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EFFECTS OF AMINOGUANIDINE AND METHYLPREDNISOLONE ON SPINAL CORD INJURY: EXPERIMENTAL STUDY

SPİNAL KORD HASARINDA AMİNOGUANİDİN VE METİLPREDNİSOLONUN ETKİSİ: DENEYSEL ÇALIŞMA

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SUMMARY

Study Design: The effects of methylprednisolone (MP) and aminoguanidine (AG) on an experimental spinal cord injury with a weight-drop technique were examined in rats, assessing the levels of malondialdehyde (MDA), antioxidant enzyme activity, and morphological changes in the spinal cord.

Objective: To create a spinal trauma model with a weight-drop technique in rats and to investigate the biochemical and histopathological changes to the spinal cord.

Summary of Background Data: Unfortunately, primary spinal cord injuries cannot be prevented, but the secondary injury that begins immediately after trauma can be treated medically. The effect of AG was investigated and compared with MP.

Methods: 35 Sprague-Dawley rats were used in this study. A spinal cord injury model was created in all rats with a weight-drop technique, and the malondialdehyde (MDA) and antioxidant enzyme activities were measured. The histopathological changes in the spinal cord were examined.

Results: MP decreased the GPX, MDA and CAT levels, and no difference was found with a combination of AG and MP in this study. The effect of AG was to decrease the levels of GPX, SOD and MDA, and to increase the CAT levels. The effect of MP was to decrease the GPX, MDA and CAT levels, and to increase the SOD levels. No significant differences were found between the effects of MP and AG by histopathological examination.

Conclusions: The levels of MDA or antioxidant enzyme activity can be helpful when testing the neuroprotective effects of any molecule in secondary injury mechanism. Molecules can show their effects by the different levels of these enzyme activities. We conclude that morphological and neurological examination would be safer to test the effects of antioxidant molecules.

Key Words: Spinal cord, spinal cord injury, medical treatment, aminoguanidine, methylprednisolone

Level of Evidence: Experimental study, Level II

ÖZET

Amaç: Sıçanlarda ağırlık düşürme tekniği ile oluşturulan deneysel omurilik yaralanmasında metilprednizolon (MP) ve aminoguaninin (AG) etkilerini malondialdehit (MDA) düzeyleri ve antioksidan enzim aktiviteleri ile ve omurilikteki morfolojik değişikliklerle değerlendirildi.

Yöntem: Otuz beş Sprague-Dawley sıçanları bu çalışmada kullanılmıştır. Ağırlık düşürme tekniği ile omurilik yaralanması modeli tüm sıçanlarda oluşturuldu ve malondialdehit (MDA) ve antioksidan enzim aktiviteleri ölçüldü. Omurilikteki histopatolojik değişiklikler incelendi.

Bulgular: Metilprednizoloni GPX, MDA, CAT düzeylerini azaltmış ve AG + MP kombinasyonu ile arasında hiç bir fark çalışmamızda tespit edilmemiştir. AG GPX, SOD ve MDA düzeylerini azaltarak ve CAT seviyelerini artırarak etkisini gösterdi. Öte yandan MP GPX, MDA ve CAT seviyelerini azaltarak ve SOD düzeylerini artırarak etkisini gösterdi. Histopatolojik incelemede MP ve AG arasında anlamlı bir fark bulunmamıştır.

Sonuç: MDA ya da herhangi bir antioksidan enzim aktivitesinin seviyesi, herhangi bir molekülün ikincil spinal kord yaralanmasında nöroprotektif etkisinin değerlendirilmesinde yardımcı olabilir. Ancak, bu moleküller bu enzim aktivitelerinde kendi etkilerini gösterebilirler. Biz morfolojik ve nörolojik muayenenin antioksidan moleküllerin etkisini test etmede daha güvenli olacağına kanaatindeyiz.

Anahtar Kelimeler: Spinal kord, spinal kord yaralanması, medikal tedavi, aminoguanidin, metilprednizolon.

Kanıt Düzeyi: Deneysel çalışma, Düzey II

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INTRODUCTION

Spinal cord injuries (SCIs) and their symptoms were first described in 500 BC as untreatable illnesses. Exact, universal and effective treatment of spinal cord injuries could not be achieved for 2500 years. The rising amount of neurologically-impaired patients and their economical load on governments pushed scientists to discover effective treatments for SCI. The prevalence of SCI is 1600–2000 patients/year in Turkey, and half of the patients have complete injury. 54% of patients are quadriplegic, and 46% are paraplegic[28,30].

Primary injury cannot be prevented, but prevention of secondary injury, which begins immediately after trauma, is a primary target[13]. Treatment with high doses of methylprednisolone (MP) has been shown to be a successful intervention for SCI, both in animal models and man[5,12,21,33]. In the NASCIS-2 and NASCIS-3 studies, a statistically significant improvement in function was found for spinal-injured humans when MP was administered within eight hours after SCI[7]. The underlying mechanism is not fully understood, but experimental data point to a protective effect from membrane peroxidation and edema[9,21]. Further research has shown that the high doses of MP required to inhibit lipid peroxidation also exert a number of other actions on the injured spinal cord that almost certainly contribute to an attenuation of post-traumatic neuronal damage, such as a reduction in ischemic area and neurofilament degradation, preserved evoked potentials, and improved spinal cord blood flow[10,19,33,35].

The neuroprotective effect of aminoguanidine (AG) was shown in an experimental study after immediate initiation[31]. The mechanism of its neuroprotective effect is still unclear. The inhibition of polyamine oxidase (PAO) after initiation in the early post-traumatic period and the relative selective inhibition of iNOS (inducible nitric oxide synthase) 24 hours after initiation have been suggested to be the mechanism of action of AG. The strongest effect of AG can be achieved 24 hours after trauma through the inhibition of iNOS[20]. The effects of MP in the first eight hours after trauma, and the combined treatment of each molecule, were tested in this experimental study. The objective of our study was to investigate the effects of AG on histopathological changes, antioxidant status and lipid peroxidationin weight-drop induced spinal cord trauma in rats, and to compare the effects of AG and MP.

MATERIALS AND METHODS

Thirty-five Sprague-Dawley rats weighing 234 ± 12.3 g were used in this study. The animals were kept under constant laboratory conditions of 18–21˚C room temperature, a 12-hour light-dark cycle, and were allowed free access to food and tap water. All experiments were approved by the Institutional Review Board of Gulhane Military Academy of Medicine and were treated according to research guidelines.

Anesthesia and Surgical Procedure:

The rats were fasted for 24 hours with free access to water before the surgical procedure. Anesthesia was induced by intramuscular administration of 90 mg/kg ketamine hydrochloride (Ketalar, Pfizer, Istanbul) and 10 mg/kg xylazine (Rompun, Bayer, Istanbul). Anesthesia was maintained with periodic administration of 20% of the initial dose. The rats were numbered with ear tags.

Their dorsal area was shaved and cleaned with 10% polyvinylpyrolidone. The rats were positioned in a prone position. Using a microscope, under sterile conditions, a median subcutaneous incision was made, starting from the midpoint of the interscapular area and extending to the lower lumbar region. The dura mater was exposed following subperiosteal dissection of the paravertebral muscles and laminectomy of the thoracic ninth or tenth vertebra. No further intervention was applied to the rats in the *sham group*, and the surgical incisions of the rats were closed in layers.

A 10 cm long Teflon tube with a 6.5 mm diameter was positioned above the dura mater vertically in the other groups. 50 g/cm of impact was produced through free-fall of a 5 g stainless steel bullet of 6 mm in diameter at the center, and 3 mm in diameter at the tip, onto the spinal cord. The surgical incisions of the rats were then closed in layers.

1 ml of 0.9% NaCl solution was given to the *vehicle group* intraperitoneally for 5 days.

For the *AG group,* consisting of seven rats, AG treatment was initiated with a dose of 100 mg/kg (in 1 ml saline solution) intraperitoneally one hour after trauma, and maintained at the same dose for 5 days.

For the *MP group*, consisting of seven rats, MP was administrated with a loading dose of 30 mg/kg intraperitoneally one hour after trauma.

For the *AG+MP group*, consisting of seven rats, 100 mg/kg of AG (in 1 ml saline solution) and 30 mg/kg of MP were initiated intraperitoneally one hour after trauma, and AG treatment was maintained at the same dose for 5 days.

Sacrificing of Animals and Sample Preparation:

The rats were sacrificed by an overdose of ketamine hydrochloride on the sixth day. Spinal cord segments between the T8 and T12 levels were excised and divided into four equal parts. The three caudal parts were stored immediately in a –76˚C freezer for homogenization. An Ultra-Turrax homogenizer (model T25, Janke and Kunkel, Germany) at 9500 rpm (4×10 sec at 4˚C) was used. The most cranial parts of the specimens obtained were processed using formalin fixation and paraffin embedding. Cross-sections of 1 micron thick were treated with hematoxylin and eosin stain, and numbered by the laboratory technician in order to blind the investigator to the group identities of the samples. The results were analyzed according to the codes given at the pathology laboratory.

Morphological evaluation of the sections was done using a light microscope by a pathologist in a blind fashion, and the changes were compared based on the amount of blood degradation products, inflammation, and edema. The pathological changes were grouped as 'none', 'mild', or 'severe'.

Measurement of Malondialdehyde (MDA) Levels and Antioxidant Enzyme Activities:

The lipid peroxidation in the spinal cord samples was determined using the MDA concentration with the method defined by Mihara and Uchiyama[24]. Briefly, 0.5 ml of homogenate was mixed with 3 ml of 1% H_3PO_4. After adding 1 ml of 0.67% thiobarbituric acid, the mixture was heated in boiling water for 45 min. The formed color was extracted into n-butanol. The mixture was centrifuged at 4000 rpm for 10 min at room temperature. The absorbance of the organic layer was read at 532 nm. Tetramethoxypropane was used as a standard, and the MDA levels

were calculated as nanomoles per gram of wet tissue.

The superoxide dismutase (SOD) activity was measured by the rate of inhibition of nitroblue tetrazolium reduction in the xanthine–xanthineoxidase system[29]. Enzyme activity leading to 50% inhibition was accepted as one unit, and the results were expressed as U/mg protein. Protein concentrations were determined according to Lowry's method[22].

The glutathione peroxidase (GSH-PX) activity was measured by the method defined by Paglia and Valentine using a RANSEL kit (Randox, Antrim, UK)[25]. In this kit, GSH-PX activity is coupled with the oxidation of NADPH by glutathione reductase. The oxidation of NADPH was followed spectrophotometrically at 37°C and 340 nm. The results were expressed as U/mg protein.

The catalase (CAT) activity was determined using the method defined by Aebi[2]. The principle of CAT activity was based on the determination of the rate constant (k, s^{-1}) or the hydrogen peroxide decomposition rate at 240 nm. Results were expressed as k/g protein.

Statistical Analysis:

Differences between the mean values measured were analyzed with one-way variance analysis (one-way ANOVA) and the post hoc Tukey test. A *p*-value of less than 0.05 was considered statistically significant. Results were expressed as mean, standard deviation (SD), median, and range.

RESULTS

The MP group had the lowest MDA levels (4.39 ± 0.82 nm/g), while the highest results were measured in the vehicle group (8.18 ± 1.85 nm/g) (Table-1). When the MDA levels of all groups were compared with a one-way ANOVA test, the results were statistically significant ($p<0.001$). When the groups were compared with a post hoc Tukey test, statistically significant differences were found between the vehicle group and the MP group ($p<0.001$), the AG+MP group ($p<0.01$), and the AG group ($p<0.05$). No significant differences were found between the MP, AG+MP, AG, and sham groups ($p>0.05$). No significant differences were found between the AG and vehicle groups, the AG and sham groups, and the MP and AG+MP groups ($p>0.05$).

The GSH-PX levels were lowest in the MP group (8.01 ± 1.15 U/mg) and highest in the vehicle group (43.68 ± 6.37 U/mg). When the GSH-PX levels were compared with a one-way ANOVA test, the results were statistically significant ($p<0.0001$). The differences between the vehicle group and all other groups were statistically significant ($p<0.0001$) when the groups were compared using a post-hoc Tukey test (Table-1). There were no significant differences between the MP and AG+MP groups and the sham group ($p>0.05$). The differences between the AG and vehicle groups and the AG and sham groups were statistically significant ($p<0.0001$).

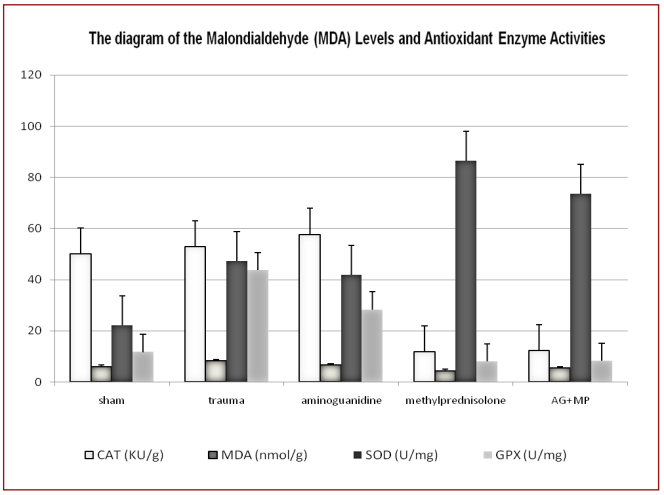
The SOD levels were lowest in the sham group (22.08 ± 10.18 U/mg) and highest in the MP group (86.48 ± 18.1 U/mg) (Table-1). When the SOD levels of the groups were compared using a one-way ANOVA test, the results were statistically significant ($p<0.0001$). When the groups were compared using a post-hoc Tukey test, no differences were found between the MP and AG+MP groups ($p>0.05$) (Table-1). The MP and AG+MP groups were significantly

different with respect to the AG, sham and vehicle groups.

No significant differences were found when the AG group was compared with the sham and vehicle groups (p>0.05). The MP group had the lowest (11.68 ± 2.72 k/g) CAT levels, while the vehicle group had the highest (57.66 ± 28.50 k/g). When the CAT levels were compared with a one- way ANOVA test, the results were statistically significant (p<0.0001). The differences between the MP and AG+MP groups and all the other groups were statistically significant when the groups were compared using a post-hoc Tukey test (p<0.001) (Table-1). Therefore, there were no significant differences between the sham and vehicle groups and the AG group (p>0.05).

Ultrastructural examination revealed minimal inflammation and edema in the sham group (Figure-1), severe blood degradation products, inflammation and edema in the vehicle group, and mild focal blood degradation products (Figure-2), inflammation and edema in the AG, AG+MP and MP groups (Figure-3).

Table-1. The different effects of AG and MP on the MDA levels and antioxidant enzyme activities.



DISCUSSION

Primary SCI can occur by various mechanisms including hyperextension, hyperflexion, burst fracture or gunshot injuries, and frequently only results in limited cell death surrounding the lesion epicenter. However, secondary injuries, which were first described at the beginning of the twentieth century, occur immediately after trauma[3]. Hemorrhage, edema, demyelinization, axonaland neuronal necrosis, and infarction occur in this process. Ducker et al.[13] showed that these changes continue to increase several days or weeks after trauma. The pathology cannot be limited and descends with the corticospinal tracts, causing atrophy, apoptosis, or necrosis in the axons. After the damage of the spinal cord, exotoxicity, neurotransmitter deposits, arachidonic acid release, production of reactive oxygen species (ROS), eicosanoids, endogen opioids and cytokines and lipid peroxidation occur and cause edema, impairment of energy metabolism, and apoptosis of neurons.

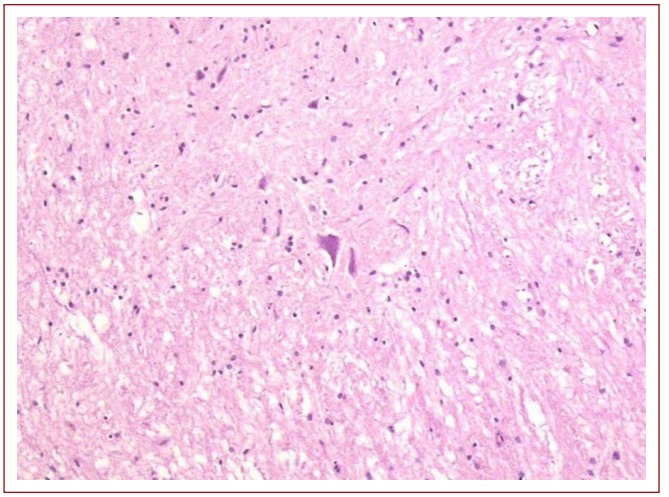


Figure-1. Minimal inflammation and edema in the sham group.

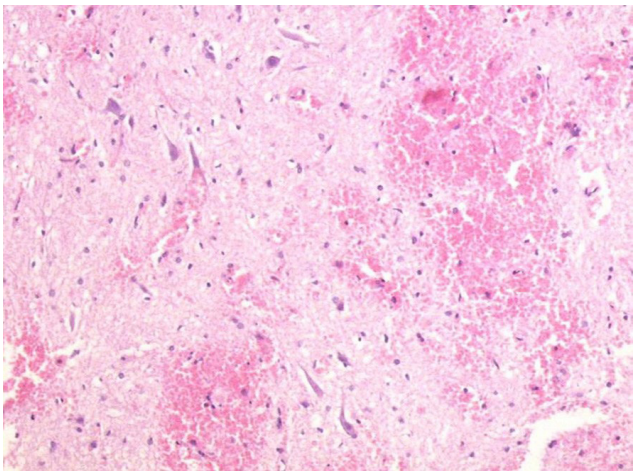


Figure-2. Severe blood degradation products, inflammation and edema in the vehicle group.

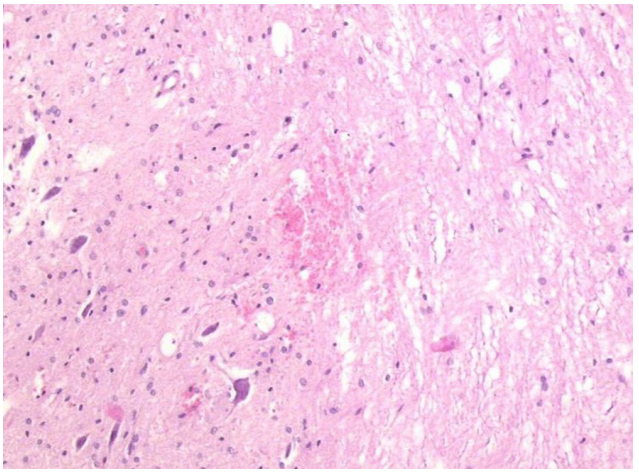


Figure-3. Mild focal blood degradation products, inflammation and edema in the AG, AG+MP, and MP groups.

The exact pathogenesis of secondary spinal cord damage has not been fully elucidated, but there is considerable evidence that it occurs within minutes and continues for days or weeks, resulting in further neurological deterioration[6].

Furthermore, secondary spinal cord damage has the propensity to worsen during the first few hours after injury, and so treatment during this time has the potential to prevent or reduce the resulting neurological deficit. Unfortunately, there is incomplete knowledge of the exact time course of many secondary mechanisms, and therefore the exact therapeutic window in which to treat many of these processes is unknown[4].

MP is currently in widespread use for the treatment of acute SCI and is widely considered a gold standard treatment. The results of recent clinical studies on neuroprotective pharmacotherapy have shown only modest improvements in neurological recovery and functional capability[11,14,15,16,17,18,26,27,38].

MP attenuates spinal cord damage in preclinical models by reducing lipid peroxidation produced by free radical and reactive oxygen species generated by cellular metabolism, especially within the milieu of ischemia followed by reperfusion[32]. MP has also been reported to reduce proinflammatory cytokine production and tissue edema[23], iNOS activity, neuronal apoptosis[36], free radical formation, and release of excitatory amino acids and vasoactive molecules, and also provides beneficial modulation of calcium and sodium transcellular fluxes. Soy et al.[27] showed the neuroprotective effects of AG in a rat SCI model.

After immediate initiation of AG, the reduction of serum NO levels and thus the prevention of lipid peroxidation has been shown. The exact neuroprotective mechanism of AG has not been described. Primarily, relative selective inhibition of iNOS and the inhibition of PAO in the early post-traumatic period are thought to be the neuroprotective mechanisms of AG[1,18,20,34].

NO has been described as one of the most important neurotransmitters in the central nervous system. However, it shows a toxic effect in pathological conditions[37]. iNOS will be activated in the late post-traumatic phase

and has a toxic effect on NO synthesis with long-term activity. Ladecola et al.[20] showed that the iNOS levels increase for 12 hours, and then show a plateau and begin to decrease on the fourth day after trauma. Soy et al.[31] showed that AG decreases lipid peroxidation due to a reductive effect on NO synthesis.

MP decreased the GPX, MDA and CAT levels, and no difference was found with the combination of AG+MP in our study. However, we have shown different effects of MP and AG on the levels of MDA and antioxidant enzyme activities. The effect of AG was to decrease the levels of GPX, SOD and MDA and to increase the CAT levels, while the effect of MP was to decrease the GPX, MDA and CAT levels and to increase the SOD levels. The combination of AG+MP showed similar effects to MP on the MDA levels and antioxidant enzyme activity. Morphological examination showed similar effects of AG, MP and AG+MP. The different effects on the levels of MDA and antioxidant enzyme activity, and the similarity onmorphological examination, can be explained by the different biochemical mechanisms of MP and AG (Table-1).

The levels of MDA or activity of any antioxidant enzymes can be helpful in testing the neuroprotective effects of any molecules on secondary injury mechanism. The molecules can show their effects by the different levels of these enzymes. We conclude that morphological and neurological examination would be a safer method to test for any effects of antioxidant molecules.

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