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Systemic Administration of Atorvastatin Improves Locomotor Functions and Hyperacute-Acute Response After Experimental Spinal Cord Injury: An Ultrastructural and Biochemical Analysis

Atorvastatinin Sistemik Uygulaması Deneysel Omurilik Hasarında Lokomotor Fonksiyonları ve Akut-Hiperakut Cevabı İyileştirir: Ultrastrüktürel ve Biyokimyasal Analiz

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ABSTRACT

AIM: Spinal cord injury (SCI) is characterized by posttraumatic inflammatory cascades including excitotoxicity, oxidative stress, and apoptosis. Agents against neuroinflammation are the current scope of studies on experimental SCI with promising results.

MATERIAL and METHODS: Thirty-two male Sprague-Dawley rats weighing 250-320 gram were used. They were randomized and divided into four groups with eight animals in each as sham, control, SCI+PEG (polyethylene glycol) and SCI+atorvastatin group. Rats were anesthetized with intraperitoneal ketamine (80 mg/kg) and xylazine (10 mg/kg) and SCI was induced by the weight-drop model. A single level laminectomy was performed at T10 and the spinal column was immobilized with a stereotactic device. Rats in the treatment group received ip atorvastatin at 0.2 mg/kg. Neurological impairments were examined through Modified Tarlov's and inclined angle scores. The SCI section was resected for electron-microscopic analysis, IL-1β and IL-6 level. All data were analyzed using one-way ANOVA and Dunnet T3 test.

RESULTS: Atorvastatin improved locomotor recovery after rat SCI. The results were further confirmed with a statistically significant decrease of IL-1β, IL-6 and lipid peroxide levels. This finding revealed the anti-inflammatory and beneficial effect of atorvastatin on rat SCI.

CONCLUSION: The present study focused on both B and T cell mediated immunity and confirmed the beneficial effect of atorvastatin with decreased expressions of IL-1β and IL-6.

KEYWORDS: Atorvastatin, Spinal cord injury, Inflammation, Rat, IL-1β, IL-6, Lipid peroxidation

ÖZ

AMAÇ: Spinal kord hasarı (SKH) eksitotoksisite, oksidatif stres ve apoptoz gibi posttravmatik enflamatuar önemli yolakları içerir. Nöroenflamasyona yönelik ajanlar umut verici sonuçlarıyla düşünüldüğünde deneysel SKH üzerine yapılan güncel deneylerin konusudur.

YÖNTEM ve GEREÇLER: Çalışmada 250-320 gram arasında olan 32 Sprague-Dawley sıçan kullanılmıştır. Randomize olarak 4 gruba ayrılan sıçanlar her grupta 8 sıçan olacak şekilde plasebo, kontrol, SKH+PEG (polietilen glikol) ve SKH+atorvastatin grubu olarak sınıflandırılmıştır. Sıçanlar intraperitoneal (ip) ketamin (80mg/kg) ve ksilazin (10mg/kg) kombine anestezisi yapıldıktan sonra SKH ağırlık düşürme modeli ile uygulanmıştır. Stereotaksik çerçevede immobilize edildikten sonra sıçanlara torakal 10 düzeyinde tek seviye laminektomi yapılmıştır. Tedavi grubunda yer alan sıçanlara ip atorvastatin 0,2 mg/kg dozunda enjekte edilmiştir. Nörolojik bozulma için Modifiye Tarlov ve eğik açı skorlamaları ile takip edilen sıçanlar sonrasında sakrifiye edilmiştir. Sonrasında SKH doku bölgesinden çıkarılan örnekler elektron mikroskopik analiz, IL-1β ve IL-6 çalışmaları için ayrılmıştır. Tüm sonuçlar tek yönlü ANOVA ve Dunnet T3 testi kullanılarak analiz edilmiştir.

BULGULAR: Atorvastatin sıçanda SKH'ında lokomotor düzelmeyi tetiklemiştir. Ayrıca bu sonuçlar IL-1β, IL-6 ve lipid peroksid ölçümleri ile desteklenmiştir. Bu, atorvastatinin sıçan SKH'da antienflamatuvar ve yararlı etkisini göstermektedir.

SONUÇ: Bu çalışma, B ve T hücreleri üzerinden işleyen bağışıklık sistemine odaklanmış ve atorvastatinin SKH üzerinde azalmış IL-1β ve IL-6 ekspresyonu ile yararlı olduğunu göstermiştir.

ANAHTAR SÖZCÜKLER: Atorvastatin, Spinal kord hasarı, Enflamasyon, Sıçan, IL-1β, IL-6, Lipid peroksidasyon

INTRODUCTION

Spinal cord injury (SCI) is an irreversible event with devastating and sometimes fatal results. The injury is a result of two-step cascade including primary mechanical insult followed by a secondary form of injury. Secondary injury includes a series of inflammatory chain of events upon which current researches are focused on. A typical posttraumatic inflammatory cascade includes several stages like excitotoxicity, oxidative stress, and apoptosis of the potentially viable cells (15). Breakdown of blood-spinal cord barrier is usually raised as the main histopathological mechanism among the cascade of events in secondary SCI (5, 14). Recent studies of acute inflammation have focused on iNOS-trigger over macrophages or astrocytes. The study presented by Pannu et al pointed to the attenuation of acute inflammatory response and a considerable neuroprotective effect by atorvastatin (24). Consequently, high levels of NO cause inhibition of mitochondrial functions and excitotoxic neuronal death (3, 18, 29). It could be predicted that agents causing inhibition of neuroinflammation may also be regarded as promising therapeutics against the mechanisms in hyperacute and acute phases of SCI.

Statins are a group of lipid lowering drugs with well known pleiotropic features like anti-inflammatory, antioxidant and neuroprotective effects (19, 31). The safety and efficacy of statins are well known and these drugs are well tolerated with minimal side effects like myopathy and seldom cases of neuropathy. Statin-induced myopathy is reported between 1 and 5% (2, 17, 26, 35). They were also found to improve the neurological functional recovery in stroke and traumatic brain injury in rats by amplification of synaptogenesis, angiogenesis and neurogenesis (7, 21). The main mechanism was claimed to be decreased level of inflammatory mediators and improved endothelial cell function or blood flow (1). Despite raised events by these HMG-CoA reductase inhibitors, a clear pathophysiological mechanism has not been established so far. In the present study, we investigated the influence of atorvastatin in hyperacute and acute phases of rat spinal cord injury through lipid peroxides, $IL1_{\beta}$, IL-6 measurements and ultrastructural level.

MATERIAL and METHODS

Animal Care and Spinal Cord Injury Induction

Animal care, surgery, and experimental protocol were all performed in accordance with the "Guide for the Care and Use of Laboratory Animals" of the US Department of Health and Humans Services (National Institutes of Health, Bethesda, MD, USA) and ethical approval of Animal Care Unit of Ankara Education and Research Hospital Ethics Committee (March 5, 2008-admission number 0269).

Thirty-two male Sprague-Dawley rats, weighing 250-320 gram were used in this study. The animals were randomized and assigned to one of the following four groups composed of eight animals each: sham (group 1), control (group 2, SCI only), vehicle (group 3, SCI+PEG (polyethylene glycol)) and treatment groups (group 4, SCI + Atorvastatin).

SCI was induced with the standardized weight-drop method, previously described by Pannu et al (12). Briefly, rats were deeply anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg) administered intraperitoneally (ip). A single level laminectomy was performed at T10 level under the operating microscope and the spinal column was immobilized under the stereotactic device. An impounder (0.3 cm in diameter) was placed gently over the dura and SCI was induced by dropping a 4 g of sterile weight from a height of 10 cm directly onto the impounder (40 g cm force). Rats' systolic blood pressure and heart rates were monitored throughout the procedure. They were returned to their cages after SCI and body temperatures were maintained at 37°C throughout the next 24 hours.

Drug Administration

Rats in the treatment group received ip injection of atorvastatin (Dr. Reddy's Laboratories Ltd, India) at a dose of 0.2 mg/kg just after SCI. In addition, rats in the vehicle group were subjected to equal volumes of the solvent of atorvastatin, polyethylene glycol (PEG) (Merck, Germany) intraperitoneally.

Motor Function Assessment

All animals were examined for neurological impairment within 24 hours after the procedure by two evaluators blinded to the study groups. Open-field behavior was assessed and scored with a Modified Tarlov's Grading Scale (1: No movement, 2: minimal hindlimb movement but unable to stand, 3: able to stand but unable to walk, 4: able to walk with mild spasticity or incoordination of the hindlimb, 5: normal motor function) as proposed by Cheng et al (8). The average score of the evaluators were accepted as the final score of the regarding rat.

The locomotor function was further evaluated by the inclined plane technique proposed by Rivlin and Tator (27). The test consisted of measuring the maximum angle at which an animal can support its weight measured in a scale from 0 to 90 degrees over the inclined plane. The animals were placed transversely on the inclined plane and the highest angle a rat can stand over for 5 seconds was regarded as the "capacity angle" for that rat.

Tissue Harvesting

After the assessment of motor functions 24 hours after SCI, the rats were immediately sacrificed using high dose anesthesia and intracardiac perfusion was performed with 0.9 % NaCl to eliminate blood remnants from the spinal cord. The section of spinal cord subjected to injury was dissected with microscissors and a medium length of half cm spinal cord tissue was resected just at the level of SCI. Tissue specimens were fixated with 2.5% gluteraldehyde for electron microscopic analysis and the remaining tissues were kept in liquid nitrogen (-196°C) for biochemical analysis.

Measurement of Lipid Peroxidation

The exposed spinal cord samples were removed, weighed

and immediately frozen at -196°C for malonyl dialdehyde (MDA) assay. The levels of lipid peroxides in spinal cord tissue samples were measured with the method proposed by Mihara and Uchiyama (22). The procedure for lipid peroxide determination in spinal cord tissue samples was as follows: Weighed tissue in 100 mM KPO_4 (pH 7.0) buffer was homogenized in an ice bath and 0.25 mL of homogenates (10% concentration) were mixed with 1.5 mL of % 1 H_3PO_4 and 0.5 mL 0.8% TBA. They were kept in boiling water for 60 minutes. After cooling, 2 mL n-butanol was added to each tube. The samples from each tube was mechanically mixed at 2000 rpm for 30 seconds and centrifuged further at 2500 rpm for another 30 minutes (Sorvall RC-5B superspeed device). The color change in n-butanol phase was read at 532 nm (Shimadzu UV 120-2 spectrophotometer). The level of lipid peroxides was calculated on a graph with a standart method of 1,1,3,3-tetramethoxypropane. Lipid peroxide levels were expressed as nano moles per gram of tissue.

Determination of IL-1β and IL-6 Levels

Spinal cords were weighed and homogenized (10 % w/v) on ice in 25 mM Tris pH 7 tamponade containing 150 mM KCl (at a concentration of 10% weight/volume). The homogenization was done with Ultrathorax homogenizator in 1 +1 minutes at 3000 rpm. A remnant from the homogenate was reserved for MDA measurements. The rest of the homogenate was centrifuged at 12000 x g at + 4°C for 25 minutes, the supernatant was removed and remaining serum was kept for ELISA and Bradford protein assays.

The levels of IL-1_β and IL-6 were measured with a commercially available AssayMax Human Interleukin-1_β and IL-6 kits in accordance with the recommendations of the manufacturer. In brief, 50µl samples were placed over a microplate to be incubated for 2 hours at room temperature. They were rinsed five times with 200µl of buffered solution and 50 µL of biotinylated IL-1_β or IL-6 was added over the microplate. After another 2 hours of incubation, the plates were washed again with 200µl of buffered solution. After elimination of liquid from the microplate, 50 µl of Streptavidin-Peroxidase conjugate was added for another incubation lasting for 30 minutes. After procedures for dehydration, further incubation was done for 8 minutes with chromogen tetramethylbenzidine. 50µl of inhibitory solution was added lastly to prevent color reaction and the results were quantified spectrophotometrically with Spectramax M2 microplate reader (Molecular Devices Inc.) at 540 nm.

Ultrastructural Examination with Electron Microscope

The spinal cord samples were placed in 2.5% glutaraldehyde for fixation lasting 24 hours. The specimens were then rinsed with Sorenson's phosphate buffer and postfixed with 1% osmium tetroxide. After dehydration in a graded series of alcohol solution, the samples were treated with propylene oxide and embedded in epoxy resin to be kept in incubator at 56^0 C for 48 hours. Ultrathin sections were cut with ultramicrotome at a thickness of 60 nm and stained with uranyl acetate and lead citrate. The sections were evaluated under transmission electron microscope (TEM) (JEM 1200EX; Jeol, Tokyo, Japan) and microphotographs were taken at 10.000X magnification level.

Statistical Analysis

Data were analyzed using the SPSS statistical program (SPSS v.16, Chicago, IL, USA). Statistical analyses among groups were performed using the one way ANOVA (Analysis of Variance) test. Post-hoc analysis among the groups was done by using Dunnet T3 test. Results were reported as means ± standard deviation (SD). A probability value of less than 0.05 was considered to be statistically significant.

RESULTS

Locomotor Findings

Before SCI induction, all rats demonstrated normal motor functions with a Tarlov score of 5. Immediately after SCI, the Tarlov score dropped to 1 in all rats indicating a complete loss of motor function. Twenty four hours after SCI, rats that received AT demonstrated a significant recovery of neurological function ($p<0.05$) compared with the sham and sham+PEG groups (Figure 3). Similar findings were obtained from the evaluation of the inclined plane results. Rats that received AT had a statistically higher capacity on the inclined angle board when compared to sham (p: 0.01) and vector (p: 0.004) groups. The results are summarized in Table I and Figure 1.

Ultrastructural Findings

Group 1: The TEM findings of the sham group did not reveal any abnormal ultrastructural changes in the gray and white matters of the spinal cord. The neurons and the mitochondria (m) were normal in appearance and the intracellular organelles, nuclei (N), membranes and perineuronal tissues did not show any histopathological changes. However, mild separations were observed in myelin sheaths of some large sized myelinated axons. This may be due to a delay in tissue fixation procedure (Figure 2A).

Group 2: The ultrastructural examination of the SCI only group revealed severe histopathological changes identified both in the gray and white matter of spinal cord. Intracellular vacuoles (v) in the cytoplasm with swollen mitochondria (m) were marked and the number of cellular organelles was

Table I: Tarlov and Inclined Angle Scores for Each Experimental Group

Groups	Tarlov Score	Inclined Angle Score
Sham	4.88 ± 0.12	67.50 ± 2.84
SCI	1.25 ± 0.16	33.75 ± 2.46
SCI+PEG	1.37 ± 0.26	35.62 ± 2.75
SCI+Atorvastatin	3.50 ± 0.27	52.50 ± 2.84

SCI: *Spinal cord injury,* ***PEG:*** *polyethylene glycol.*

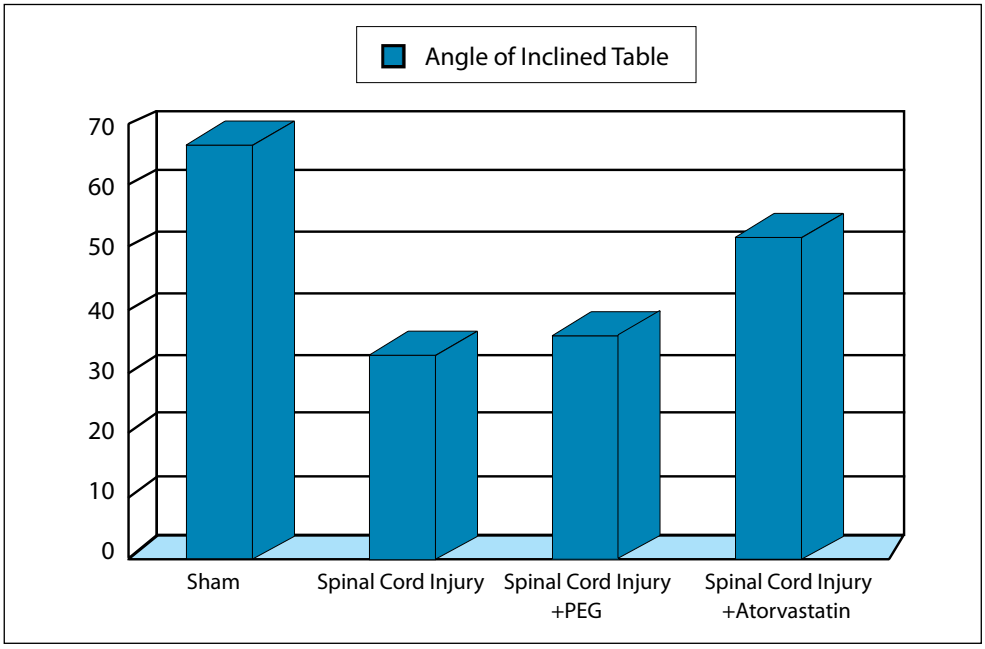
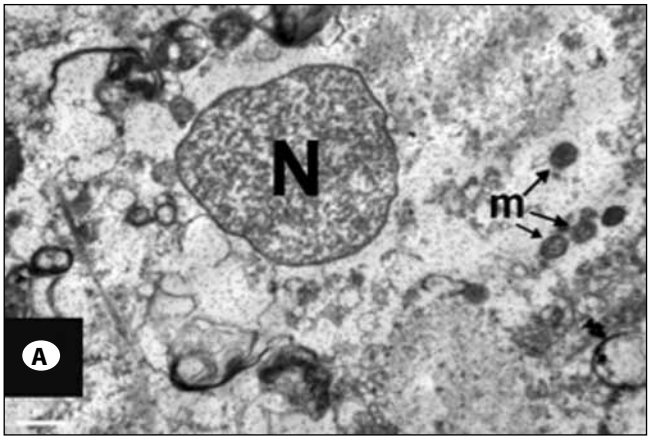
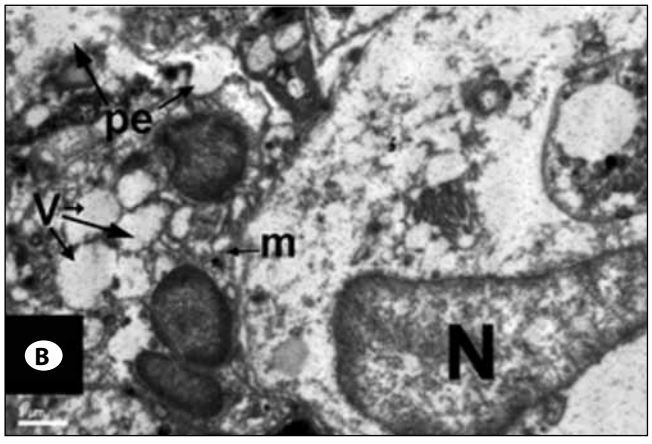


Figure 1: Comparison of angle measurements between four experimental groups over inclined table. Measurements in group 4 (SCI+Atorvastatin group) were similar to sham group.
SCI: Spinal cord injury,
PEG: polyethylene glycol.





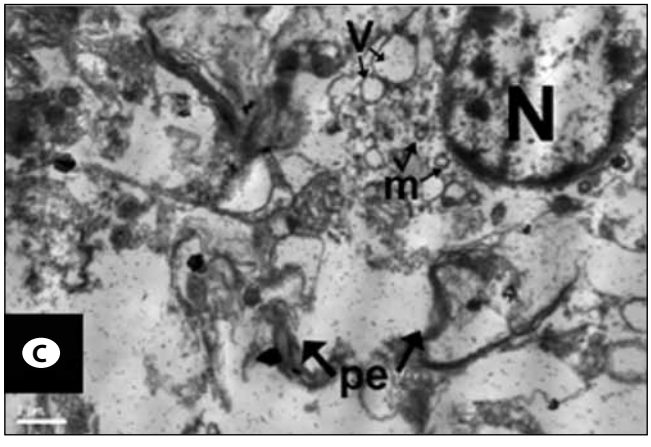


Figure 2: A) TEM findings of the sham group did not reveal any abnormal ultrastructural changes in the gray and white matters of the spinal cord. The neurons and the mitochondriae (m) were normal in appearance and the intracellular organelles, nuclei (N), membranes and perineuronal tissues did not show any histopathological changes. However, mild separations were observed in myelin sheaths of some large sized myelinated axons that may be due to delay in tissue fixation procedure. **B)** Ultrastructural examination of SCI only group revealed severe changes identified both in the gray and white matter. Intracellular vacuoles (v) in the cytoplasm with swollen mitochondria (m) were marked and the number of cellular organelles was markedly decreased in neuronal tissues. The nuclei (N) were normal. **C)** TEM examination of the atorvastatin (AT) group showed the least amount of changes in ultrastructural level. Intracytoplasmic vacuoles (v) were noticed and smaller than group 2 and 3. Additionally, swollen mitochondria (m) and perineural edema (pe) was prominent. The amount of intracellular organelles and the nuclei (N) of the neurons were ultrastructurally normal.

markedly decreased in neuronal tissues. The nuclei (N) were normal (Figure 2B).

Group 3: The ultrastructural appearance of trauma+PEG (vehicle) group did not show any marked difference from the SCI only group. Similar findings of severe ultrastructural pathological changes were typical in gray and white matter as well as intracytoplasmic vacuoles. Additionally, separation and interruption of myelin configuration in myelinated axons were prominent.

Group 4: The TEM examination of the atorvastatin (AT) group showed the least amount of changes in ultrastructural level. Intracytoplasmic vacuoles (v) were also noted in this group however the vacuoles in the present group were smaller than the ones observed in groups 2 and 3. Additionally, swollen mitochondria (m) and perineural edema (pe) was prominent. The amount of intracellular organelles and the nuclei (N) of the neurons were ultrastructurally normal (Figure 2C).

Lipid Peroxidation Levels

No statistical significance was noted between the sham and the AT treated groups ($p > 0.05$) (Figure 3). However early treatment with AT after trauma significantly decreased the lipid peroxidation compared with SCI only ($p=0.028$) and the vehicle groups ($p=0.003$) 24 hours postinjury. The results are summarized in Table II.

IL-1$_\beta$ and IL-6 Levels

The levels of IL-1$_\beta$ and IL-6 are determined at 24 after trauma and, statistically significant results were observed among the groups for both cytokines ($p<0.05$). The IL-1$_\beta$ and IL-6 levels of group IV (trauma+AT) were lower than group II (trauma) and group III (trauma+vehicle) and this was statistically significant ($p<0.05$). However no significant difference was observed among the IL-1$_\beta$ and IL-6 levels of group I (sham) and group IV (trauma+AT) ($p=0.983$ and $p=0.414$ respectively). The IL-1$_\beta$ and IL-6 values of the groups are summarised in Table II respectively.

DISCUSSION

Atorvastatin is a hypocholesterolemic agent with a proven effect on inflammation as well as antioxidant property (19, 31). Anti-inflammatory potential of this drug may aid in neurological impairment mechanisms of which are probably

Table II: Measurements of IL-1β, IL-6 and Lipid Peroxidation at Each Experimental Group. IL-1β And IL-6 were Designated in PG/ML and Lipid Peroxidation in Nmol/G Tissue Studied

Groups	IL - 1β	IL - 6	Lipid Peroxidation
Sham	12.25±1.867	0.018±0.007	51.98 ± 2.39
SCI	17.07±2.600	0.042±0.009	71.24 ± 3.53
SCI+PEG	28.34±2.429	0.034±0.003	79.14 ± 8.01
SCI+Atorvastatin	12.34±3.626	0.022±0.006	57.97 ± 5.42

SCI: *Spinal cord injury,* ***PEG:*** *polyethylene glycol.*

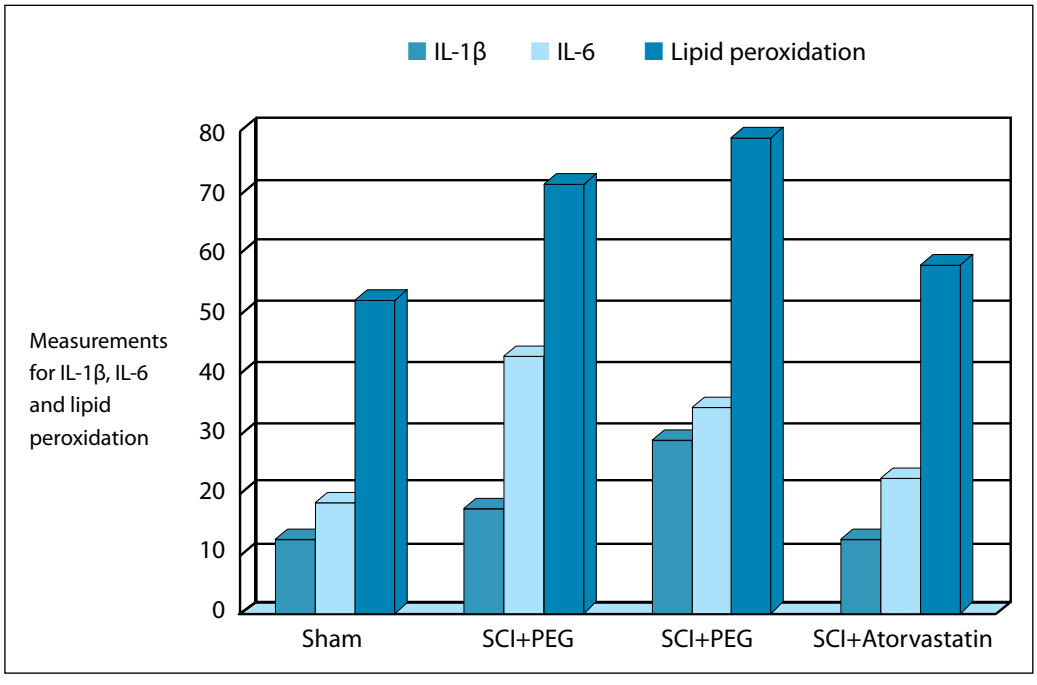


Figure 3: Comparison of measurements between four experimental groups in terms of IL-1β and IL-6 and lipid peroxidation. Measurements in group 4 (SCI+Atorvastatin group) were similar to the measurements of sham group. This finding showed the antiinflammatory and beneficial effect of atorvastatin on SCI. **SCI:** Spinal cord injury, **PEG:** polyethylene glycol.

due to unpredicted side effects of inflammatory cascades. In the present study, we demonstrated that systemic administration of atorvastatin reduced the expression of inflammatory cytokines like IL-1_β and IL-6 as well as lipid peroxides after experimental SCI in rats. The study also showed its beneficial effect in terms of Tarlov and inclined angle scores.

Pioneer studies on the inflammatory reactions after SCI mainly focuses on development of the disrupted blood spinal cord barrier or improvement of blood supply (23). The most interesting studies over the last two decades include mechanisms directed against endogeneous mechanisms: inhibition of chondroitin sulfate proteglycans, inhibition of Rho activation or inhibition of neurite outgrowth through nogo (16). Previous studies regarding anti-inflammatory neuroprotective measures include several chemotherapeutic agents like erythropoietin, tetracyclins, or methotrexate (4, 28). Statins are well known group of drugs that prevents de novo cholesterol synthesis and their neuroprotective role was recognized in previous studies against several neurological diseases like Alzheimer's disease and multiple sclerosis (9, 30). The studies reported by Pannu et al in 2005 and 2007 pointed to the neuroprotective effects of atrovastatin on SCI (24, 25). The aim of these studies was quite similar to the study we report here however the studies lack measurements of IL-6 and lipid peroxides. In the study reported by Han et al, it was found that atorvastatin provided a proper environment necessary for neural healing, in particular the drug induced the expression of BDNF (Brain derived neurotrophic factor) and GDNF (Glial cell line-derived neurotrophic factor) (13). Here we detected that atorvastatin improved the locomotor recovery after rat SCI. The results were further confirmed with the IL-1_β, IL-6 and lipid peroxide measurements. This finding revealed the anti-inflammatory and beneficial effect of the drug on rat SCI

The primary goal of the present study is to examine the inhibitory effects of atorvastatin on inflammatory cascades involved in secondary SCI (20). Proinflammatory cytokines like TNF-α and IL-1_β are found to increase within hours after SCI (6, 32). Although their pivotal role in inflammatory processes regarding SCI was demonstrated previously, the success of these agents towards SCI was not clearly shown (10, 24, 33). Immune cells and glial cells migrate to the injury site early after SCI. Myelin base protein reactive T cells are proven to produce neural inflammation and neurological findings however the role of B cells at the level of injury needs to be investigated further (11). Macrophages and microglia do participate in tissue destruction and edema at the level of SCI in that these agents contributed to the production of cytokines like TNF, IL-1, IL-6 and IL-10 (34). The present study focused on both B and T cell mediated immunity and confirmed the beneficial effect of atorvastatin with decreased expressions of IL-1_β and IL-6. However efficacy of atorvastatin on a long-term basis is not beyond the scope of this study and may be regarded as the primary limitation of this agent.

Electron microscopic observations identified severe changes after SCI both in gray and white matter. Intracellular vacuoles with swollen mitochondria and decreased number of cellular organelles were typical however examination of the AT group showed relatively mild changes in ultrastructural level. This finding showed the beneficial effect of the drug on ultrastructural level further confirming the anti-inflammatory potential of SCI. When combined together with the inhibitory role on cytokines, atorvastatin may be regarded as a future promising drug for SCI.

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