ELSEVIER

NONCYTOPATHOGENIC BOVINE VIRAL DIARRHEA VIRUS (BVDV) REDUCES CLEAVAGE BUT INCREASES BLASTOCYST YIELD OF IN VITRO PRODUCED EMBRYOS

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Received for publication: 15 May 1998
Accepted: 6 July 1998

ABSTRACT

The growing application of in vitro embryo production systems that utilize slaughterhouse tissues of animals of unknown health status conveys the risk of disease transmission. One pathogen of concern in this regard is bovine viral diarrhea virus (BVDV), and the objective of this study was to investigate the effect of BVDV on in vitro embryonic development. A bovine in vitro embryo production system was experimentally infected with BVDV at 2 stages: prior to in vitro maturation by incubating cumulus-oocyte complexes (COC) with virus (strain Pe515; titer $10^{6.2}$ tissue culture infective dose $(TCID)_{50}$/mL) or vehicle for 2 h, and then during in vitro culture by the use of BVDV infected granulosa cells. Exposure to BVDV throughout in vitro production reduced cleavage rates (P=0.01) but increased (P=0.05) the number of embryos that reached the 8-cell stage when expressed as a percentage of cleaved oocytes. Blastocyst yield was increased by the presence of virus when expressed as a proportion of oocytes (P=0.0034) or of those cleaved (P<0.0001). The percentage of total blastocyst yield on Days 7, 8 and 9 for the control and virus treatments was 20, 51, 29 and 29, 41, and 29%, respectively, indicating that the rate of blastocyst development was nonsignificantly faster in the virus-treated group (P=0.06). These results indicate that the presence of non-cytopathogenic BVDV in an *in vitro* production system may reduce cleavage rates but allow those cleaved to develop to blastocysts at a higher rate.

Key words: bovine viral diarrhea virus, in vitro production, bovine

Acknowledgments

This work was jointly funded by the Milk Marketing Board and the Meat and Livestock Commission. The authors gratefully acknowledge the sera testing performed by M.C. Clarke. The monoclonal antibodies were a gift from S. Edwards, Central Veterinary Laboratory, Weybridge, UK.

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0093-691X/98/$19.00
PII S0093-691X(98)00182-4

INTRODUCTION

In vitro produced embryos have been shown to be different from their in vivo counterparts in terms of viability, rate of development, compaction (26) and the potential to transmit pathogens (25). The latter is becoming of increasing importance as in vitro embryo production biotechnologies become applied in cattle breeding programs (15). In this regard, one such disease agent of concern is bovine viral diarrhea virus (BVDV). This virus exists as two biotypes; a non-cytopathogenic and a cytopathogenic form that may be distinguished in cell culture (10). At present, the risk of transmission of the non-cytopathogenic variant by embryos remains equivocal because only the cytopathogenic virus has been sufficiently investigated and results indicate, with one exception (2), that the latter cannot infect in vivo produced embryos exposed to BVDV in vitro (5,22,23). Unfortunately, it is the non-cytopathogenic rather than the cytopathogenic biotype which is the more likely to be transmitted by in vitro produced embryos in practice because, after mutation of the non-cytopathogenic into the cytopathogenic biotype, cattle die rapidly of mucosal disease (11). Indeed, since approximately 1% of some national herds may be persistently infected with non-cytopathogenic BVDV (16,17) and in vitro production systems often use slaughterhouse tissues of unknown health status, the risk of BVDV transmission by embryos is a real risk. In this regard, estimates indicate that 12% of in vitro production runs can be contaminated with BVDV (3). The presence of BVDV in an in vitro production system could conceivably alter in vitro embryonic development especially if it is capable of replicating in oocytes as demonstrated recently by Brownlie et al. (12). However, the effect on cleavage and subsequent in vitro development is still not clarified as BVDV appears to either have no effect (6,24,28) or reduces in vitro embryonic development (1,4,7). Therefore, in this study we have re-examined the effect of BVDV on embryonic development in our own in vitro production system, and in a detailed investigation we have demonstrated that the virus can indeed have pronounced dual effects: one being to reduce the proportion of oocytes that cleave, and the second effect being to actually increase the yield of blastocysts with an accompanying tendency to accelerate the rate of blastocyst development compared to non-infected control embryos.

MATERIALS AND METHODS

Reagents and Sera

All reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated. Estrous cow serum (ECS) was produced by the Institute for Animal Health farm. All sera and BSA used throughout the in vitro production (IVP) system, for virus propagation and titration were both BVDV negative as tested by virus isolation, and sero-negative as detected by ELISA (9).

In Vitro Production System

Cumulus-oocyte complexes (COCs) were aspirated from 2- to 10-mm diameter follicles from slaughterhouse ovaries from cows of unknown BVDV status. The COCs were selected for an unexpanded compact and complete corona radiata of at least 5 layers and for an evenly granulated cytoplasm, and were matured for 24 h in 50-μL drops of maturation medium (TCM-E199 plus 4 mmol glutamine, 0.2 mmol pyruvate, 4 μg/mL estradiol, 5 IU/mL Folligon [Intervet Ltd, Cambridge, UK] and 10% [v/v] ECS). Cumulus-oocyte complexes were fertilized with frozen-thawed spermatozoa (from a BVDV-negative bull) in 50-μL drops of IVF-TALP

(containing 10 μg/mL heparin, 10 μmol hypotaurine, 1 mmol caffeine, 6 mg/mL bovine serum albumin [BSA] and 50 μg/mL gentamycin; 21). The day of in vitro fertilization (IVF) was regarded as Day 0. After a further 24 h, the presumptive zygotes were transferred to 50-μL drops of in vitro culture medium (TCM-E199 containing 4 mmol glutamine, 0.2 mmol pyruvate, 20.9 mmol lactate and 10% [v/v] ECS) containing previously prepared granulosa cell monolayers (5 x 10^3 cells). Cleavage and development to the 8-cell stage were assessed on Day 4, and the total number of blastocysts forming was determined on Days 7, 8 and 9. Throughout IVP all incubations were performed at 38.5°C in 5% CO_2 in humidified air.

Modification of IVP Protocol during BVDV Exposure

Certain minor modifications were made to the IVF system in order to introduce BVDV and to expose both COCs and embryos to virus throughout IVP.

Cumulus-oocyte complexes that were to be exposed to either virus or vehicle (viral propagation medium) prior to in vitro maturation (IVM) were treated as follows: after aspiration, COCs were allocated to undiluted virus or vehicle for 2 h before transfer into maturation medium as normal for the remaining 22 h. This protocol was designed to expose the COCs to a concentration of virus no greater than that measured in follicular fluid (PJ Booth, MC Clarke, unpublished) and to allow the cumulus cells to become infected with virus.

No modifications to the IVF protocol were implemented as preliminary experiments had established that a 2 h preincubation period of the sperm preparation with either virus or vehicle or the addition of small quantities (6 or 15-μL) of virus or vehicle to the IVF drops significantly reduced embryonic development beyond the 1-cell stage.

Granulosa co-culture feeder cells that were to be infected with BVDV were inoculated with virus or vehicle 4 days before addition of the zygotes.

Virus Propagation, Isolation and Titration

Bovine viral diarrhea viral stocks and vehicle were prepared in parallel in roller bottles seeded with the same batch of calf testis cells. The cells were cultured in 100 mL Modified Eagles Basal Medium (ICN Flow, Thame, UK) containing 2% (v/v) fetal calf serum (FCS; Gibco, Paisley, UK), 2.34 mg/mL lactalbumin, 2 mmol/mL l-glutamine, 1.05 mg/mL sodium bicarbonate and 25 U/mL mycostatin at 37°C. One bottle was inoculated with 1 mL BVDV (Pe515; genogroup type 1a; titer $10^{6.5}$ tissue culture infective dose $(TCID)_{50}$ /mL). After 7 d the bottles were frozen at -70°C in order to release virus. The bottles were then thawed, centrifuged and stored at -70°C. The viral titer was $10^{6.2}$ $TCID_{50}$/mL.

Virus isolation was performed essentially as described by Booth et al. (9) using calf testis cells on glass coverslips. Three passages were performed on each sample representing a total period for viral isolation of 28 days in culture. Samples destined for viral titration were decimally diluted up to a dilution of 10^{-8}. The BVDV antigen was detected by immunoflorescent staining. The staining technique and the specificity studies were as described by Booth et al. (9). Monoclonal antibodies WB162 and WB103 (specific to the viral envelope glycoprotein gp53 and

the p80 nonstructural protein, respectively) were tested in combination. These antibodies were known to possess immunoreactivity to a range of field strains of BVDV and to the Pe515 strain.

The follicular fluid pool, granulosa co-culture cell preparations, the residual media remaining in the control IVM, IVF and in vitro culture (IVC) drops (i.e., those not experimentally infected with BVDV) and the blastocysts plus degenerated oocytes/embryos from the control group were submitted for virus isolation or titration. Oocytes and embryos were sonicated before virus isolation.

Statistical Analyses

Embryonic development was analyzed using a Generalized Linear Model technique (18) incorporating treatment, replicate and the interaction as the effects. When appropriate, nonsignificant effects were removed from the design in order to establish probability values for the main effects. The rate of embryonic development was analyzed for exact P-values by Pearson's Chi-square and deviance (likelihood) test in log-linear models on contingency tables.

RESULTS

Virology of IVP System

Granulosa cell coverslip cultures, follicular fluid pools and the residual media recovered from control IVM, IVF and IVC drops were negative for BVDV as assessed by the virus isolation system described. Similarly, degenerated oocytes removed from culture on Day 4 and other degenerated embryos or viable morulae and blastocysts on Day 8 that had not been experimentally exposed to virus were also negative for BVDV by virus isolation.

Titers of virus (mean and range) present in residual media recovered from BVDV-infected IVM, IVF and IVC drops were $10^{5.0}$ ($10^{3.5}$-$10^{5.5}$) (n=3 IVP replicates), $10^{6.1}$ ($10^{5.5}$-$10^{6.5}$) (n=3) and $10^{6.5}$ (n=2: both titers identical) $TCID_{50}$ /mL, respectively.

Embryonic Development in BVDV-Infected IVP System

The effect of the presence of BVDV on embryonic development throughout the duration of the IVP system is shown in Table 1. The proportion of oocytes that cleaved was significantly reduced by the presence of virus (P=0.010) and was affected by replicate (P=0.0357), but no interaction between treatment and replicate was observed (P=0.8588). Development to the 8-cell stage was not affected by treatment or replicate, although a stimulatory effect of treatment on the proportion of 8-cell embryos, expressed as a percentage of those that cleaved, was observed (P=0.05). Blastocyst yield, expressed as a proportion of total oocytes, was significantly increased by the presence of virus (P=0.0034). This effect was stronger when the blastocyst yield was calculated as a proportion of those that cleaved (P<0.0001). An effect of replicate was also observed (P=0.0391), but no interaction with replicate was recorded (P=0.1058). The percentages of total blastocyst yields on Day 7, 8 and 9 for the control and virus treatments were 20, 51, 29 and 29, 41 and 29%, respectively (Figure 1), indicating that the rate of blastocyst development was faster in the presence of virus, although the probability value fell just outside the 5% level of significance (P=0.06). Marginal P-values for the rate of blastocyst development also indicated an effect of replicate (P=0.029) and an interaction between replicate and treatment (P=0.015).

Table 1. Effect of bovine viral diarrhea virus on bovine in vitro embryonic development

Treatment[a]	No. of oocytes	% Cleavage	% 8-cell embryos/ oocytes	% 8-cell embryos/ cleaved	% blastocysts/ oocytes	% blastocysts/ cleaved
IVP Control	773	84.6[d]	58.2	68.8[b]	24.2[d]	28.6[f]
IVP Virus	769	79.6[e]	58.7	73.7[c]	30.9[e]	38.8[g]

[a]Figures are the means of 3 in vitro production replicates.
Values in the same column with different superscripts are different; b, c: P=0.05; d, e: P≤0.01; f, g: P<0.0001.

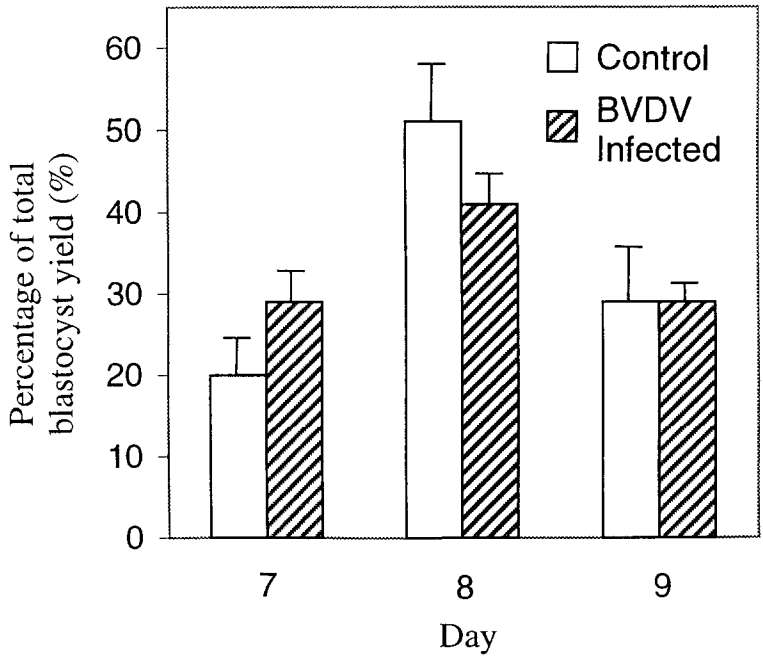

Figure 1. Percentage of total blastocyst yields on Days 7, 8 and 9 in a bovine viral diarrhea virus (BVDV) infected and vehicle (control) inoculated in vitro production system. Percentages are the means of 3 in vitro production replicates. Bars show SEMs. Control versus BVDV infected in vitro production system: P=0.06

DISCUSSION

This study revealed 2 distinct effects as a result of the experimental introduction of BVDV into the IVP system: 1) a reduced rate of embryonic cleavage, and 2) an increased yield of blastocysts plus a tendency for a faster rate of blastocyst development. These observations are contrary to those of previous reports (6,24,28), in which no differences were recorded. These dissimilarities could possibly be explained by the number of oocytes and replicates, by different *in vitro* production system protocols and by efficiencies and by different viral strains or by statistical analyses. The alteration in embryonic development due to the presence of BVDV recorded in the current investigation may have been masked in other studies in which BVDV was retrospectively screened as a contaminant in IVP systems (3,8) due to the normal variation in development between IVP runs. However, significant reductions in the in vitro produced blastocyst yields are possible under certain conditions, such as those induced following incubation of the in vitro fertilizing spermatozoa with BVDV (7), and by the use of oocytes derived from cattle acutely infected with BVDV (4). Our results suggest that although cleavage rates are reduced by the presence of BVDV, those embryos that did cleave and reached the 8-cell stage, which is notably the time of major zygotic gene activation, possessed a greater in vitro developmental potential compared with the uninfected controls. The mechanism by which BVDV reduces cleavage rate is not known but this effect may be mediated either directly during IVM or IVF by altering the proportion of fully matured oocytes or reducing fertilization rates, or perhaps indirectly by altering cumulus cell function during these stages. Furthermore, it is unclear at present if BVDV is able to replicate in the ooplasm of in vitro matured and fertilized oocytes, as has been clearly demonstrated in ovarian oocytes (12), or if the virus is incapable of penetrating the zona (27). Hence, the localization of BVDV within the oocyte and embryonic cytoplasm or merely within, on, or external to the zona cannot be ascertained in the present study. However, despite this uncertainty, it seems unlikely that BVDV affected the process of oocyte maturation since the developmental competence of cleaved embryos was enhanced rather than compromised. The presence of BVDV could also affect sperm function directly or alter the properties of the COCs to penetration and fertilization. In this respect, Bielanski and Loewen (7) recorded a nonsignificant reduction in the fertilization rate after incubating spermatozoa with noncytopathogenic BVDV, while Grahn et al. (14) demonstrated fertilization failure in cows receiving intrauterine infusions of BVDV, albeit the cytopathic biotype. In addition, Allietta et al. (1) demonstrated that although neutralization of virus in spermatozoa from a BVDV-infected bull had no effect on either in vitro fertilization or cleavage rates, blastocyst yields were decreased. Provisional experiments in our own laboratory indicated that addition of even small quantities of calf testis cell growth medium (used to propagate virus) dramatically decreased cleavage rates, as reported similarly by Neighbour and Fraser (20) who studyied the effect of cytomegalovirus on fertilization. Consequently, no calf testis cell growth medium (as either virus or vehicle) was added to the IVF drops, and hence the presence of virus in these drops was dependent upon carry-over of virus from the IVM drops and, more importantly, productive viral infection in the cumulus cells. The latter is certainly true (9), and titers measured in the residual media of not only the IVF drops but also the IVM and IVC drops indicated high levels of virus throughout the in vitro production protocol, but no higher than the concentration of BVDV in follicular fluid of persistently infected cattle (PJ Booth, MC Clarke, unpublished).

The presence of BVDV not only enhanced blastocyst yields but also tended ($P=0.06$) to increase the rate at which they developed. Such an enhancement of development is a novel observation, and could be mediated by the virus altering cell metabolism in the in vitro system. In

this respect, BVDV could alter the metabolism of the embryo directly, if it is capable of penetrating the zona and replicating in the embryonic cells, or indirectly, by modifying the metabolism of the infected granulosa co-culture cells. In the latter scenario, high levels of replicating virus, as are seen in BVDV-infected granulosa cells (9), quite likely derange the cells' normal function, as is observed in vitro in cells infected with BVDV (19), by synthesizing viral RNA and taking over the cell's ribosomal synthetic capacity and so interfering with mRNA and protein synthesis plus DNA replication. Therefore, BVDV-infected granulosa co-culture cells quite likely exhibit a disturbed metabolism that could beneficially modify the profile of substrate availabilities (13) and/or the hormonal patterns in the medium so that embryonic development is stimulated.

In conclusion, the data described above indicate that the deliberate introduction of noncytopathogenic BVDV into an IVP system, at levels that can reasonably be expected to be encountered during a natural contamination, can reduce cleavage rates but enhance subsequent embryonic development. This emphasizes the need for in vitro embryo production laboratories to continue to screen for the presence of BVDV (3,8) in order to prevent the transmission of this pathogen. Further research is needed 1) to investigate the effect of the virus on development in different in vitro production systems, 2) to identify the localization of BVDV in the in vitro produced embryos, 3) to determine if the virus can penetrate the zona and replicate in embryonic cells, as has recently been reported in ovarian oocytes (12) and 4) to identify the mechanisms by which BVDV improves embryonic development since potentially reproducing these conditions in the absence of virus could lead to improved in vitro culture media formulations.

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