European Review for Medical and Pharmacological Sciences 2014; 18: 1770-1777

Effects of montelukast and methylprednisolone on experimental spinal cord injury in rats

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Abstract. – OBJECTIVE: The development of secondary brain injury after trauma is known to involve in many cellular mediators. The aim of the study was to evaluate and compare the effects of the use of both methylprednisolone and montelukast on serum and tissue concentrations of NO, malondialdehyde (MDA) levels, superoxide dismutase (SOD) activity, and tissue glutathione peroxidase (GSH-Px) activity in rats with spinal cord injury (SCI).

MATERIALS AND METHODS: SCI was induced in Wistar albino rats by dropping a 10 g rod from a 5.0 cm height at T9-10. The 28 rats were randomly divided into four equal groups: montelukast, methylprednisolone, non-treatment and sham groups. Rats were neurologically tested at 24 hours after trauma and spinal cord tissue levels of MDA, SOD, GSH-PX, CAT levels and blood CK, CK-BB, LDH levels were measured. In addition, histopathological changes were also examined.

RESULTS: There was a significant improvement in Tarlov scores in methylprednisolone and montelukast administered group compared to the trauma group ($p = 0.001$). When compared to trauma group, methylprednisolone and montelukast groups had significant differences in MDA ($p < 0.05$), SOD ($p < 0.001$), CK-BB ($p < 0.001$) and LDH ($p < 0.05$) levels. Histopathologically, no significant changes were observed.

CONCLUSIONS: The present study shows effects of montelukast with biochemical and histopathological parameters and compares its effects with those of methylprednisolone for the first time. Our research has shown that montelukast and methylprednisolone have a neuroprotective effect on spinal cord injury.

Key Words:
Medulla spinalis, Spinal trauma, Methylprednisolone, Montelukast, Superoxide dismutase, Malondialdehyde.

Introduction

Primary pathological mechanisms in traumatic spinal cord injuries involves disruption of tissue integrity, blood vessel and axon injuries, edema and cell membrane damage. Secondary mechanisms occur within hours and include biochemical and metabolic consequences of primary injury. Medical treatment of traumatic spinal cord injuries (SCI) aims at preventing the effects of those secondary mechanisms. Most discusses secondary injury mechanisms are microvascular lesions, intracellular calcium increase, endothelial cell injury, inflammation, free radical theory, endogen opioids and ischemia-reperfuison injury.

Free radicals are formed in mitochondria but their detrimental effects are scavenged by antioxidant systems. However, antioxidant mechanisms do not function properly after trauma resulting in increased free radicals production. These radicals interact with lipids, proteins and nucleic acids forming lipid peroxides. This results in more free radicals production leading to endothelial damage and blood brain barrier disruption regionally. Central nervous system (CNS) is sensitive to free radicals damage since SOD, catalase and GSH-Px activities are low in CNS. Another reason that makes CNS more vulnerable to free radicals injury is the excess amount of iron and ascorbic acid which catalyzes free radicals formation reactions. Ischemic perfusion defect is another important factor contributing secondary injury mechanisms. Reperfusion of ischemic tissue aggravates tissue injury by leukocyte activation and free radical production. Free radicals-mediated lipid peroxidation in spinal cord neurons, organelle membranes, vascular endothelial cell membrane and myelin sheets is the leading cause

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of reperfusion injury[1]. Leukotriens, metabolites of arachidonic acid, increase in ischemia, tumour, multiple sclerosis, encephalomyelitis and aging in addition to trauma[2]. Neutrophils release reactive oxygen products, proteases, elastase, myeloperoxidase and cytokines when migrate to ischemia region[3]. Cisteinyl leukotriens are potent inflammatory mediators causing ischemia[4].

Montelukast is a leukotrien receptor antagonist that specifically inhibites sodium cisteinyl leukotrien CysLT1 receptor. Cisteinyl leukotriens (LTC4, LTD4, LTE4) are strong inflammatory eicosanoids secreted by mast cells and eosinophils. It was definitely shown that montelukast decreases neutrophil recruitment and oxidative damage induced by ischemia-reperfusion[5]. Therefore, we aimed to study the effects of montelukast and steroid administration in experimental spinal cord injury in rats.

Materials and Methods

Experimental Procedure

Twenty-eight male Wistar albino rats weighing 320-370 g were used in this study. The Local Ethics Committee for Care and Use of Laboratory Animals of Mustafa Kemal University approved the animal protocols. The rats were housed and maintained with free access to food and water on a 12-hour light/dark cycle. Animals were anesthetized with a combination of xylazine hydrochloride (2-5 mg/kg) (Rompun; Bayer, Istanbul, Turkey) and ketamine hydrochloride (40-50 mg/kg) (Ketalar, Eczaciba i, Istanbul Turkey). After anesthesia and skin incision, a laminectomy was performed at the T8-T10 level, and the SC was exposed microsurgically. The injury was performed by dropping a 10-gram stainless steel rod from a height of 5.0 cm onto the dorsal surface of SC at T9 level.

Experimental Groups

The animals were randomly divided into four groups (n = 7 each):

Group I (C=Control): The rats in this group (n=7) were subjected only to laminectomy. No SCI or treatment was performed.

Group II (T=Trauma): SCI was performed without medication.

Group III (MP=Methylprednisolone): Laminectomy and trauma was performed. Immediately after spinal trauma 30 mg/kg methylprednisolone acetate (Prednol-L 40 mg ampoul, Mustafa Nevzat laç Grubu, stanbul) was given.

Group IV (MN=Montelukast): Following laminectomy and trauma, 5 mg/kg montelukast (Singulair 10 mg pill, Merck Sharp & Dohme, Whitehouse Station, NT, USA) was diluted with sterile saline and given intraperitoneally (ip).

Neurological assessments were performed on all animals 24 h after the experimental procedure. Hind limb motor function deficit was classified according to Tarlov scale: 0 = no voluntary hind limb function, spastic paraplegia, 1 = poor hind limb motor function, 2 = joint motion present with no ability to stand, 3 = stands and walks, and 4 = complete recovery[6]. The absence of muscle tonus and contractions was defined as paraplegia. Following the neurological examination, the animals were sacrificed using the anesthetics described above. For biochemical analysis, five mililiters of blood were drawn through a cardiac puncture into a test tube with no additives. Tissue samples of injured SC segments of 1 cm in length were rapidly obtained for pathological analysis and stored in 10% formaline.

Biochemical Analysis

Blood samples were centrifuged at 1,500 *g* for 15 min. Serum samples and SC tissues were stored in a freezer at –30°C until biochemical analysis.

SC tissue samples were homogenized (for 2 min at 5,000 rpm) in four volumes of ice-cold Tris-HCl buffer (50 mM, pH 7.4) with a homogenizer (Ultra Turrax IKA T10 Basic, Werke Staufen, Germany). Protein levels were measured in the homogenate while malondialdehyde (MDA) and nitric oxide (NO) levels were measured in both the homogenate and serum. Homogenate was centrifuged at 5,000 rpm for 60 min to remove debris. Supernatant fluids were collected and analyses of glutathione peroxidase (GSH-Px) activity and protein concentrations were performed. The serum GSH-Px activity was also measured. The supernatant solutions were mixed with an equal volume of an ethanol/chloroform mixture (5/3, volume per volume). After centrifugation at 5,000 rpm for 30 min, the clear upper layer (the ethanol phase) was collected and used for the analysis of both superoxide dismutase (SOD) activity and protein levels. The same procedure was performed for the analysis of SOD in serum. All preparation procedures were performed at 4°C.

The level of MDA was determined by a method based on its reaction with thiobarbituric acid at 90-100°C. The total (Cu-Zn and Mn) SOD (EC 1.15.1.1) activity was determined according to a method of Sun et al[7] modified by Durak et al[8]. The principle of the method is based on the inhibition of nitroblue tetrazolium reduction by the xanthine-xanthine oxidase system as a superoxide generator. Catalase (CAT, EC 1.11.1.6) activity was determined according to Aebi[9]. GSH-Px (EC 1.6.4.2) activity was measured by a method that was defined by Paglia and Valentine[10]. Protein assays were conducted using the method of Lowry et al[11].

Histopathological Analysis

For histopathological analysis, tissue samples were fixed in 10% neutral buffered formalin. After fixation spinal cord tissues were dehydrated with graded alcohol series and embedded in paraffin. After obtaining tissue blocks, 5 m thick transverse sections were cut and stained with hematoxylin and eosin. Sections were examined and photographed with Olympus DP20 camera attached-Olympus CX41 photomicroscope and scored based on a method by Malinovsky et al (Table I)[12].

Statistical Analysis

Data were analyzed using a commercially available statistics software package (SPSS for Windows v. 13.0, Chicago, IL, USA). For assessment of the neurological outcome scores and biochemical data, one way Anova test was used. Kruskal-Wallis test was used to evaluate histopathological results. $p < 0.05$ was accepted as significant.

Results

Motor functions of hind legs were evaluated 24 hours after surgery by using Tarlov Scoring system. $p < 0.05$ was considered significant.

Table I. Histopathological scoring.

0	No abnormal cell Hemorrhage
1	Glial cell reaction Presence of these changes in a few location Evident necrosis in gray matter,
2	Extensive hemorrhage and demyelinization, Fibrosis and existence of inflammatory cells

There was a significant improvement in Tarlov scores in methylprednisolone and montelukast administered group compared to the trauma group ($p = 0.001$); however, there was not a significant difference between montelukast group and methylprednisolone group.

The biochemical results are shown in Table II. MDA was significantly higher in the trauma group than in the control group ($p < 0.001$). It was significantly higher in methylprednisolone group than in trauma and control groups ($p < 0.05$). Montelukast administrated group also had significantly higher MDA than the trauma and control groups ($p < 0.05$).

SOD was significantly higher in the trauma group than in the control group ($p < 0.001$). It was also significantly higher in the methylprednisolone and montelukast groups than in the trauma and control groups ($p < 0.001$).

There was not a significant difference in GPX and CAT between the groups.

CK, CK-BB and LDH values are presented in Table III. There was not a significant difference in CK between the groups.

CK-BB values were significantly higher in the trauma group than in the control group ($p < 0.01$). CK-BB values were also significantly higher in the methylprednisolone group than in the control group ($p < 0.01$) and the trauma group ($p < 0.001$). They were significantly higher in the montelukast group than in the trauma and control groups ($p < 0.05$).

Table II. Statistical evaluation of tissue MDA, SOD, GSH-Px and CAT levels with one way Anova test.

	MDA (nmol/g wet tissue)	SOD (U/mg protein)	GPX (U/g protein)	CAT (k/g protein)
Control	25.2 ± 4.7	0.144 ± 0.048	4.24 ± 0.35	0.041 ± 0.011
Trauma (Sham group)	44.5 ± 8.5[a]	0.079 ± 0.032[a]	4.37 ± 0.63	0.042 ± 0.013
Methylprednisolone	19.1 ± 3.3[b,c]	0.105 ± 0.025[a,d]	4.11 ± 0.39	0.043 ± 0.014
Montelukast	17.7 ± 3.5[b,c]	0.109 ± 0.029[a,d]	4.15 ± 0.35	0.043 ± 0.01

[a]$p < 0.001$; compared with control; [b]$p < 0.05$; compared with control; [c]$p < 0.05$; compared with trauma, [d]$p < 0.001$; compared with trauma.

Table III. Statistical evaluation of tissue CK, CK-BB, LDH levels with one way Anova test.

	CK (U/L)	CK-BB (U/L)	LDH (U/L)
Control	1344 ± 379	2045 ± 201	1462 ± 677
Trauma (Sham group)	1471 ± 428	2755 ± 582[a]	1709 ± 550[a]
Methylprednisolone	1135 ± 455	1424 ± 349[a,d]	1176 ± 362[c]
Montelukast	1304 ± 399	1510 ± 378[b,d]	1155 ± 316[c]

[a]$p < 0.001$; compared with control; [b]$p < 0.05$; compared with control; [c]$p < 0.05$; compared with trauma, [d]$p < 0.001$; compared with trauma.

LDH values were significantly increased in the trauma group than in the control group ($p < 0.01$). They significantly decreased in the methylprednisolone group and the montelukast group compared to the control group ($p < 0.05$).

Histopathologically, control group showed normal morphology (Figure 1). In trauma group (Figure 2) hemorrhagic, spongiotic changes, congestion and glial cell proliferation were observed. Montelukast administration improved tissue morphology (Figure 3). In methylprednisolone administered group (Figure 4), tissue degeneration decreased.

However no significant differences were found between the groups in terms of histological features ($p = 0.277$) (Table IV).

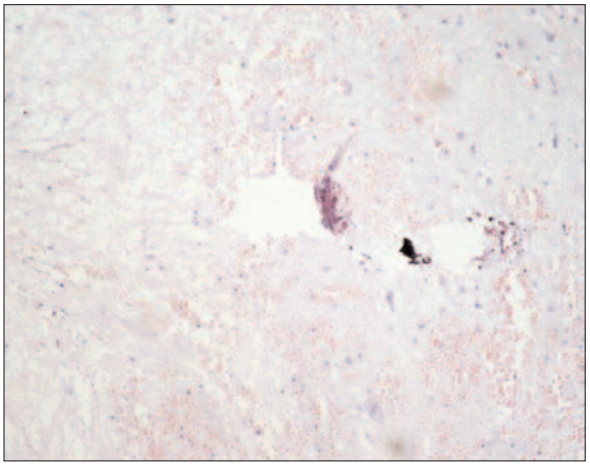

Figure 2. Hemorrhagic, spongiotic changes, congestion and glial cell proliferation in trauma group (HEX200).

Discussion

Primary injuries following spinal injuries include impairment of the tissue integrity, damage to blood vessels and axons, edema and distortion of the cell membrane. Secondary injuries developing in hours and days after primary injuries involve a number of pathophysiological changes like ischemia, ion infiltration, production of oxygen free radicals and lipid peroxidation[13].

Oxygen free radicals are normally produced in the mitochondria and their harmful effects are eliminated by antioxidant systems. However, an-

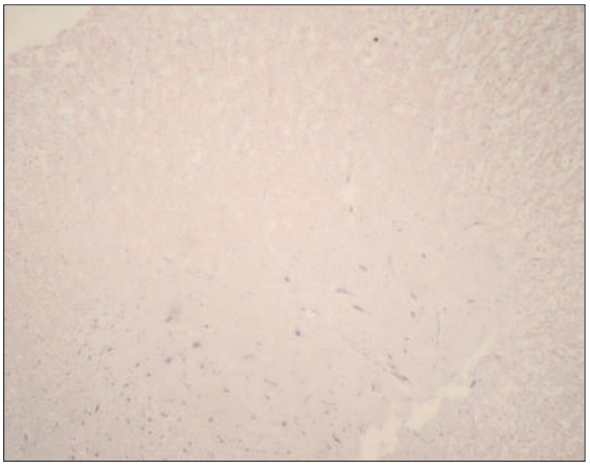

Figure 1. Normal histologic appearance in control group (HEX100).

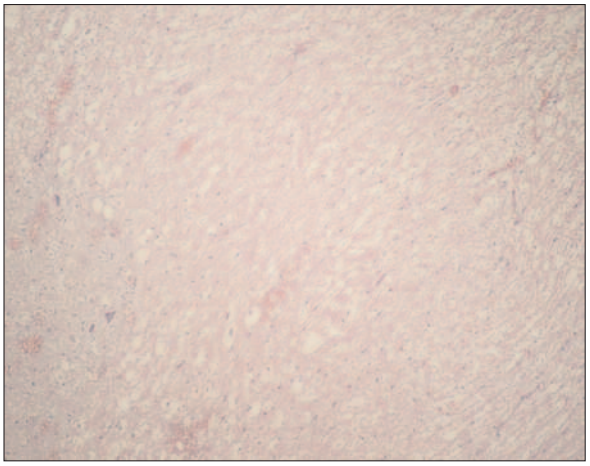

Figure 3. Improved morphology in Montelukast administrated group (HEX100).

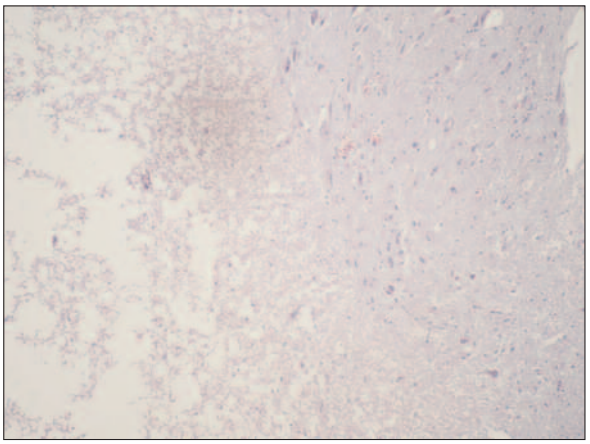

Figure 4. Decreased degeneration in Methylprednisolone administered group (HEX100).

tioxidant mechanisms rapidly decrease after traumas. Resultant oxygen free radicals react with lipids, proteins, nucleic acids and produce lipid peroxides and as a result, higher amounts of oxygen free radicals are produced.

In addition, depending on endothelial damage caused by oxygen free radicals, blood-spinal cord barrier is impaired. Then, harmful substances deposit in the damaged area. The central nervous system is prone to damage from oxygen free radicals because SOD, catalase and glutathione peroxidase activities are low at those regions. Higher amounts of unsaturated fatty acids which can easily react with oxygen free radicals and higher amounts of ascorbic acid and iron catalyzing production of oxygen free radicals with cholesterol make the central nervous system more sensitive to traumatic and ischemic injuries.

While the central nervous system has higher rates of antioxidant mechanisms such as ascorbate, glutathione and alpha-tocopherol, these mechanisms rapidly decrease after traumas and resultant oxygen free radicals react with lipids, proteins and nucleic acids, which lead to tissue repair[13]. Methylprednisolone is an antioxidant and anti-inflammatory agent whose action restricting secondary damage after injuries of the spinal cord has gained acceptance. Higher doses of methylprednisolone administered after injuries of the spinal cord inhibit posttraumatic lipid peroxidation and improve neurological functions[14].

It is believed that molecules including IL-1β, TNF-α, IL-6 and interstitial adhesion molecule-1 contribute to neuronal damage and cell death in the damaged spinal cord[15]. One of the most important factors which cause mechanisms of secondary injuries to develop is insufficient energy in the tissue. The main cause of insufficient energy is ischemia due to impaired perfusion. The central nervous system is one of the tissues most susceptible to ischemia due to its limited anaerobic metabolism and limited glycogen stores. Reperfusion in the ischemic organ increases leukocyte activation and oxygen free radical production, which in turn increase tissue damage. The most important cause of reperfusion injury is lipid peroxidation starting in cells of the spinal cord, membranes of plasma and organelles, vascular endothelial cell membranes and the myelin through increased oxygen free radicals. During lipid peroxidation developing like a chain reaction through oxygen free radicals, there is a rearrangement in the side chains of unsaturated fatty acids[1].

Cisteinil leukotrienes, a 5-lipoxygenase metabolite of arachidonic acid, are increased in various diseases of the central nervous system such as ischemia, tumors, multiple sclerosis, encephalomyelitis and aging in addition to traumas[2].

PMN leukocyte infiltration into the tissue, typical of an acute inflammation, shows that chemotactic mediators have a collective effect. When neutrophils migrate into the ischemic region, they release oxygen products, proteases, elastases, myeloperoxidase, cytokines and other cytokines involved in tissue damage[3]. Various chemokins and cisteinil LTs, a lipid mediator metabolite, are strong inflammatory mediators leading to ischemia[4].

Table IV. Statistical evaluation of histopathological scores.

	Prednisolone median (min-maks)	Montelukast Prednisolone median (min-maks)	Trauma Prednisolone median (min-maks)	Control Prednisolone median (min-maks)	*p*
Histopathological score	1 (0-2)	1 (0-1)	1 (0-1)	0 (0-1)	0.277

There is a biphasic leukocyte response which appears after traumas to the spinal cord. At the beginning, neutrophil infiltration is predominant and lytic enzymes released from leukocytes increase damage in the neuroglia and blood vessels. In the second phase, there is phagocytosis of the damaged tissue together with migration of macrophages. It is estimated that immunological activation initiates progressive tissue damage and/or inhibition of neural regeneration after damage to the central nervous system. It exacerbates demyelinization of the axons in the second phase of leukocyte infiltration, which especially peaks in the first 24 hours[16].

Creatinine kinase (CK) is a basic enzyme of the muscle metabolism and catalyzes ATP mediated creatinine phosphorylation reversibly. Creatinine kinase isoenzymes are dimeric molecules which appear as a result of combination of B and M chains. Naturally, CK has three isoenzymes; i.e. CK-MM, CK-MB and CK-BB. The brain and the kidneys contain original BB. Although skeletal muscles predominantly include MM, there is MB in 1-2% of these muscles. The heart muscle contains both MB and MM. CK-MB is responsible for 20% of the total myocardial CK activity[17]. Plasma CK-MB activity also increases in damage to the skeletal muscle. However, it is not as distinctive as in myocardial damage. It peaks earlier in MI without Q wave than in MU with Q wave[18]. CK experiments in cerebrospinal fluid have become important in recent years in terms of clinical diagnosis[19]. Studies on enzymes in the cerebrospinal fluid started in 1950-1960s for diagnosis of neurological damage[20]. CK-BB is sensitive and specific to detect damage to neurons[21]. Creatinine kinase plays an important part in energy metabolism and fulfilling needs of the heart, skeleton and brain for high amounts of energy and energy in emergency conditions because all neurons and astrocytes contain CK-BB which is released into blood and cerebrospinal fluid in traumas and cerebral diseases[19].

Nishibe[22] measured SOD, GPX and CAT activities after ischemia of the spinal cord was created in dogs and could not observe any changes in total SOD, Mn·SOD and GPX in ischemic spinal cords compared to normal spinal cords, but found a decrease in CAT activity.

Erol et al[23] detected an increase in tissue MDA levels and a decrease in SOD and GPX enzyme activities in experimental injuries of the spinal cord. Consistent with their findings, the results of the current investigation showed that MDA increased in the trauma group compared to the control group and significantly decreased in montelukast and methylprednisolone groups compared to the trauma group. In addition, SOD levels also decreased in the trauma group as compared with the control group. However, there was not a significant change in GPX and CAT levels. In Yurdal et al's report[24] on the protective effect of tadalafil on experimental injuries of the spinal cord, MDA levels increased following traumas, which is compatible with the findings of the present study. Kanter et al[25] reported an increase in MDA and a decrease in SOD and GPX enzyme activities in spinal cord tissues after damage to the spinal cord. They noted that methylprednisolone reduced MDA levels, which is congruent with the present study.

Zhang et al[26] in their study on rats showed that sisteinil LTs increased following a cerebral trauma and peaked on days 4 and 7, which were attributed to edema and cellular inflammatory response.

Taoka et al[27] demonstrated that methylprednisolone contributes to recovery of the spinal cord, which was ascribed with prevention of lipid peroxidation and increased vascular permeability in damaged tissue.

Mitsuhashi et al[28] created compression injuries in spinal cords of pigs and rats and detected a considerable increase in leukotriene C4 one hour after injuries. Akpek et al[29] observed an increase in leukotriene C4 half an hour after injuries of the spinal cord.

Genoveseve et al[16] in their study on effects of zileuton and montelukast on injuries of the spinal cord reported that these two agents reduced tissue damage, cellular infiltration into the damaged area and programmed cell death and improved motor functions. Consistent with their findings, we observed a considerable improvement in the rats administered montelukast and methylprednisolone compared to the trauma group.

It has been shown in the literature that CK-MB concentrations increase in nueuronal damage and these increases have been associated with prognosis[30]. As a result of cellular membrane damage, CK-BB travels to the extracellular space and then to blood circulation and cerebrospinal fluid[29]. CK-MB concentrations in cerebrospinal fluid measured early in an injury are indicative of neuron damage and associated with neurological scores[31]. Studies on immunohistochemical activities of CK-BB have shown that the highest amounts of CK-BB are found in astrocytes and

its lesser amounts are found in dendrites and axons[32]. This study showed that CK-BB and LDH levels were increased in the rats exposed to trauma, but that methylprednisolone and montelukast significantly reduced CK-BB and LDH levels compared to the rats exposed to trauma only. The difference was not significant between the rats administered methylprednisolone and those given montelukast though. CK levels did not differ significantly between the groups.

There have been many studies showing histopathological changes in the spinal cord in trauma models[33]. Also, this research revealed hemorrhage, spongiotic changes, congestion and glial cell proliferation in the trauma group on histopathological examinations.

Yilmaz et al[34] investigated preventive and therapeutic effects of klopidogrelin in a trauma model in rats and found hydropic degeneration and congestion in neurons following trauma, which is consistent with the results of the present study. However, unlike this study, Yilmaz et al observed liquefactive necrosis creating occasional cavitation and signs of infarct.

Alaygut et al[35] investigated effects of ketorolac tromethamine on damage to spinal cells in a medulla spinalis trauma model and showed hemorrhagic areas and glial cell reaction areas, which is compatible with the results of the present study. Unlike the present study, they found occasional necrosis in grey matter and increased vascularization areas.

Ekmekçi et al[36] investigated morphological and ultrastructural effects of ambroxol hydrochloride in a spinal cord trauma model. Consistent with our findings they observed hemorrhage. They additionally reported liquefactive necrosis creating occasional cavitation, lymphocytes, plasma cells, plenty of histiocytes and polymorphonuclear leukocytes in cavitation and an appearance suggesting infarct, axonal swelling and rare axonal spheroids in the surrounding tissue.

Conclusions

The present study is the first to show effects of montelukast based on biochemical and histopathological parameters and to compare its effects with those of methylprednisolone. Our research has shown that montelukast and methylprednisolone have a neuroprotective effect on spinal cord injury.

Montelukast administration following the spinal cord injury prevents and slows neuronal degeneration, which can be attributed to its anti-inflammatory effect. Further studies are needed to evaluate effects of montelukast at different times and to elucidate the mechanism of its action in injuries in the spinal cord. It can also be useful to compare effects of montelukast with those of other cisteinil LT2 antagonists.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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