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Original Investigation

Effect of Asiatic Acid on the Treatment of Spinal Cord Injury: An Experimental Study in Rats

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ABSTRACT

AIM: Spinal cord injury (SCI) is a devastating condition of the central nervous system. There is no proven therapeutic agent for the treatment of this complex disorder. Asiatic acid (AA) has been used as an anti-inflammatory and anti-oxidant agent in Eastern countries for many years. The aim of this study was to investigate the effectiveness of AA on the treatment of traumatic SCI in rats.

MATERIAL and METHODS: Thirty-two adult male Sprague-Dawley rats were divided into 4 groups as laminectomy, laminectomy+trauma, vehicle, and AA treatment groups. SCI was created by the modified Allen's weight-drop technique. After the injury, the levels of pro-inflammatory cytokines (IL-6, IL1-β, TNF-α) and lipid peroxidation products (MDA) were measured. Tarlov functional recovery scores were also determined for each rat.

The One-way ANOVA test was used for the analysis of difference between 4 experimental groups and the groups were compared individually by Tukey-LSD post hoc analysis test (p=0.001).

RESULTS: AA administration just after SCI attenuated the levels of lipid peroxidation products (MDA) and pro-inflammatory cytokines (TNF-α, IL1β). It also increased the Tarlov functional recovery scores of the rats.

CONCLUSION: AA administration could attenuate a number of deleterious reactions after traumatic SCI. Further studies are needed to elucidate the pathways of neuroprotective effects of AA after spinal trauma.

KEYWORDS: Asiatic acid, Spinal cord injury, Trauma

INTRODUCTION

Spinal cord injury (SCI) is a devastating condition of the central nervous system. It often results in permanent functional and neurological deficits. The prevalence of SCI varies between 236 and 1280/million (6). Methylprednisolone has been the main therapeutic agent for SCI with some controversies (2,3). Many neuroprotective agents have been investigated for the prevention of secondary injury cascades of SCI. However, no significant beneficial treatment modality has been found due to complex pathophysiology of SCI.

The aggressive pathophysiology of SCI contributes to the extension of this devastating condition. A mechanical trauma to the spinal cord triggers an immediate cascade of cellular and biochemical events. Secondary injury processes that take place after the neurological injury cause cell death by glutamate-induced exitotoxicity, inflammation, overproduction of free oxygen radicals, mitochondrial dysfunction, energy failure and apoptosis.

Asiatic acid (AA) is a form of triterpenoid, which is isolated from Centella asiatica. It has been used in far Eastern countries

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and in India as an anti-inflammatory and anti-oxidant agent for many years. It has been shown in-vivo and in-vitro that AA has neuroprotective effects. Some reports stated that AA is a promising agent in the treatment of stroke. AA exerts neuroprotection by maintaining the stability of the blood brain barrier and by protecting the mitochondrial functions (10,12,13,20).

In the present study, we hypothesized that AA administration after SCI could demonstrate neuroprotective effects and attenuate secondary injury processes. We investigated these effects in rats, by screening the levels of malonyldialdehyde (MDA) and pro-inflammatory cytokines (interleukin 6 (IL-6), interleukin1 beta (IL1-β), tumor necrosis factor alpha (TNF-α)) and by the assessment of Tarlov functional recovery scores.

MATERIAL and METHODS

This study was approved by Animal Research and Ethical Committee (50/03.09.2012 approval date) of our institution. Thirty-two adult male Sprague-Dawley rats (weighing between 250-300 g) were used for this study and randomly divided into 4 groups. They were kept in a temperature controlled room (23ºC) with a 12-hour light/dark cycle (lights on at 7:00 a.m.) with free access to water and food. Experiments were performed between 8:00 and 12:00 a.m.

Surgical Procedure

All surgical techniques were performed under aseptic circumstances. Animals were anesthetized with an intraperitoneal (ip) injection mixture of Ketamin (Ketalar, Pfizer İstanbul, Turkey) 90 mg/kg and Xylazine (Rompun, Bayer, İstanbul, Turkey) 10 mg/kg. Arterial saturation, cardiac rate, rectal temperatures were monitorized. The body temperature of the animals were fixed at a range of 37ºC±5ºC by using a heating pad.

After shaving and cleansing of the skin, animals were kept in a stereotaxic apparatus and a posterior midline incision was performed to the mid-thoracic region. The underlying muscles were retracted and total laminectomy was performed under microscopic illumination.

After the surgical procedure, the paraspinal fascia, muscle and skin were closed with 4-0 nylon suture (Ethicon, ETHILON™ nylon sutures, Reverse Cutting Size 4-0, 18" Black Monofilament Needle FS-2, 3/8 circle). The animals were placed in a heating chamber and body temperature was maintained at approximately 37° C after which they were returned to the vivarium when they were fully awake.

Traumatic SCI Model

The modified Allen's weight-drop technique was used for SCI in this study (1). The force applied for the trauma was 40 g/cm. The extent of the trauma is measured by multiplying weight and height, expressed as g/cm. Briefly, a 5-mm-diameter cylindrical glass tube was positioned at 90º to the surface of the exposed duramater, and a 4g cylindrical constant weight was dropped from a 10-cm height through the tube onto the spinal cord.

Experimental Groups

The animals were divided into four groups as shown in Table I.

1) Laminectomy (L) group: Sham-operated animals (negative control group) (n=8)

   In this group, a mid-thoracic skin incision was made, the para-vertebral muscles were dissected, and the laminae were exposed. Laminectomies were carried out at the T6-8 level as noted above without any experimental procedure. The muscles and the skin were closed with 4-0 nylon sutures.

2) Laminectomy+Trauma (LT) group: Trauma-only animals (positive control) (n=8)

   In this group, laminectomies were performed and SCI was induced as described above. The site of injury was marked with a prolene suture at the neighbouring tissue.

3) Poly Ethylene Glycol (PEG) group: Vehicle group animals (n=8)

   In this group, immediately after laminectomies and spinal trauma, rats received 0.5 ml Poly Ethylene Glycol (PEG) intraperitoneally (ip) as a carrier for AA.

4) Experimental group animals (n=8)

   In this group, rats received ip (75 mg/kg) AA immediately after trauma.

Tissue Preparation

All animals were sacrificed 24 hours after the experiment. The rats were deeply anesthetized by an overdose of sodium thiopental (100 mg/kg) intraperitoneally. The thorax of the rats

Table I: Experimental Groups' Details

Group	Procedure
Group 1: Sham-operated (negative control) (L)	Only laminectomy
Group 2: Trauma (positive control) (LT)	Laminectomy and trauma
Group 3: Vehicle (PEG)	Laminectomy and trauma, 0.5 ml, ip, PEG administered immediately after trauma
Group 4: Experimental group (AA)	Laminectomy and trauma, 75 mgr/kg, ip, AA administered immediately after trauma

was dissected for cannulating the left ventricle to perform intracardiac perfusion with 100-300 ml phosphate buffered saline (pH 7.4) and 100 ml paraformaldehyde (4% in 0.1 mol phosphate buffered saline) (16). All hemorrhagic components of spinal cord tissue were removed with NaCl 0.9% solution. Spinal cord samples were cut under the microscope using microsurgical instruments from the cranial and caudal points of the injury level (approximately 1 cm of spinal cord) and kept in – 70 °C until the analyses.

Lipid Peroxidation / MDA-TBARS Assay

The OxiSelect ™ TBARS Assay Kit was used for the measurement of lipid peroxidation products. This is a malondialdehyde (MDA)-dependent assay that uses MDA and thiobarbituric acid conjugate bonds. The results were obtained by using the Molecular Devices Spectramax M2 Microplate Reader.

Determination of Tumor Necrosis Factor (TNF)-α, Interleukin-1 β, Interleukin -6

The Invitrogen Rat ELISA kit was used in the measurement of TNF-α, IL-1β and IL -6. The Molecular Devices Spectramax M2 Microplate reader was used at the wavelength of 450nm. The results were evaluated by regression analysis.

Functional Recovery Assessment

Each rat's motor functions were evaluated by an independent observer who was unaware of the treatment modality of the animals using a 5-point scale, which was first described by Tarlov (Table II) (18).

Table II: Tarlov Model of Functional Recovery Assessment

Motor scores	
1	no voluntary hind limb movement;
2	minimal voluntary hind limb movements but unable to stand
3	able to stand but unable to walk
4	able to walk with mild spasticity or incoordination of the hind limbs
5	able to walk normally

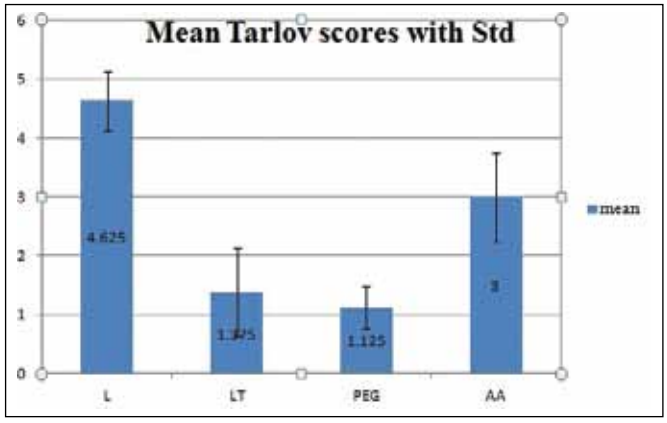

Figure 1: Mean Modified Tarlov Scores for each group with standard deviation.

RESULTS

One-way ANOVA test was used for the analysis of difference between the 4 groups and the groups were compared individually using the Tukey-LSD post hoc analysis test.

Tarlov Functional Recovery Assessment Scores

On the first day after trauma, the mean Tarlov motor scores and standard deviation (SD) in groups 1, 2, 3 and 4 were 4.625±0.51, 1.375±0.74, 1.125±0.35 and 3±0.755 respectively. The one-way ANOVA test was used for the analysis of mean Tarlov motor scores between groups and a significant difference was found (p=0.001). The 75 mgr/kg AA administration significantly increased the Tarlov motor score of AA group (3.0) with respect to LT group (1.375) (p=0.001). The Tukey-LSD post hoc test was used for analysing the PEG (vehicle) effect. There was no significant difference between the LT and PEG groups (p=0.848). The overall results are summarized in Table III, Figure 1.

Lipid Peroxidation

The one-way ANOVA test was used for the analysis of lipid peroxidation product levels between groups, which was expressed in terms of nanomole per gram tissue, and a significant difference has been found between the groups (p=0.001). Administration of AA significantly reduced the level of lipid peroxidation products (2.622) with respect to the LT group, in which only trauma was performed after laminectomy (5.525) (p=0.001). The Tukey-LSD post hoc test was used for analysing the PEG (vehicle) effect. There was a significant difference within the LT and PEG groups (p=0.002). The overall results are summarized in Table III, Figure 2.

TNF-α

TNF-α levels were expressed in terms of pictograms per milliliter tissue. The ANOVA variance test was used for detecting any significant difference between groups (p=0.001). The AA administration had significantly reduced the TNF-α levels of the AA group (32.036) with respect to the LT group where only trauma was performed after laminectomy (55.182) (p=0.026). The Tukey-LSD post hoc test was used for analysing the PEG (vehicle) effect. There was no significant difference within the LT and PEG groups (p=1.00). The overall results are summarized in Table III, Figure 3.

Interleukin-6

IL-6 levels are expressed in terms of pictograms per milliliter tissue. The ANOVA variance test was used for detecting any significant difference between groups (p=0.002). The AA administration reduced the IL-6 levels of the AA group (155.0) with respect to the LT group where no treatment modality was used (215.5), but this difference was not statistically significant (p=0.219). The Tukey-LSD post hoc test was used for analysing the PEG (vehicle) effect. There was no significant difference within the LT and PEG groups (p=0.981). The overall results are summarized in Table III, Figure 4.

Interleukin 1β

IL-1β levels are expressed in terms of pictograms per milliliter tissue. The ANOVA variance test was used for detecting significant difference between groups (p=0.001). The AA administration significantly reduced the IL-1β levels of the AA group (86.68) with respect to the LT group where no treatment modality was used (123.02) (p=0.016).

Table III: Mean Values of MDA. TNF-α. IL-1β and IL-6 for Each Group and Their P Values in Comparison

		n	Mean	Std*	p	p L-LT	p LT-AA	p LT-PEG
MDA	L	8	0.837	0.087	0.001	0.001	0.001	0.002
	LT	8	5.525	1.176				
	PEG	8	3.676	1.291				
	AA	8	2.622	0.485				
TNF-α	L	8	9.802	2.1014	0.001	0.001	0.026	1.00
	LT	8	55.182	24.343				
	PEG	8	54.842	13.323				
	AA	8	32.036	12.805				
IL1-β	L	8	62.242	14.048	0.001	0.001	0.016	0.848
	LT	8	123.02	23.044				
	PEG	8	113.871	28.821				
	AA	8	86.685	21.757				
IL-6	L	8	95.125	27.11	0.002	0.003	0.219	0.981
	LT	8	215.5	66.73				
	PEG	8	203.87	75.09				
	AA	8	155.0	63.95				
Tarlov	L	8	4.625	0.517	0.001	0.001	0.001	0.848
	LT	8	1.375	0.744				
	PEG	8	1.125	0.353				
	AA	8	3.00	0.755				

***Std*:** *Standard deviation.*

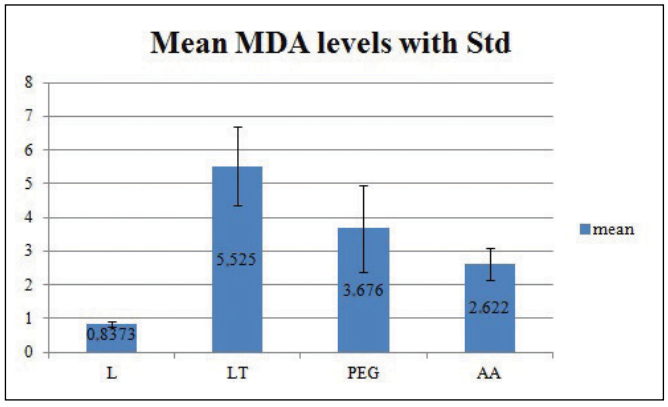

Figure 2: Mean MDA levels for each group with standard deviation.

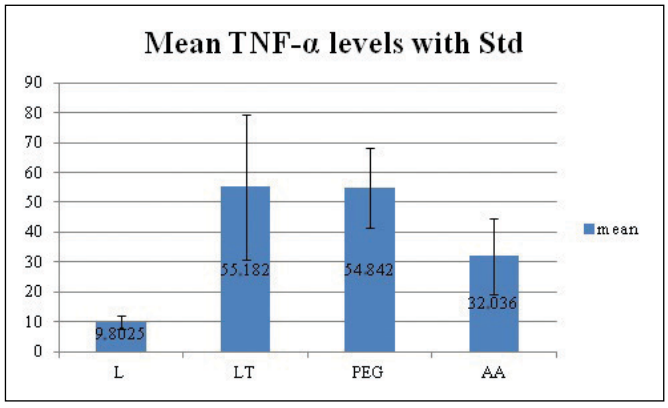

Figure 3: Mean TNF-α levels for each group with standard deviation.

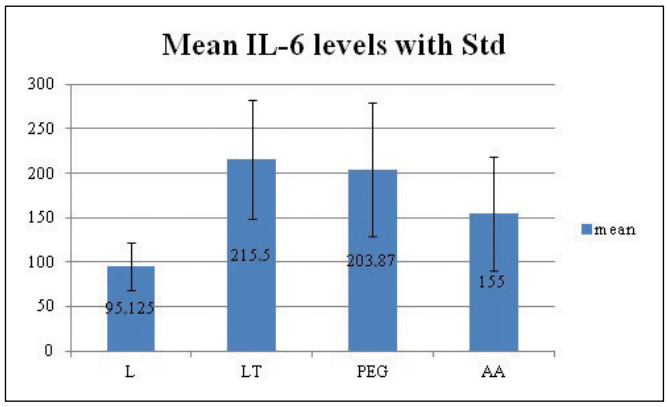

Figure 4: Mean IL-6 levels for each group with standard deviation.

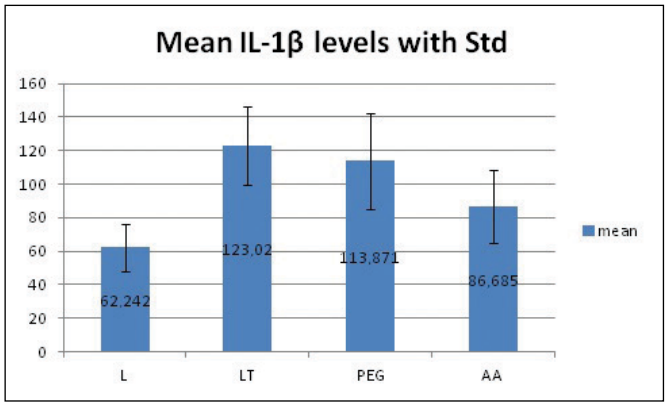

Figure 5: Mean IL-1β levels for each group with standard deviation.

The Tukey-LSD post hoc test was used for analysing the PEG (vehicle) effect. There was no significant difference within the LT and PEG groups (p=0.848). The overall results are summarized in Table III, Figure 5.

Briefly, AA administration just after the SCI, attenuated the level of lipid peroxidation products (MDA levels) and pro-inflammatory cytokines (TNF-α, IL-1β). It also increased the Tarlov functional scores.

DISCUSSION

Secondary injury cascades, which are triggered just after the trauma, have been the targets of investigations on neurotrauma in recent years. These cascades have different sub-reactions of injury such as exitotoxicity, inflammation, reactive oxygen radical injury, mitochondrial damage, apoptosis and cell death. Many therapeutic agents are currently being evaluated in the light of scientific research, but no specific agent has been demonstrated to be effective on the treatment of SCI yet.

Centella asiatica (Umbelliferae), which belongs to the Apiaceae family, is an important medical plant due to its favorable characteristics on human health. In Eastern medicine, it is widely used on the treatment of asthma, skin disorders, rheumatism, syphilis, inflammations, and wounds. Centella asiatica have a number of derivatives. These are flavonoids, triterpenes and polyacetylenes. Some of the triterpenoids compounds are madecassoside, madecassic acid, asiaticoside, and AA (7,14).

AA is a pentacyclic triterpen derivative and has neuroprotective properties. Various biological effects of AA such as anti-inflammatory and anti-oxidant effects have been reported. It also has protective effects against neurotoxixity. These effects of AA have been shown in stroke and some degenerative neurodisorders such as Parkinsonism and Alzheimer Disease (7,10,12).

During secondary injury, mitochondrial dysfunction and microglia activation play a crucial role on cellular viability by causing energy failure, uninhibiting cellular calcium influx and increasing neuro-inflammation. Neurotrauma causes an excitotoxic state, which disturbs the mitochondrial membrane potential, resulting in over-secretion of reactive oxygen species and pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, superoxide anions and hydrogen peroxide (3,9,15). Over-production of reactive oxygen species after traumatic neuronal injury has been well documented (19). The nervous system is prone to oxidative damage because of the presence of more polyunsaturated lipids in the structure of cellular membranes and insufficient anti-oxidant systems (8). Over-production of reactive oxygen species after SCI causes lipid peroxidation and cell death. MDA is a cytotoxic aldehyde. The level of MDA production after oxidative damage is widely used to determine lipid peroxidation (4). If the redox balance between reactive oxygen species and cellular free radical scavengers is disturbed, there will be an energy failure in the cell that causes apoptosis and cell death in neurological disorders such as stroke, Parkinson disease, Alzheimer disease and neurotrauma. Our results showed that spinal cord trauma caused a significant increase in the MDA level during the secondary injury with respect to the only laminectomy group as documented in the literature (3,15,16). AA administration just after the SCI reduced the MDA level compared with the laminectomy trauma group. The results between these groups were statistically significant (p=0.001).

Blood-nervous system barrier permeability increases and neuro-inflammation is triggered during the secondary injury (3,5). Microglias, neutrophils and macrophages play a role in neuro-inflammation. In the central nervous system, cytokines are vital in maintaining the neuronal homeostasis, but they will become detrimental for cell viability after trauma. TNF-α and IL-1β are oversecreted and cause inflammation just after the neurotrauma. This increases vascular permeability, over-expresses reactive oxygen species (ROS) and finally causes cell death (3,17). On the other hand, when IL-6 is over-secreted, this reaction causes leukocyte infiltration and impairs axonal growth and recovery during the secondary injury after SCI (11). In our SCI model, we investigated the pro-inflammatory cytokine levels 24 hours after trauma. Our results showed that the trauma caused a significant increase in the levels of TNF-α and IL-1β with respect to the only laminectomy group. AA administration just after the trauma reduced TNF-α, IL-

1β levels compared with the laminectomy trauma group. The difference between these groups was statistically significant ($p<0.05$). The IL-6 level was also increased 24 hours after the trauma in the laminectomy trauma group when compared with the only laminectomy group ($p=0.003$). AA reduced the IL-6 level, but this was not significant when compared with the laminectomy trauma group ($p=0.219$).

We compared all groups with modified Tarlov motor functional recovery assessment scores. We showed that there was a statistically significant difference between the laminectomy trauma group and the AA group ($p=0.001$). This means that AA administration has a positive effect on the recovery of motor functions.

Finally, our results clearly showed that trauma increased the levels of MDA, TNF-α, IL-1β and IL-6 in the spinal cord of rats at 24 hours after injury and these results are similar to those in the literature (3,5,14,16,17). AA administration just after the trauma inhibited post-traumatic inflammation and over-production of ROS and reduced MDA and pro-inflammatory cytokine (TNF-α, IL-1β) levels. On the other hand, the IL-6 level was repressed but this was not significant ($p>0.05$).

CONCLUSION

Lipid peroxidation and inflammatory process take part in neuronal damage after SCI and AA administration just after the SCI reduces the level of lipid peroxidation products and some pro-inflammatory cytokines and improves functional motor recovery. In the light of these findings, we conclude that AA administration is encouraging because it could attenuate a number of deleterious reactions that are triggered after traumatic SCI. However, more investigation is needed on the neuroprotective effects of AA after trauma.

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