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Roflumilast exerted neuroprotective effect in myelin oligodendrocyte glycoprotein$_{35-55}$ (MOG$_{35-55}$)-induced experimental autoimmune encephalitis (EAE) model

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ABSTRACT: In the recent decade, the effect of phosphodiesterase inhibitors (PDEi) in neurological diseases has been extensively investigated. In previous studies, the beneficial effects of PDEi on multiple sclerosis (MS) and oligodendroglial health have been indicated. However, as phosphodiesterase affects many different cellular and molecular pathways on both neurons and glial cells, knowledge about most PDEi is still missing. Roflumilast, a PDE4 inhibitor, is a potent anti-inflammatory drug currently used to treat chronic obstructive pulmonary disease. Recent studies have demonstrated the neuroprotective effects of roflumilast in neurological and immune diseases. Therefore, in this study, we aimed to investigate the effect of roflumilast on the experimental autoimmune encephalitis (EAE)-induced MS model in mice. The effects of roflumilast on the levels of immune semaphorins Sema3A and Sema4D, and pro-inflammatory cytokines, also motor behavior function and myelin integrity were examined. EAE model was created with myelin oligodendrocyte glycoprotein$_{35-55}$ (MOG$_{35-55}$) immunization. Animals were treated with roflumilast and FTY720 (Fingolimod hydrochloride) (as positive control) for 28 days and observed for motor impairments. Brain tissue levels of tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), semaphorin 3A (sema3A), semaphorin 4D (sema4D) were determined by enzyme-linked immunosorbent assay (ELISA). Myelin integrity was assessed with luxol fast blue staining. As a result, roflumilast prevented EAE-induced motor impairment and prevented the loss of myelin in the corpus callosum. Additionally, roflumilast suppressed EAE-induced increase in TNF-α, IL-1β, IL-6, and sema3A, sema4D levels in the brain tissue. Our results demonstrated that roflumilast exerts a neuroprotective effect and prevents EAE-induced myelin loss, possibly via decreasing inflammatory cytokines in the brain.

KEYWORDS: Roflumilast; experimental autoimmune encephalitis; multiple sclerosis; phosphodiesterase; mice.

1. INTRODUCTION

Multiple sclerosis is an inflammatory demyelinating central nervous system (CNS) disorder that affects up to two million people around the globe[1]. Typical manifestations begin after the third decade of a lifetime and are more common in women than men[2]. MS is considered the primer cause of most frequently seen neurological disability and causes social, economic, and phycological consequences. Although there are several drugs, relapsing/remitting patterns and severe side effects limit therapy options in the clinics. Therefore, identifying alternative treatment options with fewer side effects and more effective still has utmost importance. Based on the urgent need for treatment options of MS, several animal models were described that mimic clinical pictures with underlying physiopathology for investigation[3]. Experimental autoimmune encephalitis resembles MS seen in humans in many respects[4]. EAE pathophysiology is based on the immune system's reaction to brain-specific antigens. As a result of this reaction, inflammation and emerging antigen-carrying structures result in neuropathological and pathological features comparable to

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MS patients[5]. Myelin oligodendrocyte glycoprotein$_{35-55}$ (MOG$_{35-55}$)-induced EAE in C57BL/6 mice is the most frequently used model in the last twenty years[6].

In search of novel treatment options, compounds targeting both neuroinflammatory and degenerative processes are accepted as rationale alternatives. It has been shown that elevating cellular second messengers, such as cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), is essential for CNS function and directly related to regeneration pathways in the neuronal structures[7]. Additionally, the interaction of cGMP with neuronal repair proteins like semaphorins is crucial for identifying new treatment strategies[8]. Immune semaphorins and their receptors are crucially responsible for maintaining immunological homeostasis by regulating and coordinating immune cell communication. Sema4D, also known as CD100, is the first semaphorin protein that was determined to have immunoregulatory functions and involved in B-cell/dendritic cell activation. As a Class III semaphorin, Sema3A is reported to inhibit immune cell migration[9]. Phosphodiesterases (PDE) are a diverse family of enzymes (with 11 isoforms) responsible for the degradation of cAMP and cGMP. PDE4 hydrolyses cAMP and all the three isoforms (PDEA, B, D) are demonstrated to inhibit neuroplasticity. cAMP is known as a significant player in controlling the production of pro-inflammatory cytokines. Decreased levels of cAMP in the cerebrospinal fluid are linked to demyelination in MS patients. Increasing cAMP levels through PDE4 inhibition suppresses the immune response and increases remyelination[10]. Thus, clinical trials are underway investigating the efficacy of PDE4 inhibitors in MS [11]. Compounds that affect phosphodiesterase enzymes are under investigation for treating multiple sclerosis; even rolipram (a selective phosphodiesterase-4 inhibitor) reached clinical trials, but it failed due to its effect on the blood-brain barrier, which is also essential for MS physiopathology[12]. Nevertheless, its interaction with the dopaminergic system and side effects in the patients lead to a new investigation of other PDE4 inhibitors with fewer side effects[13].

Roflumilast, a selective PDE4 inhibitor, has shown to be neuroprotective in several neurological disorders[14]. Previous studies demonstrated that roflumilast could also modulate immune cells with potent anti-inflammatory action[15]. Additionally, the regulation of microglial and astroglial cells, which are also responsible for neuroinflammation seen in MS, is positively affected by roflumilast treatment[16, 17]. However, as far as we know, no study investigates the effects of roflumilast in the EAE-induced MS model. Therefore, in this study, we aimed to investigate whether roflumilast confers neuroprotection and functional recovery in the MOG-induced EAE model in mice. As PDE4 inhibition is considered to restrict neuroinflammation and stimulate neuroplasticity, we also examined the effects of roflumilast on the levels of immune semaphorins Sema3A and Sema4D, and pro-inflammatory cytokines IL-1β, IL-6, and TNF-α, as well as motor behavior function and myelin integrity.

2. RESULTS

2.1. Roflumilast alleviated motor behavior dysfunction in EAE

Daily scoring of animal motor behavior was performed for 28 days. Our results demonstrated that motor behavior dysfunction started to appear in all groups after seven days. Peaking between 11-13 days of experiments, EAE with different scores was observed till the end of the experimental schedule except for the control group, which did not show any motor behavior dysfunction throughout the experimental period, as expected (0,0±0,0). Motor behavior scores in the MS group (2,3±0,22) significantly increased compared to the control ($p<0,001$). In contrast, roflumilast (1,63±0,16) and FTY720 (1,4±0,14) both significantly prevented that increase compared to the MS group. However, the ameliorated effect of FTY720 was found to be stronger than roflumilast, as expected ($p<0,001$, Figure 1).

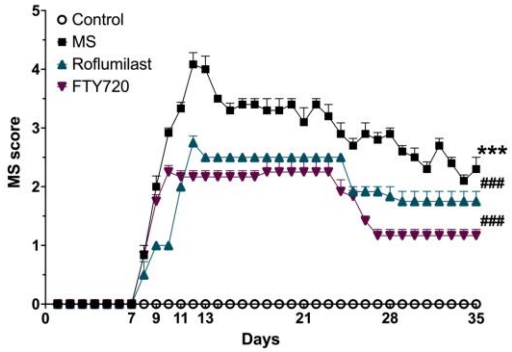

Figure 1. Behavioral motor changes which were scored through the experimental period. EAE-induced significant impairment. Roflumilast and FTY720 both prevented EAE-induced increase. Data expressed as mean±SD (n=6). ***p<0,001 versus control, ###p<0,001 versus MS.

2.2. EAE-induced increase in pro-inflammatory cytokines in peripheral blood prevented by roflumilast

Biochemical analysis was performed to assess pro-inflammatory cytokines in blood samples. EAE induced significant increase in the TNF-α (73,0±9,64 pg/mL), IL-1β (125,0±17,9 pg/mL), IL-6 (598,0±75,2 pg/mL) levels compared to control (43,7±3,98 pg/mL, 36,3±10,5 pg/mL, 123±10,6 pg/mL, respectively, p<0,001 for each cytokine, Figure 2). In contrast, roflumilast treatment significantly prevented EAE induced increase in TNF-α (56,6±3,74 pg/mL), IL-1β (85,5±5,25 pg/mL), IL-6 (425,0±29,5 pg/mL) levels compared to the MS group (p=0,027, p=0,004, p=0,001, respectively, Figure 2). Additionally, FTY720 significantly suppressed EAE-induced increase in TNF-α (57,3±2,86 pg/mL), IL-1β (56,7±5,53 pg/mL), IL-6 (312±4,65 pg/mL) compared to the control, as expected (p=0,035, p<0,001, p<0,001, respectively, Figure 2).

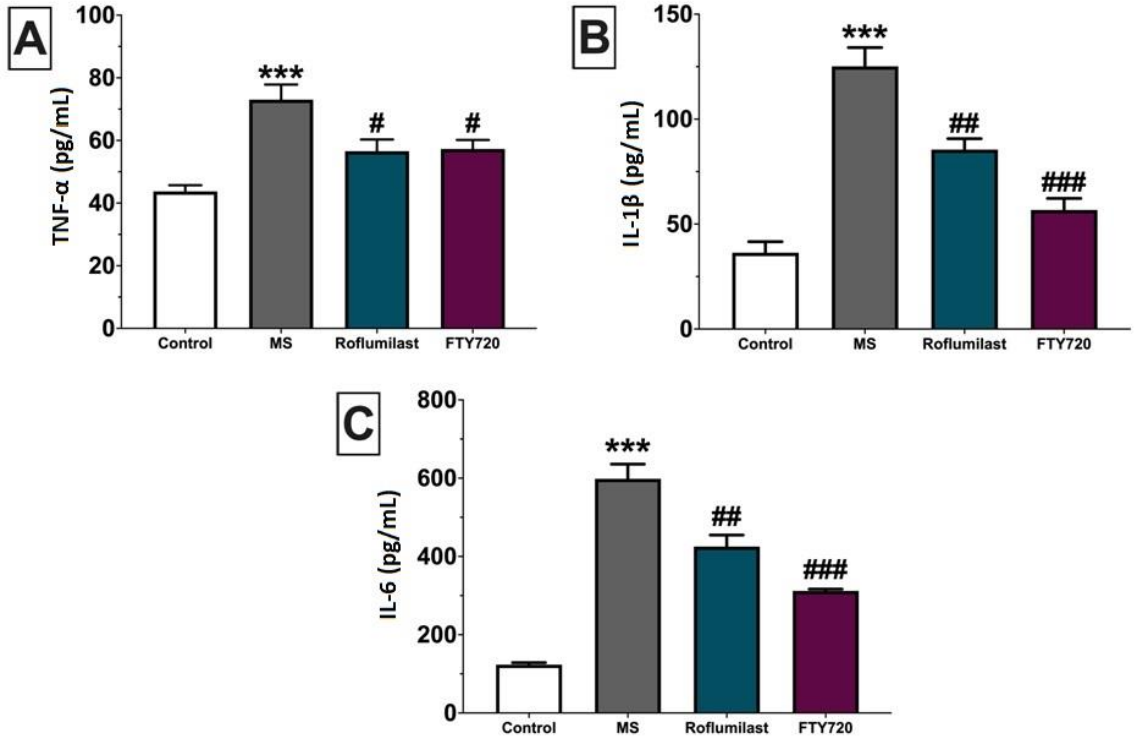

Figure 2. TNF-α, IL-1β, and IL-6 levels in the brain tissue. EAE caused a significant increase in the brain tissue levels of TNF-α (A), IL-1β (B), and IL-6 (C) levels. Roflumilast and FTY720 inhibited EAE-induced increase in TNF-α (A), IL-1β (B), and IL-6 (C) levels. Data expressed as mean±SD (n=6). ***p<0,001 versus control, ###p<0,001, ##p<0,01, #p<0,05 versus MS.

2.3. Roflumilast beneficially alters sema3A and sema4D levels

Sema3A and sema4D levels were determined in brain tissue samples. Our results demonstrated that EAE significantly increased sema3A (5,74±0,50 pg/mL) and sema4D (7,21±0,44 pg/mL) levels compared to the control (2,25±0,35 pg/mL and 3,64±0,20 pg/mL, respectively, p<0,001, Figure 3). However, roflumilast and FTY720 both significantly attenuated EAE induced increase in the sema3A (4,15±0,1 pg/mL for roflumilast, 3,18±0,20 pg/mL for FTY720) and sema4D levels (5,06±0,42 pg/mL for roflumilast and 5,27±0,150 pg/mL for FTY720) compared to the control (p=0,025 roflumilast, p<0,001 for FTY720 and p=0,003 for roflumilast and p=0,007 for FTY720, respectively, Figure 3).

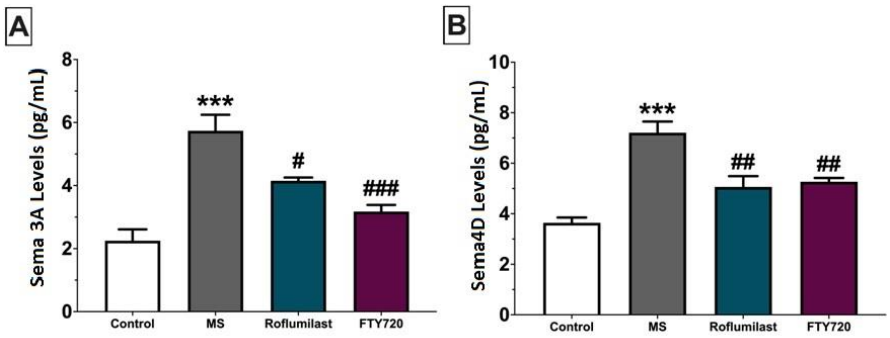

Figure 3. Semaphorin 3A and 4D levels in the brain tissue determined by ELISA. EAE significantly increased sema3A and sema4D levels. Roflumilast and FTY720 suppressed EAE-induced increase in sema3A and sema4D levels. Data expressed as mean±SD (n=6). ***p<0,001 versus control, ###p<0,001, ##p<0,01, #p<0,05 versus MS.

2.4. Roflumilast protected EAE induced decrease in myelin integrity

Histopathological analysis was performed to investigate changes in the brain myelin structures. EAE remarkably induced demyelination (Figure 4). However, attenuated demyelination and remyelination were observed in both roflumilast and FTY720 groups. Additionally, FTY720 showed stronger remyelination than the roflumilast group (Figure 4).

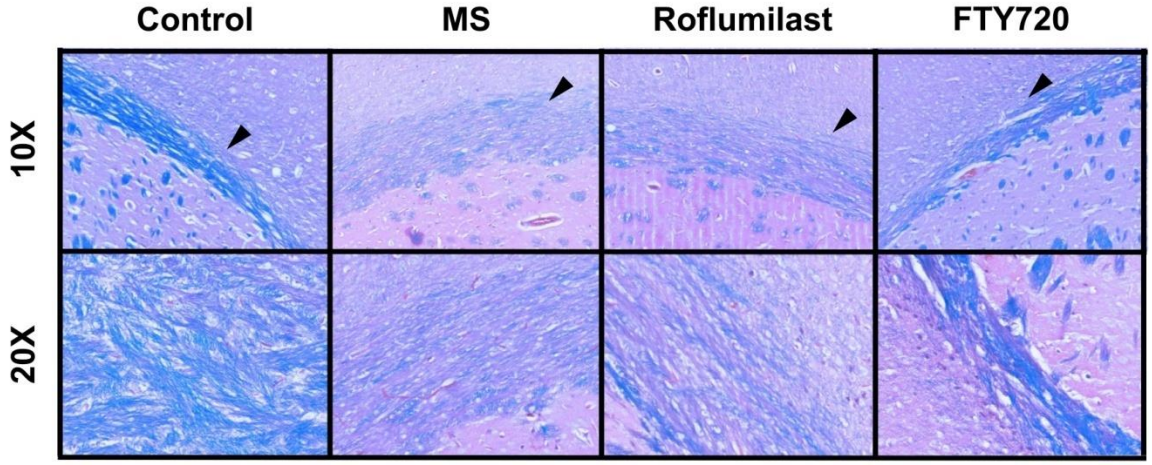

Figure 4. Luxol fast blue staining for all experimental groups. Myelinated areas stained blue (black arrowheads) and demyelinated areas-stained pinkish color with staining. Lateral parts of the corpus callosum showed strong myelination in the control group. EAE caused a significant decrease in myelinated areas. Roflumilast and FTY720 prevented EAE induced decrease. Images were taken with light microscopy.

3. DISCUSSION

Our results demonstrated that roflumilast exerts neuroprotective action in MOG-induced EAE in mice. Roflumilast prevented EAE-induced motor behavior impairment and myelin loss, and enhanced remyelination. Additionally, roflumilast suppressed EAE-induced increase in pro-inflammatory cytokines. Furthermore, roflumilast also prevented EAE-induced increase in sema3A and sema4D, which are crucial regulators of immune cell activation[18].

PDE inhibitors are a group of small compounds that bind to the phosphodiesterase enzyme and show different selectivity towards their substrates, thus increasing cellular levels of cAMP and cGMP, which are important modulators of gene expression, apoptosis, proliferation, and differentiation[19]. Previous studies have demonstrated the neuroprotective effects of vinpocetine, cilostazol, rolipram, VP3.15, sildenafil, and ibudilast in EAE-induced MS models[20]. After those inhibition effects, phosphodiesterase enzymes have been extensively investigated in the context of myelin integrity and oligodendrocyte progenitor cells which are central subjects in the MS pathologies[7]. Although the mechanisms by which cAMP modulates oligodendrocyte progenitor cell (OPC) turnover, or myelin repair are not precise, recent studies have demonstrated that PDE4 inhibitors are crucial regulators[21]. Torres et al. have demonstrated that vinpocetine modulates the expression of OPCs[22]. Another class of PDE inhibitors, sildenafil, a PDE-5 inhibitor, has been shown to inhibit inflammatory responses in the mouse model of EAE[23]. Using the remyelination model, sildenafil has been demonstrated to reduce myelin-specific damage probably regulated by metalloproteinases in cuprizone-induced MS[24]. Fujimoto et al. have demonstrated the neuroprotective action of ibudilast on myelin basic protein (MBP)-induced EAE[25]. Furthermore, rolipram and pentoxifylline have been suggested as effective in suppressing clinical signs of EAE[12, 26]. Another PDE inhibitor, cilostazol, a PDE-3 inhibitor, has been revealed to diminish inflammation symptoms in EAE models[27]. Additionally, Schepers et al. have indicated that PDE-4D specific inhibition boost remyelination in MS[28].

Although several studies have demonstrated the neuroprotective effects of roflumilast in neurological disorders, the effect of roflumilast in MS pathology is still missing. Several groups have demonstrated *in vitro* neuroprotective activity of roflumilast[29]. Roflumilast has been shown to decrease IL-1β, IL-6, and TNF-α and the number of apoptotic neurons in the brain after subarachnoid hemorrhage (SAH)[9]. Our results are in line with the previous studies that have demonstrated that roflumilast prevents EAE-induced increase in pro-inflammatory induced cytokines. In addition to the anti-inflammatory action, we also investigated the effect of roflumilast on sema3A and sema4D, which are considered essential regulators of immune cell activity[18]. Although semaphorins were initially identified as a guidance cone in neuronal development, accumulating evidence have demonstrated that sema3A and sema4D are involved in several stages of immune development in the central nervous system[30-32]. Additionally, increased sema3A and sema3F expression have been demonstrated in MS lesions, acting as a repellant or attractant for OPC migration[33]. Previous studies have also showed that sema3A has a crucial role in pathogenic response in EAE. Interestingly, sema3A is aberrantly expressed in the MS brain[34]. Moreover, sema4D is shown to be upregulated in the oligodendrocytes after spinal cord injury[35]. Sema4D is the first semaphorin that has been demonstrated with immune regulatory effects[36]. Although sema4D is a transmembrane protein, the extracellular region is proteocleaved from the surfaces by activated lymphocyte metalloproteinases which are thought to be related to the regulatory action of immune cells in the brain[37]. Elevated levels of sema4D have been demonstrated in the culture supernatant of activated lymphocytes and the sera of EAE mice or MS patients[31]. Therefore, we also investigated changes in the sema3A and sema4D levels in the brain tissue. Our results align with these studies that EAE has induced a significant increase in sema3A and sema4D. In contrast, roflumilast prevented that increase, possibly via PDE4 dependent mechanism.

Unfortunately, our study has some limitations. Firstly, confirmation of changes in the brain tissue sema3A and sema4D levels should be performed with double-staining methods to demonstrate sema3A and sema4D changes with microglial, astroglial, and oligodendroglial cells, which resident immune cells of the brain. Because sema3A and 4D regulate immune cell-neuronal interaction, it is essential to understand the role of this suggested change under microgliosis/astrogliosis. Secondly, possible interaction of PDE enzyme with semaphorins shown to be necessary, interaction with cAMP/cGMP levels with semaphorins should also be determined to confirm the regulatory role of PDE inhibitors. Lastly, as limitations of the protocol, it should be taken into account that the disease course is acute and monophasic. Moreover, the mode of immunization with the use of adjuvant and bacteria toxins both have additional influence on the immunological reaction.

4. CONCLUSION

In conclusion, our study demonstrated that roflumilast exerts a neuroprotective effect by preventing EAE-induced neuroinflammation and alleviating myelin loss, possibly affecting the semaphorin signaling pathway. However, further studies should be performed to better understand the effect of roflumilast on semaphorins in a cell type-specific manner.

5. MATERIALS AND METHODS

5.1. Animals

Animals were procured from Ondokuz Mayıs University vivarium. Experiments were conducted with twenty-four female mice (20-40 g). Ethical approval was obtained from Cumhuriyet University Ethical Committee for Experimental Animals (Ethics approval number: 2021/423). Animals were maintained under standard conditions (22±0.5°C, 55% humidity, 12/12 day-night) and fed ad libitum. All experiments were carefully conducted according to the Guide for the Care and Use of Laboratory Animals and ARRIVE guidelines[38]. All efforts were made to reduce animal suffering. For sample size calculation, a power analysis was performed with G*Power Software. The power analysis results indicated that a total sample of 24 animals, including six animals per group, would be needed to observe the effects with 95% power, which was determined by one-way ANOVA analysis between means with alpha at 0.05. Then, study groups were formed as the control group, non-treated MS group (MS), Roflumilast-treated MS group (Roflumilast), and FTY720-treated MS group (FTY720), and each group involved six mice.

5.2. Chemicals and experimental design

Roflumilast, dimethyl sulfoxide (DMSO), and FTY720 (Fingolimod hydrochloride) were purchased from Sigma Aldrich (Bethesda, USA). Saline was purchased from a local pharmacy. Roflumilast was dissolved in Saline: DMSO (98:2, v/v). FTY720 was used as positive control and dissolved in saline[9, 39]. Treatment doses of roflumilast and FTY720 were selected according to the previous studies. Animals were immunized with pre-mixed emulsion (Hooke Labs, EK-2110) containing myelin oligodendrocyte glycoprotein peptide$_{35-55}$ (MOG$_{35-55}$) and complete Freund's adjuvant (CFA) with heat-inactivated *Mycobacterium tuberculosis* (H37RA). Animals were injected subcutaneously at each of two sites over the upper and lower back. Control immunized and sex-matched littermate controls were injected with control emulsion without MOG$_{35-55}$[5]. Two hours and one day after immunization, animals were intraperitoneally injected with pertussis toxin. After immunization, mice were evaluated daily for changes in weight and clinical symptoms. Disease onset is typically correlated with a reduction of weight which might begin 1-2 days before EAE symptoms are visible. Eight days after immunization which animals started to show first signs, predominantly motoric symptoms in a caudal to rostral pattern, animals were treated with roflumilast (1 mg/kg, i.p.) and FTY720 (2,5 mg/kg, i.p.) for 28 days. All groups were monitored daily for clinical signs of disease and scored as follows; no deficit (0), partial tail paralysis (0.5), complete tail paralysis (1), hindlimb weakness (2), paralysis of one hind limb (2.5), paralysis of both hindlimbs (3), hindlimb paralysis and forelimb weakness (3.5), quadriplegia (4), death (5). Mice with low disease activity, weak symptoms or severe symptoms were taken out of the experiment.

5.3. Biochemical analysis

Twenty-four hours after the last drug treatments, animals have anesthetized with ketamine: xylazine (80:12.5 mg/kg, i.p.) and trans-cardially perfused with heparinized saline. Then, animals were decapitated, and brain tissues were carefully isolated. Three animals from each group were selected for histopathological analysis and fixed with a 4% paraformaldehyde solution. The remaining samples from each group were homogenized in Phosphate Buffered Saline (PBS) (pH 7.4). Total protein content in each sample was determined by Lowry's method. In samples, TNF-α, IL-1beta, IL-6, sema3A, and sema4D levels were quantified by commercially available ELISA kits strictly following manufacturer's instructions.

5.4. Histopathological analysis

Following 48 hours of immersion in 4% paraformaldehyde solution, samples were embedded into paraffin blocks. Then, samples were carefully cut into 5 µm thick sections to include corpus callosum

structures. After that, samples were deparaffinized in xylene and dehydrated alcohol series. Sections were stained with hematoxylin&eosin and luxol fast blue. At least ten sections were analyzed with light microscopy. The areas stained by luxol-fast blue were assessed qualitatively.

5.5. Statistical analysis

Experimental data were collected and stored in GraphPad Prism 9.0 software (California, USA). Data normalizations were determined by Shapiro-Wilk's normality test. Statistical analyses were carried out by one-way ANOVA and Kruskal-Wallis test. Differences between experimental groups were examined by Tukey's or Mann-Whitney U Post-hoc analysis. P values less than 0.05 were considered significant.

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