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ORIGINAL ARTICLE

NMDA receptor blockage with 2-amino-5-phosphonovaleric acid improves oxidative stress after spinal cord trauma in rats

M Vural[1], A Arslantaş[1], N Yazihan[2], T Köken[3], K Uzuner[4], D Arslantaş[5] and Z Özbek[1]

[1]*Eskişehir Osmangazi University, School of Medicine, Department of Neurosurgery, Eskişehir, Turkey;* [2]*Molecular Biology Research and Development Unit, Ankara, Turkey;* [3]*Kocatepe University, School of Medicine, Department of Biochemistry, Afyon, Turkey;* [4]*Eskişehir Osmangazi University, School of Medicine, Department of Physiology, Eskişehir, Turkey and* [5]*Eskişehir Osmangazi University, School of Medicine, Department of Public Health, Eskişehir, Turkey*

Study design: 2-amino-5-phosphonovaleric acid (APV) is an *N*-methyl-D-aspartate (NMDA) receptor blocker and has neuroprotective properties. This study is aimed at evaluating the effect of APV treatment on oxidative status after spinal cord injury (SCI).

Methods: The experiment was carried out on the following five groups: Group1: sham operated, non-traumatized; Group2: with injured spinal cord, no treatment; Group3: with SCI, injected with 100 $\mu g\,kg^{-1}$ APV; Group4: with SCI, injected with 200 $\mu g\,kg^{-1}$ APV; and Group5: with SCI, injected with 400 $\mu g\,kg^{-1}$ APV. SCI was inflicted by epidural compression with a cerebral vascular clip after T9–11 laminectomy. The experiments were completed after 12 h of trauma. Spinal cords were excised for evaluation of superoxide dismutase (SOD), catalase, reduced glutathione (GSH) and malonyldialdehyde (MDA) levels.

Results: After SCI, SOD and GSH levels decreased and the MDA level increased significantly. APV treatment decreased the MDA level and increased SOD, catalase and GSH levels. The maximum decrease in MDA was detected in the group treated with 100 $\mu g\,kg^{-1}$ APV compared with the other groups. The GSH level was significantly increased in the group treated with 200 $\mu g\,kg^{-1}$ APV. The SOD level was significantly increased in the group treated with 200 $\mu g\,kg^{-1}$ APV.

Conclusion: The results of this study have shown that APV treatment creates a dose-dependent antioxidant effect in rats with SCI and may be used for the treatment of SCIs.

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Keywords: experimental spinal cord injury; 2-amino-5-phosphonovaleric acid (APV); NMDA receptor blocker; oxidative stress; GSH; MDA

Introduction

Spinal cord injury (SCI) is a complex process in which neuronal tissue and vascular structures are damaged. In spite of intensive investigations, the mechanisms responsible for the destructive cascades of events in SCI are still unclear. Many potentially toxic substances are activated and released in injured spinal cords, including free radicals, phospholipases, lipid peroxidases, vasoactive eicosanoids and glutamate.

Oxygen-free radicals break apart in oxidative damage into important cellular components, including proteins, nucleic acids and lipids.[1] Oxidative stress occurs from a disequilibrium in the production of reactive oxygen species and antioxidative processes in favor of a production of oxidative radicals. The enzymatic and non-enzymatic antioxidant defense system, which includes catalase, superoxide dismutase (SOD) and glutathione (GSH) peroxidase, counteracts and regulates overall reactive oxygen species levels to maintain physiological homeostasis of cells and organ systems. Catalase and GSH peroxidase are involved in the detoxification process of H_2O_2 and peroxynitrite, peroxides. Endogenous antioxidant enzymes increase in the late stage of SCI. This process decreases the destructive potential of these free radicals. The activity of catalase and levels of GSH increase within 24 h after injury, whereas the endogenous SOD enzyme activity remains unchanged. Antioxidant enzyme levels were found unchanged at 4 h after trauma.[2–4]

In recent years, evidence supporting the involvement of excitatory amino acids in the development of secondary pathology after SCI has been reported. Glutamate is an excitatory neurotransmitter to the central nervous system; an increase in released glutamate after trauma is toxic to neuronal cells. Significant increases in tissue levels of

Correspondence: Dr M Vural, Eskisehir Osmangazi University, School of Medicine, Department of Neurosurgery, Meselik, Eskisehir 26480, Turkey.
E-mail: mvural@ogu.edu.tr
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glutamate are found in areas of traumatic or ischemic brain injury. *N*-methyl-D-aspartate (NMDA) glutamate receptors mediate Ca^{2+} accumulation in central myelin in response to chemical ischemia *in vitro*.[5] A prolonged activation of these receptors leads to increased intracellular concentrations of sodium, potassium and/or calcium ions. These changes in ionic gradients initiate a sequence of events that ultimately lead to cell death. Overstimulation of glutamate receptors has been proposed to have a significant effect in oxidative damage associated with neuronal cell death in SCI, and oxidative stress is considered to be a major contributor to the amplification of glutamate-mediated excitotoxicity.[6]

From this point of view, it has been proposed that agents that block glutamate receptors may improve functional outcomes and reduce damage to traumatized spinal cord tissue. 2-amino-5-phosphonovaleric acid (APV), the agent used in our experiment, is an NMDA receptor blocker[7–11] and acts on the glutamate transmitter recognition site of NMDA receptors, preventing receptor activation by competing with agonists for the transmitter-binding site.[12]

In this study, we aimed at determining the effects of different doses of APV on oxidative stress after an experimental SCI in rats.

Materials and methods

Surgical procedures

In this study, Wistar albino rats weighing 200–220 g were used. The rats were provided by the University Experimental and Medical Research Center, Eskişehir, Turkey, and the experimental protocol was approved by the university ethical committee for animal studies. The animals were anesthetized with thiopenthal (40 mg kg^{-1}, intraperitoneally) and were placed in a prone position on a warmed surgical plate to maintain body temperature at 37 ± 0.5 °C throughout the procedure. The back of the rats was shaved and the surgical area was disinfected with betadine solution. A midline longitudinal incision was made at the T8–12 level to expose the dorsal laminae and spinous processes. A limited multilevel posterior laminectomy (T9–11) was performed. SCI was induced by epidural compression with a cerebral vascular clip (Aesculap, Tuttlingen, Germany), which has a closing force of 40 g. Epidural compression was applied for 30 s and the surgical area was closed with primary sutures.[13]

Experimental procedures were carried out on five groups with seven animals in each ($n = 7$/group): Group1: sham-operated control, non-traumatized (water injected intraperitoneally); Group 2: with SCI, no treatment (water injected intraperitoneally); Group 3: with SCI, treated with 100 μg kg^{-1} APV (dissolved in water, Sigma, St Louis, MO, USA); Group 4: with SCI, treated with 200 μg kg^{-1} APV; and Group 5: with SCI, treated with 400 μg kg^{-1} APV. APV was administered intraperitoneally 1 h after injury.

Twelve hours after surgery, the rats were killed under anesthesia and their spinal cords were excised for a length of 2 cm rostrally and 1 cm caudally to the injury site. The excised tissue samples were immediately frozen in liquid nitrogen and deep frozen at −76 °C for biochemical studies. Until that time, the rats had free access to food and water. Bladders were emptied by manual pressure every 4–5 h.

Statement of Ethics. We certify that all applicable institutional regulations with regard to the ethical use of animals were followed during the course of this research.

Biochemical measurements

Tissues were homogenized in 0.1 M phosphate buffer (pH 7.4) with Ultra Turrax homogenizer (IKA T18 basic, Wilmington, NC, USA), then centrifuged at 5000 r.p.m., +4 °C for 10 min. After homogenization, supernatants were removed and used to determine oxidative stress markers. Tissue protein concentration was measured using the Biuret method. Chemicals used in the evaluation of tissue catalase, GSH and malonyldialdehyde (MDA) were purchased from the Sigma Chemical (St Louis, MO, USA).

SOD assay

Tissue SOD (E.C.1.15.1.1) activities were measured according to a modified method described by Winterbourn.[14] It is based on the ability of the enzyme to inhibit the reduction of nitroblue tetrazolium by superoxide, which is generated by the reaction of photo-reduced riboflavin and oxygen. The results were expressed as units of SOD per milligram of tissue protein, and 1 U is defined for a particular system as that amount of enzyme causing half of the maximum inhibition of nitroblue tetrazolium reduction. This method was calibrated using Sigma-purified bovine SOD.

Reduced GSH assay

Glutathione was measured by a colorimetric reaction, based on the oxidation of the reduced form of GSH by the aromatic disulphide compound, 5,5′-dithiobis(2-nitrobenzoic acid), to form the oxidized form of GSH and the aromatic thiol, 5-thio-2-nitrobenzoic acid.[15] The yellow color formed because of the reduction of 5,5′-dithiobis(2-nitrobenzoic acid) was measured at 412 nm, and it was proportional to the amount of GSH present in the sample.

MDA assay

Malonyldialdehyde was measured using the thiobarbituric acid method described by Ohkawa *et al*.[16] This method measures several aldehydes derived from lipid hydroperoxide and is also known as thiobarbituric acid reactive substance. MDA constitutes one indicator of oxidative stress, as it arises from the breakdown of lipid peroxyl radicals. MDA is also important in that it can cause further oxidative injury by oxidizing protein molecules.

Catalase assay

Catalase (H_2O_2: H_2O oxidoreductase, E.C.1.11.1.6) activities were measured by the methods of Beutler, in which the disappearance of peroxide is followed spectrophotometrically at 240 nm. One unit decomposes 1 μmol of H_2O_2 per minute at 25 °C and pH 7.0 under specified conditions.[17]

Statistics

All the data collected from the experiment were coded, recorded and analyzed using SPSS 10.0.1 for Windows (SPSS Inc, Chicago, IL, USA). One-way analysis of variance was used to compare differences among groups. When the analysis of variance showed a significant difference, the *post hoc* multiple comparison test was applied to show the differences. In each test, data were expressed as the mean value ± s.d., and $P<0.05$ was considered to be statistically significant.

Results

All the animals subjected to SCI by clipping developed a flaccid paraplegia immediately after surgery. A gross clinical evaluation of injury and measurement of residual limb motor functions were performed. No tests were used for clinical assessments. Neurological improvement was interpreted on the basis of our observation. In APV-administered groups, slight movements in lower extremities were observed, whereas in the non-treated group, there were nearly no functions.

Parameters of oxidative stress

Spinal cord injury significantly decreased the spinal tissue GSH level compared with the GSH level of the sham group ($P<0.001$, Figure 1) and it was increased in the APV-treated SCI groups compared with the level in the non-treated SCI group ($P<0.001$, Figure 1). The differences between the GSH levels of the non-treated SCI group and the group treated with 100 μg kg^{-1} APV, between the GSH levels of the non-treated SCI group and the group treated with 200 μg kg^{-1} APV, and between the GSH levels of the non-treated SCI group and the group treated with 400 μg kg^{-1} APV, were statistically significant ($P<0.001$, Figure 1). The GSH level was markedly increased in the group that had received

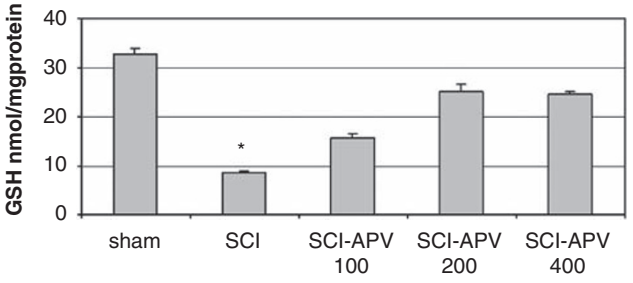

Figure 1 Tissue glutathione (GSH) levels. 2-amino-5-phosphonovaleric acid (APV) treatment (spinal cord injury (SCI)-APV) increased tissue GSH levels compared with those in non-treated rats with SCI. Data are presented as mean ± s.d. values of seven animals. '*' represents $P<0.001$ difference between the non-treated SCI group and the sham-operated group, and between the non-treated SCI group and the APV-treatment groups. The GSH level was 32.65 ± 3.39 nmol mg^{-1} protein in the sham-operated group; 8.6 ± 0.41 nmol mg^{-1} protein in the nontreated SCI group; 15.64 ± 2.51 nmol mg^{-1} protein in the group treated with 100 μg kg^{-1} APV; 25 ± 4.41 nmol mg^{-1} protein in the group treated with 200 μg kg^{-1} APV; and 24.43 ± 1.6 nmol mg^{-1} protein in the group treated with 400 μg kg^{-1} APV.

treatment with 200 μg kg^{-1} APV compared with those of the groups that received 100 μg kg^{-1} APV and 200 μg kg^{-1} APV. The GSH level was 32.65 ± 3.39 nmol mg^{-1} protein in the sham-operated group; 8.6 ± 0.41 nmol mg^{-1} protein in the non-treated SCI group; 15.64 ± 2.51 nmol mg^{-1} protein in the group treated with 100 μg kg^{-1} APV; 25 ± 4.41 nmol mg^{-1} protein in the group treated with 200 μg kg^{-1} APV; and 24.43 ± 1.6 nmol mg^{-1} protein in the group treated with 400 μg kg^{-1} APV (Table 1).

Spinal cord injury did not cause a significant change in catalase activity compared with that in the sham group ($P>0.05$). Catalase activity was increased in APV-treated groups but the difference was not statistically significant ($P>0.05$, Figure 2). Although it was not statistically significant, catalase level was higher in the group that was treated with 400 μg kg^{-1} APV ($P>0.05$). Catalase level was 5838.12 ± 422.54 U mg^{-1} protein in the sham-operated group; 5619.37 ± 521.8 U mg^{-1} protein in the non-treated SCI group; 6767.62 ± 1164.61 U mg^{-1} protein in the group treated with 100 μg kg^{-1} APV; 6541.5 ± 817.46 U mg^{-1} protein in the group treated with 200 μg kg^{-1} APV; and 7308.12 ± 1508.33 U mg^{-1} protein in the group treated with 400 μg kg^{-1} APV (Table 1).

Spinal cord injury increased and APV treatment with 100 μg kg^{-1} significantly decreased tissue MDA levels ($P<0.001$, Figure 3). At higher doses, less improvement in the MDA level was observed. The differences between the MDA levels of the non-treated SCI group and the group treated with 100 μg kg^{-1} APV ($P<0.001$) and between the MDA levels of the non-treated SCI group and the group treated with 200 μg kg^{-1} APV ($P<0.05$) were statistically significant. Although the MDA level was decreased in the group treated with 400 μg kg^{-1} APV, the difference was not statistically significant compared with the MDA level in the non-treated SCI group ($P>0.05$, Figure 3). The MDA level was 0.33 ± 0.007 nmol mg^{-1} protein in the sham-operated group; 0.98 ± 0.1 nmol mg^{-1} protein in the non-treated SCI group; 0.59 ± 0.1 nmol mg^{-1} protein in the group treated with 100 μg kg^{-1} APV; 0.76 ± 0.22 nmol mg^{-1} protein in the group treated with 200 μg kg^{-1} APV; and 0.91 ± 0.33 nmol mg^{-1} protein in the group treated with 400 μg kg^{-1} APV (Table 1). The MDA level of the group treated with 100 μg kg^{-1} APV was significantly decreased.

The SOD level of the non-treated SCI group was relatively decreased compared with the level of the sham-operated group (Figure 4). APV treatment improved SOD level. The differences between the SOD levels of the non-treated SCI group and the group treated with 100 μg kg^{-1} APV and between the SOD levels of the non-treated SCI group and the group treated with 400 μg kg^{-1} APV were statistically significant ($P<0.05$). In addition, a marked increase in the SOD level of the group treated with 200 μg kg^{-1} APV was observed compared with the SOD level of the non-treated SCI group ($P<0.001$). The SOD level was 39.98 ± 8.42 U mg^{-1} protein in the sham-operated group; 31.79 ± 7.05 U mg^{-1} protein in the nontreated SCI group; 46 ± 13.35 U mg^{-1} protein in the group treated with 100 μg kg^{-1} APV; 52.99 ± 8.14 U mg^{-1} protein in the group treated with 200 μg kg^{-1} APV; and

Table 1 Tissue GSH, catalase, MDA and SOD levels

Group	*GSH (nmol mg^{-1} protein)*	*Catalase (U mg^{-1} protein)*	*MDA (nmol mg^{-1} protein)*	*SOD (U mg^{-1} protein)*
Sham	32.65 ± 3.39	5838.12 ± 422.54	0.33 ± 0.007	39.98 ± 8.42
SCI	8.6 ± 0.41	5619.37 ± 521.8	0.98 ± 0.1	31.79 ± 7.05
SCI-APV 100 μg kg^{-1}	15.64 ± 2.51	6767.62 ± 1164.61	0.59 ± 0.1	46 ± 13.35
SCI-APV 200 μg kg^{-1}	25 ± 4.41	6541.5 ± 817.46	0.76 ± 0.22	52.99 ± 8.14
SCI-APV 400 μg kg^{-1}	24.43 ± 1.6	7308.12 ± 1508.33	0.91 ± 0.33	44.2 ± 10.17

Abbreviations: APV, 2-amino-5-phosphonovaleric acid; GSH, glutathione; MDA, malonyldialdehyde and SOD, superoxide dismutase.
[a]The data was expressed as the mean ± s.d. value.

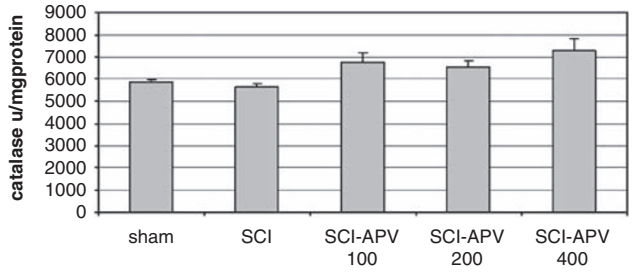

Figure 2 Tissue catalase levels. Data are presented as mean ± s.d. values of seven animals. The catalase level was 5838.12 ± 422.54 U mg^{-1} protein in the sham-operated group; 5619.37 ± 521.8 U mg^{-1} protein in the non-treated SCI group; 6767.62 ± 1164.61 U mg^{-1} protein in the group treated with 100 μg kg^{-1} 2-amino-5-phosphonovaleric acid (APV); 6541.5 ± 817.46 U mg^{-1} protein in the group treated with 200 μg kg^{-1} APV; and 7308.12 ± 1508.33 U mg^{-1} protein in the group treated with 400 μg kg^{-1} APV. Spinal cord injury (SCI) did not cause significant change in catalase activity compared with that of the sham group, but catalase activity was increased in the APV-treated groups ($P>0.05$).

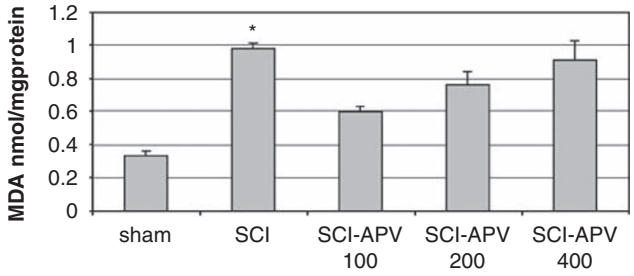

Figure 3 Tissue malonyldialdehyde (MDA) levels. Data are presented as mean ± s.d. values of seven animals. '*' represents $P<0.001$ difference between the non-treated SCI group and the sham-operated and with the 100 μg kg^{-1} 2-amino-5-phosphonovaleric acid (APV)-treated groups. The MDA level was 0.33 ± 0.007 nmol mg^{-1} protein in the sham-operated group; 0.98 ± 0.1 nmol mg^{-1} protein in the non-treated SCI group; 0.59 ± 0.1 nmol mg^{-1} protein in the group treated with 100 μg kg^{-1} APV; 0.76 ± 0.22 nmol mg^{-1} protein in the group treated with 200 μg kg^{-1} APV; and 0.91 ± 0.33 nmol mg^{-1} protein in the group treated with 400 μg kg^{-1} APV.

44.20 ± 10.17 U mg^{-1} protein in the group treated with 400 μg kg^{-1} APV (Table 1).

Discussion

Spinal cord trauma results in rapid and extensive oxidative stress. Decreasing the level of oxidative stress will minimize

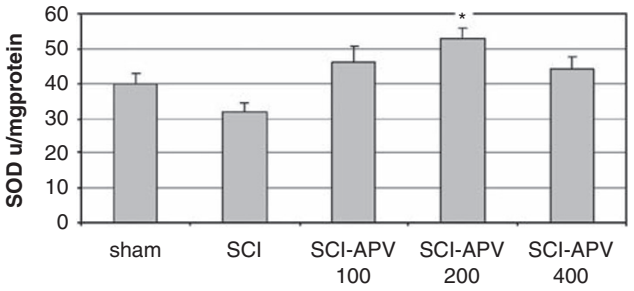

Figure 4 Spinal cord superoxide dismutase (SOD) levels. Data are presented as mean ± s.d. values of seven animals. '*' represents $P<0.001$ difference between the non-treated spinal cord injury (SCI) group and the group treated with 200 μg kg^{-1} 2-amino-5-phosphonovaleric acid (APV); $P<0.05$ signifies the difference between the non-treated SCI group and the group treated with100 μg kg^{-1} APV and between the non-treated SCI group and the group treated with 400 μg kg^{-1} APV. The SOD level was 39.98 ± 8.42 U mg^{-1} protein in the sham-operated group; 31.79 ± 7.05 U mg^{-1} protein in the nontreated SCI group; 46 ± 13.35 U mg^{-1} protein in the group treated with 100 μg kg^{-1} APV; 52.99 ± 8,14 U mg^{-1} protein in the group treated with 200 μg kg^{-1} APV; and 44.20 ± 10.17 U mg^{-1} protein in the group treated with 400 μg kg^{-1} APV.

secondary destruction after traumatic injury.[2,4] Catalase and reduced GSH are among the most important intracellular antioxidant substances against reactive oxygen species. SOD transforms O^{2-} into the less reactive molecule, H_2O_2, which is more stable but still capable of damaging cell membranes. Catalase, which acts on H_2O_2, converts it into water. Two different forms of SOD are found in eukaryotic cells. Copper-zinc SOD is found in the cytosol, whereas manganese-containing SOD is localized to the mitochondrial matrix. All forms of SOD act in the same way and catalyze the dismutation of O^{2-} to H_2O_2.[7] MDA, which arises from the breakdown of lipid peroxyl radicals, is one of the indicators of oxidative stress. MDA is also important in that it can cause further oxidative injury by oxidizing protein molecules.[18] GSH is involved in scavenging both inorganic and organic peroxides. Thus, increased MDA and protein carbonyl levels and loss or oxidation of GSH, and decreased SOD and catalase activities provide physiologically relevant estimates of oxidative stress in tissues even in the neuronal system. Kamencic *et al*.[2] reported that after a SCI, the GSH content of the site of injury and adjacent tissue was reduced to less than half of the normal within 1 h, and over the next 48 h, there was a further decrease in the GSH level of the spinal cord. Oxidative stress consumes GSH either by oxidation or through the formation of adducts with electrophilic

compounds and metals.[2] Our results indicated that APV treatment almost restored the decreased GSH level after SCI in a dose-dependent manner. The increase after treatment is more prominent in higher doses but this increase was not sufficient to improve MDA levels. In this study, we found that APV exerts a modulatory action on the antioxidant defense system in the SCI model by decreasing the lipid peroxidation product MDA and increasing SOD, catalase and GSH levels. An increase in lipid peroxidation causes a disarrangement of endothelial cell membranes, which results in increased hemorrhage and edema after trauma. Increased oxidative stress exacerbates the inflammatory process.

In vivo and *in vitro* studies have shown that ischemia of neuronal tissues activates NMDA receptors and glutamate released to the media at increasing concentrations.[19] Different models of spinal trauma have shown that glutamate receptor antagonists have therapeutic effects in both structural and functional recovery. The non-competitive NMDA ion-channel blocker MK-801 and blockade of the non-NMDA ionotropic receptors with AMPA/kainate receptor antagonist 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzol[f]quinoxaline-7-sulfonamide disodium have both demonstrated significant improvements in trauma models.[20] Our study showed that APV, which is a specific NMDA receptor blocker, may abolish oxidative damage after SCI, but direct effects of APV in glutamate, kainate and NMDA were not studied in neuronal cells. Free radicals induce glutamate release, whereas radical scavengers such as GSH, SOD and catalase reduce the ischemic release of glutamate. The simplest explanation of the protection by NMDA antagonists in ischemia is that they block NMDA receptor-mediated calcium influx into neurons. NMDA receptor blockers decrease the reflow of blood, especially during reperfusion phase. It was shown that in permanent focal ischemia, NMDA receptor blocker MK-801 reduces cytokine production.[9]

There are limitations of this study that must be addressed. First, we did not use any functional or histopathological parameters in the experiment. Second, our study lacks mechanic insights into SCI. On the other hand, our study may constitute a model for further studies in which functional and histopathological aspects of NMDA receptor blockage in SCIs can be studied.

In conclusion, the GSH level was markedly increased with $200\,\mu g\,kg^{-1}$ APV treatment. The most significant increase in the catalase level was in the group treated with $400\,\mu g\,kg^{-1}$ APV. The major decrease in the MDA level was in the group treated with $100\,\mu g\,kg^{-1}$ APV compared with the groups treated with higher doses. The SOD level was increased markedly in the group treated with $200\,\mu g\,kg^{-1}$ APV. Our study showed that APV has a dose-dependent antioxidant effect in experimental SCI. APV ($200\,\mu g\,kg^{-1}$) might be a promising therapeutic agent in the oxidative damage of spinal cord disorders such as spinal trauma. In spite of the limitations of this study mentioned above, the finding of improvement in oxidative damage after three doses of APV treatment is very important. Further *in vitro* and *in vivo* studies are required to determine the proper treatment dose of APV.

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