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Neuroprotective effects of allopurinol on spinal cord injury in rats

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Abstract
Lesion in spinal cord causes a cascade of events such as the apoptosis of neurons and eventually, neurological dysfunction. Neurologic damage developing after acute spinal cord injury is also related with necrosis and free radical formation. Allopurinol a xanthine oxidase inhibitor, was shown to have protective effects in several studies. B-cell lymphoma 2 (Bcl-2) family proteins regulate apoptosis. Apoptosis causes the death of neuronal cells, particularly neurons and oligodendrocytes in the spinal cord after lesion. Glial fibrillary acidic protein (GFAP) takes part in astrocyte and neuronal interconnection and synaptic transmission. Male Sprague Dawley rats (n:30) were divided as control, trauma, and trauma + allopurinol (i.p., 50 mg/kg of body weight) groups. Animals were applied a surgical procedure causing spinal cord injury and treated for 7 days then sacrificed under anesthesia. The spinal cords were dissected, measurements of myeloperoxidase, malondialdehyde and glutathione were performed, remaining parts were fixed in 10% formaldehyde solution for histological and immunohistochemical evaluations. Biochemical results exhibited an increase in myeloperoxidase levels in trauma group but a decrease in the allopurinol treatment group
similar to malondialdehyde levels. Degenerative changes in multipolar and bipolar neurons together with apoptotic changes in some glial cells were observed in the trauma group whereas, mild degenerative changes were observed after allopurinol treatment. In the trauma group, negative GFAP expression in multipolar ve bipolar neuronal processes with a reduction in glial processes around blood vessels and positive GFAP expression were observed but, a regular and parallel positive GFAP expression of glial processes around blood vessels in the allopurinol treated group was apparent. Trauma group depicted a positive Bcl-2 expression in glial cells and in motor and bipolar neurons. On the contrary, negative Bcl-2 expression was noticed in the trauma + allopurinol group. This study is of importance to understand the effects of allopurinol in preventing degenerative changes in nerve and glial cells related to spinal cord injuries.

**Key words: allopurinol, GFAP, Bcl-2, spinal cord injury, rat**

**INTRODUCTION**

Motor, sensory and autonomic dysfunctions are consequences of spinal cord injury (SCI) occurring on the cervical spine causing problems in cervical, thoracic, lumbar, and sacral levels under injury (1). SCI are accompanied by apoptosis of neurons and the initiation of glial cells, that result in neurological dysfunction. Lesion on spinal cord triggers infiltration of inflammatory cells and irreversible loss of neurons (2,3). Neurologic damage occurring after acute spinal cord injury involves necrosis after primary mechanic injury and secondary injury then, apoptosis takes place (4) So, previous studies reveal strong evidence demonstrating the presence of apoptosis after SCI.

Pathogenesis of spinal cord neuronal lesion after injury is also related with oxygen-derived free radical formation leading to edema and inflammatory response. After spinal cord lesion disabilities and deficits such as; loss of motor, sensory and autonomic sensory system capabilities, muscle spasms, chronic pain, urinary tract diseases may appear (5). A study on imatinib was demonstrated that functional outcomes like locomotor capacity and bladder function and also histological parameters (tissue sparing, axonal sparing, astrogliosis, inflammation and BSCB permeability) were improved following spinal cord weight-drop lesion (6).
Allopurinol, [4-hydroxy-pyrazole(3,4-d) pyrimidine], a xanthine oxidase inhibitor, was shown to have protective effects during ischemia (7) by blocking purine breakdown. It easily crosses the blood-brain barrier and throughout cerebral ischemia takes part in the protection of cells (8). Allopurinol is a free radical scavenger that is used to treat several diseases such as; vascular injury, inflammation (9), ischemic heart disease (10,11), heart failure (12), and myocardial protection during cardiac or aortic surgery or post-ischemic reperfusion (13). It is also used to treat gout (14), hyperuricemia (15), and inflammatory arthritis with relatively minor adverse effects for a long time. Allopurinol prohibits the process of axonal damage and demyelination induced by oxidative stress and proinflammatory cytokines (16,17). Palmer et al., reported that allopurinol administered 15 min after cerebral hypoxia-ischemia in neonatal rats reduced brain edema, neuronal necrosis, and cystic infarction (17). Allopurinol's neuroprotective mechanism was attributed to its ability to inhibit xanthine oxidase in previous studies that in the brain, xanthine oxidase is concentrated within endothelial cells, subject the blood-brain barrier to free radical attack (18).

Bcl-2 family proteins monitor apoptosis and regulate this complex molecular network. Various cellular events such as DNA damage, energy stress, loss of growth factor signaling and hypoxia can initiate apoptosis by activation of these proteins. The Bcl-2 family proteins takes prominent role such as; regulation of mitochondrial or intrinsic apoptotic response (19). Apoptosis causes the death of neuronal cells, particularly neurons and oligodendrocytes in the spinal cord after lesion, later corrupts axonmyelin anatomical unit and gives rise to an interruption in impulse transmission leading to neuronal loss (20,21).

Glial fibrillary acidic protein (GFAP) acts in astrocyte–neuronal interconnection and synaptic transmission (22). As an intermediate filament protein GFAP is found in the skeleton of astroglia. Previous studies indicated that increased GFAP immunoreactivity is a sensible indicator of neuronal damage in tissue and an increase in GFAP is also an indicator of reactive astrocytosis. Damage caused by a trauma or disease in cerebral tissue or spinal cord cells is known to initiate an acceleration in blood GFAP level (23-25).

In this experimental study, the role of allopurinol in rats with spinal cord injury and the immunohistochemical expression of GFAP and Bcl-2 proteins were investigated.

**MATERIALS AND METHODS**

*Animals*
Every single surgical methodology and the consequent care and healing of the animals utilized as a part of this investigation were in strict understanding with the National Institutes of Health (NIH Publications No. 8023, revised 1978) rules for animal care. All experimental protocols were approved by the Dicle University Animal Care and Use Committee. Male Sprague Dawley rats (n:30) weighing 250–290 g were kept under the conditions of 22±1°C and 12/12 hr light/dark cycles with standard pellet and water ad libitum. All rats at the end of experiment were healthy and no difference in food/water consumption and body weight gain between experimental and control rats were observed. Rats were separated into 3 groups as; control group, trauma group and trauma + allopurinol group. Isotonic saline solution (an equal volume of Allopurinol) was administered i.p. for 7 days in the control and trauma groups. Fifteen min following trauma, the allopurinol solution was injected intraperitoneally for 7 days at a concentration of 50 mg/kg of body weight (Urikoliz 300 mg, Ilsen, Turkey, 50 mg / kg). Spinal cord tissue taken from L1-L2 spinal cord segments was extracted, fixed in a 10% formalin solution, and embedded in paraffin blocks for histopathologic examination in all groups. Sections (5µm thick) were obtained from paraffin blocks and stained with Hematoxylin- Eosin for light microscopy examination.

**Surgical procedure**

Experimental animals were anesthetized intraperitoneally with ketamine and chlorpromazine 75 mg/kg and 1 mg/kg, separately (26). Each rat was then positioned on a heating pad in a prone position and a rectal test was embedded. Under aseptic conditions, following T5-12 midline skin incision and paravertebral muscle dissection, spinous procedures and laminar arcs of T5-12 were evacuated. The particular relative angulation of the spinous procedures of the T9, T10, and T11 vertebrae was utilized as an imperative intraoperative landmark: T9 points caudally, T10 points directly dorsal, and T11 points rostrally. This delivers a solid "triangle" introduction that can be promptly checked whether the animal is positioned flat on the operating table. After the T11 and T12 vertebrae had been distinguished, a laminectomy was performed at T11 and T12 with Friedman-Pearson rongeurs. The clasp was then held open with a clasp utensil, with the lower cutting edge of the clasp passed extradurally completely around the spinal cord and nerve roots at the intersection between the T11 and T12 vertebrae, comparing to the L1-L2 spinal cord segmental level. The clasp was then quickly discharged from the tool to deliver a bilateral impact force and sustained dorsal-ventral compression. The compression of the spinal cord was kept up for 60 sec before expulsion of the clasp. The muscles were then sutured utilizing 3–0 polyglactin
sutures, and the skin was shut with Michel clips (27). Following surgical system, the rats were put in warming chamber and their body temperatures were kept up at roughly 37°C until the point when they were totally conscious. An hour after the spinal cord injury, only saline was injected intraperitoneally to the trauma group.

**Histological preparation and analysis**

At the end of the experiment, all animals were anesthetized via the intraperitoneal administration of ketamine HCL (0.15 ml/100 g body weight). The spinal cords were dissected. The sections were subjected to Hematoxylen-Eosin staining for observation under a light microscope.

**Hematoxylin -EosinStaining Procedure**

1. Deparaffinize sections, 2 changes of xylene, 10 minutes each.
2. Re-hydrate in 2 changes of absolute alcohol, 5 minutes each.
3. 95% alcohol for 2 minutes and 70% alcohol for 2 minutes.
4. Wash briefly in distilled water.
5. Stain in Harris hematoxylin solution for 8 minutes.
6. Wash in running tap water for 5 minutes.
7. Differentiate in 1% acid alcohol for 30 seconds.
8. Wash running tap water for 1 minute.
9. Bluing in 0.2% ammonia water or saturated lithium carbonate solution for 30 seconds to 1 minute.
10. Wash in running tap water for 5 minutes.
11. Rinse in 95% alcohol, 10 dips.
12. Counterstain in eosin-phloxine solution for 30 seconds to 1 minute.
13. Dehydrate through 95% alcohol, 2 changes of absolute alcohol, 5 minutes each.
14. Clear in 2 changes of xylene, 5 minutes each.
15. Mount with xylene based mounting medium.

**Immunohistochemical staining**

An antigen-retrieval process was performed in citrate buffer solution (pH 6.0) two times: first for 8 min, and afterward for 5 min in a microwave oven at 700 W. They were permitted to cool to room temperature for 20 min and washed in distilled water twice for 6 min. Endogenous peroxidase action was hindered in 0.1% hydrogen peroxide for 15 min. An
ultra V block (Histostain-Plus Kit, Invitrogen, Carlsbad, CA) was connected for 10 min before the use of the primary antibodies (Bcl-2 antibody, mouse monoclonal, 1/100, Santa Cruz Biotechnology, US) and GFAP antibody (mouse monoclonal, 1/100, Abcam, UK) overnight. The secondary antibody (Histostain-Plus Kit, Invitrogen, Carlsbad, CA) was connected for 15 min. At that point, the slides were exposed to streptavidin-peroxidase for 15 min. Diaminobenzidin (DAB, Invitrogen, Carlsbad, CA) was utilized as a chromogen. Control slides were set up as specified above yet overlooking the primary antibodies. In the wake of counterstaining with Hematoxylene, washing in tap water for 5 min, and in refined water for $2 \times 5$ min, the slides were mounted.

**Measurement of myeloperoxidase (MPO) activity**

The MPO activity levels were measured using the method described by Hillegass et al. (28). Spinal cord tissue specimens were homogenized in 50 mM potassium phosphate buffer with a pH of 6.0 and centrifuged at 41,400 g for 10 min. The pellets were then suspended in 50 mM PB containing 0.5% hexadecyl trimethyl-ammonium bromide (HETAB). After three freezes and defrost cycles, with sonication between cycles, the samples were centrifuged at 41,400 g for 10 min. Aliquots (0.3 ml) were added to 2.3 ml of the response mixture containing 50 mM PB, o-dianisidine, and 20 mM H2O2 solution. One unit of enzyme action was characterized as the measure of MPO presence that caused an adjustment in absorbance, estimated at 460 nm for 3 min. MPO action was expressed as U/g tissue.

**Malondialdehyde (MDA) and glutathione (GSH) assays**

Spinal cord tissue samples were homogenized with super cold 150 mMKCl for the assurance of MDA and GSH levels. The MDA levels were tested for the products of lipid peroxidation and the outcomes are expressed as nmol MDA/g tissue (29). GSH was resolved by a spectrophotometric technique in light of the utilization of Ellman's reagent and the outcomes are expressed as μmol GSH/g tissue (30).

**Statistical analysis**

All information is expressed as means ± standard deviation. Groups of information were contrasted and an analysis of variance (ANOVA) trailed by Tukey’s various correlation samples. Estimations of p value ($p<0.05$, versus control; $p<0.001$, versus control; $p<0.01$, trauma+allopurinol versus trauma; $p<0.001$, trauma+allopurinol versus trauma) were considered as significant.
RESULTS

Control, trauma and trauma + allopurinol groups were analyzed for biochemical data. MPO action, which is acknowledged as an indicator of inflammatory cells, was fundamentally higher in the spinal cord tissues of disturbed rats than those of the control group (p<0.001). Allopurinol treatment reduced spinal cord tissue MPO levels (p<0.01) when compared to the trauma group. The injury caused a significant increment in the MDA levels (p<0.001) with a reduction in GSH levels (p<0.001). Allopurinol caused a decrease in MDA levels and reestablished the GSH content at day 7.

Table I. Biochemical results of experimental groups.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Trauma</th>
<th>Trauma+ Allopurinol</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (nmol/g)</td>
<td>27.75±0.85</td>
<td>41.95±0.72**</td>
<td>28.32±0.82**</td>
</tr>
<tr>
<td>GSH (µmol/g)</td>
<td>1.46±0.04</td>
<td>0.72±0.05*</td>
<td>1.32±0.04**</td>
</tr>
<tr>
<td>MPO (U/g)</td>
<td>3.37 ±0.05</td>
<td>7.54±0.58**</td>
<td>6.75±0.49+</td>
</tr>
</tbody>
</table>

Values are represented as mean ± SD. Each group consists of ten rats.

* p<0.05, versus control

** p<0.001, versus control

+p<0.01, trauma+allopurinol versus trauma

++p<0.001, trauma+allopurinol versus trauma
**Histopathologic findings**

In the cross-section of the control group; spinal cord, ependymal canal, which was paved with cylindrical epithelium, multipolar and bipolar neurons were diffusely distributed in substantia grisea. In the substantia alba layer, regular and nerve extension of the glial cells were observed (Figure 2a). In the trauma group, hyperplasia of epithelial cells with dilatation of the ependymal canal, and degenerative changes in multipolar and bipolar neurons together with apoptotic changes in some glial cells were observed. In the substantia grisea and alba layer, congestion in the vessels and degeneration in the endothelial cells and ruptures in the nerve extensions were observed (Figure 2b). In the group treated with allopurinol after trauma, mild degenerative changes were observed in multipolar and bipolar neurons compared to the trauma group, while the structure of the blood vessels in the endothelial cells was preserved (Figure 2c).

**Immunohistochemical findings**

In the control group, Bcl-2 was weakly expressed in the cytoplasm of neurons of anterior and dorsal horn and intermediate zone. Occasionally, positive glial cells were observed in substantia grisea layer (Figure 3a). Bcl-2 expression was positive in glial cells and degenerative changes were seen in motor neuron and bipolar neurons in the trauma group. Bcl-2 expression was positive in glial cells in some multipolar and bipolar neurons after trauma (Figure 3b). Negative Bcl-2 expression was observed in multipolar, bipolar and glial
cells in the group treated with allopurinol after trauma (Figure 3c). GFAP expression was positive in the control group sections and in the extension of multipolar bipolar neurons and glial processes (Figure 4a). In the trauma group, GFAP expressions were evaluated as positive in degenerative structures in neurons and glial processes with irregular distribution of nerve extensions around degenerative structures in dilated blood vessels. In the trauma group, a decrease in astrocyte processes and a GFAP positive reaction were observed in the substantia grisea region (Figure 4b). In post-traumatic allopurinol group, it was observed that the extension of the glial cells were in parallel with the extension of the neurons around the blood vessels in the multipolar and bipolar neurons and GFAP protein expression was observed (Figure 4c).
Spinal cord injuries may be caused by damage to the vertebrae, ligaments or spinal discs or the spinal cord itself. It has been reported that spinal cord injury may delay the repair of large thoracoabdominal aortic diseases for ischemic reasons. It was also reported that
intercostal and lumbar artery blood flow in the spinal cord and postoperative neurological deficiencies occurred in many cases (31,32).

A significant output of spinal cord injury is the formation of oxidative stress. Oxidative stress plays a critical role in the pathophysiology of SCI is long known fact (33-35). After lesion, apoptosis gives rise to the death of cells such as; neuronal cells, oligodendrocytes and also neurons in the spinal cord, and even more breaks down the axonmyelin anatomical unit and prohibits impulse conduction, leading to neuronal loss (20,21). After spinal cord lesion, inflammation of the spinal cord and obstructed vascular structure besides apoptotic changes in neurons can be seen. When apoptosis of neurons and glial cells is retained after the lesion, loss in the nerve tissue can be reduced and spinal cord lesion may become improved. Inflammatory reactions are significant components of the secondary lesion and they are supposed to be a part in controlling the pathogenesis of chronic SCI and possess a prominent role in nerve lesion and also acts in regenerative reactions (36). Inflammatory reactions may attend apoptosis of neurons and oligodendrocytes in scar formation also causing a reduction of neuronal capacity (37). In the trauma group of our study, hyperplasia of epithelial cells with dilatation of the ependymal canal, and degenerative changes in multipolar and bipolar neurons together with apoptotic changes in some glial cells and congestion in the blood vessels and degeneration in the endothelial cells and ruptures in the nerve extensions were observed (Figure 2b). In the group treated with post-traumatic allopurinol, mild degenerative changes were observed in the multipolar and bipolar neurons compared to the trauma group (Figure 2c).

A study of Chen et al. presented that in transgenic mice, over-expression of Bcl-2 by gene transfer caused to a decline in infarction after permanent and transient central ischemia. Expression of Bcl-2 gene, acting as the apoptosis inhibitor, seems to protect cells from apoptosis, especially motor neurons of the spinal cord (38). In our study, an increase in Bcl-2 expression was observed in glial cells in multipolar and bipolar neurons after apoptotic changes in glial cells in some of the endothelial cells (Figure 3a). Negative Bcl-2 expression was observed in glial cells and multipolar and bipolar neurons in the post-traumatic allopurinol treated group (Figure 3b).

Baloglu et al. found a decrease in the apoptosis of nerve cells and glial cells by the application of Potentilla fulgens after spinal cord injury (39). They suggested that P. fulgens reduced the amount of so-called inflammatory cells and stimulated angiogenetic progression by affecting the cytokine mechanism.
GFAP is a brain-specific protein that acts as the major integral component of the cell skeleton of astrocytes. After brain injury, GFAP releases the brain cells into the interstitial fluid in the environment and causes deterioration in the blood-brain barrier (40). In many pathological conditions of the Central Nervous System and astrocytes, GFAP expression is increased. Studies on experimental animals revealed GFAP positive expression in astroglial cells following traumatic brain injury (41, 25). Allopurinol is a specific inhibitor of xanthine oxidase, and it blocks the synthesis of xanthine from hypoxanthine and inhibits the formation of free radical superoxide (42). In the trauma group of our study, GFAP expression in glial cells was evaluated as positive with degenerative neuron and a decrease in astrocytic ankles around dilated blood vessels (Figure 4b). In the allopurinol treated group, glial feet around the blood vessel were regular and it was showed that GFAP protein expression increased in multipolar and bipolar neuron extensions (Figure 4c).

Studies have shown that allopurinol leads to a decrease in the levels of free radical production and reduces tissue damage associated with I/R injury (42, 43). It is both a strong xanthine oxidase inhibitor and an agent that decreases ischemia related mitochondrial dysfunction (43, 44). As a result of a study by Moorhouse et al., high doses have been suggested for the neuroprotective effects of allopurinol. It has also been shown that high levels of allopurinol and its metabolite oxypurinol in the blood can act as a scavenger and move hydroxyl radical and transition metal chelating agents (35). On the contrary, another study indicated that the high dose allopurinol had an intraperitoneal protective effect but showed no therapeutic effect in transient focal cerebral ischemia in the three-vessel occlusion model in rats (44).

Despite the results, there are a few limitations. Allopurinol may be applied for a longer period of time however, we applied for 7 days and examined its short-term effect. We also preferred a lower dose, unlike high doses, not to enable a toxic effect. Known side effects of allopurinol are dermatologic (pruritic, erythematosus, or macropapular eruptions), hematologic (leukopenia and/or eosinophilia, white blood count abnormalities), and hepatologic (increase in liver enzymes) (45) so, it was investigated whether allopurinol has an osteoblastic effect at lower dosages, since short-term administration at minimal concentrations would be more appropriate.

**CONCLUSIONS**

We think that GFAP expression in glial processes deteriorated in the multipolar, bipolar neuron extensions and blood vessel environment after spinal cord injury and in
inflammatory response after trauma and tissue damage. We are in the opinion that allopurinol has an antioxidative effect and it may induce GFAP protein activity in traumatic spinal cord injury which may affect the repairment process in nerve connections and ependymal cells after the trauma. As a conclusion, allopurinol can partially prevent degenerative changes in nerve cells and glial cells and decrease apoptotic changes due to Bcl-2 expression.

References


FIGURE LEGENDS

Figure 2a. Haematoxylin-eosin staining (Control group). Diffuse distribution of multipolar and bipolar neurons in substantia grisea, regular nerve extensions of the glial cells in substantia grisea and alba. Scale bar = 100 μm.

Figure 2b. Haematoxylin-eosin staining (Trauma group). Hyperplasia in ependymal cells, dilatation in ependymal channel, degenerative changes in multipolar and bipolar neurons,
apoptotic changes in some glial cells, congestion in the blood vessels of the substantia grisea and alba layer. Scale bar = 50 μm.

**Figure 2c. Haematoxylin-eosin staining (Trauma+Allopurinol group).** Moderate degenerative changes in multipolar and bipolar neurons. Scale bar = 50 μm.

**Figure 3a. Bcl 2 immunostaining (Control group).** Weak Bcl 2 expression in the cytoplasm of neurons of anterior horn, dorsal horn and intermediate zone. Scale bar = 50 μm.

**Figure 3b. Bcl 2 immunostaining (Trauma group).** Positive Bcl-2 expression in glial cells and in motor neuron, bipolar neurons. Scale bar = 50 μm.

**Figure 3c. Bcl 2 immunostaining (Trauma+Allopurinol group).** Negative Bcl-2 expression in multipolar, bipolar and glial cells in allopurinol group. Scale bar = 50 μm.

**Figure 4a. GFAP immunostaining (Control group).** Positive GFAP expression in multipolar bipolar neurons and glial processes. Scale bar = 50 μm.

**Figure 4b. GFAP immunostaining (Trauma group).** Negative GFAP expression in multipolar ve Bipolar neuron processes while reduction in glial processes around blood vessels and positive GFAP expression. Scale bar = 50 μm.

**Figure 4c. GFAP immunostaining (Trauma+Allopurinol group).** Regular and parallel positive GFAP expression of glial processes around blood vessels. Scale bar = 50 μm.