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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

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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 (3.12×10^2 - 2.50×10^3 $TCID_{50}$/100 μL), BVDV-2 (6.25×10^1 - 5.20×10^2 $TCID_{50}$/100 μL) or Hobi-like virus (1.90×10^2 - 1.58×10^3 $TCID_{50}$/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 aadition, 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; $P \leq 0.001$–0.014). Blastocyst rates were only reduced in the 7.9×10^2 Hobi-like virus concentration (6.9 ± 0.9% vs 15.1 ± 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.

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 conduct on the sanitary risks of IVP embryos.

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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 of bovine diarrhea, respiratory disease, immunosuppression, abortions, and a reduction in reproductive efficiency that consequently impacts herd health and production [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 [8,9]. 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-Alrich unless specified. Semen was previously certified as free of bovine herpesvirus type 1, Bluetongue virus, bovine leukosis 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 (LVB) at the Instituto Biológico (Sao 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 titered 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 (FolltropinTM, 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.

Culumus-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% CO_2, 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 −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 2×10^5 sperm alive/drop (2×10^6/mL) and cultured for 18–20 h in a humidified incubator with 5% CO_2 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 (Cristalia)), and cultured in a humidified incubator with 5% CO_2 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