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Role of glial fibrillary acidic protein (GFAP) and neurofilament (NF) expression in the pathophysiology of canine distemper encephalomyelitis

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ABSTRACT

Objectives: Canine distemper virus (CDV), a member of the genus *Morbillivirus* of the family *Paramyxoviridae*, is the causative agent of canine distemper, a fatal and highly contagious disease that affects dogs and other carnivores. This study aimed to investigate whether there is a correlation between glial fibrillary acidic protein (GFAP) and neurofilament (NF) expression in canine distemper encephalomyelitis (CDE) and the severe neuropathology that occurs.

Materials and Methods: GFAP and NF expression levels in the brain tissue of 13 dogs diagnosed with CDE were investigated by immunohistochemical method.

Results: The results of the study revealed that GFAP ($p< 0.005$) and NF ($p< 0.005$) expression levels in brain tissue were significantly increased in CDV-infected dogs compared to healthy, uninfected dogs. GFAP expression was mainly observed in endothelial cells and astrocytes, whereas NF expression was mainly found in neurons. In addition, it was found that the expression of both GFAP and NF was more pronounced in the areas with the most severe neuropathological findings.

Conclusions: This study demonstrated pathological astrocyte reactivation and neuronal degeneration at the molecular level. These findings provide information about the stage of the disease. This study clearly demonstrated that detailed information about the prognosis of the disease can be obtained from GFAP and NF expression. Since GFAP/NF levels provide information about the severity of the disease, they can be used clinically. Therefore, further research into the involvement of GFAP and NF expression in the pathophysiology of CDE has great potential to improve our understanding of this complex neurological disorder.

Keywords: *Canine distemper, GFAP, NF, Neuropathology, Encephalomyelitis*

INTRODUCTION

Canine distemper virus (CDV) is a single-stranded, negative-sense *Morbillivirus* from the *Paramyxoviridae* family, closely related to the human measles virus. It is the etiological agent of fatal distemper disease in dogs (Barrett, 1999; Murphy et al., 2012). CDV's host range includes not only dogs and other canines, but also large felines (such as leopards and tigers), raccoons, coatimundi, giant pandas, ferrets, and rhesus monkeys. Although these animals are not generally considered susceptible, they can be experimentally infected (Dalldorf et al., 1938; Qiu et al., 2011; Beineke et al., 2015; Martinez-Gutierrez and Ruiz-Saenz, 2016). In addition to neuropathology in both grey and white

matter in distemper, demyelinating leukoencephalomyelitis is considered the main neuropathological finding in dogs (Summers and Appel, 1987; Summers and Appel, 1994; Beineke et al., 2009).

Glial fibrillary acidic protein (GFAP) is considered the main intermediate filament protein in astrocytes and the building block of the cytoskeleton (De Zeeuw and Hoogland, 2015). In addition to maintaining the integrity and permeability of the blood-brain barrier, astrocytes regulate water transport via aquaporins and provide vital support to neurons/oligodendrocytes (Abbott et al., 2006; De Zeeuw and Hoogland, 2015). Astrocytes and microglia have been shown to be involved in neurodegeneration processes by rapidly and actively responding to damages occurring in neurons (Laping et al., 1994; Mongin and Kimelberg, 2005). In neural parenchymal pathology, astrocytes become activated and increase their number, size, and GFAP expression (Laping et al., 1994; Mongin and Kimelberg, 2005). Particularly, astrocytes play significant roles in CNS trauma, ischemia, parasitic (Dincel and Atmaca, 2015), viral (Dincel and Kul, 2015), and metabolic encephalopathy (Dincel and Yıldırım, 2016), and a significant upregulation in GFAP expression is a remarkable finding (Hol and Pekny, 2015). Additionally, increased GFAP expression is directly proportional to cellular hypertrophy, the degree of cellular reactivity, and especially neuropathology (Sofroniew, 2014).

When analyzing demyelinating distemper lesions, it is seen that the main target of CDV is astrocytes. In fact, it has been found that almost 95% of the infected cells are astrocytes (Summers and Appel, 1987; Mutinelli et al., 1989). Astrocyte hypertrophy, gemistocyte astrocytes (reactive astrocytes), and astrocytic syncytia formation observed in canine distemper encephalomyelitis (CDE) has been shown to be closely related to progressive myelin loss (Vandevelde et al., 1982; Summers et al., 1984; Mutinelli et al., 1989). The functional significance of astrocyte plasticity in canine distemper has not yet been fully clarified.

Neurofilaments (NF) are a basic component of the neuronal cytoskeleton and are in constant interaction with neighboring glial cells (Julien and Mushynski, 1998; Al-Chalabi and Miller, 2003). Pathological accumulation of NF is a key finding in many neurodegenerative diseases, including amyotrophic lateral sclerosis, Parkinson's, Alzheimer's, Charcot-Marie-Tooth, dementia with Lewy bodies, Border Disease, and Toxoplasmic Encephalitis (Al-Chalabi and Miller, 2003; Liu et al., 2004; Dincel and Atmaca, 2015; Dincel and Kul, 2015). Abnormal NF accumulation is mainly associated with axonal degeneration and plays a role in differentiating neuronal dysfunction (Al-Chalabi and Miller, 2003; Liu et al., 2004). Furthermore, NF release into the cerebrospinal fluid is an crucial indicator of the severity of neuropathology in neurodegenerative diseases (Norgren et al., 2003). These studies demonstrate that NF expression is closely related to neuropathology.

In this study, it was planned to show the severity of neuropathology seen in CDE by GFAP and NF expressions. Therefore, it is aimed to investigate the correlation between GFAP/NF expressions and their role in neuropathogenesis and to have information about the neurodegeneration levels of the disease with the findings obtained. It is thought that the findings obtained will give an idea for the treatment process of the disease.

MATERIALS and METHODS

Ethics Statement

This study was approved by Atatürk University Rectorate, Faculty of Veterinary Medicine, Unit Ethics Committee with the decision numbered 2023/25. The study samples comprised 13 dead dogs, ranging in age from 1 to 3 years. These animals were brought to Aksaray and Atatürk University, Faculty of Veterinary Medicine, Department of Pathology for routine necropsy. No animals studied were sacrificed for this study, and all procedures were performed with the permission of the animal owners. A total of 6 distemper-negative dogs without brain pathology and CNS-related cause of death were used as healthy control animals.

Pathologic examination

No macroscopic or histopathological findings were found in the brain tissues of 6 healthy control dogs. All steps followed the procedure described by Dincel (2017). The brains were removed and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at pH 7.4 for 48 h and then were thoroughly rinsed overnight, under tap water. After performing the routine tissue preparation procedures of dehydration using graded alcohol and xylene, the tissue samples were embedded in paraffin blocks; 4–5 μm thick paraffin

sections were then cut and mounted on glass slides. Hematoyxlin - Eosin (H&E) and immunohistochemical tests were performed, and they were analyzed using a trinocular light microscope (Olympus BX51 and DP25 digital camera). The severity of CDE in each animal was classified according to neuropathological changes: demyelination, hyperemia, mononuclear cell infiltrates, gliosis, astrocytosis, neuronal degeneration and malaise.

Antibodies

Commercial antibodies against GFAP (Abcam, Cambridge, UK) diluted to 1:250 and undiluted NF (Thermo Scientific, USA) were used.

Immunoperoxidase examinations

Immunohistochemistry was performed to observe GFAP and NF in the 4–5 μm-thick paraffin sections of the tissues by using an indirect streptavidin/biotin immunoperoxidase kit (HRP, Thermo Scientific, USA), as per the manufacturer's instructions. All steps were carried out following the procedure described by Dincel and Kul (2019). Briefly, the sections were placed onto adhesive slides, deparaffinized for 5 min. Each in the 3-step xylene series, and rehydrated using a series of graded alcohol and distilled water. The antigens were retrieved by boiling the tissue sections on glass slides in citrate buffer (pH 6.0) (Thermo Scientific, USA) for 20 min. Endogenous peroxidase activity was quenched using 3% hydrogen peroxide in absolute methanol for 7 min at room temperature (RT). The tissue sections were rinsed thrice with phosphate buffer solution (pH 7.4) for 5 min, between each consecutive step. The sections were then incubated in a blocking serum for 5 min to prevent non-specific antibody binding. Thereafter, the sections were incubated with GFAP and NF antibodies for 60 min in a humidity chamber at the RT. After treating the sections with biotin-labelled secondary antibody for 15 min and streptavidin-peroxidase enzyme for 15 min at RT, the colour reaction was performed using 3,3'-Diaminobenzidine chromogen for 5-10 min. Sections were counterstained with Mayer's hematoxylin for 1–2 min and suspended in a water-based mounting medium (Thermo Scientific, USA). For each immunoperoxidase test, three negative control tissue sections were allowed as follows; as a negative control, one of the serial paraffin sections was incubated with normal mouse serum (isotype serum control) instead of primary antibody. Additionally, the primary antibody step was omitted to control non-specific endogenous peroxidase and biotin activities in each test.

Histomorphometric analysis and statistics

The density of positive staining was measured using a computerized image system composed of a Leica CCD camera DFC420 (Leica Microsystems Imaging Solutions, Ltd., Cambridge, UK) connected to a Leica DM4000 B microscope (Leica Microsystems Imaging Solutions, Ltd.). Five representative fields were selected, and consecutive pictures were captured by Leica QWin Plus v3 software under a 20x objective lens (Leica Microsystems Imaging Solutions, N Plan) at a setting identical to the imaging system for analysing. We used the same setting for all slides. The integrated optical density of all GFAP and NF-positive staining were measured, and the mean GFAP and NF-positive area/total area was calculated by Leica QWin Plus v3. All images were collected under the same lighting conditions. To avoid observer bias, a blinded investigator quantified all sections. Data were described in terms of mean and standard deviation (mean ± SD) for area %. After calculating the proportion (% pixels) of the stained area to the whole field, each slide's mean (% pixels) staining area was determined. GFAP and NF immunohistochemical results were compared between groups using a mann whitney u test. $p<0.005$ was considered statistically significant.

RESULTS

Histopathologic findings

Although severe findings are seen in the cerebellum, lesions in brain tissues were analysed in this study. H&E-stained brain sections from healthy control animals exhibited normal architecture. Significant histopathological lesions were observed in the brain tissues of all animals. Histopathological examinations revealed areas of demyelination in the brains of the dogs, which were not very diffuse and appeared as moth-eaten areas (Figure 1A). Marginal hyperchromasia (Figure 1B) and intranuclear inclusion bodies were observed in some cells. Astrocytosis, astrogliosis and microglial cell proliferations were observed close to the areas of demyelination. Gitter cells were also found in areas with severe neurohistopathological findings. Diffuse hyperaemia and neuronal degeneration were detected (Figure 1AB). The observation of liquefaction necrosis in the parts where the lesions

were severe was considered as an important finding.

Distribution of viral antigens in the brain sections

It has been shown that all parts of the brain, including the cerebral hemisphere, cerebellum, neutrophil and midbrain, are strong diffuse and/or granular labelling of CDV antigens. In addition, linear or diffuse dense immunopositive staining was observed in endothelial (Figure 1C), neurons (Figure 1C) and glial cells (Figure 1D).

Immunoperoxidase findings

We analysed the protein expressions levels of GFAP and NF in the brain tissues from CDV-infected and healthy control animals. Immunohistochemical analysis showed significant up-regulation of GFAP and NF expressions in the CDV-infected dogs (Figure 2) unlike that in the case of the healthy control animals. Statistical analysis of the data on GFAP and NF expressions in the brain, measured by immunostaining in all the groups, are listed in Table 1.

Table 1. Immunoperoxidase test results and statistical data for healthy control and Distemper + animals

Animals	n	GFAP		*p*	NF		*p*
		Mean	SD		mean	SD	
Healthy Control Animals	6	0.726	0.367	0.001	1.727	0.312	0.001
Distemper + Animals	13	5.137	0.531		6.956	0.668	

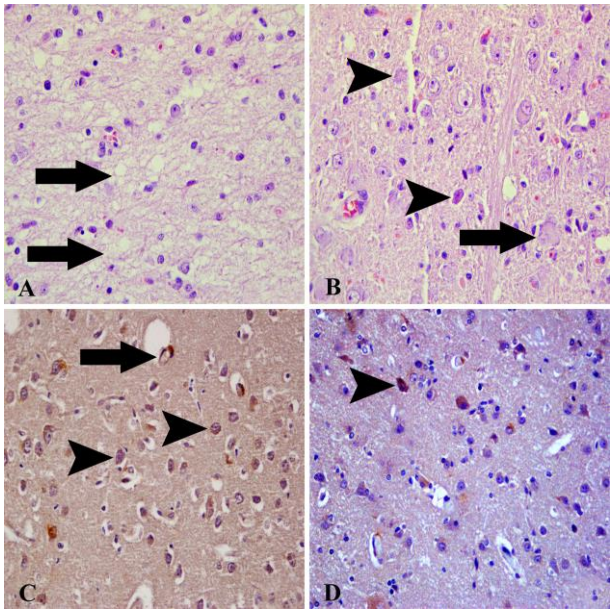

Figure 1. Large areas of moth-eaten demyelination (arrows). (A) Necrotic neurons (arrowheads) and marginal hyperchromasia (arrow). (B) Note the positive immunolabelling (red pigment) in the cytoplasm of degenerative/necrotic neurons (arrowheads) and endothelial cells (arrow). ABC technique (anti-CDV), Mayer's haematoxylin counterstain. (C) Note the positive immunolabelling (red pigment) in the cytoplasm of degenerative/necrotic glial cells (arrow). ABC technique (anti-CDV), Mayer's haematoxylin counterstain. (D)

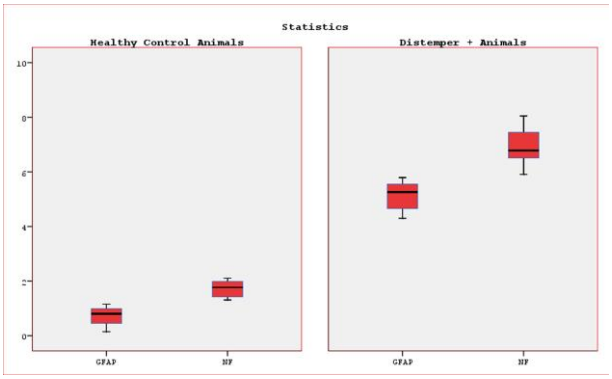

Figure 2. Significant up-regulation of GFAP and NF expressions in the CDV-infected dogs.

GFAP expressions

Fairly weak immunoreactivity for GFAP was observed in endothelial cells and neurons in healthy control animals (Figure 3A). GFAP expressions were observed especially in endothelial cells (Figure 3CD). An important finding is that these endothelial cells consist of degenerated cells. In addition to these findings, the appearance of GFAP-positive astrocytes close to necrotic neurons was considered an important finding (Figure 3BCD). The number of GFAP-expressing hypertrophic astrocytes increased in a virus-associated manner at the border of the focal gliosis. As a striking finding in the regions where viral antigens were concentrated, GFAP expressions were found to be strongly positive. There was a statistically

significantly higher incidence of positive GFAP immunoreactivity in astrocyte, neuron and endothelial cells than the levels in the healthy control animals ($p<0.005$).

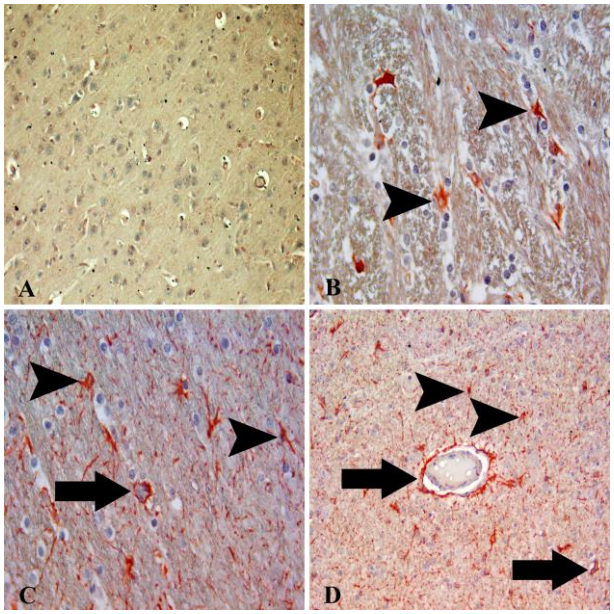

Figure 3. Weak GFAP expression in glial, endothelial cells and neurons of healthy control animals. ABC technique (anti-GFAP), Mayer's hematoxylin counterstain. (A) Strong GFAP expression in hypertrophic astrocytes (arrowheads). ABC technique (anti-GFAP), Mayer's haematoxylin counterstain. (B,C) Strong GFAP expression in hypertrophic astrocytes (arrowheads). ABC technique (anti-GFAP), Mayer's haematoxylin counterstain. (B,C) Strong GFAP expression in endothelial cells (arrows). ABC technique (anti-GFAP), Mayer's haematoxylin counterstain. (C,D)

NF expressions

Fairly weak immunoreactivity for NF was observed in the brain parenchyma and neurons in healthy control animals (Figure 4A). Abnormal NF accumulation and axonal degeneration were observed in the brain tissue of animals infected with CDV. NF was disorganised and significantly increased in some neurons and neuronal perikarya (Figure 4 BCD). The regions of increased NF immunoreactivity were localised within the lesion (demyelinated and focal gliosis area) and especially around it. Massive, regional accumulation of NF was noted in areas surrounding the cerebral blood vessels. Likewise, it is a remarkable finding that NF expressions show strong positivity in regions where viral antigens are concentrated. There was a statistically significantly higher incidence of positive NF immunoreactivity in the brain parenchyma and neurons than the levels in the healthy control animals ($p<0.005$). This pathological increase was significantly more pronounced in CDV-infected than in healthy control dogs. The increase in GFAP and NF expressions closer to the lesion areas is thought to be important evidence of neural parenchymal degeneration.

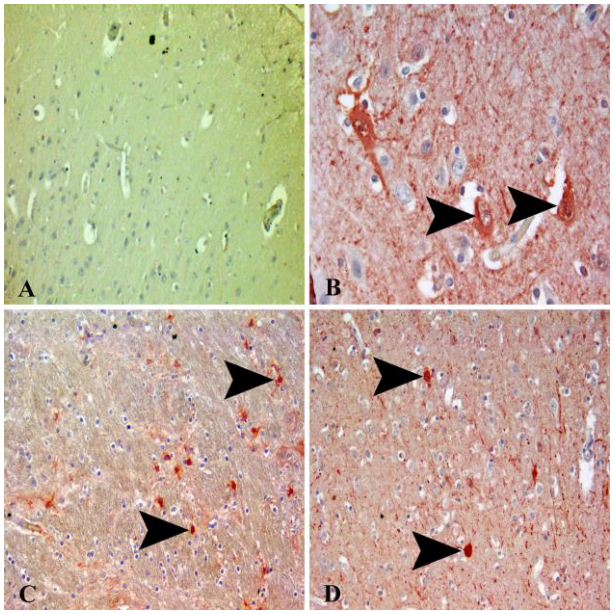

Figure 4. Weak NF expression in brain parenchyma and neurons of healthy control animals. ABC technique (anti-NF), Mayer's hematoxylin counterstain. (A) Strong NF expression in neurons (arrowheads) and brain parenchyma. ABC technique (anti-NF), Mayer's haematoxylin counterstain. (B,C,D)

DISCUSSION

CDE is a fatal disease with a poor prognosis due to severe neuropathology following infection with CDV, a *Morbillivirus* from the *Paramyxoviridae* family. Although there have been studies on the neuroimmunopathogenesis of distemper, molecular pathogenesis is still not fully understood. It remains a useful model for many demyelinating diseases. In this study, we investigated GFAP and NF expressions in CDE and revealed their correlation with observed neuropathology. The most striking finding of this study is that GFAP and NF expressions are positively correlated with neuropathology. Additionally, we found that GFAP and NF expressions are positively correlated with each other, which is another important discovery. These findings provide important insights into the neurodegeneration levels of the disease and could inform its treatment process.

Astrocytes play very important roles, including supporting neuronal function, providing communication between neurons, maintaining cerebral homeostasis, and regulating neuronal synapses (Perea et al., 2009; De Zeeuw and Hoogland, 2015). When any damage occurs in the neural parenchyma, GFAP expressions are upregulated, making it reactive against neurodegeneration (Laping et al., 1994; Mongin and Kimelberg, 2005). In this study, all CDE cases showed significant GFAP expressions, indicating the involvement of the increase in GFAP expression in the neurodegenerative process. This is a clear indication of moderate or high-level damage to astrocytes when evaluated together with histopathological findings.

NFs are a vital component of the neuronal cytoskeleton, and they interact with the surrounding glial cells to maintain healthy neuronal functions (Barrya et al., 2007; Yuan et al., 2012). Abnormal accumulation of NF occurs primarily in damaged neurons (Al-Chalabi and Miller, 2003; Norgren et al., 2003). In cerebral and neurodegenerative diseases with acute neural parenchymal destruction, pathological NF accumulation indicates the severity of neuropathology and can aid in diagnosis (Norgren et al., 2003; Liu et al., 2011). In this study, we detected a severe accumulation of NF at significant levels in CDE cases. Like GFAP expressions, the high levels of NF accumulation in cases with severe neuropathology are an important finding of the study. This finding clearly demonstrates the presence of severe neural parenchymal destruction and demyelination with neuronal degeneration at the molecular level. Therefore, it is evident that NF expressions will provide valuable information about the onset and progression of the disease.

CONCLUSION

In conclusion, this study demonstrated high levels of astrocyte reactivation and neuronal degeneration at the molecular level. The findings provide information about the stage of the disease, as GFAP/NF expressions were found to be directly proportional to the level of neural parenchymal destruction and neuropathology. Therefore, GFAP and NF expressions can be used to obtain detailed information about the prognosis of the disease. It is believed that these biomarkers can be used clinically since GFAP/NF levels can give information about the severity of the disease.

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