



Electroacupuncture and Curcumin Promote Oxidative Balance and Motor Function Recovery in Rats Following Traumatic Spinal Cord Injury

Belen G. Alvarado-Sanchez¹ · Hermelinda Salgado-Ceballos^{2,4} · Sergio Torres-Castillo^{3,4} · Juan Rodriguez-Silverio¹ · Monica E. Lopez-Hernandez³ · Salvador Quiroz-Gonzalez³ · Stephanie Sanchez-Torres⁵ · Rodrigo Mondragón-Lozano² · Omar Fabela-Sanchez⁵

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Abstract

Spinal cord injury (SCI) is a condition that puts the patient's life at risk in the acute phase and, during the chronic stage, results in permanent deficits in motor, sensory and autonomic functions. Isolated therapeutic strategies have not shown an effect on this condition. Therefore, this study aimed to evaluate the effects of electroacupuncture (EA) and curcumin, alone or combined, on the oxidative balance, motor function recovery and amount of preserved tissue following a traumatic SCI. Long-Evans rats were divided into five groups: SHAM, SCI, SCI+EA, SCI+Curcumin, and SCI+EA+Curcumin. Nitric oxide was significantly decreased in the Curcumin group; the EA, Curcumin and SCI+EA+Curcumin groups had significantly decreased hydroxyl radical and lipid peroxidation levels. Motor function recovery and the amount of preserved spinal cord tissue were significantly greater in the EA, Curcumin and EA+Curcumin groups. The results show that EA and Curcumin treatment alone or in combination decreased oxidative stress, improved functional motor recovery and increased the amount of preserved spinal cord tissue following a traumatic injury.

Keywords Electroacupuncture · Curcumin · Spinal cord injury · Oxidative stress · Lipid peroxidation · Motor function recovery

Introduction

Spinal cord injury (SCI) is a public health problem that, during its acute phase, puts patients' lives at risk and, in the long term, causes deficits in motor, sensory and autonomic functions. Pathophysiologically, SCI is divided into two stages: primary mechanical damage and secondary

injury mechanisms; the latter are primarily responsible for the destruction of the anatomical substrate necessary for normal neurological function [1]. Oxidative stress is a key mechanism in the secondary damage induced following spinal cord injury (SCI) [2–5], and it is directly associated with the structural and functional alterations of nucleic acids, proteins and lipids through the sudden formation of free radicals, including reactive oxygen species (ROS) such as superoxide anion (O_2^-), hydroxyl radical (ROH·) and nitric oxide (NO·). The importance of the damage caused by oxidative stress in SCI lies in its ability to cause disruptions in the conduction of nerve stimulation, neuronal death and permanent neurological deficits below the site of damage; these changes are predominately a result of the limited response of the antioxidant system formed by the enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) to neutralize the ROS formed during SCI [6–8].

The pathophysiology of SCI is complex, which makes it necessary to search for combined alternatives to limit, prevent or reverse damage to the spinal cord after trauma.

✉ Sergio Torres-Castillo
sergio_torres@yahoo.com

¹ Escuela Superior de Medicina, Instituto Politécnico Nacional, C.P. 11340 México City, Mexico

² Unidad de Investigación Médica en Enfermedades Neurológicas, Centro Médico Nacional Siglo XXI, IMSS, C.P. 06720 México City, Mexico

³ Universidad Estatal del Valle de Ecatepec, C.P. 55210 Ecatepec de Morelos, Estado De México, Mexico

⁴ Proyecto Camina A. C., C.P. 14050 México City, Mexico

⁵ UAM Iztapalapa, C.P. 09340 México City, Mexico

Acupuncture is an age-old alternative medicine that consists of the strategic penetration of the skin at specific points located in specific routes or channels in the body [9]. Electroacupuncture (EA) is a modality of acupuncture that utilizes electrical stimulation of acupuncture points and has been widely used in countries such as China, the United States of America, and France. Evidence indicates that EA is particularly effective in the treatment of neurological problems through the puncture of the Governor Vessel channel (GV) [10–13]. EA at the GV.4 site is a therapeutic procedure for which previous studies have demonstrated a control of oxidative stress by reducing the amount of reactive species and/or increasing the expression and activity of the antioxidant enzymes SOD and GPx in experimental rat models [14–19]. Moreover, stimulation of the GV.4 point increases the expression of aquaporin-4, which suggests that it may decrease edema following SCI [20, 21].

In recent decades, the use of active components of medicinal plants has gained popularity due to the belief that natural products are safe and exert few side effects. Curcumin is a polyphenol that is part of the active component of *Curcuma longa*, a plant of Asian origin, which belongs to the Zingiberaceae family [22]. It participates in the modulation of cytokines, growth factors and transcription [23] and promotes the expression of antioxidant enzymes and acts as a trap for free radicals [24–26]. In previous studies, curcumin has exhibited a broad pharmacological spectrum as an antineoplastic, antimutagenic, anti-inflammatory and antioxidant. In neurological diseases, such as SCI, it has exhibited a neuroprotective role because it limits the loss of neurons via the prevention of apoptosis and the activation of astrocytes [27–31].

To date, the therapeutic possibilities to revert the damage induced by SCI are limited, which leads individuals to become confined to wheelchairs, alters their psychosocial sphere and results in substantial costs for health services [32]. Thus, the present study aimed to determine the effects of EA at the GV.4 site and curcumin alone or in combination by evaluating the oxidative stress, antioxidant capacity, motor function recovery and amount of spinal cord preserved tissue in rats with traumatic SCI.

Materials and Methods

Animals

All experimental procedures were conducted according to the protocol approved by the research and ethical committee of the Valley of Ecatepec Statal University and the Mexican Social Security Institute (IMSS); the procedures were in compliance with the Mexican General Law in Health [33] and the international rules stated in the National Institutes of

Health Guide for the Care and Use of Laboratory Animals. Experiments were performed on 70 Long Evans adult female rats, which weighed between 250 and 300 g. The animals were randomly divided into five groups: (1) SHAM, (2) SCI (without treatment), (3) SCI+EA, (4) SCI+Curcumin and (5) SCI+EA+Curcumin.

Spinal Cord Injury

SCI was induced using a well-characterized technique using the New York University (NYU) weight-drop device. The rats were anesthetized intraperitoneally with a mixture of ketamine and xylazine (75 and 12.5 mg/kg of body weight, respectively). Using antiseptic procedures, a dorsal laminectomy at the thoracic nine level was performed with the aim to expose the spinal cord. The animals were subsequently placed in a stereotaxic system, and a 10 g rod was dropped onto the spinal cord from a height of 25 mm to inflict an injury via contusion with a moderate intensity, which resulted in hind limb paralysis.

Treatments

The rats were randomly assigned to receive the treatments 5 min after SCI. EA was applied to the GV.4 acupoint using an Electro-Stimulator for acupuncture (KWD-808-I Multipurpose health device). The stimulus consisted of a dense disperse current with a frequency of 2/100 Hz to 5.2 mA per 30 min, which was applied through a stainless steel needle with a diameter of 0.20 mm and a length of 13 mm (Europe-Hua tuo & CO AB) directly on the GV.4 acupoint (located over the middle posterior line beneath the lumbar spinous process-2). Curcumin was diluted with dimethylsulfoxide in a 10% physiological solution and administered at a dose of 200 mg/kg body weight (according to a dose–response curve study) via i.p. injection.

The rats assigned to the biochemical tests were euthanized 24 h after the SCI. The spinal cords, including the epicenter of the injury site plus 0.3 cm distal and caudal to this area, were subsequently extracted, and the meninges were removed. Each spinal cord was homogenized in 1 ml of physiological solution and frozen at -70°C until analysis. The radical $\text{OH}\cdot$ evaluations and lipid peroxidation were performed via spectrophotometry (Genesys 10 UV spectrophotometer). For the analysis of $\text{NO}\cdot$ and antioxidant enzyme activity, a microstrip reader was used (Stat Fax 303 Plus).

Oxidative Activities

$\text{NO}\cdot$ was conveniently measured from the total nitrate/nitrite concentration in each sample using a two-step process. The first step converted nitrate to nitrite using the nitrate reductase enzyme. The second step used Griess Reagents

to convert nitrite to a deep purple azo compound, which reflects the NO \cdot amount present in the samples expressed in nmol of nitrate (nitrite). The measurements were performed at 540 nm (Kit para NO \cdot BioVision, CAT-K262-200).

The thiobarbituric acid reactive substance (TBARS) production from 2-deoxyribose was used to estimate the radical hydroxyl production 24 h after SCI. TBARS was determined using 0.5 ml of 2.8% (wt/vol) trichloroacetic acid and 0.5 ml of 1% (wt/vol) thiobarbituric acid in 50 mM NaOH, which was added to 100 μ l of sample and heated for 10 min at 100 $^{\circ}$ C. The mixture was allowed to cool, and the absorbance was measured at 532 nm [34].

Lipid peroxidation was evaluated by the presence of malondialdehyde (MDA), a byproduct of this process. Following homogenization of the spinal cord, a 100 μ l sample was solubilized with 200 μ l of 8.1% dodecyl sodium sulfate plus 1.5 ml of 20% glacial acetic acid, pH 3.5. Then, 1.5 ml of ice-cold 0.8% thiobarbituric acid were added, and the volume was brought to 4 ml using distilled water. The samples were incubated at 95 $^{\circ}$ C for 60 min. After cooling with tap water, 1.0 ml of distilled water and 5.0 ml of a mixture of n-butanol and pyridine (15:1 v/v) were added. The solution was vigorously shaken and centrifuged at 4000 rpm for 10 min, and the absorbance of the organic layer was determined at 532 nm [35].

Antioxidant Activity

The determination of the SOD activity by Kit-WST provided a convenient SOD assay by utilizing a highly water-soluble tetrazolium salt (WST-1) that produces a water-soluble formazan dye following reduction with a superoxide anion. The rate of the reduction with O $_2$ is linearly related to the xanthine oxidase activity, and it is inhibited by SOD. The quantification was determined at 450 nm (Kit para SOD, FLUKA, CAT-19160).

The assay for reduced glutathione is based on the glutathione recycling system via dinitrothiocyanobenzene and reduced glutathione. Dinitrothiocyanobenzene and reduced glutathione react to generate 2-nitro-5-thiobenzoic acid, which has a yellow color and was measured at 405 nm (Kit para GLU, BioVision, CAT-K261).

The determination of the CAT activity was accomplished using a colorimetric method with a substituted phenol (3,5-dihloro-2-hydroxybenzene-sulfonic acid), which oxidatively couples to 4-aminoantipyrine in the presence of hydrogen peroxide and horseradish peroxidase to provide a red quinoneimine dye that absorbs at 520 nm (Kit para CAT, Sigma CAT100-1KT).

The protein determination was performed using the Bradford method, which is based on the binding of Coomassie Brilliant Blue G-250 to proteins, and the quantitation was performed at 595 nm.

Evaluation of Motor Functional Recovery

Clinical evaluations were performed using two different scales: (1) The hind limb motor score was graded using a 0–21 point scoring system referred to as the Basso, Beathide and Bresnahan (BBB) motor scale; this scale categorizes a combination of rat hind limb movements, trunk position and stability, stepping, coordination, paw placement, toe clearance and tail position. The hind limb motor function was recorded 24 h after SCI to corroborate the absence of movement, followed by every week for 2 months [36]. (2) The swimming performance was assessed using the Lowsville (LSS) score prior to injury and then 2 weeks post-injury at weekly intervals for 2 months. For each assessment, the animals swam in one 4-min session and were scored by two independent observers who were blinded to the experimental group, as well as a third individual who handled the animals and videotaped 1 min of each swim session (video-camera digital Sony, HDR-CX130/RCE23). The focus of this study was the LSS, an 18-point scale (0–17) with three ranges 0–5 (poor swimming), 6–11 (moderate swimming), and 12–17 (good swimming). It was designed to evaluate the swimming performance based on the four primary components of swimming: forelimb dependency, hindlimb activity and alternation, trunk instability and body position [37].

Determination of Preserved Tissue

At the end of the follow-up, the rats were deeply anesthetized with 3.3 ml 50% pentobarbital and were transcatheterially perfused with 100 ml of saline solution, followed by 350 ml of 10% formaldehyde in phosphate buffer (pH 7.4) at 30 ml/min. The spinal cords were then transversely cut from T8 to T10 and postfixed in 10% formaldehyde. After 3 days, the samples were embedded in paraffin blocks. Transversal paraffin sections were cut at a thickness of 8 μ m. The tissue was stained with Masson's trichrome. Following an examination of serial sections from the injury site, the section that contained the largest lesion area from each rat was selected and analyzed, as well as one slide that preceded and another slide that followed the selected slide. The amount of spared tissue was evaluated using an image analysis system (software image database Motic) connected with a microscope (Leica DM500).

Statistics

The results of the biochemical tests and the spared tissue evaluation were analyzed using a one way ANOVA followed by Tukey's test, whereas differences in the motor functional recovery (results from the BBB and LSS scores) were determined using an ANOVA of repeated-measures followed by Tukey's test. All analyses were performed using GraphPad

Prism 5.0 software. Differences were considered statistically significant at $p < 0.05$.

Results

Electroacupuncture and Curcumin Prevent SCI-Induced Oxidative Stress

The NO· levels expressed in nitrate/nitrite 24 h after SCI were significantly higher in the untreated SCI group (0.033 ± 0.010) than in the SHAM group (0.014 ± 0.005) and the Curcumin group (0.016 ± 0.005) ($p < 0.05$). The ROH· levels, expressed in TBARS, 24 h after SCI were significantly higher in the untreated SCI group (0.05 ± 0.01) than in the SHAM (0.018 ± 0.005), SCI+EA (0.030 ± 0.003), SCI+Curcumin (0.034 ± 0.009) and combination SCI+EA+Curcumin (0.031 ± 0.005) groups ($p < 0.05$). The MDA levels 24 h after SCI were significantly higher in the untreated SCI group (0.07 ± 0.01) than in the SHAM (0.03 ± 0.01), SCI+EA (0.017 ± 0.001), SCI+Curcumin (0.035 ± 0.008) and combination SCI+EA+Curcumin (0.017 ± 0.003) groups ($p < 0.05$) (Fig. 1).

Effect of Electroacupuncture and Curcumin on Antioxidant Activity

The activity of the antioxidant enzyme SOD (% control) and the levels of reduced glutathione ($\mu\text{g/ml}$ of sample) at 24 h after SCI did not exhibit significant differences among the different groups evaluated (data not shown). The activity of the antioxidant enzyme CAT ($\mu\text{mol H}_2\text{O}_2/\mu\text{g}$ of protein) 24 h after SCI exhibited a significant difference

between the SCI+Curcumin group ($0.0005 \pm 9.153\text{e}-005$) and the SHAM group ($0.0003 \pm 4.385\text{e}-005$); However, compared to the untreated SCI group, no groups showed significant differences ($p < 0.05$) (Fig. 2).

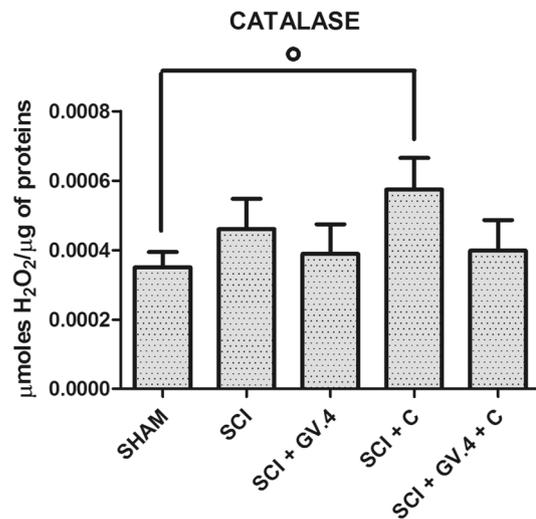


Fig. 2 Catalase. In the graph, the bars represent the levels of the antioxidant enzyme catalase 24 h after traumatic spinal cord injury (SCI) and the corresponding treatment (SHAM; SCI: spinal cord injury without treatment, EA: electroacupuncture, C: curcumin, EA + C: combination). The group data are presented as the mean \pm S.D. ($n = 7$). ANOVA followed by post hoc Tukey's test ($p < 0.05$)

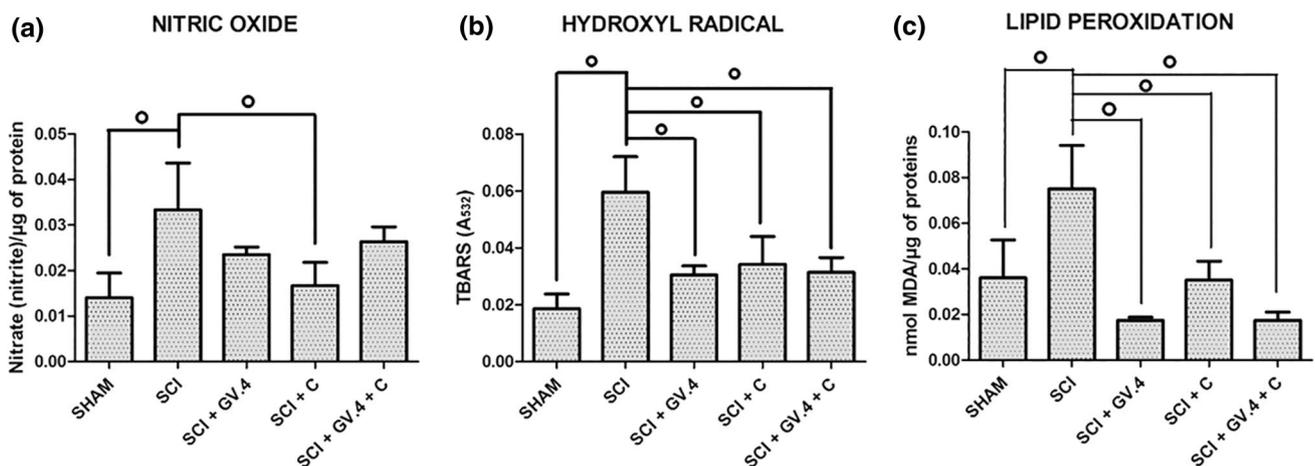


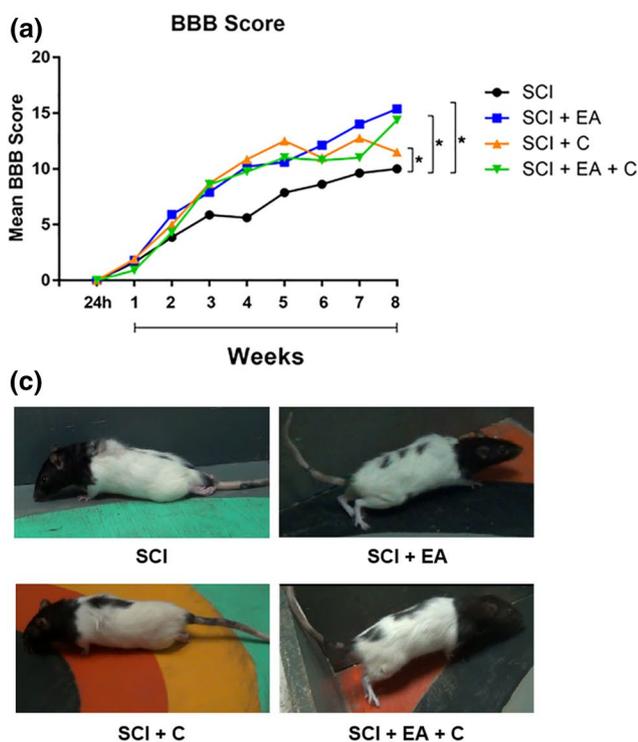
Fig. 1 Electroacupuncture and curcumin prevent SCI-induced oxidative stress. **a** nitric oxide, **b** hydroxyl radical and **c** lipid peroxidation, 24 h after traumatic spinal cord injury (SCI) in rats with different treatments (SHAM; SCI: spinal cord injury without treatment; EA:

electroacupuncture, C: curcumin, EA + C: combination). The group data are presented as the mean \pm S.D ($n = 7$). ANOVA followed by post hoc Tukey's test ($p < 0.05$)

Electroacupuncture and Curcumin They Improve Motor Function

BBB Scale

The motor functional gait evaluation indicated a significantly greater difference between the SCI + EA (14.5), SCI + Curcumin (11.5) and combination SCI + EA + Curcumin (15.8) groups and the untreated SCI group (10) ($p < 0.05$). The groups of rats treated with EA and the combination of EA + Curcumin exhibited the greatest consistency in their recovery in relation to time: at the end of the 2-month follow-up, they were able to extensively move the articulations of the hind limbs, consistently support the planting of the legs while walking and slightly coordinate the movement of the anterior and posterior limbs. Moreover, the rats in the groups treated with curcumin exhibited better functional recovery until week 5 than the rats in the remaining groups; however, at the end of the study, they did not exhibit coordination between the anterior and posterior limbs, which was exhibited in the remaining experimental groups.



LSS Scale

The functional motor evaluation of swimming through the LSS test was significantly higher in the SCI + EA (8.3), SCI + Curcumin (7.8) and combination SCI + EA + Curcumin (9.0) groups than in the group with untreated SCI; these animals were considered intermediate swimmers with occasional to frequent hindlimb movement and some retained dependency on their forelimbs for forward motion, occasional-to-consistent mild trunk instability and a moderate (21–45° tail down) body angle during forward motion compared with the untreated SCI group (5.0), which included poor swimmers for the forward motion, had little or no hind limb movement and exhibited severe trunk instability when swimming ($p < 0.05$) (Fig. 3).

Electroacupuncture and Curcumin Attenuates Tissue Damage

Representative histological sections of each experimental group and the results of the quantification of the preserved medullary tissue are summarized in Fig. 4; a significantly smaller difference was identified in the group

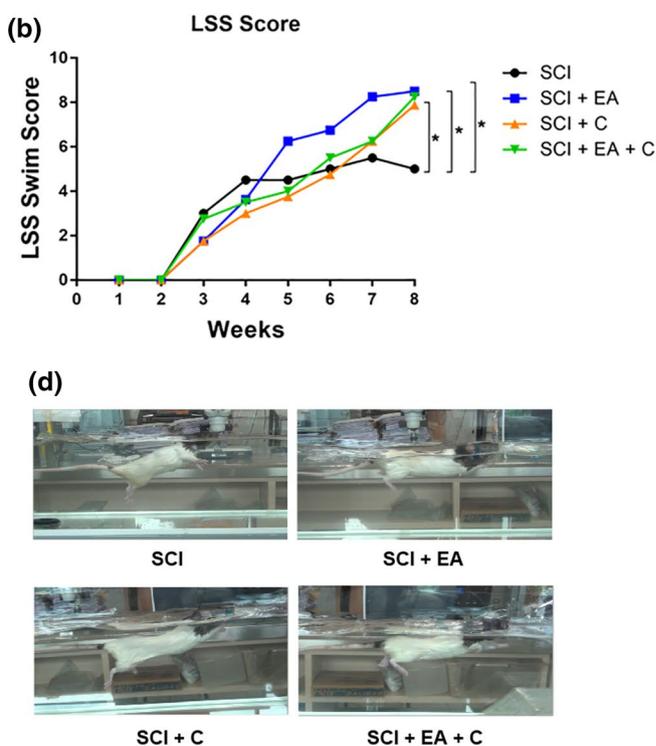


Fig. 3 Electroacupuncture and curcumin they improve motor function. **a** BBB score **b** LSS score. The graph represents the mean value of the hind limb motor function recovery of the animals during the 2 months after traumatic spinal cord injury (SCI) and the corresponding treatment (SHAM; SCI: spinal cord injury without treatment; EA: electroacupuncture, C: curcumin, EA + C: combination). The group

data are presented as the mean \pm S.D. ($n = 7$). ANOVA followed by post hoc Tukey's test ($p < 0.05$) **c** walk **d** swim. The images represent the hind limb motor function recovery of the animals 2 months after traumatic spinal cord injury (SCI) and the corresponding treatment (SHAM; SCI: spinal cord injury without treatment; EA: electroacupuncture, C: curcumin, EA + C: combination)

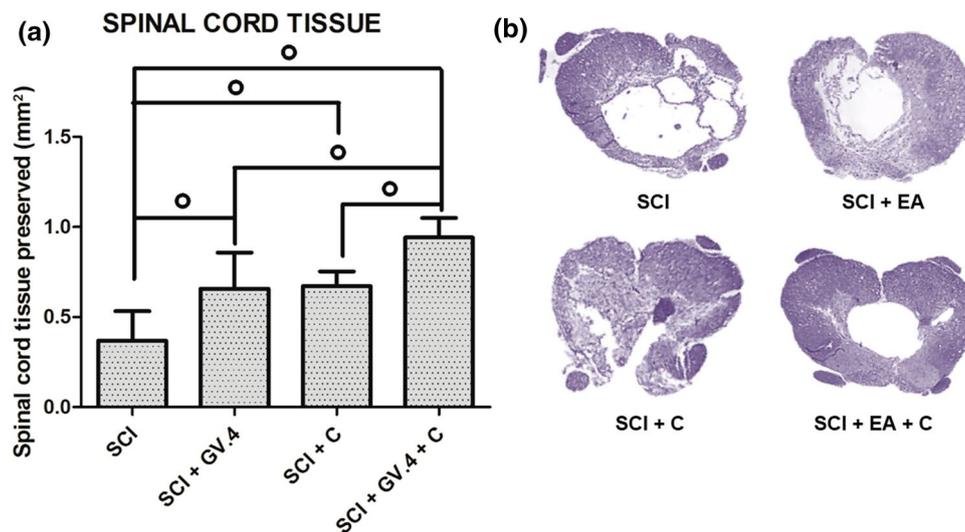


Fig. 4 Electroacupuncture and curcumin attenuates tissue damage. **a** Spinal cord tissue. The graph represents the mean value of the preserved tissue 2 months after traumatic spinal cord injury (SCI) and the corresponding treatment (SHAM; SCI=spinal cord injury without treatment; EA=electroacupuncture; C=curcumin; EA+C=combination). The group data are presented as

the mean ± S.D. (n=7). ANOVA followed by post hoc Tukey's test ($p < 0.05$) **b** Represents Hematoxylin and Eosin stained spinal cord cross sections of the animals two months after traumatic spinal cord injury (SCI) and the corresponding treatment (SHAM; SCI=spinal cord injury without treatment; EA=electroacupuncture; C=curcumin; EA+C=combination)

with untreated SCI (0.36 ± 0.16) than in the SCI+EA (0.65 ± 0.20), SCI+Curcumin (0.67 ± 0.08) and combination SCI+EA+Curcumin (0.94 ± 0.10) groups. Moreover, a significant difference was identified in the medullary tissue preserved in the combined SCI+EA+Curcumin (0.94 ± 0.10) group compared with the SCI+EA (0.65 ± 0.20) and SCI+Curcumin (0.67 ± 0.08) groups ($p < 0.05$).

Discussion

SCI results in numerous pathophysiological events that compromise cellular function and lead to neuronal cell loss and neurological dysfunction with respect to autonomic, sensitive and motor functions. One of the most important pathophysiological processes in SCI is oxidative stress because of its deleterious effects on neuronal structure and function [30, 38, 39].

In the present study, the treatment of SCI with EA at the GV.4 point caused a significant decrease in the ROH· levels and lipid peroxidation; however, it did not produce significant changes in the activity of antioxidant enzymes. The first finding may have occurred because a mitochondrial impairment occurs after SCI, which may likely be the main source of ROS generation [38], and in previous studies, the stimulation of GV.4 on the GV channel has shown an ability to decrease oxidative stress by coupling the respiratory chain and stimulating mitochondrial enzymatic activity [16]. Furthermore, an increase in the antioxidant enzyme response

occurs several hours after the generation of ROS because the expression of the genes that code for these enzymes is expressed as a negative feedback response in an environment under oxidative stress; thus, we could not confirm whether 24 h after SCI was an ideal time [17, 38].

Treatment with EA did not significantly decrease the NO· levels; however, the group administered the combination of EA + Curcumin presented the highest preservation of spinal cord tissue and degree of motor function recovery after SCI, which suggests that ROH· is the reactive species with the greatest impact on the pathophysiology of SCI, at least compared with NO·, as proposed in previous studies [22, 40]. This finding may be a result of a higher reaction rate in ROH· ($1 \times 10^{7-9} \text{ M S}^{-1}$) than NO· ($3.7 \times 10^7 \text{ M}^{-1} \text{ S}^{-1}$) [41].

Furthermore, oxidative stress has classically been considered an exclusive mechanism of damage; however, it has recently been proposed that the increase in ROS may be a positive phenomenon in pathological conditions because when ROS are at a regulated level, they may act as signaling molecules, thereby activating the intracellular transcription factors of specific genes that have a critical role in proliferation, differentiation, survival and cellular morphogenesis [42, 43]. Recent research indicates how the application of EA in GV.4 of the GV channel activates the processes of cellular metabolism related to the synthesis and secretion of endogenous neurotrophic factors at the site of injury, which is capable of promoting processes of plasticity that enable the regeneration of oligodendrocytes and nerve fibers [13, 44–46]. This finding may explain the observed increase in

the amount of nerve tissue preserved at the site of injury and the degree of functional recovery achieved by the EA-treated animals in the present study.

Another effect related to EA that is likely linked to the regulation of oxidative stress in SCI may be a result of the therapeutic stimulation that it produces on the calcitonin gene-related peptide (CGRP), which has been described as an antiapoptotic effect, as well as the ability to increase cell viability and the process of neuroprotection in the spinal cord [18, 47]. These effects may occur because calcitonin has an important regulatory role with respect to calcium levels, and in SCI, there is an intracellular increase of this ion in response to the excitotoxicity process. Moreover, the oxidative stress is intensified through the formation of NO [39, 48, 49].

Curcumin is considered a natural antioxidant associated with the phenolic hydroxyls, which may capture or scavenge free radicals [23, 50–52]. Studies indicate that curcumin treatment decreases cytochrome C release and neuronal apoptosis, favors the increase of SOD, reduces the levels of MDA and the inflammatory process, and improves neurological function [27, 30, 53]. In our study, curcumin caused a significant decrease in NO⁻, ROH⁻ and MDA; these results are related to previous studies in which the antioxidant properties of this compound protected biomembranes against peroxidative damage [23, 49, 52, 54] and induced the expression of the enzymes SOD, CAT, GPx, heme oxygenase 1, and NAD(P)H oxidase [51, 55, 56].

Regarding the functional results obtained with the BBB functional scale, a faster and more constant recovery of motor function was identified in the SCI+EA and SCI+EA+Curcumin combined groups than in the untreated SCI group; in contrast, in the LSS scale assessment, all rats in the experimental groups were rated as intermediate swimmers. The results obtained in both evaluations of motor function enable us to assume that in the experimental groups of SCI+EA, SCI+Curcumin and the combination of SCI+EA+Curcumin, when regulating oxidative stress, the ROS and MDA levels decrease, which causes a greater preservation of spinal cord tissue and, consequently, an improvement in the hind limb motor function of the treated rats. The three experimental groups significantly improved compared with the untreated lesion group; however, considering the motor function evaluated with both the BBB scale and the LSS, the combined treatment group of SCI+EA+Curcumin exhibited the highest score in these scales. The therapeutic combination of EA+Curcumin is beneficial for SCI because these factors are involved in different secondary mechanisms of damage. In oxidative stress, EA increases the expression of antioxidant enzymes, while curcumin acts mainly in the acute phase as a scavenger of free radicals. In apoptosis, EA favors the coupling and activity of mitochondrial enzymes; curcumin prevents the release of cytochrome C, which is

the key component of apoptosome formation. Finally, with regard to preserving spinal tissue and/or nerve plasticity, in the chronic phase, EA is associated with overexpression of genes related to proliferation, growth and differentiation; however, to prevent apoptosis, curcumin promotes neuronal survival and preservation of nervous tissue, which are all key mechanisms in nervous system plasticity [44–46, 49, 52, 54]. This process explains the motor function recovery in the posterior limbs of SCI rats receiving this therapeutic scheme.

Conclusions

The current findings indicate that the mechanisms of lipid peroxidation and oxidative stress generated after SCI may be significantly modulated by the application of EA in GV4 and the administration of curcumin alone or in combination. The neuroprotective effect is also evidenced by the ability of this treatment to reduce tissue damage.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest associated with the present study.

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