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Uterine tubal cells remain uninfected after culture with in vitro-produced embryos exposed to bovine viral diarrhea virus

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Abstract

Bovine viral diarrhea virus (BVDV) has been isolated from washed and sonicated, in vitro-produced embryos, but the infectivity of BVDV associated with intact, developing, embryos has not been demonstrated. The objective of this study was to determine if a dose of BVDV infective for co-culture cells was associated with individual, developing embryos, following artificial exposure to the virus and washing. In 5 replicates, zona pellucida-intact, in vitro-produced embryos were assigned to a negative control embryo group, or were incubated in 10^5–10^6 cell culture infective doses (50%, $CCID_{50}$) per milliliter of a type I, noncytopathic (strain SD-1) BVDV for 2 h. Unexposed negative control embryos and exposed positive control embryos were washed, sonicated and assayed for BVDV using virus isolation with immunoperoxidase monolayer assay. Immediately or following cryopreservation, remaining virally-exposed, washed embryos were co-cultured individually with BVDV-negative cultures of bovine uterine tubal cells in a medium free of BVDV-neutralizing activity. After two days in culture, uterine tubal cells and embryos (including the zona pellucida) were separated and washed. The culture medium, uterine tubal cells and embryos were then assayed for BVDV. Bovine viral diarrhea virus was not isolated from any negative control embryo group, but was isolated from all positive control embryo groups. Although all uterine tubal cell populations were confirmed to be susceptible to BVDV, virus was never isolated from uterine tubal cells or embryos from post-exposure culture. In conclusion, although BVDV remains associated with washed in vitro-produced embryos, the virus associated with unsonicated embryos was not infective for uterine tubal cells in vitro.

Keywords: IVF; IVC; Embryo culture; Bovine viral diarrhea virus; Cattle-viruses

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1. Introduction

The epidemiological risks involved with transfer of in vitro-produced embryos are not fully understood (Zurovac et al., 1994; Stringfellow et al., 1997; Trachte et al., 1997; Bielanski et al., 1998; Vanroose et al., 1998). A pathogen, such as bovine viral diarrhea virus (BVDV), that may not visibly affect embryo development or somatic cell viability, provides the greatest threat of distribution via in vitro-produced embryos (Avery et al., 1993; Zurovac et al., 1994; Bielanski et al., 1998). Bovine viral diarrhea virus is a significant bovine pathogen with worldwide distribution, and proven association of BVDV with gametes, bovine serum, and somatic cells provides the potential for contamination of an in vitro embryo production system (Rossi et al., 1980; Brock et al., 1991; Booth et al., 1992; Guerin et al., 1992; Avery et al., 1993; Baker, 1995). Two pathogenic biotypes of BVDV, cytopathic and noncytopathic, have been described based on the presence or absence of visible cytopathic effect in vitro when susceptible cell monolayers are infected (Perdrizet, 1990). Theoretically, transmission of undetected noncytopathic BVDV in association with bovine in vitro-produced embryos might result in infection of embryo recipients, early embryonic death, abortion, or birth of persistently infected offspring.

Previous reports by Booth et al. (1994), Bielanski and Jordan (1996), Stringfellow et al. (1997), Bielanski et al. (1998) and Trachte et al. (1998) document that standard washing procedures with, or without, trypsin treatment are not fully effective in rendering in vitro-produced embryos free of noncytopathic BVDV. Embryo-associated virus in these studies was detected by viral isolation (Booth et al., 1994; Stringfellow et al., 1997; Trachte et al., 1998) or animal inoculation (Bielanski and Jordan, 1996; Bielanski et al., 1998) after sonication of the virally-exposed, washed embryos. No previous study evaluated the infectivity of BVDV associated with intact, in vitro-produced embryos. The objective of this study was to determine if noncytopathic BVDV associated with individual, intact embryos following artificial exposure to the virus and washing is infective for susceptible uterine tubal cells in a co-culture system.

2. Materials and methods

2.1. Media for in vitro embryo production

Oocyte collection medium consisted of HEPES-buffered Ham's F10 Nutrient Mixture (GIBCO-BRL, Grand Island, NY), supplemented with 2% estrus cow serum (Cocalico, Reamstown, PA), penicillin (100 U/ml), streptomycin (100 μg/ml) and heparin (10 IU/ml). The medium for oocyte maturation (IVM medium) consisted of HEPES-buffered (25 mM) tissue culture medium 199 (TCM 199) with Earle's salts (GIBCO-BRL) supplemented with 10% (v/v) estrous cow serum, L-glutamine (0.5 mM), sodium pyruvate (1 mM), penicillin (50 U/ml), streptomycin (50 μg/ml), follicle stimulating hormone (4 μg/ml, Sioux Biochemical, Sioux Center, IA) and luteinizing hormone (6 μg/ml).

Two modifications of Tyrode's balanced salt solution (Tyrode's salts, albumin, lactate and pyruvate; TALP) were used for preparation and co-culture of gametes (Stringfellow

et al., 1997). For washing oocytes and uterine tubal cells prior to in vitro fertilization, HEPES-TALP contained modified Tyrode's salts (Tyrode, 1910), bovine serum albumin (Fraction V, 3 mg/ml), sodium pyruvate (22 μg/ml) and gentamicin (50 μg/ml). In vitro fertilization drops contained modified Tyrode's salts with bovine serum albumin (6 mg/ml), sodium pyruvate (22 μg/ml), gentamicin (50 μg/ml) and heparin (20 μg/ml) (IVF-TALP).

The culture of uterine tubal cells and presumptive zygotes prior to viral exposure was carried out in TCM 199 with Earle's salts supplemented with 10% (v/v) fetal bovine serum (HyClone Lab., Inc., Logan, UT), 0.35% (v/v) bovine serum albumin, sodium pyruvate (1 mM), L-glutamine (0.5 mM), penicillin G (50 U/ml) and streptomycin (50 μg/ml) (IVC medium). Following viral exposure, the individual culture of embryos with uterine tubal cells was carried out in TCM 199 with Earle's salts supplemented with 10% (v/v) equine serum, sodium pyruvate (1 mM), L-glutamine (0.5 mM), penicillin G (50 U/ml) and streptomycin (50 μg/ml) (IVC-eq medium).

2.2. Uterine tubal epithelial cells

Uterine tubes ipsilateral to ovaries with corpora hemorrhagica or early corpora lutea were harvested at a slaughterhouse, washed with warm (28°C), 0.85% saline supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml), and transported to the lab within 2 h in warm (28°C) Dulbecco's phosphate buffered saline supplemented with 2% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). Upon arrival at the lab and after removal of excess tissue, the uterine tubes were washed twice in maturation medium. Epithelial cells lining the uterine tubes were gently stripped free, and the sheets of cells were fragmented by sequential aspiration through a 23- and 25-gauge needle. Primary cultures of uterine tubal cells were incubated in IVC medium at 38.5°C in an atmosphere of 5% CO_2 and humidified air. During embryo production, uterine tubal cells were transferred after 24 h to fertilization drops, and after 48 h into in vitro culture drops. Uterine tubal cells were cultured for 48 h and washed in IVC-eq medium before use in uterine tubal cell susceptibility testing or co-culture with embryos previously exposed to virus.

2.3. Oocyte collection and in vitro maturation

Ovaries collected at a slaughterhouse were washed and transported to the lab as described above for uterine tubes. Upon arrival at the laboratory, the ovaries were rinsed and placed in a beaker of warm 0.85% saline until oocyte collection began. Cumulus–oocyte-complexes were collected by superficially slicing the ovarian cortex with a razor blade and rinsing the cut surfaces by agitating the ovary in a beaker containing 200 ml of oocyte-collection medium. After rinsing 20–40 sliced ovaries in the medium, the debris and oocytes were allowed to settle and the supernatant was carefully decanted and discarded. The remaining sediment was poured into a 120 mm petri dish and washed three times with fresh oocyte collection medium. Cumulus–oocyte-complexes were recovered with the aid of a stereomicroscope and washed three times in maturation medium prior to in vitro maturation. Up to 35 oocytes were matured in 50 μl droplets of

maturation medium that were covered with silicone oil. Maturation plates were cultured in an atmosphere of 5% CO_2 and air at 38.5°C for 20–24 h.

2.4. In vitro fertilization

Following removal of the cumulus–oocyte-complexes from the maturation plates, expanded cumulus cells were removed by washing and vigorously vortexing in three 35 mm petri dishes and 3 wells of a 4-well plate containing Hepes-TALP. Immediately before placement into in vitro fertilization drops, the nearly nude oocytes were washed once in IVF-TALP. Up to 20 oocytes were transferred to each 90 μl fertilization drop overlaid with silicone oil. Fertilization drops contained primary cultures of uterine tubal cells.

One straw of cryopreserved semen from a single collection of a bull was used for fertilization. This semen was previously characterized in the in vitro embryo production system and was confirmed to be free of BVDV by viral isolation and immunoperoxidase assay. Semen was thawed in a water bath at 37°C for 30–60 s. Percoll gradients were prepared by layering (sequentially starting from the bottom of a centrifuge tube) 2 ml of 90% percoll and 2 ml of 45% percoll. The thawed semen was placed on top of the percoll gradient. Following 30 min of centrifugation at 700*g*, the layers of percoll were carefully removed. The pellet of spermatozoa was resuspended in 1 ml of IVF-TALP and centrifuged for 5 min at 250*g*. After gently removing the supernatant, the sperm concentration was determined. Approximately 10^5 sperm were added to each fertilization drop. Fertilization plates were incubated for 18 h at 38.5°C in a humidified atmosphere of 5% CO_2 and air.

2.5. Initial in vitro culture

After removal from the fertilization drops, presumptive zygotes were washed and vortexed in IVC medium to remove the excess sperm in the manner described above for cumulus cell removal. Up to 35 presumptive zygotes were placed in each in vitro culture drop (90 μl of IVC medium containing uterine tubal cells). In vitro culture plates were incubated at 38.5°C in an atmosphere of 5% CO_2 and air for 7 days. After approximately 48 h of culture, each microdrop was examined for cleaved embryos and 50 μl of fresh IVC medium was added. On day 6 of in vitro culture, embryos were again examined for development, and fresh IVC medium (25 μl) was added. On day 7 of culture, developed embryos were selected for inclusion in this research project.

2.6. Bovine viral diarrhea virus exposure

Stock virus of a genotype I, noncytopathic (strain SD-1) BVDV, initially isolated from the serum of a persistently infected cow (Deng and Brock, 1992), was propagated in BVDV-free Madin Darby bovine kidney (MDBK) cells that had been cultured in minimum essential medium with Earle's salts supplemented with 10% equine serum, 0.75 mg/ml sodium bicarbonate, 0.29 mg/ml L-glutamine and antibiotics (100 U/ml

penicillin G, 100 μg/ml streptomycin and 0.25 μg/ml amphoteracin B) (supplemented MEM). Virus was harvested by freezing and thawing and stored at −80°C until needed. The cell culture infective dose (50%, $CCID_{50}$) of viral exposures was determined by the method of Reed and Muench (1938). Embryos were incubated with virus for 2 h at 38.5°C in a humidified atmosphere of 5% CO_2 and air.

2.7. Embryo washing

Embryo washing medium consisted of supplemented MEM. All embryos were washed after removal from the initial in vitro culture drop on day 7 (wash protocol A: 10 washes, 3 ml/wash). All embryos exposed to virus were washed again immediately after exposure (wash protocol B: 12 washes, 1 ml/wash). Embryos cultured after viral exposure were individually washed for the third time after removal from the secondary in vitro culture drop (wash protocol C: 12 washes, 1 ml/wash). Wash protocols A and B were consistent with International Embryo Transfer Society guidelines (Stringfellow, 1998). In these two protocols, only zona pellucida-intact embryos were washed in groups of 10 or fewer. Separate sterile micropipettes were used to move embryos between washes. The ratio of volume of medium containing embryos in the pipette to volume of medium in each wash was a minimum of 1 : 100. While wash protocol C did involve embryos that did not have intact zona pellucida due to hatching in the secondary in vitro culture drop, other conditions were consistent with International Embryo Transfer Society guidelines. All attached or unattached zona pellucida were washed with the corresponding embryos.

2.8. Embryo cryopreservation

After viral exposure and washing on day 7 of in vitro culture, embryos not assigned to be positive controls, or to be cultured immediately, were cryopreserved, as previously described (Seidel, 1990). Briefly, embryos in groups of 8 or less were subsequently placed in Emcare embryo holding solution (Immuno-chemical Products Ltd., Auckland 1, New Zealand) for 10 min, 5% glycerol (Emcare 10% glycerol mixed 1 : 1 with Emcare embryo holding solution) for 5 min, and 10% glycerol for 10 min at room temperature. Embryos were individually loaded in 0.25 ml straws during the final incubation in 10% glycerol. The straws were loaded into the Bio-Cool BC-70-4 cryopreservation unit (FTS Systems, Inc., Stone Ridge, New York) holding at 0°C. The cryopreservation unit cooled the straws from 0 to −6°C at a rate of −2°C per minute, held at −6°C for 15 min, and then cooled at a rate of −0.6°C per minute to −30°C. Straws were manually seeded after 5 min at −6°C and were plunged into liquid nitrogen upon reaching −30°C.

After storage for 14 days or less, the embryos were thawed at room temperature (25°C) for 6 s and then placed in a 37°C water bath for 15–20 s. Using Emcare thawing solutions at approximately 25°C, individual embryos were subsequently placed in 6% glycerol for 6 min, 3% glycerol for 6 min and 10% sucrose for 6 min. Each embryo was then washed three times in Emcare holding media and incubated at 38.5°C in IVC-eq medium for 10 min prior to transfer to the secondary in vitro culture drop.

2.9. Embryo culture after viral exposure

Immediately after post-exposure washing or after cryopreservation, individual embryos were added to 100 μl drops containing BVDV-negative uterine tubal cells in IVC-eq medium. The medium was free of BVDV neutralizing activity, as determined by the microtitration virus neutralization test. Individual embryos were cultured for 48 h at 38.5°C in an atmosphere of 5% CO_2 and humidified air. After incubation, embryo development was evaluated and embryos, uterine tubal cells and culture medium were prepared for viral assay.

2.10. Uterine tubal cell susceptibility testing

To determine the susceptibility of uterine tubal cells to BVDV, 50 μl of an undiluted stock virus (with a concentration of 3×10^6, 8×10^5, or 7×10^5 $CCID_{50}$/ml) and 50 μl of 5, 10-fold dilutions of stock virus in IVC-eq medium were added to sham IVC drops containing uterine tubal cells, but no embryo in IVC-eq medium. In three replicates involving 2 sham IVC drops inoculated with each concentration of virus, uterine tubal cells were incubated for 48 h after inoculation, harvested, and assayed in a manner equivalent to secondary in vitro culture drops for virally-exposed embryos. In contrast to other samples, the uterine tubal cell samples from this susceptibility study were not passaged before being declared negative. Within each replicate, the 50% endpoint for infection of uterine tubal cells was calculated by the method of Reed and Muench (1938).

To evaluate the susceptibility of primary cultures of uterine tubal cells during each weekly embryo replicate, uterine tubal cells in 90 μl microdrops of IVC-eq medium under silicone oil were inoculated in duplicate with 4×10^4 to 3×10^5 $CCID_{50}$ of BVDV. The contents of uterine tubal cell susceptibility drops were incubated for 48 h after inoculation, harvested, and assayed in a manner equivalent to secondary in vitro culture drops for virally-exposed embryos.

2.11. Sample preparation

A summary of samples assayed for BVDV is presented in Table 1. Pre- and post-exposure aliquots of stock virus were frozen (−80°C) and later titrated and assayed to determine viral presence and concentration. Madin Darby bovine kidney cells were added to the 1 ml aliquots of supplemented MEM used as the 10th, 11th, and 12th wash for groups of embryos (8 or fewer) exposed to BVDV. Washes were cultured for 5 days, frozen (−80°C) and later assayed for BVDV.

Washed negative control embryo groups were sonicated in groups of 15 or fewer. Positive control embryo groups were washed as described and sonicated in groups of 8 or fewer. Individual embryos placed into secondary culture immediately after post-exposure washing or following cryopreservation were washed and sonicated individually. All embryos were sonicated in supplemented MEM. Embryo sonication consisted of 2 bursts of 60 s duration utilizing the micro-tip of a Model 300 Artek Sonic Dismembrator (Fisher Scientific Co., Pittsburg, PA). Embryo sonicate fluids (1 ml) were inoculated onto MDBK cells in a single well of a 6-well plate (9.4 cm^2 monolayer) and incubated for 1 h before

Table 1
Samples assayed for the presence of noncytopathic bovine viral diarrhea virus and the sequence in which they were assayed

Assayed after 7 days of in vitro culture
Washed unexposed negative control embryo groups
Assayed before viral exposure of embryos
Stock virus
Assayed after viral exposure of embryos
Stock virus (embryos removed)
Embryo washes :
10th, 11th and 12th washes of positive control embryos
10th, 11th and 12th washes of embryos immediately replaced in culture
10th, 11th and 12th washes of embryos replaced in culture after cryopreservation
Washed positive control embryo groups
Assayed after 48 h of secondary in vitro culture
Uterine tubal cells from culture drops (including uterine tubal cell susceptibility samples)
Medium from culture drops (including uterine tubal cell susceptibility samples)
Washed individual embryos replaced immediately in culture
Washed individual embryos replaced in culture after cryopreservation

3 ml of supplemented MEM was added. Sonicate fluids were incubated for 5 days at 38.5°C in a humidified atmosphere of 5% CO_2 and air. After incubation, a single freeze–thaw method was used to release virus from the monolayer of cells. Three serial passages of embryo sonicate fluids were performed in this manner to provide ample opportunity for viral replication prior to determining that a sample was negative for BVDV.

After 48 h of culture in the post-exposure embryo culture drop or uterine tubal cell susceptibility drop, uterine tubal cells were separated from associated media by centrifugation at 14 000*g* for 10 s. The supernatant of IVC medium was pipetted off the uterine tubal cell pellet and stored at −80°C until being assayed. The uterine tubal cells were resuspended in 1 ml of supplemented MEM, mixed by vortical agitation and re-centrifuged to pellet the uterine tubal cells a second time. After discarding the supernatant, the pellet was resuspended in 0.5 ml of supplemented MEM prior to storage at −80°C until assay. Uterine tubal cell samples were passaged by inoculating MDBK cells in a single well of a 24-well plate (2.0 cm^2 monolayer) with 200 μl of sample and incubating for 1 h before 1 ml of supplemented MEM was added. Samples were incubated for 5 days at 38.5°C in a humidified atmosphere of 5% CO_2 and air. After incubation, a single freeze–thaw method was used to release virus from the monolayer of cells. Three serial passages of uterine tubal cell samples were performed in this manner prior to determining that a sample was negative for BVDV.

2.12. Bovine viral diarrhea virus assay

All samples were assayed for BVDV using virus isolation with virus detected by an immunoperoxidase monolayer assay developed by Afshar et al. (1991). Samples other than medium from in vitro culture drops were assayed by adding 100 μl to each of 3 wells

of a 96-well culture plate, followed by addition of MDBK cells. Due to the small volume of IVC medium following removal of the cultured embryo and uterine tubal cells, only 15 μl of this sample was added to 3 replicate wells. 50 μl of supplemented MEM were then added to samples of IVC medium prior to addition of MDBK cells. The plates were incubated for 72 h at 38.5°C in a humidified atmosphere of 5% CO_2 and air before the labeling technique was performed as follows: after fixation, potentially infected cells were incubated with anti-BVDV monoclonal antibodies D89 (Vickers and Minocha, 1990; Xue et al., 1990) specific for gp53/E2, a major envelope glycoprotein of BVDV (Xue et al., 1997) and 20.10.6 specific for p80, a conserved non-structural protein (Corapi et al., 1990). After washing with PBS and Tween 20 (Sigma Chemicals, Saint Louis, MO) to remove unbound antibodies, peroxidase-conjugated rabbit anti-mouse IgG (Jackson Immuno Research Lab, West Grove, PA) was added. After a short incubation period, unbound conjugated antibody was removed by washing with PBS and Tween 20. Finally, the enzyme substrate, aminoethyl carbazole (Zymed Laboratories, Inc., South San Francisco, CA), which produces a reddish-brown color when oxidized by horseradish peroxidase, was added. Color change was visualized using a light microscope and compared to known positive and negative controls on each plate.

2.13. Summary of experimental design

A summary of the experimental design is presented in Fig. 1. Five weekly replicates of the experiment were conducted. For each replicate, between 168 and 322 cumulus–oocyte

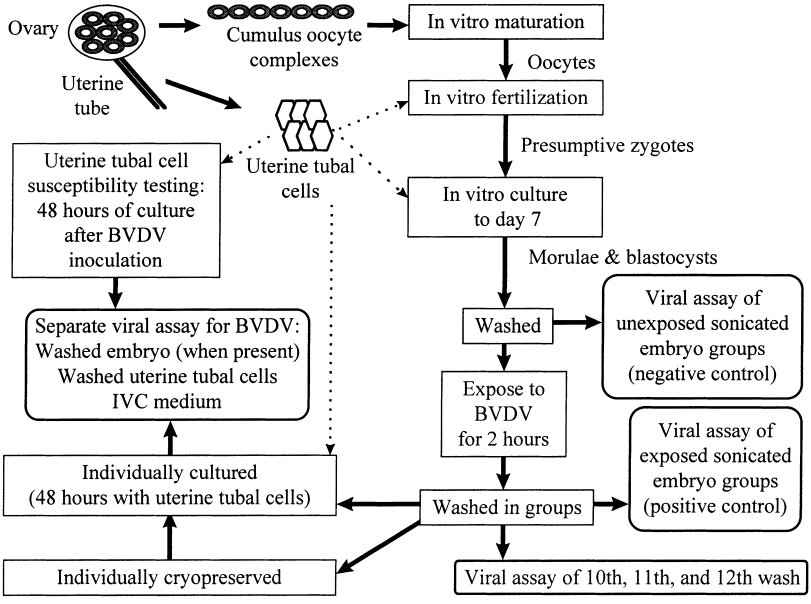

Fig. 1. Experimental design of research. Assays for bovine viral diarrhea virus (BVDV) are indicated in boxes with bold borders.

complexes were recovered, matured, fertilized, and cultured in vitro to day 7. During each replicate, between 22 and 60 morulae or blastocysts were identified and included in the research.

Embryos were assigned to each of the following categories: negative control embryo group, positive control embryo group, individual embryos to be cultured immediately, or individual embryos to be cultured after cryopreservation. Washed negative control embryos were assayed for virus to detect BVDV inadvertently introduced through another source. All embryos other than the negative controls were exposed to virus and washed. Viral concentration of exposure medium was evaluated in a pre-exposure and post-exposure stock virus aliquot. Virus isolation was attempted on the 10th, 11th, and 12th washes of each group of virally-exposed embryos. Positive control embryos were assayed for BVDV to evaluate the initial association of virus with in vitro-produced embryos. Assigned embryos were returned to culture with uterine tubal cells immediately or after cryopreservation. After co-culture of virally-exposed embryos with uterine tubal cells, washed embryos, uterine tubal cells and in vitro culture medium were assayed separately for BVDV. During each replicate, uterine tubal cell susceptibility to infection with noncytopathic BVDV was evaluated in duplicate microdrops containing uterine tubal cells inoculated with virus.

2.14. Statistical analysis

The difference between pre- and post-exposure viral concentration was analyzed using a paired *T*-test (Zar, 1984a) generated by SAS software. Embryo development of embryos returned to culture immediately, and embryos cryopreserved were compared using a Mantel Haenszel χ^2 test (Zar, 1984b) performed with EpiInfo software (Snedecor and Cochran, 1976). Because there was only a single positive result, statistical analysis of virus isolation from embryos, uterine tubal cells, and IVC medium was considered uninformative.

3. Results

During the 5 weekly replicates, 67.4% (611/906) of the presumptive zygotes cleaved and 26.8% (243/906) developed to produce 191 morulae and unhatched blastocysts, which were used for this research. Bovine viral diarrhea virus was not isolated from any negative control embryo group despite 3 serial passages. During embryo exposure to virus, the concentration of virus decreased slightly ($p = 0.09$, paired *T*-test), as indicated by pre- ($0.5–5 \times 10^6$ $CCID_{50}$/ml) and post-exposure viral samples ($5–8 \times 10^5$ $CCID_{50}$/ml). The lowest viral concentration to which an embryo may have been exposed was 5×10^5 $CCID_{50}$/ml.

Virus was isolated from 1 or more of the last 3 washes of 71% (15/21) of virally-exposed embryo groups. Virus was isolated in the last (12th) wash of 52% (11/21) of the embryo groups. Bovine viral diarrhea virus was isolated from all groups of washed, sonicated positive control embryos, despite lack of viral isolation from the last 3 washes for 43% (3/7) of positive control groups.

Table 2
Embryo development after 48 h in the secondary in vitro culture (day 9)

	Embryos in culture immediately[a] (number of embryos/total (%))	Embryos in culture after cryopreservation (number of embryos/total (%))
Degenerated	10/43 (23)	29/50 (58)
Developed, hatched	25/43 (58)	13/50 (26)
Developed, not hatched	8/43 (19)	8/50 (16)

[a] Developmental endpoints for embryos in culture immediately were significantly different from developmental endpoints for embryos in culture after cryopreservation ($p < 0.05$, Mantel Haenszel χ^2 test).

Table 3
Isolation of bovine viral diarrhea virus from IVC medium, uterine tubal cells, and individual embryos after 48 h of co-culture

	IVC medium (Pos/total (%))[a]	Uterine tubal cells (Pos/total (%))[a]	Embryos (Pos/total (%))[a]
Embryos in IVC immediately	1/43 (2)	0/43 (0)	0/43 (0)
Embryos in IVC after cryopreservation	0/50 (0)	0/50 (0)	0/50 (0)

[a] Pos, number of samples from which virus was isolated; total, total number of samples assayed for virus; %, percentage of samples from which virus was isolated.

After 48 h in the secondary in vitro culture drop, significantly fewer cryopreserved embryos hatched compared with embryos that were transferred immediately to culture after viral exposure and washing (Table 2).

After 48 h of post-exposure culture and washing, no virus was detected in association with embryos (0/43 returned to culture immediately and 0/50 returned to culture after cryopreservation) (Table 3). No virus was isolated from uterine tubal cell samples after culture with virally-exposed embryos (0/43 from drops with embryos cultured immediately, and 0/50 from drops with embryos cultured after cryopreservation). While no virus was isolated from in vitro culture medium associated with cryopreserved embryos (0/50), virus was isolated from 1 sample of in vitro culture medium surrounding a developed, but unhatched, blastocyst, which was returned to culture immediately.

The 50% endpoints for infection of uterine tubal cells during the 3 replicates of uterine tubal cell susceptibility testing were addition of 150, 40, and 1 $CCID_{50}$ in 50 μl to the sham IVC drops. In the respective replicates, all inoculations with 1500, 400, and 3.5 or greater $CCID_{50}$ produced infections of the uterine tubal cells. In results of uterine tubal cell susceptibility testing of primary cultures used during this embryo research, virus was isolated without passage from all in vitro culture medium (10/10) and uterine tubal cell samples (10/10) after inoculation and culture for 48 h.

4. Discussion

Virus has been isolated previously from 23/62 (37%) (Stringfellow et al., 1997), and 6/17 (35%) (Bielanski and Jordan, 1996) individually tested, washed in vitro-produced

embryos after artificial exposure to noncytopathic BVDV. In these studies, embryos were sonicated to release infectious virus prior to detection of virus by immunoperoxidase monolayer assay. In the present study, virus isolation from all washed positive control embryo groups, sonicated immediately after post-exposure washing, confirms that washing in vitro-produced embryos according to International Embryo Transfer Society standards is ineffective in rendering them free of BVDV. However, no reports exist of the ability of BVDV associated with virally-exposed, intact in vitro-produced embryos to infect cells in co-culture.

In previous studies, the quantity of noncytopathic BVDV isolated from individual, in vitro-produced embryos after washing and sonication was 50 $CCID_{50}$ (Bielanski and Jordan, 1996) and 16–50 $CCID_{50}$ (Bielanski et al., 1998). Also, three of five heifers in the first study (Bielanski and Jordan, 1996), and six of fifteen heifers in the other study (Bielanski et al., 1998) seroconverted after intravenous inoculation with single BVDV-exposed, washed and sonicated, in vitro-produced embryos. It was clear from these studies that sonicate fluids of single in vitro-produced embryos could contain an infective dose for cattle, when administered intravenously. However, further study is needed to determine if BVDV associated with a washed, intact, in vitro-produced embryo might constitute an infective dose for a recipient when transferred into the uterus.

In this study, after viral exposure and washing, 43 individual embryos were immediately placed into culture with uterine tubal cells to simulate the fresh transfer of embryos to recipients. An additional 50 embryos were exposed to virus, washed and cryopreserved prior to returning them individually into culture with uterine tubal cells to simulate transfer of embryos to recipients after freezing and thawing. The intent of this research was to gain a preliminary indication of whether BVDV associated with intact (not sonicated), developing embryos might constitute an infective dose for recipients.

The concentration of virus to which embryos were exposed in this study (10^5–10^6 $CCID_{50}$ per ml) is within the range of BVDV concentration detected in the follicular fluid of acutely infected cattle (10^3–10^7 $CCID_{50}$ per ml) (Bielanski and Dubuc, 1995), while slightly lower than that which was detected in the follicular fluid of persistently infected cattle ($10^{6.7}$–$10^{8.2}$ $CCID_{50}$ per ml) (Booth et al., 1998). The concentration of contaminating BVDV that might be present in culture fluids of in vitro embryo production systems will be influenced by presence or absence of anti-BVDV antibody. The viral exposure dose used in this study simulated what might be seen in media without neutralizing antibodies.

Since a minimum dilution of 1 : 100 was maintained between each wash in the post-exposure wash protocol, virus not associated with embryos (free virus) should have been diluted out by the third or fourth wash. The likely reason for isolation of virus from 1 or more of the last 3 washes of 15/21 (71%) groups of virally-exposed embryos and from the final wash of 11/21 (52%) of these groups is that a small amount of virus was tenuously adhered to the zona pellucida and was detached by continued washing. These results, including lack of isolation of virus from any of the final three washes of 3/7 positive control groups, indicate that this continued detachment of virus is not consistent among embryo groups.

While BVDV was isolated from a single sample of IVC medium, it was not isolated from any uterine tubal cells or embryos after the post-exposure culture period. Failure to

isolate BVDV from uterine tubal cells with which embryos were co-cultured may have been the result of insufficient quantity of embryo-associated virus to infect uterine tubal cells, production of antiviral agents by the developing embryo, neutralization of virus due to adherence to the zona pellucida, or a combination of these factors.

Uterine tubal cells in vivo may be infected in cattle with acute BVDV infections (Bielanski et al., 1998) or in cattle with persistent BVDV infections as demonstrated by an immunofluorescent antibody technique and in situ hybridization (Booth et al., 1992). Further, the susceptibility of uterine tubal cells to infection with BVDV in vitro has been demonstrated (Booth et al., 1992; Zurovac et al., 1994; Stringfellow et al., 1997). In this study, uterine tubal cell susceptibility cultures were always infected with BVDV, confirming that the primary cultures established each week were susceptible. Although the uterine tubal cells are less susceptible to infection than the MDBK cells used to establish a cell culture infective dose, the cells were thought to allow a reasonable simulation of what might occur after intrauterine transfer of an intact embryo.

The lack of infection of uterine tubal cells might have been partially due to bovine interferon τ (bovine trophoblast interferon), which is initially produced at days 8– 9 post fertilization by the trophectoderm of in vitro-derived bovine embryos (Hernandez-Ledezma et al., 1989). The significant anti-viral activity of bovine interferon τ in culture (Ashworth and Bazer, 1989; Cross and Roberts, 1991; Roberts et al., 1991) may have induced resistance of the uterine tubal cells to infection by the virus.

Failure to isolate BVDV from unhatched embryos indicates that viral penetration of the zona pellucida resulting in infection of embryonic cells did not occur. This finding is consistent with results of Vanroose et al. (Vanroose et al., 1998), which indicate in vitro-produced embryos enclosed in a zona pellucida are protected from infection by noncytopathic BVDV until hatching. Also, since the in vitro-derived zona pellucida is considered to be stickier than the in vivo-derived zona pellucida (Stringfellow et al., 1997), the zona may have provided a substrate to which the virus adhered. This adherence might have resulted in viral neutralization during a 48 h incubation at 38.5°C.

In this study, virus was not isolated from in vitro-produced embryos after artificial exposure to a noncytopathic BVDV, washing according to International Embryo Transfer Society standards and additional culture for 48 h. Further, virus was not isolated from uterine tubal cells in co-culture with the developing embryos. Thus, results in this in vitro simulation indicate that an infective dose of noncytopathic BVDV might not be transmitted in association with individual, virally-exposed in vitro-produced embryos that are transferred to recipients. However, additional investigation is required to determine exactly why uterine tubal cells remained uninfected. Also, presence or absence of an infective dose for recipients will eventually have to be determined by transfer of virally-exposed in vitro-produced embryos to susceptible recipients.

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