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AMIFOSTINE DECREASES LIPID PEROXIDATION ACTIVITY AFTER SPINAL CORD INJURY IN RATS

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ABSTRACT

Objective: Amifostine is a known radioprotective agent. It has been known for many years that it protects normal tissue from the undesirable effects of radiation and some chemotherapeutics due to its antioxidant effect and contains thiol. We investigated the effects of amifostine on the activity of lipid peroxidation in the spinal cord after experimental spinal cord injury in rats.

Materials and Methods: Thirty-five male Wistar albino rats were randomly divided into five groups, each containing seven rats. Group I (the control group) received laminectomies and spinal cord samples were obtained 24 h after laminectomy without trauma. Those in groups II to V all received laminectomies followed by traumatic spinal cord injury and tissue samples were taken 24 h later. Group II received no treatment; group III received 30 mg/kg methylprednisolone; group IV received 200 mg/kg amifostine; and group V received 2 mL 0.9% sodium chloride (sulfur tetrafluoride) solution. Medications were given intraperitoneally as single doses immediately after trauma. Spinal cord samples were taken 24 h post-trauma and studied for lipid peroxidation activity.

Results: Lipid peroxidation activity in the tissue samples was increased by injury. Both amifostine and methylprednisolone treatment decreased this activity, indicating a reduction in neutrophil infiltration of the damaged tissue. The effect of amifostine on lipid peroxidation activity was similar to that of methylprednisolone.

Conclusion: Amifostine may be effective in protecting the spinal cord from secondary injury.

Keywords: Amifostine, methylprednisolone, lipid peroxidase, spinal cord injury

INTRODUCTION

Recent research has revealed that most posttraumatic tissue damage and neurological disturbances are due to secondary reactive events[1,2]. This notion of a secondary mechanism was first posited by Allen[3] in 1911, who concluded that the necrotic matter left by a traumatic hemorrhage contains harmful elements that cause secondary injury and that its removal may facilitate neurological recovery. After initial studies, which indicated that neurological deficits developed because of progressive and irreversible damage in long pathways after spinal cord trauma, in 1950, it was found that damage occurred owing to decreased blood flow in the spinal cord, whereas today, tissue destruction after trauma is believed to be due to ischemia[4]. The pathophysiology of spinal cord injury is best described as a "biphasic injury," which occurs by two mechanisms: primary (direct) and secondary (indirect). Neurological damage after acute spinal cord injury occurs as a result of primary mechanical injury, necrosis following secondary injury, and later apoptosis[5-7]. While primary damage occurs through mechanical action, secondary damage occurs when primary damage is compounded by a series of biochemical and cellular reactions. Pathophysiological events that develop after primary injury constitute secondary injury in the long term. Secondary pathological events, such as ischemia, cause significant injury, including excitotoxicity, increased intracellular neuronal Ca^{2+}, free-radical formation, and increased lipid peroxidation. Ischemia after spinal cord injury is directly involved in secondary pathophysiological processes. This process of secondary injury includes increased cell permeability, apoptotic signaling, ischemia, vascular damage, edema, excitotoxicity, ionic deregulation, inflammation, lipid peroxidation, free-radical formation, demyelination, Wallerian degeneration, fibroglial scar, and cyst formation[8-10]. Although we have been unable to produce clinical improvement after severe spinal cord injury, it is encouraging that studies have begun to obtain positive results from animal experiments. Based on recent developments in the physiology and pharmacotherapy of spinal cord injury, a large number of neuroprotective substances are being tested[11-13]. So far, only methylprednisolone has increased functional recovery in humans in controlled, multicenter clinical trials[14,15]. In addition to the recent wave of experimental

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studies, several new drugs still in the preclinical study phase show promise for the treatment of spinal cord injury. A crucial factor in the success of chemotherapy treatment for cancer is the degree of cytotoxicity that it produces in normal tissues. To counter these cytotoxic effects on non-cancerous tissue, several cytoprotective drugs have been developed. One of the most frequently used drugs is amifostine (WR-2721). Compounds containing thiols, such as sodium thiosulfate and diethyldithiocarbamate, have antioxidant properties and can protect normal tissue from the unwanted effects of radiation and some chemotherapeutics[16]. However, the use of thiol compounds as a cytoprotectant in the treatment of cancer has not been possible. Thiol compounds not only protect healthy tissues but also eliminate cytotoxic anticancer effects. Amifostine is an organic thiophosphate compound that was developed as a radioprotective agent at the Walter Reed Army Institute research laboratories during the Cold War to protect military personnel from potential nuclear radiation[17]. Its chemical name is S-2-[3-(aminopropyl amine)] ethyl phosphorothioic acid. Its molecular weight is 214.23 and its molecular formula is $C_5H_{15}N_2O_3PS$. Amifostine differs from other sulfide-containing compounds in that its thiol group is covered by phosphate, so it is protected. Amifostine itself is a prodrug with little to no cytoprotective effect[17,18]. When administered, dephosphorylation of amifostine is catalyzed by the alkaline phosphatase enzyme within the cells of the organism. This enzyme removes a phosphate group, allowing free thiol conversion of the drug into its active metabolite, WR-1065. The resulting metabolite is utilized by cells for cytoprotective purposes. The free thiol group is responsible for this property. Because thiol is a known antioxidant, it can remove the free-oxygen radicals generated by platinum, alkylating drugs, and radioisotopes that damage the DNA in normal cells, thereby reducing cellular toxicity. The cytoprotective efficacy of amifostine has been demonstrated by several clinical and preclinical studies[17,18]. *In vivo* research has demonstrated the drug's ability to reduce bone marrow toxicity caused by cisplatin, carboplatin, cyclophosphamide, nitrogen mustards, bleomycin, cytarabine, etoposide, daunorubicin, paclitaxel, mitoxantrone, vinblastine, melphalan, mitomycin C, carmustine, and fluorouracil[19-21]. However, unlike other thiol compounds, amifostine does not protect cancer cells from cytotoxicity[16]. Amifostine is a radioprotective agent that prevents cellular damage due to radiation and chemotherapy through free-radical scavenging, hydrogen donation, and inhibition of DNA damage. Amifostine is metabolized and accumulates to a much greater extent in normal cells than in tumor cells. As a result, it exerts a protective effect on normal tissues due to chemotherapy- or radiotherapy-induced toxicity without reducing the antitumor effects of cancer treatment. Detailed preclinical studies have shown that amifostine protects against radiation damage and the myelotoxic, nephrotoxic, and neurotoxic effects of chemotherapeutic agents, such as alkylating agents and platinum compounds[17,18]. The clinical use of amifostine enables safer and more effective administration of radiotherapy and other anticancer therapies.

This study aimed to compare the effects of amifostine with those of methylprednisolone on tissue lipid peroxidation and cell ultrastructure after experimental spinal cord injury. While the effects of methylprednisolone are well established, those of amifostine have not yet been investigated[22-24].

MATERIALS AND METHODS

Because this is an experimental study, informed consent was not required to be obtained. The study protocol was approved by the ethics committee of Ankara Training and Research Hospital, and the test procedures were performed in compliance with the study guidelines of the animal laboratory of the same hospital (approval no: 272, date: 26.03.2005).

Groups

A total of 35 male Wistar albino rats, each weighing 210-250 g, were randomly divided into 5 groups of 7 rats as follows:

Group I (N=7) (control): Tissue samples were collected 24 h after laminectomy without trauma.

Group II (N=7) (trauma): 50 g/cm contusion injury following laminectomy was applied. After 24 h, tissue samples were collected 1 cm from the injury center.

Group III (N=7) (MPSS): 50 g/cm contusion injury following laminectomy was applied. Methylprednisolone sodium succinate (Prednol L® Mustafa Nevzat; Istanbul, Turkey; 30 mg/kg) was then administered intraperitoneally (IP). After 24 h, tissue samples were collected 1 cm from the injury center.

Group IV (N=7) (amifostine): 50 g/cm contusion injury following laminectomy was applied. Amifostine (Er-Kim Ilaç.; İstanbul, Turkey; 200 mg/kg) was then administered IP. After 24 h, tissue samples were collected 1 cm from the injury center.

Group V (N=7) (vehicle): 50 g/cm contusion injury following laminectomy was applied. NaCl solution (2 mL, 0.9%) was then administered IP. After 24 h, tissue samples were collected 1 cm from the injury center.

The tissue samples were immediately frozen and stored in liquid nitrogen at 196 °C.

Surgical Procedure

All surgical procedures were performed under general anesthesia. For this purpose, 10 mg/kg xylazine (Bayer; Istanbul, Turkey) and 60 mg/kg ketamine hydrochloride (Parke-Davis; Istanbul) were administered intramuscularly. The anesthetized rats were placed in a prone position. A 3-cm longitudinal skin incision was made along the center of the back following shaving and skin cleansing with Batticon (Adeka; Turkey). After paravertebral resection, total laminectomy was performed on thoracic vertebrae 7, 8, and 9. Dura intake was released. All subjects except those in the control group underwent 50 g/cm spinal cord trauma in accordance with the Allen method[25] as follows: a 10 cm long and 5 mm wide cylindrical glass tube was

placed perpendicular to the laminectomy area. A 5 g weight (3 mm diameter, cylindrical steel column) was reduced from within this tube to a height of 10 cm. Spinal cord trauma was thus induced at 50 g/cm (trauma intensity = weight × height). The rats were sacrificed under deep anesthesia after the tissue samples were collected.

Homogenization of Tissues

Tissue samples were weighed and homogenized in ice using glass homogenizer in 10 mm Tris buffer containing 1 mm ethylenediaminetetraacetic acid (EDTA, Tekno-Kim, İstanbul, Turkey) 10 times their wet weight, and 1 mL of tissue homogenate was transferred to tapered Eppendorf tubes and centrifuged for 5 min at 5000 rpm. The supernatant of the samples was used to determine lipid peroxidation activity.

Lipid Peroxidation Measurement

For the measurement of tissue lipid peroxidation levels, the following procedure was performed: 0.2 mL of 8.1% SDS, 0.8% NaOH, and 0.5 mL of 20% acetic acid solution were added to less than 0.2 mL of 10% homogenized tissue samples and 1.5 mL of 0.8% thiobarbituric acid aqueous solution. The mixture condenser was heated in an oil bath at 95 °C for 60 min, and 4 mL of distilled water was then added. After cooling with water, a mixture of 1.0 mL of distilled water and 5.0 mL of butanol and pyridine was added and stirred vigorously. After centrifuging for 10 min at 4000 rpm, the organic layer was collected and the absorbance of the mixture was measured at 532 nm. Tetramethylpyrazine was used as the external standard. Lipid peroxidation was expressed as nmol. Fluorometric assessment (excitation: 515 nm; emission: 553 nm) is performed when a small amount of tissue such as a small organ or biopsy specimen is examined.

Electron Microscopy Review

Spinal cord segments obtained from the thoracic level of the trauma area were placed in 2.5% glutaraldehyde and fixed for 6 h. After the first fixation with 1% osmium tetroxide, a series of immersions in solutions containing increasing ethanol concentrations was used to dehydrate the aqueous component of this fixative from within the cells. The samples were then washed with propylene oxide and placed in epochs. Ultra-thin tissue sections of 60 nm thickness were cut with a glass knife using the LKB Nova ultramicrotome (Bromma; Sweden) and placed on copper grids. These sections were stained with uranyl acetate and lead citrate and examined with a transmission electron microscope (Geol JEM 1200; Tokyo, Japan).

Statistical Analysis

A One-Way ANOVA was performed using SPSS v.11.0 software to determine differences in lipid peroxidase activity between the groups. A posthoc test was used to show which groups were different. A p-value of <0.05 was considered statistically significant.

RESULTS

Drugs

MPSS: Although a few normal mitochondria were observed, about half of the remaining mitochondria were crystalline and the other half were swollen. In the small myelinated axons, some of the myelin layers were stripped. Of those remaining, about half were normal and the remaining half showed splitting of the myelin layers. In the medium-sized myelinated axons, there was a greater number of interruptions in the myelin layers, and the myelin layer was separated from the axon in most of the remaining axons. Much fewer of the axons were normal. Among the large myelinated axons, no normal axons were found (Figure 1). Trauma was not observed in the small vacuoles of the neurons.

Amifostine: The nuclei of all cells in the tissue samples were normal. Around a sixth of the mitochondria observed were normal. A small number of swollen mitochondria were detected. All the remaining mitochondria were identifiable. The small myelinated axons were completely normal and only a small number of axons could be seen through their myelin layers. The medium-sized myelinated axons exhibited some normal axons and a small number of axons with damaged myelin. Separation of the myelin layers was seen in the majority. In the large myelinated axons, some regular axons were seen, although lesser than the other groups, and a small number of axons were stripped of myelin. The remaining large percentage of axons was still in their myelin layers (Figure 2). Trauma was not observed in the small vacuoles of the neurons (Figure 3).

There was a significant difference between the groups in tissue lipid peroxidase activity ($p<0.05$) (Figure 4). Tissue lipid peroxidase activity was significantly higher in the trauma group than in the control group ($p<0.05$). There was also a significant

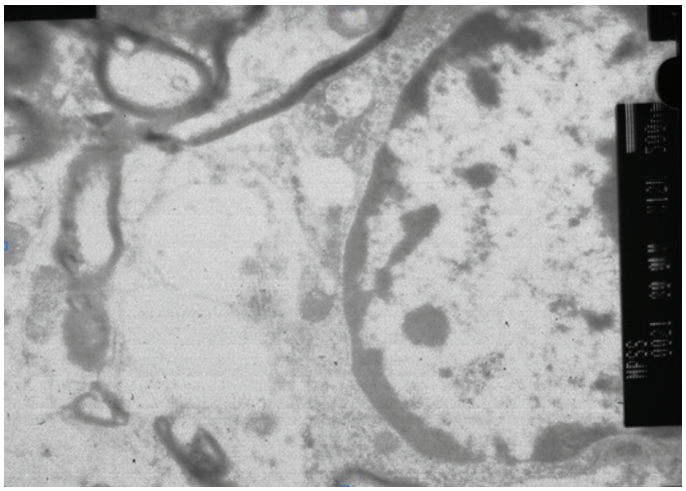

Figure 1. Electron microscopic image of spinal cord cells after damage and subsequent methylprednisolone treatment. The nuclei are normal. Swelling and crystallization of the mitochondria are apparent

difference between the control, MPSS, and amifostine groups in tissue lipid peroxidase levels ($p<0.05$) (Figure 4), but there was no difference between the trauma and vehicle groups in tissue lipid peroxidase levels ($p>0.05$). MPSS and amifostine prevented the increase in tissue lipid peroxidase activity. There was no significant difference between the tissue lipid peroxidase activity of these two groups ($p>0.05$) (Figure 4). The effect of the vehicle solution (NaCl) on tissue lipid peroxidase activity was not determined. Electron microscopy was performed on the samples from all groups to compare the intracellular structures. Approximately 300 samples were collected from each group. There was no significant difference between the control and amifostine groups in the results of small myelinated axons (Figures 2 and 3). In medium- and large-sized myelinated axons, amifostine provided significant protection (Figure 2). Cell nuclei were normal in all groups (Figures 5 and 6).

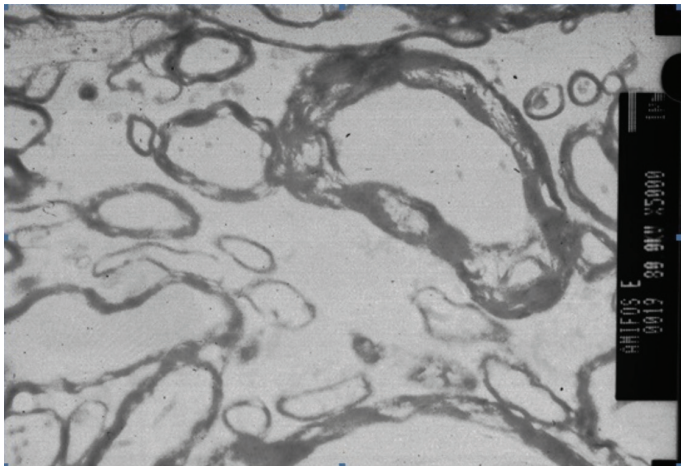

Figure 2. Electron microscopic image of spinal cord cells after damage and subsequent amifostine treatment. The cell nuclei are normal. The mitochondria are slightly swollen. There are no small vacuoles in the nuclei

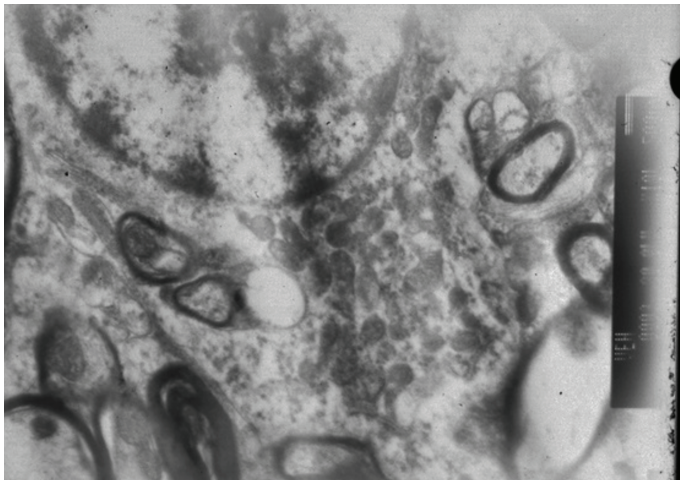

Figure 3. Electron microscopic image of spinal cord cells after damage and subsequent administration of NaCl (SF) solution. Considerable swelling of the mitochondria is apparent, with advanced crystallization. Small vacuoles can be seen in the nuclei

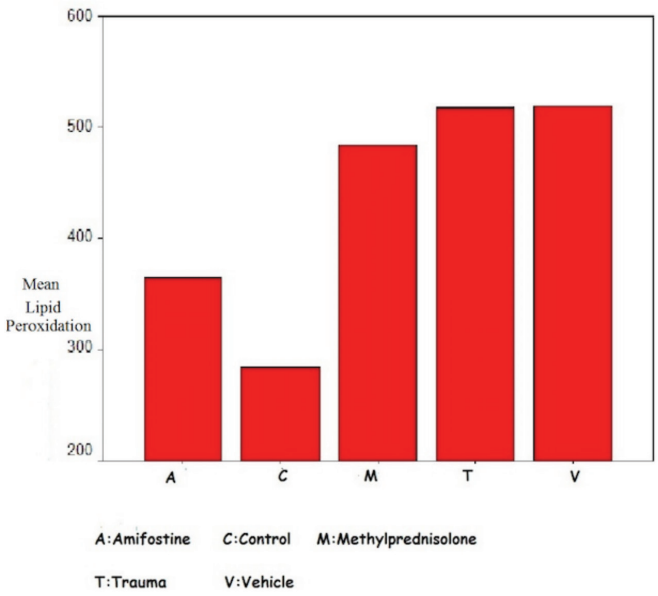

Figure 4. Mean lipid peroxidation levels of the five groups in this study

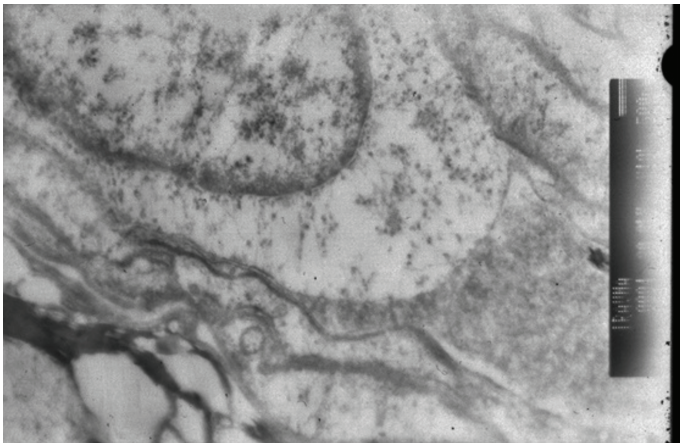

Figure 5. Electron microscopic image of spinal cord cells after damage. Considerable swelling of the mitochondria is apparent but no crystallization. Vesicular degeneration is present throughout

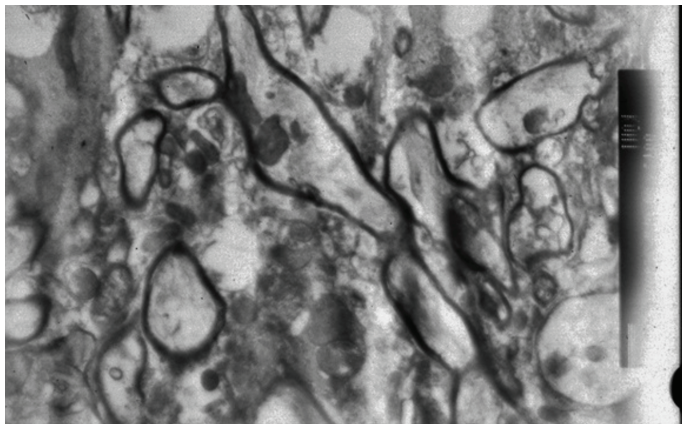

Figure 6. Electron microscopic image of normal spinal cord cells from our control group

DISCUSSION

Spinal cord injuries are a serious health problem comprising two stages[22-24]. In the first stage, primary injury occurs. In the second stage, secondary injury develops due to a series of pathophysiological processes occurring within hours or days of the primary injury. The main goal in the treatment of spinal cord injuries is to prevent secondary injury[26-27]. Pathophysiological events such as hemorrhagic necrosis, ischemia, edema, inflammation, extracellular Ca^{2+} loss, and intracellular K+ loss are responsible for the secondary injury. To prevent secondary damage, treatments including magnesium, calcium channel blockers, N-methyl-D-aspartate receptor blockers, and free-radical scavengers have been tested, but only MPSS has demonstrated any efficacy[28-31]. However, the effect of MPSS on secondary damage mediators is insufficient[32,33].

Amifostine is a cytoprotective drug used to prevent damage to the central nervous system (CNS) that may occur after radiotherapy for the treatment of cancer. It is a radioprotective agent that prevents radiation- and chemotherapy-induced cellular injury through free-radical scavenging, hydrogen donation, and inhibition of DNA damage. Radiation is known to cause microvascular damage to the CNS. Nieder et al.[34] have reported that vascular damage from post-radiation CNS toxicity induces peripheral edema in the surrounding area.

In addition, Nieder et al.[34] and Giannopoulou et al.[35] have shown that the production of blood vessels is decreased, and existing ones are damaged after irradiation of fertilized eggs. However, Kruse et al.[36] found a decrease in perivascular and interstitial fibrosis after administration of systemic amifostine in the ratio indicated by the cardiac radiation model.

These findings gave rise to the hope that systemic amifostine could be used against vascular damage, one of the most important components of CNS toxicity. In *in vitro* studies, Nieder et al.[34] also demonstrated that systemic amifostine increases post-radiation endothelial proliferation. Neuroprotection is extremely important for the spine because neurons in the spinal cord cannot regenerate. Neuroprotection may protect the axonal pathways required to heal damaged cells and provide metabolic support to damaged neurons. It may also prevent the emergence of mediators such as cytokines and free radicals that have additional toxic effects on neighboring cells and cause more neurodegeneration, cellular swelling, inflammation, and oxidative stress. The high availability of these mediators after experimental acute spinal cord injuries suggests that they have the potential to activate the neurodegeneration cycle. This includes molecules that are classically associated with CNS necrosis, including glutamate and intracellular Ca^{2+}. Glutamate is rapidly released following traumatic injury. The relationship between induced glutamate release, intrathecal Ca^{2+} increase, and cell death is unclear. Amifostine cannot pass the blood-brain barrier or may pass in very small amounts. However, it has been determined that there is a continuous transition in the blood-brain barrier after radiation. Nieder et al.[34] found that increased permeability of the blood-brain barrier after radiotherapy allows adequate penetration of amifostine. Lamproglou et al.[37] reported that 75 mg/kg and 100 mg/kg amifostine reduced neurotoxicity in the brain caused by radiation therapy by reducing systemic glutamate release. In their study on rats, Spence et al.[38] injected amifostine into the right lateral ventricle of rats. After 45 min, a single dose of radiation was given to the cervical spinal cord of the animals. Each rat was examined weekly for leg paralysis. In addition to neuroprotective effects, a histological examination found cell structures to be preserved. This was achieved through the protection of white matter and vascular elements. However, the same effect was not observed in the Schwann cells of the peripheral nerves of the cervical spinal cord. Ang et al.[39] evaluated white matter necrosis and demyelination of white matter 4-7 months after radiotherapy on rat spinal cords in 20-40 Gy intervals and confirmed the findings of Spence et al.[38].

In our study, electron microscopic examination found that, only in the rats given amifostine, were the nuclei and mitochondria preserved. In addition, we observed that intracellular structures, particularly small myelinated fibers, were preserved. The nervous system is rich in polyunsaturated lipids. Peroxidation of membrane lipids leading to the release of free radicals is an important mechanism in neuronal damage. Because free radicals are found early after traumatic injury, any effective neuroprotective agent must be given as soon as possible after trauma. In our study, the incidence of lipid peroxidation was significantly lower in the amifostine group (i.e., following administration of 200 mg/kg amifostine immediately after spinal cord injury) than in the trauma, vehicle, and MPSS groups. Nieder et al.[34] and Lamproglou et al.[37] suggested that amifostine acts as a free-radical scavenger by emitting superoxide anions that iodinate the radiation. In addition, amifostine is thought to increase endogenous glutathione concentrations. This is the major antioxidant in the mammalian CNS; it protects damaged tissue and increases the resistance of normal tissue. We found no increase in lipid peroxidase activity in our control group, which received only laminectomy with no trauma. We found no significant difference in lipid peroxidase activity between the vehicle and the trauma groups. This shows that the concentration of NaCl administered had no neuroprotective effect. This result was corroborated by the results of electron microscopic examination. MPSS is the only pharmacotherapeutic agent used clinically and is effective as a neuroprotectant following traumatic spinal cord injury. However, recent complications have led to restrictions on its use. Although MPSS has a neuroprotective effect, we found it to be less effective than amifostine.

In the literature, there are few studies on the neuroprotective effects of amifostine against radiation damage to the CNS and none on its neuroprotective effects in ischemic or trauma models of the brain or spinal cord. This study was the first to

examine the neuroprotective effect of amifostine on acute spinal cord contusion injury. We found 200 mg/kg amifostine administered IP after acute spinal cord contusion injury to be a more effective neuroprotective agent than MPSS and to reduce lipid peroxidase activity. Examination of the intracellular organelles of neurons, membranes, myelin sheaths, and axons found all to be better preserved by amifostine than by MPSS.

CONCLUSION

In this study, 200 mg/kg amifostine administered IP after acute spinal cord contusion injury was shown to have superior neuroprotective effects to MPSS that significantly reduce lipid peroxidation activity and protect the spinal cord. With further research into the effects of amifostine on spinal trauma, we hope to be able to contribute to the clinical improvement of spinal cord trauma outcomes.

Ethics

Ethics Committee Approval: The study protocol was approved by the ethics committee of Ankara Training and Research Hospital, and the test procedures were performed in compliance with the study guidelines of the animal laboratory of the same hospital (approval no: 272, date: 26.03.2005).
Informed Consent: Because this is an experimental study, informed consent was not required to be obtained.
Peer-review: Internally peer-reviewed.

Authorship Contributions

Concept: A.G., M.Ö.O., Design: A.G., M.Ö.O., Data Collection or Processing: A.G., M.Ö.O., Data analysis or Interpretation: A.G., M.Ö.O., Literature Search: A.G., M.Ö.O., Writing: A.G., M.Ö.O.
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Conflict of Interest: The authors have no conflicts of interest to declare.

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