Journal of NeuroVirology, 15: 153–163, 2009

ISSN 1355-0284 print / 1538-2443 online
DOI: 10.1080/13550280802578067

informa
healthcare

Bovine herpesvirus 5 induces an overproduction of nitric oxide in the brain of rabbits that correlates with virus dissemination and precedes the development of neurological signs

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We herein report an investigation of nitric oxide (NO) levels, a candidate molecule for neuronal toxicity and dysfunction, in the brain of rabbits during experimental neurological infection by bovine herpesvirus 5 (BoHV-5). Spectrophotometry for NO products (NO_2 and NO_3) revealed that NO levels were significantly increased ($F(4, 40) = 3.33$; $P < .02$) in several regions of the brain of rabbits with neurological disease, correlating with moderate to high BoHV-5 titers. Immunohistochemistry of brain regions revealed a group of cells with neuronal and astrocyte morphology expressing the enzyme inducible NO synthase (iNOS) close to virus antigen–positive neurons. In addition, the investigation of nitric oxide levels between 2 and 6 days post infection (d.p.i.) revealed an initial increase in NO levels in the olfactory bulb and cortex (OB/OC) and anterior cortex (AC) at day 3 p.i., correlating with the initial detection of virus. As the infection proceeded, increased NO levels—and infectivity—were progressively being detected in the OB/CO and AC at day 4 p.i. ($F(12, 128) = 2.82$; $P < .003$); at day 5 p.i. in several brain regions ($P < .003$ in the OB/OC); and at day 6 p.i. in all regions ($P < .003$) but the thalamus. These results show that BoHV-5 replication in the brain of rabbits induces an overproduction of NO. The increase in NO levels in early infection correlated spatially and temporally with virus dissemination within the brain and preceded the development of neurological signs. Thus, the overproduction of NO in the brain of BoHV-5–infected rabbits may be a component of the pathogenesis of BoHV-5–induced neurological disease. *Journal of NeuroVirology* (2009) **15,** 153–163.

Keywords: BoHV-5; rabbits; pathogenesis; nitric oxide

Introduction

Bovine herpesvirus 5 (BoHV-5) is an alphaherpesvirus associated with meningoencephalitis, a disease generally fatal of cattle (Studdert, 1989). BoHV-5 is genetically and antigenically related to another important alphaherpesvirus of cattle, i.e., BoHV-1, the agent of infectious bovine rhinotracheitis (IBR) and vulvovaginitis/balanoposthitis (IPV/IPB) (Kahrs, 2001). Neurological disease associated with BoHV-5 infection has been frequently reported in several countries, especially in Brazil, Argentina, and Uruguay, where numerous outbreaks are reported

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The authors thank the Department of Pathology of the Federal University of Santa Maria, for their assistance in immunohistochemistry staining and veterinary students in the authors' laboratory for their assistance in animal handling and NOx measurements. The authors also thank CNPq (Conselho Nacional de Desenvolvimento Tecnológico) for funding and fellowships to E.F.F., R.W., M.W., F.D.T., A.F.F., and C.F.M.; and CAPES (Conselho de Aperfeiçoamento de Pessoal do Ensino Superior) for fellowships to R.D. and M.S.O.

Received 18 June 2008; revised 11 September 2008; accepted 7 October 2008

every year (Carrilo *et al,* 1983; Weiblen *et al,* 1989; Rissi *et al,* 2006).

Rabbits have been widely used to study several aspects of BoHV-5 neuropathogenesis, because they develop neurological infection and disease resembling that occurring in cattle (Meyer *et al,* 1996; Chowdhury *et al,* 1997; Silva *et al,* 1999). After intranasal inoculation, BoHV-5 is transported by terminal regional nerves to the olfactory bulb (OB), where it replicates and subsequently disseminates into other areas of the central nervous system (CNS) (Chowdhury *et al,* 1997; Diel *et al,* 2005). Viral invasion of the brain is generally followed by spread and massive replication in different areas, which is frequently accompanied by meningoencephalitis and the development of neurological signs (Chowdhury *et al,* 1997). The neurological signs developing upon BoHV-5 inoculation of weanling rabbits include depression, excitation, tremors, incoordination, bruxism, and opisthotonus, and these signs inevitably progress to seizures and death (Meyer *et al,* 1996; Chowdhury *et al,* 1997; Silva *et al,* 1999). Histologically, the neurological disease is characterized by leptomeningitis, focal gliosis, perivascular cuffing, and to a lesser extent, neuronal degeneration, mainly in the olfactory, parietal cortices, and hippocampus (Chowdhury *et al,* 1997). The brain of many rabbits developing early onset of neurological disease (6 to 9 days after virus inoculation), and also of cattle suffering from natural disease, do not show either histological changes of inflammatory reaction or a significant number of cells positive for BoHV-5 antigens (Silva *et al,* 1999; Rissi *et al*, 2006; E. F. Flores, unpublished observations).

Nitric oxide (NO), a signaling molecule involved in a wide range of physiologic activities, has been also involved in early antiviral responses in the CNS, in cytotoxicity, and in neuronal dysfunction (Harris *et al,* 1995; Chesler and Reiss, 2002; Serrano *et al,* 2002). Nitric oxide synthase (NOS), the enzyme that converts l-arginine in NO, has been identified in three isoforms: the endothelial (eNOS), neuronal (nNOS), and inducible (iNOS), encoded by different genes and expressed in distinct cell types (Cerqueira and Yoshida, 2002; Persichini *et al,* 2006). The isoformseNOS and the nNOS are constitutive enzymes, expressed mainly in endothelial cells and neurons, respectively, both regulated by intracellular influx of calcium and by calmodulin (Cerqueira and Yoshida, 2002). The isoform iNOS is expressed in response to pathogens and their components, by the signaling of cytokines such as interleukin-1β (IL-1β) and interferon-γ (IFN-γ), through nuclear factor (NF)-κB activity (Persichini *et al,* 2006). NO synthesized by iNOS plays an important role in the innate immune response against intracellular pathogens, and is expressed mainly by activated macrophages, microglia cells, and astrocytes recruited to sites of infection and inflammation in the CNS (Saha and Pahan, 2006). However, iNOS is responsible for a large burst of NO synthesis that greatly exceeds the amount produced by the constitutive eNOS and nNOS isoforms. This burst is likely responsible for the cytotoxic effects of NO during inflammation and neuronal dysfunction leading to seizures (Kiechele and Malinski, 1993; Wong and Marsden, 1996; Akaike and Maeda, 2000).

NO has been involved in the pathogenesis of many encephalitic viral infections, and neurotoxic effects have been attributed to this molecule during viral encephalitis (Harris *et al,* 1995; Fuji *et al,* 1999; Hooper *et al,* 2001; Ubol *et al,* 2001). Virus or viral components such as nucleic acids or proteins are strong inducers of iNOS in glial cells (Saha and Pahan, 2006). Increased expression of iNOS correlates in timing, location, and magnitude with those of virus propagation in the CNS of pigs infected with pseudorabies virus (PRV) (Serrano *et al,* 2002; Marcaccini *et al,* 2007). Expression of iNOS has also been correlated with the severity of neurological signs and degree of inflammatory reaction during rabies virus infection in mice (Ubol *et al,* 2001). An increased NO synthesis was also observed in CNS regions of rats harboring active herpes simplex virus (HSV)-1 replication (Fuji *et al,* 1999). Moreover, microglia cells have been considered the major source of iNOS synthesis in the brain of mice with HSV-induced brain oxidative stress (Marques *et al,* 2008).

In early phases of infection, NO is probably involved in limiting virus spread within the brain (Serrano *et al,* 2002). However, in later stages of infection, NO likely contributes to neurological dysfunction and neurotoxicity through the production of intermediary cytotoxic products, apoptosis, and necrosis (Cerqueira and Yoshida, 2002; Serrano *et al,* 2002). The formation of peroxinitrite ($ONOO^-$), for example, results in lipid peroxidation, protein oxidation, and DNA damage, producing oxidation and nitration of several biomolecules (Bogdan, 1998; Hooper *et al,* 2001; Zaki *et al,* 2005). NO has also been implicated in mitochondrial respiratory chain inhibition, leading to mitochondrial damage (Stewart and Heales, 2003) and in the induction of electrical dysfunction and seizures in rats (Royes et al, 2005).

The neurological disease accompanying BoHV-5 replication in the brain of experimentally infected rabbits and occurring at early times after virus inoculation is not frequently associated with pronounced histological changes and/or the presence of a significant number of antigen-positive neurons. Thus, it is hypothesized that mechanisms other than a severe inflammatory reaction and/or a massive infection and destruction of neurons may contribute for the neurological signs observed during BoHV-5 infection in rabbits. Thus, the objective of this study was to investigate whether the levels of NO—a candidate molecule for neuronal toxicity and dysfunction—are increased in the brain of rabbits during neurological infection by BoHV-5.

Results

Experiment 1

Clinical signs, virus isolation, and quantitation

All inoculated rabbits excreted virus in nasal secretions until the last day of clinical monitoring, demonstrating active virus replication in the nasal mucosa. In all cases, the euthanasia for tissue collection was performed approximately 12 h after the initial manifestations of neurological disease (8 to 10 days p.i.). Three inoculated rabbits (1, 2, and 3) plus three mock infected controls were euthanatized at day 8 p.i. Inoculated rabbits 4 and 5 plus two controls where submitted to euthanasia at day 10 p.i. Brain regions collected individually were submitted to virus isolation and quantitation. Table 1 presents the viral titers in different brain areas of rabbits presenting neurological signs. Virus titers ranging from $10^{3.87}$ to $10^{5.6}$ $TCID_{50}/g$ were detected in all brain areas collected from these rabbits. Higher titers were detected in the anterior cortex (AC), parietal/ventrolateral cortices (DLC/VLC), and hippocampus/posterior cortex (HI/PC) of rabbits 1, 3, and 4. The thalamus (Th) of the animals 2 and 3 and HI/PC of animal 5 were positive for infectious virus only in the second passage in cell culture.

NOx levels in the brain of rabbits with neurological disease

Colorimetric measurement of NO intermediary products (NO_2 and NO_3) showed that NO levels were significantly increased ($F(4, 40) = 3.33$; $P < .02$) in all examined regions of the brain collected from rabbits presenting neurological disease (Figure 1). In these animals, the AC presented the highest levels, followed by the olfactory bulb and cortex (OB/OC), DLC/VLC, HI/PC, and Th. The baseline levels of NOx in the controls remained fairly steady, with minor variations among the brain regions (Figure 1). As shown in Table 1, virus isolation and quantitation from tissues revealed the presence of moderate virus titers in all these areas. These results showed that the levels of NOx were significantly increased in several areas of the brain of rabbits presenting neurological signs associated with BoHV-5 infection, and the increased NO levels correlated spatially with virus replication.

Immunohistochemistry for BoHV-5 antigens and iNOS

Antigens of BoHV-5 where detected by immunohistochemistry (IHC) exclusively in cells with characteristic neuronal morphology, predominantly of the OB/OC and AC of the brain of sick rabbits (Figure 2A). Virus-positive neurons were also observed in the HI/PC, DLC/VLC, and Th, although in a lower frequency. In general, cells positive for viral antigens were present in well-defined clusters, typically of 4 to 10 neurons; usually one to three clusters per brain region. The location of these clusters was roughly the same, probably corresponding to areas of anatomically and functionally related neurons (not shown). A few, scattered, antigen-positive neurons were also observed across some sections, outside the clusters (not shown). As expected, brain regions of control animals were negative for BoHV-5 antigens.

Clusters of cells showing strong iNOS signal—with astrocyte, microglia, and neuronal characteristic morphology—were observed in the same areas that were positive for BoHV-5 antigens (Figure 2B). The brain regions of control animals also showed some scattered cells weakly stained for iNOS. These results suggest that low levels of iNOS are expressed by cells of the brain even in the absence of inflammatory stimuli. Increased levels and well-defined clusters of cells expressing high levels of iNOS, however, were present exclusively in the brain of inoculated rabbits, demonstrating the induction of iNOS expression after BoHV-5 infection.

Histopathology

All brain regions collected from sick animals (Experiment 1) for NOx determinations were

Table 1 Infectivity in different areas of the brain of rabbits inoculated intranasally with bovine herpesvirus 5 and euthanized approximately 12 h after the onset of neurological signs, between days 8 and 10 p.i. (Experiment 1)

	Brain region				
Rabbit	OB/OC*	AC	DLC/VLC	HI/PC	Th
1	++[†]	+++	++	++	+
2	+	++	++	+	(+)[‡]
3	++	++	+++	+++	(+)
4	++	+++	+++	+++	+++
5	++	++	+	(+)	++

*OB/OC: olfactory bulb/olfactory cortex; AC: anterior cortex; DLC/VLC: parietal and ventrolateral cortices; HI/CP: hippocampus and posterior cortex; Th: thalamus.
[†]+: virus titer $<10^{3.97}$ $TCID_{50}/g$; ++: virus titer between 10^4 and 10^5 $TCID_{50}/g$; +++: virus titer between 10^5 and 10^6 $TCID_{50}/g$.
[‡](+): virus detected only in the second passage in cultured cells.

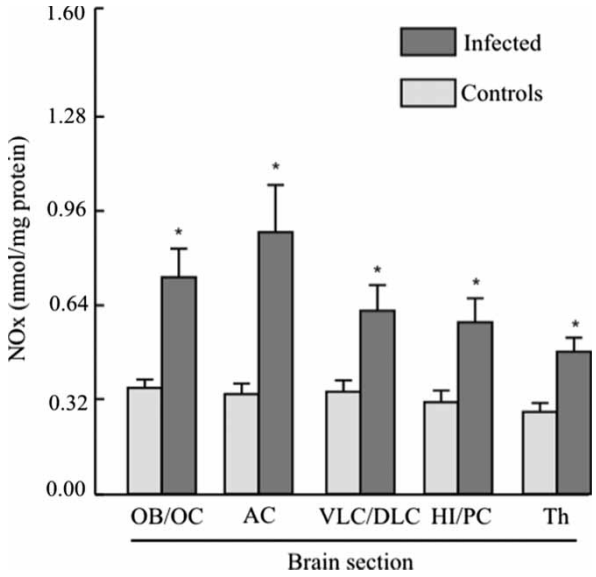

Figure 1 Nitric oxide (NOx) levels in brain regions of rabbits with neurological disease associated with bovine herpesvirus 5 infection. *Statisticaly significant, $F(4, 40) = 3.33$; $P < .02$. OB/OC: olfactory bulb and cortex; AC: anterior cortex; DLC/VLC: parietal and ventrolateral cortices; HI/PC: hippocampus and posterior cortex; Th: thalamus. The bars represent the mean of NOx levels in the respective brain regions of the groups, composed by five animals each.

submitted to histological examination. A summary of the histological changes and the clinical signs presented by sick rabbits is presented in Table 2. Histological/inflammatory changes were more pronounced in rabbits 3 and 4. At the day of euthanasia, these rabbits presented a profound depression, nasal discharge, and rabbit 3 also had bruxism. In contrast, rabbits 1, 2, and 5 presented classical, severe signs of neurological infection such as circling, opisthotonus, and seizures. Interestingly, mild, restricted or none histological changes were observed in the brain of these animals. A consistent finding among the infected rabbits (exception rabbit 2) was the presence of histological/inflammatory changes mainly in the anterior structures (OC, AC).

Experiment 2

As the NOx measurements in Experiment 1 were performed in the brain of rabbits with overt neurological signs, a question remained whether the increase in NOx levels preceded or followed the development of neurological signs, especially seizures. To answer this question, another experiment was conducted, in which the measurements of NOx levels and search for virus replication were performed concomitantly and sequentially, prior to the development of neurological signs, in the brain of rabbits euthanized at different time points after virus inoculation (Experiment 2).

Virus isolation and quantitation

Infectious virus was demonstrated in nasal secretions of the inoculated rabbits between day 1 p.i. and the respective day of euthanasia. No infectious virus was recovered from brain regions of rabbits euthanized at day 2 p.i. and from controls. The progression of virus detection in each particular day and brain region is presented in Table 3. Probably,

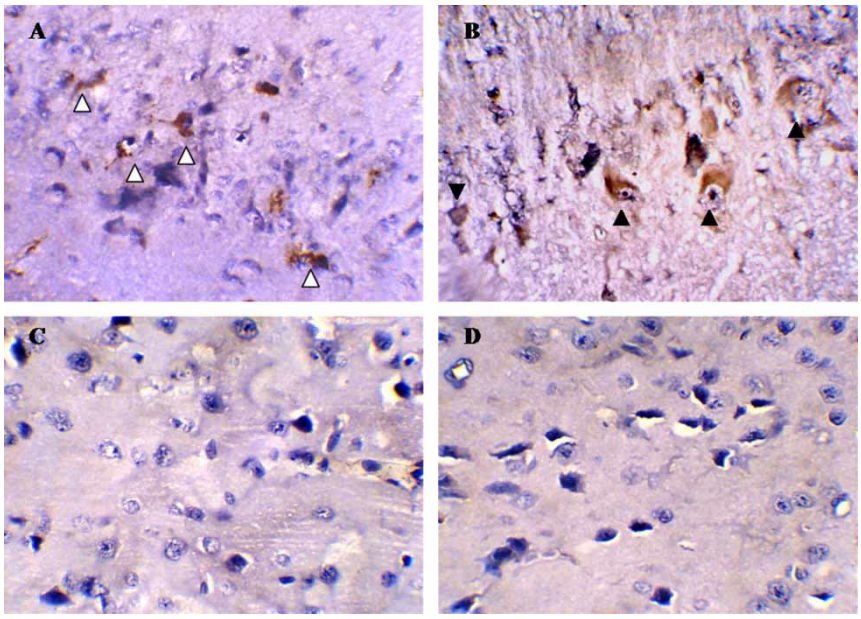

Figure 2 Immunohistochemistry for bovine herpesvirus 5 (BoHV-5) antigens and iNOS in sequential sections of the brain of rabbits with neurological disease associated with BoHV-5 infection. (**A**) Anterior cortex of rabbit 1: BoHV-5 antigens in cells with neuronal morphology (Δ). (**B**) Olfactory bulb of rabbit 5: iNOS reactivity in cells with astrocyte morphology (▼) and neuronal morphology (▲). Anterior cortex of a control rabbit stained for (**C**) BoHV-5 antigens and (**D**) iNOS.

Table 2 Clinical signs and histological findings in the brain of rabbits inoculated intranasally with bovine herpesvirus 5 and euthanized approximately 12 h after the onset of neurological signs (Experiment #1)

R	Clinical signs	Euthanasia (day)	Histopathology
1	Depression, nasal discharge, opisthotonus, bruxism, seizures.	8 p.i.[†]	OB/OC[*]: mild endothelial swelling, mild perivascular cuffing; AC: edema, neuronal necrosis; DLC/VLC and HI/CP: none; Th: neuronal necrosis.
2	Depression, nasal discharge, circling, seizures.	8 p.i.	None.
3	Depression, nasal discharge, bruxism.	8 p.i.	OB/OC, AC and Th: mild endothelial edema, mild perivascular cuffing; gliosis; OB/OC: some necrotic neurons; DLC/VLC: none.
4	Profound depression, nasal discharge.	10 p.i.	OB/OC, AC, DLC/VLC, HI/PC, Th: mild endothelial swelling, perivascular cuffing, focal and diffuse gliosis; HI/PC: edema.
5	Depression, nasal discharge, ataxia, incoordination, seizures.	10 p.i.	OC: neuronal necrosis; OB, AC, DLC/VLC, HI/PC and Th: none.
Controls ($n=5$)	None.	10 p.i.	None.

[*]OB/OC: olfactory bulb/olfactory cortex; AC: anterior cortex; DLC/VLC: parietal and ventrolateral cortices; HI/CP: hippocampus and posterior cortex; Th: thalamus.
[†]p.i.: post infection.

the HI/PC and Th are the last areas to be infected during viral dissemination within the brain, because some of these sections were still negative for virus at day 6 p.i., yet positive in Experiment 1 (examined at days 8 to 10 p.i.). The virus titers detected at late stages (5 and 6 d.p.i.) were higher than those detected at early times; and the distribution of the virus resembled that observed in Experiment 1, with the exception of Th (Tables 1 and 3). The kinetics of viral detection in different brain regions supports the findings that the main pathway of BoHV-5 transport into the brain of rabbits after intranasal inoculation is the olfactory tract (Chowdhury *et al,* 1997; Diel *et al,* 2005).

Table 3 Infectivity in different areas of the brain of rabbits inoculated intranasally with bovine herpesvirus 5 and euthanized at different intervals after virus inoculation (Experiment 2)

		Brain region			
Euthanasia	Rabbit	OB/OC[*]	AC	DLC/VLC	HI/PC
Day 2 p.i.[†]	6–9	−	−	−	−
Day 3 p.i.	10	−[‡]	−	−	−
	11	−	−	−	−
	12	(+)[§]	(+)	−	−
	13	(+)	−	−	−
Day 4 p.i.	14	−	−	−	−
	15	−	−	−	−
	16	+	++	++	−
	17	+	+	−	−
Day 5 p.i.	18	++	(+)	−	−
	19	++	++	−	−
	20	++	+	++	+
	21	+	+	++	−
Day 6 p.i.	22	++	(+)	−	−
	23	(+)	+	−	−
	24	++	++	++	+
	25	++	++	+	(+)
Controls	26–50	−	−	−	−

[*]OB/OC: olfactory bulb/olfactory cortex; AC: anterior cortex; DLC/VLC: parietal and ventrolateral cortices; HI/CP: hippocampus and posterior cortex.
[†] p.i.: post infection.
[‡]Considered negative after three passages in cell culture. Virus has not been detected in any of the thalamus (not shown).
[§](+): virus detected only in the second passage in cultured cells; +: virus titer $<10^{3.97}$ $TCID_{50}$/g; ++: virus titer between 10^4 and 10^5 $TCID_{50}$/g.

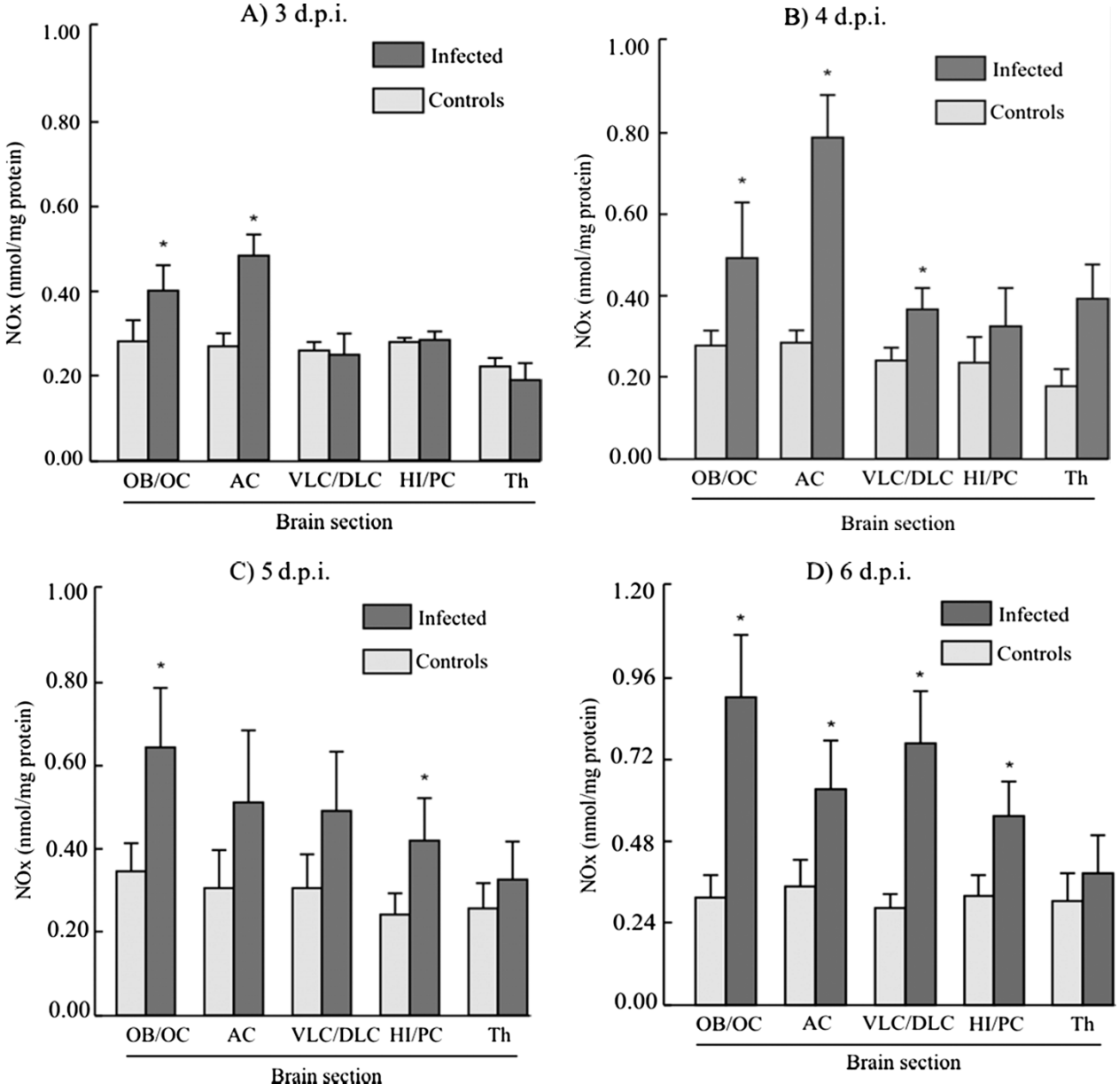

Figure 3 Nitric oxide (NOx) levels in brain regions of rabbits at different time points after intranasal inoculation of bovine herpesvirus 5. d.p.i.: days post infection. OB/OC: olfactory bulb and cortex; AC: anterior cortex; DLC/VLC: parietal and ventrolateral cortices; HI/PC: hippocampus and posterior cortex; Th: thalamus. *Statisticaly significant $F(12, 128) = 2.82$; $P < .003$. The bars represent the means of NOx measurements in the respective brain region of each group. The groups were composed by four infected and four mock-infected rabbits.

NOx levels in the brain of rabbits at different time points after virus inoculation

At day 2 p.i., the levels of NOx in different areas of the brain of infected rabbits did not differ significantly from those of control rabbits (not shown). At day 3 p.i., a slight increase in NOx levels was observed in the OB/OC and AC (Figure 3A). This increase correlated with the initial detection of virus in these structures (Table 3). No changes in NOx levels were detected in the other structures at day 3 p.i., correlating with the lack of virus detection. At day 4 p.i., high NOx levels were detected in several examined sections. The increase was more pronounced in the AC (statistically significant; $F(12, 128) = 2.82$, $P < .003$), followed by OB/OC. The other structures also showed a slight, yet not statistically significant increase (Figure 3B). Again, the increased levels of NOx were correlated with the detection of virus in these areas (Table 3). At day 5 p.i., NOx levels were increased in all examined sections, yet a statistically significant difference between inoculated and control animals ($F(12, 128) = 2.82$; $P < .003$) was observed only in the OB/OC (Figure 3C). At this day,

infectious virus was detected in several examined sections (Table 3, rabbits 18, 19, 20, and 21). In the last day of examination (day 6 p.i.), all sections but the Th presented a significant increase in the NOx levels ($F(12, 128) = 3.33$; $P < .003$; Figure 3D). These findings also correlated with the widespread distribution of the virus across these sections at day 6 p.i., and the lack of virus detection in the Th (Table 3, rabbits 22, 23, 24, and 25). These results showed that NOx levels increased progressively in most brain regions between days 3 and 6 p.i. and this increase correlated spatially and temporally with virus dissemination within the brain. In all cases, overproduction of NOx preceded the development of neurological disease.

Discussion

The results shown herein demonstrate that BoHV-5 replication in the brain of experimentally infected rabbits induces an increase in the levels of NOx. The high NOx levels correlated with the areas harboring active virus replication and were not necessarily accompanied by histological inflammatory changes. Furthermore, sequential measurements of NOx at different time points after virus inoculation revealed that the increased levels correlated temporally and spatially with virus invasion into and spread within the brain; and preceded the development of neurological signs. Thus, it is reasonable to speculate that the increased synthesis of NO that starts at early stages of virus replication in the brain—likely a component of the innate immune response to the virus—may be followed by an overproduction of NO, leading to neuronal toxicity and dysfunction and contributing for the development of neurological disease.

In our experience, the brains of many rabbits developing early onset neurological disease upon BoHV-5 inoculation (days 6 to 9 p.i.), and some cattle developing natural disease as well, do not present marked histological changes of encephalitis (Silva *et al*, 1999; Rissi *et al*, 2006; E. F. Flores, unpublished observations). Likewise, virus positive neurons (as ascertained by IHC), or necrotic neurons, are not usually present in abundant numbers in many brains from sick animals. The lack of correlation between severity of neurological disease and the degree of histological changes was also observed in the present experiment. Rabbits 1 and 5 (Experiment 1) developed severe neurological signs (seizures, opisthotonus) albeit presented only mild and/or restricted inflammatory changes. Rabbit 2 did not present any histological change in spite of the severe neurological disease (Table 2). The absence or mildness of inflammatory histological changes and the relatively low number of infected/affected neurons contrast with the severity of neurological signs, which inevitably lead to death. These observations prompted us to investigate whether the levels of NO—a candidate molecule for neuronal toxicity and dysfunction—are increased in the brain of rabbits during neurological infection by BoHV-5.

NO produced by inflammatory cells, e.g., activated macrophages and glial cells through induction of the enzyme inducible NO synthase (iNOS) has been involved in the pathogenesis of neurotropic virus infections, and both protective and neurotoxic effects have been attributed to this molecule (Harris *et al,* 1995; Fuji *et al,* 1999; Ubol *et al,* 2001; Serrano *et al,* 2002). The NO synthesized by iNOS is believed to play an important role in the innate immune response against viruses (Chesler and Reiss, 2002). In initial stages of neurological infection, NO may be important to limit the virus spread to different areas of the CNS, acting as part of an antiviral strategy during innate immune response, along with IFN-γ, TNF-α, IL-1β, and other cytokines expressed mainly by macrophages, microglia cells, astrocytes, and neurons (Minc-Golomb *et al,* 1994, 1996; Moro *et al,* 1998; Saha and Pahan, 2006; Marcaccini *et al,* 2007). These responses lead to an inflammatory reaction, necessary to control early stages of infection and activate specific immune responses (Karupiah and Harris, 1995; Kodukula *et al,* 1999; Akaike and Maeda, 2000; Chesler and Reiss, 2002). Although there is a strong neuroinflammatory response, induced by microglia cells early into the brain of HSV-infected mice, this response seems not to be enough to avoid the progression of the disease (Marques *et al*, 2006).

On the other hand, it is widely accepted that high NO levels lead to oxidative injury through oxidation and nitration reaction of biomolecules (Akaike and Maeda, 2000). Increased levels of iNOS correlates with PRV propagation in the CNS of infected pigs (Serrano *et al,* 2002; Marcaccini *et al,* 2007), correlates with the severity of neurological signs during rabies virus infection in mice (Ubol *et al,* 2001), and were also observed in CNS regions of HSV-1 experimentally infected rats (Fuji *et al,* 1999). Recent studies demonstrate the presence of oxidative damage in the brain of mice with neurological infection by HSV. In this study, microglia cells are considered the major source of iNOS (Marques *et al.*, 2008). Treatment of HSV-1–infected rats with an iNOS inhibitor (l-NMMA) resulted in reduction of morbidity and mortality, with amelioration and/or reduction of neurological signs, reinforcing the role of NO in the pathogenesis of the disease (Fuji *et al,* 1999). Taken together, these findings suggest that NO produced in early infection might be involved in limiting virus spread within the brain, yet in later stages (and in high levels) may contribute to neurological neurotoxicity and dysfunction through the production of cytotoxic products (Fuji et al, 1999; Serrano *et al,* 2002). These observations are not exclusive to neurological disease by herpesviruses, because

NO overproduction has also been demonstrated during rabies virus, borna disease virus, and reovirus neurological infections (Koprowsky *et al,* 1993; Ubol *et al,* 2001; Goody *et al,* 2005).

In our first experiment, spectrophotometry for NO_2 and NO_3 demonstrated that NOx levels were significantly increased in all examined brain regions, and that high NOx levels correlated spatially with virus replication (Figure 1, Table 1). Although the cell source of the NO burst detected by spectrophotometry could not be unequivocally determined by our IHC, it was likely derived from iNOS induction. Both nNOS and eNOS are constitutively expressed in low levels in neurons and endothelial cells, respectively, and do not contribute to the burst of NO observed in inflammatory reactions. Rather, this burst has been mainly attributed to iNOS induction and NO synthesis by inflammatory cells (Akaike and Maeda, 2000; Marcaccini *et al,* 2007). Regardless the cell source, our NOx measurements detected high NO levels in several areas of the brain of rabbits developing neurological disease upon BoHV-5 infection.

As the levels of NOx were first determined in rabbits with overt neurological disease and undergoing episodes of seizures, it was not clear whether the increased levels preceded, accompanied, or followed these neurological events. NO has been shown to act as proconvulsing agent and abnormal expression of nNOS and NO synthesis can trigger seizures in rats (Bagetta *et al,* 2002; Royes *et al,* 2005). Likewise, seizures may act as a trigger for the induction of iNOS and overproduction of NO, because seizures may induce IL-1β expression (Vezzani, 2005) and electrical impulses. Then we investigated the timing and spatial relationship between virus invasion and replication in the brain and NO synthesis. Results from Experiment 2 demonstrated that the increase in NO levels correlated spatially and temporally with viral dissemination within the brain. High NOx levels were first detected in anterior structures (OB/OC, AC) at day 3 p.i., coinciding with the first detection of virus in these areas. Following intranasal inoculation, BoHV-5 invades the brain mainly through the olfactory route, reaching first the OB and AC, where it replicates and disseminates further into the brain (Chowdhury *et al,* 1997; Diel *et al,* 2005). Indeed, both infectivity and high NO levels were first (and consistently) detected in these anterior structures (OC, AC). In the following days, high NOx levels were being progressively detected in additional areas, always correlating with infectivity. Thus, NO overproduction seemed to accompany virus dissemination within the brain and clearly preceded the development of neurological signs.

Seizures are consistently observed during neurological disease associated with BoHV-5 infection in rabbits (Meyer *et al,* 1996; Chowdhury *et al,* 1997; Silva *et al,* 1999). In our study, NOx levels were higher in the brain of rabbits with overt neurological disease (Experiment 1) than those observed early in infection, prior to the development of neurological signs (Experiment 2). Thus, as suggested in other viral systems (Fuji *et al,* 1999) it is possible that overproduction of NO during early infection may contribute to neuronal toxicity and dysfunction, leading to the production of neurological signs, including seizures. The production of seizures, in its turn, might provide stimuli for additional iNOS and nNOS activation, leading to further increase in NO levels and neuropathology (Serrano *et al,* 2002; Royes *et al,* 2005). Taken together with previous data, our results suggest that overproduction of NO and its toxic effects to neurons may be a component of the neuropathogenesis of BoHV-5 infection in rabbits. Experiments may also be performed in the future to determine whether NO levels are increased in the brain of BoHV-5 experimentally infected calves.

In summary, our results showed that NO levels are up-regulated in the brain of rabbits during BoHV-5–induced neurological disease. The increase in NO synthesis correlated spatially and temporally with the dissemination of the virus within the brain and preceded the development of clinical disease. The correlation between NO overproduction and the development of neurological signs suggests a potential role for NO in the neuropathology associated with BoHV-5 infection. In this context, a better understanding of the mechanisms leading to neuronal toxicity and/or dysfunction may help in understanding the neuropathogenesis of BoHV-5 infection in cattle. Currently, we are focusing on the investigation on the role of NO in the neuropathogenesis of BoHV-5 by means of inhibiting nitric oxide overproduction in the rabbit model.

Material and methods

Experimental design

Two independent experiments were performed to determine whether replication of BoHV-5 in the brain of experimentally infected rabbits was accompanied by increase in NO levels. In Experiment 1, five rabbits were inoculated intranasally with the virus and other five served as mock-infected controls (inoculated with minimal essential medium [MEM]). The inoculated rabbits were monitored clinically on a daily basis and submitted to euthanasia approximately 12 h after the onset of neurological signs (between days 8 and 10 post infection [p.i.]). Brain regions obtained from sick (inoculated) and healthy (control) rabbits were collected for quantitation of NO products (NOx), virus isolation and quantitation, immunohistochemistry (IHC) for viral antigens and iNOS, and for histopathological examination. In Experiment 2, five groups of four

rabbits were inoculated with the virus and together with five groups of controls (four mock-infected rabbits) were submitted to euthanasia for tissue collection and examination at days 2, 3, 4, 5, and 6 p.i. This experiment was conducted to determine whether the increase in nitric oxide levels occurred before or after the onset of neurological signs.

Cells and virus

Virus amplification, quantitation, and isolation from tissues were performed in CRIB cells (Flores and Donis, 1995), derived from Madin-Darby bovine kidney cells (American Type Culture Collection, CCL-22). Cells were routinely maintained in minimal essential medium (MEM; Cultilab, Campinas, São Paulo, Brazil) containing penicillin (1.6 mg/L), streptomycin (0.4 mg/L), nistatin (0.02 μg/L), and 5% fetal calf serum (Cultilab). The BoHV-5 SV-507/99 strain was isolated from an outbreak of meningoencephalitis in southern Brazil, submitted to nucleotide sequencing of the entire genome (Delhon *et al,* 2003), and its biological properties have been extensively characterized (Vogel *et al,* 2003; Diel *et al*, 2005).

Animals, virus inoculation, monitoring, and sampling

Thirty-day-old, weanling New Zealand rabbits were used throughout the experiments. The rabbits were inoculated by the intranasal route with 1 ml of a viral suspension containing approximately $10^{7.5}$ $TCID_{50}$ (50% tissue culture infectious dose) of SV-507/99, or received 1 ml of MEM (control groups) by the same route. Previously to virus or MEM administration, the animals were anesthetized with Zoletil 50, 200 μl SC (Virbac do Brasil Ind. E Com. LTDA, São Paulo, SP, Brazil). After virus inoculation, the rabbits were monitored clinically on a daily basis. Virus replication was monitored by submitting nasal swabs to viral isolation in CRIB cells. For tissue collection, animals were submitted to euthanasia after ventilation with halothane and oxygen. Different sections of the brain (Figure 4) were collected for quantification of degradation products of NO, virus isolation (stored at $-70^{\circ}C$ until testing), IHC (fixed in methacarn), and histopathology. All procedures during the animal experiments were conducted under veterinary supervision and according to COBEA's recommendations (Brazilian Committee on Animal Experimentation). The animal experiments were approved by the Institutional Committee on Ethics in Research (approval 23081.012136/2007-90, August, 27th, 2007).

Virus isolation and quantitation

Nasal secretions collected on a daily basis were submitted to virus isolation in CRIB cells according to standard protocols. The material was considered negative for virus after three passages of five days each without the appearance of cytophatic effect (CPE). For virus isolation from brain regions, a 1:10 (w/v) tissues homogenates were prepared, centrifuged, and the supernatant was inoculated onto CRIB cells and monitored as described above. The infectivity of positive tissues was subsequently quantitated by limiting dilution in CRIB cells and the virus titers were calculated according to Reed and Muench (1938) and expressed as $\log_{10}$ $TCID_{50}$/g of tissue. Samples positive only in the second or third passages were considered to harbor a titer of $<10^{2.87}$ $TCID_{50}$/g.

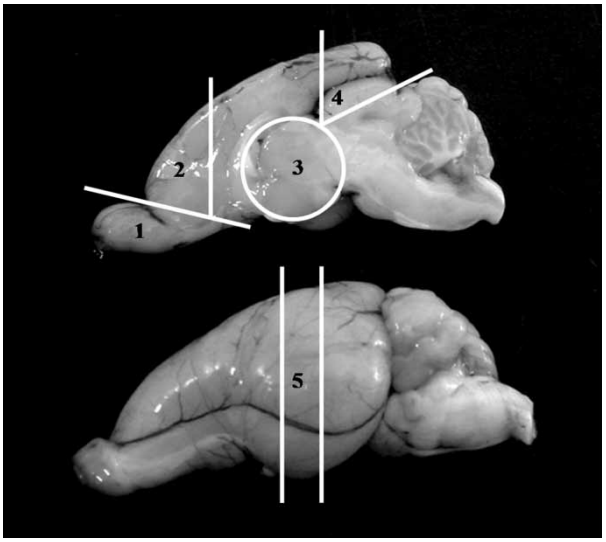

Figure 4 Sections of the brain of rabbits used for nitric oxide quantitation, bovine herpesvirus 5 isolation and immunohistochemistry for viral antigens and iNOS. (1) Olfactory bulb and cortex; (2) anterior cortex; (3) thalamus; (4) hippocampus and posterior cortex; and (5) parietal and ventrolateral cortices. The top image represents a mediolateral view of a cross section, and the bottom shows a lateromedial view.

Assay of NOx as a marker of NO levels

As NO is unstable in the environment, NO levels are generally quantified by spectrophotometry, measuring nitrite (NO_2) and nitrate (NO_3), the stable products of NO degradation. In this study, NO_2 and NO_3 levels (NOx) were colorimetrically determined according to a protocol described by Miranda *et al* (2001). All assays for NOx and protein determination were performed immediately after euthanasia and removal of the brain. A piece of 50 to 100 mg of each brain region (Figure 4) was homogenized in zinc sulphate and acetonitrile, centrifuged at $16000 \times g$ for 30 min at $4^{\circ}C$, and the supernatant was collected for analysis in triplicate. Briefly, 200 μl of supernatants was added to tubes containing 200 μl of sulfanilamide (2%), 200 μl of N-(1 naphthyl)-ethylene diamine·diHCl (NEDD) (0.1%), and 400 μl of vanadium chloride 2%. After incubation under agitation at $37^{\circ}C$ for 1 h, colorimetrical changes were evaluated spectrophotometrically at 540 nm wavelength.

Serial dilutions of NO_2 100 μM were tested in triplicate for calculation of the mean correction factor (MCF1). The mean of the three spectrophotometry values for each tissue was corrected by MCF1 and converted to nanomoles (nmol) of nitric oxide products, according to Miranda *et al* (2001).

Protein determination

The protein content of each brain region submitted to determination of NOx levels was measured colorimetrically by the method described by Bradford (1976). Each sample was tested in duplicate and the absorbance was measured in 595 nm wavelenght. Dilutions of bovine serum albumin at 1 mg/ml were tested in triplicate for mean correction factor (MCF2) calculation. Colorimetric values of the brain regions were then corrected by standard albumin MCF2, by the dilution of the samples ($\times 20$), by the volume—to the same used in NOx measure—($\times 4$). The result represents the amount of protein in each section, in milligrams.

The NOx content of each brain region was divided by the protein content of its respective pellet, resulting in the amount of NOx in nanomoles of NOx per milligram of protein (nmol NOx/mg protein).

Data analysis

The data obtained by quantitation of NO products (nmol NO_x/mg of protein) were submitted to analysis of variance, comparing the means of controls with the means of infected animals, for each particular section (Experiments 1 and 2), and for each day (Experiment 2). Two-way analysis of variance (ANOVA) was carried out using SPSS (SPSS, Chicago, IL, USA). Post hoc analysis was carried out by the Duncan test when appropriate. $P < .05$ was considered significant.

Immunohistochemistry (IHC) and histological examination

Brain regions of rabbits collected after euthanasia (Figure 4) were fixed in methacarn for 12 to 16 h, embedded in paraffin and sectioned at 5 μm. Two consecutive sections of each brain region were submitted to IHC to detect BoHV-5 antigens and iNOS expression, respectively. IHC was performed essentially as described by Oldoni *et al* (2004), using the monoclonal antibody 2F9 specific for BoHV-5 glycoprotein C (2F9; 1:500); or with the polyclonal antibody NOS2 C-19sc649 for iNOS (NOS2; 1:100), from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The presence of viral antigens was revealed by using a streptavidin-biotin-peroxidase kit (LSAB Plus, Dako, Glostrup, Denmark), followed by addition of the substrate diaminobenzidine (Sigma, St. Louis, MO, USA). Finally, the sections were stained with hematoxylin, dehydrated, and mounted for microscopic examination.

For histological examination, slides containing one section (5 μm) of each brain region were stained with hematoxilin and eosin (H&E).

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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This paper was first published online on iFirst on 24 December 2008.