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N-acetylcysteine reduces ischemia/reperfusion induced spinal cord injury: an experimental study

N-asetilsistein iskemi/reperfüzyona bağlı spinal kord hasarını azaltmaktadır: Deneysel çalışma

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Background: In this study, we investigated the protective effect of the systemic infusion of N-acetylcysteine (NAC) on spinal cord ischemia/reperfusion injury.

Methods: Sixteen rabbits with a mean weight of 2.68±0.36 kg were randomly assigned either to group NAC (n=8; receiving NAC) or group C (n=8; control group). They underwent a 30-minute period of spinal cord ischemia with double-clamp technique by clamping the abdominal aorta near below the left renal artery and near above the aortic bifurcation. Fifteen minutes before clamping, rabbits received either intravenous NAC (200 mg/kg; group NAC) or normal saline (group C). The subjects were monitored for 24 hours postoperatively and neurological scores were estimated using Tarlov scoring system. In spinal cord tissue samples, levels of malondialdehyde and gluthathione were also measured.

Results: The mean Tarlov score in the treated subjects was significantly higher compared to the controls (3.38±1.30 vs 0.25±0.46; p<0.001). Histopathological examination revealed that the integrity of the spinal cord was relatively preserved in the NAC group, whereas spinal cords from controls indicated evidences of acute neuronal injury. Spinal tissue malondialdehyde levels were significantly lower in the NAC group (21.57±1 vs 30.4±0.76; p<0.001), whereas glutathione levels were significantly higher (1.73±0.10 vs 1.41±0.05; p<0.001).

Conclusion: In this experimental model of spinal cord injury, NAC provided clinical and histopathological improvement. It is suggested that the effects of NAC are owing to its capacity to reduce oxidative stress and enhance the antioxidant properties of the tissues.

Key words: Ischemia; N-acetylcysteine; spinal cord.

Amaç: Bu çalışmada sistemik N-asetilsistein (NAC) infüzyonunun spinal kord iskemi reperfüzyon hasarı modelinde koruyucu etkisi araştırıldı.

Çalışma planı: Ortalama ağırlıkları 2.68±0.36 kg olan 16 Yeni Zelanda cinsi beyaz tavşan randomize olarak, grup NAC (n=8, NAC infüze edilen) ve grup C (n=8, kontrol grubu) olarak iki gruba ayrıldı. Sol renal arterin hemen altından ve aortik bifürkasyonun hemen üzerinden olmak üzere çift klemp tekniği ile abdominal aort klemplenerek 30 dk. süre ile spinal iskemi oluşturuldu. Klemplemeden 15 dakika önce tavşanlara grup NAC'de 200 mg/kg NAC, grup C'de ise serum fizyolojik verildi. Denekler ameliyattan sonra 24 saat süreyle takip edildi ve Tarlov skorlamasına göre nörolojik skorlama yapıldı. Spinal kord doku örneklerinde malondialdehit ve glutatyon düzeyleri ölçüldü.

Bulgular: Tedavi uygulanan deneklerin ortalama Tarlov skorları kontrol grubundakilerden anlamlı şekilde yüksekti (0.25±0.46'ya kıyasla 3.38±1.30, p<0.01). Histopatolojik değerlendirmede kontrol grubunda akut nöronal hasar bulguları saptanırken, NAC grubunda nöron bütünlüğünün kontrol grubuna oranla korunduğu gözlendi. N-asetilsistein verilen grupta spinal doku malondialdehit düzeyleri önemli ölçüde düşük iken (30.4±0.76'ya kıyasla 21.57±1, p<0.001), glutatyon seviyesi önemli ölçüde yüksekti (1.41±0.05'e kıyasla 1.73 ±0.1, p<0.001).

Sonuç: Deneysel spinal kord hasar modelinde NAC infüzyonu klinik ve histopatolojik iyileşme sağlamıştır. Bu etkilerin NAC oksidatif stresi azaltıcı ve dokunun antioksidan özelliğini artırıcı özelliğinden kaynaklandığı düşünülmektedir.

Anahtar sözcükler: İskemi; N-asetilsistein; spinal kord.

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The surgical treatment of thoracoabdominal aneurysms remains a challenging surgical procedure with a recognized incidence of significant postoperative neurological complications. Because of recent advances in anesthesia and improved surgical techniques, the incidence of intractable neurological complications has declined, but the rate of paraplegia and paraparesis still ranges from 5 to 40%.[1] Numerous techniques, including the use of the shunts, hypothermia, cerebrospinal fluid drainage, infusion of free radical scavengers, and intrathecal injection of neuroprotective agents, have been used clinically and experimentally in attempts to protect patients from neurological damage.[2,3] However, none of these have prevented this unpredictable complication in all patients.

The causative mechanism behind spinal cord ischemia/reperfusion (I/R) injury is likely multifactorial. It is known that the major proportion of the damage occurs during reperfusion when free oxygen radicals induce lipid peroxidation. This leads to functional and structural deterioration. Therefore, prevention of oxidative stress (OS)-induced lipid peroxidation seems to be an important objective among those striving to protect the spinal cord from I/R injury.[4,5] N-acetylcysteine (NAC) is a well known antioxidant that has been proven to protect against I/R-induced injury to different organs and to abrogate adult respiratory distress syndrome.[6-8] Some studies have suggested that it reduces I/R damage in the spinal cord and provides a better neurological outcome within a variable dose range.[9-13] However, clarification is still needed regarding the different aspects of spinal cord protection with NAC.

In the present study, the aim was to examine the protective effects of NAC against OS during an experimentally-induced spinal cord I/R injury in rabbits. Our assessment was controlled and included the evaluation of biochemical, morphological, and clinical parameters. To assess the drug's ability to attenuate or possibly even eliminate neurological dysfunction, we evaluated hind-limb motor function, and to evaluate the drug's effect on actual tissue injury, we performed a histopathological examination of the spinal cords. Finally, to assess the possible mechanisms behind any protection that might exist, the effects of NAC administration against OS were evaluated by measuring spinal cord levels of malondialdehyde (MDH), the main product of lipid peroxidation in neuronal tissues and other cells[14] and glutathione (GSH), an important endogenous antioxidant.[15]

MATERIALS AND METHODS

Sixteen New Zealand white rabbits (mean weight: 2.68±0.36 kg) were randomly divided into two equal groups of eight animals. Preoperatively, the animals were allowed access to standard rabbit food and water, ad libitum. All rabbits were neurologically intact before the administration of anesthesia. The experimental protocol was approved by the institutional ethics committee, and all experiments were carried out in full accordance with the Principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals (NIH Publ. No.80-23, revised 1985).

Anesthesia and monitoring

The animals were anesthetized by an intramuscular injection of ketamine (50 mg/kg) and xylazine (5 mg/kg). Supplemental intravenous doses of ketamine were administered as needed throughout the experiments. After ensuring an adequate depth of anesthesia, the abdominal and thoraco-lumbar skin of the animals was shaved, and the marginal ear vein was cannulated with a 24-G cannula to administer fluid and medication. In addition, one of the marginal ear arteries was cannulated to obtain blood samples and monitor blood pressure during the surgery. Cefazolin (10 mg/kg) was given to all animals as a single dose, and 0.9% sodium chloride (NaCl) (20 ml/kg/h) was infused during the surgery. Body temperature was regulated by means of a heating lamp used throughout the procedure. The hemodynamics, including blood pressure and heart rate, were monitored continuously with a Propaq 104EL monitor (Welch Allyn Protocol, Inc., Beaverton, Oregon, USA) and recorded at baseline and at 10-minute intervals during the cross-clamping and reperfusion periods.

Surgical procedure

After making a midline laparotomy incision of approximately 5 cm in length, the abdominal aorta was explored. After reflection of the intestine to the right, the abdominal aorta was dissected just caudal to the left renal artery and above the aortic bifurcation. The aorta was then encircled with a silk ligature, both distal to the left renal artery and proximal to the aortic bifurcation to facilitate secure occlusion. After surgical preparation, the eight rabbits in the intervention group were infused with 200 mg/kg NAC as a single dose (Hüsnü Arsan İlaçları A.Ş, İstanbul, Turkey), and the eight rabbit controls were infused with the same volume of physiological saline. These infusions were performed 15 minutes before cross-clamping the aorta. All animals were given heparin sodium (100 units/kg) five minutes before cross-clamping for anticoagulation. At the time

of clamping, the aorta, both distal to the left renal artery and proximal to the bifurcation, was occluded with atraumatic vascular clamps so that spinal cord ischemia was induced. After 30 minutes of ischemia, the ligatures and cross-clamps were removed, the abdomen was closed, and the animals were allowed to recover from anesthesia. The choice of 30 minutes of spinal ischemic insult was based upon the results of our previous experimentation using this model.[16,17] Following the recovery of the rabbits, they were returned to their cages and again permitted free access to tap water and food, ad libitum.

Neurological evaluation

The post-I/R neurological status of each animal was rated by assessing hind-limb function 24 hours after the procedure using the modified Tarlov scoring system.[18] The status of the rabbits was assessed by two researchers who were blinded to the treatment arm (intervention versus control). During neurological rating, a score of 0 to 5 was assigned to each animal as follows: 0= no voluntary hind-limb movement; 1= perceptible movement of joints; 2= active movement, but unable to sit without assistance; 3= able to sit, but unable to hop; 4= weak hop; 5= complete recovery of hind-limb function.

Tissue sampling and histopathological evaluation

All rabbits were sacrificed using sodium pentobarbital (100 mg/kg) administered intravenously through an ear vein 24 hours after reperfusion. Spinal cord specimens were resected for pathological assessment as well as for the measurement of the two biochemical markers. Lumbar segments (L4-L5) of the spinal cords were then immediately procured and flash-fixed in 10% buffered formalin. These segments were embedded in paraffin, and serial transverse sections were cut (4μ) for hematoxylin-eosin (H-E) and phosphotungstic acid (PTA) staining. The histopathologists, who were also blinded to the treatment arm, then performed their evaluations. The existence of perineuronal edema, glial cell proliferation, Nissl bodies, and new capillary proliferation was graded qualitatively. Grade 1 indicated a mild-to-moderate increase in these parameters while grade 2 indicated a severe increase in these parameters. Five serial sections from each animal were graded according to these criteria, and the results in the intervention group and control group were compared.

Analysis of the biochemical markers of oxidative reactions

Spinal tissue MDH and GSH levels were measured in the lumbar part of the spinal column (SC) by spectrophotometry,[19] and the samples were stored at -70 °C until analysis. Lipid peroxidation was quantified by measuring the formation of thiobarbituric acid reactive substances (TBARS). The tissue samples were then briefly homogenized in ice-cold trichloroacetic acid (1 g tissue in 10 ml 10% trichloroacetic acid) in a Heidolph Diax 900 tissue homogenizer (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany). Following centrifugation of the homogenate at 3.000 rpm for 10 minutes (Hermle Z 323 K, HERMLE Labortechnik GmbH, Wehingen, Germany), 750 μl of supernatant was added to an equal volume of 0.67% (m/v) thiobarbituric acid and heated at 100 °C for 15 minutes. The absorbance of the samples was measured at 535 nm using a Shimadzu UV-1208 spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, Maryland, USA). Lipid peroxide levels were expressed in terms of MDA equivalents using an extinction coefficient of $1.56x10^{-5}$ $mol^{-1}cm^{-1}$.

Glutathione levels were determined by means of a modified Ellman method.[20] After centrifugation of the homogenate at 3.000 rpm for 10 min, 0.5 ml of supernatant was briefly added to the 2 ml of 0.3M $Na_2HPO_42H_2O$ solution. Then 0.2 ml of dithiobisnitrobenzoate (0.4 mg/ml 1% sodium citrate) was added, and the absorbance at 412 nm was measured immediately after mixing. The GSH levels were then calculated using an extinction coefficient of 13.600 mol^{-1} cm^{-1}.

Statistical analysis

All analyses were performed using the Statistical Package for the Social Sciences (SPSS, Inc, Chicago, Illinois, USA) for Wíndows version 11.5, and all values were presented as means ± standard deviation. Mann Whitney U-tests were used to compare the two groups with respect to mean baseline weight, mean heart rate, and blood pressure at baseline during cross-clamping as well as the reperfusion and mean Tarlov scores. In addition, spinal cord MDH and GTH levels at 24-hour post-reperfusion were also compared. Three repeat measurements of the blood pressure and heart rate were compared using the Friedman test. The post-hoc multiple comparisons test was used to identify different pairs after statistically significant Friedman test results. The histopathological differences in serial sections were analyzed using Pearson's chi-square test. A p value of less than 0.05 was considered statistically significant.

RESULTS

All of the rabbbits tolerated the operation well, and no adverse events were observed during the surgery. The hemodynamic data, including the heart rate and systemic blood pressure, were similar in both

Table 1. Hemodynamic variables in the control group and the intervention group treated with N-acetylcysteine

	Control group	Intervention group
	p	*p*
Mean heart rate		
Basal	169.00	185.38
Cross-clamp (10 min.)	159.30	179.87
Reperfusion (10 min.)	166.75	177.50
Mean blood pressure		
Basal	63.38	66.00
Cross-clamp (10 min.)	70.50	59.50
Reperfusion (10 min.)	54.63	41.38

A *p* value of less than 0.05 was considered statistically significant; min: Minutes.

groups (Table 1). The electrocardiograms, which were monitored throughout the surgery, were normal in all of the animals.

Neurologic functional evaluation

The mean neurological score of the rabbits in the intervention group were significantly higher after 24 hours of reperfusion (Figure 1). The mean Tarlov score was 3.38±1.30 in the intervention group and 0.25±0.46 in the control group (p=0.000<0.001). Among the treated animals, two were classified as grade 5 at 24-hour reperfusion, two were grade 4, one was grade 3, and three were grade 2. The controls had six rabbits categorized as grade 0 at 24-hour reperfusion and two were grade 1.

Histopathology

A comparison of the intervention and control groups revealed significant differences in histopathology (p=0.000<0.001). The group treated with NAC appeared to be almost intact when examined with light microscopy, with only minimal evidence of cellular damage. The neurons, vascular structures, and glial cells seemed to be nearly normal, with only mild pericellular edema (Figures 2a and 2b). An examination of the control group spinal cords revealed considerable neuronal degeneration. Perineuronal edema was also increased in this group. Additionally, the ependymal cells had euchromatic nuclei and seemed to be swollen. There also was an increased proliferation of capillaries and glial cells, especially around the neurons (Figures 2c and 2d). Furthermore, there were many areas of vacuolar degeneration in the anterior and posterior horns of the gray matter in the control group. Moreover, higher numbers of Nissl bodies were noted in the PTA-stained slides from the rabbits in the control group, whereas few were observed in those treated with NAC.

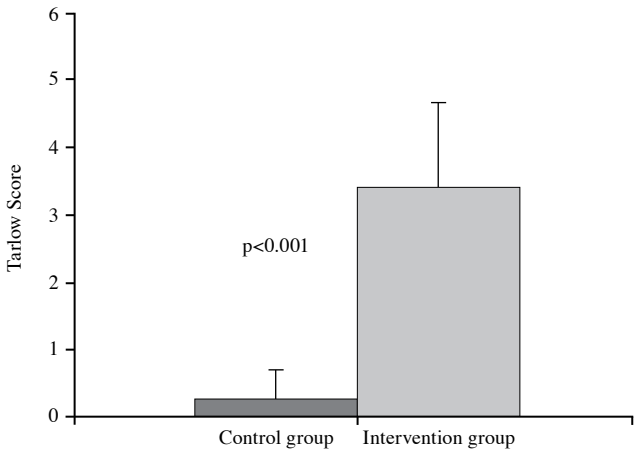

Figure 1. The mean neurological scores demonstrated a significant difference (p<0.001) between the control group and the intervention group 24 hours after reperfusion.

Biochemical markers

The mean spinal tissue MDA level was significantly lower in the intervention group (21.57±1.28 nmol/g) versus the controls (30.4±0.76 nmol/g) (p=0.000<0.001) (Figure 3). Conversely, the mean tissue GSH level was significantly higher in the intervention group (1.73±0.10 µmol/g) than in the control group (1.41±0.05 µmol/g) (p=0.000<0.001) (Figure 4).

DISCUSSION

Spinal cord injuries present a high rate of morbidity and mortality. The damage and subsequent neurological complications are primarily associated with reperfusion and the duration and severity of ischemia.[21,22] Therefore, research associated with new surgical techniques and pharmacological agents is continuing in order to prevent spinal cord injuries associated with spinal I/R.[1,16,17]

Oxidative stress has been significantly implicated in the pathogenesis of neurological injuries after spinal cord ischemia.[21-23] At the time of reperfusion, the reestablishment of flow replenishes the tissue with vital substances but also releases enormous amounts of toxic metabolites, including free oxygen radicals that were generated during the ischemic period, into the circulation. These radicals are potent initiators of protein degradation and lipid peroxidation, which can in turn lead to membrane dysfunction, alterations in cellular proteins, and cell death.[22,23]

Malondialdehyde and GSH are important in that they can be used to monitor the oxidative and anti-oxidative status of I/R states. Malondialdehyde is the main product of lipid peroxidation in spinal myelin, glial and

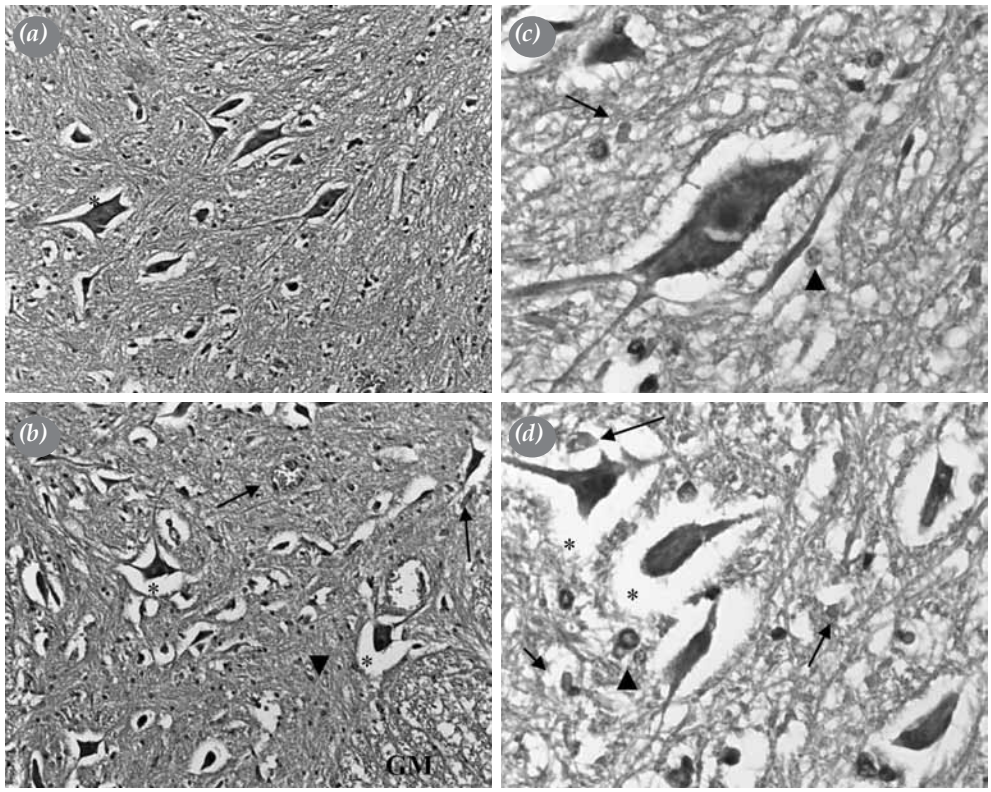

Figure 2. (a) The intervention group (x100); **(b)** The intervention group (x400); **(c)** The control group (x100); **(d)** The control group (x400). Light photomicrographs of spinal cord sections stained with H-E. The control animals exhibited severe pericellular edema, glial cell proliferation, neovascularization, and perineuronal edema. The rabbits treated with N-acetylcysteine demonstrated relative preservation of tissue architecture along with almost complete preservation of the neurons, vascular structures, and glial cells along with only mild pericellular edema. Asterisk: Pericellular edema; Arrow: Neovascularization; Arrow-head: Glial cells; GM: Gray matter.

neuronal membranes, and other cellular elements.[14,24] On the other hand, GSH is an important endogenous anti-oxidant. It reacts with free radicals and further protects cells by maintaining high cellular GSH levels so that the magnitude of the destructive potential of free oxygen radicals during reperfusion can be reduced.[25,26] It has been shown that promotion of GSH synthesis is an effective way to decrease post-traumatic OS, thereby fostering the retention of tissue integrity and function after spinal cord trauma.[27]

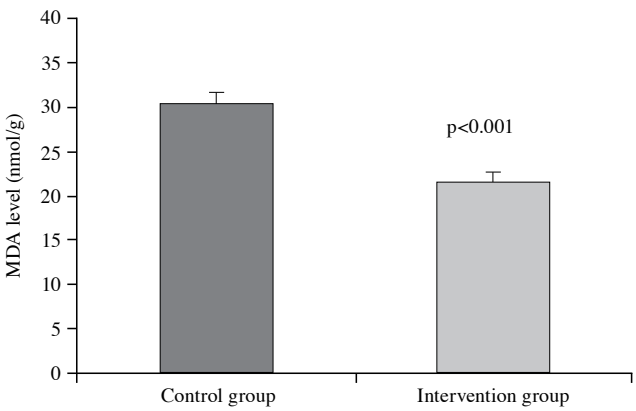

Figure 3. Malondialdehyde (MDA) levels in lumbar spinal column segments 24 hours after reperfusion.

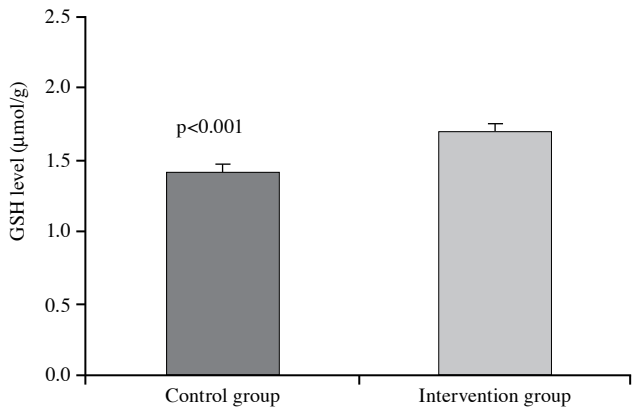

Figure 4. Glutathione (GSH) levels in lumbar spinal column segments 24 hours after reperfusion.

In our study, spinal cord MDH levels 24 hours after aortic occlusion were significantly elevated in the control group, implying that free oxygen radicals are involved in I/R neuronal injury. Conversely, we observed less of an increase in the MDH content of the spinal cord when NAC was administered before aortic occlusion. This suggests that NAC reduces OS-induced lipid peroxidation. In addition, higher GSH levels in the rabbits treated with NAC suggest that pre-ischemic NAC administration enhances anti-oxidant activity in the spinal cord.

This intervention group also exhibited better preservation of neurological functions and histological architecture 24 hours after the I/R event, which is consistent with the aforementioned biochemical marker findings.

Several studies have demonstrated that NAC administration ameliorates ischemic myocardial, liver, lung, brain, and muscle injuries.[6,28-31] For example, NAC has been used for many years in the treatment of chronic bronchitis. Cysteine, which functions as an anti-oxidant, is a precursor of GSH, a tripeptide present in high concentrations in most cells, and NAC, which is necessary for GSH regeneration, is a direct scavenger of free radicals and also inhibits inducible nitric oxide (NO) synthase expression along with the expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1).[32,33]

Khan et al.[11] reported that the best protective effect of NAC was displayed at 150-250 mg/kg in rats with temporary focal cerebral ischemia. The dose of NAC we used was average relative to the doses used in previously-reported I/R studies.[8-11,13,29,34] Çakır et al.[9] demonstrated that NAC and hypothermia protect rabbit spinal cords against ischemic injury. In their study, the combination of NAC and hypothermia offered superior protection to the use of NAC alone. Ortiz-Gómez et al.[13] reported on cerebrospinal fluid decompression in which the combination of methylprednisolone and NAC reduced the complications of acute paraplegia. In a renal I/R study, Erbaş et al.[34] found that the protective effect of NAC could be the result of the stimulatory effect that it has on arginase activity, which may result in the inhibition of inducible NO activity. This would then lead to decreased plasma NO levels.

Hancı et al.[12] investigated the biochemical effectiveness of methylprednisolone and NAC in experimental spinal cord injuries in rats and found potential biochemical benefits in preventing secondary injuries. They found lower mean MDH values and higher superoxyde dismutase values compared to the control group. These results were similar to our study, but we also showed histopathological and functional improvement in the rabbits treated with NAC.[12]

Some of the beneficial effects of NAC have been attributed to its chemical structure, which includes a compound containing thiol. In studies involving cardiopulmonary bypass (CPB) and pre-conditioning, thiol-containing compounds have been shown to be beneficial as a potential defense system against I/R reperfusion-related OS.[35] According to Fischer et al.,[8] 100 mg/kg of NAC administered 10 min before CPB followed by 20 mg/kg/h of continuous infusion until one hour after CPB reduces OS during this process. In addition, Koramaz et al.[36] reported that NAC-supplemented cold-blood cardioplegia minimizes myocardial injury in the early hours both during and after cardiac surgery. Andersen et al.[37] demonstrated that the anti-oxidant and scavenger effects of NAC reduce the neutrophil oxidative burst response usually observed in patients subjected to CPB and cardioplegic arrest. N-acetylcysteine also has been used successfully in clinical studies for the treatment of acute myocardial infarction.[38]

In our study, we demonstrated the protective effects of NAC in an experimental spinal cord injury model. New clinical studies have also shown the beneficial effects of NAC in preventing I/R injuries with severe burns and liver transplantation as well as renal and arthroscopic knee surgery.[39-43]

To receive the full potential benefit of its antioxidant properties, NAC administration should probably be initiated before aortic occlusion. Therefore, we did this before cross-clamping so that it might pre-condition the tissue and enhance the tissue's own defenses before exposure to the increased OS that occurs after clamping.

In conclusion, we demonstrated that the prophylactic use of intravenous NAC exerts a protective effect against spinal cord I/R injury in rabbits. This is most likely because of its capacity to reduce OS and enhance the tissue's antioxidant properties. However, our study does not completely elucidate the underlying protective mechanism. The protective effect of NAC is probably multifactorial, and we believe that further experimental and clinical studies are required to further explore its benefits. Once the mechanisms behind this protection are clarified and the optimum dose and dosing schedule are determined, NAC may become a useful therapeutic tool to prevent the neurological sequelae currently associated with surgery for thoracoabdominal aneurysms.

Declaration of conflicting interests

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