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Evaluation of Histopathological Changes of Oral Mucosa and Salivary Glands in Model of Demyelination (Multiple Sclerosis) in C57BL/6 Mouse

Mehrshad Mohammadi, Khadijeh Abdal, Marzieh Darvishi*, Parvin Eliasi, Ardeshir Moayeri

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Abstract

Introduction: Multiple sclerosis (MS) is an autoimmune disease, which is characterized by multifocal demyelination of axons in the CNS. Complications due to the disease, as well as the use of immunosuppressive drugs, antidepressants, etc., lead to some abnormalities. These drugs predispose to oral bleeding and are particularly susceptible to infection. The main side effects of drugs in the oral cavity are stomatitis, ulcers, gingivitis, candidiasis, and some opportunistic infections (such as herpes simplex). Dentists should also be aware of the importance of the disease in the diagnosis, treatment, and prognosis of some lesions as well as its specific conditions. Hence, in this study, the histopathological changes of the oral mucosa and salivary glands were studied on C57BL/6 demyelination mice (multiple sclerosis model).**Material & method:** The histological changes in parotid, submandibular, and sublingual glands were studied in both control (intact) and demyelination models of mice. The present study, 20 mice C57BL/6 were divided into two groups: 1) sham group (fed regular chow); 2) demyelination group (received rodent chow mixed with 0.2% cuprizone for 12 weeks). Seven weeks after demyelination inflammation (Hematoxylin-eosin), fibrosis (Masson's Trichrome), and mast cells granulation (toluidine blue) were examined. **Result:** Our results demonstrated inflammation in the major salivary gland 12 weeks after demyelination. Also, the increase in fibrosis and decrease in vascularization was observed in buccal mucosa of different areas ($P<0.05$). The keratinization reduced in demyelination group ($P<0.05$). The expression of GFAP in the major salivary gland was significantly decreased in the demyelination group ($P<0.05$). **Conclusions:** MS may be a risk factor for oral lesions such as inflammation, fibrosis, and ulcer. These data present the pathological effect of demyelination on acini and ductal region in salivary glands.

Keywords: Multiple sclerosis (MS), Major salivary glands, Inflammation, Histology.

Mehrshad Mohammadi

Department of Endodontology, Faculty of Dentistry, Ilam University of Medical Sciences, Ilam, Iran.

Khadijeh Abdal

Department of Oral and Maxillofacial Pathology, Faculty of Dentistry, Ilam University of Medical Sciences, Ilam, Iran.

Marzieh Darvishi*, Ardeshir Moayeri

Department of Anatomy, Faculty of Medical Sciences, Ilam Medical University, Iran.

Parvin Eliasi

Faculty of Dentistry, Ilam University of Medical Sciences, Ilam, Iran.

***Email:** Darvish-m@medilam.ac.ir

Introduction

Multiple sclerosis (MS) is a chronic autoimmune disorder of the central nervous system (CNS) characterized by the inflammation of nervous tissue, focal demyelination, axonal loss, and neurologic dysfunction (Preziosi, Gordon-Dixon and Emmanuel, 2018; Voet, Prinz and van Loo, 2019). The etiology of MS is not well understood, however, the genetic and environmental factors are associated with the disease(Li et al., 2019; Bigaut, De Seze and Collongues, 2019). The incidence of MS has been estimated 2 million people worldwide, and 20 per 100,000 in the Asia Pacific region, with women being at higher risk than men (3:1). Several symptoms may appear during the disease progression such as ataxia, dysarthria, fatigue, spasticity, pain, seizure, and cognitive dysfunction (Barnett and Prineas, 2004; Lucchinetti et al., 2000). The most common symptoms of oro-facial include facial palsy (Bell's palsy), trigeminal neuralgia, and sensory neuropathy. The sequel of disease and side effect of drug treatments cause oral problems including dry mouth, ulcers, tongue/mouth burning, altered taste, cheilitis, and oral thrush (Flint and Scully, 1990; Penarrocha Diago et al., 1990; Barrett and Buckley, 1986; Fukazawa et al., 1997).

In 2005, Adamashvili et al. demonstrated that the concentration of soluble human leukocyte antigen, class II (HLA class II) is significantly increased in the saliva of patients with Relapsing-Remitting MS (Adamashvili et al., 2005). Moreover, Minagar et al. reported that interferon β1-a could be effective on salivary concentrations of HLA class II (Minagar et al., 2007). On the other hand, the results confirmed the increase in the expression of oxidative stress markers, Thiobarbituric Acid Reacting Substances

(TBARS) and advanced glycation end products (AGEs) in the saliva of MS patients (Neil et al., 2017; Brandon et al., 2017).

In addition, the inflammatory infiltrates may lead to destruction, fibrosis, and change of acini with the secretory function impairment of the salivary glands (Christodoulou, Kapsogeorgou and Moutsopoulos, 2010; Voulgarelis and Moutsopoulos, 2002). Although there have been findings in clinical studies representing the orofacial changes in MS depending on different stages of the disorder, there have been no histological evaluations of oral mucosa and salivary glands.

Due to the inability of obtaining serial biopsies, it is not possible to investigate histopathological changes in the major salivary glands and oral mucosa, for the reason few data are available in this area. The animal models of MS provide a proper condition for histological examination with several advantages (WU QZ et al., 2007; Irvine K-A and Blakemore, 2006). These models show the complications of MS and give researchers a view into the disease pathogenesis without the interference of drug effect and sequel of disorder (Skripuletz et al., 2010; Wergeland et al., 2012).

The purpose of the study was to investigate the histopathological changes of oral mucosa and the major salivary glands (parotid, submandibular, and sublingual) in the demyelination model of (multiple sclerosis) c57bl/6 mouse.

Materials and Methods

Supply of animals and Demyelination

Female C57BL/6 mice were obtained from Pasteur Institute, Tehran, Iran, and housed in the Laboratory Animal Facility at the Ilam University of Medical Sciences. Twenty C57BL/6 mice with body weight ranging between 19 and 21 g (aged 8 to 9 weeks old) were used in this study. The animals were kept at room temperature (20–22 °C) and 40-50% humidity with a 12-h light/dark cycle. The mice were divided into two groups of ten animals. Group I, consisted of animals (n=10) that were fed with normal powdered chow (Control) and Group II consisted of animals (n=10) with induced demyelination. To induce demyelination, cuprizone 0.2 % (w/w) was mixed with ground standard rodent chow. This diet was fed for a prolonged period of 12 weeks (Berghoff et al., 2017).

Obtaining samples

The mice in two groups were sacrificed by cardiac perfusion under anesthesia induced by ketamine/xylazine (50/10 mg/kg). The head of mice was separated and prefixed in 10% formalin for 24h. Samples were then incubated with 5% nitric acid for decalcification. Subsequently, samples were washed and then performed routine dehydration and paraffin embedding. 7µm of the sections were used in immunohistochemical and histopathological evaluations.

Histological evaluation of salivary gland and oral tissues

7µm of thick tissue sections were stained with Hematoxylin-eosin (H&E) and toluidine blue to study inflammation and detect mast cells in the submucosal layer. The histological change of acini and duct glands, as well as the morphology and disperses of vessels, were investigated by H&E staining. Also, Masson's Trichrome staining was used to detect collagen fibers surrounding the acini of the salivary gland and submucosal layer of oral tissue. The collagen fibers were stained blue, the nuclei were stained black, and the background was stained red. The stained sections were investigated using the light microscope (Kern-Germany).

Immunohistochemical examination

According to the previous studies, glial fibrillary acid protein (GFAP) reactivity is a marker for the evaluation of pleomorphic cells in salivary glands.

For the evaluation of metaplasia, paraffin sections were stained with Immunohistochemical technique. The deparaffinized tissue sections were dehydrated and rinsed in distilled water and then were boiled in citrate buffer solution for 5 minutes to perform antigen retrieval. They were incubated with H_2O_2 to suppress endogenous peroxidase activity. Finally, the sections were treated with mouse anti-GFAP monoclonal primary antibody (1:500; Abcam, Cambridge, UK) for overnight at 4°C. After primary antibody application, the secondary antibody was applied for 45 minutes (FITC -1:100; Abcam, Cambridge, UK) and nuclear counterstaining was carried out using propidium iodide. Negative control was performed by removing the primary antibody. The prepared slides were examined and photographed with a Kern microscope. The intensity of GFAP immunoreactivity was evaluated by using the Image J software. Each slide was investigated in five randomly selected areas in the light microscope (10X magnification). Two researchers identified the percentage of cells in each area. The mean scores of both researchers were calculated.

Results

Oral mucosal changes

a. Control group

Figure 1 demonstrated the histological structure of the oral mucosa in control and demyelination groups. In the control group, the mice oral mucosal tissue showed a normal keratin thickness and epithelium thickness without changes in the vascularity and depth of fibrosis. The gingiva and hard palate were lined with the keratinized stratified squamous epithelium and lamina propria rests directly on the periosteum of the underlying bone. In addition, the soft palate, cheeks, and floor of the mouth were covered with parakeratinized squamous epithelium with a thick submucosa containing many minor salivary glands (in this article tongue was studied as an example of the oral mucosa (Fig 1A and C)).

b. Demyelination group

In the tongue of the demyelination group, an increase was observed in the fibrotic tissue deposition in the submucosal layer and thin keratin layer with epithelium thickness in comparison to the control group. In addition, we observed a decreased number of blood vessels in comparison to the control group (Fig 1B and D).

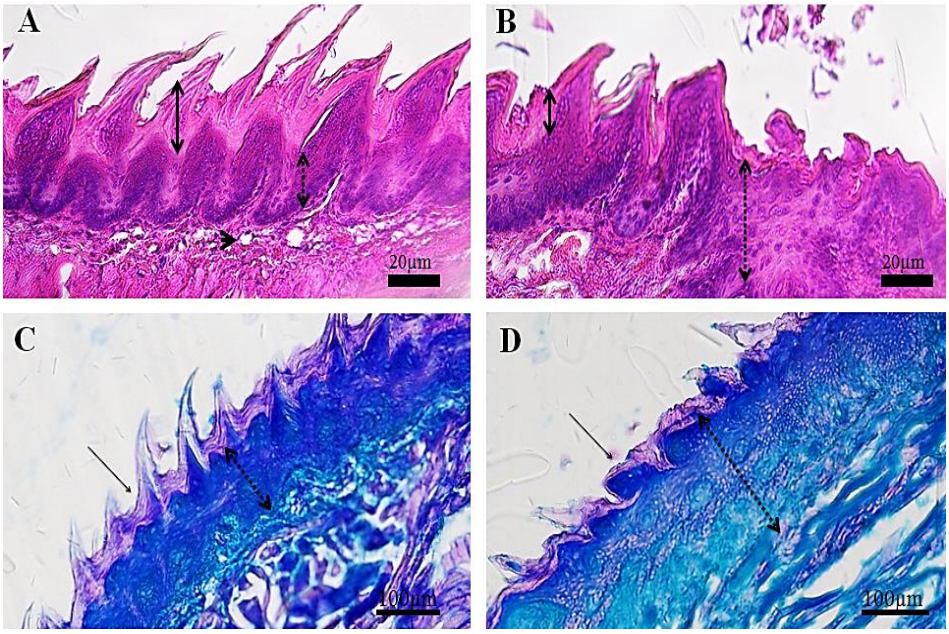

Figure 1. The keratin thickness and epithelium thickness in control (A and C) and demyelination groups (B and D). Arrow: keratin layer, two-direction arrow: epithelium thickness and fibrosis in connective tissue (Masson's Trichrome)

Table 1. The histological characters of the oral mucosa

Group	Area	Number Of Epithelial Layer	Keratin	Connective Tissue Density	Vascularization
Intact	Ventral Surface Of Tongue	7-9	++	++	+++
	Mouth Floor	7-9	+	++	++
	Cheek	7-13	+	++	++
Demyelination	Ventral Surface Of Tongue	4-6	+	+++	+
	Mouth Floor	3-6	+	+++	+
	Cheek	7-12	+	++	+

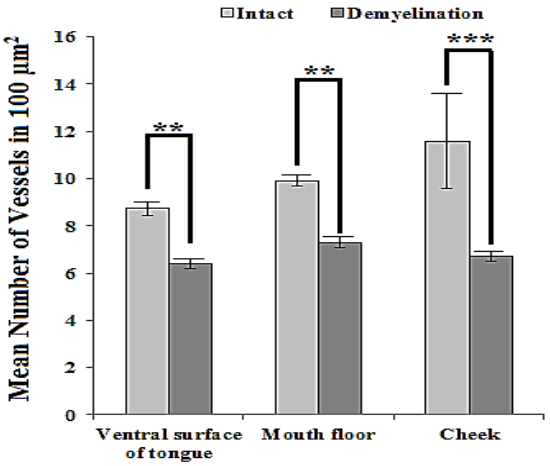

Figure 2. The mean number of vessels in 100 μm^2 on the ventral surface of the tongue, mouth floor, and cheek in two control and demyelination groups. Each symbol indicates mean ± SEM, $^{**}P < .01$, $^{***}P < .001$.

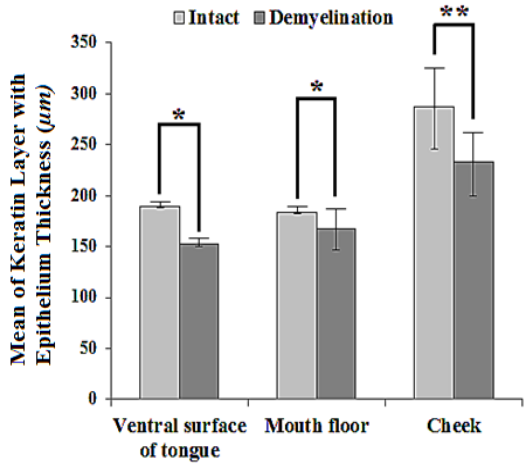

Figure 3. The thickness of keratin and epithelium layers in several areas of the oral mucosa. Each symbol indicates mean ± SEM, $^{*}P < .05$, $^{**}P < .01$.

Salivary gland changes

a. Control group

Parotid gland: The control group showed no significant changes in the major salivary gland (acini and the granular ducts). In the

parotid gland stained with H&E demonstrating serous acini with cytoplasm contained basophilic and eosinophilic granules and the basal spherical nuclei (Fig. 4A). In addition, swollen mucosal cells with cuboidal shape, basal nuclei, and pale cytoplasm, surrounded by the serous cells in a demilune position, were observed in the parotid gland (Fig. 4B). The figure of parotid gland stained with Masson's Trichrome shows thin connective tissue collagen fibers (Fig. 6B). The toluidine blue showed the metachromatic secretory granules with different staining intensities in the acinar cells and unstained duct cells (Fig. 7B). The Immunohistochemical study also showed the high expression of GFAP marker in the parotid glands.

The submandibular (5A) and sublingual (5C) glands have pale bubble-like cytoplasm with the basal nuclei (mucosal cells) and the serous cells have spherical nuclei and their cytoplasm is apically filled with basophilic zymogen secretory granules. Acini of the parotid and submandibular salivary gland are composed of serous cells. While in the sublingual gland, the distal end of swollen mucosal cells is surrounded by serous demilune. The expression of GFAP marker was observed in submandibular and sublingual glands similar to the parotid gland. The assessment of collagen fiber with Masson's Trichrome showed fine connective tissue fiber in both glands. In addition, metachromatic secretory granules were observed in the section of two glands stained with toluidine blue. There was low metachromatic staining of serous demilune and duct granules.

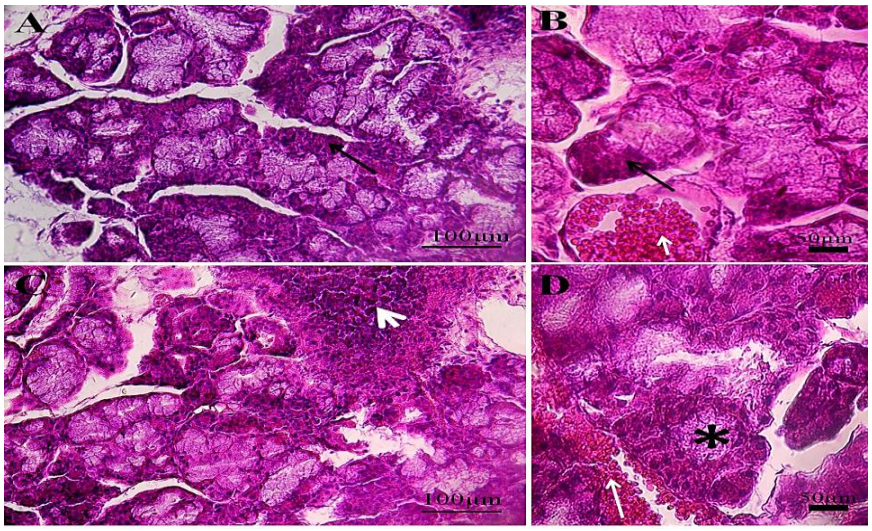

Figure 4. The control group (A and B) and demyelination group (C and D) in the parotid gland stained with H&E indicating serous cells (SC) and mucous acini (MA). Arrow: mucosal cells with serous demilune, stare: The striated ducts (*). White arrow showed moderate inflammation.

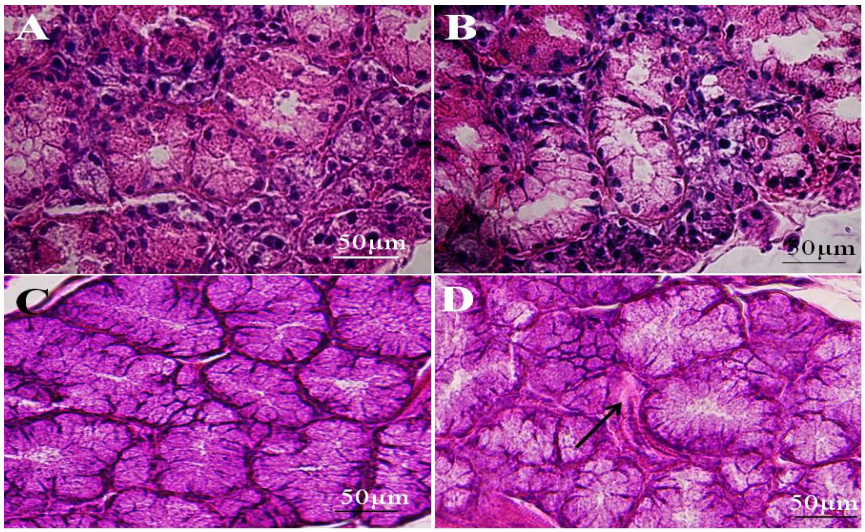

Figure 5. A and C showed the control group of submandibular and sublingual glands, respectively. B and D presented the demyelination group of submandibular and sublingual glands, respectively. Arrow: muscle fiber in the sublingual gland

b. Demyelination group *Parotid gland*

The architecture appeared divided the parenchymal tissue into several lobules by the stromal connective tissue. Inflammation surrounded granular ducts and the low number of blood vessels were detected in stromal tissue. We observed dilation of interlobular channels that were covered by pseudostratified epithelium (Fig 4C and D). Fumy cells, shrunken nuclei, promotion of collagen fiber in stromal tissue (Fig.6B), and deceased of GFAP expression showed in serous and mucosal acini (Fig.8B). The fat tissue detected in the parotid gland. The toluidine blue staining detected metachromatic secretory granules with an eosinophilic stain in the acinar cells (Fig.7B).

Submandibular gland

Abnormal salivary gland architecture was observed with significant changes in acini and granular ducts. The tissue demonstrated dilatation and fusion of acini and granular ducts with the increasing number of mucous acini in the submandibular gland. Degenerative and edematous changes in the mucus and serous acini were observed in the submandibular gland (Fig. 5B). Immunohistochemical staining of GFAP showed a moderate immunoreactivity in comparison to the control group (Fig. 8D). The Masson's Trichrome staining showed that the total collagen fiber was higher and these fibers were disorganized in demyelination group in comparison to the control group (Fig. 6D). Also, in the toluidine blue staining, some atrophic acini and loss of their metachromatic secretory granules were observed (Fig.7D).

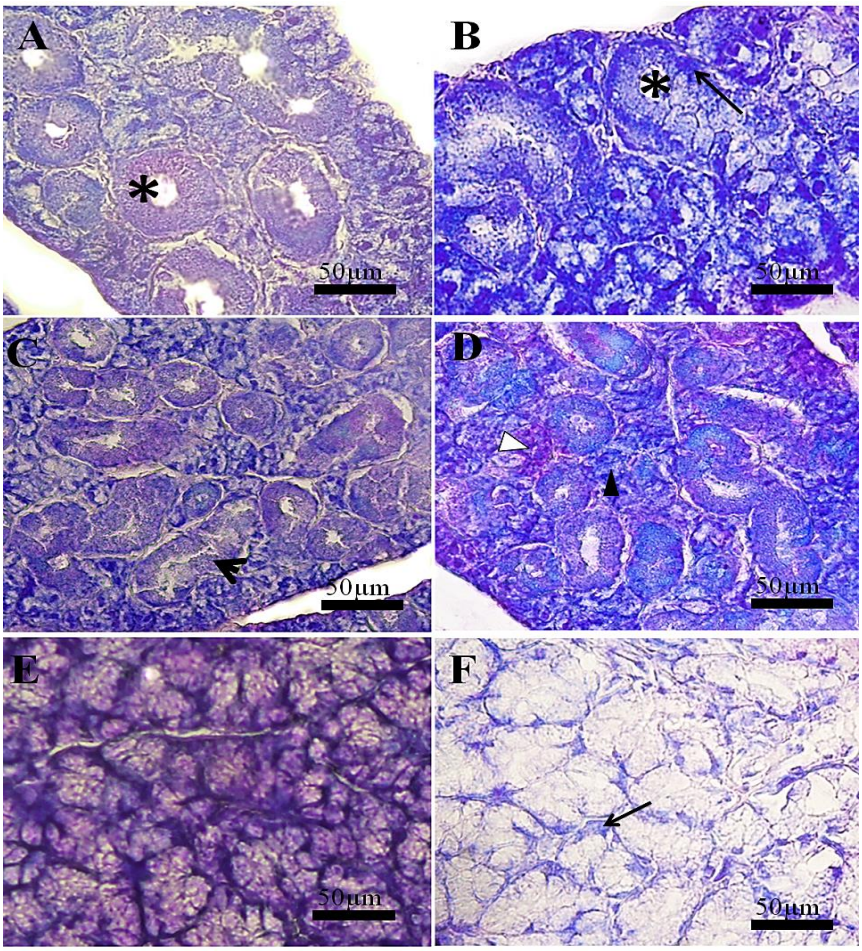

Figure 6. Parotid (A and B), submandibular (C and D) and sublingual (E and F) salivary gland Masson's trichrome staining. A) Parotid gland of intact mice; B) Parotid gland of demyelination mice; C) Submandibular gland of intact mice; D) Submandibular gland of demyelination mice; E) Sublingual gland of intact mice; F) Sublingual gland of demyelination mice; *: Granular ducts, Black arrow: nuclei, Arrowhead: Acini, Black triangle: Collagen fibers

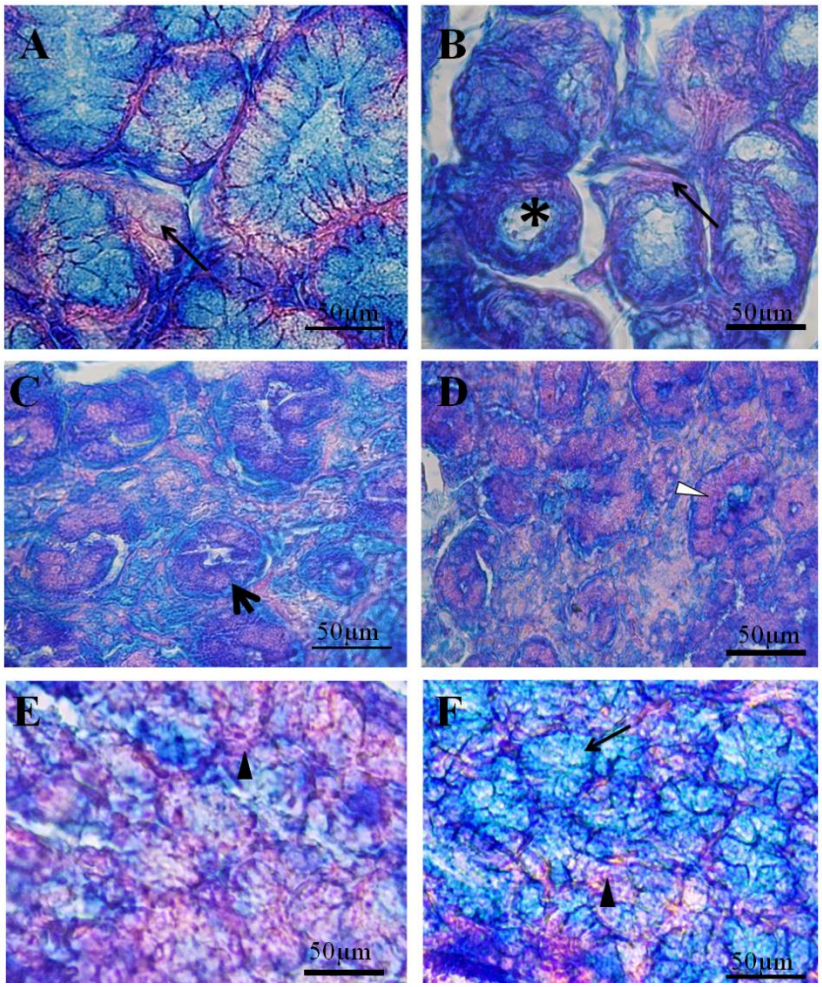

Figure 7. Parotid (A and B), submandibular (C and D) and sublingual (E and F) salivary glands stained with toluidine blue. A) Parotid gland of intact mice; B) Parotid gland of demyelination mice; C) Submandibular gland of intact mice; D) Submandibular gland of demyelination mice; E) Sublingual gland of intact mice; F) Sublingual gland of demyelination mice; *: Granular ducts, Black arrow: metachromatic secretory granules, Arrowhead: Acini

Sublingual gland: The architecture appeared distorted in acini and granular ducts (Fig. 7D). Masson's Trichrome staining showed the loose and disorganized collagen fibers in the sublingual gland (Fig. 6F). Moreover, acinar atrophy with the shrunken nuclei and dilated and fusion interlobular ducts were seen in demyelination mice. High granular convoluted tubules due to inflammation were demonstrated. The pyknotic and peripheral nuclei, enlarged cytoplasm, reduced intracellular space, and disturbing cell boundaries were discovered in the acini cells. The replacement of the fat tissue and the reduction of blood vessels were other changes in the sublingual gland (Fig. 7D).

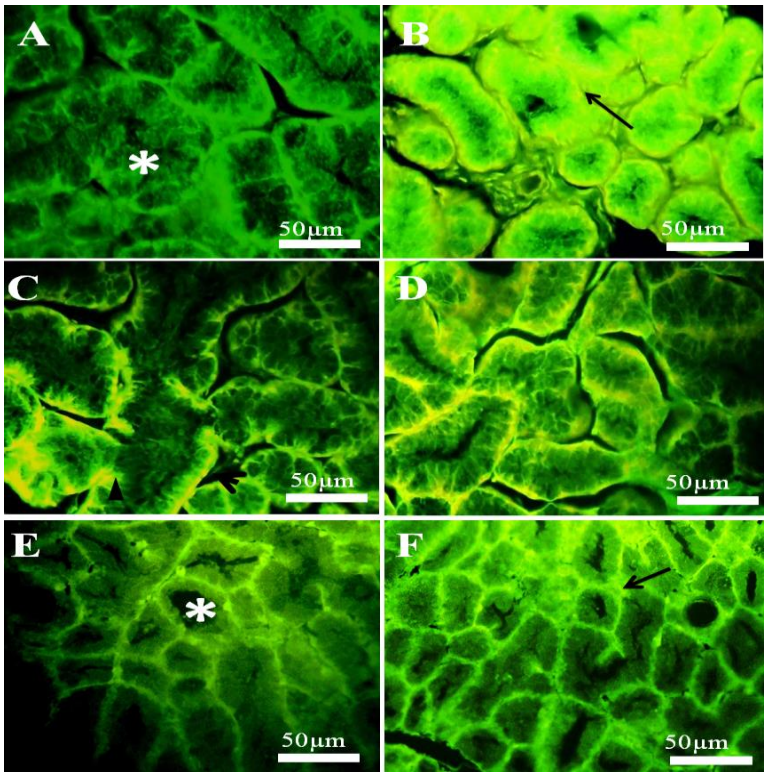

Figure 8. Parotid (A and B), submandibular (C and D), and sublingual (E and F) salivary glands Immunohistochemical staining for GFAP. A) The parotid gland of intact mice; B) Parotid gland of demyelination mice; C) Submandibular gland of intact mice; D) Submandibular gland of demyelination mice; E) Sublingual gland of intact mice; F) Sublingual gland of demyelination mice; *: Granular ducts, Black arrow: GFAP positive cells

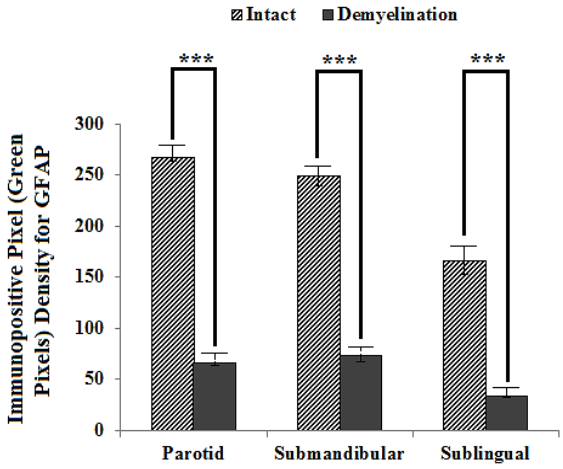

Figure 9. The immunopositive pixel density of GFAP. Each symbol indicates mean ± SEM, **P* <0.05, ***P* < 0.01, ****P* < 0.001.

Discussion

Salivary secretion may be changed by physiological and pathological conditions. Multiple sclerosis (MS) is one of the main pathological factors for the change of salivation (Giannobile et al., 2009; Miller et al., 2006; Nanci A. Salivary Glands, 2012; Proctor, 2016). Since the salivary secretion is very effective in protecting the integrity of oral tissues, teeth against decay, tasting, swallowing, and talking, changes in salivation can be problematic (Humphrey and Williamson, 2001; Tiwari, 2011). In this study, the histological changes of oral mucosa and major salivary gland were observed in intact and demyelination models of mice. In previous studies, there was no histological evaluation of salivary gland or oral mucosa in this neurological disorder. This study is the first study to compare the histological status (epithelial thickness, keratinization, fibrosis, and vascularization) in the oral mucosa of intact and demyelination mice. We performed morphometric and quantitative evaluations of oral mucosa in vivo using the mouse model. In addition, an immunohistochemical technique was used to compare GFAP in order to assess the pathological changes in the major salivary gland in two experimental groups (Gnepp and el-Mofty, 1997; Curran et al., 2001). A mouse model of chronic demyelination was used in the experimental studies because this chronic process impairs oligodendrocyte regeneration (Skripuletz et al., 2008; Berghoff et al., 2017). The microscopic changes in the oral mucosa and major salivary glands were reported, which were related to demyelination of CNS. The demyelination of CNS affects almost all organs of the human body including the oral cavity, esophagus, heart, liver, lungs, and reproductive organs (Ito, 1960; Garrett, 1987). Previous studies confirmed that parasympathetic impulses lead to increased salivation from the salivary glands (Dawes, 1969; Greabu et al., 2009).

Sympathetic neurotransmitter activates alpha and beta-adrenergic receptors, while parasympathetic neurotransmitter activates cholinergic receptors (Iorgulescu et al., 2009). The stimulation of alpha-adrenergic receptor leads to the protein secretion from the salivary glands while beta-adrenergic or cholinergic stimulation causes the decrease of protein and increases of water and electrolytes secretion. Watery saliva is released with parasympathetic stimulation while the thicker saliva is in response to the sympathetic stimulation (Kaufman and Lamster, 2002; Ellison, Massimo and Mandel, 1960).

This saliva is diluting is discharged through the contraction of myoepithelial cells from the acinar cells (Iorgulescu et al., 2009; Kaufman and Lamster, 2002). MS influences the afferent and efferent component of the autonomic system (Ellison, Massimo and Mandel, 1960).

In the previous study, the findings showed Sjogren's syndrome that characterized by inflammation and dysfunction of salivary and lacrimal glands, leading to fibrosis, atrophy, and damage in acini and granular ducts. These findings are in agreement with the data obtained in this study (Thorne and Sutcliffe, 2017; Bayetto and Logan, 2010).

Conclusion

Our findings indicated that demyelination in multifocal of the brain and spinal cord leads to various types of inflammation, atrophy, promotion of fibrosis, and high polymorphic cells in the major salivary gland. The parotid glands are involved much in the course of MS with highly severe inflammation, while the sublingual glands are rarely involved in the inflammation or metaplasia. Based on these findings, we hypothesized that human MS may affect the histological and physiological functions of the major salivary and may lead to early diagnosis of oral lesion and thereby treatment of these disorders.

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