Comparative Immunology, Microbiology and Infectious Diseases 57 (2018) 8–14

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

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

Bovine herpesvirus type 5 replication and induction of apoptosis *in vitro* and in the trigeminal ganglion of experimentally-infected cattle

Daniel E. Rensetti[a], Maia S. Marin[b,c], Pedro E. Morán[a], Anselmo C. Odeón[c], Andrea E. Verna[b,c], Sandra E. Pérez[d,*]

[a] Facultad de Ciencias Veterinarias, Universidad Nacional del Centro de la Provincia de Buenos Aires, Paraje Arroyo Seco S/N, Tandil, 7000, Argentina
[b] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Rivadavia 1917, C1033AAJ, Buenos Aires, Argentina
[c] Instituto Nacional de Tecnología Agropecuaria (INTA), Estación Experimental Agropecuaria Balcarce, Ruta 226 Km 73.5, 7620, Balcarce, Buenos Aires, Argentina
[d] Centro de Investigación Veterinaria de Tandil (CIVETAN)-CONICET, Facultad de Ciencias Veterinarias, Universidad Nacional del Centro de la Provincia de Buenos Aires, Paraje Arroyo Seco S/N, Tandil, 7000, Argentina

ARTICLE INFO

Keywords:
Apoptosis
Bovine herpesvirus 1 and 5
Trigeminal ganglion
Neuropathogenesis

ABSTRACT

Bovine herpesvirus (BoHV) types 1 and 5 are neuroinvasive. Cases of BoHV-1-induced encephalitis are not as frequent as those caused by BoHV-5. In this study, the capability of BoHV-5 to induce apoptosis in cell cultures and in the trigeminal ganglion during acute infection of experimentally-infected cattle was analyzed. Apoptotic changes in cell cultures agree with the ability of the viral strains to replicate in each cell line. Marked differences were observed between the *in vitro* induction of apoptosis by BoHV-1Cooper and BoHV-5 97/613 strains. Apoptotic neurons were clearly evident in the trigeminal ganglion of BoHV-1-infected calves. For BoHV-5 a fewer number of positive neurons was observed. There is an association between the magnitude of bovine herpesviruses replication and the induction of apoptosis in trigeminal ganglion. These findings suggest that the induction of apoptosis and the innate immune response orchestrate the final outcome of alpha herpesviruses infection of the bovine nervous system.

1. Introduction

Bovine herpesvirus types 1 (BoHV-1) and 5 (BoHV-5) are two closely related alpha-herpesviruses that infect cattle. BoHV-1 causes significant economic losses to the cattle industry worldwide [1] since it is responsible for a variety of clinical syndromes, including respiratory disease, conjunctivitis, abortion and genital infections. Acute infection of the respiratory tract by BoHV-1 causes immunosuppression, leading to secondary bacterial infections, pneumonia and death [2]. BoHV-5 is highly prevalent in South America, and it is the primary etiological agent of non-suppurative meningoencephalitis in calves [3], a condition which is usually fatal [4]. Although both viruses are neuroinvasive, cases of BoHV-1-induced encephalitis are not as frequently reported as those caused by BoHV-5. The reasons why they differ in their ability to cause neurological disease have not been completely elucidated.

Herpesviruses' life cycle is characterized by stages of acute infection, latency and reactivation. Sensory neurons of the trigeminal ganglion (TG) are the main site of latency of alpha-herpesviruses [5]. During latency, viral gene expression is restricted to the latency-related (LR) gene [6]. Sporadic virus reactivation from latency can occur under conditions of natural stress or it can be experimentally induced by glucocorticoids administration. These episodes of reactivation are the main source of virus dissemination [5]. BoHV-1 and BoHV-5 share a high level of amino-acid identity, particularly in those proteins involved in viral DNA replication. However, Delhon et al. [7] reported marked differences in the coding and transcriptional regulatory regions of the LR gene. Previous studies [8,9] have demonstrated that BoHV-1 LR products have anti-apoptotic activity *in vitro* and *in vivo*. The anti-apoptotic activity of the LR gene is required to reach a high number of latently-infected neurons available for reactivation. Recently, Silvestro and Bratanich [10] have shown that components which are essential for the anti-apoptotic functions of BoHV-1 LR gene are not expressed in BoHV-5-infected-cells, suggesting that, at least *in vitro,* BoHV-1 LR gene may have roles different from the homologous BoHV-5 gene.

The purpose of this study was to analyze the capability of BoHV-5 to induce apoptosis in cell cultures and in the trigeminal ganglion of acutely-infected calves, as differences in the apoptotic potential of BoHV-5 and BoHV-1 might be related to the distinct features of their neuropathogenesis.

* Corresponding author.
E-mail address: seperez@vet.unicen.edu.ar (S.E. Pérez).

https://doi.org/10.1016/j.cimid.2018.01.002
Received 4 June 2017; Received in revised form 4 December 2017; Accepted 18 January 2018

2. Material and methods

2.1. Viruses

For *in vitro* assays and animal inoculations, the reference BoHV-1 Cooper strain and the field BoHV-5 isolate 97/613 were used. Furthermore, infection of cell cultures was also performed with the strain A663 of BoHV-5. This strain was considered as a BoHV-5 subtype *b* strain, a subtype only described in Argentina [11]. Recently, it was demonstrated that strain A663 is indeed a natural, field recombinant virus between BoHV-5 and BoHV-1 [12] which was isolated from a case of bovine encephalitis [11]. From here on this strain will be designated as BoHV-5 nrecA663. BoHV-5 strain 97/613 is classified as subtype *a* BoHV-5 strain [10], which was isolated from the brain of a 2 year-old Brangus cow with neurological signs. This strain has been previously characterized [3]. Titers of virus stocks for experimental inoculations and virus titers for the different bovine herpesviruses strains in each cell line at 6 and 24 hpi were determined by the end-point titration method, in 96 well-plates and expressed as $TCID_{50}$/ml, according to Reed and Müench [13]. Determination of virus titers in the different cell cultures was performed by triplicate.

2.2. Experimental inoculation of cattle

All procedures for animal handling and experimentation were performed according to the Animal Welfare Committee of the University of the Center of Buenos Aires Province (Res. 087/02). Five BoHV-1- and BoHV-5-free and seronegative cross-bred, 1 year-old calves were used. Calves were randomly assigned to each group. For the study of primary acute infection, two calves were intranasally inoculated with a high dose ($10^{6.3}$ $TCID_{50}$ in 10 ml) of BoHV-1 Cooper strain and two calves were inoculated with $10^{6.3}$ $TCID_{50}$ (in 10 ml) of BoHV-5 97/613 isolate. One calf was intranasally inoculated with 10 ml of cell culture medium as placebo. Calves were euthanized at 6 days post-infection (dpi). At necropsy, TG samples were collected and properly stored for further processing.

2.3. Cells

Two cell lines of bovine origin (Madin-Darby bovine kidney [MDBK] and bovine turbinate [BT] cells) and a human cell line (HeLa, cervical carcinoma cells) were selected for this study. HeLa cells were chosen to compare the findings in routine substrates for bovine herpesviruses growth with the findings in cells which are susceptible to infection but do not sustain high levels of virus replication. Cells were grown in Minimum Essential Medium (MEM, Sigma-Aldrich) supplemented with 10% fetal calf serum (Natocor SRL, Argentina), L-glutamine (10 µl/ml), penicillin (100 µg/l) and streptomycin (200 µg/l). Cultures were incubated at 37 °C in a 5% CO_2 atmosphere.

2.4. Inoculation of cell cultures

Cell lines were grown in 24 well-plates containing coverslips in each well, for cell attachment. Cells were infected with BoHV-1 or BoHV-5 strains at a low multiplicity of infection (moi = 0.1). Infected monolayers were fixed at 6 or 24 h post-inoculation (hpi). Cells were also treated with deoxynivalenol (DON, Sigma-Aldrich) (2.8 µg/ml in MEM) and 5% dimethyl-sulfoxide (DMSO, Sigma-Aldrich), as controls for the induction of apoptosis and fixed at the same time-points. Evaluation at 6 h was chosen as a starting point to determine initial alterations in nuclear morphology after infection or chemical treatment. DON is a mycotoxin which has been previously demonstrated to induce apoptosis in intestinal and immune cells [14] and in several cell culture lines [15]. Apoptosis induced by treatment with DMSO has been also previously described [16]. Virus strains, DMSO and DON were added to the preformed cell monolayer and incubated for 1 h at 37 °C. Then, cells were washed with PBS and culture medium was added for the remaining incubation time.

2.5. Identification of nuclear changes in cell cultures

Nuclear morphological changes (chromatin condensation, nuclear fragmentation and presence of apoptotic bodies) were evaluated by staining with 4′, 6-diamidino-2-phenylindole (DAPI) after fixing infected or treated cells with 4% paraformaldehyde. Experiments were done by triplicate and cells with nuclear alterations in six microscopic fields per sample were counted. The percentages of cells with altered nuclear morphology were determined.

2.6. Detection of apoptotic cells by TUNEL (Terminal dUTP Nick End-Labeling)

For detection of cells with fragmented DNA, a TUNEL assay (DeadEndColorimetric TUNEL System, Promega) was performed according to the manufacturer's instructions. Cells were seeded on coverslips in 24 well-plates and treated as described for DAPI staining. Cells were fixed with 1% paraformaldehyde. Experiments were done by duplicate.

2.7. Immunohistochemistry for detection of cleaved caspase 3 in bovine TG

Tissue sections were deparaffinized and rehydrated in graded ethanol. Sections were incubated in 0.03% hydrogen peroxide solution to block endogenous peroxidase. For antigen unmasking, sections were treated with proteinase K (20 µg/ml, Sigma-Aldrich) for 20 min at 37 °C. After blocking, tissues were incubated overnight at 4 °C with diluted primary antibody (1:100, cleaved caspase 3, Cell Signaling # 9661). The secondary antibody was applied for 30 min at room temperature. The peroxidase substrate system (DAB, SK-400, Vector Laboratories) was used according to the manufacturer's instructions. Sections were counterstained with hematoxylin, mounted and observed under microscope.

2.8. Statistical analysis

Differences in the percentages of cells with nuclear changes were evaluated for each virus or treatment within a cell line and among the different cell lines. Virus titers for the different bovine herpesvirus strains were also evaluated in the different cell lines. The percentages of cells with nuclear changes and virus titers were analyzed by ANOVA followed by Tukey test. A P value ≤ 0.05 was considered statistically significant.

3. Results

3.1. Nuclear morphological changes in BoHV-1 and BoHV-5-infected cell cultures

Marginated nuclei and chromatin condensation were easily visualized in infected or treated cells, particularly after 24 hpi. Apoptotic bodies, which are membrane bound portions of chromatin [17] were also observed, particularly after infection with strain BoHV-5 nrecA663. On the contrary, nuclei in mock-infected cells remained uniformly stained (Fig. 1).

As expected, the percentages of cells with nuclear morphological changes observed at 6 hpi were lower when compared with the percentages detected at 24 hpi (Fig. 2A and B). At 6 hpi, only significant changes ($P \leq 0.05$) were detected in BT cells infected with the strain BoHV-5 nrecA663. In mock-infected BT, DAPI staining also revealed significantly higher numbers of cells with nuclear changes with respect to the other mock-infected cell lines ($P \leq 0.05$) (Fig. 2A). Differences were not detected after chemical treatment or infection of the other cell

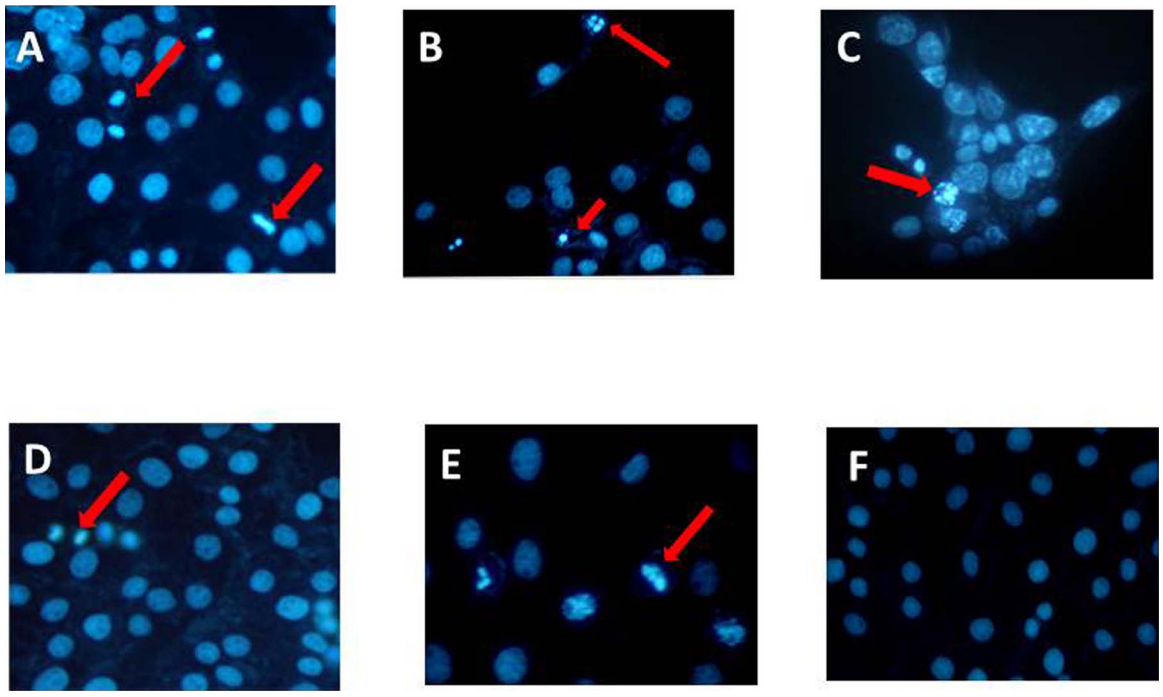

Fig. 1. Representative images of nuclear changes in DAPI-stained MDBK cells at 24 h post-infection or treatment. A. BoHV-5 97/613, B. BoHV-1 Cooper, C. BoHV-5 nrecA663, D. DMSO, E. DON, F. mock-infected. Magnification: 200×.

lines with any of the viral strains ($P \geq 0.05$). Low percentages of HeLa cells with nuclear changes were detected at this time-point (Fig. 2B).

Then, as shown in Table 1, the magnitude of the nuclear changes induced by each virus strain within a particular cell line was evaluated at the same time-points previously mentioned. In MDBK cells, at 6 hpi, the percentages of apoptotic nuclear changes were significantly higher ($P \leq 0.05$) after infection with any of the strains or after treatment with DON or DMSO with respect to mock-infected cells. Nevertheless, differences among treatments or virus strains were not detected ($P \geq 0.05$). The percentages of nuclear changes detected in BoHV-1-Cooper and BoHV-5 nrecA663 infected-BT at 6 hpi were significantly higher ($P \leq 0.05$) with respect to mock-infected cells. Furthermore, in this cell line, infection with BoHV-5 nrecA663 induced levels of apoptotic nuclear changes that were significantly higher ($P \leq 0.05$) than

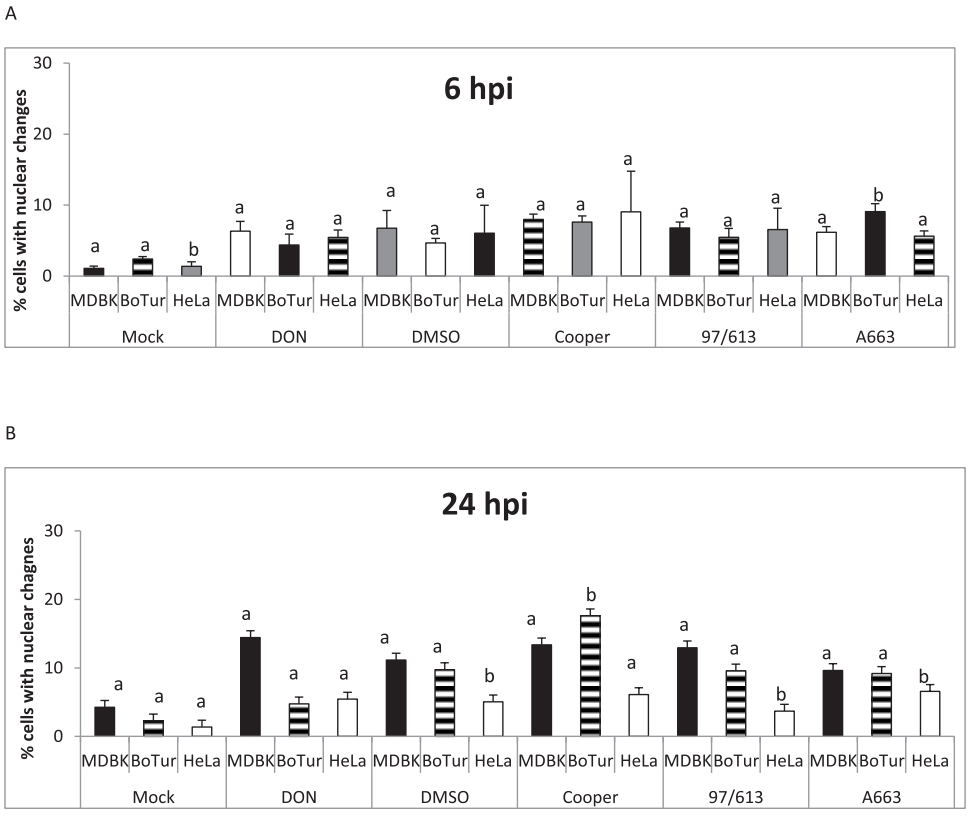

Fig. 2. Percentage of cells with nuclear changes after 6 (A) or 24 (B) hours of BoHV-1 and BoHV-5 infection or chemical treatment of different cell lines. Experiments were performed by triplicate. Different superscripts indicate statistically significant differences ($P \leq 0.05$) among cell cultures for each virus strain or treatment.

Table 1
Percentage of cells with nuclear morphological changes at 6 and 24 h after virus inoculation or treatment.

Cell culture	Virus strains and cell treatment					
	BoHV-5 97/613	A663	BoHV-1 Cooper	DON	DMSO	Mock
6 hpi						
MDBK	6.8 ± 0.8[b]	6.2 ± 0.8[b]	7.9 ± 0.7[b]	6.3 ± 1.4[b]	6.7 ± 2.5[b]	1.10 ± 0.3[a]
BoTur	5.4 ± 1.3[a]	9.1 ± 1.1[b]	7.6 ± 0.9[b]	4.4 ± 1.5[a]	4.7 ± 0.6[a]	2.40 ± 0.3[a]
HeLa	6.5 ± 3[a]	5.6 ± 0.7[a]	9.1 ± 5.7[a]	5.4 ± 1.0[a]	6.1 ± 3.9[a]	1.4 ± 0.6[a]
24 hpi						
MDBK	12.9 ± 2.6[b]	9.6 ± 2.5[b]	13.4 ± 3.3[b]	14 ± 3.7[b]	11 ± 2.6[b]	4.3 ± 1.5[a]
BoTur	9.6 ± 4.1[a]	9.2 ± 6.4[b]	17.6 ± 2.8[b]	4.8 ± 1.5[a]	9.7 ± 3[a]	2.3 ± 2.5[a]
HeLa	3.7 ± 1.8[a]	6.6 ± 2.6[b]	6.1 ± 1.8[b]	3.9 ± 1.7[a]	5.1 ± 1.8[b]	1.0 ± 0.9[a]

The results shown are mean ± standard deviation of three independent experiments. Different superscripts in the same line indicate statistically significant differences ($P \leq 0.05$) for virus strains or chemical treatments in comparison with mock-infected or mock-treated cells within each cell line.

those detected in DON-treated cells. Differences were not recorded ($P \geq 0.05$) among mock-infected and virally infected or treated HeLa cells.

At 24 hpi, the percentage of nuclear changes in MDBK cells was significantly higher ($P \leq 0.05$) in virus infected cells when compared with mock-infected cells. However, differences among strains were not detected ($P \geq 0.05$). The level of apoptotic changes detected in DON-treated MDBK cells was only significantly higher ($P \leq 0.05$) when compared with the infection by BoHV-5 nrecA663. At this time-point, BoHV-1 Cooper strain showed the highest percentage of apoptotic changes in BT cells ($P \leq 0.05$) with respect to the other viral strains or treatments. Furthermore, the percentage of cells with altered nuclear morphology in BoHV-1 Cooper-infected BT cells was significantly higher ($P \leq 0.05$) than the changes observed after infection by BoHV-5 97/613 or BoHV-5 nrecA663. Significantly higher levels of nuclear changes were also recorded for BoHV-1 Cooper ($P \leq 0.05$) with respect to mock-infected or DMSO-treated BT cells. As previously described, at 24 hpi, the lowest percentage of apoptotic changes was detected in HeLa cells, independently of the virus strain or treatment. Nevertheless, the nuclear apoptotic changes detected in Cooper-, BoHV-5 nrecA663- or in DMSO- mock-infected cells were significantly higher ($P \leq 0.05$) when compared with uninfected or untreated cells.

3.2. Replication of bovine herpesvirus strains in the different cell lines

Bovine herpesviruses replication was assessed in the different cell types used in this study. At 6 hpi, significant differences in the virus titers for the strains within a specific cell culture were not recorded ($P \geq 0.05$). However, at 24 hpi, significant differences ($P \leq 0.05$) in the levels of virus replication were detected in MDBK and BoTur cells. In both cell types, Cooper BoHV-1 strain titer was significantly higher ($P \leq 0.05$) with respect to the titers reached by BoHV-5 strains.

Virus replication in HeLa cells, which are from human origin, was lower than in cells from bovine origin, as expected (Table 2).

3.3. Detection of apoptotic cells by TUNEL assay

Changes in nuclear morphology occur early after exposure to an apoptotic stimulus. However, significant TUNEL staining is observed later during the apoptotic process. In this study, the percentage of TUNEL positive cells was determined at 24 hpi in MDBK cells, the standard substrate for the isolation and propagation of bovine herpesviruses (Fig. 3). At this time-point, the level of TUNEL- positive cells was strikingly higher after infection with BoHV-1 Cooper strain (48.9%) when compared with mock-infected (1.6%), DON-treated (22.1%) and BoHV-5-infected cells (97/613: 6.25%, BoHV-5 nrecA663: 3.9%).

3.4. Neuronal apoptosis in the trigeminal ganglion of BoHV-1 and BoHV-5-experimentally-infected calves

The results demonstrating that the experimental inoculation of calves in this study was successfully achieved have already been published [18,19]. BoHV-1 was isolated and viral DNA detected by PCR [18] in TGs used for the analysis of neuronal apoptosis. Although the presence of BoHV-5 DNA was detected (Table 3), as previously reported [20], isolation of BoHV-5 from this tissue was not achieved. Caspase 3 is a key executioner of apoptosis. In the cells, caspase 3 is present as an inactive procaspase which is activated by proteolytic cleavage [21]. Most of the neurons per microscopic field in TG from BoHV-1-infected calves showed clear nuclear cleaved caspase 3 staining. TGs from calves infected with BoHV-5 also showed positively-stained neurons. However, the presence of apoptotic cells was not as evident as in BoHV-1-infected cattle. In BoHV-5-infected animals, some neurons showed granular, perinuclear staining. Neuronal apoptosis was not detected in the TG from the mock-infected calf (Fig. 4).

Table 2
Summary of the percentage of nuclear changes in relation to virus titers for BoHV-1 and BoHV-5 strains in the different cell cultures at 6 and 24 h post-inoculation.

Virus strains	MDBK		BoTur		HeLa	
	% nuclear change	Virus titer	% nuclear change	Virus titer	% nuclear change	Virus titer
	6 hpi					
BoHV-1 Cooper	7.9 ± 0.7[a]	3.95 ± 0.2[a]	7.6 ± 0.9[a]	5.00 ± 0.9[a]	9.1 ± 5.7[a]	0.0 ± 0.0[a]
BoHV-5 nrecA663	6.2 ± 0.8[a]	3.80 ± 0.9[a]	9.1 ± 1.1[a]	3.60 ± 0.7[a]	5.6 ± 0.7[a]	1.5 ± 2.1[a]
BoHV-5 97/613	6.8 ± 0.8[a]	3.60 ± 0.4[a]	5.4 ± 1.3[b]	2.57 ± 1.7[a]	6.5 ± 3[a]	1.9 ± 2.7[a]
	24 hpi					
BoHV-1 Cooper	13.4 ± 3.3[a]	7.5 ± 1.1[a]	17.6 ± 2.8[a]	6.77 ± 1.7[a]	6.1 ± 1.8[a]	3.90 ± 0.8[a]
BoHV-5 nrecA663	9.6 ± 2.5[a]	6.3 ± 0.0[b]	9.2 ± 6.4[ab]	2.67 ± 0.0[b]	6.6 ± 2.6[a]	4.57 ± 0.1[a]
BoHV-5 97/613	12.9 ± 2.6[a]	6.8 ± 0.1[b]	9.6 ± 4.1[ac]	3.10 ± 2.2[c]	3.7 ± 1.8[a]	5.37 ± 1.0[a]

The results shown are mean ± standard deviation of three independent experiments. Different superscripts in the same column indicate statistically significant differences ($P \leq 0.05$). Virus titers are expressed as $TCID_{50}$/ml.

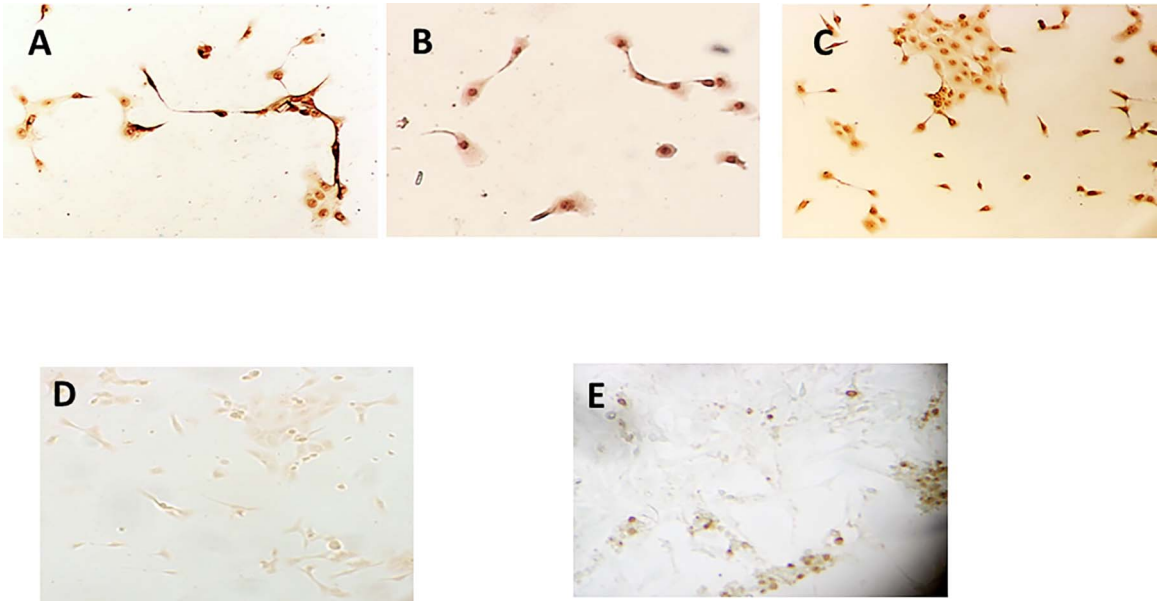

Fig. 3. Representative images of TUNEL staining of BoHV-5 97/613 (A), BoHV-5 nrecA663 (B), BoHV-1 Cooper (C), uninfected MDBK cells (D) and DON-treated MDBK cells (E) at 24 h post-inoculation or treatment. Experiments were done by duplicate. Magnification: 200×.

Table 3
Caspase 3-positive neurons after infection with BoHV strains, virus isolation and DNA detection in TG from experimentally-infected cattle.

	BoHV-5 97/613		BoHV-1 Cooper		Mock
	Calf1	Calf2	Calf3	Calf4	Calf5
Caspase 3	−/+	−/+	+	+	−
Virus isolation	−	−	−	5.65[a]	−
Genome detection	+	+	+	+	−

[a] Expressed as $TCID_{50}$/g of tissue [18].

4. Discussion

The removal of damaged cells by apoptosis is an essential process in homeostasis and development. However, this mechanism of cell death may also play a pathogenic role in infectious diseases. It is considered that apoptosis is an important process in many viral infections of the central nervous system and, frequently, the severity of the disease is associated with the level of apoptosis [22]. In this study, it was investigated whether apoptosis is a relevant mechanism in the pathogenesis of BoHV-5. Furthermore, it was analyzed whether differences in the apoptotic potential of BoHV-5 and BoHV-1might be associated with the distinct features of their neuropathogenesis.

For some viral infections, the number of apoptotic cells has been correlated with the levels of virus replication. In this study, the apoptotic changes observed in cell cultures were in agreement with the ability of BoHV-1 and BoHV-5 strains to replicate in each cell line. MDBK and BT are frequently used for replication and isolation of bovine alpha-herpesviruses [23,24]. However, HeLa cells, which are not an adequate substrate for bovine alpha-herpesviruses' replication showed significantly lower levels of altered nuclear morphology compatible with apoptosis. Regarding the viral strains, differences were observed between the *in vitro* induction of apoptosis by BoHV-1 Cooper and BoHV-5 strains. This was evident in BoTur cells at 24 h post-infection, particularly for BoHV-1. The higher ability of BoHV-1 to induce apoptosis *in vitro* was evidenced by the nuclear morphological alterations observed in the different cell types and confirmed by TUNEL assay in MDBK cells. These findings are in contrast with the observations by Cardoso et al. [25] who demonstrated low levels of apoptosis after infection of neuronal-like cells with BoHV-1 Cooper. Nevertheless, the same authors demonstrated that BoHV-5 does not induce high levels of apoptosis in neurons, which is in agreement with the findings from this study using different substrates. This has also been shown for other viruses, such as varicella-zoster virus, which is able to induce apoptosis in fibroblasts but not in neurons, demonstrating that apoptosis is a cell-type specific process [26].

In a previous study of experimental inoculation of cattle, we demonstrated that, in neural tissue, two BoHV-1 strains (Cooper and Los Angeles) replicated at titers which were higher than those observed for BoHV-5 [18]. These low BoHV-5 titers in the nervous system have been associated with the difficultness of virus isolation after natural or experimental infections [3]. Although viral DNA was detected in the trigeminal ganglion from all infected animals, in this study, only BoHV-1 was readily isolated from this tissue. Samuel et al. [27] determined a positive correlation with the peak West Nile virus titers in central nervous system and detection of activated caspase 3. Similar findings have been observed for the infections by reovirus [28] and sindbis virus [29] and the presence of apoptotic neurons in neural tissue. Delhon et al. [30] demonstrated that inhibition of BoHV-1 replication prevented neuronal apoptosis. This type of study has not been conducted with BoHV-5. However, the results of this study, considering the levels of replication of BoHV-1 and BoHV-5 and the presence of apoptotic neurons in trigeminal ganglion, demonstrate that virus growth is required to trigger the apoptotic programme for neuronal death. As previously suggested by Delhon et al. [30], it can also be inferred by the results from this study that it is necessary a critical level of viral DNA replication to induce apoptosis.

It is well-known that the induction of apoptosis limits the inflammatory response. Phagocytosis of apoptotic cells suppresses inflammation by the production of anti-inflammatory mediators and the inhibition of pro-inflammatory cytokines [31]. In this work, the number of caspase 3-positive neurons was not estimated since the number of trigeminal ganglion neurons in each microscopic field is not large enough to obtain a reliable counting. Nevertheless, at the peak of acute infection, cleaved caspase 3 was clearly evident in trigeminal ganglion neurons of BoHV-1-infected calves, whereas a fewer number of positive neurons was observed after BoHV-5 infection. TLRs allow the recognition of pathogens by the innate immune system [32]. They induce a series of events that result in the production of cytokines leading to the stimulation of the adaptive immune response [24]. In previous studies from our group, Rensetti et al. [19] demonstrated a strong stimulation

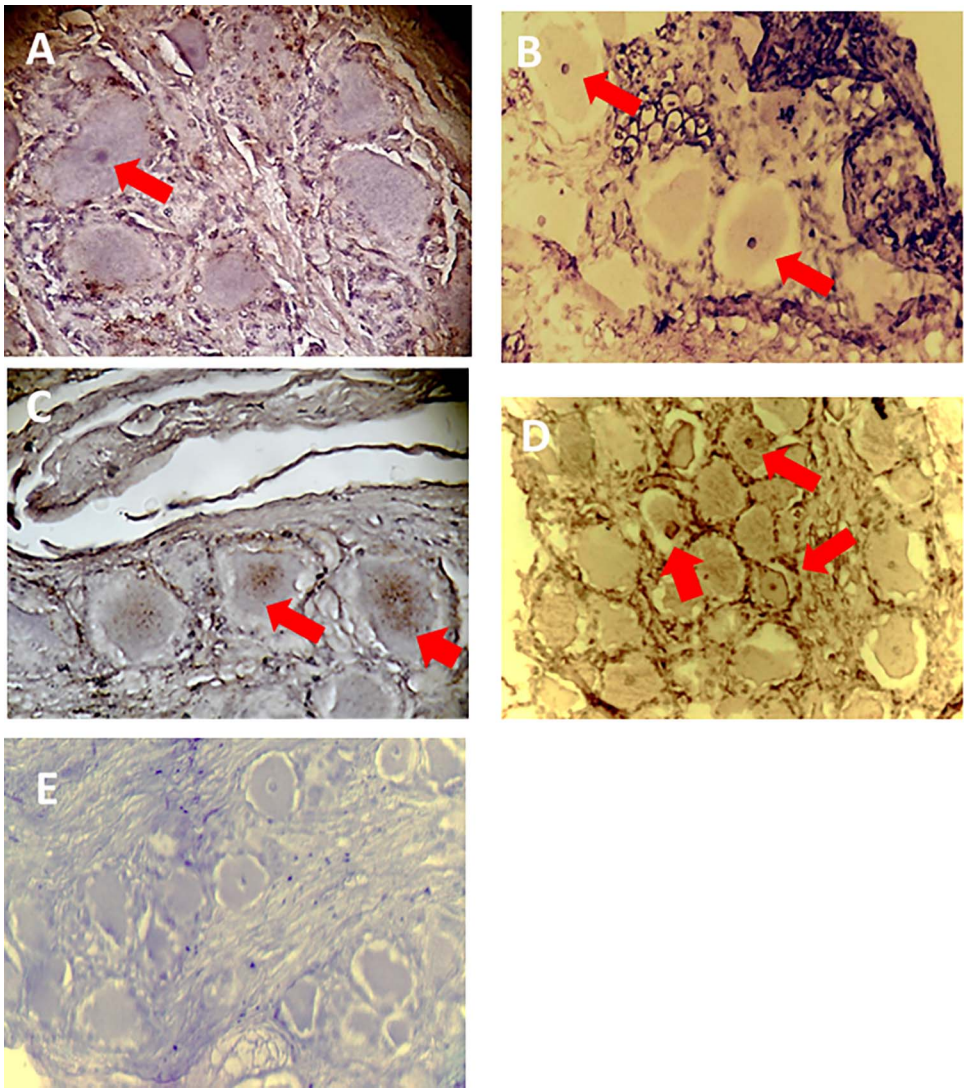

Fig. 4. Detection of cleaved caspase 3 in trigeminal ganglia of cattle experimentally- infected with BoHV-1 Cooper (A and B), BoHV-5 97/613 (C and D) and mock-infected (E). Magnification. 400× (A–C), 200× (D and E).

of TLR7 during acute BoHV-5 infection of the central nervous system and Marin et al. [20] observed an up-regulation of TLR7 in trigeminal ganglion at this stage of BoHV-5 infection. On the contrary, TLR7 levels are undetectable in trigeminal ganglion of BoHV-1-infected calves [20]. Stimulation of this receptor triggers the production of pro-inflammatory cytokines and induces a negative feedback on viral replication *in vitro*, in MDBK cells infected with BoHV-1 and BoHV-5 [33] and in neurons infected with Langat virus [34]. Thus, it is likely that in BoHV-5 infection the cytokine response plays a predominant role in neuropathology and virus growth within neural tissue.

The results from this study, in conjunction with the previous reports from our research group, suggest that there might be an association between the magnitude of BoHV-5 replication and the induction of apoptosis in trigeminal ganglion. It is also apparent that the stimulation of TLRs plays an additional role in the neuropathogenesis of BoHV-5.

5. Conclusions

Overall, the findings from this study suggest that differences in the levels of apoptosis induced by BoHV-5 and BoHV-1 can be related to their distinct pathogenic potential. It is likely that a combination of factors, such as the induction of apoptosis and the innate immune response, orchestrates the final outcome of BoHV-1 and BoHV-5 infection of the bovine nervous system.

It would be interesting to analyze whether differences in the magnitude of apoptosis are relevant at further time-points, during the transition from acute infection to latency to establish the importance of apoptotic cell death in the life cycle of BoHV-5.

Conflict of interest statement

The authors declare no potential conflicts of interest with respect to the research, authorship, publication of this article and/or financial or personal relationships that could inappropriately influence this work.

Funding

This work was supported by ANPCyT (PICT 2008-1421) and CONICET (PIP 098).

References

[1] B. Muylkens, J. Thiry, P. Kirten, F. Schynts, E. Thiry, Bovine herpesvirus 1 infection and infectious bovine rhinotracheitis, Vet. Res. 38 (2007) 181–209.
[2] S. Tikoo, M. Campos, L. Babiuk, Bovine herpesvirus 1 (BHV-1): biology, pathogenesis, and control, Adv. Virus Res. 45 (1995) 191–223.
[3] S. Pérez, G. Bretschneider, M. Leunda, F. Osorio, E. Flores, A. Odeón, Primary infection, latency, and reactivation of bovine herpesvirus type 5 in the bovine nervous system, Vet. Pathol. 39 (2002) 437–444.
[4] K. Cascio, E. Belknap, P. Schultheiss, A. Ames, J. Collins, Encephalitis induced by bovine herpesvirus 5 and protection by prior vaccination or infection with bovine herpesvirus 1, J. Vet. Diagn. Invest. 11 (1999) 134–149.

[5] D. Rock, J. Lokensgard, T. Lewis, G. Kutish, Characterization of dexamethasone induced reactivation of latent bovine herpesvirus 1, J. Virol. 66 (1992) 2484–2490.
[6] C. Jones, Herpes simplex virus type 1 and bovine herpesvirus 1 latency, Clin. Microbiol. Rev. 16 (2003) 79–95.
[7] G. Delhon, M. Moraes, Z. Lu, C. Afonso, E. Flores, R. Weiblen, G. Kutish, D. Rock, Genome of bovine herpesvirus 5, J. Virol. 77 (2003) 10339–10347.
[8] J. Ciacci-Zanella, M. Stone, G. Henderson, C. Jones, The latency-related gene of bovine herpesvirus 1 inhibits programmed cell death, J. Virol. 73 (1999) 9734–9740.
[9] M. Inman, L. Lovato, A. Doster, C. Jones, A mutation in the latency-related gene of bovine herpesvirus 1 disrupts the latency reactivation cycle in calves, J. Virol. 76 (2002) 6771–6779.
[10] C. Silvestro, A. Bratanich, The latency related gene of bovine herpesvirus types 1 and 5 and its modulation of cellular processes, Arch. Virol. 161 (2016) 3299–3308.
[11] S. Maidana, M. Ladelfa, S. Pérez, P. Lomónaco, M. Del Médico Zajac, A. Odeón, J. Blanco Viera, G. Combessies, N. Fondevila, M. Palacios, J. Thiry, B. Muylkens, E. Thiry, S. Romera, Characterization of BoHV-5 field strains circulation and report of transient specific subtype of bovine herpesvirus 5 in Argentina, BMC Vet. Res. 7 (2011) 1–8, http://dx.doi.org/10.1186/1746-6148-7-8.
[12] S. Maidana, A. Mauroy, E. Thiry, S. Romera, Bovine herpesvirus 5 (BoHV5) subtype b is unexpectedly a recombinant virus between BoHV5 and BoHV1, the agent of infectious bovine rhinotracheitis, Proceedings of the World Buiatrics Congress, Dublin, Ireland, July 3rd to 8th, 2016 p. 416.
[13] L. Reed, H. Müench, A simple method of estimating fifty percent endpoints, Am. J. Hyg. 27 (1938) 493–497.
[14] W. Awad, K. Ghareeb, A. Dadak, M. Hess, J. Böhm, Single and combined effects of deoxynivalenol mycotoxin and a microbial feed additive on lymphocyte DNA damage and oxidative stress in broiler chickens, PLoS One (2014) e88028, http://dx.doi.org/10.1371/journal.pone.0088028.
[15] D. Pérez Gaudio, G. Martínez, M. Fernández Paggi, M. Riccio, J. Decundo, S. Dieguez, M. Tapia, A. Soraci, Protective effect of fosfomycin on deoxynivalenol-treated cell cultures, Eur. J. Biomed. Pharmacol. Sci. 3 (2016) 99–106.
[16] M. Chateau, C. Ginestier-Verne, J. Chiesa, R. Caravano, J. Bureau, Dimethyl sulfoxide-induced apoptosis in human leukemic U937 cells, Anal. Cell. Pathol. 10 (1996) 75–84.
[17] Y. Guo, C. Shen, A. Cheng, M. Wang, N. Zhang, S. Chen, Y. Zhou, Anatid herpesvirus 1 CH virulent strain induces syncytium and apoptosis in duck embryo fibroblast cultures, Vet. Microbiol. 138 (2009) 258–265.
[18] M. Marin, M. Leunda, A. Verna, P. Morán, A. Odeón, S. Pérez, Distribution of bovine herpesvirus type 1 in the nervous system of experimentally infected calves, Vet. J. 209 (2016) 82–86.
[19] D. Rensetti, M. Marin, S. Quintana, P. Morán, A. Verna, A. Odeón, S. Pérez, Involvement of toll-like receptors 3 and 7/8 in the neuropathogenesis of bovine herpesvirus types 1 and 5, Res. Vet. Sci. 107 (2016) 1–7.
[20] M. Marin, S. Quintana, C. Faverín, M. Leunda, A. Odeón, S. Pérez, Toll-like receptor expression in the nervous system of bovine alpha-herpesvirus-infected calves, Res. Vet. Sci. 97 (2014) 422–429.
[21] H. Chang, X. Yang, Proteases for cell suicide: functions and regulation of caspases, Microbiol. Mol. Biol. Rev. 64 (2000) 821–846.
[22] P. Clarke, K. Tyler, Apoptosis in animal models of virus-induced disease, Nat. Rev. Microbiol. 7 (2009) 144–155.
[23] B. Chandranaik, S. Shivaraj, S. Kumar, C. Renukaprasad, Isolation of BoHV-1 from bovine semen and application of Real Time PCR for diagnosis of IBR/IVP from clinical samples, Veterinaski Arhiv 80 (2010) 467–475.
[24] N. Taborda, L. Acevedo, C. Patiño, J. Forero, A. López-Herrera, Actividad antiviral in vitro de extractos de Hura crepitans y Codiaeum variegatum en la replicación de herpes virus bovino tipo-1 y virus de estomatitis vesicular, Rev. Colomb. Ciencias Pecuarias 20 (2007) 3.
[25] T. Cardoso, H. Ferreira, L. Okamura, B. Oliveira, A. Rosa, R. Gameiro, E. Flores, Comparative analysis of the replication of bovine herpesvirus 1 (BHV1) and BHV5 in bovine-derived neuron-like cells, Arch. Virol. 160 (2015) 2683–2691.
[26] S. Pugazhenthi, S. Nair, K. Velmurugan, Q. Liang, R. Mahalingam, R. Cohrs, M. Nagel, G. Gilden, Varicella-zoster virus infection of differentiated human neural stem cells, J. Virol. 85 (2011) 6678–6686.
[27] M. Samuel, J. Morrey, M. Diamond, Caspase 3-dependent cell death of neurons contributes to the pathogenesis of West Nile virus encephalitis, J. Virol. 81 (2007) 2614–2623.
[28] S. Oberhaus, S. Smith, G. Clayton, T. Dermody, K. Tyler, Reovirus infection and tissue injury in the mouse central nervous system are associated with apoptosis, J. Virol. 71 (1997) 2100–2106.
[29] L. Labrada, X. Liang, W. Zheng, C. Johnston, B. Levine, Age-dependent resistance to lethal alphavirus encephalitis in mice: analysis of gene expression in the central nervous system and identification of a novel interferon-inducible protective gene, mouse ISG12, J. Virol. 76 (2002) 11688–11703.
[30] G. Delhon, M. González, P. Murcia, Susceptibility of sensory neurons to apoptosis following infection by bovine herpesvirus type 1, J. Gen. Virol. 83 (2002) 2257–2267.
[31] V. Fadok, D. Bratton, S. Frasch, M. Warner, P. Henson, The role of phosphatidylserine in recognition of apoptotic cells by phagocytes, Cell Death Differ. 5 (1998) 551–562.
[32] S. Akira, S. Uematsu, O. Takeuchi, Pathogen recognition and innate immunity, Cell 124 (2006) 783–801.
[33] M. Marin, S. Quintana, C. Faverín, M. Leunda, A. Odeón, S. Pérez, Toll-like receptor activation and expression in bovine alpha-herpesvirus infections, Res. Vet. Sci. 96 (2014) 196–203.
[34] D. Baker, T. Woods, N. Butchi, T. Morgan, R. Taylor, P. Sunyakumthorn, P. Mukherjee, K. Lubick, S. Best, K. Peterson, Toll-like receptor 7 suppresses virus replication in neurons but does not affect viral pathogenesis in a mouse model of Langat virus infection, J. Gen. Virol. 94 (2013) 336–347.