The biochemical effectiveness of N-acetylcysteine in experimental spinal cord injury in rats

Sıçanlarda deneysel spinal kord hasarlanmasında N-asetilsisteinin biyokimyasal etkinliği

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BACKGROUND

In this study, we investigated the biochemical effectiveness of methylprednisolone, N-acetylcysteine (NAC) and methylprednisolone combined with NAC treatment in experimental spinal cord injury in rats.

METHODS

Thirty-two Sprague-Dawley male rats weighing 250-300 g were divided into four groups. Spinal cord injury was created extradurally with an aneurysm clip at the T4-T5 level. Following the trauma, Group C (Control group, n:8) was not given any treatment. Group M (methylprednisolone group, n:8) was treated with 30 mg.kg^{-1} methylprednisolone followed by a maintenance dose of 5.4 mg.kg^{-1} per hour. Group N (NAC group, n:8) was given 150 mg.kg^{-1} NAC. Group MN (methylprednisolone and NAC group, n:8) was given 30 mg.kg^{-1} followed by an hourly maintenance dose of 5.4 mg.kg^{-1} methylprednisolone and 150 mg.kg^{-1} NAC intraperitoneally. Twenty-four hours after the trauma, the rats were decapitated under anesthesia, and their spinal cord samples were taken for biochemical examination.

RESULTS

Mean malonyldialdehyde (MDA) values in Groups M, N and MN were significantly reduced compared to Group C. Mean superoxide dismutase (SOD) values in Groups M, N and MN were significantly higher than in Group C ($p<0.05$). No difference existed between Groups M and N with respect to mean MDA and SOD values.

CONCLUSION

Methylprednisolone, NAC and methylprednisolone plus NAC treatments have potential biochemical benefits in preventing secondary injury in experimental spinal cord injury in rats.

Key Words: Experimental spinal cord injury; N-acetylcysteine; methylprednisolone.

AMAÇ

Çalışmamızda, metilprednizolon, N-asetilsistein (NAC) ve metilprednizolonla kombine NAC tedavisininin, sıçanlarda oluşturulan deneysel spinal kord hasarlanmasındaki biyokimyasal etkinliği karşılaştırıldı.

GEREÇ VE YÖNTEM

Spraque-Dawley cinsi, ortalama 250-300 gram ağırlığında 32 erkek sıçan dört gruba ayrıldı. Spinal kord hasarlanması ekstradural olarak T4-T5 seviyesine yerleştirilen anevrizma klibi ile uygulandı. Grup C'ye (Kontrol grubu, n=8) travma uygulanması ardından hiçbir tedavi uygulanmadı. Travma ardından Grup M'ye (Metilprednizolon grubu, n=8) 30 mg.kg^{-1} ardından idamede saatte 5,4 mg.kg^{-1} dozunda metilprednizolon, Grup N'ye (NAC grubu, n=8) 150 mg.kg^{-1} NAC, Grup MN'ye (Metilprednizolon ve NAC grubu, n=8) 30 mg.kg^{-1} ardından idamede saatte 5,4 mg.kg^{-1} dozunda metilprednizolon ile 150 mg.kg^{-1} NAC intraperitoneal olarak verildi. Travmadan 24 saat sonra sıçanlar anestezi uygulanarak dekapite edildi ve spinal kord örnekleri alınarak biyokimyasal inceleme yapıldı.

BULGULAR

Gruplarda ortalama malondialdehid (MDA) değerleri Grup M, Grup N, Grup MN'de, Grup C'ye göre anlamlı olarak düşüktü ($p<0,05$). Ortalama süperoksit dismutaz (SOD) değerleri ise, Grup M, Grup N, Grup MN'de, Grup C'ye göre anlamlı olarak yüksekti ($p<0,05$). Grup M ile Grup N arasında ortalama MDA ve SOD değerleri açısından anlamlı farklılık yoktu.

SONUÇ

Metilprednizolon, NAC ve metilprednizolon ile kombine NAC tedavisinin, sıçanlarda deneysel spinal kord hasarında, sekonder hasarın önlenmesinde biyokimyasal olarak faydalı olabileceği düşünüldü.

Anahtar Sözcükler: Deneysel spinal kord hasarlanması; N-asetilsistein; metilprednizolon.

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Spinal cord injuries cause serious social and economic problems. Despite the advanced surgical techniques of our day, neurological recovery of spinal cord injury cases generally remains incomplete. This has led to many studies on post-trauma biochemical pathological processes that exacerbate functional loss, or in other words, on secondary autodestructive mechanisms, as well as on the primary injury caused by the trauma. Studies on the physiopathology of spinal cord injuries have shown edema, free oxygen radicals and lipid peroxidation to play a major role in the development of secondary injury. Such studies have led to others focusing on using neuroprotective agents against secondary injury. Today, methylprednisolone is still the most common clinical agent used in spinal cord injuries. Despite the neurological benefits of high-dose corticosteroids, their serious side effects have necessitated studies focusing on the use of other neuroprotective agents against secondary injury.[1,2]

N-acetylcysteine (NAC) is a strong glutathione precursor with antioxidant effects to prevent the formation of free radicals. The positive effects of NAC on central nervous system ischemia and ischemia/reperfusion models have been documented. Studies on the use of NAC in central nervous system trauma, and particularly against spinal cord injury, are rare.[3]

In our study, we used biochemical data to investigate the treatment effectiveness of using methylprednisolone, NAC, and the two together in rats with experimentally induced spinal cord injury.

MATERIALS AND METHODS

After obtaining ethical consent from the Ethics Committee, 32 male adult Sprague-Dawley rats weighing 250-300 g were included in the study. The animal study was performed at Sarıkamıs Military Hospital and Kafkas Universty Veterinary and Medicine Faculty from April to October 2007. All rats were kept and fed under veterinary control for 12 hours (h) in line with the light/dark cycle. Temperature was maintained at 22-25°C with appropriate humidity, and animals were given sufficient fluids. All stages of the experiment were conducted under veterinary control, following ethical rules. All rats were anesthetized intraperitoneally with 8 $mg.kg^{-1}$ xylazine hydrochloride and 10 $mg.kg^{-1}$ ketamine, and divided into four groups as: Control group (Group C), methylprednisolone treatment group (Group M), NAC treatment group (Group N), and combined methylprednisolone and NAC treatment group (Group MN). Each group comprised 8 rats. Following the application of anesthetic agents, the rats were placed on the operating table in the prone position. Local area disinfection and environment isolation were done at the T3-T6 region. The skin was incised in the midline. The fascia was cut open and paravertebral muscles were removed subperiostally. Laminectomy was done at T4 and T5. Bleeding control was done with bipolar coagulator; none of the subjects was given an additional intervention for bleeding. Spinal cord trauma was created with the clip method using the FE 740 K Yaşargil aneurysm clip with the strength of 1.43 Newton (N). The clip was used for 60 seconds epidurally. Paraplegia was identified in all subjects using painful stimulus in the tail. Following the use of the clip, the development of hemorrhagic contusion was seen at the point in the spinal cord where the clip was used. Following the trauma caused by the clip, no treatment was given to Group C (n=8). Immediately after the clip, Groups M and MN were given 30 $mg.kg^{-1}$ methylprednisolone intraperitoneally. As a maintenance dose, these groups were given a total of 5.4 $mg.kg.hour^{-1}$ intraperitoneal methylprednisolone treatment at 6-h intervals, with total dose being 23 h. A single dose of 150 $mg.kg^{-1}$ NAC was given to Groups N and MN intraperitoneally. The subjects were kept alive for 24 h under appropriate conditions and veterinary control, after which decapitation took place after anesthetization using the same anesthetic agents. The spinal cord of the subjects was cut 0.5 cm distal to the observed hemorrhagic contusion line. One cm proximal to the distal incision was removed and kept at -20°C for biochemical measurements. Biochemical data was obtained by measuring the malonyldialdehyde (MDA) and superoxide dismutase (SOD) levels in spinal cord tissue. The lipid peroxidation level in the tissue samples was expressed by MDA. MDA measurement in the tissue was determined in accordance with the Ohkawa method.[4] The reaction mixture contained 0.1 ml of tissue sample, 0.2 ml of 8.1% sodium dodecyl sulfate (SDS), 1.5 ml of 20% acetic acid, and 1.5 ml of a 0.8% aqueous solution of thiobarbituric acid. The mixture pH was adjusted to 3.5, the volume was finally made up to 4.0 ml with distilled water, and a 5.0 ml mixture of n-butanol and pyridine (15:1, v/v) was added. The mixture was shaken vigorously. After centrifugation at 4000 rpm for 10 minutes (min), the absorbance of the organic layer was measured at 532 nm. Calculated MDA levels were presented as $pmol.mg^{-1}$ protein.[4]

Superoxide dismutase (SOD) activity was determined in accordance with the method defined by Winterbourn et al.[5] The tissue was homogenized with Ultra-Turrax in 10 volume 0.02 M phosphate tampon. Following the centrifuge, 0.05 ml supernatant was added to 0.1M EDTA (ethylenediaminetetraacetic acid) containing 1.5 mg NaCN in 100 ml, 1.5 mM NBT (nitroblue-tetrazolium) and 2.6 ml phosphate tampon, and the tube was warmed to 20-22°C. Riboflavin 0.05 ml was added. The mixture was kept under ultraviolet light for 12 min and absorbance was read immediately at 560 nm. One SOD unit was defined

Table 1. MDA values in groups (p.mol^{-1} protein)

Subject No	Group C (n:8)	Group M (n:8)	Group N (n:8)	Group MN (n:8)
1	118.70	118.60	145.33	81.33
2	104.20	75.60	85.83	73.60
3	163.60	45.50	50.66	35.50
4	103.30	88.30	65.60	68.00
5	119.40	113.70	103.3	17.33
6	130.33	96.00	75.51	65.60
7	99.50	35.66	93.33	44.36
8	85.30	15.50	25.70	22.50

Table 2. SOD values in groups (U.mg^{-1} protein)

Subject No	Group C (n:8)	Group M (n:8)	Group N (n:8)	Group MN (n:8)
1	12.07	20.76	31.14	26.46
2	5.54	15.10	23.84	29.67
3	2.80	9.30	10.18	38.75
4	4.70	20.6	11.30	27.80
5	2.93	27.00	18.83	36.50
6	4.03	21.05	14.19	30.90
7	10.8	13.50	16.87	18.09
8	6.10	11.48	5.04	24.30

as the amount of enzyme ensuring half the maximum inhibition of NBT reduction. The enzyme activity was given as U.mg^{-1} protein.

Statistical Analysis Methods: Statistical analyses were computed on SPSS 10.0 package program. All results are presented in mean ± standard error. Mann-Whitney U test was used in analyzing the biochemical data of SOD and MDA values. A level of $p<0.05$ was considered statistically significant.

RESULTS

Biochemical results are presented in Tables 1, 2, 3, and 4.

In the statistical analysis of biochemical data, mean MDA values were significantly reduced in Groups M, N and MN compared to Group C ($p<0.05$). With respect to MDA values, no significant difference was seen between Groups N and M. Although mean MDA values were lower in Group MN than in Groups M and N, the difference was not significant.

Mean SOD values were significantly higher in Groups M, N and MN when compared to Group C ($p<0.001$). No difference existed between Groups N and M with respect to SOD values. Mean SOD values were significantly higher in Group MN than in Groups M and N ($p<0.05$).

DISCUSSION

Spinal cord injuries present a high rate of morbidity and mortality. Fortunately, the experiences in spine biomechanics and surgical techniques have increased and rehabilitation techniques have developed significantly in recent years. However, no breakthrough has yet happened in neurological recovery in spinal cord injuries. Scientific studies thus focus on the use of different treatment options in the early stages of spinal traumas to prevent the development of edema, ischemia and tissue damage.[1,2,6]

The formation of spinal cord injuries is defined in two pathophysiological ways. Primary injury happens during the trauma as a result of the damage in cord

Table 3. Means and standard deviation in MDA values

	N	Mean ± SD
Group C	8	115.54±23.88
Group M	8	73.60±37.71*
Group N	8	80.65±35.96†
Group MN	8	51.11±24.45‡

*: $p<0.05$ (between Group C and Group M);
†: $p<0.05$ (between Group C and Group N);
‡: $p<0.05$ (between Group C and Group MN).

Table 4. Means and standard deviation in SOD values

	N	Mean ± SD
Group C	8	6.12±3.48
Group M	8	17.34±5.95*
Group N	8	16.42±8.25†
Group MN	8	29.05±6.59$^{\ddagger\#\S}$

*: $p<0.05$ (between Group C and Group M);
†: $p<0.05$ (between Group C and Group N);
‡: $p<0.05$ (between Group C and Group MN);
#: $p<0.05$ (between Group M and Group MN);
§: $p<0.05$ (between Group N and Group MN).

unity due to mechanical reasons. Secondary injury happens in the late stages as a result of cell death and tissue damage due to cellular and biochemical processes. Secondary injury pathophysiology includes the formation of free radicals, lipid peroxidation, formation of eicosanoid and prostaglandin, protease activation, glutamate and other excitotoxic molecules, and intracellular calcium increase. A neuroprotective strategy aims to protect from the effects of secondary injury and tissue and functional capacity loss.[2,6]

Today, methylprednisolone is the only commonly accepted drug in the treatment of acute spinal cord injury.[1,2,6-8] Corticosteroids have been shown to be effective in a significant number of experimental studies,[1,2,6] and are thought to work with mechanisms such as lipid peroxidation, the inhibition of inflammatory cytokines, and inflammatory immune cell modulation.[9-13] In our study, biochemical examination of the spinal cord tissue samples of the rats with experimentally induced spinal cord injury showed the neurprotective effectiveness of methylprednisolone through significantly lower mean MDA and higher SOD values in the groups that used methylprednisolone alone and combined NAC and methylprednisolone. This finding corroborates those of other studies.

As much as high-dose corticosteroids seem to have neurological benefits in acute spinal cord injury, they also involve serious side effects. Therefore, there has been an increase in the number of studies on alternative neuroprotective agents in preventing secondary injury. Phase III clinical studies have been conducted using drugs such as GM1 (Sygen), tirilazad, thyrotropin-releasing hormones (TRH) analogs, and naloxan.[1] Experimental studies have reported that coenzyme Q10,[2] magnesium sulphate,[14] minocycline,[15] antithrombin III,[16] erythropoietin,[17] gacyclidine, MK801,[18-20] combination of methylprednisolone, α-tocopherol (TOC) and selenium,[21] ebselen,[22] resveratrol,[23] riluzol, mexiletine,[24] N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone,[25] quercetin,[26] citicolin,[27] chlotrimazole,[28] agmatin,[29] etomidate,[30] melatonin,[31] beta-glucan,[32] indomethacin,[33] mannitol,[34] and thiopental and propofol[35] have potential neuroprotective effects in spinal cord injury. Apart from the use of different drugs, hyperbaric oxygen treatment[36] and immune regulatory treatment with interferon beta[37] have also been emphasized as having neural protective effects on spinal cord injury.

Tissue SOD and MDA levels are frequently used in the measurement of lipid peroxide levels, and provide a good correlation with the degree of lipid peroxidation and secondary injury in experimental spinal cord injury models.[2,22,32]

Endogenous glutathione has a significant preventive effect against secondary injury in experimental spinal cord injury.[38] NAC is a precursor of glutathione and an agent with frequently cited neuroprotective effects as a potent antioxidant.[3,39] Lin et al.,[40] in a study on the neuroprotective effects of NAC on the apoptosis-inducing effects of synthetic gingerdion compounds on cortical neuron cultures, found that NAC takes on a neuroprotective effect by affecting genes such as p42 and p44 extracellular signal-regulator kinase (ERK), p38 mitogen-activator protein kinase (MAPK) and p53. In a study where they compared the effects of NAC, α-TOC, ebselen, and S-allyl-L-cysteine (SAC) on the toxicity induced in the cerebellar granular neurons by the neurotoxic compound of 4-hydroxynonenal (HNE). Arakawa et al.[41] reported that NAC prevents the neural death induced by HNE in the cerebellar granular neurons by protecting the mitochondrial membrane potential and intracellular glutathione levels, but also that they were unable to identify any similar effects of other antioxidants such as TOC, SAC and ebselen. In a different study on the same topic, Arakawa et al.[42] concluded that low concentration NAC pretreatment also protects cerebellar granular neurons against HNE damage. Jayalakshmi et al.[43] stated that they used NAC against the oxidative stress induced by the hypoxy in hippocampal neuron cell culture and that it had significant neuroprotective activity. In a study by Hart et al.,[44] a daily dose of 150 $mg.kg^{-1}.day^{-1}$ NAC was shown to decrease glial cell death after sciatic nerve injury when the TUNEL method was used. The researchers stated that even a daily dose of 30 $mg.kg^{-1}.day^{-1}$ NAC had significant neural protective effect. They reported that NAC had neural and mitochondrial protective effects after nerve injury and that it prevented sensorial neural death.

N-acetylcysteine has been shown to have favorable effects on central nervous system ischemia and ischemia/reperfusion models. Khan et al.[45] found that when a dose of 150 $mg.kg^{-1}$ NAC was used intraperitoneally in rats with temporary focal cerebral ischemia immediately and 6 h after the perfusion, it protected against free oxygen radical injury, apoptosis and inflammation. In a study conducted by Cakır et al.,[46] the effects of 50 $mg.kg^{-1}$ NAC and hypothermia following spinal ischemia and reperfusion caused by aortic clamping in rabbits were compared, and it was found that NAC and hypothermia together and separately protected against spinal cord injury following ischemia and reperfusion. Similarly, Boga et al.[47] also underlined the favorable effects of NAC, which they used in an ischemia-reperfusion model in rats.

However, the effects of NAC on traumas of the central nervous system are not completely understood. Hicdonmez et al.[3] assessed the effects of single dose 150 $mg.kg^{-1}$ NAC on tissue MDA, SOD, glutathione

peroxidase and catalase activity in rats given experimental closed head trauma. They reported favorable effects of NAC treatment on the oxidative brain tissue injury induced by the trauma. However, Thomale et al.[48] found NAC to be ineffective against posttraumatic perfusion, brain edema or the contusion volume after brain injury in rats with moderate left focal cortical contusion trauma when 163 mg.kg^{-1} NAC was intraperitoneally applied 2-4 h after the trauma.

In accordance with these results, we also found that a dose of 150 mg.kg^{-1} NAC used alone and in combination with methylprednisolone in experimental spinal cord injury created a significant decrease in the MDA and increase in the SOD values when compared to the control group.

There are few studies on the neural effects of NAC in humans. Lin et al.[49] used 1200 mg oral NAC in patients receiving oxyplatin-based adjuvant chemotherapy due to colon cancer and concluded that the oxyplatin they used induced neuropathy.

Although the positive effects of NAC on experimental neural protection and injuries have been documented in many studies, those reporting otherwise also exist in the literature. Similar to our study, Kaynar et al.[50] reported to have found a single dose NAC ineffective in rats given experimental spinal cord injury with an aneurysm clip. However, in contrast with our study, the rats in this study were decapitated an hour after spinal cord injury and biochemical examinations were performed.

While the algorithm of methylprednisolone treatment in our day is identified through randomized studies using large patient series,[51-54] a consensus has not been reached yet about the timing, effective dose intervals and maintenance dose of other drug groups effective against secondary injury. Khan et al.[45] tried using different doses of NAC ranging between 50 mg.kg^{-1} and 500 mg.kg^{-1} applied intraperitoneally at 6-h intervals after perfusion in rats with temporary focal cerebral ischemia, and reported that the best protective effect was displayed at 150-250 mg.kg^{-1}. In clinical toxicology applications of NAC as well, acceptable doses that can be used in humans for paracetamol overdose are reported to be 150 mg.kg^{-1}.[39,55] In this study, we thus used 150 mg.kg^{-1}, which is currently used in humans and has been shown to be useful through experimental studies.

Spinal cord injuries are generally comorbid with pulmonary complications. It was found in one study that 51.1% of patients with T1-T6 spinal cord injury developed respiratory complications. These patients had significantly increased intubation needs, risk for pneumonia and mortality rates. Patients with respiratory complications are known to carry 26.7 times increased relative mortality risk.[56] Agents such as NAC have also been suggested for the treatment of atelectasis, which is among the respiratory complications that accompany spinal cord trauma, and the developing mucosa flaps.[57]

We have thus concluded that methylprednisolone, NAC and the two agents in combination may be useful in preventing secondary damage in experimental spinal cord injury. Prospective clinical studies are needed about the neural protective effects of NAC, which should be used more routinely in the treatment of spinal cord injury and against respiratory problems patients may have.

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