COMPARATIVE STUDY OF THE CLASTOGENIC ACTION OF BOVINE HERPESVIRUS 1 (BoHV-1) IN PRIMARY AND ESTABLISHED CELL CULTURES.

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ABSTRACT

The concern about the transmission of infectious diseases by the use of biotechnologies, like in vitro fertilization (IVF), has been increasing. The aim of this study was to verify the clastogenic alterations in the DNA of Madin-Darby Bovine Kidney (MDBK) and Bovine Oviduct Epithelial Cells (BOEC) infected or not with bovine herpesvirus 1 (BoHV-1), for 24 hours, by the use of the Comet Assay or Single-Cell Gel Electrophoresis (SCGE). The nuclear change was evaluated by measuring the extent of DNA damage, where it was possible to observe 1.7% of level II comet and 2.1% of level IV in the BOEC group “in situ“. However, in BOEC infected by the virus the damages observed were 13.8% comet level I, 0.6% level III and 1.2% level IV. The MDBK cells not infected group showed displayed 1.9% level II and the infected group 26 % level I, 12.3 % level II and 6.1 % level III. All groups, regardless if they were infected or not by the virus, were considered positive by the comet assay, maybe because there has not been virus association with the cell DNA. The number of comets detected in the BOEC “in situ“ corresponds to spontaneous mutations that use to be present in organisms with failure of DNA repair. In vitro established and primary cells show greater failure frequency in DNA repair mechanisms, and may present more mutations. Therefore, in vitro models provide better conditions to mimic in vivo conditions. These data have demonstrated that not always the IVF problems are due to pathogens infection.
INTRODUCTION

In recent years, the increasing worldwide population and food demand has served as a stimulus for the use of new reproductive technologies (Gonçalves et al. 2007, Parra et al. 2008, Ricarte et al. 2008, Stringfellow & Wrathall 1995). The *in vitro* production of embryos (IVP) coupled with embryo transfer (ET), is in the process of accelerated development and diffusion. Also, its use has enabled the production of embryos with high genetic potential (Gonçalves et al. 2007, Ricarte et al. 2008). Accordingly, there has been a major concern about the risks of spreading infectious agents from the reproductive sphere as bovine herpesvirus type 1 (BoHV-1) may be present in ovaries, uterine tubes and follicular fluid of apparently healthy animals. Also the risk of transmission can be facilitated by these techniques (Bielanski & Dubuc 1994, D'Angelo et al. 2002, 2005, 2009, Ferreira et al. 2005, Rufino et al. 2006).

Embryos fertilized *in vivo* show differences from those fertilized *in vitro* with regard to development, physiological traits and, especially, the characteristic and composition of the zona pellucida. These differences may facilitate the infection of IVP embryos, mostly because the health status of the donor is unknown. Therefore the introduction of pathogens during the collection of oocytes, the maturation process and capacitation *in vitro* and thus the use of IVP embryos might lead to a higher risk of disease transmission (D’Angelo et al. 2009, Gonçalves et al. 2007, Parra et al. 2008, Rufino et al. 2006, Stringfellow & Wrathall 1995).

Several cytogenetic and biochemical methods have been used in order to assess the damage of the DNA from cells subjected to aggression imposed by chemical, physical and biological agents (Fairbain et al. 1995, McKelvey Martin et al. 1993).

A simple and fast method used to quantify DNA damage in individual cells is the Comet Assay, also known as microgel electrophoresis. Briefly, the test consists of embedding the cells in agarose, lysis, and electrophoresis under low voltage, so that fragmented and relaxed DNA migrate farther than intact or cross-linked DNA, resembling the image of a comet. The extent of migration of the “tail” of the comet is related to increased DNA damage. These images can be analyzed and compared in a cell-to-cell basis (Fairbairn et al. 1995, McKelvey-Martin et al. 1993). Because it is a method which directly analyzes DNA damage at individual cell level (and hence a particular cell
subpopulation) and requires a very small amount of cells, its use in reproduction techniques is of great importance (D’Angelo et al. 2000, 2005).

The aim of this study was to apply the comet assay to assess possible clastogenic changes in the DNA of primary epithelial cells from Bovine Oviduct Epithelial Cells (BOEC) and cells established from Madin Darby Bovine Kidney (MDBK) exposed experimentally to bovine herpesvirus type-1.

MATERIAL AND METHODS

The Madin Darby Bovine Kidney (MDBK) cells and the BoHV-1 virus were obtained from the Bovine Viruses Laboratory, Animal Health Research Center, São Paulo Biological Institute, SP, Brazil. Fetal calf serum (FCS, Gibco™, Invitrogen Corporation, Grand Island, NY, USA), uterine tube epithelial and MDBK cells used in all experiment were tested by virus isolation to ensure that they have not been already contaminated by BoHV-1.

Madin Darby Bovine Kidney (MDBK) cells culture.

The MDBK cells were plated at a density of $5 \times 10^5$ cells per 100 mm$^2$ plastic dish in minimum essential medium (MEM) with Earle’s modified Eagle’s (Gibco™) supplemented with 10% FCS, penicillin (100 units/mL) - streptomycin (100 µg/mL) (Gibco™) at 37.5 ºC in a humidified atmosphere of 5% CO$_2$ in air.

Bovine Oviduct Epithelial Cells isolation and culture.

Bovine Oviduct Epithelial Cells were collected at the slaughterhouse, immersed in sterile phosphate balanced solution (PBS, Gibco™) with 0.1% penicillin-streptomycin, and transported to the laboratory within 2 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). Briefly, the mucosal tissues containing epithelial cells were washed 3 times with 5 mL of PBS with 0.1% penicillin-streptomycin and the cells recovered after passive sedimentation. The cellular suspension was diluted 50 times in Tissue Culture Medium-199 (TCM-199, Gibco®) with 10% of fetal calf serum (FCS, Gibco®), 0.1% penicillin-streptomycin, 34 mg of sodium pyruvate (Gibco®) and incubated at 37°C in a humidified atmosphere of 5% CO$_2$ in air. The medium was
renewed every 2 days. The development of the primary cultures was assessed daily by phase-contrast inverted microscope and monolayer reached total confluence in 5 to 7 days.

**Virus.**

The reference strain of Bovine Rhinotracheitis Virus, Colorado sample (ATCC VR-864), 10th passage in MDBK cells, with the title of $10^{4.5}$ TCID$_{50}$/ml kept in Eagle MEM medium without serum at -80°C was used in this study.

**Exposure of cells to the virus.**

Confluent monolayers from both cells were prepared in 25cm$^2$ flasks. The culture medium was replaced by PBS for the washing procedure. After its removal, the cells were inoculated with 100µL of the viral strain suspension. The inoculum was homogenized twice over the monolayer and maintained for 15 minutes at 37.5°C in an atmosphere of 5% CO$_2$. Later, 8mL of TCM 199 medium was added, the flasks were kept at 37.5°C with 5% CO$_2$ for 24 hours, and then observed for cytopathic effect. The supernatant was discarded and the cells were removed using trypsin, centrifuged and the cell pellet was subjected to the Comet assay. The BOEC "in situ" group was subjected to the test immediately after collection from the uterine tube, and thus being considered the control group.

**Comet Assay.**

All groups were subjected to the Comet assay, following the protocol described by Singh *et al.* (1988). The cells were placed on microscope slides precoated with 300µL normal electrophoresis grade agarose (Sigma®, St. Louis, MO, USA) in 0.7% in PBS free of Ca$^2+$ and Mg$^2+$ at 65°C. The coverslips were removed and cells were added to 100mL of "low melting" agarose (Sigma®) in 0.5% in PBS free of Ca$^2+$ and Mg$^2+$ at 37°C and covered with a glass coverslip. Gels were left to set for 5 minutes at 4°C. The coverslips were then removed and the cells carefully submerged in lysing solution consisting of 2.5M NaCl, 100mM EDTA, 10mM Tris, 1% sodium sarcosinate, pH 10, 1% Triton X-100, 10% DMSO for 2 hours at 4°C. Slides were then placed in a horizontal gel electrophoresis unit filled with fresh, chilled electrophoresis buffer (pH> 12: 1mM EDTA pH 10, 300mM MAOH) to a level ~0.25 cm above the slides and left for 30 min to allow
the DNA to unwind fully. Electrophoresis was conducted for 30 minutes at 25V-300 mA at 4°C. The slides were then neutralized three times using Tris buffer (0.4M, pH 7.5), 5 minutes each wash, stained with 50µL of ethidium bromide (Sigma®), covered with coverslips and kept protected from light in a moist chamber.

**Analysis of slides.**

The slides were examined under a fluorescence microscope (Olympus BX51) using a 200x magnification. The evaluation of nuclear modification was performed by the category of damage, based upon the visual appearance of comets, grouping them into 5 classes (0, I, II, III, IV). Comets with bright heads and no apparent tails were classified as class 0 (cells without DNA migration), comets with very small heads and long and diffuse tails were classified as Class IV (severely damaged cells). Comets with intermediate characteristics were divided and classified as Class I, II and III, respectively (Visvardis et al. 1997).

**RESULTS**

The results were analyzed by ANOVA assuming P <0.05 as significant. The change in the category of nuclear damage was evaluated in each group, where in the BOEC “in situ” group 1.7% comets Level II and 2.1% Level IV were observed. In the BOEC uninfected group 13.8% comets Level I, 0.6% Level III and 1.2% Level IV were found, while in the BOEC infected group, 7.7% Level I, 2.8% Level II, 1.4% Level III and 0.7% Level IV were observed. The MDBK uninfected group showed 1.9% comets Level II, and the infected group showed 26% Level I, 12.3% Level II and 6.1% Level III (Table 1).

**DISCUSSION**

The comet assay is a technique used to detect the DNA break produced by genetic changes in cells. Due to the low operating cost, it is a test used in the analysis of genotoxicity (Fairbairn et al. 1995, McKelvey Martin et al. 1993). It has some advantages over biochemical and cytogenetic tests, as it can be used for any cell type (any material that can be extracted from nucleated cells), requiring only a small number of them and do not require dividing cells (Ostling & Johanson 1987).
Table 1. Comparative study of the clastogenic action of bovine herpesvirus 1 (BoHV-1) in primary and established cell cultures by Comet Assay.

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>LEVEL I</th>
<th>LEVEL II</th>
<th>LEVEL III</th>
<th>LEVEL IV</th>
</tr>
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<tbody>
<tr>
<td>BOEC in situ</td>
<td>_</td>
<td>1.7 %</td>
<td>_</td>
<td>2.1 %</td>
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<tr>
<td>BOEC uninfected</td>
<td>13.8 %</td>
<td>_</td>
<td>0.6 %</td>
<td>1.2 %</td>
</tr>
<tr>
<td>BOEC infected</td>
<td>7.7 %</td>
<td>2.8 %</td>
<td>1.4 %</td>
<td>0.7 %</td>
</tr>
<tr>
<td>MDBK uninfected</td>
<td>_</td>
<td>1.9 %</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>MDBK infected</td>
<td>26 %</td>
<td>12.3 %</td>
<td>6.1 %</td>
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</table>

Abbreviations: MBDK = Madin Darby bovine kidney cells; BOEC = uterine tube epithelial cells.

* P <0.05

As it was demonstrated in the present study, that cells in the control group displayed comets Level II. It was also possible to observe the presence of comets in the other groups, despite the presence of the virus. However, there was an increase in the amount and level of cellular damage in the infected groups, when compared to the control. It was observed in uninfected MDBK cells, that the nuclei displayed less DNA damage than in uninfected BOEC cells, however the infected MDBK cells showed more damaged nuclei than those of BOEC. This is the first report about Comet Assay and BoHPV-1 infected cells.

The primary cells have characteristics closely resembling those of cells in vivo (Gonçalves et al. 1999), explaining the number of comets detected in BOEC “in situ”, indicating spontaneous mutations that occur in organisms when there are failures in the repair of DNA. It should be highlighted that there is no cell without DNA damage,
whereas the cellular metabolism can cause daily lesions in the cell’s DNA.

According to Geiser et al. (2008), four types of cell death occur in eukaryotes: apoptosis, autophagy, oncosis, and pyroptosis. Necrosis is the predominant type of cell death in infected MDBK cells. Established or primary cells (in vitro) have an increased frequency of failures in DNA repair mechanisms, therefore, being more susceptible to mutations. However, experimental models that use the primary culture provide better conditions to mimic the situation in vivo.

It was conclude that, not always, the viability of the technique of IVP (embryo production in vitro) is caused by pathogens infection. When using the ovaries of slaughtered cows, the lack of good management practices could be interfering with the quality and even possibly be triggering clastogenic changes in the epithelial cells of the oviducts collected for the co-culture of embryos. When subjected to in vitro conditions, the manipulation in the laboratory and environmental alterations, such as light, pH, temperature, among others, these cells become more susceptible to external factors and hence mutations occur.

REFERENCES


Ostling O, Johanson KJ 1987. Bleomycin, in contrast to gamma irradiation, induces


