IN VITRO FERTILIZATION AND IN VITRO CULTURE OF BOVINE EMBRYOS IN THE PRESENCE OF NONCYTOPATHIC BOVINE VIRAL DIARRHEA VIRUS

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ABSTRACT

In vitro embryo production has been used extensively in research and is now offered as a commercial service, yet the hazards of introducing specific infectious agents into in vitro embryo production systems have not been completely defined. The introduction of noncytopathic bovine viral diarrhea virus (BVDV) is a special concern. One objective of this study was to determine if noncytopathic BVDV-infected uterine tubal cells in IVF and IVC systems affected the rate of cleavage and development. An additional objective was to determine if either degenerated ova or embryos produced in the presence of the infected cells had virus associated with them after washing. Follicular oocytes (n=645) collected from slaughterhouse ovaries were matured and fertilized in vitro, and presumptive zygotes were cultured for 7 d. Primary cultures of uterine tubal cells for use during IVF and IVC were divided into 2 groups. One-half of the cultures was infected with noncytopathic BVDV while the other half was not exposed to the virus. Approximately equal groups of mature oocytes were inseminated, and the presumptive zygotes were cultured with infected or noninfected uterine tubal cells. After 7 d in IVC, zona pellucida-intact (ZP-I) morulae and blastocysts and degenerated ova were washed and assayed for the presence of infectious virus. Infections of uterine tubal cells were not apparent and did not reduce rates of cleavage and development (P > 0.05; Chi-square test for heterogeneity). After washing, BVDV was isolated at a significantly higher rate from groups of virus-exposed degenerated ova (79%) than from individual virus-exposed morulae and blastocysts (37%; P = 0.0002; Mantel-Haenszel summary, Chi-square).

Key words: IVF, IVC, bovine viral diarrhea virus

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Correspondence and reprint requests.
INTRODUCTION

Components of in vitro bovine embryo production systems have been used in research for more than a decade. The technology for commercial calf production is now available. Current applications include calf production from genetically superior, infertile cows (15); calf production from terminally ill, injured or prematurely culled cows (26,31); and superior beef calf production through fertilization of oocytes from slaughterhouse ovaries (14).

Sera and essential cells and tissues used for in vitro production of bovine embryos are potential sources of infectious agents. Noncytopathic (NC) bovine viral diarrhea virus (BVDV) is one pathogen of concern, since it has been associated with key components of IVF systems, including fetal bovine serum (19), serum of adult cattle (7), cumulus cells, uterine tubal cells, semen (3,5,12) and cell lines (19).

In a preliminary study, (34) no virus was isolated from IVF/IVC-produced embryos that had been co-cultured with BVDV-infected uterine tubal cells. However, in that study, the anti-BVDV antibody was present in the IVC medium, and while virus was not isolated from the small number of morulae and blastocysts that were produced, a portion of ZP-I nonfertile and degenerated ova were virus positive. The present study was designed to test a worst-case scenario in which virus was present and there was no anti-BVDV antibody in the IVC medium.

MATERIALS AND METHODS

Media for IVM, IVF, and IVC

Oocytes were matured in tissue culture medium 199 (TCM199) with Earle's salts (GIBCO-BRL, Grand Island, NY; Cat. No. 12340-014) supplemented with 10% (v/v) fetal bovine serum (FBS; HyClone Lab., Inc., Logan, UT), 4 μg/ml FSH and 6 μg/ml LH (NOBL Lab., Inc., Sioux Center, IA).

Three modifications of Tyrode's balanced salt solution (29) were used for preparation and co-culture of gametes. These media, commonly referred to as TALP (16; Tyrode's salts, albumin, lactate and pyruvate) were custom-prepared in our laboratory as previously described (11). Briefly, Hepes-TALP contained modified Tyrode's salts (29), BSA (Fraction V, 3 mg/ml), sodium lactate (21.6 mM), sodium pyruvate (1.0 mM) and gentamicin (50 μg/ml). Sperm-TALP contained modified Tyrode's salts (29), BSA (Fraction V, 6 mg/ml), sodium lactate (21.6 mM), sodium pyruvate (1.0 mM) and gentamicin (50 μg/ml). The IVF-TALP contained modified Tyrode's salts (29) with BSA (Fraction V, 6 mg/ml), sodium lactate (10.0 mM), sodium pyruvate (1.0 mM), gentamicin (50 μg/ml) and heparin (20 μg/ml).

The culture of uterine tubal cells and IVC of zygotes were carried out in TCM 199 with Earle's salts supplemented with 10% (v/v) estrous cow serum (ECS), sodium pyruvate (2.5 mM), penicillin G (50 U/ml) and streptomycin (50 μg/ml).
Estrous Cow Serum

Estrous cow serum used in IVC medium was produced from a cow housed at the Ohio Agricultural Research and Development Center in Wooster, Ohio. Blood was collected from the cow when she was in standing estrus and was allowed to clot. Then, serum was separated by centrifugation, prefiltered through a 0.8-μm membrane, filtered through a 0.22-μm membrane and heat-inactivated (56°C for 30 min). The processed serum was tested to confirm that it was free of BVDV by virus isolation (17) and of the anti-BVDV antibody by virus neutralization (10). Then the serum was aliquoted and stored at -60°C until used.

Oocyte Collection and In Vitro Maturation

Cumulus-oocyte-complexes were matured while in transit between the laboratory of Em Tran, Inc. in Elizabethtown, Pennsylvania and the Animal Health Research Laboratory at Auburn University in Auburn, Alabama. Ovaries were obtained from an abattoir and were transported in an insulated container (28 to 30°C) to Em Tran in Dulbecco's PBS (GIBCO-BRL, Grand Island, NY; Cat No 14010-026). There the oocytes were aspirated from 3- to 5-mm follicles using a vacuum pump and an 18-g needle and rinsed in a collection dish with modified Tyrode's medium (11, 29) with 3% BSA. They were evaluated, washed 5 times in the same medium, and placed in groups of 50 in approximately 2 ml of IVM medium in screw-cap tubes. The tubes were equilibrated in an atmosphere of 5% CO₂ and air, then tightened and shipped in a portable incubator (Minitube, Inc., Verona, WI) set at 39°C. Upon arrival at the Auburn laboratory, the tubes were placed in an incubator set at 38.5°C and were allowed to equilibrate in an atmosphere of 5% CO₂ and air for 1 to 2 h before processing. The entire maturation period ranged between 20 and 22 h. In preparation for fertilization, the contents of each tube were pipetted into a 35-mm Petri dish where the cumulus-oocyte-complexes (COCs) were examined and counted before washing.

Collection and Culture of Uterine Tubal Epithelial Cells

Uterine tubes were removed surgically from cross-breed beef and dairy cows from production research herds maintained by Auburn University. Sheets of epithelial cells were stripped from uterine tubes, fragmented and washed as described previously (34). Briefly, sheets of epithelium were stripped by drawing the uterine tubes through a narrow gap created by slight pressure on thumb forceps. The sheets were fragmented by aspiration and discharge through a 23-g and then a 25-g needle. Fragments of uterine tubal cells were washed 4 times through a sequence of sedimentation and aspiration of supernatant and the resuspension of sedimented cells in fresh IVC medium. After washing, primary cultures of uterine tubal epithelial cells were incubated in IVC medium for 24 or 48 h before use in IVF and IVC, respectively. Prior to use in IVF and IVC, uterine tubal epithelial cells were washed 3 times in either Hepes-TALP or IVC medium, respectively.
In Vitro Fertilization

Frozen semen from a single collection of a bull previously determined to be free of BVDV was used. The swim-up procedure developed by Parrish et al. (16) was modified for use in our study. Swim-up was carried out in four 12x75-mm polypropylene snap-cap tubes, each containing 1 ml Sperm-TALP. Fertilization was carried out in IVF-TALP. In preparation for IVF, matured COCs were washed 6 times in Hepes-TALP and 1 time in IVF-TALP. During the washing procedure, expanded cumulus cells were removed by vortical agitation, which involved repeated vigorous aspiration and discharge through a small bore glass pipette until cumulus cells were removed (approximately 2 min). Prior to addition of spermatozoa, oocytes were divided into 2 groups and transferred to 100-µl drops of IVF-TALP under silicone oil containing either BVDV-infected uterine tubal cells or noninfected cells. Uterine tubal cells were washed as described (34) and were added to drops 30 to 60 min before fertilization. Aliquots of pelleted spermatozoa were added to IVF drops at a concentration of approximately 1 x 10^6 sperm/ml. The oocytes were then exposed to spermatozoa for 16 to 18 h at 38.5 °C in a highly humidified atmosphere of 5% CO₂ and air.

In Vitro Culture

After the IVF period, cultures were examined for the presence of hyperactivated spermatozoa. The presumptive zygotes were then washed 7 times in IVC medium. In Wash 4, presumptive zygotes were additionally treated by vortical agitation to remove excess spermatozoa. Vortical agitation involved repeated vigorous aspiration and discharge through a small bore glass pipette (approximately 2 min). After washing, the presumptive zygotes were added to 100-µl drops of IVC medium containing uterine tubal epithelial cells. Infected and noninfected uterine tubal cells were washed separately as described and added to drops 30 to 60 min before the addition of the zygotes. Finally, the presumptive zygotes were cultured for 7 d at 38.5°C in humidified air and 5% CO₂. Additional IVC medium was added to each drop at 48 h (50 µl) and at 6 d (25 µl). Cleavage at 48 h and development at 6 and 7 d were observed. The condition, morphology and ciliary activity of uterine tubal cells were also noted at 48 h and at 6 and 7 d. On the seventh day of IVC, the final numbers of morulae and blastocysts and nonfertile and degenerated ova were recorded.

Embryo/Ova Washing

One milliliter of minimum essential medium (MEM) supplemented with 10% equine serum (HyClone Lab. Inc., Logan, UT) and antibiotics (100 U/ml penicillin G, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B) in each of 12 wells of a 24-well cell culture plate constituted the washes for developed embryos or degenerated ova. The washing procedures conformed to guidelines recommended by the International Embryo Transfer Society (2) for the washing of in vivo-derived bovine embryos. Briefly, only ZP-I ova or embryos were washed in groups of 10 or fewer. In addition, separate sterile micropipettes were used to move ova/embryos between
washes. The ratio of volume of medium containing ova/embryos in the pipette to volume of medium in each wash was a minimum of 1:100.

Viral Stocks and Assays

The virus used in this study was a noncytopathic biotype of BVDV (SD-1; 9) that had initially been isolated from the serum of a persistently-infected cow at the Ohio Agricultural Research and Development Center in Wooster, Ohio. The stock virus was propagated in BVDV-free Madin Darby bovine kidney (MDBK) cells that had been cultured in MEM 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 amphotericin B), harvested using a single freeze-thaw method, aliquoted and frozen at -60°C until used. The tissue culture infective dose (50%, TCID50) of stock virus was determined by the method of Reed and Muench (18) to be 1x10^5/ml prior to the beginning of the experiments.

Viral presence was confirmed by an immunoperoxidase labeling technique that was described previously (1). Briefly, the procedure involved the incubation of exposed MDBK cells with the anti-BVDV monoclonal antibody (D89; from Dr. Harish Minocha, Kansas State University, Manhattan, KS; 32), washing with PBS and Tween 20 (Sigma Chemicals, Saint Louis, MO) to remove unbound antibody, addition of peroxidase-conjugated rabbit anti-mouse IgG (Jackson Immuno Research Lab, West Grove, PA), washing with PBS and Tween 20 to remove unbound conjugated antibody, and the addition of aminoethyl carbazole (Zymed Laboratories, Inc., South San Francisco, CA), which produces a reddish brown color when oxidized by horseradish peroxidase. Thus, a reddish brown color is considered positive for the presence of virus and the lack of color is considered negative. Color changes were evaluated by observation with a light microscope and comparison with known infected and uninfected controls. Samples were assayed by inoculating triplicate 100-μl portions in 96-well cell culture plates followed by addition of MDBK cells. The plates were incubated for 72 h at 37°C in humidified air and 5% CO2 before the labeling technique was performed.

Virus Isolation

A summary of samples assayed for virus is presented in Table 1. The small samples from IVF and IVC drops were assayed directly in 96-well plates as follows: Uterine tubal epithelial cells were separated from associated media by centrifugation in microcentrifuge tubes at 15,600 x G for 5 min. Supernatants were assayed immediately, but pelleted uterine tubal cells were frozen (-60°C) and thawed once to enable virus release prior to assay. Then 3 replicate wells containing 99 μl of MEM (supplemented as described earlier) were inoculated with 11-μl aliquots of BVDV-exposed or control sample. Virus isolation was attempted from both control (nonexposed) samples and exposed samples. Each sample was serially diluted (10^1 to 10^6) and overlaid with 26 μl of MDBK cells suspended in the culture medium. Virus-free negative controls were also inoculated in 3 replicates while titration of the stock
virus served as a positive control. Plates were incubated for 72 h at 37°C in humidified air and 5% CO₂ before the immunoperoxidase labeling was performed.

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

<table>
<thead>
<tr>
<th>Assayed after IVF (16 to 18 hours)a</th>
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<tbody>
<tr>
<td>1) Uterine tubal epithelial cells from IVF drops</td>
<td></td>
</tr>
<tr>
<td>2) Medium from IVF drops</td>
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</table>

<table>
<thead>
<tr>
<th>Assayed after IVC (7 days)a</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Uterine tubal cells from IVC drops</td>
<td></td>
</tr>
<tr>
<td>2) Medium from IVC drops</td>
<td></td>
</tr>
<tr>
<td>3) Washed and sonicated degenerated ova (groups)</td>
<td></td>
</tr>
<tr>
<td>4) Washed and sonicated morulae and blastocysts (individually)</td>
<td></td>
</tr>
<tr>
<td>5) Last 3 washes for groups of embryos or degenerated ova</td>
<td></td>
</tr>
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</table>

aBoth control (nonexposed) and virus-exposed samples were assayed.

Degenerated ova were washed as described and sonicated in groups of 10 or fewer (2 bursts at 35% for 60 sec each time with a Model 300 Artek Sonic Dismembrator, Fisher Scientific, Co, Pittsburgh, PA). Morulae and blastocysts were washed as described and sonicated individually using the same method. Sonicate fluids (1 ml) were inoculated onto MDBK cells in a single well of a 6-well plate (9.4 cm² monolayer) and passaged 3 times (5-d incubation per passage) to provide maximum opportunity for viral replication before virus was released by a single freeze-thaw method. Aliquots from each wash culture were assayed for virus in a 96-well plate.

Experimental Design

Four trials were conducted during 4 different weeks. For each trial, 4 primary cultures of uterine tubal epithelial cells were established. Two cultures were exposed to noncytopathic BVDV (1 x 10⁴ TCID₅₀) while the remaining 2 cultures were not exposed to the virus. One set of exposed and unexposed uterine tubal epithelial cell cultures was incubated 24 h and used in IVF drops, and the other set was incubated for 48 h and used in IVC drops. Importantly, as described earlier, ECS that was used in the IVC medium was tested and proved to be free of anti-BVDV antibodies by virus neutralization (10) and also free of infectious BVDV by virus isolation (17).

For each trial, approximately 150 to 200 matured COCs were received from Em Tran, Inc. and divided into approximately equal groups. Treated groups were cultured with exposed uterine tubal cells while control groups were cultured with unexposed uterine tubal cells during IVF and IVC. On Day 7 of IVC, groups of 10 or fewer ZP-I nonfertile and degenerated ova, or ZP-I morulae and blastocysts were washed and
sonicated as described. Nonfertile and degenerated ova were sonicated and assayed in groups while morulae and blastocysts were sonicated and assayed individually. Sonicate fluids were passaged 3 times (5 d incubation per passage) to maximize the opportunity for viral replication before samples were considered to be negative. Also, the last three washes for each group of nonfertile or degenerated ova or morulae and blastocysts were assayed for presence of infectious virus to provide assurance that the washing procedure was effective for removal of free virus. After removal of presumptive zygotes or nonfertile and degenerated ova as well as morulae and blastocysts at the end of the IVF and IVC periods, respectively, viral presence or absence in the drops was determined by collecting the uterine tubal cells and media from the drops and assaying for virus as described. All samples listed (Table 1) from both virus-exposed and unexposed groups were assayed for the presence of virus. The materials from unexposed groups were assayed to ensure that these controls did not have BVDV that was inadvertently introduced through another source.

Contingency table analysis was used to analyze group differences (33). Differences among all trials were analyzed using heterogeneity Chi-square analysis of 2 x 2 tables. If differences among trials were detected, the data were stratified by trial. Differences in rates of cleavage and development between virus-exposed and unexposed groups were compared by Chi-square analysis. The rates of virus isolation from degenerated ova and from morulae and blastocysts were analyzed using Mantel-Haenszel summary Chi-square analysis (24). All possibilities were generated using EpilInfo software (8).

RESULTS

During the 4 weekly trials, a total of 645 matured oocytes of acceptable quality were divided into virus-exposed (n = 323) and unexposed (n = 322) groups. Cleavage and development in the 2 groups are compared in Table 2. Rates of cleavage and development did not differ (P > 0.05; Chi-square test for contingency table analysis) between the two groups. Condition, morphology and ciliary activity of uterine tubal cells in treated and control cultures also did not differ.

For virus-exposed groups in each of the 4 trials, noncytopathic BVDV was always isolated from oviductal cells that were in the IVF and IVC drops as well as in the media of IVF and IVC drops. These same samples collected from drops in which the unexposed groups were produced were always negative for BVDV. Likewise, BVDV was not isolated from any degenerated ova (27 groups assayed) or from morulae and blastocysts (60 assayed individually) of the unexposed control groups. A summary of attempts to isolate BVDV from groups of degenerated ova or morulae and blastocysts produced in the presence of virus-infected uterine tubal cells is presented in Table 3.

Finally, BVDV was never isolated from the last 3 washings of groups of ZP-I degenerated ova or from morulae and blastocysts produced in the presence of uterine
Table 2. Cleavage (48 hours) and development (7 days) of ova fertilized and cultured in the presence of uterine tubal cells infected or not infected with noncytopathic bovine viral diarrhea virus (BVDV)

<table>
<thead>
<tr>
<th>Trial</th>
<th>BVDV-infected (Treated)</th>
<th>Uninfected (Control)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Cleavage</td>
<td>Development</td>
</tr>
<tr>
<td>I</td>
<td>26/96 (27%)</td>
<td>10/96 (10%)</td>
</tr>
<tr>
<td>II</td>
<td>59/99 (60%)</td>
<td>29/99 (29%)</td>
</tr>
<tr>
<td>III</td>
<td>46/60 (77%)</td>
<td>10/60 (17%)</td>
</tr>
<tr>
<td>IV</td>
<td>53/88 (60%)</td>
<td>14/88 (16%)</td>
</tr>
<tr>
<td>Total</td>
<td>184/323 (57%)</td>
<td>62/323 (19%)</td>
</tr>
</tbody>
</table>

*Estrous cow serum in IVC medium failed to neutralize BVDV at a dilution of 1:2.*

*aCleavage in BVD-infected cultures was not significantly different from cleavage in control cultures (P = 0.09; Chi-square test).*

*bDevelopment in BVDV-infected cultures was not significantly different from development in control cultures (P = 0.90; Chi-square test).*

Table 3. Isolation of bovine viral diarrhea virus (BVDV) from groups of degenerated ova and individual morulae and blastocysts produced in the presence of uterine tubal cells infected with noncytopathic BVDV

<table>
<thead>
<tr>
<th>Trial</th>
<th>Groups(^a) of degenerated ova</th>
<th>Individual morulae and blastocysts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. Positive/Total (% Positive)</td>
<td>No. Positive/Total (% Positive)</td>
</tr>
<tr>
<td>I</td>
<td>7/9 (78%)</td>
<td>2/10 (20%)</td>
</tr>
<tr>
<td>II</td>
<td>4/7 (57%)</td>
<td>16/29 (55%)</td>
</tr>
<tr>
<td>III</td>
<td>4/5 (80%)</td>
<td>1/10 (10%)</td>
</tr>
<tr>
<td>IV</td>
<td>8/8 (100%)</td>
<td>4/13 (31%)</td>
</tr>
<tr>
<td>Total(^b)</td>
<td>23/29 (79%)</td>
<td>23/62 (37%)</td>
</tr>
</tbody>
</table>

*One group of 4, one group of 5, one group of 8, and 26 groups of 10 degenerated ova. Positive = virus isolated.*

*Isolation rate from degenerated ova was significantly greater than isolation rate from morulae and blastocysts (P = 0.0002; Mantel-Haenszel summary Chi-square).*
tubal epithelial cells that were not infected with BVDV; however, the tenth, eleventh, and/or twelfth washes for 9 of the 36 groups (25%) of ZP-I degenerated ova or morulae and blastocysts produced in the presence of infected uterine tubal cells were positive for BVDV. Virus was isolated in the last (twelfth) wash for only 2 of these groups (6%).

DISCUSSION

The high degree of freedom of the in vivo-derived bovine embryos from infectious agents was due to a variety of factors, including the resistance of the intact zona pellucida of these embryos to adherence or penetration by many of the pathogens (20,21,27,28). The efficacy of established procedures for washing these embryos (2) is based on the barrier provided by their zona pellucidae. When in vivo-derived, ZP-I, bovine embryos were exposed in vitro to a cytopathic strain of BVDV and then properly washed, they remained free of the virus (23). Similar studies have not been conducted in which in vivo-derived, ZP-I, bovine embryos were exposed in vitro to noncytopathic BVDV. However, results of our previous study indicated that the noncytopathic virus may associate with in vitro-derived, ZP-I, bovine embryos and that washing might not be effective in removing the virus (34).

As stated, sera and essential cells and tissues used for in vitro bovine embryo production are known to be potential sources of noncytopathic BVDV (3,5,6,13,19). Thus, the noncytopathic biotype of BVDV was used in our study. Infected uterine tubal cells were used to provide a steady source of infectious BVDV throughout the periods of IVF and IVC, and care was taken to ensure that no specific anti-BVDV antibody was present in the serum used in media. The continuous supply of virus with no virus neutralizing activity created conditions for a worse case scenario.

Despite the lack of interference from any anti-BVDV antibody and continuous exposure to virus, there was no observable detrimental effect on uterine tubal cells, sperm activity or the total number of ova that was fertilized, cleaved and developed normally through 7 d of IVC. Thus, visual observation under a light microscope was unreliable for detecting the presence of this virus.

The fact that 79% of the groups (i.e., groups of 4 to 10) of degenerated ova were virus-positive versus 37% of individual morulae and blastocysts was probably due to the quantity of material sonicated and assayed for virus. Whether the virus adhered to the ZP of positive ova and embryos or penetrated the ZP and infected embryonic cells could not be determined from this study. Regardless, noncytopathic BVDV remained associated with some groups of ZP-I degenerated ova (range 57 to 100%) and some individual ZP-I morulae and blastocysts (range 10 to 55%) after in vitro exposure and washing during each of the 4 trials, indicating that thorough washing alone is not sufficient for ensuring freedom from this virus (SD-1).

Our experience indicates that the ZP of in vitro-derived bovine embryos is sticky compared with the ZP of in vivo-derived bovine embryos, thus possibly
increasing the tendency of pathogens to adhere. We have found it to be more difficult to wash IVF embryos free of adherent material that can be observed at x50 magnification than it is to wash in vivo-derived embryos free of adherent material. To date, only 2 viruses, infectious bovine rhinotracheitis virus and BVDV, have been shown to associate with in vitro-derived ZP-I embryos (4,13,34); however, as stated, BVDV does not remain associated with in vivo-derived, ZP-I, bovine embryos after proper washing (7). Since there is a high reliance on washing in vivo-derived bovine embryos free of specific pathogens and since washing effectiveness is directly related to the capability of ZP of these embryos to resist adherence or penetration by pathogens, it is essential to determine the degree of resistance to adherence or penetration of pathogens provided by the ZP of in vitro-derived bovine embryos.

Regarding the BVDV-positive ova in our study, it is possible that the virus was carried through the ZP with the spermatozoa at the time of fertilization. Otherwise, the virus might have adhered to the zona pellucida. Although we cannot be certain, it is not likely that the virus passed through the ZP of the in vitro-derived embryos. Future studies need to include the use of trypsin (22,25) or anti-BVDV antibody to assess their efficacy for insuring freedom from the virus. If either of these treatments were to be effective, it could be concluded that the virus does not enter embryonic cells.

The results must be viewed in their proper perspective. The source of virus in this study consisted of artifically infected uterine tubal cells. While this was a realistic representation of a natural hazard, since uterine tubal cells have been shown to be infected naturally (3,5), not all IVF embryo production systems use these cells. For example, many laboratories use established cell lines in IVC. While such cell lines may not constitute the hazard of primary cells collected from an abattoir, they might nevertheless be susceptible to infection with noncytopathic BVDV that is introduced through serum, and thus need to be regularly confirmed to be free of the virus.

It is also important to recognize that most if not all commercially available sera used in IVM and IVC media contain the anti-BVDV antibody, and some lots of BSA used in IVF might contain the anti-BVDV antibody as a contaminant (34). These antibodies are likely to provide some protection against contamination of IVF embryos with BVDV, but further studies are needed to determine if high titers of antibody can provide a consistent level of protection. Of course, if IVF systems are developed in which serum-free media are used exclusively, treatment with serum containing high titers of antibody would not be possible.

Finally, it should be noted that in studies such as this only a single system of IVF embryo production is used, usually the system commonly used in the laboratories of the investigators. A legitimate consideration would be that a variety of in vitro embryo production systems are in use, and this variety could result in variation among systems in the hazards of producing pathogen-associated embryos. It is likely that additional technical modifications are needed to increase the efficiency of IVF embryo production. Until technical consistency is achieved, we need to evaluate results of
embryo-pathogen interactions judiciously and not be too quick to extrapolate the results to all systems.

In this study, continuous exposure to noncytopathic BVDV during IVF and IVC in media with no anti-BVDV antibody resulted in the production of morphologically normal, ZP-I morulae and blastocysts that were virus-positive after thorough washing. While it is recognized that ours was a fabricated worst-case scenario using a single system for IVF embryo production, it is clear that the potential for production of virus-associated embryos exists. Appropriate quality controls in the form of consistently reliable treatments or assays to ensure that IVF embryos are free of noncytopathic BVDV should be investigated.

REFERENCES


