Effects of oocytes exposure to bovine diarrhea viruses BVDV-1, BVDV-2 and Hobi-like virus on in vitro-produced bovine embryo development and viral infection


a Laboratório de viroses de bovídeos, Instituto Biológico de São Paulo, Avenida Conselheiro Rodrigues Alves, 1,252, São Paulo, SP, CEP 04014-900, Brazil
b In Vitro Brasil, Rodovia SP 340, Km 166, Mogi Mirim, SP, CEP 13800-970, Brazil

ABSTRACT
As production of in vitro (IVP) bovine embryos steadily increases, the sanitary risk associated with IVP embryos remains a concern. One of the greatest concerns is how BVDV may be transmitted through IVP embryos. The objective of this study was to evaluate the effects caused by BVDV-1, BVDV-2 and Hobi-like virus exposure during in vitro maturation on embryo development and viral infection. Abittior-derived oocytes were randomly assigned for in vitro maturation with serial concentrations of BVDV-1 (3.12 - 2.50 x 10^2 TCID50/100 μL), BVDV-2 (6.25 - 5.20 x 10^2 TCID50/100 μL) or Hobi-like virus (1.90 - 1.58 x 10^2 TCID50/100 μL) for 22-24 h. After maturation, oocytes were fertilized and embryo cultured following standard in vitro procedures. Embryo development was evaluated and percentage of respective, positive BVDV degenerated and viable embryos were evaluated by RT-qPCR. No concentration of BVDV-1 altered embryo development as measured by cleavage and blastocyst rates, compared to negative control group. However 100% of degenerated embryos and 50–100% of viable embryos tested positive for BVDV-1, depending on the viral concentration. BVDV-2 exposed oocytes had higher cleavage rates than the negative control group (60.2 - 64.1% vs 49.8%; P = 0.003 - 0.032). However, no difference was detected for blastocyst rates. In addition, 100% of degenerated embryos and 20–50% of viable embryos tested positive for BVDV-2. Hobi-like virus treated oocytes had reduced cleavage rates for the three highest viral concentrations (60.2 - 64.1% vs 49.8%; P = 0.003 - 0.032). However, no difference was detected for blastocyst rates. In addition, 100% of degenerated embryos and 20–50% of viable embryos tested positive for BVDV-2. Hobi-like virus treated oocytes had reduced cleavage rates for the three highest viral concentrations (33.3 - 38.0% vs 49.8% for negative controls; P ≤ 0.001–0.014). Blastocyst rates were only reduced in the 7.9 x 10^2 Hobi-like virus concentration (6.9 ± 0.9% vs 15.4 ± 1.6%; P = 0.009), when calculated by oocyte number. 50–80% of degenerated embryos tested positive for Hobi-like virus. No viable embryos from the Hobi-like virus treated oocytes tested positive. These results suggest that IVP embryos from BVDV-1 and -2 infected oocytes develop normally, but carry the virus. However, Hobi-like virus infected oocytes had reduced cleavage and cause pre-implantation embryo loss, but viable embryos did not carry the virus.

© 2017 Published by Elsevier Inc.

1. Introduction

In 2013, global production of ovum-pick-up (OPU), IVF embryos grew significantly to 517,587 embryos. This was an increase of 16.7% from 2012, and the first time over 500,000 bovine OPU/IVF embryos were collected and over 400,000 transferred in one year [1]. However, a total of 712 IVP embryos were exported internationally that same year from only two countries, Canada and Dominican Republic [1]. While global production of IVF embryos dramatically grows, the slow growth of international trade in IVF embryos is primarily due to governmental regulation to insure minimal risks of disease transmission via IVP embryos.

While several studies have been conducted and acceptable washing protocol approved for in vivo produced embryos, fewer studies have been conducted on the sanitary risks of IVP embryos.
In vivo fertilized embryos differ with IVP embryos in regards to development, physiological characteristics and especially differences in the zona pellucida [2,3]. Changes in the zona pellucida appear to be one reason washing protocols are less effective for IVP embryos [4].

Viruses, bacteria and protozoa can infect and multiply in cells during culturing of IVP embryos. Also, contamination can be introduced through the use of un-tested semen, infected donors or animals by-products utilized, particularly fetal bovine serum (FBS) [5]. Viral contamination is considered one of the greatest risks since it can be present without causing any cytopathic effects to cells. One of the more common pathogens is bovine viral diarrhea virus (BVDV) [6].

BVDV is a RNA virus that causes bovine diarrhea, respiratory disease, immunosuppression, abortions, and a reduction in reproductive efficiency that consequently impacts herd health and productivity [7,8]. Studies in recent years indicate that several subgenotypes have been distributed within the BVDV genotype-1 and BVDV-2, such as BVDV-1a NADL, BVDV-2 NY93, among others [9,10]. BVDV is also classified as cytopathic (CP) or non-cytopathic (NCP) [10,11]. The NCP, as found in nature, is responsible for the emergence of animals being persistently infected [10,11]. In addition, a pestivirus was recently reported that appeared to be a new species of BVDV. This new genotypic group, called Hobi-like virus or BVDV-3, is an atypical or new pestivirus [12]. This virus was first isolated from samples of FBS and vaccines originating from Brazil [12].

While little is known about Hobi-like virus effects on embryos, BVDV-1 and -2 have been reported to infect recipient animals that have received embryos cultured with the virus [13]. To minimize the risk of transmission, the World Organisation for Animal Health (OIE) requires that all in vivo embryos for international sale be treated to the trypsin wash protocol [14]. However, this wash protocol is ineffective to remove BVDV-1 and BVDV-2 from IVP embryos [15].

Studies on the risk of disease transmission by infected gametes and/or embryos are of extreme importance to the national and international market of bovine embryos. The current study was designed to simulate possible viral contamination during oocyte collection, via non-diagnosed, infected donors or introduce during the collection procedure. The objective of this study was to evaluate the effects caused by BVDV-1, BVDV-2 and Hobi-like virus exposure during in vitro maturation on embryo development and viral infection.

2. Materials and methods

All chemical were purchased from Sigma-Altich unless specified. Semen was previously certified as free of bovine herpesvirus type 1, Bluetongue virus, bovine leukemia virus and BVDV by PCR. The same semen batch was utilized for all experiments. In addition, BSA, media, and follicular fluid were verified free of BVDV by RT-qPCR.

2.1. Viral strains

Different concentrations of three non-cytopathic (NCP) biotypes of BVDV (BVDV-1, BVDV-2 and Hobi-like virus) were utilized. BVDV strains were isolated in cell cultures of MDBK (Madin-Darby bovine kidney) at the Laboratory of Virus Diseases of Cattle (LABV) at the Instituto Biológico (São Paulo, Brazil), between 2014 and 2015 and genotyped by RT-qPCR and genetic sequencing (data not shown). BVDV-1 and BVDV-2 strains originated from bovine blood samples from the State of São Paulo, Brazil. The Hobi-like virus strain originated from a bovine lung fragment that presented clinical signs of respiratory disease from the State of Minas Gerais, Brazil. Serial dilutions were made from the stock solution of BVDV-1, BVDV-2 and Hobi-like virus, previously titrated by ELISA and RT-qPCR, and prepared in maturation media.

2.2. Viral exposure during in vitro maturation

The oocytes were aspirated and selected from ovaries at a local abattoir. Selection was based on morphological evaluation of cytoplasm and layers of granulosa cells surrounding the oocyte. The selected oocytes were transported in TCM-199 maturation media (GIBCO BRL; Grand Island, NY, USA) supplemented with 2% synthetic substitute serum, 0.5 µL/mL FSH (FollitropinTM, Bioniche Animal Health, Belleville, Ont, Canada), 50 IU/mL of hCG (ProfasiTM, Serono, São Paulo, Brazil), 1 µL/mL 0.20 mM estradiol sodium pyruvate and 83.4 µg/mL amikacin (Instituto Biochimico, Rio de Janeiro, Brazil), to the laboratory at the Instituto Biológico.

Cumulus-oocytes complexes (n = 20–25) were placed in 100 µL micro-drops and randomly assigned to co-cultures with viral treatments during maturation in a humidified atmosphere with 5% CO₂, to 38.5 °C for 22–24 h.

2.3. In vitro fertilization and culture

After maturation, oocytes were washed three times, prior to in vitro fertilization. Maturation media along with cellular debris that remained, from each treatment, were collected and stored in freezer at −80 °C until further processed for RT-qPCR.

Frozen semen was processed and in vitro fertilized was conducted as previously described [16]. Briefly, frozen semen was thawed and prepared by centrifugation with a percoll gradient. After assessment of sperm concentration and motility, semen was diluted in TALP-IVF medium [TALP] with 30 µg/mL of heparina, 18 µM of penicillamine, 10 µM hipotaurina and 1.8 µM epinephrine, containing sodium pyruvate 0.2 mM, 83.4 µg/mL amikacin sulfate and supplemented with 6 mg/mL BSA. Diluted semen was added to drops of IVF media containing oocytes, with the final concentration of 2 × 10⁶ sperm alive/drop (2 × 10⁸/mL) and cultured for 18–20 h in a humidified incubator with 5% CO₂ at 38.5 °C.

After fertilization, cumulus cells were removed with gentle pipetting and zygotes were washed in SOF medium (+0.5% BSA, 0.20 mM sodium pyruvate and 83.4 µg/mL amikacin sulfate (Cristalina)), and cultured in a humidified incubator with 5% CO₂ at 38.5 °C for 7 days.

On day 7 of culture, embryos were evaluated and classified according to IETS guidelines. Viable embryos of degree 1 and 2, from each concentration were randomly divided into two groups; washed according to IETS protocol [17] with or without trypsin. Briefly, embryos were washed (maximum ten embryos at a time, dilution factor of 1:100) with five baths of phosphate buffered saline (PBS) containing antibiotics and 0.4% BSA (BSA), two washes with either only PBS (-Trypsin group) or 0.25% trypsin (Amresco®) in PBS (+Trypsin group) for a total of 90 s, followed by five more PBS + antibiotic and 0.4% BSA washes to de-activate and remove remaining enzyme.

Viable embryos (- and +Trypsin groups), and culture media containing degenerated embryos from the same drops were collected and stored at −80 °C until further processed by RT-qPCR.

2.4. Viral detection by RT-qPCR

Extraction of viral RNA was carried out using TRizol® Reagent (Ambion®), according to manufacturer’s instructions, adjusting for samples volume. The VetMax®—Gold BVDV Detection Kit (Applied Biosystems) was utilized for detection of BVDV-1, BVDV-2 and...
Hobi-like virus by RT-qPCR. To valid kit, specific oligonucleotide primers and probes for BVDV-1 and -2 were utilized, as previously described [18]. To validate the use of detection kit for Hobi-like virus, RT-qPCR was conducted with serial dilutions of stock virus.

Standard curves for each BVDV genotype were constructed and utilized to evaluate the analytical sensitivity of RT-qPCR reactions and quantify positive samples. RT-qPCR reactions were run on a Light Cycler 480 (Roche Applied Sci; Penzberg, Germany). Reaction and cycling conditions were followed according to manufacturer’s instruction VetMaX Gold kit. Detection threshold and cutoff point instruction were determined based on Ct cutoff levels of the negative standard curve samples (37, 38 and 35 Ct for BVDV-1, BVDV-2 and Hobi-like virus, respectively). Positive samples were determined based on Ct cutoff levels of the negative standard curve samples (37, 38 and 35 Ct for BVDV-1, -2 and Hobi-like virus, respectively).

2.5. Experimental design

Experimental design was similar for each viral strain. Briefly, immatured cumulus-oocyte complexes (COCs) were cultured without (negative control) or with four concentrations of the virus for 22–24 h in maturation media. After maturation, COCs were washed, subjected to in vitro fertilization and culture. Cleavage rates and development data were collected. Samples of IVM media (n = 11–12 per treatment), degenerated embryos (n = 11–12 pools per treatment) and viable embryos subjected to the IETS washing protocol -Trypsin (n = 5–6 pools per treatment) and +Trypsin (n = 5–6 pools per treatment) were collected for viral detection by RT-qPCR, as described above.

Experiments were conducted in triplicate.

2.6. Statistical analysis

Cleavage and blastocyst rates for oocytes exposed to different types and concentrations of BVDV were analyzed by logistic regression of XLSTAT Statistical Program (Addinsoft SARL, New York, NY). BVDV types were analyzed separately with replication and viral concentration as dependent variables. Difference were considered significant with P < 0.05.

3. Results

3.1. BVDV-1

Exposure of maturing oocytes to BVDV-1 had no statistically significant effect on cleavage rates (P > 0.097 for all viral concentrations) or blastocyst rates when calculated by blastocyst/oocyte (P > 0.106 for all viral concentrations) or blastocyst/cleaved (P > 0.064 for all viral concentrations), compared to the non-viral, control group (Table 1).

Maturation media with cell debris after IVM, exposed to BVDV-1, tested positive by RT-qPCR in all samples but one of the lowest viral concentration replicates (Fig. 1). Regardless of viral concentration, 100% of degenerated embryos tested positive for BVDV-1 (Fig. 1). The percentage of viable embryos positive for BVDV-1 was viral dose dependent with 100% of the viable embryos produced from oocytes exposed to the highest dose (2.50 × 10^2) tested positive and 50% of the embryos positive at the lowest viral dose (3.12 × 10^2). Washing viable embryos with trypsin had no effect on reducing the percentage of BVDV-1 positive embryos when compared to unwashed embryos (Fig. 1).

3.2. BVDV-2

Exposure to BVDV-2, caused an increase in cleavage rates (P-values = 0.015, 0.004, 0.003 and 0.032 for viral concentrations 6.25 × 10^1, 1.25 × 10^2, 2.50 × 10^2 and 5.20 × 10^2 TCID50/100 μL, respectively) for all viral concentrations compared to controls (Table 2). No significant differences in blastocyst rates, either calculated by blastocyst/oocyte (P > 0.425 for all cases) or blastocyst/cleaved (P > 0.324 for all cases) were detected among the groups (Table 2).

Similarly, 96–100% of IVM media with cell debris and degenerated embryos tested positive for BVDV-2 (Fig. 2). All viral concentrations produced positive viable embryos, but to a lesser percentage than BVDV-1 (20–50% vs. 50–100%, respectively). Trypsin washing protocol did not reduce the percentage of BVDV-2 positive embryos (Fig. 2).

3.3. Hobi-like virus

Exposure of oocytes to Hobi-like virus during maturation caused a reduction in cleavage rates for oocytes exposed to 3.90 × 10^2 TCID50/100 μL (P = 0.014), 7.90 × 10^2 TCID50/100 μL (P < 0.001) and 1.58 × 10^3 TCID50/100 μL (P = 0.002) of the virus. No difference in cleavage rate was detected for the 1.90 × 10^2 TCID50/100 μL concentration (P = 0.174; Table 3). Blastocyst rates were statistically lower for oocytes treated to the 7.90 × 10^2 TCID50/100 μL of Hobi-like virus, compared to control (6.9 ± 0.9% vs 15.1 ± 1.6%; P = 0.009), when rates were based on total oocytes, but

<table>
<thead>
<tr>
<th>Viral Concentration (TCID50/100 μL)</th>
<th>Number of Oocytes</th>
<th>Number of Cleaved Embryos (% per Oocytes)</th>
<th>Number of Blastocysts (%)</th>
<th>Percentage of Blastocyst/Cleaved</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>225</td>
<td>112 (49.8 ± 7.5%)</td>
<td>34 (15.1 ± 1.6%)</td>
<td>30.4 ± 7.1%</td>
</tr>
<tr>
<td>3.12 × 10^1</td>
<td>214</td>
<td>116 (54.2 ± 7.9%)</td>
<td>22 (10.3 ± 1.5%)</td>
<td>19.0 ± 5.0%</td>
</tr>
<tr>
<td>6.25 × 10^1</td>
<td>221</td>
<td>100 (45.2 ± 5.3%)</td>
<td>22 (10.0 ± 2.5%)</td>
<td>22.0 ± 9.1%</td>
</tr>
<tr>
<td>1.25 × 10^2</td>
<td>213</td>
<td>123 (57.7 ± 7.3%)</td>
<td>32 (15.0 ± 1.9%)</td>
<td>26.0 ± 6.7%</td>
</tr>
<tr>
<td>2.50 × 10^2</td>
<td>213</td>
<td>114 (53.5 ± 7.1%)</td>
<td>33 (15.5 ± 2.4%)</td>
<td>28.9 ± 4.3%</td>
</tr>
</tbody>
</table>

*Different superscripts refer to a significance of P < 0.05.*
not when rates were based on cleaved embryos (P = 0.165; Table 3). Blastocyst rates were insignificant for concentrations 1.90 × 10^2 (P = 0.208 and 0.444), 3.90 × 10^2 (P = 0.356 and 0.843) and 1.58 × 10^3 TCID_{50}/100 μL (P = 0.116 and 0.786) when based on either the number oocytes or cleaved embryos, respectively (Table 3).

For Hobi-like virus, 96–100% of IVM media with cell debris were positive (Fig. 3). 60–90% of degenerated embryos tested positive for Hobi-like virus, regardless of viral concentration. Unlike BVDV-1 and -2 treated oocytes, 0% of viable embryos produced from Hobi-like virus treated oocytes were positive, regardless of viral concentration or trypsin washing (Fig. 3).

4. Discussion

While IVP of bovine embryos has grown dramatically in the last decade, international sales have been restricted due to concerns about disease transmission. These concerns are based mainly on the lack of research in this area and lower effectiveness of preventive disinfection protocols in regards to IVP embryos. The current study was conducted to determine the effects of BVDV exposure during oocyte maturation on subsequent embryo development and test viral transmission. The current model mimics in field conditions, such as collected oocytes from a non-diagnosed, infected donor or viral exposure during the OPU process. The current study was based on the range viral loads that have been found in infected animals tested for BVDV (personal communication). In another study, no difference in polar body extrusion was detected between oocytes exposed to BVDV-NCP (2.5 × 10^7 TCID_{50}/ml) during maturation compared to non-treated oocytes [23].

The lack of a viral effect on embryo development suggests two possible explanations. 1) The virus did not infect or propagate in the oocyte and/or embryo, or 2), BVDV-1 NCP virus does not interfere in oocyte maturation and embryo development to the blastocyst stage. To determine if BVDV infected the subsequent embryos, poor quality or degenerated embryos and viable embryos were collected and tested for BVDV-1 by RT-qPCR. BVDV-1 was found in all degenerated samples, regardless of viral concentration. In addition, 50–100% of viable embryos also tested positive for BVDV-1. Bielanski and co-authors [13] reported that 25% of embryos tested positive for BVDV-1 non-cytopathogenic biotype when d7 blastocyst IVP embryos were exposed to the virus for only 1 h. Other researchers reported infection rates up 42% when d7 IVP blastocysts were exposed for 2 h [25]. In addition, these authors reported that 99% of positive embryos contained ≤3.44 copies/5 μL of the virus [25]. While these studied treated blastocyst stage embryos, the current study demonstrates that infected oocytes transmit the transmission of BVDV is of great concern to the international market.

In the current study, co-incubation of BVDV-1 with maturing bovine oocytes had no statistically significant effect on cleavage or blastocyst rates of the subsequent embryos, regardless of viral concentration. Booth and co-authors [22], reported a reduction in cleavage, but an increase in blastocyst production, in response to BVDV-1 exposure. However, the viral concentration was 1000-fold higher than utilized in the current study. The concentrations in the current study were based on the range viral loads that have been found in infected animals tested for BVDV (personal communication). In another study, no difference in polar body extrusion was detected between oocytes exposed to BVDV-NCP (2.5 × 10^7 TCID_{50}/ml) during maturation compared to non-treated oocytes [23]. However, Altamiranda and co-authors [24] reported reduced cleavage and embryo development rates from oocytes collected from BVDV-infected heifers.

Table 2

Embryo development of bovine oocytes exposed to different concentration of BVDV-2 during maturation.

<table>
<thead>
<tr>
<th>Viral Concentration (TCID_{50}/100 μL)</th>
<th>Number of Oocytes</th>
<th>Number of Cleaved Embryos (% per Oocytes)</th>
<th>Number of Blastocysts (% per Oocytes)</th>
<th>Percentage of Blastocyst/Cleaved</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>225</td>
<td>112 (49.8 ± 7.5%)</td>
<td>34 (15.1 ± 1.6%)</td>
<td>30.4 ± 7.1%</td>
</tr>
<tr>
<td>6.25 × 10^4</td>
<td>203</td>
<td>125 (61.6 ± 11.0%)</td>
<td>36 (17.7 ± 2.8%)</td>
<td>28.8 ± 4.9%</td>
</tr>
<tr>
<td>1.25 × 10^5</td>
<td>223</td>
<td>141 (63.2 ± 8.6%)</td>
<td>40 (17.9 ± 1.7%)</td>
<td>28.4 ± 7.7%</td>
</tr>
<tr>
<td>2.50 × 10^5</td>
<td>209</td>
<td>134 (64.1 ± 12.1%)</td>
<td>37 (17.7 ± 2.0%)</td>
<td>27.6 ± 10.4%</td>
</tr>
<tr>
<td>5.20 × 10^5</td>
<td>201</td>
<td>121 (60.2 ± 9.2%)</td>
<td>30 (14.9 ± 2.2%)</td>
<td>24.8 ± 7.4%</td>
</tr>
</tbody>
</table>

*a,bDifferent superscripts refer to a significance of P < 0.05.
BVDV-1 virus to the subsequent developing embryo.

To determine if a trypsin washing would reduce the number of infected embryos, viable embryos were divided into two wash groups (- and +Trypsin). The percentage of BVDV-1 positive embryos was not different between the two wash groups (50–100% positive), regardless of initial viral concentration. These results are similar to previous work comparing in vivo versus in vitro produced embryos [26]. This lack of effect may be due to the zona pellucida characteristics of IVP embryos [26]. It was not determined if BVDV-1 was only attached to the zona pellucida or also in the growing embryo in the present study.

While BVDV-1 exposed oocytes did produce BVDV positive embryos, the transmission to recipients or the subsequent offspring was not tested in the current study. Bielsani and co-authors [13] reported that when d.7 IVP embryos were exposed to BVDV-1 for 1hr prior to transfer, no difference in d30 pregnancy loss, (compared to non-treated embryos), no seroconversion in the recipients (and no live calves born with BVDV-1) [13]. These results suggest that BVDV-1 positive IVP embryos may not infect the recipients or the subsequent offspring or the viral load of embryos is not sufficient to cause infection. Further studies are needed to test these conclusions.

While BVDV-1 is more prevalent, BVDV-2 has been found to present in 37% of cases in the US [18]. In Southern Brazil, it has been reported that 42% (14/33) of the BVDV positive animals tested in the state of Rio Grande do Sul were infected with BVDV-2 [27]. Even though BVDV-2 appears to less prevalent, its effects are just as devastating.

Exposure of maturing bovine oocytes to BVDV-2 caused a statistically significant increase in cleavage rate in the current study (Table 2). This increased cleavage was observed for all viral concentrations. However, the increased cleavage rates did not result in a statistically significant increase in blastocyst development (Table 2). Little data is available in regards to the effect of BVDV-2 during IVP of bovine embryos. One study demonstrated that IVP embryos exposed to BVDV-2 for 1 h had harmful effects on subsequent pregnancies [13]. BVDV-2 exposed embryos caused lower pregnancy rates (35–41% versus 57% for non-treated embryos) and higher pregnancy loss at d.30 (50–52% versus 25%) [13]. In addition, 12–26% of pregnancy recipients seroconverted. However, 0% of live offspring tested positive for BVDV-2 [13].

Similar to BVDV-1, 92–100% of degenerated embryos tested positive for BVDV-2 by RT-qPCR. It is unclear if BVDV-2 caused these embryos to not develop successfully, or after the embryos stop developing, the virus was able to attach/infect more easily. Viable embryos were also detected as positive by RT-qPCR, but to a lesser extend than BVDV-1 (33–75% vs 50–100%, respectively). In addition, trypsin had no effect on removing BVDV-2 from the viable embryos, based on positive PCR results for each viral concentration (Fig. 2). These results suggest alternative methods of washing are required for IVP embryos, in regards to BVDV.

Due to the relatively recent identification, Hobi-like virus has not been studied to the extent as BVDV-1 and -2. It was first identified in fetal bovine serum (FBS) originating from Brazil, shipped to Europe [28]. Hobi-like virus has also been identified in Italy and Thailand [12]. This new pestivirus is being detected in FBS from North America and Australia [29]. With the international sale of FBS from countries identified as non-free, the increased risk of spreading to non-infected countries is great [29]. One hypothesis to the emergence of this new pestivirus is that it was past cross-species from water buffalo to cattle, this theory would explain its presence in countries with high number of water buffalo [12].

It must be noted that a serum supplement was utilized throughout the experiment to eliminate any potential contamination. The use of a serum substitute for IVM and IVF may explain the overall lower maturation and embryo development rates [30,31] found in the current study.

Most infections in cattle is caused by biotype BVDV NCP, which does not cause cytopathic effect visible under a microscope in growing cells and, therefore, during IVP, infection may go unnoticed [19]. In the current study, no characteristic cytopathic effects were observed in the developing embryos. However, it was observed that after maturation, the Hobi-like virus treated cumulus-oocyte complexes were visibly different than control or other BVDV-treated groups (data not shown). Irregular cumulus expansion and darkened oocyte cytoplasm may suggest that this particular BVDV virus has some characteristics different than the other non-cytopathic biotypes. While cleavage rates were reduced, only the higher viral doses reduced blastocyst rates, when based on the number of oocytes cultured.

Similar to both BVDV-1 and -2, Hobi-like virus degenerated embryos were positive for the virus by RT-qPCR. However, all viable embryos tested were negative for Hobi-like virus. Since both viable and degenerated embryos were taken from the same micro-drop, these results suggest that Hobi-like virus may cause IVP embryos to stop developing. Further studies are needed to determine if Hobi-like virus does induce embryo loss during IVP.

Using the current model to mimic how BVDV exposure, either

---

**Table 3**

Embryo development of bovine oocytes exposed to different concentration of Hobi-like virus during maturation.

<table>
<thead>
<tr>
<th>Viral Concentration (TCID₅₀/100 μL)</th>
<th>Number of Oocytes</th>
<th>Number of Cleaved Embryos (% per Oocytes)</th>
<th>Number of Blastocysts (% per Oocytes)</th>
<th>Percentage of Blastocyst/Cleaved</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>225</td>
<td>112 (49.8 ± 7.5%)⁴</td>
<td>34 (15.1 ± 1.6%)⁴</td>
<td>30.4 ± 7.1%</td>
</tr>
<tr>
<td>1.90 × 10⁴</td>
<td>226</td>
<td>98 (43.4 ± 4.7%)⁴</td>
<td>25 (11.1 ± 3.5%)⁴</td>
<td>25.5 ± 10.4%</td>
</tr>
<tr>
<td>3.90 × 10⁴</td>
<td>208</td>
<td>79 (38.0 ± 5.3%)⁴</td>
<td>25 (12.0 ± 1.0%)⁴</td>
<td>31.6 ± 8.1%</td>
</tr>
<tr>
<td>7.90 × 10⁴</td>
<td>204</td>
<td>68 (33.3 ± 4.9%)⁴</td>
<td>14 (6.9 ± 0.9%)⁴</td>
<td>20.6 ± 4.7%</td>
</tr>
<tr>
<td>1.58 × 10⁵</td>
<td>210</td>
<td>74 (35.2 ± 3.4%)⁴</td>
<td>21 (10.0 ± 2.7%)⁴</td>
<td>28.4 ± 8.7%</td>
</tr>
</tbody>
</table>

⁴Different superscripts refer to a significance of P < 0.05.
from infected oocytes donors or during oocyte collection, it was demonstrated that different BVDV genotypes (BVDV-1, BVDV-2 and Hobi-like virus) have different effects/infection on production of IVP bovine embryos. BVDV-1 had no significant effect on cleavage or embryo development. BVDV-2 exposure caused a significant increase in cleavage rates, but no significant effect on embryo development. However, viable embryos from both BVDV-1 and -2 were positive for the viruses. Which could potentially lead to transmission to the recipient or calf. Hobi-like virus significantly reduced cleavage rates, but had not significant effect on embryo production. However, no viable embryos were detected with the virus, suggesting Hobi-like virus infection may cause embryo loss prior to blastocyst development. While the potential of transmitting BVDV by IVP is possible, the risk appears minimal. Using computer modeling with abattoir-derived oocytes (ie. unknown infection status of the oocyte source), it was calculated that the probability of transmitting BVDV was 0.0006 and $1.2 \times 10^{-5}$ for embryos either co-cultured or not with granulosa cells, respectively [32]. In addition, these results support previous reports that the IETS trypsin washing protocol is not effective in removing BVDV-1 and -2 from IVP embryos, suggesting new wash protocols need to be investigated.

5. Conclusion

In summary, the current results demonstrate that BVDV exposed oocytes (Hobi-like) have to potential to reduced embryo development or (-1, -2) produced BVDV positive embryos in an IVP system. With vigilant sanitary standards (by testing oocyte donors, IVP medias and degenerated embryos) it may be possible to certify the safety of IVP embryos for international trade.

Acknowledgments

The authors thank FAPESP (Fundaçao de Amparo à Pesquisa do Estado de São Paulo) for financial support (PIPE no 2014/50169-4).

References