infectious doses (TCID50) of BTV-8 and incubated for 1 h at 39°C in an atmosphere of 5% CO2 in air. At the same time, 2 groups of hatched control embryos were incubated under the same circumstances in 800 μL of SOF and 800 μL of MEM, respectively. After infection, all embryos were washed according to IETS guidelines with the exception that they were not zona pellucida intact and cultured in new SOF. At 48, 60, 72, and 96 h post-infection (hpi), one-fourth of the embryos of each group were fixed in 4% paraformaldehyde for 12 to 24 h and subsequently stained for BTV-8 with double immunofluorescent staining using a BTV-8 monoclonal antibody (8A3B.6, ID-Vet, Montpellier, France). All control embryos (CTRL and MEM) were negative for BTV-8 virus antigen at all time points. At 48 hpi, only 1 out of 7 infected embryos was positive for virus antigen (in all its cells). At 60 hpi, all remaining embryos \((n = 6)\) were negative, whereas at 72 hpi and 96 hpi all embryos had 25% to 100% BTV-8-positive cells \((n = 6\) at 72 hpi and \(n = 7\) at 96 hpi). Furthermore, 1
embryo at 72 hpi and 2 embryos at 96 hpi showed morphological signs of degeneration. This study has showed for the first time that hatched in vitro-produced blastocysts are susceptible for BTV-8 virus infection and replication in vitro. The relatively long time between virus infection and detection of viral antigen is in accordance with the slow replication cycle of the virus. Further research is ongoing to investigate the importance of BTV-8 infection in early embryonic death.


Bovine herpesvirus-1

The aim of this study was to evaluate the efficiency of trypsin treatment on the inactivation of bovine herpesvirus type 1 (BoHV-1) on in vitro produced by fertilization and artificially infected bovine embryos. Bovine embryos on day 7 were exposed with 10 microl of BoHV-1, Los Angeles strain 10^7.5 TCID. These embryos and control embryos were divided in two groups: submitted to the sequential washes or to the trypsin treatment according to the International Embryo Transfer Society (IETS) guidelines. The embryos and the last washing drop of each group were used as inoculum to infect Madin Darby bovine kidney (MDBK) cells and submitted to nested PCR reaction using the primer that encodes the gene conserved region of virus glycoprotein gB. The data have shown that the control embryos and their last washing drop were negative. The exposed embryos that were treated with trypsin have shown positive results on the n-PCR and MDBK culture, and their last washing drop were negative. Our data have demonstrated that the trypsin treatment was not able to eliminate the BHV-1 of the embryos, suggesting an interaction between virus and embryo.

D'Angelo M, Visintin JA, Richtzenhain LJ, Gonçalves RF. Evaluation of trypsin treatment on the inactivation of bovine herpesvirus type 1 on in vitro produced pre-implantation embryos. Reprod Domest Anim. 2009;44(3):536-9. (Note that the pdf of this article was forwarded with the 2009 update to facilitate discussion on Agenda item #5a.)

Bovine herpesvirus-5

Bovine herpesvirus-5 (BoHV-5), which is the second most important infectious brain disease affecting livestock in Latin America, has been detected in bull semen and aborted fetus; however, no reports are found regarding its presence in bovine embryos. Because it has 90% genomic similarity to BoHV-1, it is possible that BHV-5 can infect the genital system after viral reactivation, leading to reproductive disorders. This study was designed to investigate the effects of infection of bovine blastocysts (B) by BoHV-5. Hormones and fetal calf serum were tested by PCR and considered free of virus. Selected oocytes, obtained from ovaries at a local slaughterhouse, were washed in PBS with 10% fetal calf serum (Nutricell®, Campinas, Brazil). The oocytes were transferred to 100-μL drops of maturation medium consisting of TCM 199 (Gibco®, Grand Island, NY, USA), 0.5 μg mL^-1 of FSH (Pluset®, Calier, Spain), and 50 μg mL^-1 of LH (Lutropin®-V, Bioniche
Inc., Belleville, Ontario, Canada) for 24 h at 39°C and 5% CO2 in air. Afterward, frozen semen (500 μL) was thawed and placed on a Percoll gradient (45 and 90%) and centrifuged at 700 × g for 30 min. The resultant pellet was centrifuged (200 × g for 5 min) in TALP medium and the sperm pellet was then diluted in TALP plus phenylalanine (PHE) and heparin. After an 18-h fertilization period, presumptive zygotes were transferred to culture in CR2 medium up to Day 7 post-fertilization. The procedures were carried out under the same conditions previously reported for IVM. A total of 150 intact B were assigned into 2 experimental groups: I (free of virus) and II (with virus; 102 TCID50/mL for a 1-h period). Then, B of both groups were washed and re-cultured for 72 h in drops of CR2 without virus. The percentage of embryos reaching the hatched blastocyst (HB) stage was observed and analyzed by the chi-square test. At this time, HB were fixed to investigate the presence of virus, degree of apoptosis, and oxidative stress. The virus detection was performed by using an in situ hybridization assay with a specific probe to the glycoprotein C gene of BoHV-5 labeled to biotin. The apoptosis was determined by the annexin V, 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI), and propidium iodide (PI) markers, using immunofluorescence technique. The oxidative stress was realized by using monoclonal anti-AOP1 (antioxidant-like protein 1; Sigma®, St. Louis, MO, USA) through immunoassay. More HB (P > 0.05) were found in group II (75.0%) than for group I (55.0%). In both groups, positive signs for the presence of the apoptosis and oxidative stress markers were observed. The mechanism of apoptosis was initiated independently of virus presence, as evidenced by positive signs also observed in group I. However, oxidative stress was intense in group II, suggesting an evident viral effect on the host cell without compromising embryonic development. These findings might indicate that BoHV-5 uses some mechanisms that keep the cell viable to allow its replication, as seen by the greater hatching rate of infected embryos (75%) compared with the control (55%).

Silva-Frade C, Martins Jr. A, Borsanelli AC, Frade MC, and Cardoso TC. In vitro development of blastocysts continues after artificial infection with bovine herpesvirus type 5. Reproduction, Fertility and Development. 2010; 22(1): 254 (abstr.). (Note that the pdf of this article was forwarded with the 2009 update to facilitate discussion on Agenda item #5a.)
Neospora caninum

Neospora caninum, an intracellular protozoon, causes encephalomyelitis in dogs (Bjerkas I et al. 1984 Zentralblat fur Parasitenkunde 70, 271-274). For the past decade, neosporosis has been a main cause of abortion in dairy cattle worldwide (Anderson M et al. 2000 Anim. Reprod. Sci. 60-61, 417-431; Dubey JP 2003 Korean J. Parasitology 41, 1-16). Vertical transmission has been indicated as an important way of spreading neosporosis (Hall CA et al. 2005 Vet. Parasitology 31, 231-41); thus, we investigated whether the protozoon could be transferred by embryo production techniques. Blood samples were collected from 92 dairy cows with history of reproductive failure and abortion within the previous 90 days at 7 dairy farms in Tizayuca, Mexico. For serology evaluation, a commercial indirect ELISA kit (Civtest Bovis Neospora, Laboratories Hipra S.A, Girona, Spain), yielded 46.74% (43/92) positive results, 46.74% (43/92) negative results, and 6.52% (6/92) suspicious to N. caninum infection. Thirteen positive cows were chosen for uterine flush (UF), ovum pickup (OPU), and a blood sample collection. Lymphocytes from blood and cells within the UF and OPU collection fluids were collected after centrifugation and DNA was extracted. All samples were tested for the presence of N. caninum by PCR, using primers and protocols that amplified a 275-bp fragment of the genomic region (5'-GGGTGAACCGAGGGAGTTG-3' and 5'-CCTCCCAATGCGAACGAAA-3'). The N. caninum vaccine (Bovilis® NeoGuard, Intervet, Santiago Tianguistenco, Mexico) was used as a positive control and water as a negative control. Uterine flush could not be obtained from 1 cow. From 13 cows seropositive to N. caninum, only 38% were positive to PCR from blood lymphocytes. In contrast, PCR amplification was obtained from OPU cell sediment in 92.31% (12/13) and in 33.33% (4/12) of UF. Of these 12 OPU- and 4 UF-positive samples, only 5 and 3 of their corresponding blood lymphocytes were positive. Our results using uterine and follicular fluid were contradictory to those published by Moskwa et al. (2008 Vet. Parasitology 158, 370-375) where oocytes and embryos were evaluated. These results indicate that N. caninum is present in the ovary and uterine lumen of the cows, suggesting a possible risk of neospora transmission during oocyte and embryo collection and transfer techniques.

Prevention and regulation of equine infectious anemia virus (EIAV) disease transmission solely depend on identification, isolation, and elimination of infected animals because of lack of an effective vaccine. Embryo production via the somatic cell nuclear transfer (SCNT) technology uses oocytes collected mainly from untested animals, which creates a potential risk of EIAV transmission through infected embryos. The current review examines the risk of EIAV disease transmission through SCNT embryo production and transfer. Equine infectious anemia virus is a lentivirus from the family Retroviridae. Because of a lack of direct reports on this subject, relevant information gathered from close relatives of EIAV, such as human immunodeficiency virus (HIV), bovine immunodeficiency virus (BIV), feline immunodeficiency virus (FIV), and small ruminant lentiviruses (SRLVs), is summarized and used to predict the biological plausibility of EIAV disease transmission through transfers of the equine SCNT embryos. Based on published information regarding interaction of oocytes with lentiviruses and the sufficiency of oocyte and embryo washing procedures to prevent lentivirus transmission from in vitro-produced embryos of various species, we predicted the risk of EIAV transmission through SCNT embryo production and transfer to be very small or absent.


**Horses**

**Equine infectious anemia virus**

The risk of transmission of mouse minute virus (MMV) to recipients of murine embryos arising from in vitro fertilization (IVF) of cumulus-enclosed oocytes (CEOs) or without cumulus cells (CDOs) in the presence of MMV-exposed (10(4) TCID(50) [mean tissue culture infective dose]/ml MMVp [prototype strain of MMV]) spermatozoa was evaluated. Also, the time after embryo transfer to detection of MMV antibody and the presence of MMV DNA in the mesenteric lymph nodes of recipients and pups were investigated. All mice were MMV free, but two seropositive recipients and four seropositive pups were found in the group with CDOs. With regard to the CEOs, two of 11 holding drops and five of 11 groups of embryos were MMV positive using PCR, while neither holding drops nor embryos carried infectious MMVp, as evidenced by the in vitro infectivity assay. From IVF with CDOs, five of 14 holding drops and four of nine groups of embryos were MMV positive, while one of 14 holding drops and no embryos carried infectious MMVp. When 10(5) cumulus cells were analyzed 5 h after exposure to 10(4) TCID(50)/ml MMVp, cells had an average titer of 10(4) TCID(50)/ml MMVp. The
present data show that, in contrast to CDOs, 2-cell embryos from CEOs did not transmit infectious MMVp to the holding drops and to recipients. This observation is due to the presence of cumulus cells during the IVF process that reduce entry of MMV into the zona pellucida and absorb some of the virus. These data further confirm the efficacy of the IVF procedure in producing embryos that are free of infectious virus, leading to virus-free seronegative recipients and rederived pups.


Mouse Minute Virus (MMV) & Mouse Hepatitis Virus

The aim of this study was to determine the susceptibility of murine embryonic stem (mESCs) to mouse hepatitis virus (MHV-A59) and mouse minute virus (MMVp) and the effect of these viruses on germline transmission (GLT) and the serological status of recipients and pups. When recipients received 10 blastocysts, each injected with 10(0) TCID(50) MHV-A59, three out of five recipients and four out of 14 pups from three litters became seropositive. When blastocysts were injected with 10(-5) TCID(50) MMVp, all four recipients and 14 pups from four litters remained seronegative. The mESCs replicated MHV-A59 but not MMVp, MHV-A59 being cytolytic for mESCs. Exposure of mESCs to the viruses over four to five passages but not for 6 h affected GLT. Recipients were seropositive for MHV-A59 but not for MMVp when mESCs were cultured with the virus over four or five passages. The data show that GLT is affected by virus-contaminated mESCs.


Human Immunodeficiency Virus (HIV-1)

Acquired immunodeficiency syndrome (AIDS) is a major public health problem worldwide. This study was performed to explore the feasibility of vertical transmission of human immunodeficiency virus-1 (HIV-1) gag gene via oocyte. The recombinant plasmid (pIRES2-EGFP-gag) was injected into mouse ovaries to transfected germ cells. Induction of superovulation and then animal mating were performed to collect oocytes and two-cell embryos. Positive FISH signals for HIV-1 gag DNA were detected in the nuclei of oocytes and embryos, and in chromosomes of mature oocytes, indicated integration of the gene into the oocyte genome and gene replication in the embryo. HIV-1 gag cDNA positive bands detected by RT-PCR in oocytes and embryos indicated successful gene transcription, while positive immunofluorescence signals for HIV-1 gag protein indicated successful translation in both oocytes and embryos. The HIV-1 gag gene was transmitted vertically to the next generation via oocytes and it retained its
function in replication, transcription and translation following at least one mitotic division in embryos.


Miscellaneous topics

Antiviral research

Bovine viral diarrhea virus (BVDV) can associate with in vitro fertilized (IVF) bovine embryos despite washing and trypsin treatment. An antiviral compound, DB606 (2-(4-[2-imidazolinyl]phenyl)-5-(4-methoxyphenyl)furan), inhibits the replication of BVDV in bovine uterine tubal epithelial cells, Madin Darby bovine kidney cells, and fetal fibroblast cells. As well, DB606 in in vitro culture medium does not affect embryonic development. Antiviral-treated-IVF embryos placed into recipients developed into clinically normal calves. The objective of this project was to determine if these resultant heifer calves were capable of reproducing. Seven heifers from each of the treatment groups (natural breeding, IVF embryo, and IVF embryo cultured in DB606) of the previous study were used. At 20-27 months of age, the heifers were exposed to a fertile bull in a single pasture during a 63 d breeding season. Five of the seven heifers originating from natural breeding were pregnant 35 d after removal of the bull and calved. All of the heifers resulting from transfer of untreated IVF embryos were pregnant at 35 d; however, one aborted the fetus at 5-7 months of gestation. All of the heifers derived from transfer of IVF embryos cultured in DB606 were pregnant and calved. Offspring from dams of all treatment groups were clinically normal at birth. Adjusted 205 d weaning weights were not significantly different among the offspring of the treated and untreated dams. These results indicate that culture of bovine-IVF embryos in DB606 does not impair future reproductive capacity of resulting heifers.


Bovine herpesvirus 1 (BoHV-1) is widely distributed among cattle populations and has been associated with cells, fluids, and tissues collected from donor animals for use in reproductive technologies. The purpose of this study was to determine if lactoferrin would inhibit BoHV-1 in cell culture and to evaluate if embryos could develop normally when cultured in vitro with lactoferrin. In Experiment 1, lactoferrin (10 mg/mL) inhibited up to 25,000 plaque forming units (PFU)/mL of BoHV-1 in Madin Darby bovine kidney (MDBK) cell culture. In Experiment 2, lactoferrin (10 mg/mL) combined with cidofovir (62.5 microg/mL) inhibited up to 100,200 PFU/mL of virus in cell culture. In Experiment 3, following fertilization, presumptive zygotes were cultured in media containing lactoferrin (10, 5, and 2.5 mg/mL). Embryonic development and quality were assessed,
and embryonic viability was determined by counting the nucleated cells of developed blastocysts. While lactoferrin did not affect the nucleated cell count of the treated embryos, it did significantly decrease blastocyst development. In conclusion, lactoferrin from bovine milk can inhibit BoHV-1 in cell culture. However, supplementation of in vitro culture medium with lactoferrin inhibits blastocyst development of in vitro-produced embryos.


Contaminating microorganisms

Murine embryonic stem cells (mESCs) inoculated at passage P13 with the mycoplasma species M. hominis, M. fermentans and M. orale and cultured over 20 passages showed reduced growth rate and viability (P < 0.0001) compared to control mESCs. Spectral karyotypic analysis of mycoplasma-infected mESCs showed a number of non-clonal chromosomal aberrations which increased with the duration of infection. The differentiation status of the infected mESCs was most affected at passage P13+6 where the infection was strongest and 46.3% of the mESCs expressed both POU5F1 and SSEA-1 markers whereas 84.8% of control mESCs expressed both markers. The percentage of germline chimeras from mycoplasma-infected mESCs was examined after blastocyst injection and embryo transfer to suitable recipients at different passages and, compared to the respective control group, was most affected at passage P13+5 (50% vs. 90%; P < 0.07). Further reductions were obtained at the same passage in the percentage of litters born (50% vs. 100%; P < 0.07) and in the percentage of pups born (22% vs. 45%; P < 0.001). Thirty three chimeras (39.8%) obtained from blastocyst injection with mycoplasma-infected mESCs showed reduced body weight (P < 0.0001), nasal discharge, osteoarthropathia, and cachexia. Flow cytometric analysis of plasma from chimeras produced with mycoplasma-infected mESCs revealed statistically significant differences in the proportions of T-cells and increased levels of IgG1 (P < 0.001), IgG2a (P < 0.05) and IgM (P < 0.05), anti-DNA antibodies (P < 0.05) and rheumatoid factor (P < 0.01). The present data indicate that mycoplasma contamination of mESCs affects various cell parameters, germline transmission, and postnatal development of the resulting chimeras.


Cryopreservation

The objective was to determine the effect of cryopreservation by conventional slow controlled cooling (0.5 degrees C/min) and by vitrification on the presence of bovine viral diarrhea virus (BVDV) and bovine herpesvirus-1 (BHV-1) infectivity associated
with frozen-thawed Day 7 bovine embryos. In this study, Day 7 embryos generated by in-vitro fertilization (IVF) were exposed in vitro for 1.5h to BVDV (N=393) and BHV-1 (N=242) and subsequently tested before and after cryopreservation for the presence of infectivity. Exposure of embryos to viral agents resulted in 72% of them infected prior to cryopreservation. Stepwise exposure of embryos to cryoprotectants, as well as their removal, substantially reduced the proportion of contaminated embryos (46% vs. 72%, P<0.05). Overall, both freezing methods reduced the percentage of infectious embryos compared with that of embryos similarly exposed to viruses but not cryopreserved (31% vs. 72%, respectively; P<0.001). The percentage of embryos with infectious viruses was not significantly higher after vitrification than after slow cooling (38% vs. 22%). In addition, after cryopreservation, a higher percentage (P<0.002) of embryos exposed to BHV-1 (42%) remained infectious than did embryos exposed to BVDV (24%). In conclusion, cryopreservation reduced the proportion of infected embryos but did not render all of them free from infectious pathogens.


Pathogens in Materials of Animal Origin

Both continuous UV lights and pulsed UV lasers have potentials to inactivate known and emerging viruses. Bovine viral diarrhea virus (BVDV), from the Pestivirus genus, is known to be a common viral contamination in (fetal) bovine serum (FBS). Also, BVDV has been used in the blood product industry as a surrogate for Hepatitis C virus (HCV), due to its similarity in structure and genome. Germicidal UV lamp with the wavelength of 254 nm and Nd:YAG laser (pulsed UV laser) in its third and fourth harmonic with the wavelengths of 355 and 266 nm, respectively, were used. BVDV suspended in PBS or FBS were exposed to different intensities and doses and then reduction in BVDV titer were calculated. To complete inactivation of BVDV suspended in PBS and PBS containing 5% FBS, 1.6 (t=30 min) and 3.2 (t=60 min)J/cm(2) were used. The minimum doses for inactivation of BVDV suspended in PBS with the 355 and 266 nm of pulsed UV laser were 352 and 92.25 J/cm(2). Also, the minimum doses for inactivation of BVDV suspended in FBS with 355 and 266 nm wavelengths of pulsed UV laser were 704 and 127 J/cm(2). To evaluate the irradiated FBS quality to support cell culture growth, FBS was treated with the dose of 190.5 J/cm(2) and 266 nm pulsed UV laser and was used to grow Vero cells, in comparison with a control group. The viability of cells in two groups was identical and the statistical evaluation showed no significant difference in 12 passages.

Multiplex testing

The objective of this study was to develop and validate a duplex quantitative polymerase chain reaction (qPCR) for simultaneous detection of bovine herpesvirus 1 (BHV-1) and bovine viral diarrhea virus (BVDV) type I and II. Follicular fluid was collected from a BHV-1 acutely infected heifer, a BVDV I persistently infected heifer, and from 10 ovaries recovered from an abattoir. Both the BHV-1 and BVDV contaminated follicular fluid were diluted 1:5 to 1:10^7 using the pooled, abattoir-origin follicular fluid. Each dilution sample was analyzed using the duplex qPCR, virus isolation, reverse transcription nested PCR and BHV-1 qPCR. The duplex qPCR was able to simultaneously detect diluted BHV-1 (1:100) and BVDV I (1:1,000). These results corresponded with the reverse transcription nested PCR and BHV-1 qPCR. Therefore, the duplex qPCR might be used for quality assurance testing to identify these two viruses in cells, fluids and tissues collected from donor animals and used in reproductive technologies.


Diagnostic assays on embryos

The objective was to develop a method to accurately and efficiently detect minute amounts of bovine viral diarrhea virus (BVDV) associated with a single embryo. There are two major challenges for BVDV detection in a single embryo: the test sensitivity and the efficiency of viral molecule recovery. These become even more critical when attempts are made to detect BVDV infections that occurred naturally, not through artificial exposure of the embryos to high affinity BVDV strains. We have developed a one-step sample preparation method that has increased the viral molecule recovery rate compared to the standard RNA isolation procedure by 7-100-fold. Instead of using the traditional virus exposure approach, we generated BVDV positive embryos via somatic cell nuclear transfer (SCNT) technology using BVDV positive donor cells. By combining the highly efficient sample preparation procedure with a sensitive one-step, real-time PCR system, we have developed a sensitive test that allows detection of as low as two copies of BVDV in a single embryo. This method will allow systematic risk assessment for BVDV transmission during in vitro embryo production via IVF or SCNT procedures.

After vitrification of oocytes, fertilization rates and subsequent development are unsatisfactory, possibly due in part to zona hardening. Foetal calf serum (FCS) can prevent zona hardening because of its fetuin content, but FCS composition varies among batches, and may contain viruses. In this study, we therefore compared media supplemented with different sources of macromolecules, 2% bovine serum albumin (BSA), 2% BSA + 1 mg/ml fetuin and 20% FCS, for handling oocytes for 10-30 min prior to vitrification. None of the treatments resulted in developmental rates comparable with the non-vitrified controls, but FCS inclusion in pre-vitrification handling medium resulted in higher blastocyst production per oocyte (p < 0.05) (10.8%) on day 9 of culture than BSA (5.3%) or BSA + fetuin (6.4%). Blastocysts developing from oocytes from all vitrification treatments were somewhat retarded relative to those developed from non-vitrified oocytes. We also tested the use of fetuin during vitrification as well as two different exposure times with cryoprotectants, 180 and 30 s. There was no significant effect of fetuin or exposure time on rates of subsequent blastocyst production.

Interspecies embryos

The low efficiency of somatic cell nuclear transfer may be related to the ultrastructural deviations of reconstructed embryos. The present study investigated ultrastructural differences between in vivo-produced and cloned goat embryos, including intra- and interspecies embryos. Goat ear fibroblast cells were used as donors, while the enucleated bovine and goat oocytes matured in vitro as recipients. Goat-goat (GG), goat-cattle (GC) and goat in vivo-produced embryos at the 2-cell, 4-cell, 8-cell and 16-cell stages were compared using transmission electron microscopy. These results showed that the three types of embryos had a similar tendency for mitochondrial change. Nevertheless, changes in GG embryos were more similar to changes in in vivo-produced embryos than were GC embryos, which had more extreme mitochondrial deviation. The results indicate the effects of the cytoplast on mitochondria development. The zona pellucida (ZP) in all three types of embryos became thinner and ZP pores in both GC and GG embryos showed an increased rate of development, especially for GC embryos, while in vivo-produced embryos had smooth ZP. The Golgi apparatus (Gi) and rough endoplasmic reticulum (RER) of the two reconstructed embryos became apparent at the 8-cell stage, as was found for in vivo embryos. The results showed that the excretion of reconstructed embryos was activated on time. Lipid droplets (LD) of GC and GG embryos became bigger, and congregated. In in vivo-produced embryos LD changed little in volume and dispersed gradually from the 4-cell period. The nucleolus of GC and GG embryos changed from electron dense to a fibrillo-granular meshwork at the 16-cell stage, showing that nucleus function in the reconstructed embryos was activated. The broken nuclear envelope and multiple nucleoli in one blastomere illuminated that the nucleus function of reconstructed embryos was partly changed. In addition, at a later stage in GC embryos the nuclear envelope displayed infoldings and the chromatin was concentrated, implying that the blastomeres had an obvious trend towards apoptosis. The gap junctions of the three types of embryos changed differently and GG and GC embryos had bigger perivitelline and intercellular spaces than did in vivo-produced embryos. These results are indicative of normal intercellular communication at an early stage, but this became weaker in later stages in reconstructed embryos. In conclusion, inter- and intraspecies reconstructed embryos have a similar pattern of developmental change to that of in vivo-produced embryos for ZP, rough ER, Gi and nucleolus, but differ for mitochondria, LD, vesicles, nucleus and gap junction development. In particular, the interspecies cloned embryos showed more severe destruction. These ultrastructural deviations might contribute to the compromised developmental potential of reconstructed embryos.

PART C UPDATE

****References to Part A Update****


Bruyas JF, Hebia-Fellah I.  Risk of pathogen transmission through breeding in horses.  Reproduction in Domestic Animals. 2009; 44(3);71-72 (abstr.).


