

# In vitro interaction of bovine herpesvirus 1 with uterine tube epithelial cells and oocytes

## *Interação in vitro do herpesvírus bovino tipo 1 com células epiteliais da tuba uterina e oócitos*

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**ABSTRACT:** The aims of this study were to assess in vitro if bovine oocytes and oviductal epithelial cells from slaughterhouses for in vitro fertilization use may be infected with bovine herpesvirus 1; to analyze whether the treatment with trypsin according to the International Embryo Transfer Society guideline is efficient to inactivate the bovine herpesvirus 1; to morphologically study the virus-oocyte interaction through optical microscopy. In this study, Madin Darby Bovine Kidney (MDBK) cells that were co-cultured with oocytes matured in vitro and exposed to bovine herpesvirus 1 showed a cytopathic effect. The nested polymerase chain reaction for the supernatant was positive for the bovine herpesvirus 1, thus suggesting that the cytopathic effect observed in the MDBK monolayer was seen due to virus replication and not because of any culture toxicity. It was also observed cytopathic effect and positive nested polymerase chain reaction in MDBK cells co-cultured with in vitro matured oocytes free of virus, but that were co-cultured in uterine epithelial cells pre-infected with bovine herpesvirus 1 and washed or not with trypsin, demonstrating an oocyte contamination by the virus. When trypsin-washing efficacy was evaluated, we could observe that the trypsin treatment was not able to eliminate the bovine herpesvirus 1 of the oocytes, and it was not observed any morphological difference in the infected oocytes.

**KEYWORDS:** bovine herpesvirus 1; oocytes; uterine tube epithelial cells; nested polymerase chain reaction; bovine.

**RESUMO:** Os objetivos do presente estudo foram avaliar *in vitro* se oócitos bovinos e células epiteliais de oviduto provenientes de abatedouros para uso em fertilização *in vitro* podem ser infectados com o herpesvírus bovino tipo 1; analisar se o tratamento com tripsina padronizado pelo *International Embryo Transfer Society* é eficiente para inativar o herpesvírus bovino tipo 1; estudar morfologicamente a interação vírus e oócito pela microscopia óptica. Neste estudo, as células *Madin Darby Bovine Kidney* (MDBK), que foram cocultivadas com oócitos maturados *in vitro* e expostos ao herpesvírus bovino tipo 1, apresentaram efeito citopático. A reação em cadeia da polimerase aninhada ao sobrenadante foi positiva para o herpesvírus bovino tipo 1, sugerindo que o efeito citopático observado na monocamada MDBK foi em função da replicação do vírus, mas não devido a qualquer toxicidade da cultura. Também foram mostrados efeito citopático e reação em cadeia da polimerase aninhada positivos em células MDBK cocultivadas com oócitos maturados *in vitro* isentos de vírus, porém que foram cocultivados em células epiteliais uterinas previamente infectadas com herpesvírus bovino tipo 1, que se lavou ou não com tripsina, demonstrando uma contaminação pelo vírus do oócito. Quando foi avaliada a eficácia de lavagem com a tripsina, foi possível notar que este tratamento não foi capaz de eliminar o herpesvírus bovino tipo 1 dos oócitos, e não foi observada qualquer diferença morfológica nos oócitos infectados.

**PALAVRAS-CHAVE:** herpesvírus bovino tipo 1; oócitos; células epiteliais da tuba uterina; reação em cadeia da polimerase aninhada; bovinos.

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## INTRODUCTION

Embryo cryopreservation techniques have improved over the last decade, providing a safe opportunity for embryo storage and increasing the commercial scale for embryo transfer (ET) and in vitro fertilization (IVF) process. Nevertheless, this fact certainly brought more attention for the potential risks in terms of infectious diseases transmission, such as bovine rhinotracheitis.

The disease agent is the bovine herpesvirus 1 (BoHV-1), which can be found in the ovaries, uterine tube, and follicular fluid. It is apparently more seen in healthy animals, which increases the risk of transmission by the use of these techniques (D'ANGELO, 1998; WRATHALL et al., 2006).

In cows, BoHV-1 infection may lead to respiratory and reproductive disorders, including rhinotracheitis, vulvovaginitis, endometritis and abortions (WRATHALL et al., 2006). In bulls, the virus is known to be replicated in the preputial mucosa, penis, urethra and the semen can be contaminated during ejaculation (MURPHY et al., 1999; WRATHALL et al., 2006; MUYLKEN et al., 2007). GUERIN et al. (1990), using BoHV-1 infected semen and oocytes, found that BoHV-1 did not influence oocyte maturation, but it significantly decreased IVF rates. BIELANSKI; DUBUC (1994) demonstrated that oocytes recovered from BoHV-1 infected cows could be matured and in vitro fertilized, resulting in transferable embryos; however, the ratio of morphologically normal transferable blastocysts was decreased. According to VANROOSE et al. (2000), the zone *pellucida* (ZP) can protect against viral infection. Nonetheless, there is a risk that viral particles may be trapped on the outer layers of the ZP. One way to remove BoHV-1 from the surface of embryos is through a procedure using trypsin baths, which was firstly reported by THIBIER; NIBART (1987), and has been recommended by the International ET Society – IETS (STRINGFELLOW, 1998). However, neither washing nor trypsin treatment seem to completely remove the virus from embryos (EDENS et al., 2003; D'ANGELO et al., 2009).

BoHV-1 has been the focus of risk contamination associated with virus transmission via embryos produced by IVF or somatic cell nuclear transfer, due to its presence in serum and reproductive tissues (WRATHALL et al., 2006).

The aims of this study were to verify if: BoHV-1 can interact with the ZP from oocytes with or without cumulus cells; trypsin washing after oocytes were co-cultured with uterine tube epithelial cells (UTEc) pre-inoculated with BoHV-1 can prevent virus penetration inside the oocyte; trypsin washing after oocyte BoHV-1 exposure can prevent virus penetration inside the oocyte and subsequent virus-induced damages; and there are morphological alterations assessed by phase-contrast inverted microscope, when the oocytes were exposed to BoHV-1.

## MATERIAL AND METHODS

The Madin Darby Bovine Kidney (MDBK) cells and the BoHV-1 were obtained from the Bovine Viruses Laboratory, in the Animal Health Research Center, São Paulo Biological Institute, in São Paulo, Brazil.

The fetal calf serum (FCS), bovine serum albumin (Fraction V), follicular fluid, cumulus cells, oocytes, uterine tube epithelial, and MDBK cells used in all experiments were tested by virus isolation to ensure that they were not already contaminated by BoHV-1.

### Media

Unless otherwise noted, all chemicals and media were purchased from Sigma-Aldrich® (St. Louis, MO, USA) and Invitrogen Life Technologies (Grand Island, NY, USA).

### Madin Darby Bovine Kidney cells culture

The MDBK cells were plated at a density of  $5 \times 10^5$  cells per a 100 mm<sup>2</sup> plastic dish in minimum essential medium (MEM) with Earle's modified Eagle's (Gibco™) supplemented with 5% FCS, penicillin (100 units/mL) – streptomycin (100 µg/mL) (Gibco™) at 38°C in a humidified atmosphere of 5% CO<sub>2</sub> in the air.

### Bovine uterine tube epithelial cells isolation and culture

Bovine uterine tubes were collected in the slaughterhouse immersed in sterile phosphate balanced solution (PBS, Gibco™) with 0.1% penicillin-streptomycin, and transported to the laboratory within two hours. Epithelial cells from the ampulla and isthmus of the uterine tubes were isolated and cultured as previously described (EYESTONE; FIRST, 1989; GONÇALVES et al., 1999).

### Bovine herpesvirus type 1 – stock virus

Stocks of BoHV-1 (Los Angeles strain) were propagated in MDBK cells. The virus was harvested by freezing and thawing the infected cell cultures, and stored in cryovials at -80°C until necessary ( $10^{7.5}$  TCID<sub>50</sub> virus/mL). The plaque forming units per milliliter of stock virus was determined by quantifying plaques on MDBK cells (D'ANGELO, 1998).

### Oocyte collection and in vitro maturation

Bovine ovaries were harvested at a slaughterhouse and placed into Dulbecco's PBS (Life Technologies, Grand Island, NY) at 37°C prior to transport back to the laboratory (GONÇALVES et al., 2007). Once they were there, the ovaries were rinsed twice with 37°C PBS. Oocytes were collected by aspiration of 3-8 mm follicles and washed four times in Hepes buffered TCM-199, containing 0.4 mg/mL BSA, 25 mM Hepes (Sigma®), 0.022 mg/mL

sodium pyruvate and 0.05 mg/mL gentamicin (Gibco™). Then, they were matured in 90 µL bicarbonate buffered TCM-199 containing 10% FCS, LH (6 µg/mL), FSH (8 µg/mL) (Sioux Biochemical®, Sioux Center, IA, USA), and penicillin-streptomycin in an atmosphere of 5% CO<sub>2</sub> in humidified air (BAVISTER et al., 1983). The criteria for oocyte selection was based on previous experience with oocyte quality for IVF, and data reported by HASLER et al. (1995), in which embryo production was improved if oocytes with multiple layers of cumulus cells were used.

## Experimental design

### Experiment 1 – Evaluation of cumulus cells and bovine herpesvirus 1 interaction

The cumulus-oocyte complexes (COCs) submitted to in vitro maturation were divided into two groups, with 20 units each: no exposed to BoHV-1 (Control Group) and exposed to BoHV-1. During all procedures, only oocytes with intact ZP were used. The oocytes groups were separately submitted to sequential washes 12 times in drops of 100 µL in the washing medium (TCM 199, with 0.4% BSA, 0.34 mg/mL pyruvate, 25 mM of Hepes and 100 g/mL gentamicin), changing the tips in all washing steps. Each group was inoculated onto confluent monolayer of MDBK cells free of BoHV-1, and incubated at 38°C in an atmosphere of 5% CO<sub>2</sub> in humidified air. The MDBK cells were daily examined for evidence of cytopathic effect (CPE). After 48 hours, the supernatants from each culture medium were collected, centrifuged at 1,000 rpm for 10 minutes, and stored at -80°C. The dishes were fixed in 20% formaldehyde, stained with gentian violet, daily assessed by phase-contrast inverted microscope (Olympus IX51), and photographed.

### Experiment 2 – Effect of co-culturing oocytes with uterine tube epithelial cells previously inoculated with bovine herpesvirus 1 and subsequently treated with trypsin

Confluent monolayers of UTEC, without virus (Control) and pre-inoculated for 24 hours with BoHV-1, separately received groups of 10 matured oocytes with cumulus and without virus in a co-culture (38°C in an atmosphere of 5% CO<sub>2</sub> in humidified air). After 24 hours of incubation, the oocytes were collected and placed into a vortex for cumulus removal. In one of the non-control groups without cumulus, sequential washes were performed and the other group of oocytes without cumulus was treated with trypsin according to the IETS guidelines (STRINGFELLOW, 1998). This involved five washes in Dulbecco's PBS (Gibco™) with 0.4% BSA, penicillin G (100 IU/mL), streptomycin (100 µg/mL), and amphotericin (0.25 µg/mL). This was followed by other two washes with 0.25% trypsin (Gibco™) in Hank's balanced salt solution (Gibco™) for a total of 60 to 90 seconds of exposure, and later five additional washes that were identical to the first five ones. All groups were inoculated in confluent monolayer of MDBK

cells, and incubated at 38°C in an atmosphere of 5% CO<sub>2</sub> in humidified air for 48 hours. After such period of incubation, the CPE presence was determined. The supernatants were stored as described for subsequent titration of the virus on a microplate and the PCR tests. The dishes were fixed, stained, and photographed as already described.

### Experiment 3 – Effect of trypsin treatment on oocytes previously exposed to bovine herpesvirus 1

The cumulus cells complexes (COCs) were divided into two groups with 20 oocytes each during in vitro maturation: no exposure to BoHV-1 (Control Group) and exposed with BoHV-1. After 24 hours, all groups were vortexed to remove cumulus cells and separated in two subgroups: those submitted to the sequential washes and those treated with trypsin, as in Experiment 2. Each group of oocytes were co-cultured with confluent monolayers of UTEC free of virus at 38°C, in an atmosphere of 5% CO<sub>2</sub> in humidified air. After 48 hours, the supernatants of the cultures were collected, centrifuged at 1,000 rpm for 10 minutes, and inoculated in confluent monolayers of MDBK cells kept under the conditions described. The CPE was seen after 24 hours of incubation. The supernatants of these cultures were collected and stored as mentioned for the virus titration on a microplate and the PCR test. The dishes were fixed with 20% formaldehyde, stained with gentian violet, daily assessed by phase-contrast inverted microscope, and photographed.

### Experiment 4 – Morphological evaluation of in vitro matured oocytes inoculated with bovine herpesvirus 1

The COCs were divided into three groups with 20 oocytes each, during the in vitro maturation: none were exposed with BoHV-1 (Control Group), exposed with BoHV-1 (10<sup>7.5</sup> TCID<sub>50</sub>virus/mL) and exposed with BoHV-1 (10<sup>8.5</sup> TCID<sub>50</sub>virus/mL). After 24 hours, the oocytes were washed in buffered TCM-199, assessed by phase-contrast inverted microscope, and photographed.

## Nested polymerase chain reaction

For DNA extraction, oocytes, cells, and MEM medium were assayed separately for BoHV-1. Briefly, 400 µL from each sample were washed in Tris-EDTA (TE) buffer (10 mM Tris HCl, pH = 8.0; 1 mM EDTA), and digested by overnight incubation at 37°C in lyses buffer (10 mM Tris-HCl, pH = 8.0; 100 mM NaCl; 25 mM EDTA; 1% SDS; 400 µg/mL proteinase K). DNA was extracted via phenol, phenol/chloroform steps, and then it was precipitated with two volumes of 100% ethanol. Following overnight incubation at -20°C and centrifugation, each pellet was re-suspended in 30 µL of TE buffer, incubated for 10 minutes at 56°C and stored at -20°C until amplification.

The PCR assay was carried out through a 50 µL solution with 10 µL of DNA, Tris HCl 10 mM, pH = 9.0, MgCl<sub>2</sub> 1.5 mM, 200 µM of each nucleotide (dNTPs), 5% glycerol, 2.5 U Taq

DNA polymerase enzyme, and 50 pmol of each sense and anti-sense primers (5' CCT CTG TGA ACT GCA TCG TGG A 3'; 5' TAG CCC TCG ATC TGC TGG AGG C 3'). Those primers amplified a 175 bp conserved region of BoHV-1 gB gene (VILCEK et al. 1994; D'ANGELO, 1998; D'ANGELO et al., 2009). Such amplification included denaturation at 95°C for one minute, annealing at 57°C for 1 minute, synthesis at 72°C for 1.5 minutes, and a final extension at 72°C for 10 minutes. In each reaction, a negative control (water), positive control RNA, and external DNA standards were included for evaluation and comparison in order to be able to detect the viral RNA in a sample. Data were displayed and analyzed by electrophoresis in 2% agarose gel in TBE buffer (Tris, boric acid, EDTA 0.5 mM, pH = 8.0). The gel was stained with bromide ethidium (5 µg/mL), and visualized through the UV transilluminator. The pictures were taken by Kodak Digital Science DC-40 camera.

This work was performed according to the ethical principles of animal experimentation established by the Brazilian Society of Science in Laboratory Animal/Brazilian College of Animal Experimentation (SBCAL/COBEA), as stated in certificate number 55/08 (08/22/2012), issued by the Animal Experimentation Ethics Committees – CETEA-IB.

## Statistical analysis

Ten replicates of the trial were performed, and for each, at least 200 oocytes were matured. Proportion of the different treatments during all experiments was compared using the chi-square analysis with the program Epi-Info, 2000 version.

## RESULTS

### Evaluation of cumulus cells and bovine herpesvirus 1 interaction – Experiment 1

The MDBK culture inoculated with in vitro matured oocytes infected with BoHV-1, showed CPE evidence. The BoHV-1 presents a focal CPE with rounding cells, and lyses. The MDBK cells that co-cultured with the control oocytes, free of virus, did not show evidence of CPE. The n-PCR was positive for the supernatants from oocytes in vitro matured with BoHV-1.

### Effect of co-culturing oocytes with uterine tube epithelial cells previously inoculated with bovine herpesvirus 1 and subsequently treated with trypsin – Experiment 2

It was observed CPE in MDBK cells that were co-cultured with free virus oocytes, but from infected monolayers of

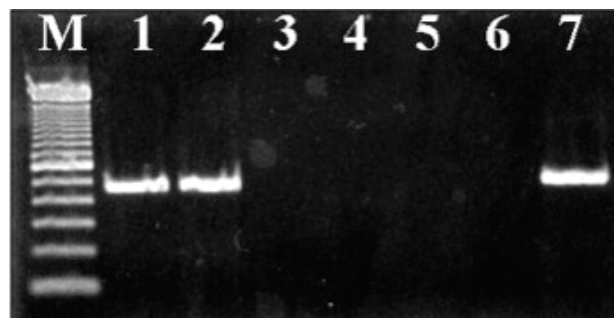
UTEC and washed or not with trypsin. CPE in the MDBK co-cultured with free virus oocytes was not observed. It was originated from UTEC, but not inoculated with BoHV-1. The n-PCR was positive for the supernatants with CPE, and negative for the samples without CPE (Fig.1).

### Effect of trypsin treatment on oocytes previously exposed to bovine herpesvirus 1 – Experiment 3

The monolayers of bovine epithelial cells (BOEC) that co-cultured with in vitro matured oocytes pre-infected with BoHV-1, washed or not with trypsin, showed CPE. The CPE from trypsin treatment was less intense. The BOEC control did not present CPE. The n-PCR was positive for the samples that showed CPE and negative for the samples without CPE (Fig. 2).



**Figure 1.** Amplification by nested polymerase chain reaction of BoHV-1 in supernatant of MDBK cells co-cultured with oocytes pre-cultured with uterine epithelial cells pre-inoculated with BoHV-1 (Experiment 2). M: weight molecular markers; 1: oocytes treated with trypsin; 2: oocytes submitted to sequential washes; 3: control (sequential washes); 4: supernatant of non-infected uterine epithelial cells culture; 5 and 6: negative controls (water); 7: positive control (BoHV-1 from MDBK cells).



M: weight molecular markers; 1: oocytes treated with trypsin; 2: oocytes submitted to sequential washes; 3: control (trypsin treatment); 4: control (sequential washes); 5 and 6: negative controls (water); 7: positive control (BoHV-1 from MDBK cells).  
**Figure 2.** Amplification by nested polymerase chain reaction of BoHV-1 in bovine oocytes pre-inoculated with BoHV-1.

## Morphological evaluation of in vitro matured oocytes inoculated with bovine herpesvirus 1 – Experiment 4

The oocytes in vitro matured with BoHV-1 in different concentrations, and the oocytes free of virus were morphologically similar when assessed by phase-contrast inverted microscope. The cumulus cells did not show any morphological alteration.

### DISCUSSION

In our study, we observed whether: BoHV-1 can interact with the ZP of oocytes with or without cumulus cells; trypsin washing after oocytes co-cultured with UTEC pre-inoculated with BoHV-1 can prevent virus penetration inside the oocyte; trypsin washing after oocyte BoHV-1 exposure can prevent virus penetration in the oocyte and subsequent virus-induced damages; and there are morphological alterations assessed by phase-contrast inverted microscope when the oocytes were exposed with BoHV-1.

The mammalian ZP is formed around the oocyte during the early stages of follicular development, and remains with the egg during uterine tube transport, fertilization, and early embryonic development. The ZP is comprised of three to five glycoproteins depending on the species. ZP proteins have been implicated in specific sperm binding of species, the prevention of polyspermy, and embryo protection between fertilization and implantation (DUNBAR et al., 1991; LASIENE et al., 2009; GUPTA et al., 2012; YONEZAWA, 2014). However, there is a risk that virus particles could be trapped in the layers of the ZP (VANROOSE et al., 2000; VAN SOOM et al., 2010). SINGH et al. (1982) demonstrated that bovine embryos (from 16-cell to blastocyst stage) with intact ZP that had been exposed to BoHV-1 for 24 hours did not show embryonic infection; but the virus was recovered from most of these embryos even after extensive washing. These observations mean that although the ZP is an effective barrier to BoHV-1, the virus may stick to this structure.

Previous studies were conducted with in vitro fertilized embryos and have included the use of infected semen and/or exposure of oocytes to the virus at the time of fertilization to evaluate the risks of BoHV-1 transmission. GUERIN et al. (1990), using BoHV-1 exposed oocytes or sperm in an in vitro embryo production procedure, found that BoHV-1 did not influence oocyte maturation, but it significantly reduced the IVF and increased the frequency of sperm abnormalities, such as sperm head decondensation. Similarly, BIELANSKI; DUBUC (1994) tested in vitro embryo production with oocytes from experimental and also natural infected donor cows and observed that the blastocyst rate decreased, and showed the presence of virus from both donors. EDENS et al. (2003) observed the susceptible recipients by the BoHV-1, studying in vitro produced embryos infected by BoHV-1, washed with trypsin and co-cultured with

uterine tubal cells. They concluded that trypsin washing might prevent infection of recipients if individual day-seven exposed embryos were transferred into the uterus.

D'ANGELO et al. (2009) studied the efficiency of trypsin treatment on the inactivation of BoHV-1. They exposed bovine embryos produced in vitro with HPV-1, Los Angeles strain, and submitted them to sequential washes or to the trypsin treatment according to the IETS guidelines. The study demonstrated that the trypsin treatment was not able to eliminate the BoHV-1 of the embryos. However, BIELANSKI et al. (2013) concluded that recombinant bovine trypsin (RBT<sub>r</sub>) was effective at the inactivation of BoHV-1 from ZP intact, in vivo produced embryos. The RBT<sub>r</sub> showed reduced infectivity of the BoHV-1 associated with the intact ZP, which prevented transmission of the disease to ET recipients and their offspring.

In this study, MDBK cells co-cultured with in vitro matured and exposed to BoHV-1 oocytes showed a CPE more intense when cumulus cells were present than without cumulus cells. The n-PCR for the supernatant was positive for the BoHV-1, suggesting that CPE observed in the MDBK monolayer was seen due to the virus replication, but not because of any culture toxicity. It was also observed CPE and positive n-PCR in MDBK cells co-cultured with in vitro matured oocytes free of virus, but that were co-cultured in uterine epithelial cells pre-infected with BoHV-1 and washed or not with trypsin, demonstrating an oocyte contamination by the virus. When the efficacy of trypsin washing was evaluated, we could observe that the trypsin treatment was not able to eliminate the BoHV-1 of the oocytes, and no morphological difference was found. The known potential for natural exposure to BoHV-1 and the relative ineffectiveness of oocyte processing procedures (washing and trypsin treatment) create legitimate concerns that they might be transmitted with exposed oocytes, MBDK, or UTEC. However, it has not been determined that the quantity of infectious virus associated with these oocytes would constitute an infective dose for susceptible recipients via the intrauterine tube route.

Here are the conclusions taken from this study:

- The cumulus cells have an important role in the virus-oocyte interaction, and maybe they can have receptors for BoHV-1.
- The uterine tube epithelial and MBDK cells are susceptible to BoHV-1.
- The trypsin washing was not able to eliminate the BoHV-1 of the oocytes;
- The BoHV-1 may interact with the ZP, and there is a risk that virus particles could be trapped in the layers of the ZP.
- The absence of morphological alterations, observed by contrast phase microscope, can contribute to dissemination of the virus in animal biotechnologies, like in vitro embryo production or cloning.

Finally, full understanding of the exact interaction between BoHV-1 and oocyte, sperm, and embryo requires further studies.

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