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eNOS Expression in the Cerebellum of Dogs Naturally Infected with Canine Distemper Virus and Relationship with Apoptosis

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ABSTRACT
Objective: In this study, the role of eNOS expression in the neuropathology of canine distemper disease was investigated. **Materials and Methods:** In the study, cerebellar tissues of 20 dogs brought for necropsy and diagnosed with distemper by histopathological and immunohistochemical methods were used. Cerebellum tissues from 7 dogs that died of other diseases in which the central nervous system was not affected were used as controls. All tissues were fixed in 10% buffered formalin solution for 48 h then washed thoroughly in tap water overnight. After dehydration in graded alcohols, cleared in xylene and embedded in paraffin. Paraffin blocks of cerebellum were cut at 5 µm and stained with hematoxylin and eosin (HE) and Immunohistochemistry (IHC) was performed according to the manufacturer's protocol. **Results:** Expression of eNOS and cells undergoing apoptosis in infected tissues were increased compared to control tissues. This difference was statistically significant. **Conclusion:** As a result, increased eNOS expression may be one of the factors that trigger apoptosis in neuropathology of canine distemper disease. However, further studies are needed for other effects that contribute to the neuropathology of the disease**.**
Keywords: Apoptosis, Distemper, Dog, eNOS.

Köpek Distemper Virus ile Doğal Enfekte Köpeklerin Serebellumunda eNOS Ekspresyonu ve Apopitoz ile İlişkisi

ÖZ

Amaç: Bu çalışmada köpek distemper hastalığının nöropatolojisinde eNOS ekspresyonunun rolü araştırıldı. **Gereç ve Yöntem:** Araştırmada nekropsi için getirilen ve histopatolojik ve immunohistokimyasal yöntemlerle distemper tanısı konan 20 köpeğin beyincik dokuları kullanıldı. Merkezi sinir sisteminin etkilenmediği diğer hastalıklardan ölen 7 köpeğin beyincik dokuları ise kontrol olarak kullanıldı. Tüm dokular %10'luk tamponlu formalin solüsyonunda 48 saat süreyle fikse edildikten sonra çeşme suyu altında bir gece yıkandı. Dereceli alkollerde dehidrasyondan sonra ksilende şeffaflaştırıldı ve parafine gömüldü. Parafin bloklardaki serebellum dokularından 5'er mikronluk kesitler alındı ve hematoksilen ve eozin (HE) ile boyandı ve üretici firmanın protokolüne göre immunohistokimya (IHC) yapıldı. **Bulgular:** Enfekte dokularda eNOS ekspresyonu ve apopitoza giden hücrelerin kontrol dokulara göre arttığı görüldü. Bu fark istatistiksel olarak anlamlıydı. **Sonuç:** Sonuç olarak artan eNOS ekspresyonu apoptozu tetikleyen faktörlerden biri olabilir. Ancak hastalığın nöropatolojisine katkıda bulunan diğer etkiler için daha fazla çalışmalara ihtiyaç vardır.
Anahtar Kelimeler: Apopitoz, Distemper, Köpek, eNOS.

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INTRODUCTION

Canine Distemper Virus (CDV) is a virus belongs to the morbillivirus genus from the paramyxoviridia family, as well as rinderpest and measles viruses (Norrby et al., 1992). CDV causes multifocal demyelination in the central nervous system in infected dogs (Graber et al., 1995). Clinical findings such as apathy, behavioral disorders, muscle atrophy, hyperesthesia, ataxia and seizures occur in infected animals. Histopathological findings of the disease include demyelination in the substantia alba of the cerebellum, gemistocytic astrocytes, and intranuclear and occasionally cytoplasmic inclusions in glial cells (Tipold et al., 1996).

Nitric oxide (NO) is a molecule synthesized from L-arginine by the enzyme nitric oxide synthase (NOS) (Stuehr, & Griffith, 1992). It acts as a neurotransmitter and is part of the signaling pathways between cerebral blood vessels, neurons and glial cells. Three isoforms of NOS have been identified. Endothelial NOS (eNOS), which balances vascular hemostasis (Heiss et al., 2015), neuronal NOS (nNOS), which affects neurological regulation and neuronal function and is mainly found in the nervous system (Zhou & Zhu, 2009), inducible NOS (iNOS) that is expressed in macrophages when there is a pathogenic condition for host defense (Cinelli et al., 2020). It has been confirmed that low levels of NO can have positive effects on the proliferation and survival of cells. However, it is known that high levels of NO trigger oxidative and nitrosative stress, which can cause pathological effects (Thomas et al., 2008).

Apoptosis is physiologically necessary for cell turnover and normal development. When exposed to excessive apoptosis, tissue damage occurs, however, in cases where apoptosis does not occur at a physiological level, pathological cellular growth occurs. (Kim et al., 1999). NO and its reaction products can either promote or prevent apoptosis in a multitude of settings. It has been reported that many cells, especially macrophages and central nervous system cells, undergo apoptosis in response to NO (Albina et al., 1993). To our knowledge, NO expression and its relationship with apoptosis in the neuropathology of distemper disease has not been reported. Therefore, in this study, we investigated the expression of eNOS and its relationship with apoptosis in the cerebellum of dogs with distemper.

MATERIALS AND METHODS

Tissue Samples

Twenty canine cerebellum tissues embedded in formalin-fixed paraffin blocks were used in the study. Tissue blocks from the archives of Cukurova University, Faculty of Ceyhan Veterinary Medicine, Department of Pathology were used in the study. Cerebellum tissues from 7 dogs that died of another disease and whose central nervous system was not affected were used as control. Infected and control tissues were fixed in buffered formaldehyde for 2 days, washed in tap water over night, then passed through a graded alcohol series and embedded in paraffin blocks. 5 µm sections from the tissues were taken on a slide with a microtome (Leica RM2125) and stained histopathologically with hematoxylin and eosin (HE), and immunohistochemically (IHC) with distemper and eNOS antibodies. In addition, staining was done with the TUNEL method to determine apoptosis and examined under a light microscope (Nikon YS 100).

Immunohistochemical Examinations

Immunohistochemical staining was performed using the routine streptoavidin-biotin peroxidase technique according to the manufacturer's recommendations. [Anti rabbit streptoavidin/biotin immunoperoxidase kit (Histostain-Plus Kits, California, USA)]. The selected 5 µm paraffin tissue sections were stained immunohistochemically in order to elucidate the expressions of anti-eNOS polyclonal antibody [Invitrogen PA3-031A, (diluted 1/250)] and anti-CDV monoclonal antibody (DV2-12) [Invitrogen, MA1-82327, (diluted 1/250)]. The red color reaction was enhanced using 3-amino-9-ethylcarbazole (AEC) (Zymed AEC RED substrate kit, ABD) as the chromogen. All sections were counterstained with Gill hematoxylin (HX71788774, Meck, USA) solution and then washed in water. Coverslips were applied with the water-based mounting medium (Shandon Immuno-mounting). In addition, all infected and control tissues were stained by the TUNEL method to determine apoptotic cells that undergo extensive DNA degradation during the late stages of apoptosis (In Situ Cell Death Detection Kit, Roche, Basel, Switzerland).

Evaluation of Immunostaining

Positive stained cells were measured by a camera system attached to the microscope (Leica CCD camera DFC420, Leica Microsystems Imaging Solutions, Ltd., Cambridge, UK). Five representative areas were randomly selected, and consecutive images were taken with a 20x objective. All eNOS and TUNEL stainings were measured using the same integrated optical density setting, and the ratio of stained area to total area was calculated using Leica QWin Plus v3. An investigator blinded to the identity of the sections quantified all sections.

Statistical Analysis

The obtained data were statistically analyzed using the Mann Whitney U test in the SPSS version 26 package program. The results were presented in the format of mean (median) ± standard deviation (SD). P-value of <0.05 was considered statistically significant **(**Table 1, Figure 1).

Ethical Considerations

Since this study used tissues archive our laboratory, ethics committee approval is not required. A document was received from the faculty ethics committee that the study does not require ethics committee approval (Date and Number of Document: 14/03/2024-32889)

RESULTS

Histopathological Findings

In histopathological examination, demyelination in substantia alba, gitter cells, gemistocytes and intranuclear inclusion bodies in astrocytes were detected in the cerebellum of infected animals (Figure 2).

Immunohistochemical Findings

In the immunohistochemical examination performed with CDV antibody, immunopositive CDV antigens were found in the cytoplasm and nucleus of glial cells and occasionally extracellular free antigens in the substantia alba (Figure 2). In eNOS immunohistochemical staining, intense eNOS expression was observed in vascular endothelium, glial cells and Purkinje neurons in infected animals compared to controls (P<0.001) (Figure 3).

Additionally, strong TUNEL positive staining was obtained in infected animals and this result was significiant compared to controls (P<0.001) (Figure 4).

Table 1. Immunohistochemical expression of eNOS and TUNEL staining.

	eNOS				TUNEL staining			
Groups	**n**	**Mean (Median)**	**SD**	**P**	**n**	**Mean (Median)**	**SD**	**P**
Healthy control	7	0.42 (0.00)	0.53	0.000	7	0.43 (0.00)	0.53	0.000
Canine Distemper	20	2.35 (2.00)***	0.59		20	2.00 (2.00)***	0.46	

***P<0.001 shows significance when comparing healthy control to CDV infection according to the **Mann-Whitney Test.**

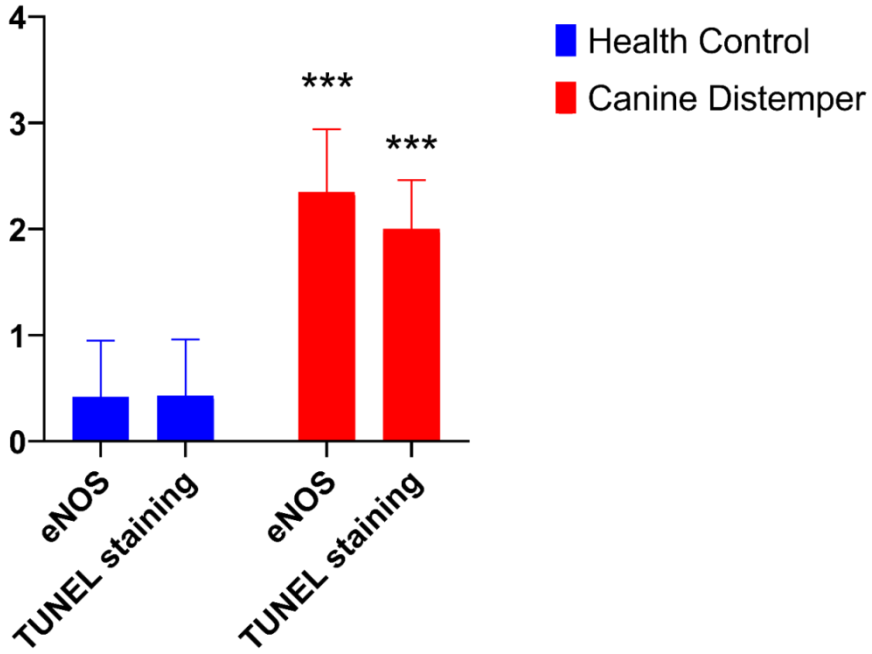

Figure 1. Comparison of eNOS expression and TUNEL staining intensity in control and CDV infection.

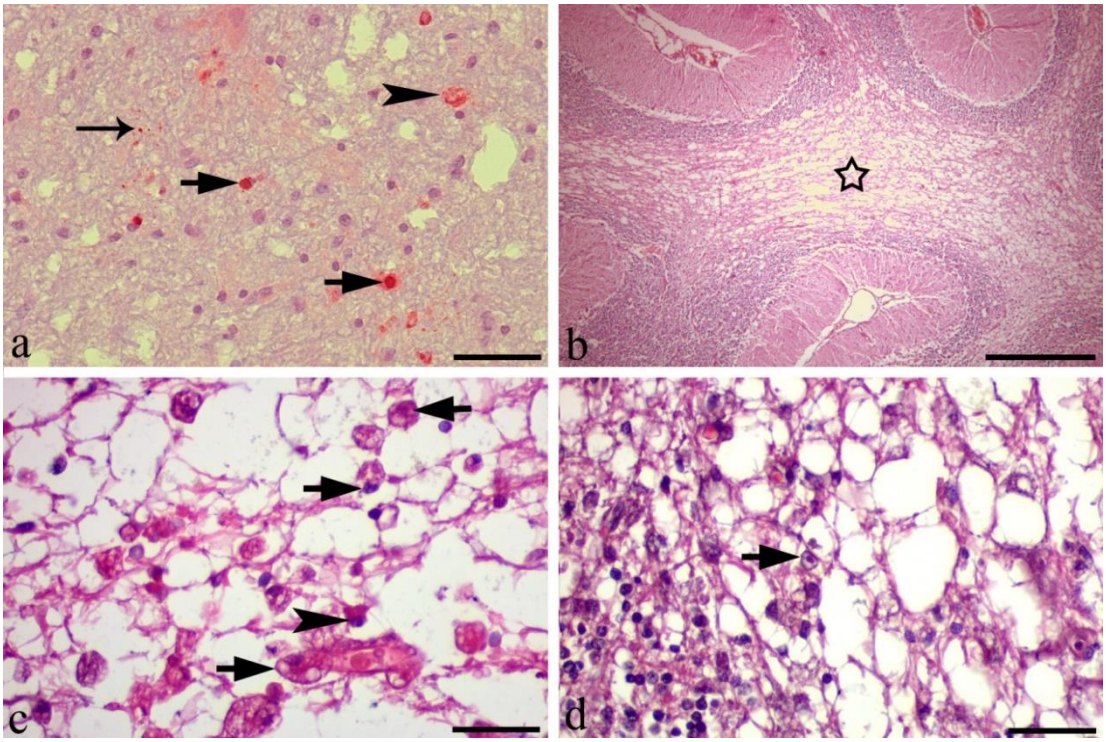

Figure 2. Immunohistochemical staining with CDV antibody and histopathology with HE stain in canine distemper. a) Nuclear (thick arrows) and cytoplasmic (arrowhead) CDV antigens in glial cells and extracellular (thin arrow) CDV antigens in the neuropil. Distemper, IHC, Bar: 25µm. b) Demyelination in the substantia alba of the cerebellum (star). Distemper, HE, Bar: 100µm**.** c) Gitter cells (arrows) and gemistocyte (arrowhead) in the substantia alba. Distemper, HE, Bar: 25µm**.** d) Intranuclear inclusion in astrocytes (arrow). Distemper, HE, Bar: 25µm**.**

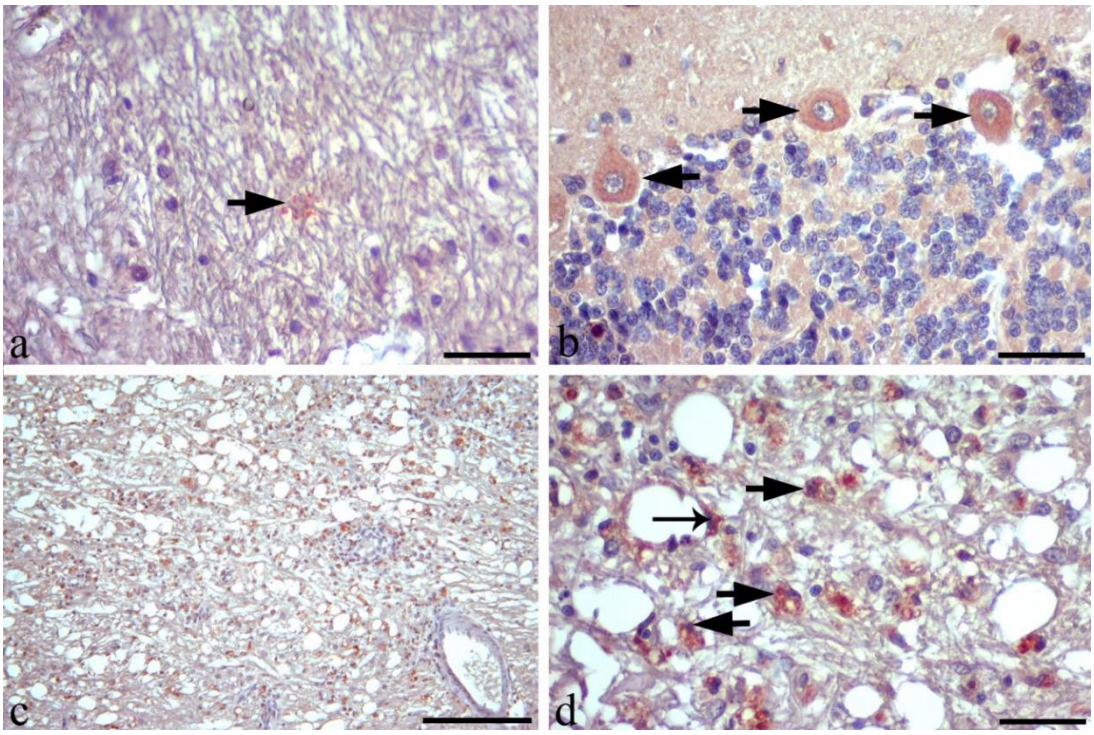

Figure 3. Immunohistochemical staining with eNOS antibody in canine distemper and control tissues. a) Weak eNOS immunoreactivity in control tissues (arrow). Control, IHC, Bar: 25µm. b) Strong eNOS expression in the Purkinje neurons (arrows). Distemper, IHC, Bar: 25µm**.** c) Strong eNOS expression in glial cells in the substantia alba. Distemper, IHC, Bar: 100µm**.** d) Intense eNOS expression in glial cells (thick arrows) and vascular endothelium (thin arrow). Distemper, IHC, Bar: 25µm**.**

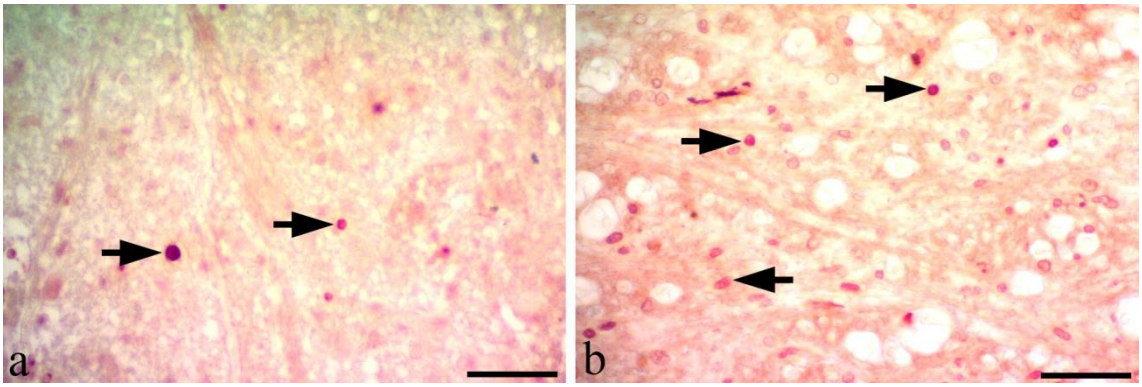

Figure 4. TUNEL staining in canine distemper and control tissues. a) Weak TUNEL staining in control tissues (arrows). Control, TUNEL, Bar: 25µm**.** b) Strong TUNEL staining in canine distemper infection (arrows). Distemper, TUNEL, Bar: 25µm**.**

DISCUSSION

Canine distemper is a highly contagious disease characterized by high mortality and morbidity. CDV causes severe immunosuppression and neurological findings in dogs and a model of the human disease multiple sclerosis, which is associated with demyelination (Vandevelde, & Zurbriggen, 2005). Neurodegeneration occurring in distemper disease is a subject of research. We suggest that there are many factors that trigger vascular lesions and inflammation in the cerebellum that have not yet been investigated. In our study, we investigated the contribution of eNOS in neurodegeneration and cellular destruction in distemper. NO plays a role in the regulation of various cellular functions (Nathan, 1992). NO is thought to be beneficial in many situations, such as preventing vascular thrombosis (Yao et al., 1992), inflammatory cell-mediated damage (Fridovich, 1983), and reperfusion injury (Kubes, 1993; Siegfried et al., 1992). Stimulation of NO may also cause adverse events such as hypotension (Moncada et al., 1991), inhibition of intermediate metabolism (Hibbs et al., 1988) and production of the strong oxidant peroxynitrite. Despite reports that overproduced NO in the presence of oxygen will lead to cytotoxic events, NO has also been reported to limit damage to target molecules or tissues during events associated with overproduction of reactive oxygen species, by often unidentified mechanisms (Kubes, 1993; Siegfried et al., 1992). When NO production exceeds physiological levels, it has a neurotoxic effect rather than a neuroprotective effect (Calabrese et al., 2007; Dawson et al., 1991; Boje, & Aroa, 1992). High levels of NO during inflammation suggest that it plays a critical role in regulating tissue destruction that occurs during oxidative stress (OS).

eNOS is one of three isoforms of NOS that produce NO (Stuehr, & Griffith, 1992). eNOS plays a role in maintaining and protecting microcirculation in the brain (Toda et al., 2009), preventing platelet accumulation, leukocyte adhesion and migration (Broos et al., 2011), and reducing smooth muscle proliferation (Toda et al., 2009). Disruption of eNOS inactivity is involved in many cellular mechanisms such as neuronal damage, subaortic hemorrhage, traumatic brain injury, and ischemic stroke (Srivastava et al., 2012). eNOS expressions increase in neurodegenerative disease (Cole et al., 2012; Karayiğit, 2018). It has been reported that the expression of eNOS in the brain increases in viral and bacterial neurodegenerative diseases (Dincel, & Kul, 2015; Karayigit, 2018). Previous studies have reported that eNOS plays a partially protective role by keeping the blood flow level high against ischemia that occurs after brain injury (Endres et al., 2004). We found no reports of immunohistochemical expression of eNOS and associated with apoptosis in cerebellum tissues of dogs with naturally infected with CDV. In this study, the relationship between eNOS expression and apoptosis in neuropathology of canine distemper disease was investigated. According to the results obtained from our study, higher levels of eNOS expression were obtained in the cerebellum of infective animals compared to controls ($P<0.001$). To our knowledge, this finding has not been demonstrated before in distemper disease. However, it is consistent with results from other reported viral and bacterial neurodegenerative diseases (Dincel, & Kul, 2015; Karayigit, 2018).

NO and OS cause apoptosis in central nervous system cells as well as other cells (Nicotera et al., 1995; Chandra et al., 2000; Dincel, & Kul, 2015; Karayiğit, 2018). Pathological levels of NO can cause loss of mitochondrial membrane potential, leading to apoptosis (Bonfoco et al., 1995; Brookes et al. 2000; Karayiğit, 2018). Similarly, OS damages mitochondrial DNA by disrupting the respiratory chain balance, which impairs Ca2+ homeostasis and the mitochondrial defense system by increasing membrane permeability (Annunziato et al., 2003; Bhat et al., 2015). It has been previously reported that glial cells in the cerebellum undergo apoptosis in distemper disease (Moro et al., 2003). Our most interesting finding was the strong eNOS expression and TUNEL staining in the cerebellum during infection. It has been suggested that mitochondrial damage is caused by OS of nitrosative origin, because the cells may have been exposed to high

levels of NO. Increased OS and NO in distemper may have triggered apoptosis. Certainly, the relationship between apoptosis and neuropathology in distemper disease is a complex process and involves unknown factors. There are many molecules that affect this process and a complex chain of neuropathological events that stimulate each other. However, according to the findings of our study and previous reports supporting our findings, increased eNOS expression in distemper infection may contribute to apoptosis and OS or NO induced mitochondrial DNA damage. Similar and more comprehensive studies on the subject will contribute to revealing other factors affecting neuropathology and cell death and further elucidating the pathogenesis.

CONCLUSION

As a result, in our study, eNOS expression and TUNEL positivity were found to be significant higher in CDV infection compared to control tissues. This suggests that increased eNOS expression in CDV infection may trigger apoptosis. Further research on this subject is needed to develop new treatment approaches.

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Conflict of Interest

The author declares no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

Author Contributions

All stages of the study were carried out by the corresponding author.

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Ethical Considerations

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