The Neuroprotective Effects of Ebselen in Experimental Spinal Cord Injury

Deneysel Spinal Kord Travmasında Ebselenin Nöroprotektif Etkilerinin Araştırılması

YUSUF ATİLLA ÜNLÜ, EKREM KAYA, HAFİZE UZUN, ŞEREF BARUT, AHMET BELCE, BÜGE ÖZ, AHMET ÇOLAK

Taksim Education and Research Hospital, Neurosurgery Service, Istanbul, Turkey (YAÜ, EK, ŞB, AÇ)
Istanbul University Cerrahpaşa Medicine Faculty, Department of Biochemistry, Istanbul, Turkey (HU, AB)
Istanbul University Cerrahpaşa Medicine Faculty, Department of Pathology, Istanbul, Turkey (BÖ)

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Abstract: *Objective:* The neuroprotective effects of ebselen were investigated in a rat spinal cord trauma model.
Methods: Thirty-six Wistar albino rats were studied in four groups of nine animals each: sham-operated controls; trauma-only controls; and two ebselen treatment groups (administered 1 hour after injury [E1] and administered 12 hours after injury [E12]). Spinal cord trauma was produced using the clip compression method of Rivlin and Tator. The effects of the injury and the efficacy of ebselen were determined based on histopathological findings and levels of lipid peroxidation, superoxide dismutase, and catalase in the spinal cord tissue. The lipid peroxidation, superoxide dismutase, and catalase levels were determined 24 hours after injury.
Results: The mean lipid peroxidation level in the tissue from the trauma-only group was significantly higher than that in the sham-operated controls (p=0.001), but there were no significant differences between the trauma-only group and the ebselen-treated groups (p=1.00 for E1; p=0.565 for E12). The mean superoxide dismutase activity in the trauma-only group was significantly lower than that in the sham-operated control group (p=0.01). The level in the trauma-only group was also significantly lower than the means in the ebselen-treated groups (p=0.030 for E1; p=0.033 for E12). Regarding catalase, the mean level in the trauma-only group was significantly lower than that in the sham-operated controls (p=0.004), but there were no significant differences between the findings in the trauma-only group and the ebselen-treated groups

Özet: *Amaç:* Bu deneysel çalışmada ratlarda oluşturulmuş spinal kord travma modelinde ebselenin nöroprotektif etkileri araştırıldı.
Metod: Deneyler, kontrol grubu, travma oluşturulan grup, travmadan 1 saat sonra ebselen verilen grup E1 ile travmadan 12 saat sonra ebselen verilen grup E12 olmak üzere 4 eşit gruba ayrılan 36 wistar albino rat üzerinde yapıldı. Spinal kord travması Rivlin ve Tator'un klip kompresyon modeline göre oluşturuldu. Yaralanmanın etkileri ve ebselenin etkinliği, doku lipid peroksidasyonu, superoksid dismutaz düzeyi, katalaz düzeyi ve histopatolojik incelemelerle ölçüldü. Doku lipid peroksidasyon, superoksid dismutaz ve katalaz seviyeleri travmadan 24 saat sonra ölçüldü.
Bulgular: Travma oluşturulmuş grup, kontrol grubu ile kıyaslandığında ortalama lipid peroksidasyon değerlerinde istatistiksel olarak anlamlı farklılık vardı (p=0,001); ancak ebselen tedavi grupları ile travma oluşturulmuş grup arasında anlamlı farklılık gözlenmedi (E1 için p=1,00 ve E12 için p=0,565). Travma oluşturulmuş grup ile kontrol grubu arasında superoksid dismutaz aktivitesinde istatistiksel olarak anlamlı farklılık gözlendi (p=0,01). Aynı anlamlı farklılık travma oluşturulmuş grup ile ebselen tedavi grupları arasında da gösterildi (E1 için p=0,030 ve E12 için p=0,033). Travma oluşturulmuş grup kontrol grubu ile kıyaslandığında ortalama doku katalaz aktivitesinde anlamlı farklılık bulundu (p=0,004); Bununla birlikte travma oluşturulmuş grup ile ebselen tedavi grupları arasında anlamlı farklılığa rastlanmadı (E1 için

(p=1.00 for E1; p=1.00 for E12). Both ebselen-treated groups showed significant histopathological improvement with respect to findings in the trauma-only group.
Conclusion: Administration of a single 10-mg/kg dose of ebselen did not prevent high levels of lipid peroxidation or catalase depression after spinal cord injury in the rat. However, the treatment did raise superoxide dismutase levels, and this helped to repair tissue and preserve tissue architecture. The findings indicate that ebselen may have a potential role in the treatment of acute spinal cord injury.

Key Words: Catalase, ebselen, lipid peroxidation, neuroprotection, spinal cord injury, superoxide dismutase

p=1.00 ve E12 için p=1.00). Buna karşın, ebselen tedavi gruplarında kontrollere oranla önemli histopatolojik iyileşmeler gözlendi.
Sonuçlar: Bu sonuçlara göre 10 mg/kg-tek doz ebselen tedavisi lipid peroksidasyon üretimini ve katalaz depresyonunu önlememiş, ancak superoksid dismutaz düzeyini arttırmış ve histopatolojik iyileşmeye neden olmuştur. Tüm bu bulgular akut spinal kord yaralanmasının tedavisinde ebselenin potansiyel role sahip olabileceğini göstermiştir.

Anahtar Kelimeler: Ebselen, katalaz, lipid peroksidasyon, nöroprotektif, spinal kord yaralanması, superoksid dismutaz

INTRODUCTION

Blunt trauma to the spinal cord produces significant problems that are difficult or impossible to treat. Although most of the primary effects of this type of injury are irreversible, the secondary effects such as tissue edema, ischemia, ion fluxes, and free radical damage can be prevented or reversed. Research has shown that the process of secondary injury involves release of excess glutamate and aspartate (15), intracellular calcium overload (5), activation of the arachidonic acid cascade (6), and initiation of free radical-induced lipid peroxidation (12). Experimental studies and clinical observations have revealed that these secondary events significantly worsen spinal cord lesions; however, the molecular and cellular mechanisms behind them are still not clearly understood. The available evidence suggests that oxygen radical formation and cell membrane lipid peroxidation play important roles in secondary spinal cord injury (9).

Ebselen (2-phenyl-1,2-benzisoselenasol-3(2H)-one) is a synthetic lipid-soluble selenium-containing organic compound that has multiple pharmacological effects. This agent has been investigated in patients with cerebral vasospasm after aneurysmal subarachnoid hemorrhage (26,31), and has proven effective at attenuating free radical-induced damage in a rodent model of permanent middle cerebral artery occlusion (30). To date, ebselen has not been evaluated in any experimental model of traumatic spinal cord injury. The purpose of this study was to investigate the time-dependent effects of a single dose of ebselen after experimental spinal cord injury, and to assess the potential therapeutic efficacy of this agent in this type of trauma. To best of our knowledge, this is the first study that has demonstrated the neuroprotective effect of this agent in spinal cord injury.

MATERIALS AND METHODS

Thirty-six adult male Wistar albino rats weighing 230-320 g were studied. The animals were fed a normal diet throughout the study period. Prior to surgery, each rat was anesthetized with an intramuscular injection of 9-mg/kg of xylazine (Rompun, Bayer, İstanbul, Turkey) and 50-mg/kg of ketamine (Ketalar, Parke-Davis, Eczacıbaşı, İstanbul), and the dorsal region was shaved and cleaned with povidone iodine solution (Batticon, Adeka, Samsun, Turkey). Body temperature was monitored with a rectal thermometer, and was maintained at 37°C during the procedure with a heating pad and lamp. Laminectomies were performed at T7-9 with the aid of a surgical microscope (Mentor, Japan) under x10 magnification. Care was taken to avoid damaging the dura mater during these procedures.

Experimental Protocol

The rats were randomly divided into four groups of nine animals each.

Group **1** (Sham-operated control group): After the skin incision, the paravertebral muscles were dissected and the laminae were exposed. As noted above, laminectomies were carried out at T7-9. Once these were completed, the paravertebral muscles and the skin were closed with 3/0 silk. Twenty-four hours after the operation, each rat was sacrificed by

intraperitoneal administration of an overdose of thiopental. The area where the laminectomies had been performed was exposed, and 1 cm of spinal cord was excised under the microscope. The dura mater, leptomeninges, and blood vessels were dissected from the spinal cord. Three of the nine spinal cord samples were fixed in 10% formaldehyde solution for histopathological examination. The remaining six specimens were embedded in liquid nitrogen for biochemical analysis.

Group 2 (Trauma-only group): Laminectomies were carried out as described above, and spinal cord trauma was induced using the model of Rivlin and Tator (25). In each case, a temporary aneurysm clip (Yaşargil aneurysm clip, Aesculap, Catalog no: FE 753K, Latek, İstanbul) which had 70 g closing pressure was applied extradurally to the spinal cord for 60 seconds and then removed. The paravertebral muscles and the skin were then closed with 3/0 silk. At 24 hours post-injury, the rats were sacrificed and their spinal cords were removed and prepared for analysis as detailed above.

Group 3 (Ebselen treatment group E1): Each rat underwent both the laminectomies and trauma procedures described above. A single 10-mg/kg dose of ebselen granules (SIGMA, Yeni Farmakim, İstanbul) suspended in water was administered by gavage 1 hour after spinal cord injury was induced. At 24 hours post-injury, the animals were sacrificed and cord specimens were removed and prepared as stated above.

Group 4 (Ebselen treatment group E12): Each rat was subjected to the same combination of laminectomies and trauma as described for Group 3, and the same single dose of ebselen granules was administered in the same way 12 hours after cord injury. As in the other groups, at 24 hours post-injury, the animals were sacrificed and cord tissues were removed and prepared as described above.

Histopathological Examination

The spinal cord specimens were fixed in 10% formaldehyde solution and embedded in paraffin. Five micrometer-thick coronal sections of the injury site were obtained, and these were mounted on slides and stained with hematoxylin and eosin. The slides were examined under the light microscope (Mentor, Japan) at x10 and x40 magnification.

Biochemical Analysis

To assess lipid peroxidation, the malonic dialdehyde (MDA) content of the tissue was measured by the thiobarbituric acid (TBA) method (10). The level of MDA was recorded as nmol/g of wet spinal cord tissue. Superoxide dismutase (SOD) activity was determined by the nitroblue tetrazolium method, and was recorded as U/mg protein in the spinal cord tissue (4). Catalase activity was measured using the method of Beers and Sizer, and was also recorded as U/mg protein in the cord tissue (8).

Statistical Analysis

Data were analyzed using the SPSS PC+statistical solving pocket program (Microsoft). One-way ANOVA was used. Differences between groups were determined by Bonferroni's *Post Hoc* tests. P values <0.05 were considered to indicate statistical significance.

RESULTS

Lipid Peroxidation

At 24 hours after spinal cord injury, the mean concentrations of MDA in the tissues from Groups 1 through 4 were 26.76 ± 1.46 nmol/g wet tissue, 33.72 ± 2.22 nmol/g wet tissue, 35.36 ± 2.65 nmol/g wet tissue, and 31.40 ± 2.60 nmol/g wet tissue, respectively (Figure 1). The MDA level in Group 2 was significantly higher than that in Group 1 (p=0.001), but was not statistically different from the levels in Groups 3 and 4 (p=1.00 and p=0.565, respectively).

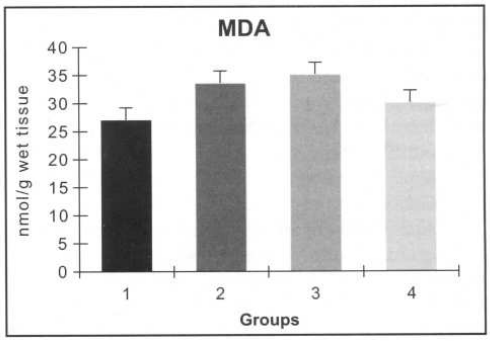

Figure1: The groups' lipid peroxidation levels at 24 hours after spinal cord injury. (MDA: malonic dialdehyde)

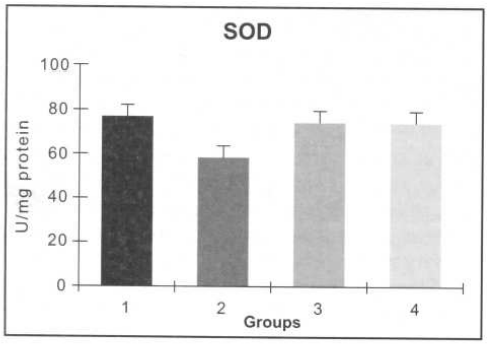

Figure 2: Superoxide dismutase (SOD) activity in the groups at 24 hours after spinal cord injury.

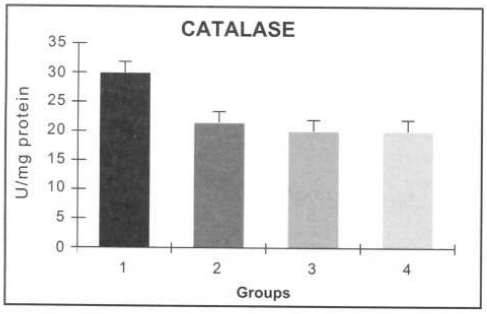

Figure 3: Catalase activity in the groups at 24 hours after spinal cord injury.

Superoxide Dismutase

At 24 hours post-injury, the mean SOD activity findings in Groups 1 through 4 were 75.83 ± 8.12 U/mg protein, 57.29 ± 13.49 U/mg protein, 73.33 ± 6.45 U/mg protein, and 73.12 ± 4.45 U/mg protein, respectively (Figure 2). Group 2 showed significantly lower SOD activity than Group 1 (p=0.01). The levels of SOD activity in Groups 3 and 4 were both significantly higher than the level in Group 2 (p=0.030 and p=0.033, respectively).

Catalase

At 24 hours post-injury, the mean catalase activity findings in Groups 1 through 4 were 29.79 ± 4.83 U/mg protein, 21.87 ± 3.03 U/mg protein, 20.20 ± 2.55 U/mg protein, and 20.20 ± 2.67 U/mg protein, respectively (Figure 3). The catalase activity in Group 2 was significantly lower than that in Group 1 (p=0.004), but did not differ significantly from the levels in Groups 3 and 4 (p=1.00 and p=1.00, respectively).

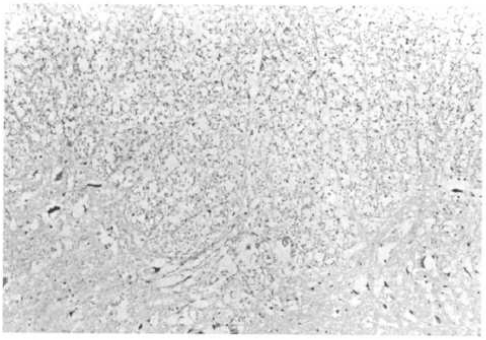

Figure 4: Cord tissue from the sham-operated control rats (Group 1): Light microscopic examination revealed normal gray and white matter (H&E x 115).

Histopathological Findings

As expected, microscopic study of the cord specimens from Group 1 revealed normal gray and white matter (Figure 4). The Group 2 tissues showed diffuse hemorrhagic necrosis, infiltration by polymorphonuclear leukocytes (PMLs), thrombi inside vessel lumens, and vacuolar cystic degeneration in the white matter (Figure 5 A and B). There was also diffuse hemorrhage in the gray matter around the central canal. The specimens from Group 3 showed much less hemorrhage around the central canal and minimal vacuolar changes in the white matter. There was very mild infiltration with PMLs, and fewer thrombi were present than in Group 2 tissues (Figure 6). The tissues from Group 4 showed similar gray matter hemorrhage to that in Group 3 and moderate vacuolar changes in the white matter. No necrosis or intravascular thrombi were observed in the gray or white matter (Figure 7).

DISCUSSION

Increased free radical generation and lipid peroxidation have been implicated in various pathological conditions, including trauma, ischemia, and inflammation. Lipid peroxidation, phospholipid hydrolysis with production of eicosanoids, and depletion of energy stores with increased lactic acid formation are well-known early biochemical events in spinal cord injury (1,2,3). It is believed that lipid peroxidation is catalyzed by free radicals that are generated by ischemia and hemorrhage (17). Peroxidation of lipids in the spinal cord membrane can lead to decreased activity of specific enzymes

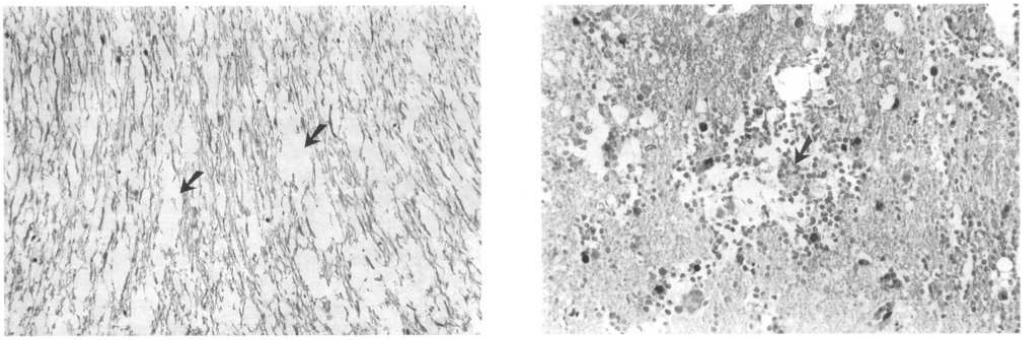

Figure 5: Cord tissue from the trauma-only rats (Group 2): A: Photomicrographs of white matter show severe vacuolar cystic defects, edema, and nerve fiber necrosis (arrow) (H&E x230). B: Light microscope examination revealed severe degeneration of neurons and glial cells, and widespread edema. Note the presence of hemorrhage and necrosis in the gray matter (arrow) (H&E x460).

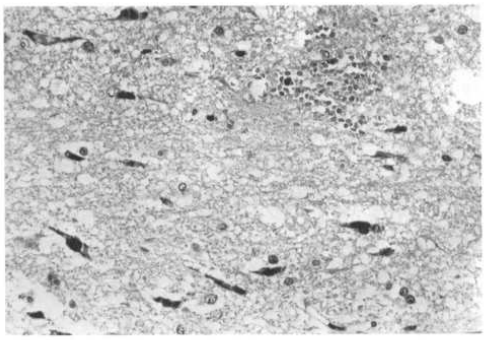

Figure 6: Ebselen-treated rats (Group 3, E1): Light microscope examination showed mild tissue edema in some areas. Note the polymorphonuclear leukocytes and macrophages in the hemorrhagic areas, as well as the preservation of spinal cord architecture and the absence of necrosis (H&E x460).

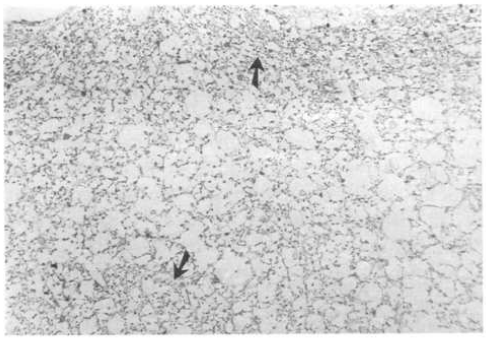

Figure 7: Ebselen-treated rats (Group 4, E12): Light microscope examination showed multifocal petechial hemorrhage, moderate edema, and no necrosis (arrow) (H&E x230).

that are critical to the maintenance of neuronal excitability, and can ultimately lead to tissue dissolution (32).

The extent of lipid peroxidation is a useful parameter for evaluating the cellular disturbance caused by spinal cord injury. İnci et al. investigated time-level relationships for lipid peroxidation in mild and severe experimental brain injury (19). Their results showed that the level of lipid peroxidation increases rapidly after both these types of damage. Barut et al. researched spinal cord tissue after clip compression, and demonstrated that the levels of MDA rise significantly starting at 15 minutes post-trauma, peak at 1 hour, and then tend to decrease (7). In our study, we choose extradural clip application as the method for producing acute spinal damage because it allowed us to induce the same amount and form of trauma in every animal. Also, this technique causes a severe lesion that is similar to the damage seen in the human subjects with serious spinal injuries (14,23). To evaluate lipid peroxidation in cord tissues, we measured MDA levels 24 hours after injury. The mean MDA level in the trauma-only group was 20.6% higher than that in the sham-operated controls (Figure 1), but was not

significantly different from the MDA levels in the ebselen-treated groups. Thus, a single 10-mg/kg dose of ebselen at 1 hour or 12 hours after spinal cord trauma did not reduce lipid peroxidation post-injury. This finding can be explained in a number of ways. First, the dose and timing of ebselen administration may have limited the efficacy of the agent. Second, the extent of lipid peroxidation may not be an appropriate parameter for investigating the efficacy of molecules in this type of injury. Also, the TBA method for measuring lipid peroxidation has been widely criticized because of the lack of specificity of the reactions involved, and the fact that other mechanisms of lipid peroxidation are likely at play (16,21).

Living organisms have many means of protecting themselves from the injurious effects of free radicals. Among these are endogenous enzyme systems and anti-oxidants that control the production and effects of these species (4). One example is superoxide dismutase, which catalyzes dismutation of the superoxide anion radical. Cells are capable of increasing SOD synthesis in response to hyperoxidant stress. Another example is catalase, which acts in one of two enzyme systems that breakdown hydrogen peroxide (18). At low concentrations of hydrogen peroxide, glutathione is reduced by a reaction with glutathione peroxidase to form oxidized glutathione and water. At high concentrations, catalase plays an important role.

The brain and spinal cord may be prone to oxidative stress for several reasons (23). The membrane lipids are rich in cholesterol and polyunsaturated fatty acids, which are susceptible to attack by oxygen free radicals. Deng et al. observed decreased SOD activity in brain tissue after experimental focal cerebral ischemia-reperfusion, and Islekel et al. documented similar results (13,20). Our study assessed SOD and catalase levels in the spinal cord after trauma and ebselen treatment. The findings showed that the mean SOD activity at 24 hours post-injury in the trauma-only group was 24.44% lower than the level in the sham-operated controls (Figure 2). Also, compared to the mean SOD activity in the trauma-only specimens, the activity of this enzyme was 27.99% higher in the E1 tissues and 27.93% higher in the E12 tissues. These differences show that a single 10-mg/kg dose of ebselen increases SOD activity in the rat spinal cord after trauma.

Regarding catalase, Nishibe reported decreased activity of this enzyme in the cord tissue after spinal injury (24). Sampath et al. investigated the effect of spinal cord photolesion injury on catalase in the rat (27). They observed significantly depleted levels of catalase mRNA in the spinal cord tissues at 6 days post-lesion, and a return to normal values at 14 days. In our study, at 24 hours post-injury the catalase activity in the trauma-only group was 26.58% lower than that in the sham-operated controls (Figure 3) ($p=0.004$), and the activity levels in the ebselen-treated groups were similar to the level in the trauma-only tissues ($p=1.00$ for both). This indicates that a single dose of ebselen at 1 hour or 12 hours post-trauma does not increase catalase activity after spinal cord injury in the rat.

Previous work has shown ebselen to be effective at attenuating free radical-induced damage both *in vitro* and *in vivo* (11,22,28). This agent has also been found to protect tissue against oxidative attack by mimicking glutathione peroxidase and phospholipid hydroperoxide glutathione peroxidase (29). The neuroprotective effect of ebselen has been investigated in vasospasm after aneurysmal subarachnoid hemorrhage, lipid peroxidation, and permanent middle cerebral artery occlusion (26,30). In one study on the effect of ebselen on vasospasm of the rabbit basilar artery, Takase et al. reported that ebselen suppressed ET-1-induced contraction, and also documented a synergistic interaction between oxyhemoglobin and ET-1 (31). Saito et al. evaluated the effect of ebselen on the outcome of subarachnoid hemorrhage in a multicenter human trial (26). They found that this agent reduced brain damage in patients who had delayed neurological deficits after subarachnoid hemorrhage. Our literature search revealed no reports on the efficacy of ebselen in experimental spinal cord injury.

The histopathological findings in our study indicate that ebselen treatment results in significant tissue recovery after cord injury in the rat. In the trauma-only group, the most prominent findings were diffuse hemorrhagic necrosis, PML infiltration, intravascular thrombi, and white matter edema. Both the E1 and E12 groups showed comparatively little hemorrhage in the gray matter around the central canal. We also noted that white matter edema was mild in the E1 group and moderate in the E12 tissues. This significant neuroprotective effect of ebselen is likely related to its anti-vasospasmodic features. This

mode of action would increase the local blood supply to the spinal cord, which could help preserve tissue integrity.

Considering the results overall, our study shows that administering ebselen 1 or 12 hours after spinal cord injury in the rat increases SOD activity but has no effect on lipid peroxidation or catalase activity. This reflects an imbalance between anti-oxidant systems and the lipid peroxidation process occurring in the cord. We also observed histological evidence that ebselen provides neuroprotection against free radical-induced damage. The findings indicated that significant tissue recovery occurred after the agent was given. As mentioned, we suspect that this improvement may be linked to ebselen's anti-vasospasmodic effects. The SOD activity and tissue-recovery benefits of ebselen signify its promise for future neuroprotective treatment of acute spinal cord injury. However, further experiments are needed to determine the exact protective mechanisms involved.

Correspondence: Ahmet ÇOLAK
Kartaltepe Mahallesi, Terakki caddesi,
No: 47/7 Bakırköy, Istanbul, TURKEY.
Phone: (+90) (212) 543 55 30
Fax: (+90) (212) 252 63 00
E-mail:drahmetcolak@yahoo.com

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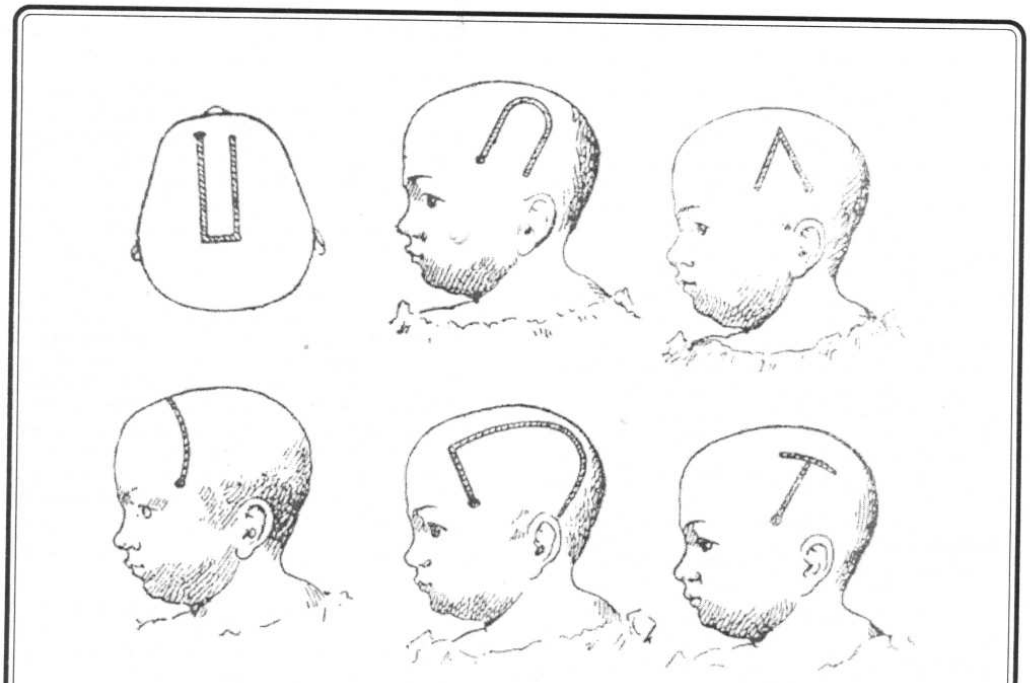

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