Molecular and Cellular Probes 28 (2014) 113–117

Contents lists available at ScienceDirect

Molecular and Cellular Probes

journal homepage: www.elsevier.com/locate/ymcpr

Short communication

Programmed cell death-associated gene transcripts in bovine embryos exposed to bovine *Herpesvirus* type 5

Camila Silva-Frade [a], Roberto Gameiro [a], Lucas Hidenori Okamura [a], Eduardo Furtado Flores [b], Tereza Cristina Cardoso [a,*]

[a] UNESP-São Paulo State University, Laboratory of Animal Virology and Cell Culture, College of Veterinary Medicine, São Paulo 16050-680, Brazil
[b] UNESP-São Paulo State University, Departament of Preventive Veterinary Medicine, Federal University of Santa Maria, UFSM, College of Veterinary Medicine, Santa Maria, RS 97115-900, Brazil

ARTICLE INFO

Article history:
Received 16 August 2013
Received in revised form
25 September 2013
Accepted 2 October 2013
Available online 13 December 2013

Keywords:
Programmed cell death
Apoptosis
Alphaherpesvirus
Bovine embryos
Mitochondrion

ABSTRACT

In vitro-produced bovine embryos become infected after exposure to bovine *Herpesvirus* type 5 (BoHV-5), yet no changes in developmental rates, mitochondrial activity and inhibition of apoptosis are detected in comparison to unexposed embryos. Thus, the aim of the present study was to assess the transcription of mitochondria-mediated apoptosis genes using TaqMan real-time polymerase chain reaction. Transcripts of mcl-1, caspase-2, -3, Apaf-1 and Bax genes were measured after exposure to BoHV-5 *in vitro*. Mitochondrial dehydrogenase activity was evaluated by MTT test and compared between groups of exposed and unexposed embryos, at day 7 of development. The rate of oocyte maturation was assessed by the extrusion of the first polar body. In summary, BoHV-5 exposed embryos retained their viability, mitochondrial dehydrogenase activity and displayed up-regulation of transcription of survival mcl-1 gene and down-regulation of Bax transcription in relation to mitochondria-mediated pathway which might improve embryo viability. These findings demonstrate that BoHV-5 exposed embryos maintain their viability and mitochondrial dehydrogenase activity with no compromise of embryos produced *in vitro*.

Bovine *Herpesvirus* type 5 (BoHV-5) belongs to the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Varicellovirus* and is associated with meningoencephalitis in cattle and, to a lesser extent, also incriminated in reproductive disorders [1]. BoHV-5 replication *in vitro* is characterized by a rapid lytic cycle especially in Madin–Darby bovine kidney (MDBK) cells. In vivo, acute BoHV-5 replication in the nasal mucosa is followed by establishment of latent infection in sensory nerve ganglia [2]. Different virus replication behavior *in vitro* has been described for different field isolates [3,4].

Several mammalian DNA and RNA viruses have been shown to be associated with induction of cell apoptosis [5]. Apoptosis is a genetic and biochemical process that plays an essential role in morphogenesis, host defense and homeostasis of cell tissues. Caspases are a family of cysteine proteases that mediate apoptosis induced by a variety of stimuli [6]. Based on their structure and order in cell death pathways, caspases can be divided into initiators (such as caspase-2, -8, -9, -10, and -12) and effectors (such as caspase-3, -6, and -7) [6]. Viruses possess various biochemical and genetic mechanisms to evade and/or to induce apoptosis modulation through virus-encoded proteins [7,8]. Two death pathways, intrinsic and extrinsic, have been identified in most cases of caspase-dependent apoptosis. The intrinsic death pathway involves mitochondrial release of cytochrome *c* which interacts with Apaf-1 and dATP to promote procaspase-9 auto activation, which in turn, activates downstream effectors such as caspase-3, -6, and -7. The extrinsic death pathway is initiated by engagement of cell surface death receptors (CD95/Fas/APO-1) and tumor necrosis factor receptor [9]. The complex of death receptors and ligands leads to the recruitment of the adapter molecule FAAD and the activation of caspase-8 [6]. In some cells, active caspase-8 is sufficient to lead to caspase-3 activation by cleaning the proapoptotic BCl-2 family members, including mcl-1, which induces mitochondrial cytochrome c release and, thereby, links the two pathways [10,11]. After activation, caspases cleave various cellular substrates resulting in membrane blebbing, chromatin condensation and formation of apoptotic bodies [12]. Modulation of apoptosis seems to be a key step in *herpesviruses* pathogenesis [12,13].

Recently, we demonstrated that experimental exposure of bovine gametes to BoHV-5 led to the infection of *in vitro*-produced embryos without interference in embryonic development. In addition, derived embryos revealed less apoptotic cells detected by annexin-V and TUNEL analysis [14,15]. Moreover, in another study,

* Corresponding author. Tel.: +55 18 36361379.
E-mail address: tcardoso@fmva.unesp.br (T.C. Cardoso).

0890-8508/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.mcp.2013.10.005

BoHV-5 exposed bovine blastocysts seemed to display an increase on mitochondrial membrane potential [16]. Determining how BoHV-5 interferes with cell-death pathways will not only improve our knowledge of viral pathogenesis but also has the potential to advance our understanding of the processes that control cellular death pathways. Besides, BoHV-5 has been, in some cases, associated with reproductive disorders worldwide [17–22].

The objective of this study was to investigate the role of mitochondrial intrinsic pathway of apoptosis *in vitro* produced bovine embryos exposed to BoHV-5. Caspase-2, -3, mcl-1, Bax and Apaf-1 expression was investigated by assessing gene transcription. Both mitochondrial dehydrogenase activity and embryo development were also investigated after virus exposure.

BoHV-5 isolates obtained from animals affected during an outbreak in Araçatuba, SP, Brazil in 2007 were propagated in "Madin Darby Bovine Kidney" cells (MDBK, ATCC CCL-2). Cells were grown under the culture conditions described previously [2]. After virus amplification, virus titers were calculated and expressed as mean tissue culture infective dose per 50 μl ($TCID_{50}$). Aliquots of virus stock (100 μL) with $10^{3.4}$ $TCID_{50}$/50 μL were frozen at −86 °C until embryo exposure. Bovine oocytes used were collected from ovaries of crossbred cows obtained in local slaughterhouses. Ovaries were transported to the laboratory within 1 h *post mortem* in physiological solution at 37 °C. The *cumulus*-oocytes complexes (COC) were recovered by aspiration of the follicles with a diameter of up to 2.6 mm, with the aid of a syringe (10 ml) and needle (40 × 12). Only the COC with 3–4 or more cell layers of compact *cumulus* and homogeneous cytoplasm were selected.

The oocytes were divided into two groups, depending on the treatment: unexposed ($n = 357$) and exposed ($n = 388$) to BoHV-5. Before virus exposure the oocytes were conditioned in cryotubes containing 400 μL of maturation medium (MM). The unexposed group received 40 μl of fresh MEM (Sigma–Aldrich®, St. Louis, MO, USA) and the exposed group received 40 μl of viral suspension, derived from the virus stock containing $10^{3.4}$ $TCID_{50}$/ml. Virus adsorption was carried out at humid atmosphere and 5% of CO_2 for 1 h at 38.8 °C. After virus adsorption, oocytes were washed three times in MM and allocated in drops of 70 μl of MM, distributed in Petri dishes (35 × 10 mm) under mineral oil, with each drop containing a maximum of 20 oocytes. A drop of culture was the experimental unit of work, there were 23 replicates, or 23 drops (experimental units), for each group. At each step the same number of drops was made for each group, and the dishes were kept under the same conditions described before for a period of 23 h. After 23 h of maturation, 100 oocytes from each group (unexposed and exposed) were washed three times in droplets 400 μl of PBS solution with hyaluronidase 5 mg/ml (Sigma–Aldrich®). The oocytes were pipetted successively to promote complete removal of cumulus cells in order to analyze the first polar body extrusion by inverted microscope (IX70, Olympus, Tokyo, Japan). Polar bodies were counted in both unexposed and exposed oocytes.

Frozen bovine semen from a single bull and in the same sample was used in the *in vitro* fertilization (IVF) in 0.5 ml vanes. The semen was centrifuged in a Percoll gradient 700× g for 20 min. The spermatozoid pellet was washed in TALP-FERT (Tyrode albumin, lactate and pyruvate) media, supplemented with 6 mg/ml of BSA, 30 μg/ml of heparin and PHE solution (2 mM of penicillamine, 1 mM of hipotaurine and 250 mM of epinephrine) and centrifuged at 200× g for 5 min. The pellet was diluted in TALP-FERT medium to a final concentration of 1×10^5 sperm/ml in drops of 100 μl. After 24 h of maturation, oocytes were washed and transferred to drops with 100 μl of TALP-FERT medium. In the IVF, oocytes and sperm were co-incubated for 20 h. After this period, presumptive zygotes (pz) were denuded by repeated pipetting and placed on drops with 100 μl of *in vitro* culture medium SOFm (synthetic oviduct fluid modified) until day seven, being considered day zero the day of fertilization. The cleavage rate was evaluated at day three, and the feeding realized for removal and addition of 50 μl of fresh medium of the same composition. The procedure of feeding was repeated on day five. IVF and culture were carried out under the same conditions described for IVM. Embryo development was monitored, and cleaved oocytes and blastocyst stages (BL), expanded blastocyst (BX) and hatched blastocyst (BE) were evaluated at days three and seven, respectively. On day seven, only embryos of grade 1 (excellent or good) or grade 2 (good or reasonable) according to the IETS instructions (1998), were stored for further analysis. All animal reagents used in this study were tested for the presence of BoHV-1 and 5 [23] in order to ensure the absence of non-experimental viral contamination.

Table 1
Effects of experimental exposure of bovine oocytes to BoHV-5 during *in vitro* embryo development.

Groups	Oocytes (n)	Embryo development − n (%)	
		Cleaved 72 h (mean ± S)	bl/bx/be 168 h (mean ± S)
Unexposed	357	313 ± 6.5 (87.7)[a]	195 ± 3.2 (54.6)[a]
Exposed	388	328 ± 8.9 (84.5)[b]	193 ± 3.2 (49.7)[a]

[a,b]Values followed by the same letter, in the column, do not differ by t test ($p < 0.05$).
blastocyst stages (BL), expanded blastocyst (BX) and hatched blastocyst (BE).
h = hours post *in vitro* fertilization.

Mitochondrial activity was assessed using the "*In vitro* toxicology assay kit based MTT" (Sigma–Aldrich®) using spectrophotometry. The system measures the MTT activity of viable cells through detect the mitochondrial dehydrogenases activity. The key component is (3 − [4,5-dimethylthiazol-2-yl] -2,5-diphenyl tetrazolium bromide) or MTT. The MTT solution dissolved in saline balanced salt solution without serum or phenol red shows a yellowish color. The mitochondrial dehydrogenases activity of viable cells cleave tetrazolium ring to form formazan crystals, which have purple and are insoluble in water. The crystals are dissolved in acidified isopropanol, resulting in a purple solution is measured by spectrophotometry at 600 nm.

Total RNA, from unexposed/exposed embryos was extracted using TRIzol® reagent (Invitrogen®, California, USA) following the manufacturer's recommendations. A total of 2 ng of each RNA sample was treated with 1 μl of DNAse (Sigma–Aldrich®, DNAse 1 mg) and reverse-transcribed using the kit high capacity RNA-to-cDNA™ (Applied Biosystems™). The expression of apoptotic and anti-apoptotic regulating genes was quantified using software on a StepOnePlus® real time instrument (Applied Biosystems™). The real time PCR mix (50 μl) contained 1.2 μg of cDNA, 400 nM primers and 200 nM probes FAM-MGB (5′region) customized for Apaf-1 (Bt03210919_g1), Bax (Bt03211776_m1), caspase-2 (Bt03817113_m1), caspase-3 (Bt03250955_g1) and mcl-1 (Bt03276965_g1), bovine sequences (Applied Biosystems™). The PCR was initiated by 40 cycles of amplification at 95 °C (15 s) and 60 °C (60 s). The results were obtained from three replicates of each sample to ensure representative and accuracy pipetting. The expression of bovine histone 2a gene was quantified in a similar way. The comparative delta–delta C_t method was used to analyze the results with the expression level of the respective target genes at the corresponding time point in exposed/unexposed embryos and in comparison to histone 2a C_t values described previously [24].

Cleavage, embryo development, mitochondrial viability and expression of genes mcl-1, caspase-2, caspase-3, Apaf-1 and Bax were analyzed by unpaired t test, with differences considered significant at $p < 0.05$. The maturation rate was evaluated by χ^2 test. The analysis of reference genes was performed by linear correlation coefficient and determination of the.