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Curcumin reduces pain after spinal cord injury in rats by decreasing oxidative stress and increasing GABAA receptor and GAD65 levels

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About 70% of spinal cord injury (SCI) patients experience neuropathic pain (NP), posing an important medical challenge. Painkillers are used to manage pain today and often have undesirable side effects. Curcumin's antioxidant properties may help alleviate NP following SCI (NP-SCI). We decided to study curcumin's effects on NP-SCI for the first time. Male Wistar rats were divided into five groups ($n = 8$): Control (no injury/no treatment), Sham (laminectomy), SCI (spinal cord compression at T11-T12 using a clip), Curcumin100 and Curcumin200 (Curcumin at 100 and 200 mg/kg administered 30 min after SCI for 10days). Motor function, allodynia, and hyperalgesia were assessed using the BBB scale, acetone, and tail-flick until six weeks after SCI. H&E staining for assaying cavity, western blot for measuring GAD65 and GABA-A receptors, and biochemical kits for assaying SOD, catalase, total antioxidant capacity, and MDA were used. PRISM software analyzed data. Results showed significant improvements in motion, allodynia, hyperalgesia, cavity, urinary retention ($P < 0.0001$), and weight in curcumin treatments. There was also a reduction in MDA, with increasing GABA-A receptors, GAD65, and antioxidants in them. Findings suggest curcumin may provide good analgesic effects through its antioxidants, and extensive studies are needed to confirm it as a treatment for NP-SCI in the clinic.

Keyboards: spinal cord injury, curcumin, antioxidant, pain, GABA, GAD65 enzyme.

Abbreviations

SCI	Spinal Cord Injury
CNS	Central Nervous System
ROS	Reactive Oxygen Species
GABA	Gamma-Aminobutyric Acid
TAC	Total antioxidant capacity
CAT	Catalase
GAD65	Glutamic Acid Decarboxylase 65-Kilodalton Isoform
BBB scale	Basso, Beattie, and Bresnahan locomotion scale
NP	Neuropathic pain
MDA	Malondialdehyde
SOD	Superoxide dismutase

69.1% of patients with spinal cord injury (SCI) report neuropathic pain (NP) symptoms which have a terrible impact on their physical and psychological conditions[1]. Hyperalgesia (extreme response to usual pain stimuli), allodynia (painful reaction to non-noxious stimuli), and spontaneous pain (pain without external stimuli) are diagnostic hallmarks of NP.

NP does not respond to surgery, neurostimulation, or physical or psychological therapy. Therefore, pharmacologic interventions (antidepressants, anticonvulsants, and psychotropic medications) are more effective methods for controlling SCI pain than nonpharmacologic methods[2,3]. Unfortunately, none of these interventions have long-term positive effects, and in the long term, analgesics have low or moderate efficacy in

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controlling NP[4]. Multiple side effects of painkillers make the treatment of NP a medical challenge[5]. Therefore, it seems necessary to replace analgesics that have fewer side effects.

NP illustrates an imbalance of the excitatory and inhibitory conditions in the nervous system[6–8]. For instance, reactive oxygen species (ROS) and cell stress have a remarkable function in the pathophysiology of the central nervous system (CNS) and primarily affect the function of the gamma-aminobutyric acid (GABA)[9,10]. GABA is the natural neurotransmitter that controls the pain gates in the CNS under normal conditions. SCI and peripheral nerve injury (e.g., sciatic nerve) degenerate GABAergic interneurons of laminae I-III in the spinal cord and play crucial roles in developing allodynia and hyperalgesia[11]. Therefore, GABA suppression in the CNS is a hallmark of NP[12]. GABA induces analgesic effects by stimulating GABA-A and GABA-B receptors[13,14]. GABA binds to the GABA-A receptors (chloride ion channel) on sensory neurons, hyperpolarizing mature neurons and suppressing neuronal excitability. Thus, GABA-A receptors are critical modulators of pain processing[15]. Consequently, any factor affecting GABA synthesis, release, and activity can influence NP[16].

Glutamic acid decarboxylase 65-kilodalton isoform (GAD65) synthesizes GABA from L-glutamic acid in the spinal cord and is severely affected by SCI[17]. After SCI, the reduction of GAD65 in the spinal cord significantly decreases the inhibitory effect of GABA and induces NP[18,19].

Plants with antioxidant properties improve many diseases[20,21]. Turmeric (*Curcuma longa*) is a species from the ginger family; Curcumin is one of its active components[22]. Curcumin applies valuable therapeutic effects through its antioxidant, anti-inflammatory, and neuroprotective properties[23]. Based on preclinical and clinical results, it can play a role in reducing or preventing nerve pain[24,25], especially in the initial stages of peripheral neuropathy[26] and improving motor function after SCI[27]. Additionally, turmeric and curcumin inhibit or desensitize transient potential receptor ion channels and eliminate spinal free radicals. Curcumin increases GABA content and facilitates its response in hippocampal neurons[28,29]. Therefore, curcumin has been suggested as a neuroprotective agent to relieve NP[23].

NP involves different mechanisms for peripheral and central sensitization after nervous system injuries[30]. Unlike the peripheral nervous system (PNS), neurons in the adult CNS do not spontaneously regenerate after injury in adult mammals due to inhibitory barriers like glial scars and the poor intrinsic regenerative capacity of the spinal cord[31]. Consequently, treatments that successfully regenerate the PNS and reduce peripheral NP may not help CNS injuries. On the other hand, Curcumin is cost-effective and readily available. Therefore, we decided to investigate curcumin's influence on NP caused by SCI and its related protein expression (GABA-A receptors and the GAD65 enzyme) for the first time.

Materials and methods

Study design

Experimental research protocol on animals was approved by the ethics committee of Kerman University of Medical Sciences (IR.KMU.AEC.1402.015.) and followed by the Animal Research Reporting of In Vivo Experiments (ARRIVE) guidelines. The methods were done with these relevant guidelines and regulations including a statement in the methods section to this effect. Forty mature male Wistar rats were kept under controlled conditions (12 h light/12 h darkness) and temperature (22 ± 2 °C) at the Physiology Research Center of KMU. Before surgery, three rats were housed in each cage. After surgery, they remained in the same cage to control adaptation conditions and minimize confounding factors. Two rats in the SCI group with bedsores were excluded from the study and replaced. Euthanasia was performed by anesthetizing with an intramuscular injection of a mixture of ketamine and xylazine (50 mg/kg and 10 mg/kg, respectively)[32]. Behavioral tests were conducted from 10 AM until 3 PM to ensure homogeneous circadian rhythms.

The study conducted the Basso, Beattie, and Bresnahan locomotion scale (BBB scale)[33] to confirm the severity of SCI. The scale categorizes injury severity as severe (BBB 0–7), moderate (BBB 8–14), and low (BBB 15–21)[34]. All injured animals had a BBB scale from 0 to 4 in the thirty minutes post-surgery included in our study.

After adaptation to conditions, the animals (190–220 g) were randomly placed in five groups ($n = 8$): 1- Control (rats that did not undergo surgical procedure and treatment), 2- Sham (removing the lamina without SCI induction), 3- SCI (removing the lamina with clip compression), 4- Curcumin100, and 5- Curcumin200.

Curcumin was obtained from Merck Aldrich, and 100 and 200 mg/kg of it were used in treatment groups. Curcumin was calculated for each animal and separately suspended in 1 mL of normal saline, then this volume was administered to fasting animals (3 h) via oral gavage 30 min post-injury for 10 days based on a previous study by Yardım et al.[23]. The experimental timeline is in Fig. 1.

SCI model induction

An SCI induced using the method of Behroozi et al.[35]. Rats were deeply anesthetized with a mixture of ketamine and xylazine (100 and 10 mg/kg, respectively, IP), and the last rib attached to the T13 vertebra was used as a guide for the injury location[36]. After a midline incision, the shaved skin and superficial muscles were held aside a laminectomy was done to expose the spinal cord at the T11-T12 areas where an aneurysm clip was applied (force 35–45 g/cm^2, RS6474) for 120 s for severe SCI. The muscles and skin were separately sutured (0.3 suture thread). Surgery duration was 15–20 min. After surgery, tetracycline spray prevented infection at the injury site, and 70% alcohol was used daily in the cage until the wound healed[35].

The evaluation of bladder function (BF)

Animals' bladders were manually emptied twice a day until they could urinate voluntarily[35]. Bladder dysfunction, especially weakness or inactivity of the bladder muscles, can lead to urinary retention[37]. Duration of urinary retention (DUR), and the time needed to empty the bladder (TEB) were measured daily to assess bladder dysfunction, and the means were included in the analysis.

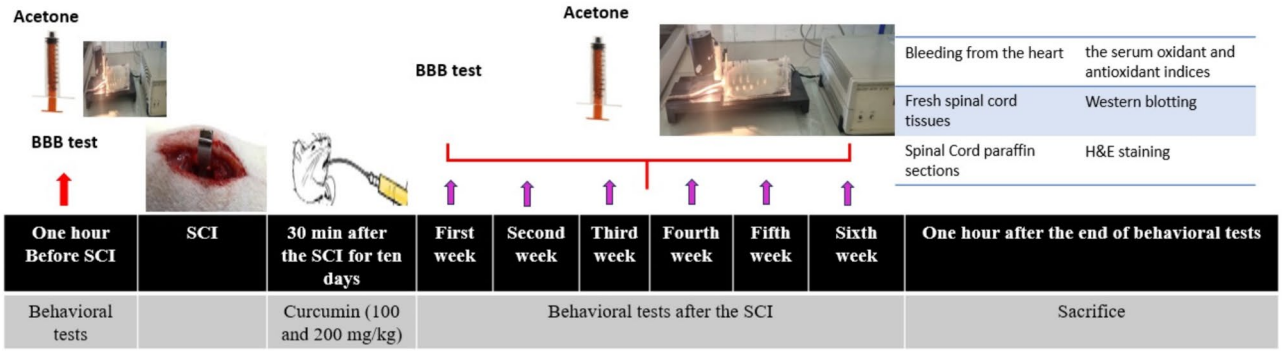

Fig. 1. Schematic diagram of the experimental timeline. Motor function, allodynia, and hyperalgesia were assessed using the BBB scale, acetone, and tail-flick one hour before the injury and then weekly for six weeks afterwards. Curcumin at 100 and 200 mg/kg doses was calculated for each animal and separately suspended in 1 mL of normal saline, then this volume was administered to fasting animals (3 h) via oral gavage 30 min after SCI for 10 days. Animals were sacrificedone hour after the end of behavioral tests. H&E staining for assaying cavity, western blot for measuring GAD65 and GABA-A receptors, and biochemical kits for assaying SOD, catalase, total antioxidant capacity, and MDA were used.

Weight measurement

Rats were weighed weekly on a digital weighing scale (accurate at 0.01 g) after a 2-hour fasting period. The weight was recorded from 9 AM to 10 AM when an animal was motionless.

Behavioral tests

All behavioral tests were performed one hour before the injury and then weekly for six weeks afterwards.

The evaluation of recovery motor function

The BBB scale was performed to assess locomotor performance after SCI with scores ranging from 0 (absolute paralysis) to 21 (standard function)[33]. two independent observers, unaware of grouping, monitored the animal's behaviors for four minutes and scored them. Scoring done based on the motion of joints, weight-bearing, disposition of paws, harmony of the limbs, and toe clearance were assessed. The mean of scales was included in the analysis.

Cold allodynia measurement test

The acetone test was performed to assess the cold sensation of the foot. The animal was situated in an acrylic cage with a metal mesh surface. An acetone drop was delicately sprayed on the rat's paw via a blunt plastic needle connected to a syringe. The test was repeated five times with approximately 2-minute intervals for each paw. Hind paw withdrawal, licking, stamping, leaning posture, and jumping were recorded as positive reactions[38]. The withdrawals were reported as a percentage of responses using the formula: (number of paw withdrawals / total number of trials) × 100[39].

The withdrawals were calculated as a percentage of responses.

Tail Flick measurement test

The tail-flick response is a radiant heat stimulus on the animal's tail that likely involves the supraspinal centers[40,41]. Initially, the heat intensity of the tail flick device (ANALGESY_METER LE 7106, Panlab s.l. Spania) was set to 6 Focus. After animal adaptation (30 min), the animal was held in a rat restraint device. The light source was applied to the center of the first two-fifths of the tail. the time was recorded as soon as the tail moved. This test was repeated three times at 5-minute intervals. Finally, the mean of three latency values was calculated as the pain threshold. A maximum cut-off time of 15 s ensured the animal was not harmed if it did not respond[42].

Tissue assessment

Blood collection and trans-cardiac perfusion and fixation for tissue

One hour after finishing behavioral tests at the end of the sixth week, the animals were administered deep anesthesia (ketamine 100 mg/kg and xylazine 10 mg/kg, IP). Then, Animals ($n = 4$ per group) were placed on a surgical fence for trans-cardiac perfusion. Then, a horizontal incision was made under the sternum to access the heart. Blood was collected from the heart apex using a 23 G needle. Then, the syringe was quickly removed from the needle without disturbing the needle in the heart. An infusion set was rapidly attached to the needle and when the heart was filled with normal saline, the right atrium was incised, and injection continued until whole body blood was removed (150 ml, 15 min for a rat weighing 250 g, injection rate:10 ml/min). Afterwards, the vertebral column was dissected, and the spinal cord was fixed in 10% formaldehyde (pH = 7.2–7.4) for 72 h. A 0.5 mm section from the L1-L2 region (T11-12 vertebra) was paraffinized[36]. Tissues were cut into cross-Sect. (4 µm thickness) and stained with hematoxylin and eosin (H&E). On the other hand, Eppendorf microtubes

containing blood samples were centrifuged at 3500 rpm for 15 min. The separated serums were saved and frozen at − 80 °C.

H&E staining

H&E staining was applied to confirm that the spinal cord injury model was induced correctly and to estimate the cavity size at the injury site. Each animal's cross-sectional slices (beginning, middle, and end of the cavity) were chosen after H&E staining, and images were taken (Olympus, 4x magnification). The cavity size was calculated using Image J software.

The formula for calculating the cavity size of each animal is as follows:

Cavity size for every slice (%) = Cavity size (μm) / Total area of the section (μm) × 100.

The mean percentage of cavity size for each animal = The total percentage of three slices of cavity size for each animal (%) / 3.

Western blotting

At the end of the study, four animals underwent deep anesthesia (ketamine 100 mg/kg and xylazine 10 mg/kg, IP). Their spinal cords were separated in a 5 mm section of the T11-T12 vertebra (L1-L2 of the spinal cord) and kept at −80 °C. The diaphragm ruptured during deep anesthesia and the animals died in less than 5 s.

After the protein was extracted according to the instructions[39]. The supernatants containing 50 μg protein were loaded into the wells of a 10% SDS-polyacrylamide gel for electrophoresis. The protein was moved from the gel to a polyvinylidene-difluoride (PVDF) membrane under semi-dry conditions. Then, it was kept in a blocking buffer (containing 5% skimmed milk and 0.1% Tween-20 in Tris-buffered saline, pH 7.4) at room temperature for 2 h (to prevent non-specific interactions). The primary antibodies, including GAD65 antibody (1:1000, sc-293,133, MW: 65 kDa), β-actin antibody (1:500, sc-47,778, MW: 43 kDa), and Anti-GABA A Receptor alpha 1 (1:1000, ab86320, MW: 52 kDa), were incubated (overnight at 4 °C). Next, the PVDF membranes were cleaned with TBST (3 times) and mixed with goat anti-rabbit horseradish peroxidase-conjugated IgG (1/1000, sc-516,102). Protein bands were observed using an immunoreactivity detector as enhanced chemiluminescence (ECL). β-actin, an internal standard for cytoplasmic protein, was used. The proteins were normalized with β-actin. ImageJ software was employed to quantify results.

Preparation of serum samples and measurement of serum oxidant and antioxidant indices

Malondialdehyde (MDA) is a lipid peroxidation biomarker. The thiobarbituric acid (TBA) test is used to measure MDA. 100μL of samples was utilized. MDA reacts with TBA and yields a pink color. A wavelength of 535 nm is suitable for reading absorbances[43]. The data were quantified using an MDA standard curve[44].

Total antioxidant capacity (TAC)

Benzie and Strain suggested that the ferric reducing/antioxidant power (FRAP) assay procedure be used to measure the ferric-reducing ability of serum for TAC level assessment[45]. Briefly, 5μL of serum was mixed with 70 μL of FRAP reagent. The mixture was incubated at 37 ℃ for 5 min, and the absorbance was read at 593 nm. The serum can reduce the ferric tripyridyl triazine (Fe III–TPTZ) complex to an intense blue-colored ferrous (Fe II) form at low pH. Distilled water was used as a blank[44].

Superoxide dismutase (SOD) was estimated with commercial colorimetric assay Randox kits (RANDOX et al., UK). 50μL of serum was used for each animal.

Catalase (CAT) activity was assayed following the method described by Sinha[46]. The absorbance of samples was determined at 570 nm spectrophotometrically.

Statistical analysis

Data was analyzed using Graph Pad Prism software. 9. The Kolmogorov-Smirnov test was used to assess the normal distribution of the data. If the distribution was normal, a Two-way ANOVA with repeated measure analysis with Bonferroni post hoc test was done for behavioral assessments and weight, while a one-way ANOVA with Tukey post hoc test was utilized to evaluate the proteins and histological findings. P value of less than 0.05 was statistically significant. Finally, data was shown as mean ± SD. All assessments were conducted blindly without knowledge of the grouping.

Results

Improvement of bladder function (BF)

A: Curcumin treatment reduces the DUR

Urinary retention is a condition where the bladder does not empty completely or at all when an animal urinates. In this study, severe injury was induced. The statistical analysis indicated that the injured animal experienced a long DUR. ($DF = 4, 35$; $F = 92.28.$; $P < 0.0001$). In the 100 mg/kg and 200 mg/kg curcumin-treated groups, curcumin resulted in a significant decrease in DUR (mean ± SD: 5.75 ± 2.05 days, $P < 0.0001$, and 3.25 ± 1.7, $P < 0.0001$, respectively) compared to the SCI group (mean ± SD: 11.75 ± 1.83). There was a significant difference in DUR between treatment groups ($P = 0.013$) (Fig. 2-A).

B: Curcumin treatment reduces the TEB

Statistical analyses showed dramatic differences in the average time to perform bladder massage among the groups ($DF = 4, 35$; $F = 28.45.$; $P < 0.0001$). TEB in the SCI group was higher than in other groups (122.9 ± 50.56 s, $P < 0.0001$). Animals received the 100 mg/kg and 200 mg/kg curcumin significantly decreased TEB (40 ± 22.8 s, $P < 0.0001$, and 31.38 ± 22.7 s, $P < 0.0001$, respectively) compared to the SCI group. No significant differences were in TEB between treatment groups (Fig. 2-B).

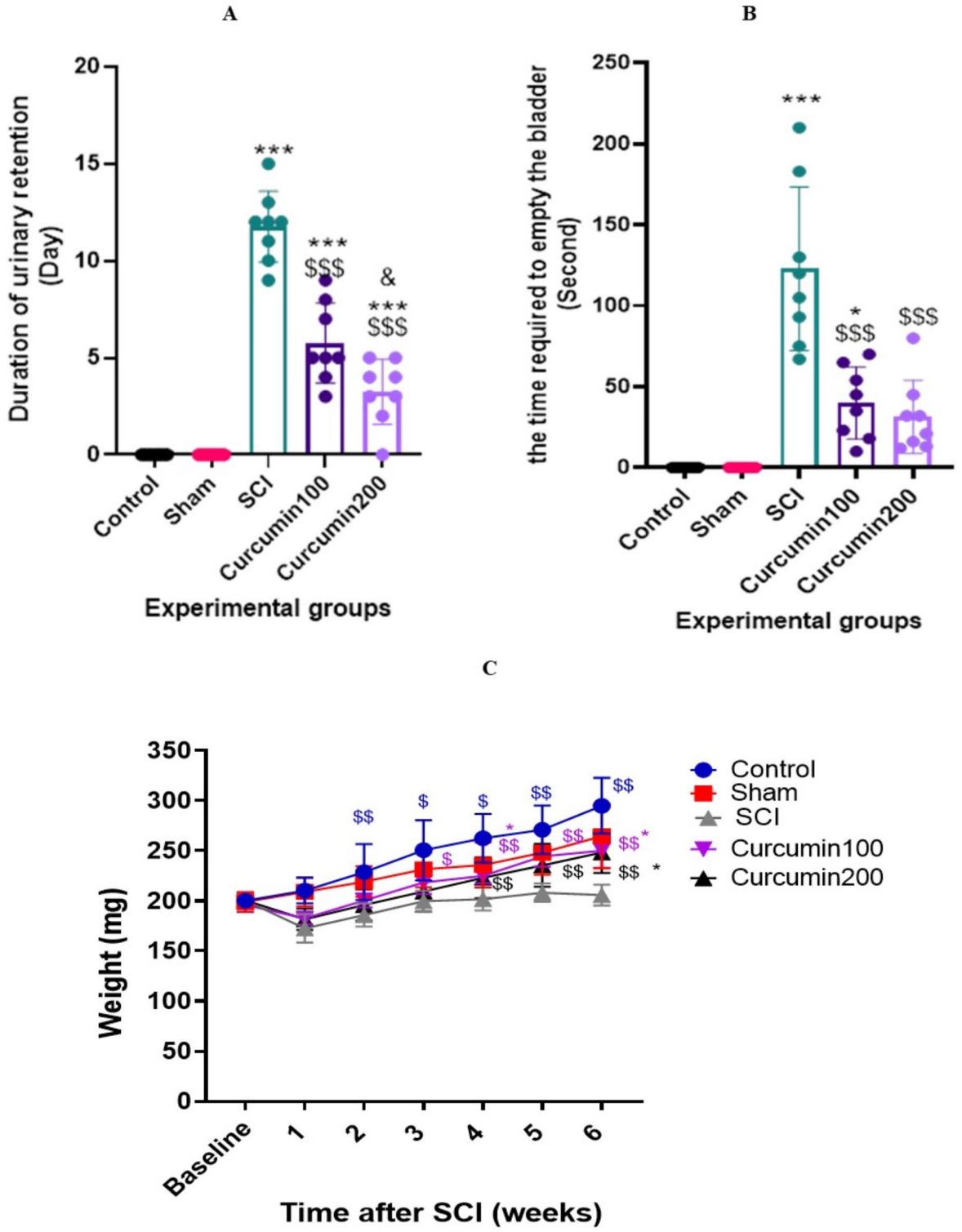

Fig. 2. The study examined the effects of oral curcumin on bladder function (BF) and weight loss in animals with spinal cord injury (SCI). Duration of urinary retention (DUR) (**A**), Time needed to empty the bladder (TEB) (**B**), and weight loss (**C**) Results showed that curcumin treatment at 100 and 200 mg/kg doses significantly reduced DUR and the TEB, indicating improved bladder function. Additionally, animals receiving curcumin had higher weights compared to the SCI group. The experimental groups included a Control (no injury/no treatment), Sham (laminectomy), SCI (Spinal cord compression in the T11-T12 vertebrae using a clip), Curcumin100 and Curcumin200 (Curcumin at100 and 200 mg/kg doses, respectively, administrated 30 min after SCI for ten days). Data are expressed as mean ± SD ($n = 8$). *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus control. $ $P < 0.05$, $$ $P < 0.01$ and $$$ $P < 0.001$ versus SCI group, & $P < 0.05$ vs. Curcumin100.

Curcumin treatment improved post-injury weight loss
The result showed that animals with SCI experienced significant weight loss in the first week ($P = 0.0007$), which continued until the sixth week ($P = 0.0002$). The therapy effects of curcumin 100 and curcumin 200 significantly started in the third week and the fourth week respectively continued until the end of the study ($P = 0.043$, $P = 0.003$) (Fig. 2-C).

Curcumin treatment decreased the cavity size post-injury
A wide cavity in the spinal cord of SCI animals was observed (DF = 4. 15; F = 60.73; $P < 0.0001$), with a mean cavity size of 51 ± 8.71%. However, animals that received curcumin at doses 100 and 200 mg/kg indicated a considerably smaller cavity size than the SCI group (17.4 ± 7.39, $P < 0.0001$, and 12.6 ± 3.6, $P < 0.0001$, respectively) (Fig. 3).

Behavioral tests

Curcumin improved the motor function
Evaluation data of the BBB scores stated that animals with SCI had severe motor disabilities compared with the control and sham (DF = 24,10; F = 43.18; $P < 0.0001$). All the injured animals with scores of 0 to 4 entered the study. Those scores did not change between the SCI and treatment groups in 3 days. Curcumin administration (100 and 200 mg/kg) after surgery significantly improved the locomotor function of animals compared to the SCI group from the third week until the end of the study ($P < 0.0001$). Also, there was no significant difference between the treatment groups during the study. Despite the improvements, neither treatment group fully improved motor function to that of the control ($P = 0.0001$ and $P = 0.026$, respectively) (Fig. 4).

Curcumin increased the threshold of cold allodynia
The acetone test results indicated that injured animals had a higher reaction (or lower pain threshold) versus normal and sham (DF = 24, 210; F = 4.194; $P < 0.0001$). Curcumin at doses of 100 and 200 mg/kg significantly decreased the percentage of animal reaction to acetone beginning in the second and fourth-weeks post-SCI ($P = 0.005$ and $P = 0.003$, respectively) and continued until the end of the sixth week ($P < 0.0001$). Also, significant differences were not observed between the Curcumin200 and control groups in this period. Curcumin 200 mg/kg (22.5 ± 30.11) had a lower paw withdrawal percentage than curcumin 100 mg/kg (27.5 ± 15.81), but the difference was not statistically significant (Fig. 5-A).

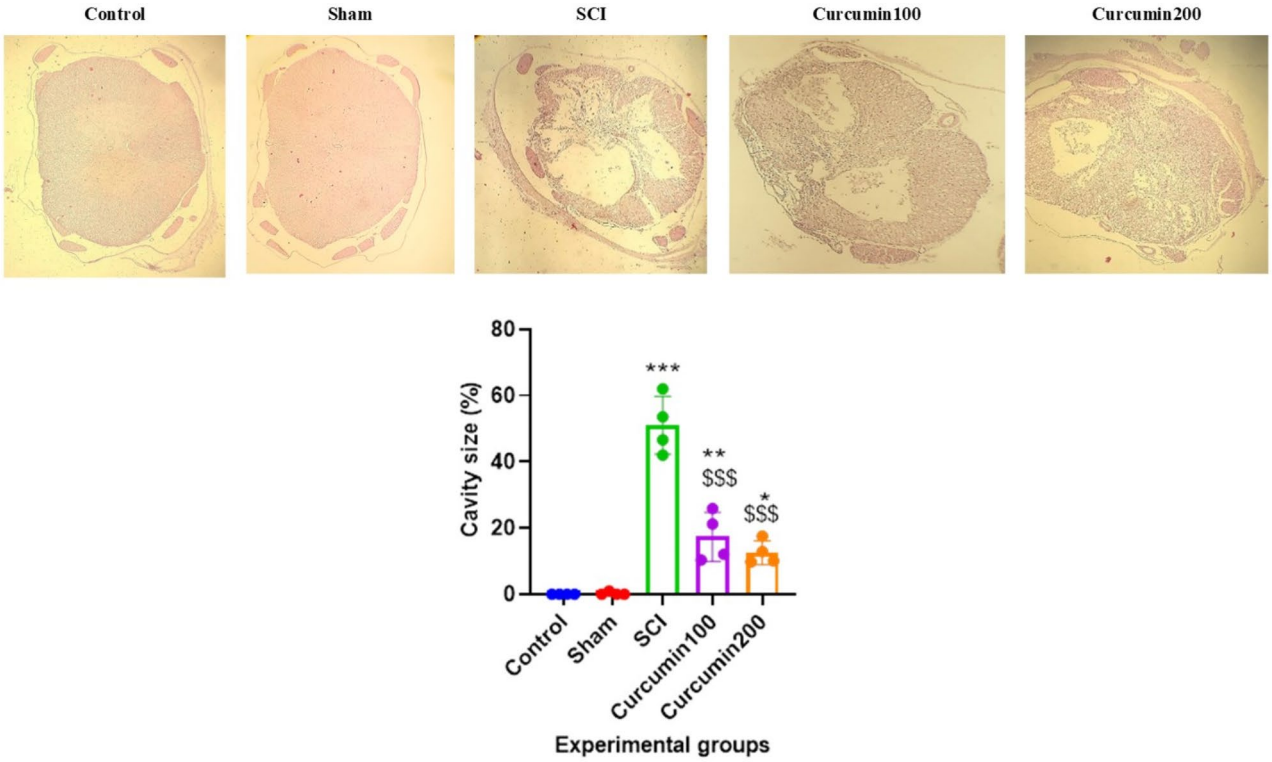

Fig. 3. The study examined the effect of oral curcumin on cavity size in rats with spinal cord injury (SCI). Results showed that curcumin treatment at 100 and 200 mg/kg doses significantly decreased cavity sizes compared to the SCI group. The experimental groups included a Control (no injury/no treatment), Sham (laminectomy), SCI (Spinal cord compression in the T11-T12 vertebrae using a clip), Curcumin100 and Curcumin200 (Curcumin at100 and 200 mg/kg doses, respectively, administrated 30 min after SCI for ten days). Data are presented as mean ± SD ($n = 4$ in each group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus control groups. $$$ $P < 0.001$ versus SCI group.

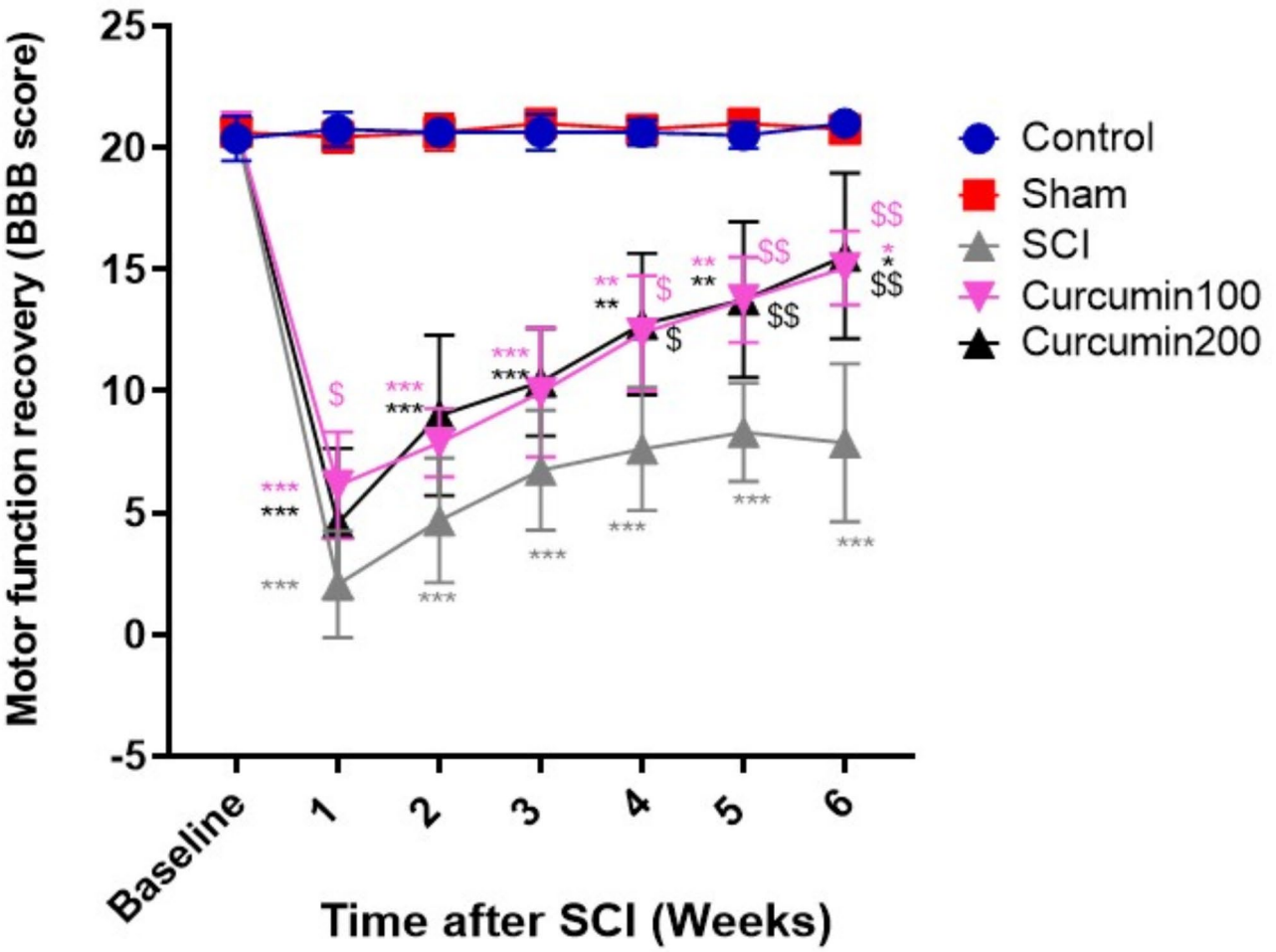

Fig. 4. The study examined the effect of oral curcumin on BBB score in rats with SCI. Results showed that curcumin treatment at 100 and 200 mg/kg doses significantly improved locomotor function after SCI. The experimental groups included a Control (no injury/no treatment), Sham (laminectomy), SCI (Spinal cord compression in the T11-T12 vertebrae using a clip), Curcumin100 and Curcumin200 (Curcumin at100 and 200 mg/kg doses, respectively, administrated 30 min after SCI for ten days). Data is expressed as mean ± SD ($n = 8$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus control group. $ $P < 0.05$, $$ $P < 0.01$ and $$$ $P < 0.001$ versus SCI group.

Curcumin increased the threshold of hyperalgesia

The analysis of the tail-flick test confirmed that in the SCI group, the pain threshold decreased versus the normal and sham groups (DF = 24, 21; F = 5.39; $P < 0.0001$). Animals that orally received curcumin (100 and 200 mg/kg) after surgery had significantly increased the threshold of noxious stimulus versus the SCI group from the first week until the end of the study ($P < 0.0001$). Also, no significant difference was observed between the treatment and health groups, except in the second week (Fig. 5-B).

Curcumin increased GAD 65 protein expression

In the sixth week post-injury, GAD65 protein expression significantly decreased in the injured animals (0.605 ± 0.093) versus the normal animals ($P < 0.0001$). However, GAD65 levels in groups treated with curcumin at doses of 100 and 200 mg/kg significantly improved compared to the SCI groups (0.832 ± 0.047; $P = 0.0137$ and 0.875 ± 0.079; $P = 0.003$, respectively). There was no significant difference between the treatment groups and the control (Fig 6-A and 6-C).

Curcumin increased the GABA-A receptor alpha1 expression

In the sixth week post-injury, the GABA-A receptor expression significantly decreased in the SCI group (0.6475 ± 0.134) compared to the control and sham animals (1 ± 0.0; $P = 0.0038$). However, GABA-A receptor expression significantly improved in groups treated with curcumin 100 and 200 mg/kg (0.895 ± 0.086; $P = 0.048$ and 1.04 ± 0.189; $P = 0.0014$, respectively) compared to the SCI. No significant differences were found between the treatment and control groups in the sixth week (Fig 6-B and 6-C).

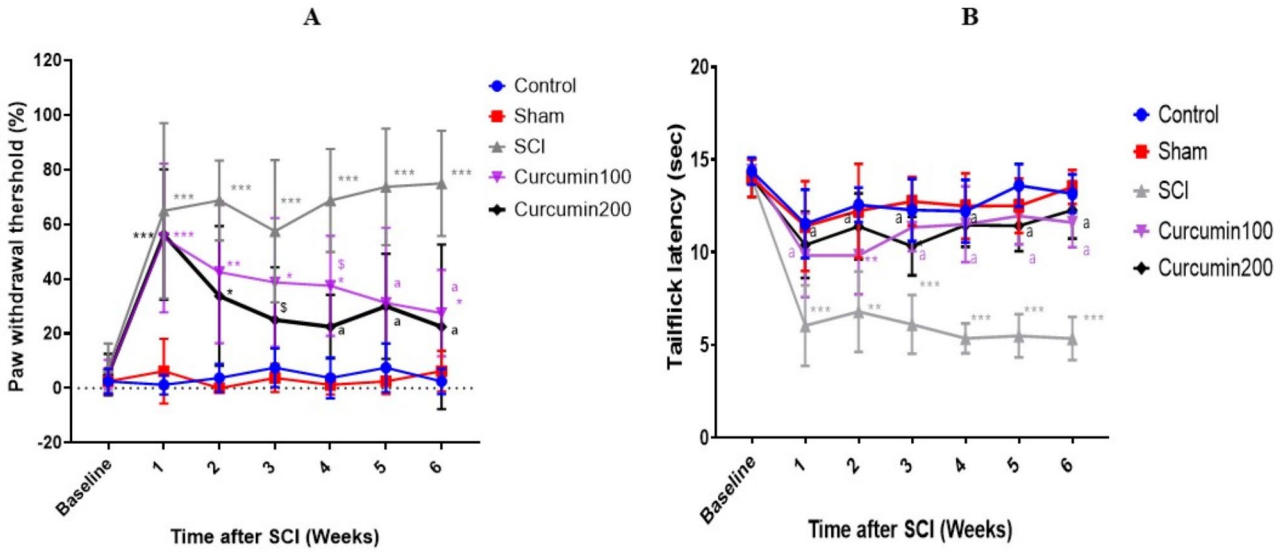

Fig. 5. The study examined the effect of oral curcumin on cold allodynia (**A**) and tail flick (**B**) in rats with spinal cord injury (SCI). Results showed that curcumin treatment at 100 and 200 mg/kg doses significantly increased threshold pain after SCI. Control (no injury/no treatment), Sham (laminectomy), SCI (Spinal cord compression in the T11-T12 vertebrae using a clip), Curcumin100 and Curcumin200 (Curcumin at100 and 200 mg/kg, respectively, administrated 30 min after SCI for ten days). Data are expressed as mean ± SD ($n = 8$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus control group. $ $P < 0.05$, $$ $P < 0.01$ and $$$ $P < 0.001$ versus SCI group, a: $$$.

The effect of Curcumin on serum antioxidant and oxidant factors

The SOD level, as a representative antioxidant enzyme, significantly decreased in the SCI group versus the normal animals ($P = 0.0002$). Contrary to expectation, none of the curcumin doses could significantly increase SOD levels compared to SCI animals (Fig. 7-A).

CAT levels in the SCI group significantly decreased compared to the control group ($P = 0.027$). Curcumin at the 200 mg/kg dose increased CAT levels in the serum compared to the SCI group ($P = 0.045$) (Fig. 7-B).

TAC levels in the SCI group significantly decreased compared to the control group ($P = 0.0009$). Curcumin 100 and 200 mg/kg increased the TAC serum level versus the injured animal group ($P = 0.010$ and $P = 0.015$, respectively) (Fig. 7-C).

MDA was higher in the SCI group compared to other groups ($P < 0.0001$). Both treatment groups decreased the MDA level compared to the SCI group ($P = 0.0002$ and $P = 0.0001$, respectively) (Fig. 7-D).

Discussion

Our findings showed that in the SCI group, the GAD65 and GABA A receptor expression and antioxidant factors decreased, while oxidants like MDA increased. This unbalanced condition between the production of oxidants and antioxidants induced oxidative stress conditions.

These animals showed heightened sensitivity to both painful and non-painful stimuli. Although spontaneous recovery was observed in them, cavities were still large. It confirms that this recovery was not enough to decrease injury severity and motor disability, stating our model's correctness[34]. This recovery is mainly due to the sprouting of remaining axons in the grey matter[47]. Produced stress oxidative in our study likely with the contribution of factors like ion imbalance, mitochondrial dysfunction can lead to the production of more reactive radicals. Unpaired electrons of free radicals react with normal tissue around the cavity, resulting in the expansion of tissue damage and enlargement of the cavity[48,49].

There is a relationship between the severity of SCI and neurogenic bladder dysfunction[50,51]. In our study, the time of emptying the bladder and the number of days until urinating voluntarily were greater in the severe SCI compared to the treatment groups. Stress oxidative can induce CNS degeneration by inducing demyelination, axonal dieback, Wallerian degeneration, and apoptosis. Also, glial scars around injury sites create barriers that inhibit axonal growth[52]. Big cavity size, glial scars, and oxidative stress can reduce innervation to the limbs and bladder, resulting in motor disability and bladder dysfunction[39,53].

SCI leads to the destruction of the GABAergic neurons[54,55] and a reduction of GAD65 in specific spinal cord laminae[18,19], which increases pain sensitivity. Large cavities in SCI confirm axonal degeneration. Therefore, the decreased levels of GAD65 and GABA-A receptors in the SCI group may reflect tissue damage[56]. Also, The studies illustrated that knocking out GAD65 reduces GABA content[57–60]. Based on these studies, NP in our injury group may be caused by a decrease in GABA levels, which follows a reduction in GAD65 content.

Oxidative stress can decrease GABA and increase glutamate neurotransmitters in the CNS injuries[9,61–63]. MDA, as a major marker of oxidative stress, reacts strongly with proteins and nucleic acids, compromising membrane integrity and permeability[64], This disruption affects the Na-K ATPase pump activity[65,66], resulting

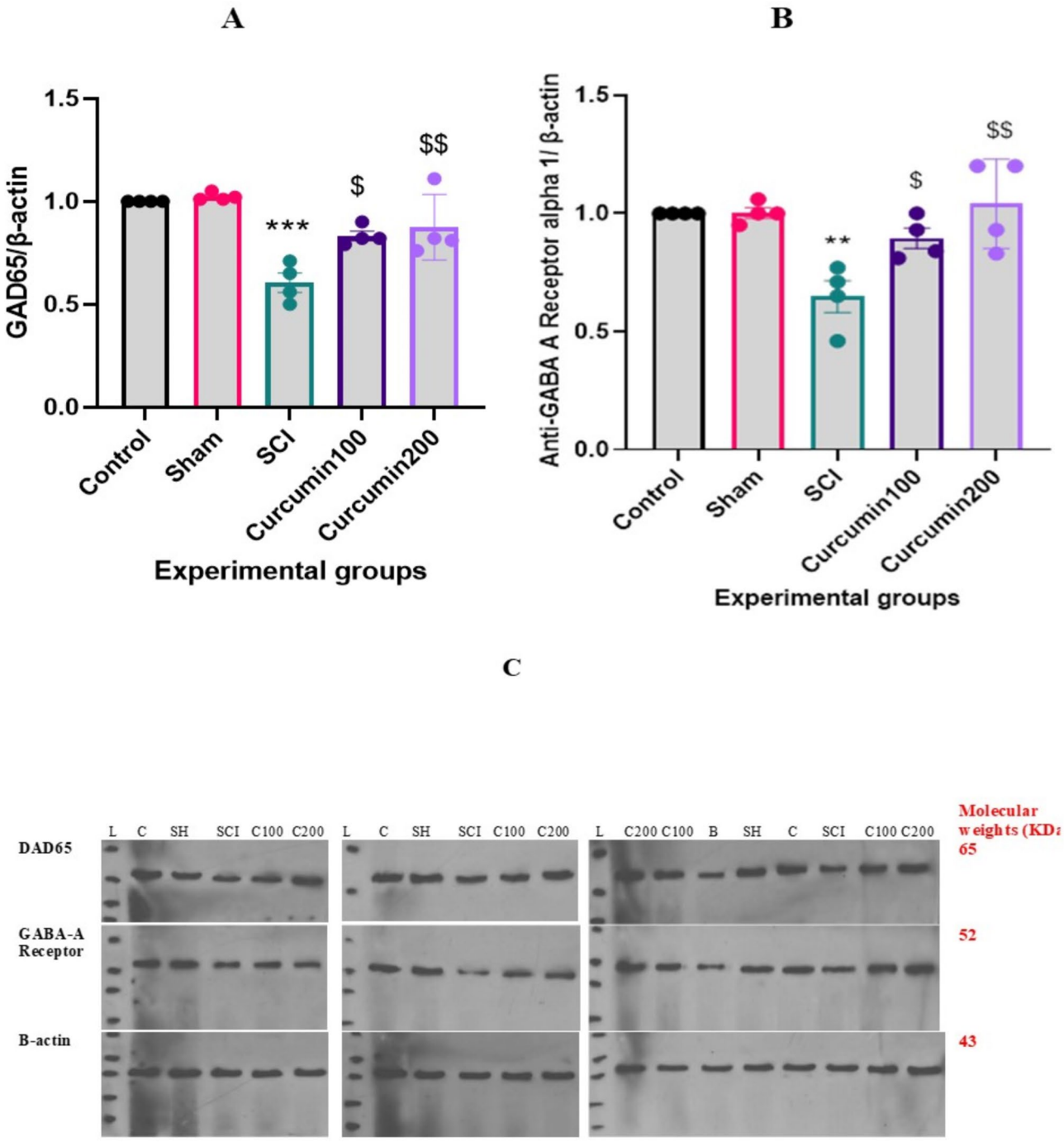

Fig. 6. The study examined the effect of oral curcumin on the expression of GAD65 protein (**A**), GABAA receptor (**B**) in rats with spinal cord injury (SCI). Western blot bands were shown in (**C**). Results showed that curcumin treatment at 100 and 200 mg/kg doses significantly increased GAD65 and GABAA receptors after SCI at the end of the study. The experimental groups included a Control (no injury/no treatment), Sham (laminectomy), SCI (Spinal cord compression in the T11-T12 vertebrae using a clip), Curcumin100 and Curcumin200 (Curcumin at100 and 200 mg/kg doses, respectively, administrated 30 min after SCI for ten days). Data are expressed as mean ± SD. $n = 4$ in each group. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. L: Ladder, A: Control, SH: Sham, SCI: Spinal Cord Injury, C100: Curcumin100 and C200: Curcumin200.

in decreased glutamate uptake[67]. Elevated glutamate in synaptic space may induce NP through two pathways[68]. First, Excessive activation of glutamate receptors in postsynaptic neurons[69].Second, Decreased GABA synthesis .Glutamate is essential for the GABA synthesis. Glutamate uptake into inhibitory synaptic terminals is an important source for saving GABA in the synaptic pools[70]. Furthermore, GABA attenuates the neurotoxicity

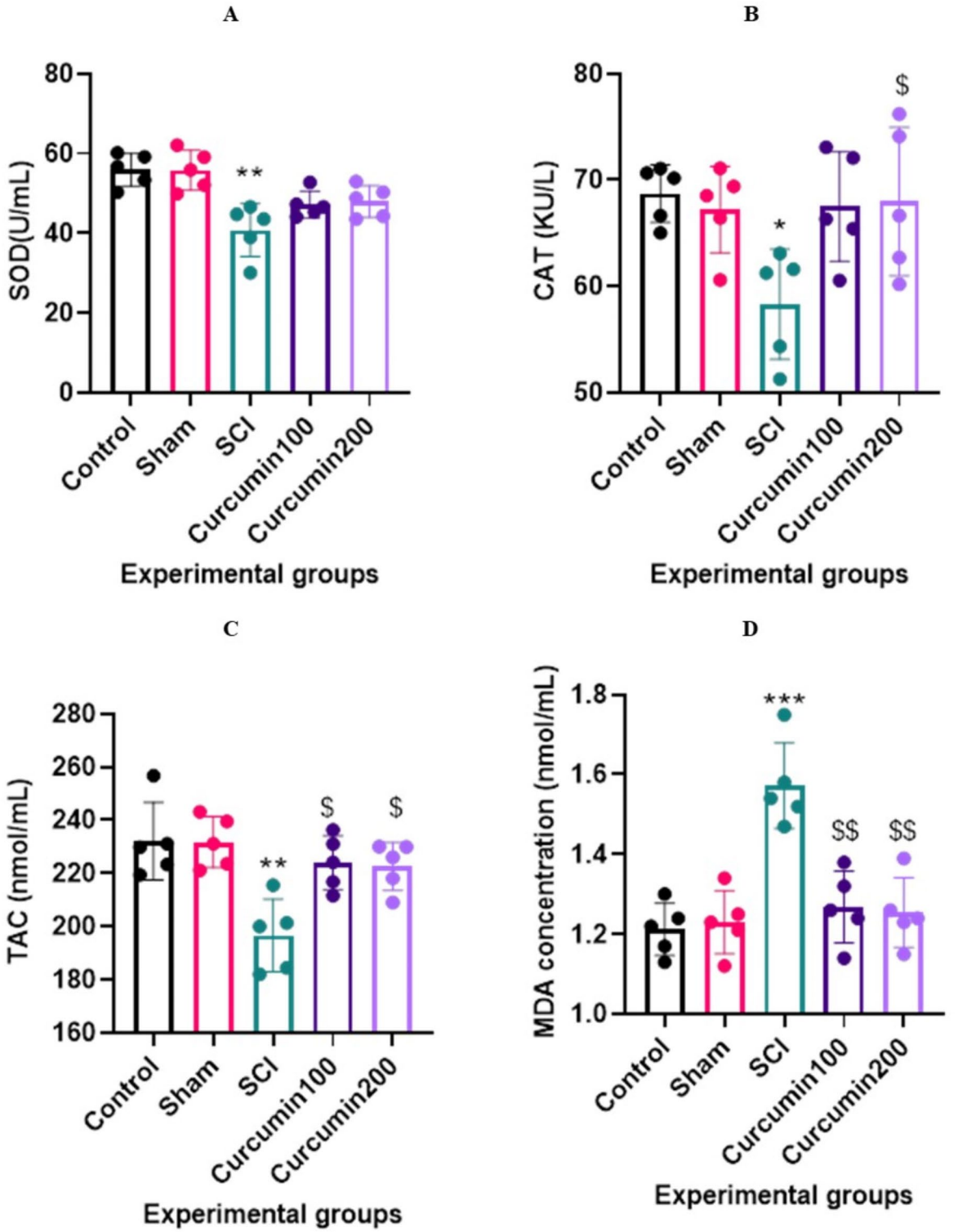

Fig. 7. The study examined the effect of oral curcumin on the serum level of (**A**) SOD (superoxide dismutase), (**B**) CAT (catalase), (**C**) TAC (total antioxidant capacity), and (**D**) MDA (malondialdehyde) in rats with spinal cord injury (SCI). Results showed that curcumin treatment at 100 and 200 mg/kg doses significantly increased antioxidant factors expect SOD and decreased oxidant index such as MDA versus SCI group at the end of the study. The experimental groups included a Control (no injury/no treatment), Sham (laminectomy), SCI (Spinal cord compression in the T11-T12 vertebrae using a clip), Curcumin100 and Curcumin200 (Curcumin at100 and 200 mg/kg doses, respectively, administrated 30 min after SCI for ten days). $n = 5$ in each group. $**P < 0.01$, $***P < 0.001$ vs. control group; $\#P < 0.05$, $\#\#P < 0.01$, vs. SCI.

of oxidative stress[71], and depletion in GABA can lead to increased oxidative stress and sustain this harmful feedback loop[72].

In our study, curcumin demonstrated beneficial effects by increasing antioxidant factors, enhancing the expression of GAD65 and GABA A receptors, and decreasing cavity size, urinary retention, and MDA levels compared to the spinal cord injury (SCI) group. Smaller cavity sizes in the treatment groups can be attributed to neuroprotective properties related to curcumin's anti-oxidant function[73]. A meta-analysis has confirmed the positive effects of curcumin on axonal growth and motor function recovery in SCI models[27]. Our data were in line with previous research, including Sanchez's study, also noted curcumin (200 mg/kg) reduced lipid peroxidation and improved antioxidant factors, although it did not significantly affect SOD one day after SCI[74]. Curcumin's phenolic structure allows it to react with ROS directly and neutralizes free radicals. For this reason, curcumin can enhance the cellular tolerance to oxidative damage and protect the nervous system by reducing lipid peroxidation[75]. Our study found that curcumin improved spinal cord function and NP by reversing SCI-induced events. The efficacy of curcumin at 100 and 200 mg/kg doses on the cold allodynia started two and four weeks post-surgery, respectively, and continued until the end of the study. However, curcumin 200 had more efficacy in the sixth week than curcumin100. Both doses reduced cold allodynia sooner than hyperalgesia, with no significant difference in hyperalgesia between the two doses. This difference may be attributed to the type of nerve fibers involved in allodynia and hyperalgesia[76]. Additionally, curcumin could ameliorate GABA content in the animals' hypothalamus[29]. Based on the studies[57–60]., it can be suggested that curcumin likely enhances GABA levels by increasing GAD65 content[17]. Curcumin may alleviate NP through GABAA receptors[15].

Our study has several limitations that should be considered in the next studies. First, we focused on curcumin's effects on injured male rats during the acute phase of SCI without considering the influence of Female sex hormones[77] and the complexities of different injury phases[78] which could affect curcumin's therapeutic properties. Second, curcumin's low bioavailability, low absorption, quick metabolism, and rapid systemic removal of curcumin can limit its therapeutic applications[22,79], highlighting the need for dosage optimization and delivery mechanisms for human studies are necessary and we ignored them. Lastly, our study did not clarify whether the observed increase in GAD65 and GABAA receptor content was due to curcumin's neuroprotective effects or their direct synthesis. Also, our study lacked an assessment of GABA levels but previous research indicates that curcumin's pain-relieving effects may be linked to an increase in GABA synthesis facilitated by GAD65.

We studied the effect of curcumin on NP following SCI (NP-SCI) for the first time. Given the similarity between SCI in rats and humans, such as the forming of the cavity and inhibitory barriers[80], Fibroblast invasion, and failure to fully regenerate from injury[81], we hope our results will be practical for clinical trials. But the differences between them, such as compensatory mechanisms such as the sprouting of the spinal cord in rats[82], differences in corticospinal pathways[47,81] and different events in the injury phases make us hesitant to generalize the results to humans. extensive studies are needed to confirm it as a treatment for NP-SCI in the clinic.

Conclusions

Our results showed that in SCI animals, the level of GAD65, GABA A receptors, antioxidants, and pain threshold decreased, and oxidant factors like MDA increased. Curcumin significantly reversed these changes. the analgesic effects of curcumin after SCI seem associated with its antioxidant properties. Studies have shown that curcumin has neuroprotective aspects and powerful effects against oxidants, inflammation, and apoptosis. It can likely regulate the microenvironment after SCI and improve this injury with its multiple properties. However, the current study on the positive effects of curcumin on NP-SCI is still in its infancy, and sufficient research on its functional mechanism after SCI is yet to be conducted.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions

Designing the experiment, methodology, surgery of animals western blotting and statistical analysis were done by [Z. B]. Care of animals, behavioral tests and collection of tissues were performed by [M.H] and [MM. F]. Editing and material providing were done by [M. M]. writing and statistical analysis were performed by [M. TM]. The authors declare that all data were generated in-house and that no paper mill was used.

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Declarations

Competing interests

The authors declare no competing interests.

Ethics approval

The experimental research protocol on animals was approved by the ethics committee of Kerman University of Medical Sciences (IR.KMU.AEC.1402.015) and followed the Animal Research Reporting of In Vivo Experiments (ARRIVE) guidelines. The methods were done by these relevant guidelines and regulations, and a statement to this effect was included in the methods section.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-93726-7.

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