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Research Paper

Effect of repeatedly given CDP-choline on cardiovascular and tissue injury in spinal shock conditions: investigation of the acute phase

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Abstract

Objectives The protective effect of CDP-choline in spinal cord transection and the mediation of its cardiovascular effects were investigated.

Methods Spinal cords of rats were transected at the T1–T2 levels. CDP-choline (250 mg/kg; intravenous) was administered 2 h and/or 24 h after the injury.

Key findings Spinal cord transection caused severe tissue damage, decreased mean arterial pressure, heart rate, plasma adrenaline, and noradrenaline but increased plasma vasopressin levels. Repeated CDP-choline treatment attenuated the degree of tissue injury. Administration of CDP-choline at 2 h after transection transiently increased blood pressure and decreased heart rate, while it produced a small decrease in blood pressure and heart rate when it was given at 24 h. Plasma adrenaline levels were higher in the group where CDP-choline was given repeatedly. Plasma noradrenaline and vasopressin levels did not change additionally after CDP-choline injections in all groups. In order to determine if CDP-choline attenuates the oxidative injury induced by transection, we measured blood superoxide dismutase, glutathione peroxidase activity and malondialdehyde levels. Repeated CDP-choline administration decreased blood superoxide dismutase and glutathione peroxidase activity without any effect on malondialdehyde levels.

Conclusions Data indicate that repeated intravenous CDP-choline treatment prevents tissue damage in spinal shock conditions in the acute phase. The cardiovascular effects of the drug do not seem to be responsible for this protection but the drug-induced attenuation of the oxidative stress may play a role.

Keywords catecholamines; CDP-choline; injury; oxidative stress; spinal shock; vasopressin

Introduction

Spinal cord transection is a devastating outcome of trauma, leading to muscle paralyses and autonomic deficits that include cardiovascular dysfunctions through the loss of central motor and autonomic control of peripheral organs distal to the injury.[1] Although it is widely accepted that the loss of neuronal connections is irreparable after complete injury, recent studies focused on strategies to increase the regenerative response of the central nervous system in animal models of spinal cord injury have provided some promising results. For example, the use of peripheral nerve grafts with the appropriate environment, such as α fibroblast growth factor (αFGF) treatment[2–4] or implantation of Schwann cells, olfactory ensheathing cells, stem cells or trophic factor-secreting fibroblasts,[5–7] may regenerate the damaged axons of the catecholaminergic system, protect spinal neurons and reduce autonomic dysfunction in chronic spinal cord injured animals. These studies demonstrate the beneficial effects of the treatments in chronically injured animals. Realistically, however, there is an interval period between the time of primary injury and the beginning of proper treatment, which promotes tissue damage and leads to neuronal death. Effective resuscitation during the acute phase is therefore critical to keeping the injured neurons stable and the patient alive until the available treatments can be performed.

In the acute phase, as early as 15 min after the primary traumatic injury, secondary mechanisms, including membrane depolarization and glutamate release, production of free

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radicals, initiation of the inflammatory process in response to ischaemia, and oxidative stress, are activated and cause progressive tissue damage, neuronal dysfunction and eventually death in most cases.[8] Furthermore, following the complete transection of the spinal cord, tonic activation of sympathetic preganglionic neurons by the descending input from the supraspinal structures is disrupted, resulting in acute neurogenic shock.[1,9,10] Hypotension is the main symptom of neurogenic shock, which further complicates the injury by perturbing the spinal blood flow and cerebral perfusion, and severely affects mortality rates.[9] Although several strategies have been suggested to reduce secondary injury by restoring blood pressure, or by attenuating oxidative stress or the inflammatory process, preclinical treatment algorithms including corticosteroid use have had limited success.[11]

Cytidine-5-diphosphocholine (citicoline or CDP-choline) is an endogenously synthesized mononucleotide and an intermediate product of membrane phosphatidylcholine. After oral or intravenous administration, it is rapidly hydrolysed to cytidine and choline by membrane phosphodiesterases.[12,13] These metabolites are taken up by the cells and mediate several physiological and pharmacological effects. They also contribute to the intracellular resynthesis of CDP-choline.[14] The beneficial effect of CDP-choline in ischaemia and hypoxia is well documented.[15] It is used for the treatment of head trauma and stroke in several countries. Proposed mechanisms of the protective effects of CDP-choline are the reduction of the membrane phospholipid breakdown, restoration of the intracellular enzyme sensitivity and function, and the attenuation of glutamate toxicity and oxidative stress.[16,17] As we have previously reported, CDP-choline may also increase blood pressure in normotensive rats and reverse hypotension in haemorrhagic shock conditions by activating central cholinergic neurotransmission, leading to an increase in plasma catecholamine and vasopressin levels.[18,19] It may therefore be possible that CDP-choline has positive impacts on both the restoration of blood pressure during the shock period and the attenuation of tissue injury in spinal cord transected animals. Considering the importance in the early period of complete spinal cord injury and the lack of an effective treatment, it is important to examine whether CDP-choline might be helpful during this critical stage. In the present study, CDP-choline was intravenously administered as early as 2 h after the injury, with or without a second administration 24 h later, or simply one injection 24 h after the injury. To our knowledge, this is the first study in which CDP-choline is given repeatedly to explore its beneficial role during the acute phase of spinal cord transection.

Materials and Methods

Animals

Adult female Wistar Albino rats (250–300 g), obtained from the Experimental Animals Breeding and Research Center at Uludag University, Bursa, Turkey, were used in the present study. Rats were housed in well-ventilated and temperature-controlled rooms under a 12-h light/dark cycle with free access to food and water. The surgical and experimental protocols were approved by the Animal Care and Use Committee of Uludag University and are in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Surgical procedures

The surgical procedures were carried out under sevofluorane (2–4% in 100% O_2) anaesthesia. The animals were kept on a heating pad and the rectal temperature was monitored and maintained at 37°C during surgery. Surgical procedures were performed under sterile conditions. The left common carotid artery and left jugular vein of rats were cannulated with PE 50 tubing filled with heparinized saline (250 U/ml). During the arterial cannulation procedure, the vagus nerve and the cervical sympathetic trunk were carefully separated. The catheters were exteriorized at the nape of the neck and sealed until use. Following the midline skin incision and paravertebral muscle dissection, the spinous process and laminar arcs of C7–T2 were explored. Sham-operated rats received a laminectomy without spinal cord injury. In the transection groups, complete transverse cuts were made through the spinal cord at the T1–T2 level. Further examination by surgical microscope was carried out to ensure lack of any remaining axons or neuronal tissue within the transected area. The muscles and incision area were then sutured.

The spinal cord transected rats were randomized into four treatment groups with eight rats each. All treatments were made intravenously. Group 1 received saline (0.9% NaCl) (1 ml/kg) at both 2 and 24 h after the injury. Group 2 received saline (1 ml/kg) at 2 h and CDP-choline (250 mg/kg) at 24 h after the injury. Group 3 received CDP-choline (250 mg/kg) at 2 h and saline (1 ml/kg) at 24 h after the injury. Group 4 received CDP-choline (250 mg/kg) at both 2 and 24 h after the injury (see Table 1).

Blood pressure recording

For cardiovascular monitoring, the arterial cannulae of rats were connected to a volumetric pressure transducer (BPT 300) attached to a DA1 00B general purpose transducer amplifier (Commat Ltd, Ankara, Turkey). The blood pressure and heart rate of rats were recorded and analysed using the MP100 system and AcqKnowledge software (BIOPAC Systems Inc., CA, USA). Blood pressure was reported as mean arterial pressure (MAP) (mmHg) and heart rate (HR) was expressed as beats per minute (BPM).

Experimental protocol

Following surgical procedures, rats were placed in their individual cages with thick soft bedding material. Their body

Table 1 Treatment groups of spinal cord transected rats

Spinal cord transected rats	Treatment	
	2 h	24 h
Group 1	Saline	Saline
Group 2	Saline	CDP-choline
Group 3	CDP-choline	Saline
Group 4	CDP-choline	CDP-choline

n = 8 per group.

functions were closely monitored for a 90-min period. The arterial lines were then connected to the transducer and baseline cardiovascular parameters were recorded as described above. Rats were allowed to stabilize for 30 min, after which intravenous (i.v.) injections (saline or CDP-choline) were made. The blood pressure and heart rate were monitored for an additional 60 min. At the end of this period, a 1-ml blood sample was collected through the arterial line and replaced with 2 ml saline. Animals were allowed to stay in their individual cages with free access to food and water for the rest of the day. Manual compression was used to empty the bladder twice within 24 h. All animals were observed carefully to ensure lack of infection around the surgical site. At 24 h after spinal cord transection, the arterial line of each rat was connected to the transducer and cardiovascular monitoring began. All procedures described above were repeated. After blood samples were collected, spinal cord samples were extracted from 1 cm rostral to 1 cm caudal to the transected level for histopathological evaluation and malondialdehyde (MDA) measurement. Samples for histopathological examination were immediately placed in a neutral formalin solution for light microscopic examination.

Measurement of plasma catecholamines and vasopressin levels

A total of 1 ml of blood was removed from the arterial catheter and placed into ice-cold tubes containing EDTA. Samples were placed on ice immediately. Plasma was separated by centrifugation at 1800 rev/min for 20 min at 4°C. Plasma noradrenaline and adrenaline were determined using a commercially available radioimmunoassay kit (Biosource Europe S.A., Belgium). Adrenaline and noradrenaline standard solutions were extracted and assayed simultaneously with samples. Values are expressed as pg/ml.

Plasma samples (0.3 ml) were used for vasopressin measurement. First, vasopressin was extracted with ethanol and extracts were dried in a vacuum concentrator (Jouan, Saint-Herblain, France). The dried residues of the extracts were resuspended with 0.3 ml of assay buffer and aliquots were assayed in duplicate using a commercially available radioimmunoassay kit (Biosource). Values are expressed as pg/ml.

Determination of blood superoxide dismutase and glutathione peroxidase activity

Blood samples were immediately centrifuged at 1500*g* for 10 min. After separation of plasma, the packed erythrocytes were washed three times with normal saline and haemolysed with ice-cold water. The erythrocyte lysate of each sample was used for the measurement of superoxide dismutase (SOD) and glutathione peroxidase (GSHPx) activity. The commercially available Superoxide Dismutase Assay Kit (Cayman Chemical Company, MI, USA) and Glutathione Peroxidase Assay Kit (Randox, Antrim, UK) were used for the respective measurements. Briefly, the determination of SOD activity was based on the production of O_2^- anions by the xanthine/xanthine oxidase system. Activity of GSHPx was catalysed by the oxidation of reduced glutathione in the presence of cumene hydroperoxide. The SOD results are expressed as units per gram of haemoglobin. Haemoglobin values were measured by Drabkin's method.

Determination of plasma and tissue malondialdehyde levels

Plasma MDA concentrations were determined by a reaction with thiobarbituric acid (TBA) and high performance liquid chromatography (HPLC) separation of the MDA–TBA conjugate.[20] A calibration curve was prepared for each day by using 1,1′,3,3′-tetramethoxypropane as the standard. The mobile phase contained 50% methanol and 50% 25 mM phosphate buffer at pH 6.5. A flow rate of 0.8 ml/min was used. The spectrophotometric detector was set at 532 nm.

For the determination of spinal cord MDA levels, tissue samples were weighed and homogenized in ten times their volume of 1.15% potassium chloride with a Janke–Kunkel tissue homogenizer (IKA-Labortechnik, Staufen, Germany). The tissue concentration of MDA was measured as previously described[21] and was expressed as nmol MDA per gram of tissue.

Light microscopic evaluation

Starting at the transected level, 10 mm segments of the vertebral column rostral and caudal to the transection were carefully freed of soft tissue, dissected and placed into a large volume fixative (neutral formalin) for histological studies. After 5 days of fixation, the vertebral arches were removed along the entire fragment and the prepared spinal cord tissues were placed in fresh fixative for 2 days. Spinal cord tissues were dehydrated and embedded in paraffin wax. Serial 5-μm cross-sections were cut and two consecutive serial cross-sections were collected at 75-μm intervals (15 serial sections) and placed on poly-L-lysine-coupled slides. The slides were stained using the haematoxylin–eosin (H&E) and Kluwer–Barrera methods for morphological evaluation and examined under a light microscope (Olympus BX-50 and Olympus DP-71 CCD).

To assess the overall injury, each spinal cord cross-section stained with H&E was divided into eight distinct areas (left and right ventral horns and left and right dorsal, ventral and lateral funiculi). The posterior horns are too narrow to allow reliable assessment.[22] The presence of petechiae, extensive bleeding, hypereosinophilic staining in motor neurons (condensation of mitochondria, acute neuronal necrosis), fragmentation, ischaemia, necrosis (identified by tissue pallor and loss of cellular architecture) and perineuronal vacuolization was evaluated in the grey and white matter regions in the rostral and caudal tissues. The percentages of the pathological parameters were then calculated.

The other spinal cord cross-sections were stained with the Kluwer–Barrera method for evaluation of Nissl bodies in motoneurons. In the quantification, neurons were classified as normal or chromatolytic. Normal-basophilic neurons had a triangular shape, mottled nucleus, dense nucleolus and a clearly defined cytoplasm containing distinct Nissl bodies. Motoneurons exhibiting the classic signs of chromatolysis have a granular dispersion of Nissl bodies, eccentric nuclei, rounded and ballooned perikarya and faintly stained cytoplasm, resulting in a 'ghostly appearance'.[22] The percentage

of injured motoneurons was calculated as the ratio of the number of injured neurons divided by the total number of neurons. Double counting of neurons was unlikely because of the 75-μm spacing selected between consecutive sections.[23]

Drugs

CDP-choline was purchased from Sigma-Aldrich (St Louis, MO, USA) and was dissolved in NaCl (0.9%).

Data and statistical analysis

Data are represented as mean ± standard error of the mean (SEM). A Student's *t*-test was used to test differences between values from different groups of rats. An analysis of variance (one-way ANOVA) or repeated measures of analysis of variance (RM-ANOVA, one-way or two-way) was performed for appropriate groups. A Tukey's test was performed when significant interactions were found. $P < 0.05$ was considered significant.

Results

Effects of CDP-choline treatment on tissue injury in spinal shock conditions

In the uninjured control rats there were no histopathological abnormalities identified. In spinal cord transected rats treated with saline and/or CDP-choline, specimens contained various degrees of tissue fragmentation, petechiae (particularly in the grey matter and dorsal horns), haemorrhages and/or haemorrhagic necroses. The injured neurons were shrunken, eosinophilic and their nuclei had become pyknotic. Numerous polymorphonuclear neutrophils were widely spread throughout the damaged tissue. Perineuronal satellitis was observed. The semi-quantitative assessment of overall tissue damage in grey and white matter of rostral and caudal segments to the center of the lesion revealed significant histopathological damage in saline-treated spinal cord transection (SCT) groups (Figure 1, a-1 and a-2). Although the histopathological scores gradually decreased in the groups receiving CDP-choline as a first or second treatment, the score was significantly lower only in group 4, which repeatedly received CDP-choline (Figure 1, d-1, d-2, j and k).

In the intact grey matter, several changes in the anterior horn motoneurons, including ballooned neuronal bodies, were observed in saline-treated SCT rats. The cytoplasm of the neurons became smooth due to the lysis of the Nissl substance (chromatolysis) and their nuclei were displaced toward the periphery of the cell (central chromatolysis) (Figure 1, f–h). In the SCT groups, (especially 'saline+saline' and 'saline+CDP choline') a small cell with homogenously dark and shrunken cytoplasm and nucleus was observed in the motor neurons (Figure 1, f–g, white arrow). This cytoplasmic and nuclear basophilia was described as the change from the chromatolytic to the attritional stage. The percentage of the injured number of neurons was significantly low in SCT groups 3 and 4 (Figure 1, l).

Effects of CDP-choline treatment on cardiovascular parameters in spinal shock conditions

Two hours after spinal cord transection

The MAP and HR values of sham control rats were 125 ± 3 mmHg and 400 ± 24 BPM, respectively. The values remained at these levels throughout the monitoring period (Figure 2 and Figure 3). Blood pressure and HR of SCT rats were 93 ± 2 mmHg ($n = 30$) and 280 ± 6 BPM ($n = 30$), respectively. There were no significant MAP or HR differences between the treatment groups before the injections. Intravenously injected CDP-choline (250 mg/kg) increased blood pressure and decreased the HR (Figure 2 and Figure 3). Both effects were time-dependent and reached their maximal levels within 3–5 min. The MAP values in CDP-choline-injected rats returned to basal levels within 20–30 min, while the duration of CDP-choline-induced bradycardia was only 10 min. Saline (1 ml/kg; i.v.) injections did not affect the MAP or HR levels in related groups (Figure 2 and Figure 3).

Twenty-four hours after spinal cord transection

Blood pressure and HR levels in the sham group were very similar to those observed in the first measurement (Figure 2 and Figure 3). In SCT rats, however, these measurements were lower than those observed 2 h after transection (Figure 2 and Figure 3). The blood pressure and HR levels of all transected rats at 24 h were 84 ± 3 mmHg ($n = 28$) and 195 ± 5 BPM ($n = 28$), respectively. Interestingly, the i.v. injection of CDP-choline (250 mg/kg) did not induce the pressor effect in these animals, but instead produced a small and transient decrease in blood pressure (Figure 2). HR decreased immediately in response to the CDP-choline injection (250 mg/kg; i.v.) and returned to basal levels within 10 min (Figure 3). Saline injections (1 ml/kg; i.v.) did not alter the MAP or HR levels of related groups.

Effects of CDP-choline treatment on plasma catecholamines and vasopressin levels in spinal shock conditions

Two hours after spinal cord transection

Plasma adrenaline and noradrenaline levels in sham control rats were 1041 ± 127 and 382 ± 11 pg/ml ($n = 6$), respectively. Complete transection of the spinal cord decreased plasma adrenaline levels to approximately one-tenth of those observed in sham control rats (Table 2). Adrenaline levels were comparable in all treatment groups (Table 2).

Plasma noradrenaline levels tended to decrease after spinal cord transaction. However, levels observed in saline and CDP-choline-injected rats were not significantly different from levels observed in sham animals or between the treatment groups at 2 h after transection (Table 2).

Plasma vasopressin levels increased almost six-fold in SCT rats (Table 2). These levels were not significantly different between saline and CDP-choline-injected rats (Table 2).

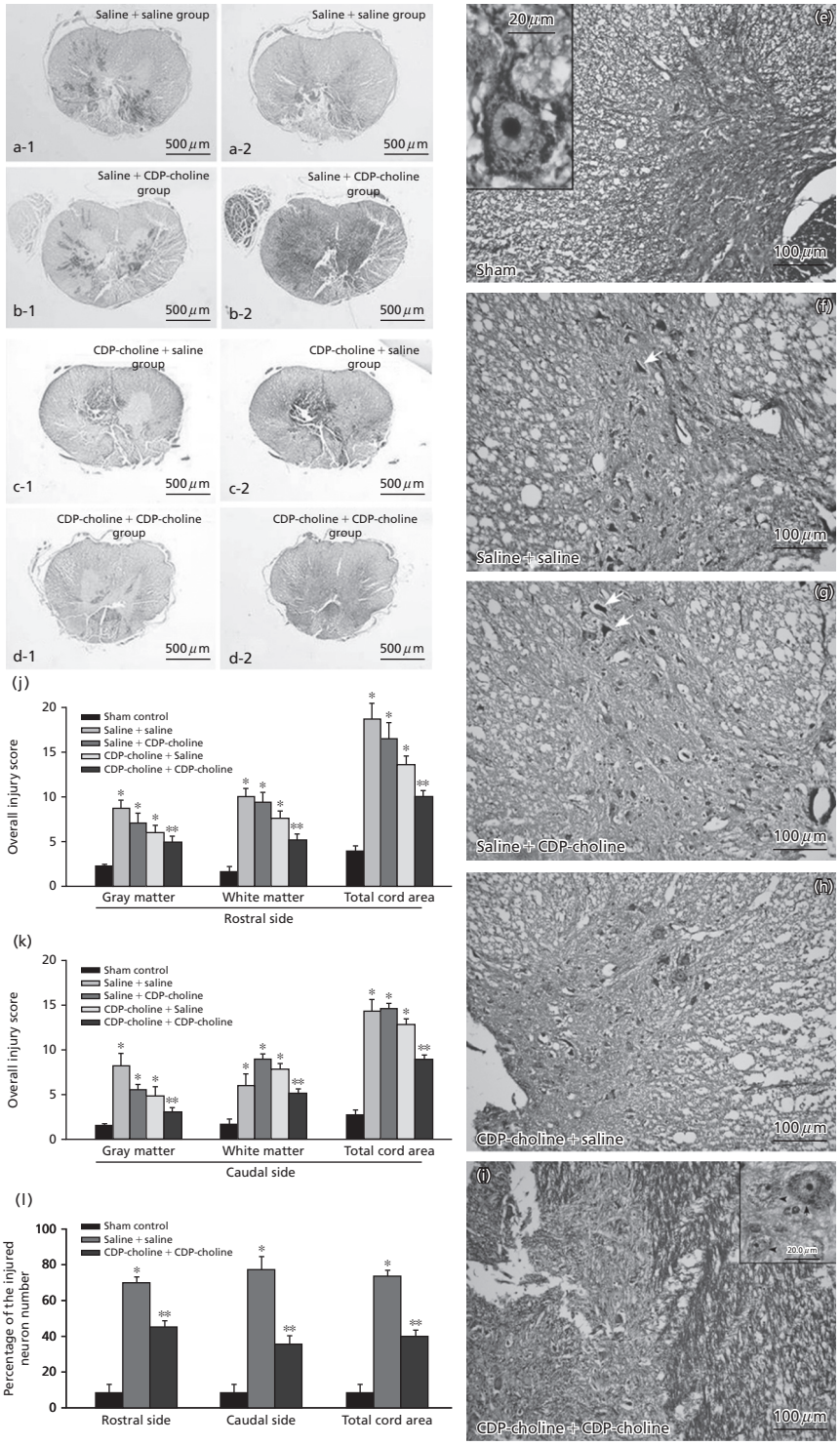

Figure 1 Histopathological changes at the lesion site (pictures) and morphological scores (graphs) in spinal cord transected rats receiving saline or CDP-choline treatments. a–d, caudal spinal cord sections stained with haematoxylin–eosin (left column) and Kluwer-Barrera's method (right column); h–i, caudal spinal cord sections stained with Kluwer-Barrera's method presented in a bigger format; h, sham group. Normal motor neurons have a well-defined nucleus and Nissl substance distributed throughout the cytoplasm (inlet). f–i, Study groups. The white arrows indicate a progressively small, homogenously dark and shrunken cytoplasm and nucleus in injured motor neurons. i, The arrow indicates a healthy appearing neuron and the arrowheads indicate chromatolysis (inset). j, k, The graphs demonstrate the morphological scores of each treatment group of the spinal cord transected animals. l, The graph shows the percentage of the injured neuron number according to the Kluwer–Barrera's method. Bars represent the mean ± SEM of evaluation of the five to seven animals. Statistical analysis of the groups was performed by using ANOVA with post-hoc Tukey test. $^{*}P < 0.05$ significantly different from the value of the sham control group; $^{**}P < 0.05$ significantly different from the value of the group 1

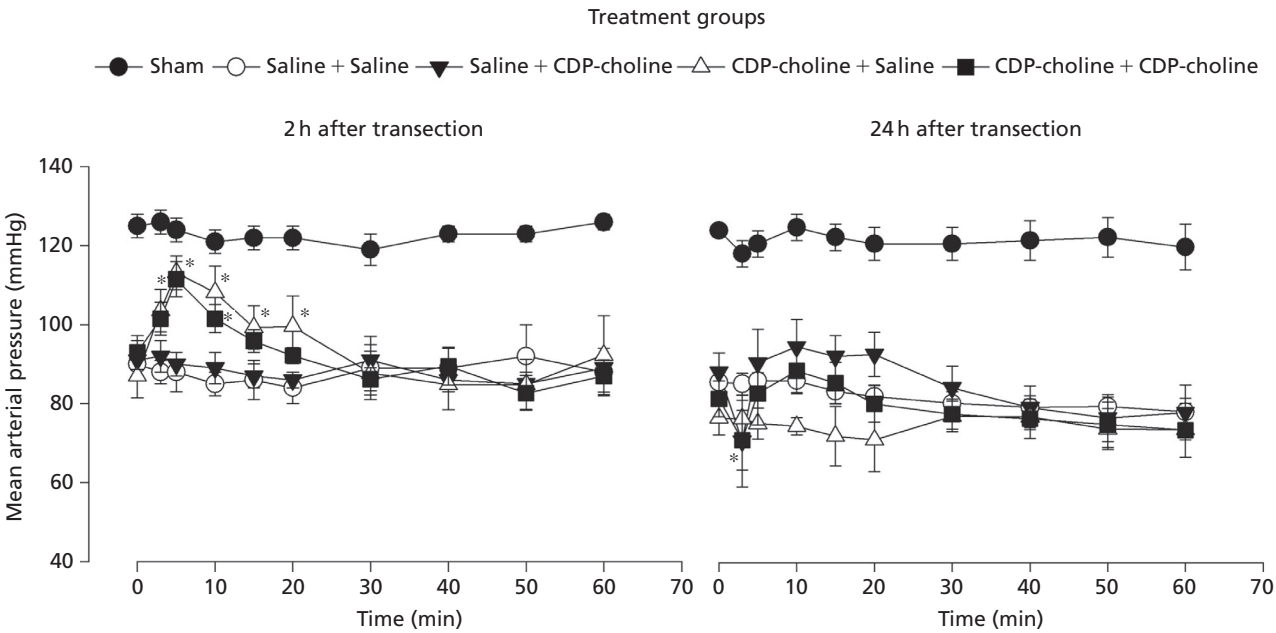

Figure 2 Mean arterial pressure responses to CDP-choline in spinal cord transected animals. Rats were administered saline (1 ml/kg; i.v.) or CDP-choline (250 mg/kg; i.v.) at 2 h and 24 h after spinal cord transection. Blood pressure was monitored during the 60-min period after the injections. Data are given as means ± SEM of eight measurements. Statistical analysis was performed by using RM-ANOVA with post-hoc Tukey test. $^{*}P < 0.05$ significantly different from the value for the animals that received saline at the corresponding treatment interval

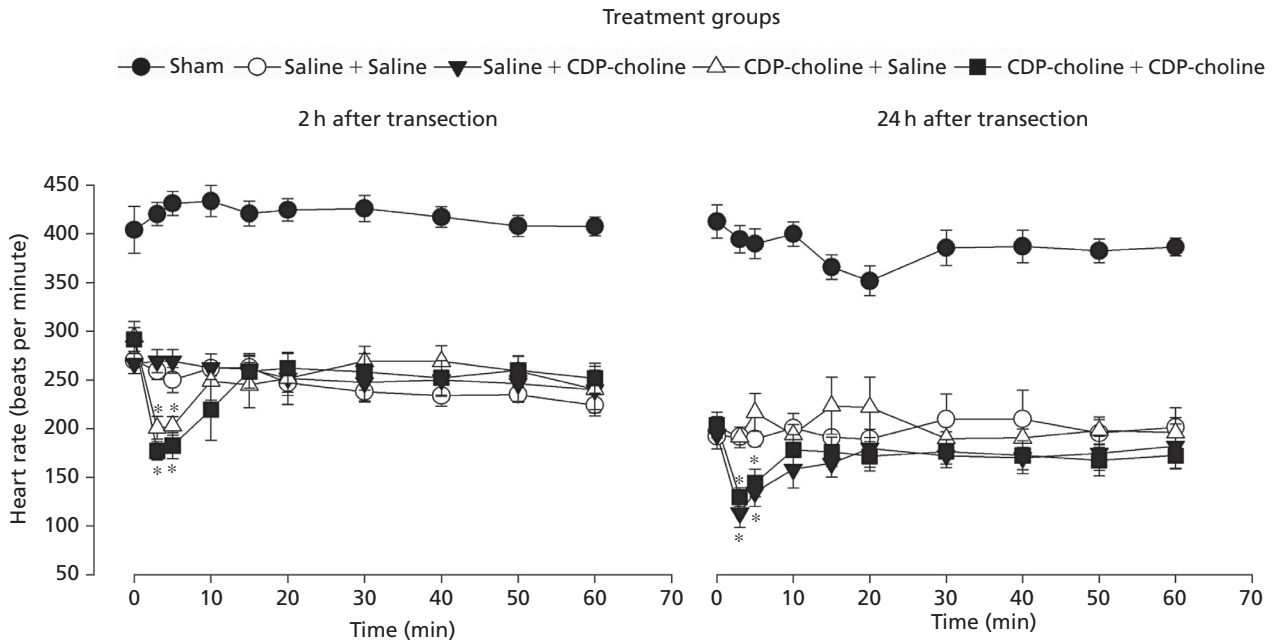

Figure 3 Heart rate responses to CDP-choline in spinal cord transected animals. Rats were administered saline (1 ml/kg; i.v.) or CDP-choline (250 mg/kg; i.v.) at 2 h and 24 h after spinal cord transection. The heart rate of rats was monitored during the 60-min period after the injections. Data are given as means ±SEM of eight measurements. Statistical analysis was performed by using RM-ANOVA with post-hoc Tukey test. $^{*}P < 0.05$ significantly different from the value for animals given saline at the corresponding treatment interval

Table 2 Plasma adrenaline, noradrenaline and vasopressin levels in spinal cord transected rats receiving saline or CDP-choline treatments

Treatment groups	Plasma adrenaline levels (pg/ml)		Plasma noradrenaline levels (pg/ml)		Plasma vasopressin levels (pg/ml)	
	2 h	24 h	2 h	24 h	2 h	24 h
Sham control	1042 ± 127	1747 ± 438	374 ± 16	189 ± 35	2 ± 0.2	2 ± 0.3
Group 1	86 ± 15*	107 ± 44*	268 ± 20	87 ± 24	12 ± 1*	7 ± 1*
Group 2	52 ± 21*	133 ± 36*	261 ± 12	153 ± 41	11 ± 2*	10 ± 2*
Group 3	22 ± 9*	194 ± 49*	253 ± 63	160 ± 69	12 ± 2*	7 ± 1*
Group 4	20 ± 12*	417 ± 125**	198 ± 35	261 ± 36	10 ± 2*	8 ± 1*

Rats were administered saline (1 ml/kg; i.v.) or CDP-choline (250 mg/kg; i.v.) at 2 h and 24 h after spinal cord transection. Blood samples were taken via arterial line 60 min after each injection. Data are given as means ± SEM of six to eight measurements. Statistical analysis was performed by using ANOVA with post-hoc Tukey test. $^{*}P < 0.05$, significantly different from the sham values of the corresponding time scale. $^{**}P < 0.05$, significantly different from their own respective 2 h values.

Twenty-four hours after spinal cord transection

Plasma adrenaline levels did not significantly change in any treatment groups at the corresponding time interval. However, the levels were significantly higher in group 4, in which CDP-choline was given repeatedly, than in the same group at 2 h (Table 2). Plasma noradrenaline and vasopressin levels were not significantly different from their own respective 2-h values after SCT in all groups (Table 2).

Effects of CDP-choline treatment on oxidative stress parameters in spinal shock conditions

Two hours after spinal cord transection

In order to examine if CDP-choline treatment can attenuate the oxidative stress in spinal shock conditions, we measured blood SOD, GSHPx activity and plasma and tissue MDA levels in the repeatedly CDP-choline injected group, since the tissue protective effect of the drug was seen clearly in this group of animals. Blood SOD activity significantly increased in rats that underwent SCT compared to sham operated animals (Figure 4). Levels were similar between saline and CDP-choline-injected rats (Figure 4). There were, however, no significant changes in the activity of GSHPx (Figure 5) or the plasma MDA levels (1131 ± 151, 956 ± 63, 1321 ± 43 nmol/ml; sham control, 'saline + saline' group, 'CDP-choline + CDP-choline' group, respectively) 2 h after SCT.

Twenty-four hours after spinal cord transection

Blood SOD levels were not significantly different from levels observed 2 h after SCT, while GSHPx activity in saline-treated groups significantly increased on day 1 compared to sham operated animals (Figure 5). Both SOD and GSHPx activity were significantly lower in the CDP-choline groups (Figure 4 and Figure 5). Plasma (1236 ± 111, 999 ± 87, 1146 ± 69 nmol/ml; sham control, 'saline + saline' group, 'CDP-choline + CDP-choline' group, respectively) and tissue (25 ± 2, 26 ± 1, 25 ± 1 nmol/g; sham control, 'saline + saline' group, 'CDP-choline + CDP-choline' group, respectively) MDA levels did not change after SCT and CDP-choline treatment.

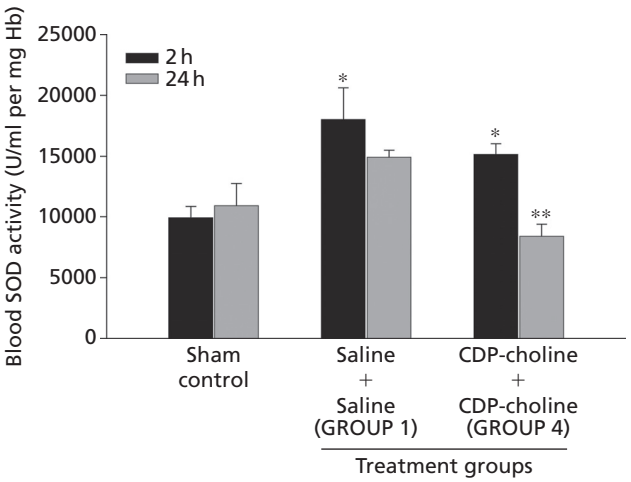

Figure 4 Blood superoxide dismutase activity in spinal cord transected rats receiving saline or CDP-choline treatments. Rats were administered saline (1 ml/kg; i.v.) or CDP-choline (250 mg/kg; i.v.) at 2 h and 24 h after spinal cord transection. Blood samples were taken via arterial line 60 min after each injection. SOD, superoxide dismutase. Data are given as means ± SEM of 6–8 measurements. Statistical analysis was performed by using ANOVA with post-hoc Tukey test. $^{*}P < 0.05$ significantly different from the value of the sham group. $^{**}P < 0.05$ significantly different from its own respective 2 h values and the group 1 value for the corresponding time interval

Discussion

The present data show that repeated intravenous injection of CDP-choline at 2 and 24 h after spinal cord transection can prevent tissue injury, transiently alter blood pressure and heart rate, and decrease blood SOD and GSHPx activity in spinal shock conditions.

The dose of CDP-choline chosen for this study was based on our earlier experiments. Our previous reports demonstrated that CDP-choline can increase blood pressure and reverse hypotension induced by haemorrhagic shock,[18,19,24] as well as prevent arrhythmias and death in response to short-term myocardial ischaemia–reperfusion injury.[25] These studies have implied that the pressor- and tissue-protective effect of CDP-choline is dose-dependent. Since we were interested in investigating whether the pressor response to CDP-choline influences the drug's protective effects, the i.v. 250 mg/kg dose of CDP-choline employed in this study was the most appropriate one for these observations.

Acute complete transection of the spinal cord, particularly at a high level (cervical and high thoracic), results in severe hypotension and persistent bradycardia. This phenomenon is

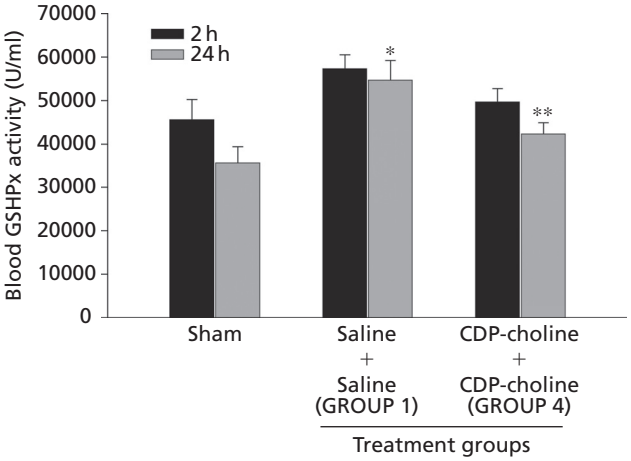

Figure 5 Blood GSHPx activity in spinal cord transected rats receiving saline or CDP-choline treatments. Rats were administered saline (1 ml/kg; i.v.) or CDP-choline (250 mg/kg; i.v.) at 2 h and 24 h after spinal cord transection. Blood samples were taken via arterial line 60 min after each treatment period. GSHPx, glutathione peroxidase. Data are given as means ± SEM of 6–8 measurements. Statistical analysis was performed by using ANOVA with post-hoc Tukey test. $^{*}P < 0.05$ significantly different from the sham value of the respective time interval; $^{**}P < 0.05$ significantly different from the group 1 value of the corresponding time interval

known as neurogenic shock.[1] Consistent with this knowledge, the transection of the spinal cord at the T1–T2 level caused prompt and sustained decrease in blood pressure. The decrease in MAP was approximately 30 mmHg at 2 h after the transection and an additional 10–20 mmHg decrease in blood pressure was observed on day 1 in all rats. Studies attempting to explain the mechanism of this hypotension have shown that there is a generalized reduction in tonic sympathetic activity associated with the low levels of plasma adrenaline and noradrenaline[26] and compensatory increases in plasma vasopressin levels.[27] Our observations of plasma catecholamines and vasopressin levels in SCT rats correspond to previous findings and demonstrate the main results of neurogenic shock. In these conditions, CDP-choline (250 mg/kg; i.v.) that was injected just 2 h after SCT significantly increased MAP but produced a small and very transient decrease in blood pressure when injected 24 h after the transection (Figure 2). Our previous studies showed that the pressor response to CDP-choline is mediated by enhancement of the central cholinergic transmission followed by an activation of the sympathoadrenal pathway, which results in an increase in plasma catecholamines as well as an increase in plasma vasopressin levels.[18,19] In the present study, we observed no significant difference in plasma noradrenaline and vasopressin levels at all time and treatment periods. On the other hand, in groups that received CDP-choline injections repeatedly, plasma adrenaline values were higher after the second injection than their respective 2 h values. However, CDP-choline-induced pressor response was observed only after the first injection (2 h after SCT) and there was no pressor effect after the second injection of the drug. Since the blood samples of rats were taken 1 h after the injections, we may assume that the present levels of catecholamines and vasopressin do not reflect the immediate effect of the drug and may not explain the mechanism of the pressor response to CDP-choline observed after the first injection. We therefore performed additional experiments to measure the levels of catecholamines and vasopressin 5 min after the first CDP-choline injection. We observed small increases in plasma noradrenaline levels in CDP-choline treated rats. This could be one explanation for this prompt and transient pressor response.

In the present study, the heart rate of SCT rats was approximately 100 BPM lower than that observed in sham animals 2 h after spinal cord transection. Heart rates of the rats were additionally decreased (by about 100 BPM) 24 h after the injury. Injection of CDP-choline (250 mg/kg; i.v.) caused immediate and short-term bradycardia (observed within 3 min and disappearing at 10 min) after each injection (Figure 3). The profile of the heart rate responses to CDP-choline was quite different to those we observed previously in normal and hypotensive rats.[18,19] In those studies, intravenously or intracerebroventricularly injected CDP-choline did not change or tended to increase the heart rates of animals during the pressor effect. In the intact animals, the cardiac effect of elevated plasma catecholamines may be the leading effect, however in spinal cord transected rats, with the total disruption of the descending sympathetic pathways, the cardiac effect of the parasympathetic nervous system becomes dominant since the parasympathetic control of the cardiovascular system exits at the level of the brain stem via the vagus nerve. Hence, in the present conditions, the activation of central cholinergic transmission due to CDP-choline administration mainly results in the activation of the efferent vagal pathways,[25] leading to a decrease in heart rate in rats. On the other hand, CDP-choline is rapidly hydrolysed to its final metabolites, choline and cytidine, after its intravenous injection. Our previous report clearly demonstrated that plasma choline concentrations increase almost 30-fold after a 250 mg/kg CDP-choline injection,[19] therefore we may also assume that plasma choline derived from CDP-choline may directly activate cardiac muscarinic receptors and cause bradycardia.[28] Moreover, this behaviour of the drug may be the primary reason for the observation of a small CDP-choline-induced decrease in MAP after the second injection.

Transection of the spinal cord resulted in various histopathological incidences, including extensive bleeding, necrosis (identified by tissue pallor and loss of cellular architecture) and oedema (characterized by tissue microvacuolation) in saline-treated SCT animals. Although there was a gradual reduction in tissue damage in other groups given CDP-choline treatment at 2 or 24 h after the injury, the morphological damage was significantly reduced only in group 4, which was given repeated CDP-choline treatments. Our observation that the percentage of the injured number of neurons was also significantly reduced in the same group suggests that the repeated administration of CDP-choline also has a neuroprotective effect.

Cardiovascular results of the present study suggest that the tissue protection induced by CDP-choline in spinal shock conditions is not mediated by its cardiovascular responses since the drug only transiently increased blood pressure after its first injection and did not produce any pressor response at 24 h after SCT.

On the other hand, oxidative stress is one of the early results of primary spinal cord injury and leads to tissue injury and neuronal dysfunction.[8] The release of oxygen-derived free radicals by several blood cells and microglial cells causes cellular degeneration by damaging the critical cellular components, such as lipids, proteins, phospholipids and nucleic acids.[29] Superoxide dismutase is the primary enzyme that converts the superoxide, the first generated reactive oxygen species in the body, to hydrogen peroxide. Glutathione peroxidase is the enzyme that converts hydrogen peroxide to water and molecular oxygen. In the presence of excessive lipid peroxidation and superoxide anions, hydrogen peroxide can be converted to extremely reactive and cytotoxic free radicals, hydroxyl radicals. The increased activity of these enzymes implies the presence of oxidative stress and the body's reaction against it. Studies investigating the role and progression of oxidative stress in several types of cord injuries have determined the activity of these enzymes, mostly in the spinal cord tissue surrounding the transection or injury sites.[30,31] Once lipid peroxidation is stimulated by the primary injury, however, it will not be limited to the injury site but will generate oxidative stress all over the body, particularly in shock conditions. In the present study therefore we determined the activity of these enzymes in the circulation. Interestingly, the blood SOD activity obtained from rats 2 h after the SCT is significantly higher than the levels obtained from sham animals. Furthermore, the SOD levels remained the same on day 1 while GSHPx activity increased significantly. Conversely, we did not observe any change in plasma and tissue MDA levels, which is not consistent with some of the previous reports that demonstrated the increase in spinal tissue MDA levels 24 or 48 h after spinal cord trauma.[32,33] In contrast to previous studies, however, we have used the transection method instead of the weight dropped trauma model. We can explain this difference by suggesting that the lipid peroxidation induced by our model or the time interval of 24 h after the injury may not be sufficient to observe the end product of this pathology.

Repeated CDP-choline treatment significantly decreased SOD and GSHPx activity, indicating that repeated drug treatment alleviates oxidative stress under spinal shock conditions. Morover these findings correspond to the morphological data observed in group 4 and suggests that limiting oxidative stress results in a decrease in tissue damage.

Conclusions

The present data show that the intravenous administration of CDP-choline can decrease lipid peroxidation and protect tissue and neuronal injury in spinal cord transected rats. The pressor effect of CDP-choline does not appear to be the mechanism underlying the observed tissue protection, as this was a temporary response at 2 h after the injection and did not occur at the 24 h time point. On the other hand, the limitation of oxidative injury by the drug appears to be one of the protective mechanisms. We found that in order to obtain the most protective effect, it must be administered repeatedly, starting at a very early point. CDP-choline is a very safe drug, which has been used in the treatment of several cerebral ischaemic situations with virtually no side effects. Since the neuroprotective effect of CDP-choline was responsible for about half of the injury attenuation, it can be considered as one component of a combination treatment in the early phase of spinal cord trauma.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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