Dantrolene can reduce secondary damage after spinal cord injury

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Abstract The aim of this experimental study was to investigate the possible protective effects of dantrolene on traumatic spinal cord injury (SCI). Twenty-four New Zealand rabbits were divided into three groups: Sham (no drug or operation, $n = 8$), Control (SCI + 1 mL saline intraperitoneally (i.p.), $n = 8$), and DNT (SCI + 10 mg/kg dantrolene in 1 mL, i.p., $n = 8$). Laminectomy was performed at T10 and balloon catheter was applied extradurally. Four and 24 h after surgery, rabbits were evaluated according to the Tarlov scoring system. Blood, cerebrospinal fluid and tissue sample from spinal cord

were taken for measurements of antioxidant status or detection of apoptosis. After 4 h SCI, all animals in control or DNT-treated groups became paraparesic. Significant improvement was observed in DNT-treated group, 24 h after SCI, with respect to control. Traumatic SCI led to an increase in the lipid peroxidation and a decrease in enzymic or non-enzymic endogenous antioxidative defense systems, and increase in apoptotic cell numbers. DNT treatment prevented lipid peroxidation and augmented endogenous enzymic or non-enzymic antioxidative defense systems. Again, DNT treatment significantly decreased the apoptotic cell number induced by SCI. In conclusion, experimental results observed in this study suggest that treatment with dantrolene possess potential benefits for traumatic SCI.

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Introduction

Traumatic spinal cord injury (SCI) is one of the most serious consequences of accidents that human beings suffer. Owing to a motor vehicle accident, violence or falling, each year thousands of peoples are diagnosed with SCI. Permanent neurological deficit and a broad range of secondary complications following SCI result from the damage of the axons, death of neuronal and glial cells, and demyelination. The pathophysiology of acute SCI is not clear, but it is suggested that there are primary and secondary injury mechanisms. Mechanical damage (contusion and compression) is called the primary injury and it is inevitable. After primary injury, a series of pathological events such as hypoxia, edema and inflammation, altered

blood flow and changes in microvascular permeability arise; thus, lesions greatly enlarge and worsen by the secondary injury [43]. The excessive release of excitatory neurotransmitters (especially glutamate) can trigger destructive processes, and cause death of neuronal cells. Previous reports stated that increase in lipid peroxidation and reactive-oxygen species (ROS) generation mediate significant secondary developments, resulting in demyelination and further cell death by necrotic and apoptotic pathways [2, 21]. Furthermore, the release of the inflammatory mediators after SCI is believed to play an important role in the pathogenesis of secondary injury [8, 23].

Primary injury is inevitable in SCI; however, preventive measures may be taken against development of the secondary injury. Because of this, researchers are especially interested in the prevention of the secondary injury. Prevention of excitotoxicity and apoptosis, controlling of inflammatory response and decrease in oxidative stress may improve neurological outcome in acute SCI. Nowadays, high-dose methylprednisolone is the most extensively used drug for the treatment of acute traumatic spinal cord injuries, if the injury occurred within 8 h (National Acute Spinal Cord Injury Studies (NASCIS) II and III), but harmful side effects shade its functional efficacy in patients [30]. On the other hand, there are some contrary claims for methylprednisolone [19, 41]. Several pharmacological agents are screened against secondary injury after experimental spinal cord trauma. Beneficial effects of melatonin [40], resveratrol [4], etomidate [14], magnesium sulfate [37] and sodium channel blockers mexiletine, phenytoin and riluzole [5] have been shown in traumatic SCI in rodents. However, none of these agents have reached a point that warrants their use in the clinical care of human SCI.

Dantrolene (1-[[[5-(4-nitrophenyl)-2-furanyl] methyl-ene] amino]-2,4-imidazolidine-dione sodium salt hydrate), a hydantoin derivative, is a peripherally acting skeletal muscle relaxant that is used clinically in the treatment of muscle spasticity, malignant hyperthermia and neuroleptic malignant syndrome [46]. It depresses excitation–contraction coupling in the muscle fiber by inhibiting the calcium release from the sarcoplasmic reticulum and affecting the calcium channel in the smooth muscle membrane [34, 46]. The neuroprotective effects of dantrolene in cell culture [16] or aortic ischemia/reperfusion-induced SCI [29] have been demonstrated in a variety of in vivo and in vitro experimental studies. It also exerts radioprotective and antioxidative properties [11, 12]. The effect of dantrolene in traumatic SCI has not yet been studied. Thus, in the present study, we tested whether the administration of dantrolene after SCI has beneficial effects on behavioral, biochemical and morphological recovery in rabbits.

Materials and methods

The investigation was conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996) and approval has been received from institutional Animal Ethics Committee at Afyon Kocatepe University.

Chemicals

Hydrogen peroxide, reduced glutathione (GSH), thiobarbituric acid, phosphate buffer, butylated hydroxytoluene, trichloroacetic acid, EDTA [5,5-dithiobis-(2-nitrobenzoic acid)], disodium hydrogen phosphate, phenylendiamine, sodium azide, 2,4-dinitrophenylhydrazine, ethanol, hexane, sodium nitrite, sodium nitrate, sulfanilamide, *N*-(1-Naphthyl) ethylenediamine dihydrochloride, dantrolene (DNT) and vanadium (III) chloride were purchased from Sigma Chemical Co (Germany). All other chemicals and reagents used in this study were of analytical grade. In addition, superoxide dismutase (SOD) and glutathione peroxidase (GPx) commercial kits (Randox, UK) were used.

Animals

Twenty-four New Zealand male and female rabbits, weighing between 2.5–3.0 kg were divided into three groups: Sham (no drug or operation, $n = 8$), Control (SCI + single dose of 1 mL saline intraperitoneally, $n = 8$) and DNT (SCI + 10 mg/kg dantrolene in 1 mL, intraperitoneally, $n = 8$). Owing to the ease of application, we preferred the intraperitoneal route for treatment. The animals were allowed access to water and food ad libitum, presurgery and postsurgery period. The animals kept at the Animal Care Facility of Afyon Kocatepe University Experimental Research Centre.

Surgical procedures

All rabbits, in control and DNT groups, were anesthetized via intramuscular injection of xylazine (Bayer, Istanbul Turkey) 5 mg/kg and ketamine hydrochloride (Parke Davis, Istanbul, Turkey) 50 mg/kg; breathing was continued spontaneously with room air. Rabbits were positioned prone on operating table. Under a sterile technique, a midline dorsal incision was done. The laminae and transverse processes of T6–L2 were exposed by gentle blunt dissection of paravertebral muscles. A self-retaining retractor was placed in operation area, and then laminectomy was performed at T10. A balloon angioplasty catheter (Medtronic-146671, 2.0 × 20 mm, USA) was placed extradurally and sublaminary on thoracic spinal cord, upwards below T9. Inflation,

slowly until 2 atm pressures was achieved and then was waited for 5 min in 2 atm pressure, and balloon was deflated. Following the careful removal of balloon catheter, paravertebral fascia and skin were sutured with silk stitches. Just after trauma, animals in control group were given 1 mL of saline, in DNT group were given 10 mg/kg dantrolene (dissolved in saline). A complete closure of surgical wound was achieved.

The main reason for the use of balloon compression model was to form a partial spinal cord lesion [3].

Neurological evaluation

Four and 24 h after surgery, rabbits were evaluated by an independent observer according to the Tarlov scoring system as described in Table 1 [39]. After last neurological evaluation, the rabbits in all groups were anaesthetized with ketamine (50 mg/kg) and cerebrospinal fluid (CSF), tissue samples from spinal cord and blood (from vena cava inferior) were taken. At the end of these procedures, all rabbits were killed under deep anesthesia.

Biochemical analysis

Whole blood was collected into heparinized tubes, and malondialdehyde (MDA) and GSH levels were studied on the same day of admission. Blood was also collected into a polystyrene microtube, and after clotting, centrifuged at 1,000g for 10 min at +4°C, and the serum was removed using EDTA-washed Pasteur pipettes. The red blood cells that remained after the removal of plasma were washed with isotonic saline (0.89% NaCl), and the buffy coat was removed. The red blood cells were washed again with isotonic saline and further processed for the preparation of hemolysate. The studied tissues were homogenized in tenfold volume of physiological saline solution using a homogenizer (Ultra-Turrax T25, IKA; Werke 24,000 rpm; Germany). The homogenate was centrifuged at 10,000g for 1 h to remove debris. Clear upper supernatant was taken, and tissue analyses were carried out in this fraction. The serum, erythrocyte and tissue samples were stored in polystyrene plastic tubes at −70°C until the time of

analysis. MDA, GSH, nitrate, nitrite, ascorbic acid, retinol, β -carotene and erythrocyte SOD, GPx and catalase (CAT) activities were studied by spectrophotometer (Jenway 6305 UV/VIS).

MDA assay

Malondialdehyde (as an important indicator of lipid peroxidation) levels were measured according to a method of Jain et al [25]. The principle of the method was based on the spectrophotometric measurement of the color that occurred during the reaction of thiobarbituric acid with MDA. The concentration of thiobarbituric acid reactive substances (TBARS) was calculated by the absorbance coefficient of malondialdehyde–thiobarbituric acid complex and is expressed in nmol/ml.

GSH assay

Estimation of the reduced glutathione was measured by the method of Beutler et al. by a spectrophotometric method [9]. After lysing whole blood and the removal of precipitate, disodium hydrogen phosphate and DTNB solution were added and the color formed was read at 412 nm. The results were expressed in mg/dl.

Ascorbic acid, retinol and β -carotene analyses

Serum vitamin C (ascorbic acid) level was determined after derivatization with 2,4-dinitrophenylhydrazine [36]. The levels of β -carotene at 425 nm and vitamin A (retinol) at 325 nm were detected after the reaction of serum: ethanol: hexane at the ratio of 1: 1: 3: respectively [42].

Nitrate and nitrite analyses

The concentrations of nitrate and nitrite were detected by the methods of Miranda et al. [33]. Nitrite and nitrate calibration standards were prepared by diluting sodium nitrite and sodium nitrate in pure water. After loading the plate with samples (100 μ l), the addition of vanadium (III) chloride (100 μ l) to each well was rapidly followed by the addition of the Griess reagents, sulfanilamide (50 μ l) and *N*-(1-naphthyl) ethylenediamine dihydrochloride (50 μ l). The Griess solutions may also be premixed immediately prior to the application to the plate. Nitrite mixed with Griess reagents forms a chromophore from the diazotization of sulfanilamide by acidic nitrite, followed by coupling with bicyclic amines, such as *N*-(1-naphthyl) ethylenediamine. Blank sample values were obtained by substituting a diluting medium for Griess reagent. Nitrate was measured in a similar manner, except that samples and nitrite standards were only exposed to Griess reagents. The

Table 1 Criteria in Tarlov scoring

Score	Neurological outcome
0	Spastic paraplegia and no movement of the lower limbs
1	Spastic paraplegia and slight movement of the lower limbs
2	Good movement of the lower limbs but unable to stand
3	Able to stand but unable to walk normally
4	Complete recovery and normal gait/hopping

absorbance at 540 nm was read to assess the total plasma level of nitrite and nitrate in all samples.

CAT, SOD and GPx analyses

Catalase activity was measured according to the method of Aebi [1]. The principle of the assay is based on the determination of the rate constant [$k(s-1)$] of hydrogen peroxide decomposition by catalase enzyme. The rate constant was calculated from following formula:

$$k = \left(\frac{2.3}{\Delta t} \right) \left(\frac{a}{b} \right) \log \left(\frac{A1}{A2} \right)$$

where, A1 and A2 are the absorbance values of hydrogen peroxide at times of t_1 (0th s) and t_2 (15th s), “a” is the dilution factor, and “b” is the hemoglobin content of erythrocytes. Erythrocyte SOD and GPx activities were studied on hemolysates by using commercial kits (Randox Laboratories, UK) [17, 38].

Spinal cord immunohistochemistry

A terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling (TUNEL) assay was used to identify double-stranded DNA fragmentation, characteristic of DNA degradation by apoptosis. An ApopTag in situ apoptosis detection kit (Oncor, Gaithersburg, MD) was used according to the manufacturer’s directions. In brief, tissue slides were deparaffinized, treated with proteinase K (20 μ g/mL) for 15 min at room temperature, and then quenched in 3% hydrogen peroxide for 5 min. After rinsing in phosphate-buffered saline (PBS), pH 7.4, specimens were incubated in 1 $\times$ Equilibration Buffer (Oncor) for 10 min. Slides were next incubated with terminal deoxynucleotidyl-transferase (Tdt) for 1 h at 37°C, blocked with Stop/Wash Buffer (Oncor), and then incubated with peroxidase-conjugated antidigoxigenin antibody for 30 min at room temperature. Finally, slides were developed using diaminobenzidine (DAB; Sigma, St Louis, MO) and counterstained with methyl green.

On each slide, six fields were randomly selected and positive cells were counted at the healthy tissue which is situated at the peripheries of damaged areas. To quantitate extents of apoptosis, we recorded numbers of TUNEL-positive cells in each group. Finally, the overall mean counts for each set of specimens in each group were calculated, and mean group values were compared [20].

Statistical analysis

Statistical analysis was performed with the Statistical Package for the Social Sciences for Windows (SPSS version 10.0, Chicago, IL, USA). All values were expressed as

mean $\pm$ standard deviation. Statistical analysis of data was performed using a one-way analysis of variance (ANOVA) and Tukey’s post test. A value of $P < 0.05$ was considered statistically significant.

Results

Neurological outcome

Animals in Sham group had normal neurological outcome (mean Tarlov score was 4). After 4 h of SCI, all animals in control or DNT-treated groups became paraparetic (mean Tarlov scores were 1.88 and 2.00, respectively) and there was no significant difference between control and DNT. On the other hand, 24 h after SCI, partial improvements were observed in both control and DNT-treated groups; neurological improvements were significantly higher in DNT group when compared with control (Fig. 1).

Biochemical analysis

Effects on whole blood MDA and GSH levels

The levels of MDA and GSH in whole blood of experimental groups were presented in Table 2. Significantly different MDA levels were observed for DNT and control groups. As for GSH levels in the blood, DNT

Fig. 1 The result of Tarlov scoring in the experimental groups ($n = 8$, mean $\pm$ SD, DNT 10 mg/kg dantrolene). * $P < 0.01$ versus control

Table 2 Effects of 10 mg/kg dantrolene (DNT) on whole blood malondialdehyde (MDA) and reduced glutathione (GSH) levels (mean $\pm$ SD) in rabbits

Groups	<i>n</i>	MDA (nmol/ml)	GSH (mg/dl)
Sham	8	3.11 $\pm$ 0.27	35.05 $\pm$ 3.42
Control	8	3.71 $\pm$ 0.24	33.04 $\pm$ 3.70
DNT	8	1.30 $\pm$ 0.33**	37.71 $\pm$ 1.50*

* $P < 0.05$, ** $P < 0.001$ versus control

Table 3 Effects of 10 mg/kg dantrolene (DNT) on serum nitrate, nitrite, ascorbic acid, retinol and β -carotene levels (mean $\pm$ SD) in rabbits

Groups	<i>n</i>	Nitrite (mg/l)	Nitrate (mg/l)	Ascorbic acid (mg/dl)	Retinol (μ g/dl)	β -carotene (μ g/dl)
Sham	8	1.76 $\pm$ 0.06	6.25 $\pm$ 0.23	1.12 $\pm$ 0.17	47.76 $\pm$ 2.35	13.80 $\pm$ 1.75
Control	8	1.57 $\pm$ 0.22	5.92 $\pm$ 0.32	1.28 $\pm$ 0.16	48.58 $\pm$ 1.26	16.23 $\pm$ 1.66
DNT	8	1.52 $\pm$ 0.06	6.38 $\pm$ 0.80	1.31 $\pm$ 0.25	53.20 $\pm$ 3.17*	20.28 $\pm$ 1.54**

* $P < 0.05$, ** $P < 0.01$ versus control

Table 4 Effects of 10 mg/kg dantrolene (DNT) on the activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) in rabbits erythrocytes (mean $\pm$ SD)

Groups	<i>n</i>	SOD (U/ml)	CAT (kU/l)	GPx (U/l)
Sham	8	258.29 $\pm$ 27.52	2,480.86 $\pm$ 341.25	7,837.71 $\pm$ 1,156.31
Control	8	172.29 $\pm$ 16.53	2,956.244 $\pm$ 250.69	5,540.62 $\pm$ 671.93
DNT	8	208.43 $\pm$ 16.24*	3,521.54 $\pm$ 317.70*	7,355.11 $\pm$ 1112.42*

* $P < 0.05$ versus control

Table 5 Effects of 10 mg/kg dantrolene (DNT) on cerebrospinal fluid malondialdehyde (MDA) and reduced glutathione (GSH) levels (mean $\pm$ SD) in rabbits

Groups	<i>n</i>	MDA (nmol/mL)	GSH (nmol/mL)	Nitrite (mg/l)	Nitrate (mg/l)
Sham	8	0.39 $\pm$ 0.03	15.76 $\pm$ 2.02	1.74 $\pm$ 0.15	8.26 $\pm$ 0.43
Control	8	0.64 $\pm$ 0.08	3.04 $\pm$ 0.96	2.17 $\pm$ 0.35	8.87 $\pm$ 0.71
DNT	8	0.38 $\pm$ 0.03**	4.74 $\pm$ 1.02	1.59 $\pm$ 0.14*	8.62 $\pm$ 0.79

* $P < 0.05$, ** $P < 0.001$ versus control

administration significantly increased the GSH levels with respect to control.

Effects on serum nitrite, nitrate and vitamins levels

Comparison of nitrite, nitrate and ascorbic acid levels in the serum revealed that there were no significant differences between experimental groups (Table 3). On the other hand, DNT administration significantly augmented the raises in the retinol and β -carotene levels, with respect to control.

Effects on antioxidant enzymes levels

Table 4 shows the activities of enzymatic antioxidants (SOD, CAT and GPx) in the erythrocytes of normal and experimental animals in each group. SOD and GPx activities significantly decreased in traumatized rabbits when compared with those in normal (Sham) rabbits. The treatment of traumatized rabbits with DNT significantly prevented the decrease in the SOD and GPx activities. On the other hand, DNT treatment significantly increased the CAT activity when compared with control group.

Effects on MDA, GSH, nitrite and nitrate levels in CSF

Table 5 shows the levels of MDA in the CSF of normal and experimental animals in each group. DNT treatment resulted in significant decrease in the CSF MDA levels with respect to control. The GSH levels significantly decreased in the experimental rabbits when compared with Sham. DNT treatment significantly prevented the increase in nitrite level, with respect to control. Nitrate levels in Sham and experimental groups were very close, and there was no significant difference between the groups.

Effects on spinal cord MDA and GSH levels

Table 6 shows the spinal cord MDA and GSH levels in normal and experimental animals in each group. There were no significant differences between MDA and GSH levels of the groups.

Immunohistochemical study

The results of this study showed that the number of apoptotic cell significantly increases after SCI. Furthermore, DNT

Table 6 Effects of 10 mg/kg dantrolene (DNT) on spinal cord malondialdehyde (MDA) and reduced glutathione (GSH) levels (mean $\pm$ SD) in rabbits

Groups	<i>n</i>	MDA (nmol/mg tissue)	GSH (nmol/g tissue)
Sham	8	17.50 $\pm$ 2.89	272.60 $\pm$ 62.70
Control	8	19.39 $\pm$ 5.15	337.72 $\pm$ 34.04
DNT	8	15.54 $\pm$ 3.88	356.80 $\pm$ 80.23

Fig. 2 Quantitative analysis of immunohistochemical staining (TUNEL) in spinal cords of the experimental groups (*n* = 8, mean $\pm$ SD, DNT 10 mg/kg dantrolene). **P* < 0.05 versus control**Fig. 3** Apoptosis in spinal cord. DNT (SCI + dantrolene), S Sham (no drug or operation), C control (SCI + saline), C+ positive control. Arrow TUNEL (+) reaction in cell. TUNEL staining bar 100 μ m

treatment could attenuate the SCI-induced apoptosis (Figs. 2, 3).

Discussion

Spinal cord injury is still a major clinical problem with a permanent neurological deficit and a broad range of secondary complications. Secondary injury in spinal cord trauma is believed to be a result of a several destructive process, and all of them can cause dysfunction and death in neuronal cells. Thus, a number of studies have been focused on the treatment of secondary injury. Although some therapeutic agents are used in SCI, but there is still no effective treatment for the prevention of secondary injury. In the present study, we tested whether the treatment of DNT immediately after experimental SCI has protective effect on behavioral, biochemical and histopathological recovery. The current study is the first to investigate the effects of DNT on traumatic SCI. The goal in this work was to reveal the effect of DNT on oxidative stress-related secondary damage in the early stage of traumatic SCI. Therefore, the effect of DNT was examined for the first 24 h after trauma.

Used method in the present study for SCI led to the significant neurological deficit in rabbits. Some neurological

deficits after traumatic SCI may arise from the first hours on, up to the first week and neurological recovery is seen after a long time. Tarlov's scoring is simple and appropriate behavioral test for the evaluation of neurological deficit, and it demonstrates functional recovery after SCI in animals [39]. The results obtained from present study demonstrated that DNT treatment significantly prevented traumatic SCI-related neurodeficit. Thus, beneficial effect of DNT in SCI was also supported by behavioral test.

Lipid peroxidation is well known that one of the most important precipitating component of neuronal degeneration in the SCI. The increase in lipid peroxidation may be the cause of insufficiency in enzymatic and non-enzymatic antioxidative of defense mechanisms. Because of large lipid content and high oxygenation, lipid peroxidation-related cellular damage in central nervous system might be easily formed by ROS. Furthermore, it is believed that antioxidative defense capacity of neurons is insufficient than that of many other cells. Thus, susceptibility of the neurons to oxidative stress is very high and permanent neuronal damage caused by ROS is more than that of other cells. Prevention of lipid peroxidation may be important for neurological recovery. MDA is one of the most commonly used indicators of lipid peroxidation and following oxidative stress, its level increases in the tissues. Numerous studies have demonstrated that MDA level increases in animals exposed to traumatic SCI [4, 5, 14]. The results presented in this study have also revealed that lipid peroxidation increases in all blood, CSF and spinal cord tissue of the rabbits. Some neuroprotective agents with antioxidant activity have been investigated in traumatic SCI and some of them have been found useful [4, 31]. Owing to the neuroprotective and anti-lipid peroxidative [11, 12, 16, 29] properties, we used the dantrolene in SCI and found that it significantly reduced lipid peroxidation in all samples of the rabbits, except for spinal cord tissue. Interestingly, anti-lipid peroxidative activity of dantrolene was very strong; moreover, the MDA level in DNT-administered rabbits was less than that of Sham, and we do not know the reason for this activity.

Thiol-containing tripeptide GSH is known important cellular antioxidant and has various biological functions in the defense against oxidative stress [32]. It is also the substrate for antioxidant and detoxifying enzyme GPx [35]. Its level is often increased in the tissues and blood as an adaptive response after increased oxidative stress. GSH depletion results in enhanced lipid peroxidation or excessive lipid peroxidation and can cause GSH consumption. In the present study, decreased GSH levels of whole blood and CSF were observed in untreated rabbits, but decrease is significant only in CSF compared with those of Sham group. On the other hand, insignificant increase in the GSH level was observed in spinal cord

tissue. DNT administration significantly elevated GSH amount in whole blood, partly and insignificantly restored GSH levels in the CSF with respect to control. Again, DNT administration augmented trauma-induced GSH increase in spinal cord tissue. It seems that the consumption of GSH in CSF after SCI is very high than those of whole blood and spinal cord tissue, and DNT could not prevent decrease in GSH level of CSF.

It is well known that nitric oxide (NO) possesses both antioxidant and pro-oxidant properties [10, 44]. An antioxidative property of NO has been shown by some investigators [24, 26]. NO is an effective chain-breaking antioxidant in free radical-mediated lipid peroxidation, and reacts rapidly with peroxy radicals as a sacrificial chain-terminating antioxidant. In the present study, we also found that blood lipid peroxidation was increased while the serum levels of nitrate and nitrite were decreased in the SCI-subjected rabbits, and DNT administration insignificantly restored nitrate level in the serum, with respect to control. On the other hand, unlike serum, the levels of nitrate and nitrite in CSF increased after SCI in rabbits, and DNT treatment significantly prevented nitrite increase. Based on the above-mentioned effects of SCI on NO pathway, it may be mediated either by an activation or inhibition of NO synthase. Furthermore, it may be suggested that the effect of DNT against SCI-induced oxidative stress in CSF, at least in part, may be related to inhibition of nitrosative stress.

Antioxidant vitamins ascorbic acid, retinol and β -carotene play an important acute and chronic role in reducing or eliminating the oxidative damage produced by ROS [22]. Protective effect of DNT against oxidative stress in aortic ischemia/reperfusion-induced SCI and the role of antioxidant vitamins have been shown in previous study [29]. In the present study, values of serum ascorbic acid levels were very close and there was no significant difference between groups. On the other hand, vitamins A levels insignificantly increased following SCI. The cause of increase in the retinol and β -carotene levels of serum in SCI groups might be due to the adaptive response against SCI-induced oxidative stress. The mean retinol and β -carotene levels in the serum of DNT-administered rabbits increased, compared with those of untreated group. DNT administration significantly augmented this increase, with respect to control. Thus, it may be suggested that the protective effect of dantrolene against oxidative stress in SCI, at least in part, may be related to the restoration of antioxidant vitamins availability.

As for enzymatic antioxidants, SOD, CAT and GPx play an important role in preventing the cells from oxidative damage. SOD is an enzymatic antioxidant which catalyzes the conversion of superoxide radical to hydrogen peroxide and molecular oxygen. While CAT catalyzes the reduction

of hydrogen peroxides and protects the tissues against reactive hydroxyl radicals. GPx, is selenoprotein and it oxidizes GSH to glutathione disulfide (GSSG) which is then reduced to GSH by glutathione reductase, and reduces the hydroperoxides. Decreased activities of enzymatic antioxidants SOD and GPx have been well demonstrated in SCI [27]. The current study revealed that SCI leads to significant decrease in the SOD and GPx activities when compared with those in Sham group ($P < 0.001$ and $P < 0.01$, respectively). Moreover, there were significant changes in SOD and GPx activities in DNT-administered group when compared with those in control. The decreased activity of SOD and GPx in SCI, as reported previously, which could be due to increased consumption for free radicals' detoxification. In a previous study, increased CAT activity in SCI has been demonstrated [6, 28]. In the present study, we determined that CAT activity was insignificantly elevated as a result of SCI and this elevation may be related to defense response of organism. Again, treatment with DNT significantly increased the level of CAT, compared to those of the untreated group. Thus, it may be suggested that antioxidative activity of DNT is partly related to upregulation of CAT which eliminates free radicals by the generation of water and oxygen.

Apoptosis or programmed cell death occurs physiologically during development and aging, and it is necessary for maintaining the normal cell populations in tissues. At the same time, it occurs pathologically as a defense mechanism when cells are damaged by noxious stimuli and conditions. Thus, organism gets rid of unwanted cells. In a previous study, apoptosis following SCI has been determined in neurons and glial cells in the zone of the lesion 1 h after trauma; between 4 and 8 h postinjury, the number of apoptotic cells increased, but, early administration of a single dose of methylprednisolone decreased the apoptotic cells after SCI [45]. In the present study, used traumatic SCI model significantly increased apoptotic cell numbers and early administration of 10 mg/kg DNT following SCI significantly decreased the number of apoptotic cells, 24 h after injury. According to this finding, anti-apoptotic activity of DNT may play a role in reducing secondary damage in injured spinal cord tissue.

As mentioned above, the release of the inflammatory mediators after SCI is believed to play a major role in the pathogenesis of secondary injury [8, 23]. Migration of macrophages and activation of glial cells, release of cytokines are an important component of inflammatory responses which contribute to the secondary injury [8]. High-dose methylprednisolone is the most extensively used drug for the treatment of acute traumatic SCI and it has been shown to reduce acute inflammation [15]. Furthermore, non-steroidal anti-inflammatory drugs have been determined to promote axon regeneration [18]. However,

pain following SCI is an important healthcare problem, so far, there is no adequate cure for this pain [7]. Anti-inflammatory and antinociceptive properties of DNT have been demonstrated in rodents [13]. Thus, protective effect of DNT against SCI, besides being the antioxidative and antiapoptotic properties, at least in part, may depend on the reduction in the inflammatory reactions. In addition, DNT may cure trauma or SCI-related detrimental pain.

In conclusion, traumatic SCI was found to increase the lipid peroxidation and decrease enzymatic or non-enzymatic endogenous antioxidative defense systems. Furthermore, it was observed that SCI led to apoptosis in spinal cord tissue. This work demonstrates for the first time the effect of DNT on SCI. DNT treatment clearly prevented lipid peroxidation, augmented endogenous antioxidative defense systems and prevented apoptosis or neurodeficit following traumatic SCI. Inhibition of oxidative stress or apoptosis by DNT may have potential therapeutic benefits for reducing secondary damage and improving the outcome after traumatic SCI. The beneficial effects of DNT administration on traumatic SCI at the early stages was studied here. If the further studies focus on to obtain the similar effects at different terms after traumatic SCI, and by administering different doses of DNT producing similar successful results, it would be much more realistic to adapt the proposed method to the clinical applications.

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