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FULL PAPER

Pathology

Brain-derived neurotrophic factor is down regulated after bovine alpha-herpesvirus 5 infection in both wild-type and TLR3/7/9 deficient mice

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ABSTRACT. Neurotrophic factors have been implicated in the control of neuronal survival and plasticity in different brain diseases. Meningoencephalitis caused by bovine alpha-herpesvirus 5 (BoHV-5) infection is a frequent neurological disease of young cattle, being the involvement of apoptosis in the development of neuropathological changes frequently discussed in the literature. It's well known that Toll-like receptors (TLRs) can activate neuroinflammatory response and consequently lead to neuronal loss. However, there are no studies evaluating the expression of neurotrophic factors and their association with brain pathology and TLRs during the infection by BoHV-5. The current study aimed to analyze brain levels of neurotrophic factors along with neuropathological changes during acute infection by BoHV-5 in wild-type (WT) and TLR3/7/9 (TLR3/7/9$^{-/-}$) deficiency mice. The infection was induced by intracranial inoculation of 1×10^4 $TCID_{50}$ of BoHV-5. Infected animals presented similar degrees of clinical signs and neuropathological changes. Both infected groups had meningoencephalitis and neuronal damage in CA regions from hippocampus. BoHV-5 infection promoted the proliferation of Iba-1 positive cells throughout the neuropil, mainly located in the frontal cortex. Moreover, significant lower levels of brain-derived neurotrophic factor (BDNF) were detected in both BoHV-5 infected WT and TLR3/7/9 deficient mice, compared with non-infected animals. Our study showed that BDNF down regulation was associated with brain inflammation, reactive microgliosis and neuronal loss after bovine alpha-herpesvirus 5 infection in mice. Moreover, we demonstrated that combined TLR3/7/9 deficiency does not alter those parameters.

KEY WORDS: bovine herpesvirus 5, brain-derived neurotrophic factor, toll-like receptor

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Meningoencephalitis caused by bovine alpha-herpesvirus 5 (BoHV-5) infection is a frequent neurological disease of young cattle, being most frequently reported in Southern Brazil and Argentina [27, 40, 43]. The affected animals can present serous nasal

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and ocular discharges, anorexia with progression to ataxia, convulsions, and ultimately death [19, 40]. Histopathological changes include meningitis, perivascular cuffing, gliosis, hemorrhage and neuronal death [35, 40]. The involvement of apoptosis in the development of neuropathological changes after BoHV infection has been frequently discussed in the literature [10, 19, 41]. Some authors demonstrated that the replication of BoHV-1 and BoHV-5 is required to trigger the apoptotic program for neuronal death [15, 16, 36]. Moreover, bovine herpesviruses can induce different cell death forms in neuronal and glial-derived tumor cell cultures [10, 46].

Activated microglia induced by expression of Toll-like receptors (TLRs) promotes secretion of type I interferons [13, 52], leading to neuroinflammatory response [12, 26, 30]. Type I interferons play an important role in innate immune control of viruses, but also promote neurotoxicity and subsequent apoptosis in patients with HIV-associated neurocognitive disorders [8].

Neurotrophic factors such as BDNF, nerve growth factor (NGF) and glial cell line-derived neurotrophic factor (GDNF) produced by glial cells have been involved in the control of neuronal survival and plasticity [7, 28]. Sellner and colleagues (2005) observed overexpression of neurotrophic factors during acute as well as remote course of experimental herpes simplex virus encephalitis, suggesting a critical role of these mediators in the pathogenesis of the disease [45]. Interestingly, the reduction of BDNF has been associated with neuronal apoptosis in preclinical models of HIV infection [36]. The pre-treatment with IFN-alpha or IFN-beta inhibited brain-derived neurotrophic factor (BDNF) signaling and neurotrophic activity and induced neuronal damage in neuroblastoma cells and primary mouse cortical neurons [18]. However, to best of our knowledge there are no studies evaluating the levels of the neurotrophic factors BDNF, NGF and GDNF during the meningoencephalitis caused by BoHV-5 infection in mice. Additionally, we will carry out the investigation of effects of combined TLRs3/7/9 deficiency in the measurement of these neurotrophic factors in the brain associated with neuropathology and microglial patterns during the BoHV-5 infection.

MATERIALS AND METHODS

Virus and cell culture

BoHV-5 Mutum sample (GenBank AY916517) was isolated from central nervous system of an adult cow presenting neurological symptoms [5]. The strain was maintained with minimal essential medium (Sigma-Aldrich Brasil Ltd.., São Paulo, Brazil), containing 5% inactive fetal bovine serum (FBS). Inactive FBS was free for Mycoplasm and Bovine Viral Diarrhea Virus (Thermo Fisher Scientific Inc., Wilmington, DE, USA) and treated with penicillin (1.6 mg/l), streptomycin (0.4 mg/l) and fungizone (2.5 mg/l) at 37°C in 5% CO_2. The virus was propagated in CRIB-1 cells (CRL-11883, ATCC, Manassas, VA, USA; Flores & Donis, 1995) at a low multiplicity of infection (m.o.i 0.01); titrated by the End-point method and calculated by Reed and Muench (1938). The viral titer obtained was $10^{8.79}$ Median Tissue Culture Infectious Dose ($TCID_{50}$)/ml.

Animals and infection

Sixteen male C57BL/6 wild-type (WT) mice and sixteen male TLR3/7/9$^{-/-}$ mice, with 7 to 9-weeks-old were distributed into four groups: uninfected WT group, uninfected TLR3/7/9$^{-/-}$ group, BoHV-5 infected WT group and BoHV-5 infected TLR3/7/9$^{-/-}$ group. C57BL/6 WT mice were obtained from the Animal Care Facilities of Instituto de Ciências Biológicas (ICB-UFMG), and combined TLR 3/79$^{-/-}$ mice were kindly provided by Dr. Marco Antônio Campos (Centro de Pesquisas René Rachou, Fiocruz, Minas Gerais, Brazil). The experimental protocol was approved by the Committee on the Ethics of Animal Experiments of the Universidade Federal de Minas Gerais (CEUA/UFMG, Permit Protocol Number 388/2015). Animals were anesthetized by intraperitoneal injection of a mixture of ketamine and xylazine. A 1×10^4 $TCID_{50}$ inoculum of the purified BoHV-5 was resuspended in 10 µl of phosphate-buffered saline (PBS) and injected intracranially in the right side of a sagittal suture at the level of the eyes [49]. The control group received 10 µl of PBS. Mice were housed in microisolator cages in our Bio Safety Level-2 facility with water and food *ad libitum* and were observed for 3 days following the infection.

Histopathology and Iba-1 immunohistochemistry

Mice were euthanized with an overdose of sterilized mixture with 150 mg/Kg ketamine and 10 mg/Kg xylazine in PBS. We performed necropsy of all animals. Brains from non-infected and infected mice were collected and fixed in 10% buffered formalin solution, dehydrated, cleared, and embedded in paraffin. Sections of 4 µm thickness were obtained and stained with hematoxylin-eosin (H&E). All analyzed sections had areas of cerebrum, brainstem, hippocampus and cerebellum. The degree of meningitis was evaluated as 0: without inflammation; 1: a layer of inflammatory cells; 2: two layers of inflammatory cells; 3: three layers of inflammatory cells; 4: four to six layers of inflammatory cells; 5: seven or more layers of inflammatory cells [3]. Histopathological scores for degenerative and hippocampal changes were graded: 0 =absence of significant alterations; 1 =minimal; 2 =mild, 3 =moderate and 4 =intense alterations. Other sections of these fragments were used to evaluate positive cells for anti-ionized calcium binding adapter molecule 1 antibody (Iba-1), in order to detect the active microglia [21]. Antigen retrieval was done using sodium-citrate buffer (pH 6), moist heat by pressure cooking at 120°C for 8 min. The sections were blocked for endogenous peroxidase activity (Novolink™ Polymer Detection System–Leica Biosystems, Wetzlar, Alemanha) and for unspecific proteins (Novolink™ Polymer Detection System). After that, sections were incubated with rabbit monoclonal antibody against Iba-1 (Wako Chemicals, Richmond, VA, USA), diluted in 1:2,000 and incubated overnight at 4°C. Biotinylated polyclonal link and streptavidin-HRP (Novolink™ Polymer Detection System) were applied and the sections were incubated with diaminobenzidine (Novolink™ Polymer Detection System). After, the sections were counterstained with Harris hematoxylin.

ELISA of neurotrophic factors

Brains from non-infected and infected groups were collected and stored at −80°C for detection of neurotrophic factors by sandwich Enzyme Linked Immunonosorbent Assay (ELISA). Then brain homogenates were obtained using an extraction solution (100 mg of tissue per milliliter), containing 0.4 M NaCl, 0.05% Tween 20, 0.5% BSA, 0.1 mM phenyl methyl sulphonyl fluoride, 0.1 mM benzethonium chloride, 10 mM EDTA, and 20 KIU aprotinin, using Ultra-Turrax. Lysates were centrifuged at 13,000 g for 10 min at 4°C. Concentrations of brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and glial cell line-derived neurotrophic factor (GDNF) from the supernatants were assayed in an ELISA (R&D Systems, Minneapolis, MN, USA) setup, according to the manufacturer's procedures. The data were expressed as picogram per 100 mg of tissue.

Statistical analysis

Results obtained were presented as mean ± standard error of the mean (SEM) and differences were compared by analysis of variance (ANOVA). Statistical significance was set at $P<0.05$.

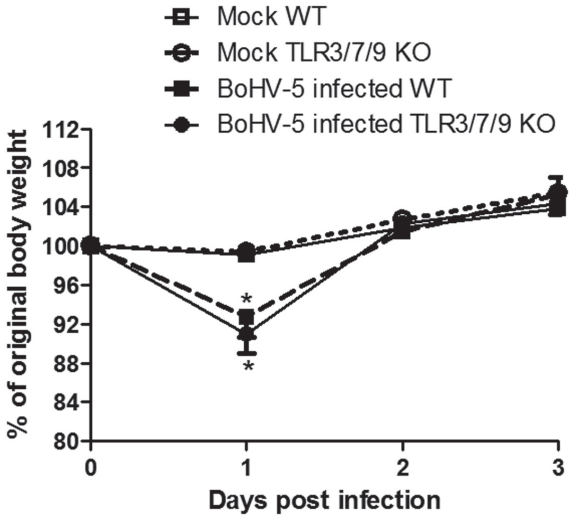


Fig. 1. Percentage of original body weight of non-infected and Bovine alpha-herpesvirus 5 (BoHV-5) infected mice. 7-week old male wild-type (WT) and TLR3/7/9$^{-/-}$ animals were inoculated with 10^4 Median Tissue Culture Infectious Dose ($TCID_{50}$) of Bovine alpha-herpesvirus 5 (BoHV-5), Mutum sample, or phosphate-buffered saline by the intracranial route and weighed for three days post-infection. Both infected groups exhibited significant weight loss at day 1 post infection and recovery from days 2 and 3, in comparison to non-infected groups. Values were expressed as the percentage of original weight. *$P<0.05$, Non-infected WT and TLR3/7/9$^{-/-}$ mice versus BoHV-5 infected WT and TLR3/7/9$^{-/-}$ mice.

RESULTS

BoHV-5 infection in Wild-type and TLR3/7/9 deficient mice promoted similar clinical signs

Non-infected WT (n=5) and TLR3/7/9$^{-/-}$ (n=5) animals did not show any clinical signs. In the other hand, BoHV-5 infected WT (n=11) and TLR3/7/9$^{-/-}$ (n=11) mice presented significant weight loss at day 1 post infection and recovery from days 2 and 3 (Fig. 1). All infected animals presented clinical signs characterized by serous ocular discharge, ruffled fur and apathy in a similar frequency. No death was recorded.

BoHV-5 infection in Wild-type and in TLR3/7/9 deficient mice had similar neuropathological findings

No histopathological changes were observed in non-infected WT (n=5) and TLR3/7/9$^{-/-}$ (n=5) animals (Fig. 2A, B, 2G–H). Both mice groups infected with BoHV-5 (n=5 per group) exhibited infiltration of mononuclear cells in the meninges and neuropil (Fig. 2C–D), occasional vacuolization of neuropil (Fig. 2E–F) adjacent to inflamed areas, mainly located in the cerebrum and brainstem. In addition, CA1 pyramidal layer from hippocampus was thinner and disorganized and had several shrunken neurons, suggestive of apoptosis (Fig. 2I–L). BoHV-5 WT and TLR3/7/9$^{-/-}$ infected mice presented similar degrees of meningitis, degenerative and hippocampal changes (Fig. 2M).

BoHV-5 infection leads to reactive microgliosis in both wild-type and TLR 3/7/9 deficient mice

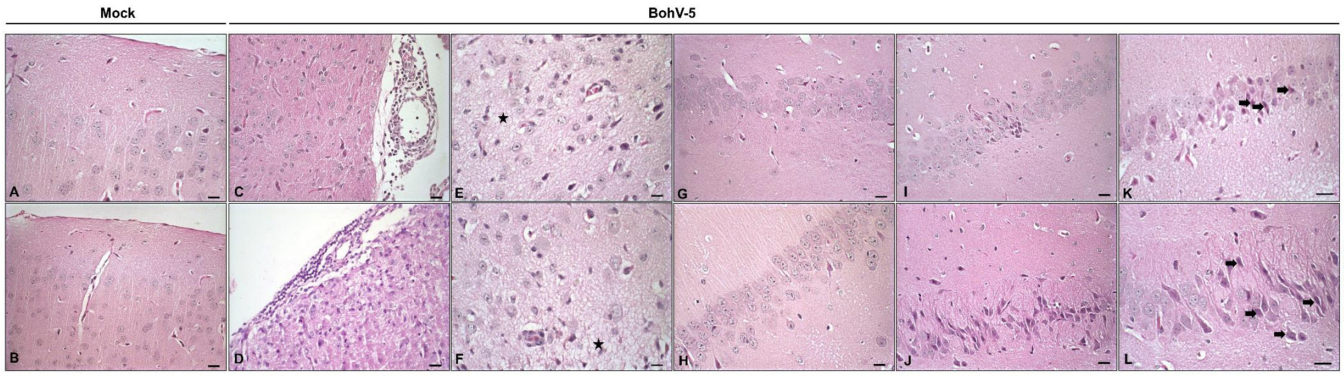
Non-infected WT and TLR3/7/9$^{-/-}$mice presented immunopositive cells for Iba-1 sparsely distributed throughout the brain parenchyma. Both infected groups exhibited focal areas of microgliosis, mainly located in cerebral cortex (Fig. 3).

BoHV-5 infection promoted down regulation of BDNF and the absence of TLR3/7/9 does not change this pattern

The brain levels of the neurotrophic factors BDNF, NGF e GDNF were measured at 3 days post infection, n=6 per group (Fig. 4). Similar levels of BDNF were detected in both non-infected WT and TLR3/7/9 deficient mice. BoHV-5 infection promoted significantly reduction in the brain levels of BDNF ($P<0.05$), compared with non-infected animals. TLR3/7/9 deficient mice infected with BoHV-5 also had decreased amounts of BDNF ($P<0.05$), compared with non-infected TLR3/7/9 deficient mice. Similar levels were observed in both infected groups (Fig. 4A). All evaluated groups did not show any significant difference in the brain concentrations of NGF (Fig. 4B) and GDNF (Fig. 4C).

DISCUSSION

This study was focused on the evaluation of neurotrophic factors levels in the brain after bovine herpesvirus 5 infection in wild-type and TLR3/7/9 deficient mice. Furthermore, we studied the neuropathological changes and microglial activity associated with the brain expression of BDNF, NGF and GDNF. BoHV-5 infected WT and TLR3/7/9$^{-/-}$ mice presented significant weight loss at day 1 post infection and recovery from days 2 and 3. BoHV-5 infected animals presented similar clinical signs to those observed in an early phase of naturally infected cattle [40]. Additionally, the present findings corroborated our previous study in which C57BL/6 mice infected with 10^4 $TCID_{50}$ of Mutum strain presented weight loss, ruffled fur and hunched posture associated with



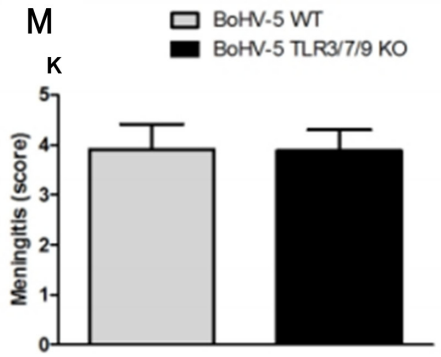


Fig. 2. Representative photomicrographs of H&E-stained brain sections and pathology score. 7-week old male wild-type (WT) and TLR3/7/9$^{-/-}$ mice were inoculated with 10^4 Median Tissue Culture Infectious Dose ($TCID_{50}$) of Bovine alpha-herpesvirus 5 (BoHV-5), Mutum sample, or phosphate-buffered saline by the intracranial route and evaluated three days post-infection. Non-infected WT (A) and TLR3/7/9$^{-/-}$ mice (B) showing frontal cortex with normal histological appearance; BoHV-5-infected WT (C) and TLR3/7/9 deficient mice (C) with infiltration of immune cells in the meninges. Both BoHV-5 infected animals (E, F) exhibited mild spongiosis (asterisks). Non-infected WT (G) and TLR3/7/9$^{-/-}$ (H) animals with hippocampal Cornu Ammonis (CA) region with regular morphology and preserved neurons. BoHV-5-infected WT (I, K) and TLR3/7/9 deficient mice (J, L) showed shrinkage neurons (arrows) in CA region. Scale bars: 20 µm. Similar pathological score of meningitis was observed in both BoHV-5 WT and TLR3/7/9 deficient groups (M).

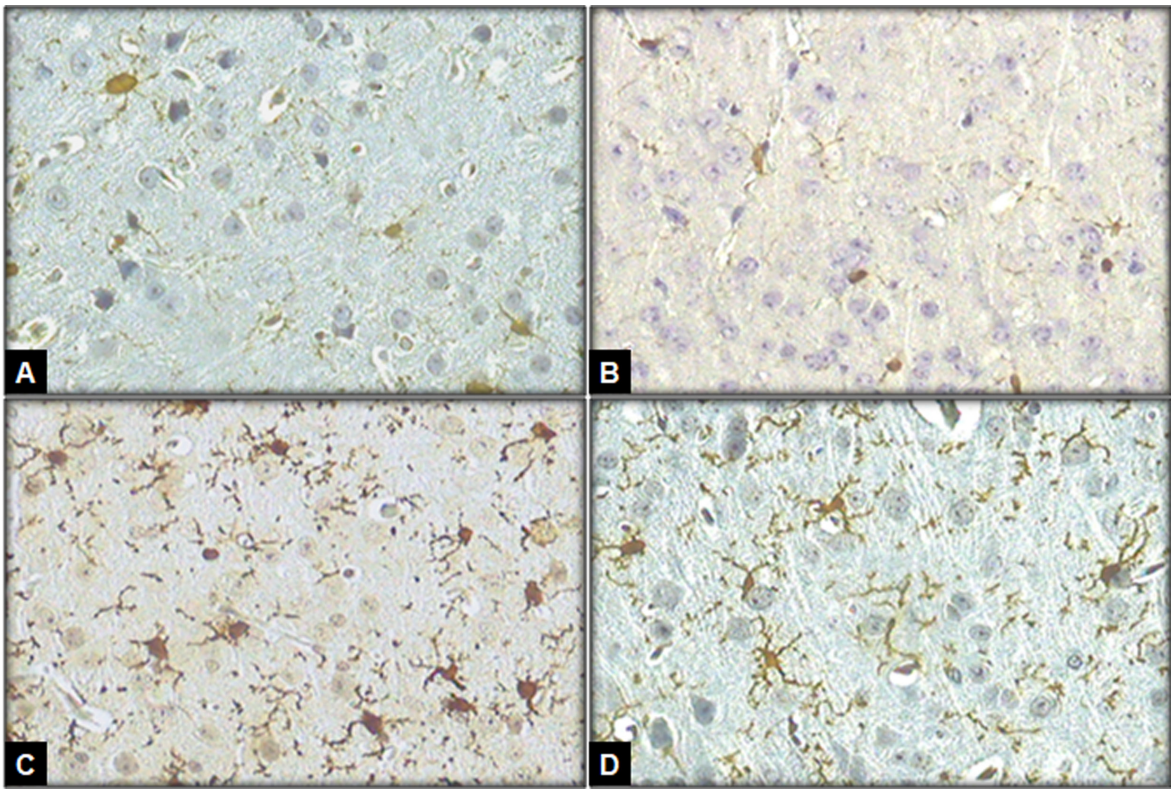


Fig. 3. Representative photomicrographs of Iba-1 immunohistochemical-stained brain sections. 7-week old male wild-type (WT) and TLR3/7/9$^{-/-}$ mice were inoculated with 10^4 Median Tissue Culture Infectious Dose ($TCID_{50}$) of Bovine alpha-herpesvirus 5 (BoHV-5), Mutum sample, or phosphate-buffered saline by the intracranial route and evaluated three days post-infection. Cerebral cortex of non-infected wild-type (WT) (A) and TLR3/7/9$^{-/-}$ mice (B) showing occasional immunopositive cells; BoHV-5-infected WT (C) and TLR3/7/9 deficient mice (D) with focal accumulation of Iba-1 positive cells in the frontal cortex. Magnification: A–D: ×400.

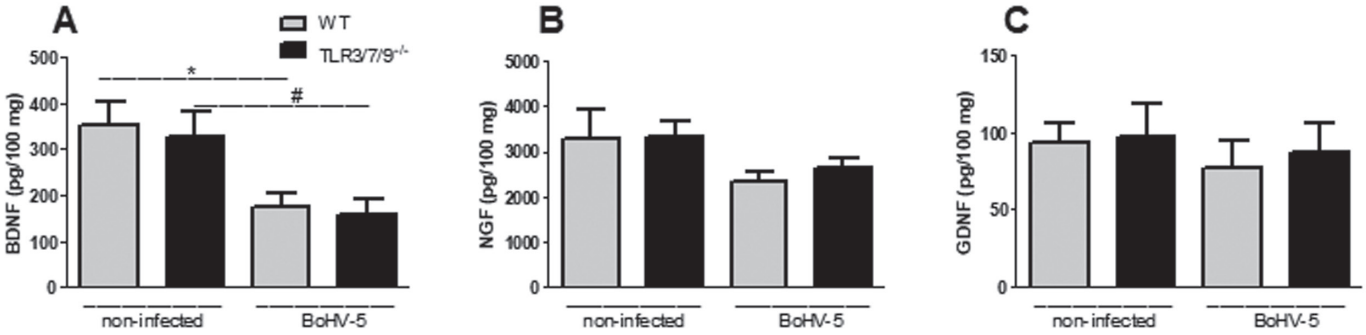


Fig. 4. Comparison of neurotrophic factors of brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and glial cell line-derived neurotrophic factor (GDNF) in the brain of non-infected wild-type (n=5), non-infected TLR3/7/9$^{-/-}$ (n=5), Bovine alpha-herpesvirus 5 (BoHV-5) infected wild-type (n=11) and Bovine alpha-herpesvirus 5 (BoHV-5) infected TLR3/7/9$^{-/-}$ mice (n=11). 7-week old male wild-type (WT) and TLR 3/7/9$^{-/-}$ mice were inoculated with 10^4 Median Tissue Culture Infectious Dose ($TCID_{50}$) of Bovine alpha-herpesvirus 5 (BoHV-5), Mutum sample, or phosphate-buffered saline by the intracranial route and evaluated three days post-infection. Both wild-type and TLR 3/7/9$^{-/-}$ infected mice exhibited lower brain levels of BDNF (P<0.05), compared with non-infected groups (A).

meningitis eight days post infection [4]. Triple TLR3/7/9 knockout mice did not alter those parameters.

Microscopically, the non-suppurative meningoencephalitis caused by BoHV-5 is characterized by leptomeningitis, focal gliosis, perivascular cuffing, and neuronal degeneration in infected cattle [27] and rabbits [30]. We also observed meningeal and parenchymal infiltration of lymphocytes, plasma cells and macrophages associated with gliosis, mainly located in the cerebrum and brainstem. Likewise, we showed neuronal damage in the CA regions from hippocampus. Previous studies already demonstrated the vulnerability of the hippocampus to neuronal damage after HSV-1 infection [48]. Apoptosis has been considered a relevant mechanism in the pathogenesis of BoHV-5. A large number of viral antigens was already visualized in hippocampal neurons from rabbits infected with BoHV-5 by intranasal route [2]. Moreover, trigeminal ganglions from calves infected with BoHV-5 showed positive neurons for cleaved caspase 3 [41]. Studies *in vitro* also demonstrated that BoHV-5 induced apoptosis in bovine neuron-like cells [11]. Additionally, lesions characterized by a locally extensive area of spongiosis, an increase in the number of glial cells and meningoencephalitis were described in BALB/c mice infected by intracranial route with BoHV-5. BoHV-5 antigens were observed within the cytoplasm of inflammatory and glial cells, within the vascular endothelium and macrophages [34].

In the present work, we observed several microglial cells with amoeboid morphology, similar to described in other viral infections [23, 25]. Microgliosis can trigger neuronal damage by the direct virus infection in CA areas of the hippocampus [39]. Additionally, increased TLR expression in reactive microglia activates the production of inflammatory mediators that can compromise brain function during viral infection [22, 24, 31]. On the other hand, it is important to note that microglia can also mediate neuroprotection by secretion of anti-inflammatory cytokines and neurotrophic factors [16]. In the current study, concurrent with the neuronal loss and brain inflammation, we observed that BoHV-5 infection promoted a significant decrease in the BDNF concentrations in wild-type and TLR3/7/9$^{-/-}$ mice. Neurotrophic factors stimulate survival of brain cells, being the BDNF one of the most relevant to prevent neuronal death and to modulate the neurogenesis [14, 47]. BDNF modulates brain plasticity and plays an essential role in the neuronal survival [28, 37]. BDNF prevented apoptosis by inhibition of caspase-3 activation in neurons infected with HIV-1 [37]. Furthermore, previous studies indicated the participation of BDNF in anti-inflammatory and anti-apoptotic effects in experimental models of *S. pneumoniae* meningitis [9]. In this context, reduction in BDNF levels has been correlated with the pathology severity in different brain disorders, such as Alzheimer's disease, HIV encephalitis and brain stroke [6, 37, 51]. Likewise, studies have demonstrated that the neuronal degeneration observed in NeuroAIDS patients may be promoted by lowering BDNF levels [37].

The intracerebral route used in the present study is a way to elicit an immune response directly from the central nervous system and determines lesions similar to those seen in cattle [4, 20]. When virus is inoculated in the periphery, by intraperitoneal route in Swiss mice, immune response of the host efficiently controls virus infection [1]. Viral central nervous system infection triggers activation of microglia and astrocytes and can elicit both innate and adaptive immune responses. Microglial cells express various TLRs such as TLR1, TLR2, TLR3, TLR4, TLR5, TLR7, TLR8, TLR9 [26]. This is the first study evaluating the effects of BoHV-5 infection in the absence of TLRs 3, 7 and 9 in C57BL/6 mice. There are few works analyzing the role of TLRs during acute bovine herpesvirus-5 infection and those works were restricted to *in vitro* or *in vivo* inoculation in cattle so far [32, 33, 38, 42]. Calves infected by intranasal route with $10^{6.3}$ $TCID_{50}$ of BoHV-5 showed an increase in the brain expression of TLRs 3, 7, 8 and 9 [33]. Additionally, a significant increase of TLR3, TLR7 and TLR8 mRNA was detected in different brain regions of cattle after inoculation with $10^{6.3}$ $TCID_{50}$ of BoHV-5. Nevertheless, the TLR9 expression was not affected by BoHV-5 infection of the central nervous system [42]. An *in vitro* study showed that the agonist stimulation of TLR 7/8 expressed by peripheral blood leukocytes promoted anti-viral activity on BoHV-5 infected MDBK cells [32]. The role of TLRs has been also evaluated in Herpes Simplex Virus 1 (HSV-1) infection in mice. TLR9 or TLR2/9 deficient mice showed higher susceptibility to HSV-1 infection by intranasal inoculation [29]. However, controversial results regarding the role of TLRs have been observed after the infection with West Nile virus (WNV) in mice. Some authors described that the absence of TLR3 resulted in higher susceptibility to WNV [17]. In contrast,

the survival rate of WNV-infected TLR3$^{-/-}$ mice was higher than WNV-infected wild-type mice [50]. Interestingly, TLR3 seems to be dispensable for the innate miRNA response to WNV infection [15]. In addition to TLR3, the cytosolic dsDNA-sensing machinery consisting of cGAS and STING was indispensable in combatting infection with HSV-1 [44]. Based on these findings we speculated that other pattern recognition receptors could be relevant to identification and response to BoHV-5 infection in C57BL/6 mice, acting as a compensatory multiple innate sensing pathways.

We suggested that BDNF down regulation was associated with brain damage and reactive microgliosis after BoHV-5 infection in mice. Moreover, we demonstrated that combined TLR3/7/9 deficiency does not alter those parameters. There are some limitations in the present study. The results are largely descriptive and does not show proof of causality. Further studies aimed to examine the mechanisms involved with neuronal damage as well as the neuroprotective role of BDNF during BoHV-5 infection are warranted.

CONFLICT OF INTEREST. The authors report no conflict of interests.

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