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Sophocarpine Attenuates Chronic Constriction Sciatic Nerve Injury-induced Neuropathic Pain in Mice by Inhibiting the HMGB1/TLR4/NF-κB Signaling Pathway

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Abstract

Background: Sophocarpine (SC) is a major alkaloid extracted from *Sophora alopecuroides* L. Our previous study showed that SC has analgesic effects on neuropathic pain induced by chronic constriction injury (CCI). However, the exact analgesic mechanism of SC on neuropathic pain has not yet been elucidated.

Objectives: The current study aimed to examine the anti-neuropathic pain effects of SC on the HMGB1/TLR4/NF-κB signaling pathway to explore its analgesic mechanism in neuropathic pain.

Methods: In this experimental study, the neuropathic pain mouse model was established by CCI of the sciatic nerve in a university-affiliated animal lab, China, 2016. Mechanical withdrawal threshold (MWT), thermal withdrawal latency (TWL), cold withdrawal threshold (CWT), and tail-curling latency (TCL) were used to assess the antinociceptive effect of SC in neuropathic pain mice. The mRNA and protein expression levels of HMGB1, TLR4, NF-κB, p-NF-κB, TNF-α, and IL-6 in the spinal cord of neuropathic pain mice were detected by reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting.

Results: Treatment with 40 and 20 mg/kg of SC was effective in increasing MWT, lengthening TWL, reducing CWT, and prolonging TCL in mice with neuropathic pain induced by CCI. Compared to the neuropathic-pain model group, treatment with 40 mg/kg of SC could effectively down-regulate HMGB1, TLR4, NF-κB, p-NF-κB, TNF-α, and IL-6 mRNA and protein expression levels in the spinal cord of mice with neuropathic pain induced by CCI.

Conclusions: Our results showed that SC has analgesic effects on neuropathic pain induced by CCI, and its analgesic mechanism may be related to down-regulating the HMGB1/TLR4/NF-κB signaling pathway.

Keywords: HMGB1Protein, Interleukin-6, Messenger, Mice, NF-κB, Neuralgia, RNA, Sophocarpine, *Sophora alopecuroids* L, TLR4

1. Background

Neuropathic pain is defined as "pain arising as a direct consequence of a lesion or disease affecting the somatosensory system". As a form of chronic pain, neuropathic pain is characterized by spontaneous pain, hyperalgesia, allodynia, abnormal pain, and paresthesia (1-3). Neuropathic pain can be induced by many factors, such as autoimmune disease, metabolic disease, inflammation, trauma, cancer, chemotherapy, neural compression, vascular disease, and so on (4-7). Neuropathic pain is a prolonged condition that is difficult to cure, thus severely affecting the patient's quality of life and work efficiency (8, 9). The pathogenesis of neuropathic pain is complex and remains to fully understand. However, sustained chronic inflammation has been considered to play an important role in neuropathic pain (10). Toll-like receptor 4 (TLR4) is the most common receptor for high mobility group box-1 (HMGB1) protein. In the central nervous system (CNS), TLR4 is primarily expressed in microglial cells and is involved in their activation and central immune inflammation (11). Once activated, many pro-inflammatory cytokines (such as TNF-α, IL-6, etc.) are generated and released via the NF-κB signaling pathway, enhancing inflammation, and increasing pain (12). HMGB1 is an endogenous ligand for TLR4 that is highly expressed in the spinal dorsal horn of rats

with neurological pain or bone cancer pain (13, 14). Based on these studies, we hypothesized that HMGB1/TLR4/NF-κB signaling pathway might participate in the occurrence and development of neuropathic pain.

Sophocarpine (SC) is an important alkaloid extract from *Sophora alopecuroides* L. (leguminous sophora plants) (15, 16). Recent studies have shown that sophocarpine possesses analgesic, anti-arrhythmic, anti-inflammatory, anti-tumor, central inhibitory, anti-colitis, anti-cardiac fibrosis, anti-liver fibrosis, anti-hepatocyte steatosis, and anti-viral properties (17-20). Our preliminary experiments revealed that sophocarpine exerts analgesic effects against neuropathic pain induced by chronic constriction injury (CCI) in mice and that the underlying mechanisms are related to GABA and GAD65 up-regulation and GAT1 down-regulation (21). However, whether the mechanism by which sophocarpine alleviates neuropathic pain is related to HMGB1/TLR4/NF-κB signaling has not been reported. Therefore, the current study aimed to observe the anti-neuropathic pain effects of sophocarpine on the HMGB1/TLR4/NF-κB signaling pathway to explore its analgesic mechanism in neuropathic pain.

2. Objectives

The current study aimed to observe the anti-neuropathic pain effects of SC on the HMGB1/TLR4/NF-κB signaling pathway to explore its analgesic mechanism in neuropathic pain.

3. Methods

3.1. Reagents

Sophocarpine (≥ 98.0% purity, confirmed by high-performance liquid chromatography analysis) was purchased from Ningxia Bauhinia Pharmaceutical Co. Ltd. (Yanchi, Ningxia, China) dissolved in normal saline at a stock concentration of 400 mg/mL and stored at 4°C. Pregabalin (Pre) was obtained from Pfizer Manufacturing Deutschland GmbH (Betriebsstatte Freiburg, Germany). Pentobarbital sodium was provided by Sigma-Aldrich Co. LLC (St. Louis, MO, US).

3.2. Experimental Animals

Specific pathogen-free (SPF)-grade male ICR mice (five-weeks-old weighing 20 ~ 22 g) were obtained from the Experimental Animal Center of Zhengzhou Medical University (Certificate number SYXK 2010 - 0002). The animal room temperature was maintained at 20 ± 2°C, and the relative humidity was kept at 55 ± 10% under a 12-h light/dark cycle. During the experiment, all mice were given free access to food and tap water. Animal experiments were approved by the Animal Ethics Committee of Luohe Medical College (Code: 2016007; Date: March 10, 2016). The experiment started in March, 2016. The sample size was calculated with a power of 0.95 and type I error of 0.05. We allocated 10 mice to the effected groups to increase the power of the study (22).

3.3. Chronic Constriction Injury of the Neuropathic Pain Mouse Model

Neuropathic pain mouse models were established via CCI of the sciatic nerve. Briefly, the mice were weighed and given pentobarbital sodium (60 mg/kg, intraperitoneal injection, supplemented as necessary) for anesthesia. The mice were tied to the operating table on their left side. The right hind leg was disinfected with iodophor. The skin of the right thigh was incised longitudinally and the biceps femoris and gluteus were bluntly separated. Finally, approximately 5 - 7 mm of nerve tissue proximal to the sciatic trifurcation was freed and exposed. Three ligatures (4 - 0 chromic gut ligatures) spaced 1 mm apart were tied loosely around the sciatic nerve until a slight twitch of the right hind limb was aroused. In sham groups, an identical surgical procedure was performed, except that the sciatic nerve was not ligated (23).

3.4. Experimental Protocol

The male ICR mice were randomly divided into six groups (10 mice per group): Sham-operated, model (CCI), CCI + Pre, CCI + sophocarpine 40 mg/kg (24, 25), CCI + sophocarpine 20 mg/kg, and CCI + sophocarpine 10 mg/kg groups. The sham and CCI mice received normal saline, the CCI + Pre mice received pregabalin 10 mg/kg, and the three different CCI + sophocarpine groups were treated with sophocarpine (40, 20, 10 mg/kg). All mice received intraperitoneal injections of the corresponding drugs (0.1 mL/10 g body weight) on the eighth-day post-operation, once per day for seven consecutive days.

3.5. Mechanical Withdrawal Threshold (MWT) Test

The methods of grouping and model making were the same as the 3.3 and 3.4 sections. MWT was measured using von Frey hairs (Ugo Basile Srl, Italy) one day before the CCI surgery (baseline), on the seventh-day post-surgery, and on the first, third, fifth, and seventh days after treatment. The mice were placed in a transparent Plexiglas cylinder (height, 20 cm; diameter, 9 cm) with a wire mesh bottom (grid size, 5 mm × 5 mm) that allowed for the mouse' paws full access to the von Frey hairs and made them acclimate to this controlled environment and temperature

for 20 min before the experiment. Different forces of von Frey hairs were utilized to perpendicularly stimulate the mid-plantar surface (the sciatic nerve distribution area) of the right hind paw until a clear withdrawal or foot-lick response was observed and held for approximately 5 s. Failure to elicit withdrawal or foot-lick response until the von Frey hairs distorted to 90° was considered a negative response. The subsequent force of the Frey hair application was increased or decreased according to the previous response of the mouse. A von Frey hairs force of 4.0 g was used as a cutoff.

3.6. Thermal Withdrawal Latency (TWL) Test

The methods of grouping and model making were the same as the 3.3 and 3.4 sections. TWL was evaluated using an XR-1102 thermal sting apparatus (Shanghai Xinruan Information Technology Co. Ltd., China) according to the method of Hargreaves et al. (26) on the day before CCI surgery, on the seventh-day post-surgery, and on the first, third, fifth, and seventh days after treatment. The mice were placed in a transparent Plexiglas box (height, 20 cm; diameter, 9 cm) with a transparent glass floor (thickness, 3 mm) that allowed mice to acclimate to this controlled environment and temperature for 20 min before the experiment. The radiant heat source of the thermal sting apparatus was used to vertically irradiate the plantar skin of the CCI-side hind paw of each mouse. TWL was recorded as the time from the application of the thermal sting apparatus to the induction of an obvious withdrawal. To prevent tissue damage, the cutoff time was set at 20 s.

3.7. Cold Withdrawal Threshold (CWT) Test

The methods of grouping and model making were the same as the 3.3 and 3.4 sections. CWT was detected using a BW-YLS-21A cold/hot plate analgesia test apparatus (Shanghai Bio-will Co., Ltd., China) on the day before CCI surgery, on the seventh-day post-surgery, and on the first, third, fifth, and seventh- days after medication. The temperature of the cold/hot plate analgesia test apparatus was maintained at 4 $\pm$ 0.5°C. The mice were placed on the plate and acclimated for 5 min, and the numbers of hind-paw withdrawal and licking or shaking events on the CCI side were recorded for 5 min as the CWT or the counts of paw withdrawal.

3.8. Tail-Curling Latency (TCL) Test

The methods of grouping and model making were the same as the 3.3 and 3.4 sections. TCL wa detected on the day before CCI surgery, on the seventh-day post-surgery, and on the first, third, fifth, and seventh days after treatment. The mice were fixed in a holder, and the tail was allowed to rest on the table. The terminal 2 ~ 3 cm of the tail was immersed in hot water at a temperature of 50 $\pm$ 0.5°C until the tail drew back from the water; the duration was recorded as the TCL. To avoid skin injury, a cutoff time of 10 s was established.

3.9. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

The HMGB1, TLR4, NF-κB, TNF-α, and IL-6 mRNA expression levels were analyzed by RT-PCR. All mice were sacrificed by cervical dislocation 24 h after the last drug administration. Total RNA was extracted from the mouse spinal cord (lumbar 4/5) tissue using TRIzol (BBI, Kitchener, Ont., Canada) and reverse transcribed using a RevertAidTM First Strand cDNA Synthesis Kit (Thermo Scientific Fermentas, USA) according to the manufacturer's instructions. The primers of HMGB1, TLR4, NF-κB, TNF-α, and IL-6 were obtained from Sangon Biotech Co., Ltd. (Shanghai). The sequences are listed in Table 1. Amplification was performed in a 25 μL reaction containing 2 μL primer, 3 μL cDNA, 12.5 μL Taq Master Mix (Novoprotein Scientific Inc. Jiangsu, China), and 7.5 μL nuclease-free water. Cycling conditions were pre-denaturation at 95°C for 5 min, 35 cycles of 95°C for 30 s, 58°C for 30 s, and 70°C for 30 s, and extension at 72°C for 10 min. The PCR products were confirmed on a 1.2% agarose gel and the semi-quantitative analysis of the target mRNAs was normalized to the reference gene β-actin by Quantity One software packet version 4.6.2 (Bio-Rad Laboratories, California, USA).

3.10. Western Blotting

All mice were sacrificed by cervical dislocation 24 h after the final treatment. Total proteins were extracted from the mouse spinal cord (Lumbar 4/5) tissue using the radioimmunoprecipitation assay (RIPA) lysis buffer (Boster Biological Technology, Wuhan, China) at 4°C according to the manufacturer's instructions. Protein concentrations were detected using a BCA protein assay kit (Boster Biological Technology, Wuhan, China) and adjusted to obtain equal loading. The proteins were separated via 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membranes (Boster Biological Technology, Wuhan, China), blocked with PBS containing 2% bovine serum albumin for 2 h at room temperature and incubated with anti-HMGB1, TLR4, NF-κB, p-NF-κB (S529), TNF-α, IL-6, and β-actin antibodies (Boster Biological Technology, Wuhan, China) at room temperature for 3 h. Subsequently, the

Table 1. Primer Sequences and Product Length

Gene	Forward Primer (5' - 3")	Reverse Primer (5' - 3")	Product Length (bp)
HMGB1	ACTTGGCAAAGCAAGGAGTGA	GCCAGCGTTCTTGTGATAGC	283
TLR4	CAGTGGGTCAAGGACCAGAA	ACCACAATAACCTTCCGGCT	437
NF-κB	GCAAGCCGGTTCCAAATTCC	AGGGTACATCTGTGAAGGAGGA	355
TNF-α	GGTGCCTATGTCTCAGCCTC	ATAGCAAATCGGCTGACGGT	403
IL-6	GTCCGGAGAGGAGACTTCAC	CTGCAAGTGCATCATCGTTGT	166
β-actin	TGTGACGTGGACATCCGTAA	GCTAGGAGCCAGAGCAGTAATCT	117

membranes were incubated with a secondary antibody (labeled by horseradish peroxidase) at room temperature for 1.5 h. The signal was detected using an enhanced chemiluminescence kit (Boster Biological Technology, Wuhan, China) on a Bio-Rad ChemiDoc XRS + chemiluminescence imaging system (USA). The protein expression levels of HMGB1, TLR4, NF-κB, p-NF-κB, TNF-α, and IL-6 were normalized to β-actin and quantified using Quantity One software packet version 4.6.2 (Bio-Rad Laboratories, California, USA).

3.11. Statistical Analysis

All data were presented as the mean $\pm$ SD. The normality assumption was checked for the tests. The comparison between groups was analyzed by one-way ANOVA followed by Tukey post hoc test for multiple comparisons. $P < 0.05$ was considered statistically significant. The statistical analysis was performed using SPSS software packet, version 19.0 (SPSS Inc., Ill., Chicago, USA).

4. Results

4.1. Sophocarpine Increased the MWT in CCI Mice

The effects of sophocarpine on the MWT in CCI mice are shown in Table 2. The MWT of the CCI model group was lower than that of the sham group and the MWT of sophocarpine 40 mg/kg, 20 mg/kg and pregabalin groups was increased on the third, fifth, and seventh days after medication ($P < 0.05$, $P < 0.01$). However, there were no obvious changes after sophocarpine 10 mg/kg administration ($P > 0.05$).

4.2. Sophocarpine Increased the TWL in CCI Mice

The TWL was significantly shorter in the CCI group than in the sham group on the seventh day following surgery ($P < 0.01$). After sophocarpine (40, 20, and 10 mg/kg) and pregabalin 10 mg/kg administration, the TWL became markedly longer than that of the CCI group on the third, fifth, and seventh days after medication ($P < 0.05$, $P < 0.01$, Table 3).

4.3. Sophocarpine Reduced the CWT in CCI Mice

As shown in Table 4, seven days after the operation, the CWT was significantly higher in the CCI group than in the sham group ($P < 0.01$). After sophocarpine (40 mg/kg, 20 mg/kg) and pregabalin 10 mg/kg administration, the counts of paw withdrawal were significantly lower than those in the CCI group on the fifth and seventh days after medication ($P < 0.05$, $P < 0.01$).

4.4. Sophocarpine Increased the TCL in CCI Mice

On the seventh day after surgery, the TCL was markedly shorter in the CCI group ($P < 0.01$) than in the sham group. The TCL was significantly longer in sophocarpine (40 mg/kg, 20 mg/kg) and pregabalin 10 mg/kg treated groups on the third, fifth, and seventh days after medication than in the CCI group ($P < 0.05$, $P < 0.01$, Table 5).

4.5. Sophocarpine Down-Regulated HMGB1, TLR4, NF-κB, TNF-α, and IL-6 mRNA Expression Levels in CCI Mouse Spinal Cord Tissue

HMGB1, TLR4, NF-κB, TNF-α, and IL-6 mRNA expression levels were detected by RT-PCR in mice spinal cord tissue. On the 14th day after surgery, HMGB1, TLR4, NF-κB, TNF-α, and IL-6 mRNA expression levels were markedly up-regulated in the CCI group compared to those in the sham group ($P < 0.01$). After sophocarpine (40 mg/kg) administration, HMGB1, TLR4, NF-κB, TNF-α, and IL-6 mRNA expression levels in the mouse spinal cord tissue were significantly down-regulated compared to those in the CCI group ($P < 0.05$, $P < 0.01$, Figure 1).

4.6. Sophocarpine Down-Regulated the HMGB1, TLR4, NF-κB, TNF-α, and IL-6 Protein Expression Levels in CCI Mouse Spinal Cord Tissue

HMGB1, TLR4, NF-κB, TNF-α, and IL-6 protein expression levels were detected by western blotting in mouse spinal cord tissue. On the 14th day after surgery, HMGB1, TLR4, NF-κB, p-NF-κB, TNF-α, and IL-6 protein expression levels were markedly up-regulated in the CCI group compared

Table 2. Effect of Sophocarpine on Mechanical Withdrawal Threshold (MWT) in Neuropathic Pain Mice Induced by CCI (N = 10)[a]

Group	Dose (mg/kg)	MWT (g)					
		1 d before CCI	7 d after CCI	SC administration			
				1 d	3 d	5 d	7 d
Shame	-	1.63 ± 0.15	1.50 ± 0.20	1.57 ± 0.32	1.53 ± 0.21	1.43 ± 0.42	1.47 ± 0.38
CCI	-	1.60 ± 0.20	0.11 ± 0.03^{B}	0.11 ± 0.04^{B}	0.12 ± 0.04^{B}	0.11 ± 0.03^{B}	0.12 ± 0.04^{B}
CCI + Pre	10	1.67 ± 0.25	0.12 ± 0.03^{B}	0.25 ± 0.07$^{B, C}$	0.62 ± 0.06$^{B, D}$	0.94 ± 0.12$^{A, D}$	1.44 ± 0.23^{D}
CCI + SC	40	1.47 ± 0.31	0.13 ± 0.03^{B}	0.21 ± 0.02$^{B, C}$	0.54 ± 0.06$^{B, D}$	0.85 ± 0.14$^{A, D}$	1.31 ± 0.19^{D}
	20	1.53 ± 0.42	0.11 ± 0.03^{B}	0.18 ± 0.02^{B}	0.39 ± 0.05$^{B, C}$	0.74 ± 0.10$^{B, D}$	0.91 ± 0.10$^{A, D}$
	10	1.57 ± 0.31	0.10 ± 0.04^{B}	0.13 ± 0.02^{B}	0.13 ± 0.02^{B}	0.18 ± 0.03^{B}	0.13 ± 0.04^{B}
F value		0.192	128.511	53.253	89.521	20.448	29.161
P value		0.960	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

[a]Compared to the sham group, ^{A}P < 0.05 and ^{B}P < 0.01; compared to the CCI group, ^{C}P < 0.05 and ^{D}P < 0.01.

Table 3. Effect of Sophocarpine on Thermal Withdrawal Latency (TWL) in Neuropathic Pain Mice Induced by CCI (N = 10)[a]

Group	Dose (mg/kg)	TWL (s)					
		1 d Before CCI	7 d After CCI	SC Administration			
				1 d	3 d	5 d	7 d
Shame	-	15.13 ± 1.60	14.61 ± 1.16	14.47 ± 2.82	13.53 ± 0.92	14.69 ± 0.98	14.13 ± 2.50
CCI	-	15.05 ± 1.85	5.62 ± 0.62^{B}	5.70 ± 0.80^{B}	5.24 ± 0.46^{A}	5.37 ± 0.65^{B}	5.23 ± 0.60^{B}
CCI + Pre	10	13.86 ± 2.72	5.77 ± 0.87^{B}	6.65 ± 0.69^{B}	11.54 ± 1.11$^{A, D}$	13.76 ± 1.07^{D}	13.65 ± 1.94^{D}
CCI + SC	40	14.57 ± 2.61	5.72 ± 0.66^{B}	6.40 ± 0.69^{B}	9.28 ± 0.55$^{B, D}$	10.59 ± 0.78$^{A, D}$	12.67 ± 2.24^{D}
	20	14.69 ± 0.78	5.46 ± 0.76^{B}	6.14 ± 0.62^{B}	8.28 ± 0.37$^{B, D}$	9.13 ± 0.64$^{A, D}$	9.79 ± 0.35$^{A, D}$
	10	14.45 ± 1.57	5.87 ± 0.91^{B}	5.96 ± 0.15^{B}	7.47 ± 0.48$^{B, C}$	8.31 ± 0.41$^{B, C}$	8.51 ± 0.60$^{B, C}$
F value		0.163	55.584	20.943	53.331	59.319	13.607
P value		0.972	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

[a]Compared to the sham group, ^{A}P < 0.05 and ^{B}P < 0.01; compared to the CCI group, ^{C}P < 0.05 and ^{D}P < 0.01.

Table 4. Effect of Sophocarpine on Cold Withdrawal Threshold (CWT) in Neuropathic Pain Mice Induced by CCI (N = 10)[a]

Group	Dose (mg/kg)	CWT (Times)					
		1 d Before CCI	7 d After CCI	SC Administration			
				1 d	3 d	5 d	7 d
Shame	-	15.33 ± 2.52	16.33 ± 4.16	14.33 ± 4.04	15.00 ± 6.08	13.67 ± 4.04	17.00 ± 2.00
CCI	-	16.67 ± 3.51	50.67 ± 6.66^{B}	51.67 ± 6.03^{B}	50.67 ± 7.51^{B}	49.33 ± 7.37^{B}	52.67 ± 6.51^{B}
CCI + Pre	10	13.33 ± 3.21	49.67 ± 6.03^{B}	45.00 ± 5.57^{B}	34.67 ± 4.51$^{B, C}$	27.33 ± 2.52$^{A, D}$	22.33 ± 4.16^{D}
CCI + SC	40	14.67 ± 3.79	47.67 ± 7.02^{B}	45.67 ± 5.51^{B}	38.00 ± 6.56$^{B, C}$	30.33 ± 3.51$^{A, D}$	28.67 ± 2.52$^{A, D}$
	20	16.33 ± 4.16	50.33 ± 8.39^{B}	46.67 ± 7.64^{B}	42.33 ± 6.11^{B}	39.00 ± 3.61$^{B, C}$	31.33 ± 3.51$^{B, D}$
	10	15.67 ± 3.06	51.33 ± 7.37^{B}	47.33 ± 4.04^{B}	51.33 ± 6.66^{B}	51.33 ± 8.62^{B}	52.33 ± 5.69^{B}
F value		0.377	12.613	17.770	13.006	20.861	35.954
P value		0.855	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

[a]Compared to the sham group, ^{A}P < 0.05 and ^{B}P < 0.01; compared to the CCI group, ^{C}P < 0.05 and ^{D}P < 0.01.

to those in the sham group (P < 0.01). After sophocarpine (40 mg/kg) administration, HMGB1, TLR4, NF-κB, p-NF-κB, TNF-α, and IL-6 protein expression levels in mouse spinal cord tissue were significantly down-regulated compared to those in the CCI group (P < 0.05, P < 0.01, Figure 2).

5. Discussion

Neuropathic pain is a common chronic pain syndrome that is primarily caused by nervous system dysfunction and seriously affects human life and health (27). Immune activation in the central nervous system and peripheral nerve inflammation play key roles in neuropathic pain (28,

Table 5. Effect of Sophocarpine on Tail-Curling Latency (TCL) in Neuropathic Pain Mice Induced by CCI (N = 10)[a]

Group	Dose (mg/kg)	TCL (s)					
		1 d Before CCI	7 d After CCI	SC Administration			
				1 d	3 d	5 d	7 d
Shame	-	5.36 ± 0.93	5.06 ± 0.74	5.45 ± 0.76	5.62 ± 0.68	5.71 ± 0.48	5.96 ± 0.46
CCI	-	5.28 ± 0.79	1.98 ± 0.28^{B}	2.01 ± 0.23^{B}	2.13 ± 0.49^{B}	2.24 ± 0.40^{B}	2.10 ± 0.32^{B}
CCI + Pre	10	5.41 ± 0.58	1.93 ± 0.33^{B}	4.02 ± 0.54$^{A, D}$	4.88 ± 0.63^{D}	4.93 ± 0.36^{D}	5.12 ± 0.29$^{D, D}$
CCI + SC	40	5.27 ± 0.93	2.11 ± 0.39^{B}	3.66 ± 0.45$^{B, D}$	4.47 ± 0.44^{D}	4.61 ± 0.42$^{A, D}$	4.81 ± 0.41$^{A, D, D}$
	20	5.45 ± 0.78	2.02 ± 0.45^{B}	3.49 ± 0.51$^{B, C}$	4.16 ± 0.47$^{A, D}$	4.20 ± 0.23$^{A, D}$	4.58 ± 0.55$^{A, D, D}$
	10	5.34 ± 0.66	2.06 ± 0.43^{B}	2.22 ± 0.29^{B}	2.07 ± 0.11^{B}	2.32 ± 0.34^{B}	2.34 ± 0.33^{B}
F value		0.024	21.698	19.667	25.478	42.380	45.585
P value		1.000	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

[a]Compared to the sham group, ^{A}P < 0.05 and ^{B}P < 0.01; compared to the CCI group, ^{C}P < 0.05 and ^{D}P < 0.01.

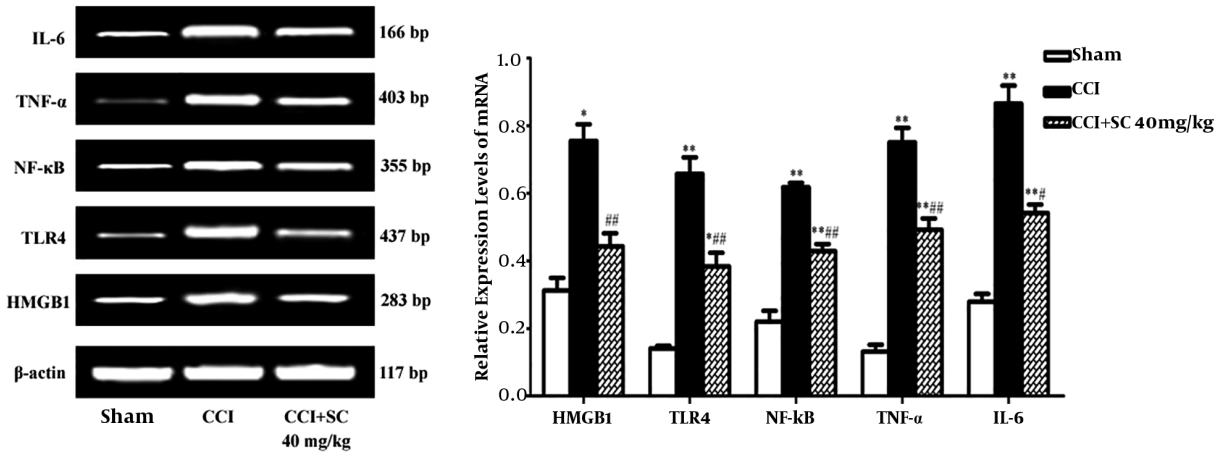

Figure 1. Sophocarpine down-regulated the expression levels of HMGB1, TLR4, NF-κB, TNF-α, and IL-6 mRNA in the spinal cord tissue of neuropathic pain mice based on RT-PCR. Data are expressed as the mean ± SD. and each group consisted of five mice (n = 5). Compared to the sham group, *P < 0.05 and **P < 0.01; compared to the CCI group, #P < 0.05 and ##P < 0.01.

29). Although many medications or remedial techniques have provided symptomatic relief in patients with neuropathic pain, strategies for improving the quality of life are still lacking (30). Research to find novel, efficacious, and safe agents with limited side effects to treat neuropathic pain is imperative. Traditional Chinese medicine has great potential in analgesia as a rich source of agents with multi-targeting mechanisms, few adverse reactions, and no drug resistance (31). Many Traditional Chinese Medicine extracts are increasingly being used to ameliorate the symptoms of neuropathic pain (32). Sophocarpine is an important alkaloid extracted from *Sophora alopecuroides* L., which is a traditional Chinese herb (15). Recent studies have shown that sophocarpine has anti-nociceptive and anti-inflammatory activities (33). MWT is an important index for evaluating the mechanical hyperalgesia of CCI mice; TWL and TCL are used to evaluate the thermal hyperalgesia of CCI mice; and CWT can be used to detect the cold pain hypersensitivity on CCI mice (34, 35). In the present study, we found that sophocarpine (40, 20 mg/kg) increased the MWT, lengthened the TWL and TCL, and decreased the CWT in neuropathic pain mice. This result suggests that sophocarpine has analgesic effects on neuropathic pain. However, the potential analgesic mechanism of sophocarpine against neuropathic pain has thus far not been elucidated.

The occurrence of neuropathic pain is closely related to immune inflammation. Many inflammatory factors such as HMGB1, TLR4, TNF-α, IL-6, and NF-κB play an important role in the development of neuropathic pain (36, 37). HMGB1 is a member of the HMG family that is broadly expressed in eukaryotic cells. As a potent inflammatory mediator, HMGB1 is involved in numerous inflammations.

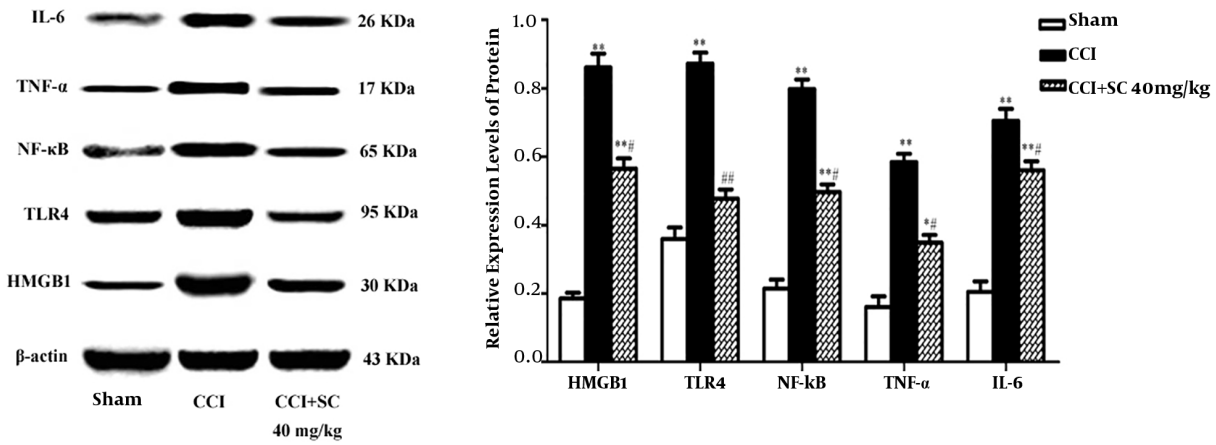

Figure 2. Sophocarpine down-regulated the expression levels of HMGB1, TLR4, NF-κB, p-NF-κB, TNF-α, and IL-6 protein in the spinal cord tissue of neuropathic pain mice based on western blotting. Data are expressed as the mean $\pm$ SD. and each group consisted of five mice (n = 5). Compared to the sham group, *P < 0.05 and **P < 0.01; compared to the CCI group, #P < 0.05 and ##P < 0.01.

Both RAGE and TLR4 are the HMGB1 receptors. HMGB1 is combined with RAGE or TLR4, thus activating the NF-κB signaling pathway to induce and promote the inflammation. HMGB1, TLR4, and RAGE are expressed in the spinal dorsal horn and dorsal root ganglion, and are involved in regulating the occurrence and development of various chronic pain. HMGB1-mediated RAGE activation of NF-κB differs from HMGB1-mediated TLR4 activation of NF-κB pathway. The former activates IKKβ while the later activates IKKα and IKKβ (38, 39). In this study, we mainly studied the TLR4 activation pathway. NF-κB is an important transcription factor involved in inflammation, immunoreactions, oxidation reaction, and cell apoptosis and plays a vital role in neuropathic pain (40). Studies have shown that the activation of the NF-kB pathway in the spinal cord may contribute to the pathogenesis of neuropathic pain induced by CCI and that the suppression of NF-kB protein expression by antisense ODN (NF-kB antisense oligodeoxynucleotides) alleviates hyperalgesia and allodynia in CCI model rats (41). Moreover, intrathecal infusions of pyrrolidine dithiocarbamate (PDTC, an inhibitor of NF-κB) inhibited the activation of microglia and astrocytes and attenuated CCI-induced allodynia and hyperalgesia (42). HMGB1 interacts with TLR4 to induce the activation of NF-κB signaling and stimulates the release of TNF-α, IL-6, etc. When a nerve is injured, for example in sciatic nerve CCI, the damaged nerve and dorsal root ganglia tissue release TNF-α, IL-6, and other pro-inflammatory factors, activating HMGB1 to bind TLR4, which, in turn, activates the NF-κB signaling pathway. Then, NF-κB dissociates from NF-κB/I-κB complexes and rapidly enters the nucleus, binds to specific sites in DNA, and activates the transcription of target genes (e.g., TNF-α, IL-6). TNF-α, IL-6, and other cytokines also activate NF-κB, which further sustains or amplifies inflammation and pain, and make neuropathic pain aggravate ceaselessly (13, 36). In our study, we found that treatment with sophocarpine 40 mg/kg could suppress the HMGB1, TLR4, NF-κB, p-NF-κB, TNF-α, and IL-6 expression levels in the spinal cords of mice with neuropathic pain. The results suggested that sophocarpine could inhibit the HMGB1/TLR4/NF-κB signaling pathway in mice with neuropathic pain.

5.1. Conclusions

Collectively, our results suggest that sophocarpine has analgesic effects on neuropathic pain induced by CCI, and its mechanism is related to down-regulating the HMGB1/TLR4/NF-κB signaling pathway. Sophocarpine may be developed as a potential and effective agent for relieving neuropathic pain.

Footnotes

Authors' Contribution: Shaoju Jin, Junjun Zhou, and Yongchao Ma designed the experiment. Shaoju Jin, Rong Wang, and Songtao Xu drafted the manuscript. Shaoju Jin, Rong Wang, Tingting Wang, Kunpeng Guo, Liucheng Guo, and Songtao Xu conducted the experiment. Shaoju Jin and Junjun Zhou contributed to the analysis of data. Shaoju Jin and Junjun Zhou contributed to the revision of the manuscript. Yongchao Ma and Junjun Zhou provided the scientific advice. Shaoju Jin, Rong Wang, and Songtao

Xu contributed equally to this paper. All authors read and approved the manuscript.

Conflict of Interests: The authors declare that there are no conflicts of interest.

Ethical Approval: Animal experiments were approved by the Animal Ethics Committee of Luohe Medical College (Code: 2016007; Date: March 10, 2016).

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