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Immunohistochemical expression of MMP–2 and MMP–9 in the brain tissue of sheep naturally infected with *Listeria monocytogenes* and relationship with cell death in the Listerial encephalitis

Expresión inmunohistoquímica de MMP–2 y MMP–9 en el tejido cerebral de ovejas infectadas naturalmente con *Listeria monocytogenes* y relación con la muerte celular en la encefalitis por listeria

***Mehmet Önder Karayigit*[1]*, *Mehmet Halıgür*[1], *Mehmet Ekici*[2]**

[1]*Cukurova University, Faculty of Ceyhan Veterinary Medicine, Pathology Department. Adana, Türkiye.*
[2]*Cumhuriyet University, Faculty of Veterinary Medicine, Physiology Department. Sivas, Türkiye.*
**Corresponding author: karayigit09@hotmail.com*

ABSTRACT

Listeria monocytogenes is an intracellular, food–borne bacterium. Silage is an important source of this pathogen causing listeriosis. Listeriosis is an important health problem for both animals and humans in the world. The disease comprises three clinical syndromes: meningoencephalitis, septicemia and metritis with abortion. Encephalitis is frequently observed and the factors that play a role in its pathogenesis are the subject of research. In this study, the immunohistochemical expression of MMP–2 and MMP–9 together with TUNEL staining was investigated in the pathogenesis of meningoencephalitis in sheep naturally infected with *L. monocytogenes*. The brains of 25 sheep with Listerial meningoencephalitis were used in this study. Brain material from 10 sheep provided from the slaughterhouse was also used as a control. Tissue sections were stained immunohistochemically with *L. monocytogenes*, MMP–2 and MMP–9 antibodies. Additionally, TUNEL staining was performed to determine apoptosis in the disease. As a result of the study, it was observed that TUNEL staining in neurons and glial cells, MMP–2 and MMP–9 expressions in vascular endothelial cells, inflammatory cells, microglia and especially neurons in the infected brain tissue were significantly increased compared to controls. These results suggested that MMP–2 and MMP–9 play an active role in the neurodegeneration and cell death that occur in Listerial encephalitis.

Key words: Immunohistochemistry; *Listeria monocytogenes*; MMP–2, MMP–9; TUNEL

RESUMEN

Listeria monocytogenes es una bacteria intracelular transmitida por los alimentos. El ensilaje es una fuente importante de este patógeno causante de listeriosis. La listeriosis es un importante problema de salud tanto para los animales como para los humanos en el mundo. La enfermedad comprende tres síndromes clínicos: meningoencefalitis, septicemia y metritis con aborto. La encefalitis se observa con frecuencia y los factores que influyen en su patogénesis son objeto de investigación. En este estudio, se investigó la expresión inmunohistoquímica de MMP–2 y MMP–9 junto con la tinción TUNEL en la patogénesis de la meningoencefalitis en ovejas infectadas naturalmente con *L. monocytogenes*. En este estudio se utilizaron los cerebros de 25 ovejas con meningoencefalitis por listeria. También se utilizó como control material cerebral de 10 ovejas procedente del matadero. Las secciones de tejido se tiñeron inmunohistoquímicamente con anticuerpos de *L. monocytogenes*, MMP–2 y MMP–9. Además, se realizó tinción TUNEL para determinar la apoptosis en la enfermedad. Como resultado del estudio, se observó que la tinción de TUNEL en neuronas y células gliales, las expresiones de MMP–2 y MMP–9 en células endoteliales vasculares, células inflamatorias, microglía y especialmente neuronas en el tejido cerebral infectado aumentaron significativamente en comparación con los controles. . Estos resultados sugirieron que MMP–2 y MMP–9 desempeñan un papel activo en la neurodegeneración y muerte celular que se producen en la encefalitis por Listerial.

Palabras clave: Inmunohistoquímica; *Listeria monocytogenes*; MMP–2; MMP–9; TUNEL

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INTRODUCTION

Listeria monocytogenes is a gram-positive, facultative anaerobic, rod-shaped intracellular and ubiquitous bacterium that causes Listeriosis, affecting both animals and humans [1, 2]. Ruminants such as cattle (*Bos taurus*), goats (*Capra hircus*) and sheep (*Ovis aries)* play an important role in the maintenance and spread of this pathogen in the farm environment [3]. The bacterium may cause septicemia by invading the intestinal tissue of herbivorous animals, and also causes neurological disorders [2, 4]. Typical histopathological findings of encephalitic Listeriosis are microabscesses consisting of macrophages and microglial cells and neutrophil leukocytes [4, 5, 6, 7]. Matrix metalloproteinases (MMPs), especially gelatinous MMPs (MMP-2 and MMP-9) play a critical role during inflammation and healing processes in mammals [8, 9, 10]. In a healthy central nervous system, small amounts of gelatinous MMPs are known to be expressed under normal physiological conditions. However, a remarkable increase has been reported under various neuropathological conditions [11, 12]. Gelatinous MMPs disrupt the structure of the extracellular matrix (ECM) and therefore play a significant role in the pathogenesis of diseases in the nervous system. In general, gelatinous MMPs lead to some pathological changes after decreased cerebral blood flow including loss of ionic homeostasis, energy deficiency increased oxidative stress, apoptosis, irreversible tissue/organ damage, and neurological disorders [13, 14]. It is shaped by the disruption of the complex interaction of the inflammatory reaction and the ECM model after the decrease of blood flow in the cerebral tissue [15]. It has been reported that MMP-2 and MMP-9 are expressed in vascular endothelial cells, meninges, inflammatory cells, microglia and especially neurons in neuroinflammatory changes in the central nervous system [16, 17].

Apoptosis is involved in the physiological processes of many cells in the body during and after the embryonal period. These physiological processes include embryonic development, organ metamorphosis, cell cycle, development and activation of cells, and cell aging. Thus, it is important to maintain tissue homeostasis under normal physiological conditions [18, 19]. However, abnormal apoptosis (either too little or too much) is a critical factor in the explanation of pathogenesis of some pathological conditions including neurodegenerative diseases, autoimmune disorders and many types of cancer. The mechanism of Listerial encephalitis is a complex process accompanied by many cellular interactions and expressions. Although some of the cellular effects that play a role in this mechanism have been revealed, it appears that there are new factors that contribute to the neurodegeneration and cell death that occur in the disease over time. In this context, the relationship between gelatinous MMPs and apoptosis has not been previously studied in Listerial encephalitis. Therefore, in this study, it was aimed to evaluate the roles of gelatinous MMPs and apoptosis in the formation of lesions in the central nervous system of sheep naturally infected with *L. monocytogenes* in terms of pathogenesis.

MATERIAL AND METHODS

Ethical statement

Because the experiment did not involve any invasive procedures for animal experiment Ethics Committee permission is not required. A decision was taken from the Cukurova University Faculty of Ceyhan Veterinary Medicine Research Ethics Committee stating that ethics committee approval was not required for the study (Document Date and Number: 01/02/2024-31330).

Tissue samples and histopathology

Twenty-five sheep brain tissues samples were obtained from the archive of the Department of Pathology, Faculty of Ceyhan Veterinary Medicine, University of Cukurova. The samples were from sheep of different breeds in different farms at different times. The sheep were 2–5 years old. The breeds of sheep included Akkaraman (fifteen cases), Kivircik (one cases), Merinos (four cases) and crossbreed (five cases). Although symptoms such as droopy ear, drooping eyelid, fever, lack of coordination, salivation were reported in sheep, circling and silage history were common anamnesis information. Hyperemia and opacification of the meninges were common necropsy findings. Listeriosis was diagnosed based on immunohistochemical and histopathological findings in the tissues. The 10 healthy animals comprising the control group had been slaughtered for human consumption and the heads of these animals were purchased from the Slaughterhouse. The infected and control brain tissues were comprised by cerebral cortex, midbrain, cerebellum and brain stem. All brain tissues were fixed in 10% buffered formalin solution for 48 hours (Sigma, Darmstadt, Germany), then washed thoroughly in tap water overnight. After dehydration in graded alcohols, were cleared in xylene and embedded in paraffin (Merck, Darmstadt, Germany). Paraffin blocks of cerebral cortex, midbrain, cerebellum and brain stem were cut at 5 μm (Leica, RM 2125) and stained with hematoxylin and eosin [20] (HE) Immunohistochemistry (IHC) method was performed according to the manufacturer's protocol. Stained sections were examined and photographed using a trinocular light microscope (Olympus BX51) with a DP25 digital camera (Tokyo, Japan). The severity of *L. monocytogenes* infection in each animal was classified based on occurrence of the following neuropathological changes: gliosis; neuronal necrosis; perivascular cell infiltration; and bacterial antigen immunostaining in the neurons, leuykocytes and glial cells.

Immunohistochemistry

Immunohistochemical staining was performed using the routine streptavidinbiotin-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-*L. monocytogenes* polyclonal antibody [Novus NB100-65667, (diluted 1/250)], anti-MMP-9 [orb13583,Biorbyt (diluted 1/250)] and anti-MMP-2 [GeneTex, GTX104577, (diluted 1/500)] The red color reaction was enhanced using 3-amino-9-ethylcarbazole (AEC) (Zymed AEC RED substrat 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 water-based mounting medium (Shandon Immuno-mounting). In additon, all infected and control tissues were stained by 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). Routine IHC period was applied to the sections until the antibody stage, and then the ready-use TUNEL kits were gently mixed and dropped onto the tissues and kept at room temperature for 1 hour. Then In Situ Cell Death Detection Kit-POD was added and left for 30 min, it was washed and stained with AEC and covered with a coverslip using water-based adhesive (Shandon Immuno-mounting).

Histomorphometric analysis

Positive labelled neuronal and glial cells was measured using a computerized image system comprising 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 images were captured by Leica QWin Plus v3 software using a 20× objective lens (N Plan; Leica Microsystems Imaging Solutions). Same setting used for all integrated optical density of MMP–2, MMP–9 and TUNEL staining was measured and the mean stained area/total area was calculated using Leica QWin Plus v3. All images were collected under the same lighting conditions. An investigator blinded to the identity of the sections quantified all sections.

RESULTS AND DISCUSSIONS

Statistics Result

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 less than 0.05 was considered statistically significant (TABLE I, TABLE II, FIG. 1, FIG. 2)

TABLE I
Neuronal immunohistochemical expression of MMP–9, MMP–2, and TUNEL staining

	MMP–9			MMP–2			TUNEL staining		
Groups	**n**	**Mean (Median)**	**SD**	**n**	**Mean (Median)**	**SD**	**n**	**Mean (Median)**	**SD**
Healthy control lambs	10	0.50 (0.50)	0.53	10	0.60 (1.00)	0.52	10	0.70 (1.00)	0.48
Listeria monocytogenes infected lambs	25	2.60 (3.00)***	0.58	25	2.20 (2.00)***	0.65	25	2.00 (2.00)***	0.50

***: *P*<0.001 shows significance when comparing healthy control lambs to those infected with *L. monocytogenes* according to Mann–Whitney Test

TABLE II
Glial immunohistochemical expression of MMP-9, MMP-2 and TUNEL staining

	MMP–9			MMP–2			TUNEL staining		
Groups	**n**	**Mean (Median)**	**SD**	**n**	**Mean (Median)**	**SD**	**n**	**Mean (Median)**	**SD**
Healthy control lambs	10	0.60 (1.00)	0.52	10	0.40 (1.00)	0.52	10	0.50 (0.50)	0.53
Listeria monocytogenes infected lambs	25	2.52 (3.00)***	0.51	25	2.12 (2.00)***	0.60	25	2.16 (2.00)***	0.62

***: *P*<0.001 shows significance when comparing healthy control lambs to those infected with *L. monocytogenes* according to Mann–Whitney Test

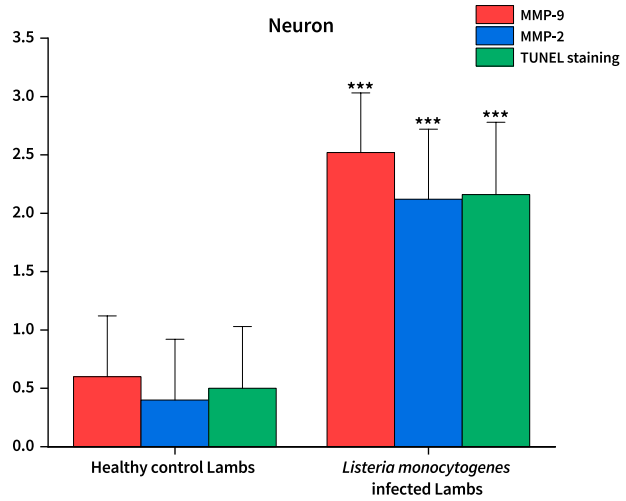

FIGURE 1. Immunohistochemical expression of MMP–9, MMP–2, and TUNEL staining in neuronal cells was assessed in both healthy and *Listeria monocytogenes* infected lambs

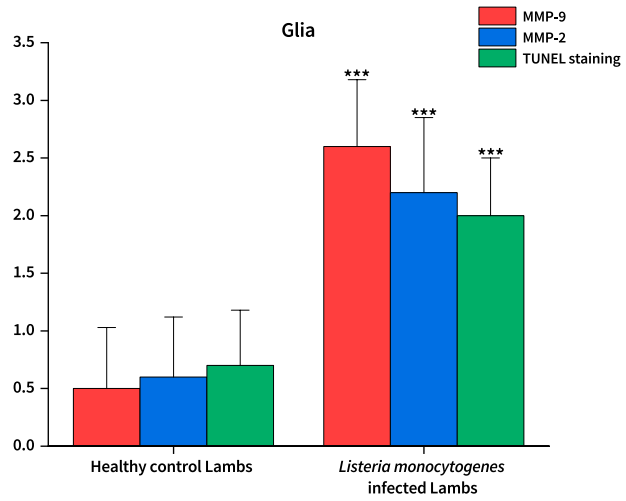

FIGURE 2. Immunohistochemical expression of MMP–9, MMP–2 and TUNEL staining in glial cells was assessed in both healthy and *Listeria monocytogenes* infected lambs

The reason for the differences in neuronal and glial cells undergoing apoptosis may depend on the relationship between the physiological activities of the cells and neuropathology. The difference between MMP-2 and MMP-9 expression may depend on the diversity of signaling molecules and the number of cells expressing these gelatinous cells in the affected area.

Histopathology Results

At histopathological examination, edema was detected in the Virchow–Robin spaces and hyperemia was seen in the vessels in the central nervous system. Common findings associated with *L. monocytogenes* infection were found in the cerebellum and brain stem especially in the pons and medulla oblongata. It was observed that the lesions associated with the disease varied from mild to severe leptomeningitis. Neurodegenerative reaction and liquefaction necrosis were detected in all cases especially in the brain stem. In addition, meningitis and perivascular inflammatory cell infiltration including lymphocytes, plasma cells and macrophages, mostly neutrophil leukocytes were observed in all cases (FIG. 3). This inflammatory cell infiltration was mostly localized in the pons, medulla oblongata and cerebellum. Multiple microabscesses, which are the typical histopathological finding of the disease, were seen in the same regions (FIG. 3).

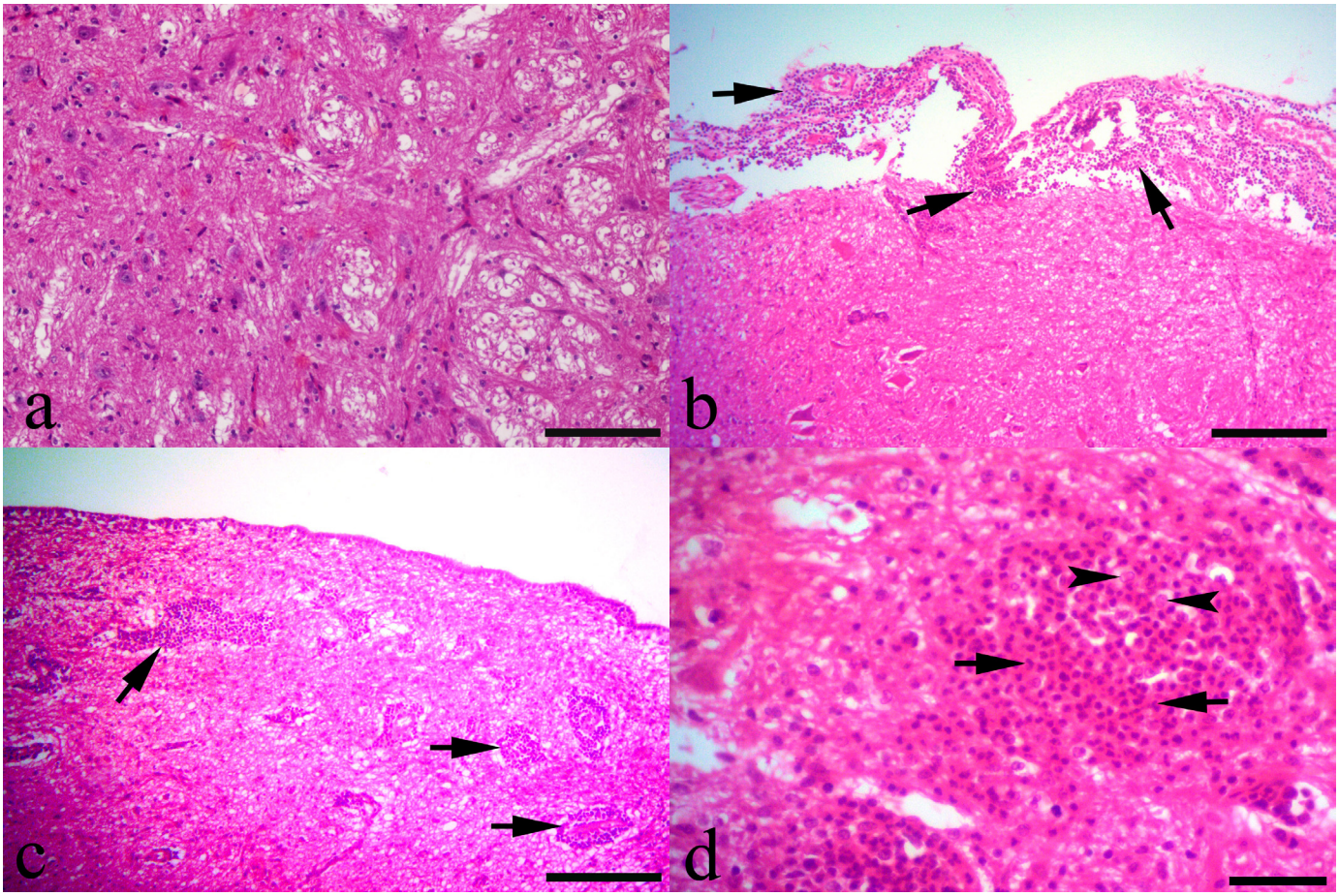

FIGURE 3. Histopathological findings of Listerial encephalitis a) Control. Medulla oblongata, HE, bar: 100 µm. b) Thickened meninges and severe lymphocyte infiltration (arrows). Medulla oblongata, HE, bar: 100µm. c) Perivascular inflamatory cell infiltratration (arrows). Medulla oblongata, HE, bar: 100µm. d) Severe inflammation, inflammatory exudate, and neutrophils (arrows) and macrophage infiltration (arrow heads). Medulla oblongata, HE, bar: 25µm

Immunohistochemistry results

Immunopositive reactions for MMP-2 were detected in the cytoplasm of neurons and glial cells (FIG. 4). Additionally, a strong cytoplasmic immunopositive reaction of MMP-2 was observed in perivascularly infiltrated leukocytes (FIG. 4). However, weak MMP-2 expressions were seen in control brains ($P<0.001$) (FIG. 4).

MMP-9 immunoreactivity was detected in the cytoplasm of glial cells, neurons, perivascularly infiltrated leukocytes and endothelial cells (FIG. 5). It was seen that the immunoreaction was very strong in the pons and medulla oblongata. On the other hand, a weak immunoreactivity was observed in the control preparations ($P<0.001$) (FIG. 5).

Listeriosis is a seasonal disease in ruminants. Farm management practices, animal health and hygiene, feed quality and storage play an important role in the emergence of the disease. One of the most common pathological findings of Listeriosis is encephalitis. It is

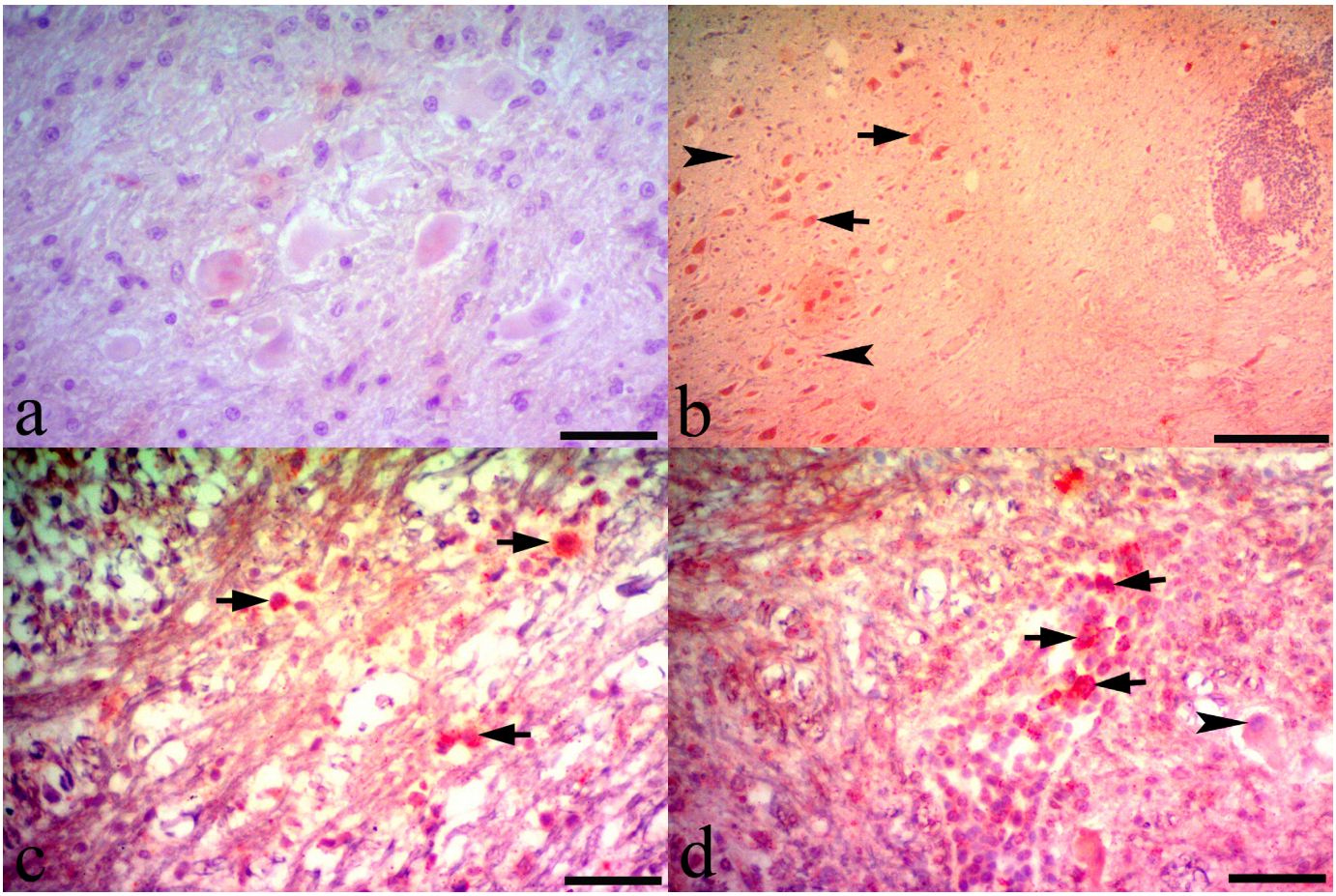

FIGURE 4. Immunohistochemical staining for MMP–2 antibody of brain with Listerial encephalitis. a) Weak MMP–2 expression in control tissue. Medulla oblongata, IHC, bar: 25 µm. b) Strong expression of MMP–2 in neurons (arrows) and glial cells (arrow heads). Medulla oblongata, IHC, bar: 100 µm. c) Strong expression of MMP–2 in glial cells (arrows). Medulla oblongata, IHC, bar: 25 µm. d) MMP–2 positive perivascular leukocytes (arrows) and neuron (arrow head). Medulla oblongata, IHC, bar: 25 µm

assumed that disease agents reach the brain via the bloodstream or via the branches of the trigeminal nerve ending in the oral cavity, nasal cavity or conjunctiva [21, 22, 23]. Therefore, pathological examination and immunohistochemical analysis in the central nervous system will contribute to the diagnosis and treatment of the disease. Best of our knowledge no reports of immunohistochemical expression of MMP–2 and MMP–9 and associated neuronal apoptosis in brain tissues of sheep with naturally occurring Listerial encephalitis. In this context, this is the first immunohistochemical study to report the expression of MMP–2 and MMP–9 and associated neuronal apoptosis in brain tissues of sheep with naturally occurring Listerial encephalitis.

Cortical neuron and astrocyte cultures show that in pathological conditions, astrocytes and neurons may express more MMP–2 and MMP–9. [24]. Zeng *et al.* (2018) reported that the release of MMP–2 and MMP–9 from the brain increased during ischemia–perfusion in rats and at the same time, apoptosis was occurred in neurons [25]. There are also reports that MMP–2 and MMP–9 expressions play an important role in the destruction of the blood brain barrier [26]. Studies have emphasized that various neurotropic disease agents develop strategies to cross the blood–brain barrier. These strategies include, crossing the blood brain barrier directly, crossing the blood brain barrier via paracellular, and crossing the blood brain barrier with infected leukocytes [27, 28] (Trojan horse mechanism). In the study, the presence of leukocytes immunohistochemically positively labeled with listeria in the inflammatory area may be related to these strategies. In addition, it was determined that macrophages also play an important role in the penetration of the agents through the blood–brain barrier. It is observed that in Listeriosis infection perivascular cellular infiltration in the vessels may cause ischemia and therefore lead to neurodegeneration. The most interesting finding in this study was the strong MMP–2 and MMP–9 expressions in neurons, glial and endothelial cells in the brains of sheep with Listeriosis. It has been suggested that increased MMP–2 and MMP–9 expressions may contribute to neurodegeneration and neuropathology in Listeriosis through extracellular matrix degradation and associated endothelial cell damage.

Immunopositivity reactions of *L. monocytogenes* antigens were detected in the cytoplasm of microglia, neurons and perivascular inmflamatory cells (FIG. 6). It was determined that TUNEL positive cells mainly consisted of neurons and microglia especially in the caudex cerebri (FIG. 6).

TUNEL positive areas were seen especially in the regions of microabscess, perivascular and meningeal inflammatory reaction areas in Listeriosis. Weak TUNEL reaction was detected in the control brain tissues ($P<0.001$) (FIG. 6).

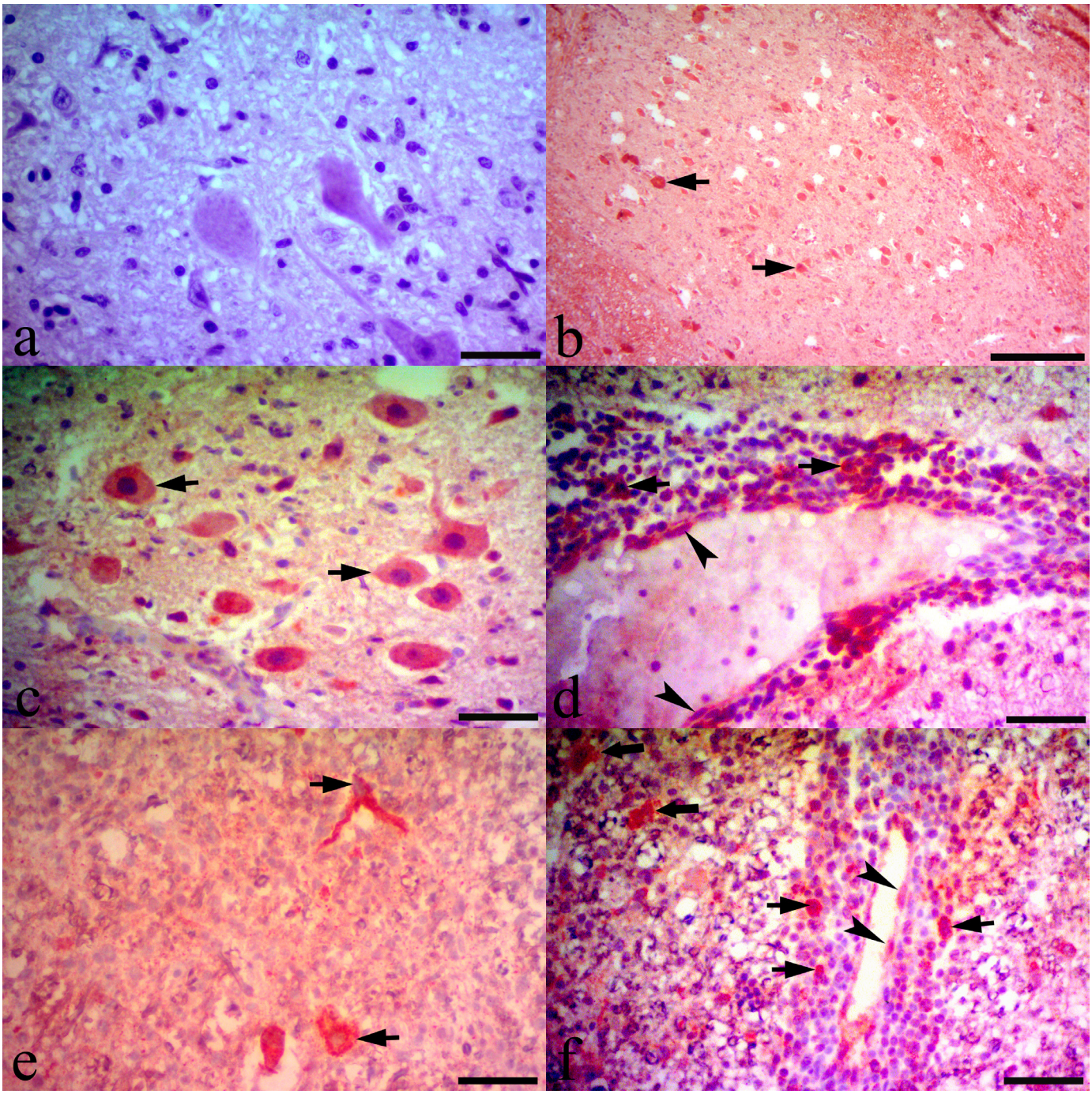

FIGURE 5. Immunohistochemical staining for MMP–9 antibody of brain with Listerial encephalitis a) MMP–9 immunostaining in healty control tissue. Medulla oblongata, IHC, bar: 25μm. b) Strong MMP–9 expression in neurons (arrows). Medulla oblongata, IHC, bar: 100μm. c) Strong MMP–9 expression in neurons (arrows). Medulla oblongata, IHC, bar: 25μm. d) MMP–9 immunolabelling in perivascular leukocytes (arrows) and endothelial cells (arrow heads), Medulla oblongata, IHC, bar: 25μm. e) Strong MMP–9 expression in glial cells (arrows). Medulla oblongata, IHC, bar: 25μm. f) MMP–9 immunolabelling in leukocytes (arrows) and endothelial cells (arrow heads) and glial cells (thick arrows). Medulla oblongata, IHC, bar: 25μm

Previous studies have reported that neurological symptoms occur in neuronal and glial damage or central nervous system.infections [29, 30]. It has also been stated that infections cause apoptosis of neural stem and progenitor cells, thus impairing neurogenesis [31]. Additionally, bacteria cause apoptosis in neural and immune system cells in the central nervous system [32]. MMP–9 contributes to caspase–mediated cytotoxicity that promotes apoptosis, finally leading to brain cell death in focal and global ischemia [33]. Apoptosis has a complex pathogenesis that occurs as a result of the interaction of many factors. Disruption of blood brain barrier function may reverse many physiological conditions in the brain and lead to consequences that directly affect the microenvironment. One of these is that cells

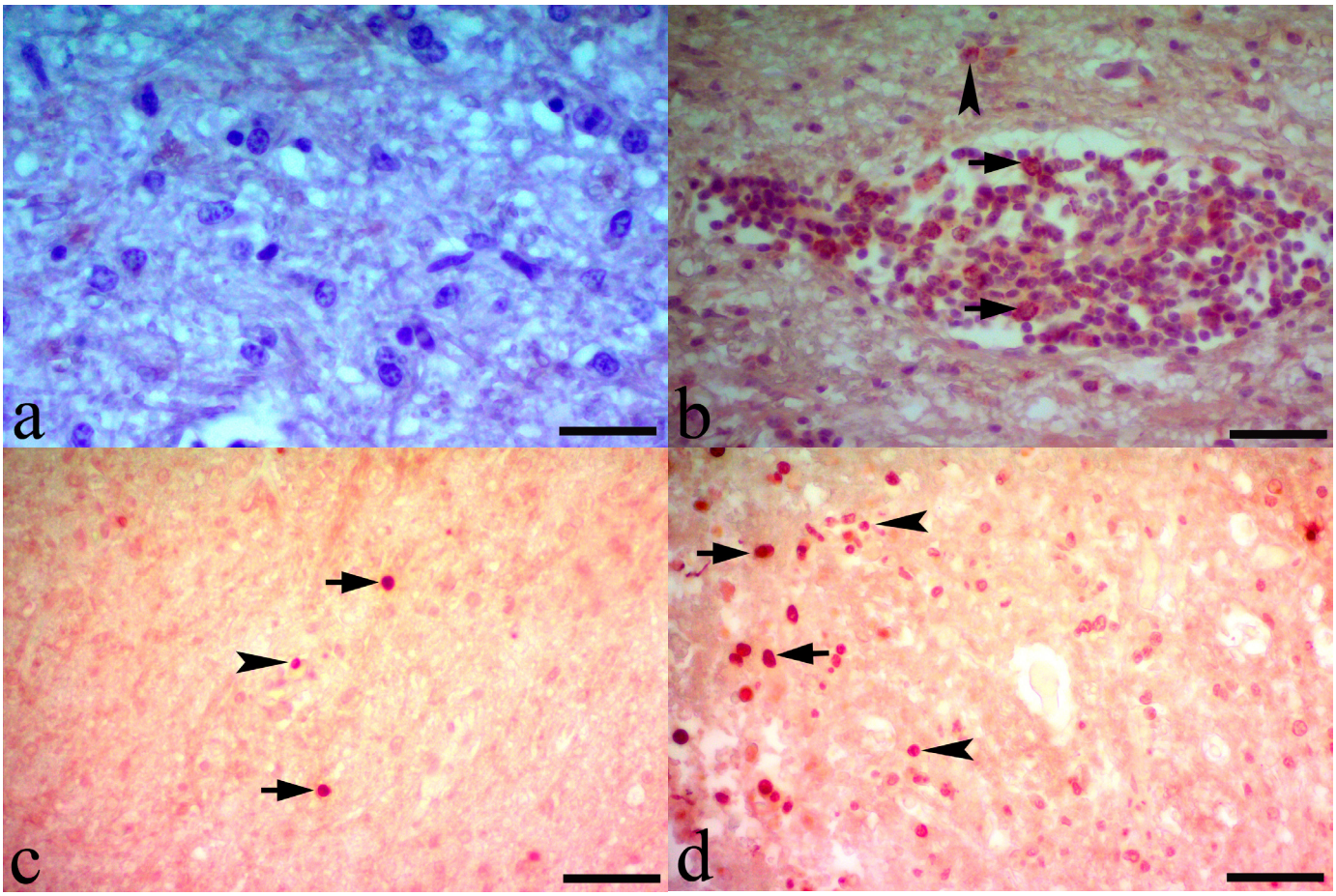

FIGURE 6. *Listeria monocytogenes* and TUNEL immunostaining of brain with Listerial encephalitis. a) Control,, Medulla oblongata, IHC, bar: 25 µm. b) Immunostaining of *L. monocytogenes* antigens in perivascular inflamatory cells (arrows) and glial cells (arrow head), Medulla oblongata, IHC, bar: 25 µm. c) TUNEL positive neurons (arrows) and glial cells (arrow head) of healty control animals, Medulla oblongata, IHC, bar: 25 µm. d) Strong TUNEL positive neurons (arrows) and glial cells (arrow head), Medulla oblongata, IHC. Scale bar 25 µm

are exposed to oxidative stress. Increased gelatinous MMPs causes ionic homeostasis and energy deficiency after decreased cerebral blood flow [13, 34]. As a result, increased oxidative stress, apoptosis, irreversible tissue/organ damage, and neurological and behavioral disorders appear to occur [14]. There are former reports that show oxidative stress triggers apoptosis in the pathogenesis of Listeriosis [5, 7]. The data obtained from this study showed that increased gelatinous MMPs expressions in Listeriosis may be one of the first steps in a chain of neuropathological mechanisms that trigger each other.

CONCLUSION

In the presented study, MMP 2 and MMP 9 expressions were found to be high in brain damage caused by encephalitic Listeriosis. The damage in the brain cells was demonstrated pathologically by TUNEL staining. There were also differences in expression between animals in general. However, almost all of them showed higher expression than controls. This difference may depending on the individual sensitivity of the animals and the severity of infection. However, statistically significant results were obtained when compared to controls. As a result of all neuronal changes, it was thought that MMP-2 and MMP-9 were especially effective in neurovascular cells and would shed light on the pathogenesis of *L. monocytogenes* infection. In addition, using this information, it was concluded that the prevention of MMP-2 and MMP-9 activation, which play a role in the pathophysiology of neurodegenerative diseases such as Listeriosis, that is, MMP-2 and MMP-9 inhibitors may be useful in the treatment. It has been suggested that apoptosis occurring in the cells of the central nervous system in *L. monocytogenes* infection may help the diagnosis in terms of determining the level in the pathogenesis and severity of the disease.

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Conflict of Interest

The authors declare no conflict of interest.

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