

Intrathecal injection of ozone alleviates CCI-induced neuropathic pain via the GluR6-NF- κ B/p65 signalling pathway in rats

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Abstract. Ozone is widely used to relieve chronic pain clinically, but the precise mechanisms governing its action have yet to be elucidated. The present study aimed to investigate the mechanisms underlying the pain-alleviating effect of ozone in the chronic constriction injury (CCI) model of sciatic nerve in rats. Pain behaviours of rats were assessed by mechanical allodynia and thermal hyperalgesia. The expression of spinal glutamate receptor 6 (GluR6) and NF- κ B/p65 was detected by western blotting and reverse transcription-quantitative PCR. Meanwhile, the expression of spinal IL-1 β , IL-6 and TNF- α was detected by ELISA. GluR6 short interfering (si)RNAs were used intrathecally immediately following CCI once per day. Ozone (10, 20 or 30 μ g/ml) or oxygen was injected intrathecally on day 7 after CCI. The expression level of spinal GluR6 increased on day 3 and reached a peak on day 7 after CCI. The expression level of spinal IL-1 β , IL-6, TNF- α and NF- κ B/p65 also increased on day 7 after CCI. In addition, pre-intrathecal injection of GluR6 siRNAs inhibited pain behaviours and suppressed the expression of spinal GluR6, IL-1 β , IL-6, TNF- α and NF- κ B/p65 in CCI rats on day 7. Intrathecal injection of ozone was also observed to inhibit pain behaviours and suppress the expression of spinal GluR6, IL-1 β , IL-6, TNF- α and NF- κ B/p65 in CCI rats on day 7. The present study suggested that GluR6 served a pivotal role in neuropathic pain and that intrathecal injection of ozone may alleviate neuropathic pain via the GluR6-NF- κ B/p65 signalling pathway.

Introduction

Neuropathic pain is the most frequently reported form of chronic pain and seriously affects the quality of life of patients (1). However, there are few effective methods for treating neuropathic pain, hence the requirement for pharmacological research into new analgesics (2). Neuropathic pain arises from primary lesions or dysfunctions of the peripheral and central nervous systems, which often result in a long-lasting excitability of spinal dorsal horn neurons (central sensitization) (3,4). In general, hyperalgesia, allodynia and spontaneous pain are the commonest symptoms of neuropathic pain (5). Chronic constriction injury (CCI) of the sciatic nerve is a classic model used to study neuropathic pain over a long period (6); it can imitate the clinical neuropathic pain conditions resulting from lumbar disk herniation or chronic entrapment of the peripheral nerve (7). In recent years, ozone has been widely used to relieve chronic pain in clinical practice (8). For example, multicentre clinical trials have indicated that ozone therapy can generate valid effects and low morbidity rates when applied percutaneously for the treatment of chronic low back pain (9-11). Accumulating research has also indicated that ozone can treat prolapse of the lumbar intervertebral disc or failed back surgery syndrome effectively (12-15). Despite the wide use of ozone in the treatment of clinical pain conditions, it is necessary to pay attention to its potential toxicity due to its strong oxidizing capacity (16). It has been verified that ozone has useful antiseptic, antiviral and disinfectant effects (17). Previous studies have indicated that ozone may provide long-lasting anti-inflammatory effects and reduce inflammation through the immunomodulation and activation of cellular metabolism (18) and that it could also activate the endogenous antioxidant system in endotoxic and septic shock models (19). Although multiple studies have been carried out in the past several years, the precise analgesic mechanisms of ozone have yet to be elucidated (17-19). Therefore, further research is warranted to illuminate the exact biological mechanisms of ozone and to avoid potential detrimental effects.

Kainate receptors are a type of ionotropic glutamate receptor and serve important roles in mediating excitatory synapse transmission in the central nervous system (20).

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These receptors have been implicated in the pathogenesis of a number of neurological diseases, such as stroke, Alzheimer's disease, epilepsy and neuropathic pain (21). To date, five types of kainate receptor subunits have been confirmed: Glutamate receptor (GluR)5, GluR6, GluR7, KA1 and KA2 (22). Accumulating evidence has indicated that GluR6 is expressed in the superficial dorsal horn and is involved in nociception transmission (23). Our previous study demonstrated that GluR6 is associated with visceral pain (24). However, few studies have investigated the role of GluR6 in neuropathic pain and the analgesic effect of ozone.

As a crucial nuclear transcription factor, the NF- κ B/p65 heterodimer has been demonstrated to be related to the development and maintenance of neuropathic pain. This transcription factor is expressed widely in the central nervous system (25,26).

It is natural to consider the question of whether GluR6 is involved in neuropathic pain and the analgesic effect of ozone. The present study investigated the role of GluR6 in neuropathic pain and whether intrathecal injection of ozone could alleviate pain through the GluR6-NF- κ B/p65 signalling pathway in the spinal cord of CCI rats.

Materials and methods

Animals. A total of 108 adult male Sprague-Dawley rats (weight, 200-250 g; age, 8-10 weeks) from Experimental Animal Centre, Shandong University, China) were used for the present study. The rats were housed 4-5 per cage on a 12-h light/dark cycle with *ad libitum* access to food and water and kept under controlled environmental conditions (temperature, 23-25°C; humidity, 60-70%). The rats were acclimated to the circumstances for at least 5 days before the experiments. The experimental surgeries were accomplished under anesthesia with sodium pentobarbital (40 mg/kg, i.p.). All animal studies were approved by the Ethics Committee of Clinical Medical College at Shandong University (China). The experimental procedures were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (27), and efforts were made to minimize the pain and discomfort of rats.

CCI of the sciatic nerve model. Neuropathic pain was induced by CCI of the sciatic nerve, as described previously (28). In brief, rats were anaesthetized with sodium pentobarbital (40 mg/kg, i.p.), then the left sciatic nerve was bluntly dissected at mid-thigh level proximal to the sciatic trifurcation without stretching the nerve structures and the connective tissue was freed. Later, the sciatic nerve was ligated loosely (4-0 chromic gut) with four ties (1 mm interval). After slight shrinkage of the left posterior limb was observed, the muscles and skin were sutured. Rats in the sham group were also subjected to identical surgery but without nerve ligation.

Intrathecal (i.t.) implantation of catheter. Rats were implanted with catheters intrathecally for administration of drugs. In brief, rats were first anaesthetized with sodium pentobarbital (40 mg/kg, i.p.). After blunt separation of the occipital muscles, the cisternal membrane was exposed. A polyethylene catheter (PE-10, 7.0-8.0 cm in length) was inserted into the subarachnoid space through an incision in

the cisterna magna, and the cannula was advanced 7.0-7.5 cm caudally to the level of lumbar enlargement in the spinal cord. After rats recovered from anesthesia, 10 μ l lidocaine was injected through the catheter to confirm the correct catheterization site at the end of the experiment. The catheter was verified as being correctly implanted if paralysis and dragging of bilateral hind limbs appeared within 30 sec after the injection. Rats with obvious motor impairments were excluded from the experiment (29,30). After recovering from the surgery for 5-7 day, the rats were used for the following experiments.

GluR6 siRNAs and ozone injection. The GluR6 siRNAs (0.066 nmol dissolved in 10 μ l diluent; cat. no. sc-270102; Santa Cruz Biotechnology, Inc.) were injected intrathecally using a microinjection syringe once every day following CCI, over a period of 20 sec and followed by a flush with 5 μ l normal saline. The same dosages of negative GluR6 siRNAs and vehicle were used as controls (30). Ozone at different concentrations (10, 20, 30 μ g/ml; 20 μ l), generated by a medical ozone generator (cat. no. CHY-31; Yuehua Co.), was injected intrathecally over 20 sec on day 7 after CCI through polyethylene catheters. The same volume of oxygen was used as a control (31).

Assessment of neuropathic pain behaviours. Mechanical allodynia was measured by the Von Frey filaments test. In brief, after habituation for 30 min, the plantar surface of each left hind paw of the rat was stimulated with a sharp, cylindrical filament provided by an Electro Von Frey (American Semmes-Weinstein Monofilaments Inc.), and the incidence of foot withdrawal in response to mechanical stimulation was recorded. The test was performed according to previously reported procedures (32). The threshold of mechanical withdrawal for each rat was obtained to assess mechanical allodynia.

Thermal hyperalgesia was measured by the hot plate test. In brief, rats were placed on a smooth glass floor of a plastic cage. After habituation for 30 min, the plantar surface of each left hind paw of the rat was stimulated with a heat source provided by a Plantar Test Apparatus (Series 8 Model 390; IITC Life Science Inc.) and the heat source was shut off when hind paw movement occurred, or after 25 sec, to prevent tissue damage. The intensity of the heat source (60A) was modulated to maintain the paw withdrawal latency at 13 ± 2 sec in normal rats. The thermal stimuli were carried out 5 times to the same hind paw at 7-min intervals, and the averaged seconds were obtained as the thermal paw withdrawal latency to assess thermal hyperalgesia (20).

Sample preparation. The rats of each group were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and decapitated immediately after pain behaviour assessment. The spinal cord segments (L3-6) were carefully isolated from each animal and snap frozen in liquid nitrogen. The isolated spinal cord segments were homogenized with ice-cold homogenization buffer that contained 50 mM 3-(N-morpholino) propanesulfonic acid (Sigma-Aldrich; Merck KGaA; pH 7.4), 100 mM KCl, 1 mM Na_3VO_4 (Sigma-Aldrich; Merck KGaA), 20 mM sodium pyrophosphate, 1 mM EDTA, 1 mM p-nitrophenyl

phosphate, 0.5 mM $MgCl_2$, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 0.2 mM dithiothreitol, 320 mM sucrose, 1 mM EGTA, 50 mM NaF, 20 mM β -phosphoglycerol, and 5 μ g/ml pepstatin A, leupeptin and aprotinin. The homogenates were then centrifuged at 4°C for 10 min at 800 x g. The supernatants (the cytosol portion) were collected, and the protein concentrations were confirmed with Lowry's method (33). After that, the samples were stored at -80°C and thawed only before use.

Reverse transcription-quantitative (RT-q)PCR. RT-qPCR was used to measure GluR6 and NF- κ B/p65 mRNAs. β -actin served as the internal control. The sequences of the PCR primers were as follows: GluR6, forward, 5'-TTCCTGAATCCTCTCTCCCCT-3' and reverse, 5'-ACCTCGCAATCACAAACAGTACA-3'; NF- κ B/p65, forward, 5'-AGAGCAACGATTCCACCAA-3' and reverse, 5'-GCAGTCTTTTCCCACAGC-3'; β -actin, forward, 5'-TACAACCTCCTTGACAGCTCC-3' and reverse, 5'-GGATCTTCATGAGGTAGTCAGTC-3'.

First, the total RNAs were separated from homogenized samples using TRIzol® reagent (Thermo Fisher Scientific, Inc.). Then, the total RNAs were inversely transcribed into the cDNAs using the MML-V reverse transcriptase kit (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Then, the RT-qPCR master mix kit (Fermentas; Thermo Fisher Scientific, Inc.) was used to detect GluR6 and NF- κ B/p65 mRNAs with the PCR system (FTC2000q PCR System; Conrem). The following thermocycling conditions were used for the qPCR: Initial denaturation for 15 min at 95°C, followed by 35 cycles of denaturation at 95°C for 15 sec, annealing at 55°C for 30 sec and elongation at 72°C for 30 sec. Finally, GluR6 and NF- κ B/p65 mRNA levels were quantified with the relative quantification $2^{-\Delta\Delta C_q}$ method (34).

Western blotting. Total protein was extracted from tissues on ice using RIPA lysis buffer (Beyotime Institute of Biotechnology) supplemented with protease and phosphatase inhibitors. Total protein was quantified using a BCA protein assay kit, and 80 μ g protein per lane was separated by 12% SDS-PAGE and electrotransferred onto a PVDF membrane (Amersham; Cytiva). After blocking in TBS-0.05% Tween-20 (TBST) for 1.5 h at room temperature and 3% BSA (Beijing Solarbio Science & Technology Co., Ltd.), the membranes were incubated with the following primary antibodies in TBST containing 3% BSA overnight at 4°C: Anti-GluR6 (1:1,000; cat. no. EPR6307; Abcam), anti-NF- κ B (1:1,000; cat. no. ab16502; Abcam) and anti- β -actin (1:5,000; cat. no. ab8227; Abcam). Following the primary antibody incubation, the membranes were washed and incubated a goat anti-rabbit IgG secondary antibody (1:20,000; cat. no. ab97051; Abcam) for 2 h in TBST. After being visualized using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate color substrate (Promega Corporation), the bands on the membranes were scanned and analysed with an image analyzer and LabWorks software (version 4.5, UVP). β -actin served as the internal control.

ELISA. ELISA was used to detect the protein levels of TNF- α , IL-1 β , and IL-6 in the spinal cord. Briefly, the spinal

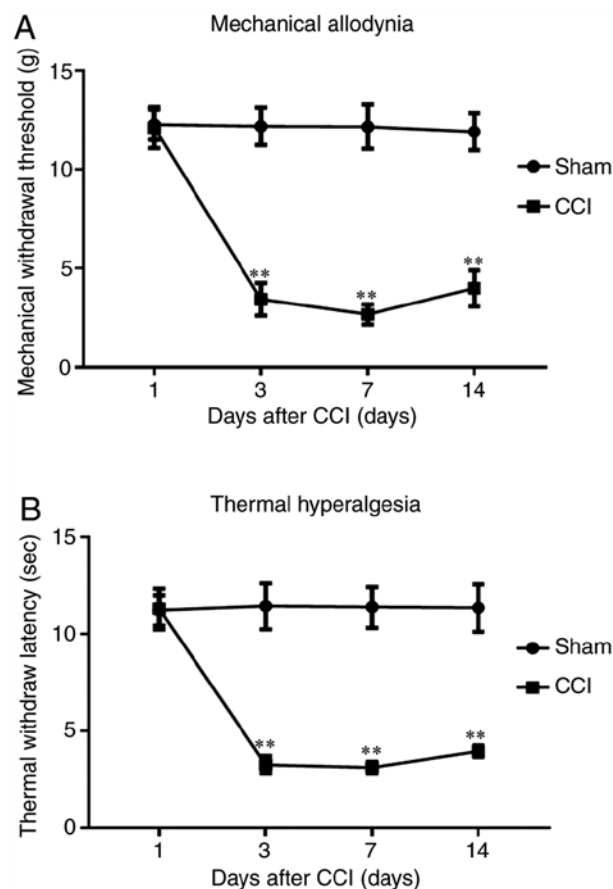


Figure 1. Alterations in pain scores in rats at different time points following CCI. Rats treated with CCI demonstrated obvious mechanical allodynia and thermal hyperalgesia behaviours as compared with the sham group. (A) The alterations in the pain scores of mechanical allodynia in rats following CCI. (B) Alterations in the pain scores of thermal hyperalgesia in rats following CCI. Pain scores were measured on days 1, 3, 7 and 14 following CCI. '1' is the time point 1 day after CCI. Data are presented as the mean \pm standard deviation. ** $P < 0.01$ vs. sham group ($n = 6$ in each group). CCI, chronic constriction injury.

cord tissues were pooled and homogenized in ice-cold PBS solution (pH 7.4, containing 1 mM phenylmethylsulfonyl fluoride, 1% Triton-X100, 1 g/ml leupeptin, and 10 g/ml aprotinin). Following centrifugation at 10,000 x g at 4°C for 30 min, the supernatants were aliquoted and stored at -80°C for future protein quantification. Cytokine protein levels were analysed by rat TNF- α (cat. no. SRTA00), IL-1 β (cat. no. SRLB00) and IL-6 (cat. no. SR6000B) ELISA kits (R&D Systems, Inc.) according to the manufacturer's protocols.

Statistical analyses. Data are expressed as the mean \pm standard deviation. SPSS 23.0 (IBM Corp.) was used to perform the statistical analyses in the present study. Comparisons were performed by one-way analysis of variance followed by Tukey's test. $P < 0.05$ was considered to indicate a statistically significant difference. For Fig. 1, in combination with data characteristics, the indicators of pain behavior scores, time, and groups were standardized. The results demonstrated that the pain behavior scores of CCI group were statistically significant compared with sham group at d3, d7 and d14 time points ($P < 0.01$).

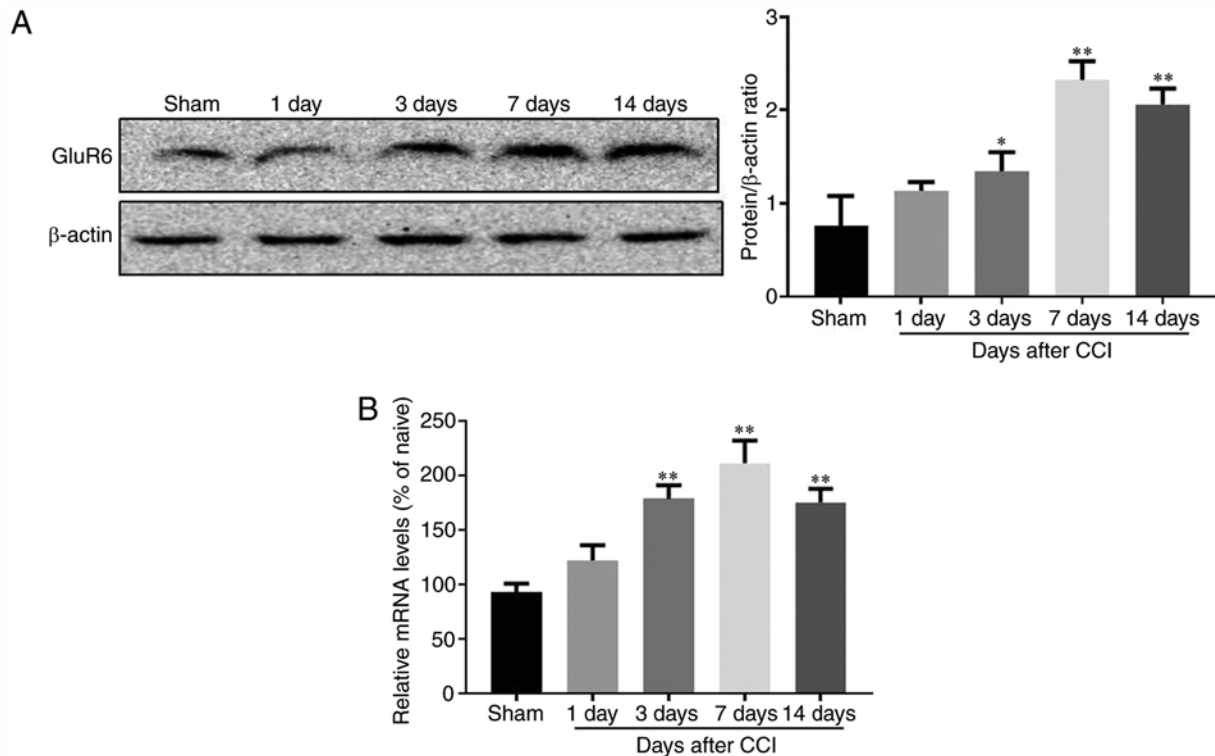


Figure 2. Alterations in spinal GluR6 levels in rats at different time points following CCI. The expression of GluR6 began to increase on day 3 and reached its peak on day 7 after CCI as compared with the sham group. (A) Alterations in spinal GluR6 protein levels in rats following CCI. (B) Alterations in spinal GluR6 mRNA levels in rats following CCI. GluR6 expression was measured on days 1, 3, 7 and 14 following CCI. '1d' is the time point 1 day after CCI. Naïve means sham group data. Data are presented as the mean \pm standard deviation. * $P < 0.05$ and ** $P < 0.01$ vs. sham group ($n = 6$ in each group). GluR6, glutamate receptor 6; CCI, chronic constriction injury.

Results

Changes in pain behaviours and GluR6 expression in the spinal cord of rats following CCI at different time points. In the present study, rats treated with CCI of the sciatic nerve demonstrated obvious mechanical allodynia and thermal hyperalgesia behaviours, and the pain behaviours were mostly obvious on day 7 after CCI, which was in contrast to the sham group (Fig. 1). To survey the change in spinal GluR6 expression following CCI, the spinal cords of rats at different time points were obtained and homogenized. GluR6 protein expression was then measured by immunoblotting and GluR6 mRNA was measured by RT-qPCR analysis. As shown in Fig. 2, the expression of GluR6 began to increase on day 3 and reached its peak on day 7 after CCI.

Pre-intrathecal injection of GluR6 siRNAs attenuates CCI-induced pain behaviours and reduces spinal GluR6 expression in rats on day 7 after CCI. To further ascertain the role of GluR6 in CCI-induced neuropathic pain, we used GluR6 siRNAs to knock down GluR6 expression through intrathecal injection once per day following CCI. The alteration of GluR6 protein expression was detected by immunoblotting, and GluR6 mRNA was measured by RT-qPCR analysis. As shown in Figs. 3 and 4, pre-intrathecal injection of GluR6 siRNAs significantly inhibited mechanical allodynia and thermal hyperalgesia behaviours and decreased the expression of spinal GluR6 in rats, which was in contrast to the CCI

rats without pretreatment with GluR6 siRNAs. Meanwhile, pre-intrathecal injection of negative GluR6 siRNAs or vehicle had no effect on the changes in pain behaviours or GluR6 expression in rats.

Pre-intrathecal injection of GluR6 siRNAs reduces spinal NF- κ B/p65 expression in rats on day 7 after CCI. The change in NF- κ B/p65 protein expression in the spinal cord of rats was measured by immunoblotting, and NF- κ B/p65 mRNA was measured by RT-qPCR analysis. As shown in Fig. 5, the expression of NF- κ B/p65 protein and mRNA decreased significantly in the CCI group with pretreatment with GluR6 siRNAs, which was in contrast to the CCI group without pretreatment with GluR6 siRNAs.

Pre-intrathecal injection of GluR6 siRNAs reduces spinal IL-1 β , IL-6 and TNF- α protein expression in rats on day 7 after CCI. The changes in spinal IL-1 β , IL-6 and TNF- α protein expression in rats were measured by ELISA. As shown in Fig. 5, the expression of IL-1 β , IL-6 and TNF- α protein decreased significantly in the CCI group with pretreatment of GluR6 siRNAs, which was in contrast to the CCI group without pretreatment with GluR6 siRNAs.

Intrathecal injection of ozone attenuates CCI-induced neuropathic pain behaviours in rats. In the present study, ozone at different concentrations was administered intrathecally on day 7 after CCI in rats. Mechanical allodynia and thermal hyperalgesia of rats were measured. As shown

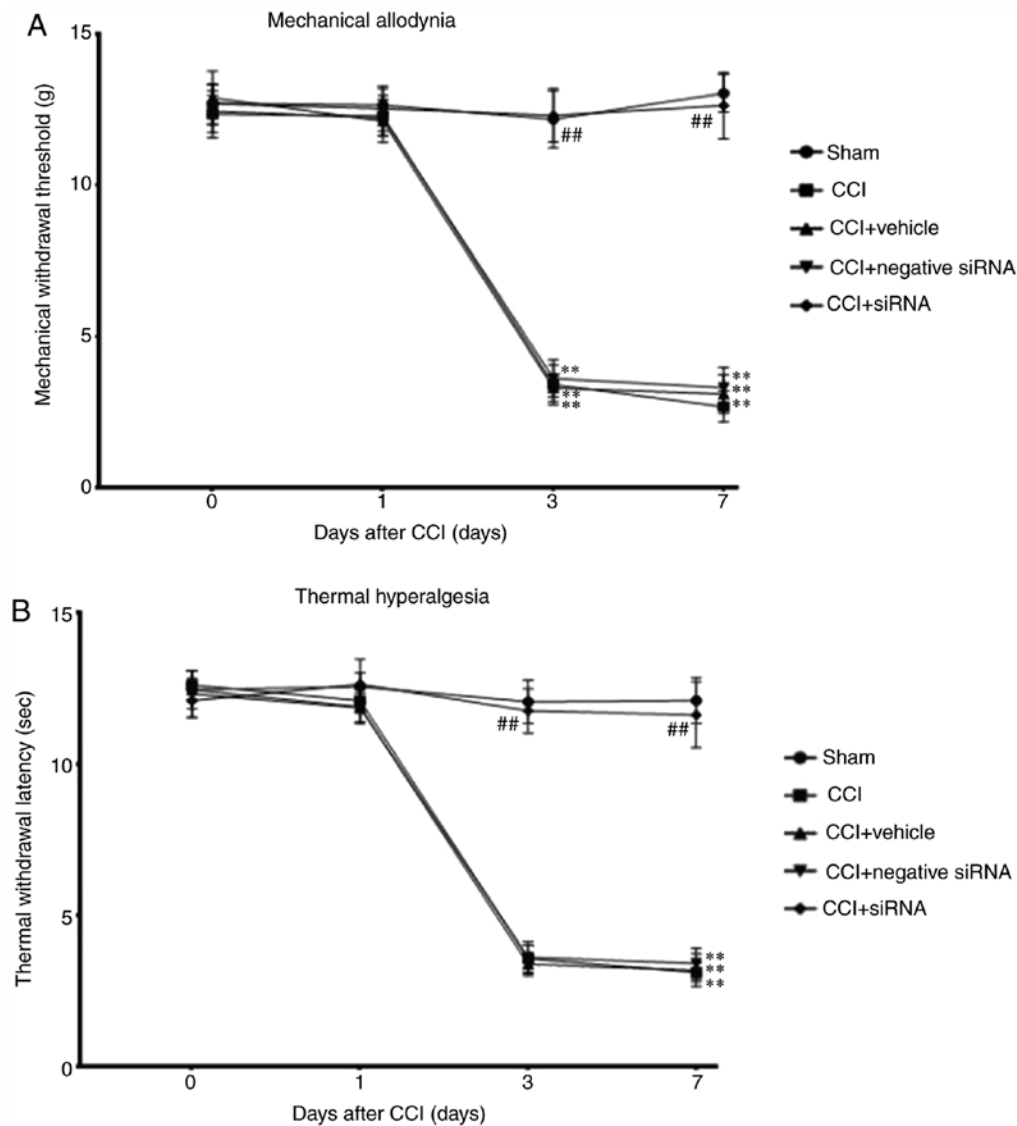


Figure 3. Pre-intrathecal injection of GluR6 siRNAs reduces pain scores in rats on day 7 after CCI. (A) The alterations in the pain scores of mechanical allodynia in rats following CCI in the indicated groups. (B) The alterations in the pain scores of thermal hyperalgesia in rats following CCI in the indicated groups. Data are presented as the mean \pm standard deviation. ** $P < 0.01$ vs. sham group; ## $P < 0.01$ vs. CCI group (n=6 in each group). GluR6, glutamate receptor 6; CCI, chronic constriction injury; si, short interfering.

in Fig. 6, intrathecal injection of ozone clearly attenuated mechanical allodynia and thermal hyperalgesia induced by CCI in rats, in contrast to the CCI group with intrathecal injection of oxygen, and ozone at 20 $\mu\text{g/ml}$ had the most evident effect.

Intrathecal injection of ozone decreases GluR6, NF- $\kappa\text{B/p65}$, IL-1 β , IL-6 and TNF- α expression in the spinal cord of rats. To further investigate the mechanisms underlying the pain-relieving effect of ozone, spinal GluR6 and NF- $\kappa\text{B/p65}$ expression levels were measured by immunoblotting and RT-qPCR assays. Spinal IL-1 β , IL-6 and TNF- α protein expression was measured by ELISA. As shown in Fig. 7, intrathecal injection of ozone clearly decreased GluR6, NF- $\kappa\text{B/p65}$, IL-1 β , IL-6 and TNF- α expression in the spinal cord of rats, which was in contrast to the CCI group with intrathecal injection of oxygen, and ozone at 20 $\mu\text{g/ml}$ had the most evident effect.

Discussion

The present study demonstrated for the first time, to the best of the authors' knowledge, that GluR6 participated in the neuropathic pain induced by CCI and that intrathecal injection of ozone suppressed the GluR6-NF- $\kappa\text{B/p65}$ pathway to alleviate neuropathic pain in the spinal cord of CCI rats. In recent years, ozone has been widely used in clinical practice and is suggested to be effective in treating a number of chronic pain diseases (35,36). Nevertheless, in contrast to the effective use of ozone in clinical practice, it is necessary to pay close attention to its side effects due to its powerful oxidizing capacity (37). It has been reported in our previous study that ozone at 60 $\mu\text{g/ml}$ can damage astrocytes *in vitro*, while ozone of 20 $\mu\text{g/ml}$ or 40 $\mu\text{g/ml}$ has no damaging effect (38). In addition, ozone at a high-concentration (>40 $\mu\text{g/ml}$) can induce neurotoxicity in spinal cord neurons due to ER Ca^{2+} release and CaMKII/MAPK signalling pathway activation, while

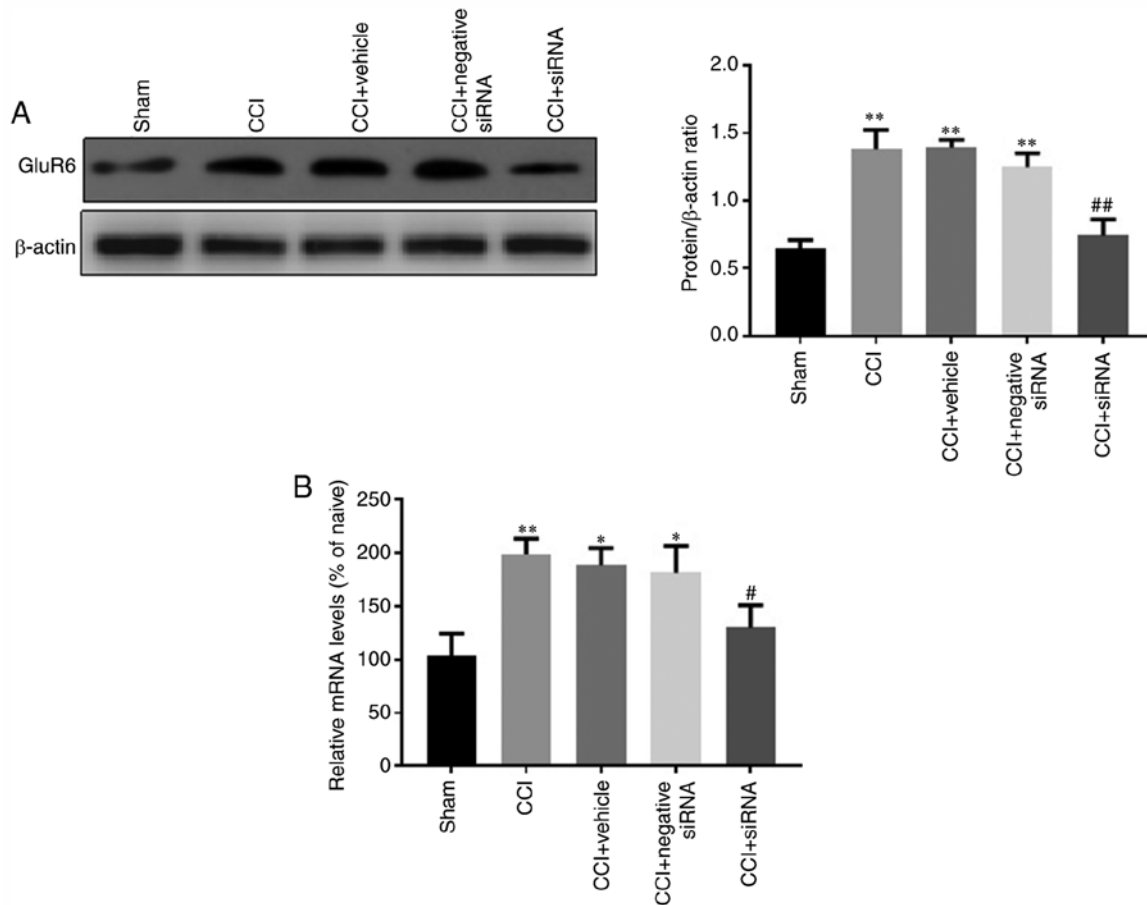


Figure 4. Pre-intrathecal injection of GluR6 siRNAs reduced spinal GluR6 levels in rats on day 7 after CCI. (A) The expression of spinal GluR6 protein in rats on day 7 after CCI in the indicated groups. (B) The expression of spinal GluR6 mRNA in rats on day 7 after CCI in the indicated groups. Data are presented as the mean \pm standard deviation. * P <0.05 and ** P <0.01 vs. sham group; # P <0.05 and ## P <0.01 vs. CCI group (n =6 in each group). GluR6, glutamate receptor 6; si, short interfering; CCI, chronic constriction injury.

ozone at low concentration (<40 μ g/ml) exhibits no neurotoxic effect (39). Furthermore, ozone can reduce apoptosis of nerve roots by blocking NF- κ B signalling pathway in a radiculoneuritis rat model (40). Wei *et al* (41) found that ozone can inhibit the necrosis of the endometrial epithelial cells and reduce expression of inflammatory factors, including IL-6 and TNF- α , in rats with pelvic inflammatory disease. Re *et al* (42) reported that the analgesic effect of ozone may involve two different mechanisms; a short-term mechanism that may be associated with the direct oxidation of biomolecules, and a long-term mechanism that may involve the activation of antioxidant pathways. Although a number of studies have been performed (38-42), the exact biological mechanisms governing the efficacy of ozone remain to be elucidated.

In the present study, it was observed that rats with CCI demonstrated obvious pain behaviours, which started on day 3, reached their peak on day 7 and could persist for ≥ 14 days after CCI. Meanwhile, intrathecal injection of low-concentration ozone could alleviate CCI-induced neuropathic pain on day 7 after CCI and ozone at 20 μ g/ml had the most evident effect.

As one subunit of kainate receptors, GluR6 serves a crucial role in neuronal cell death induced by cerebral ischaemia/reperfusion, as demonstrated in previous studies (33,43-45). It has also been shown that GluR6 is expressed in the superficial dorsal horn in adult rats and

participates in nociceptive transmission (23,46). However, thus far, the role of GluR6 in neuropathic pain has not been studied. Therefore, the present study investigated whether GluR6 participated in the neuropathic pain induced by CCI. It was also investigated whether intrathecal injection of ozone could alleviate pain through the GluR6 pathway in the spinal cord of CCI rats. In the present study, pain behaviours and GluR6 expression were examined. It was demonstrated that the expression of GluR6 in the spinal cord of rats increased on day 3 and reached a peak on day 7 after CCI, which was consistent with the alteration of pain behaviours. As the pain behaviours of rats and the expression of GluR6 were significantly changed on day 7 after CCI, the time point of day 7 was selected to investigate the role of GluR6 in neuropathic pain. As demonstrated in the present study, pre-intrathecal injection of GluR6 siRNAs not only markedly inhibited the expression of GluR6 but also clearly alleviated the pain behaviours induced by CCI, which demonstrated that GluR6 might serve a crucial role in the neuropathic pain induced by CCI.

The NF- κ B/p65 heterodimer is a crucial transcription factor in the central nervous system, which has been demonstrated to be associated with the initiation and maintenance of inflammation and neuropathic pain (26,31,47,48). NF- κ B/p65 is expressed in spinal dorsal horn neurons and

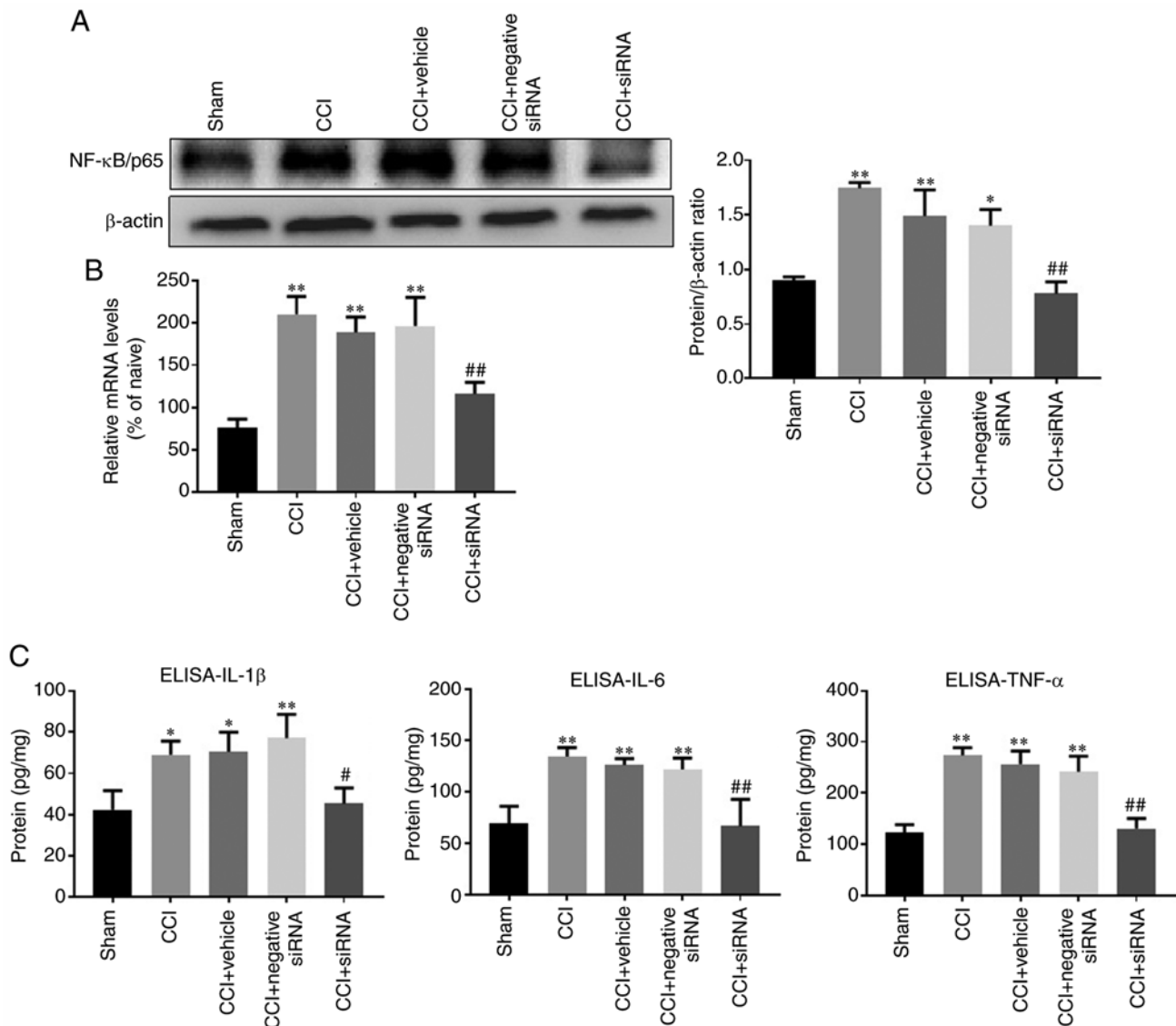


Figure 5. Pre-intrathecal injection of GluR6 siRNAs reduced spinal NF-κB/p65, IL-1β, IL-6 and TNF-α expression levels in rats on day 7 after CCI. (A) The expression of spinal NF-κB/p65 protein in rats on day 7 after CCI in the indicated groups. (B) The expression of spinal NF-κB/p65 mRNA in rats on day 7 after CCI in the indicated groups. (C) The expression of spinal IL-1β, IL-6 and TNF-α proteins in rats on day 7 after CCI in the indicated groups. Data are presented as the mean ± standard deviation. *P<0.05 and **P<0.01 vs. sham group; #P<0.05 and ##P<0.01 vs. CCI group (n=6 in each group). GluR6, glutamate receptor 6; si, short interfering; CCI, chronic constriction injury.

has been revealed to be activated by peripheral nerve injury or inflammation (1,25,26,30,31). NF-κB/p65 can interact with pro-inflammatory factors (IL-1β, IL-6 and TNF-α), which may amplify the neuroinflammatory responses and give rise to central sensitization and hyperalgesia (25). A previous study by our group demonstrated that suppression of spinal NF-κB/p65 expression could clearly relieve mechanical allodynia and thermal hyperalgesia in CCI rats (30). Furthermore, spinal NF-κB/p65 activation in CCI rats has been reported to be inhibited by MK-801, an NMDA receptor antagonist, which indicates that the NMDA receptor may activate the NF-κB/p65 pathway (49). The present study investigated whether GluR6 could activate the NF-κB/p65 pathway. In accordance with the results above, the current study demonstrated that peripheral nerve constriction injury could significantly activate the expression of spinal NF-κB/p65, IL-1β, IL-6 and TNF-α in CCI rats, and the

alterations could be inhibited predominantly by knockdown of GluR6 with intrathecal injection of GluR6 siRNAs, which indicated that GluR6 could activate the spinal NF-κB/p65 pathway in rats with CCI.

Our previous study demonstrated that intrathecal injection of low-concentration ozone could attenuate radiculitis in rats with non-compressive lumbar disc herniation, probably through the PDE2A-cGMP/cAMP-NF-κB/p65 pathway (31). The present study further investigated whether intrathecal injection of low-concentration ozone could alleviate neuropathic pain induced by CCI and mechanism. It has been shown that oxygen has no analgesic effect on the neuropathic pain induced by CCI (50), and in our preliminary experiment, it was found that there was no statistical significance in pain behaviors between CCI group and CCI + oxygen group (data not shown), so the CCI + oxygen group was selected as the control group. As shown in the present study,

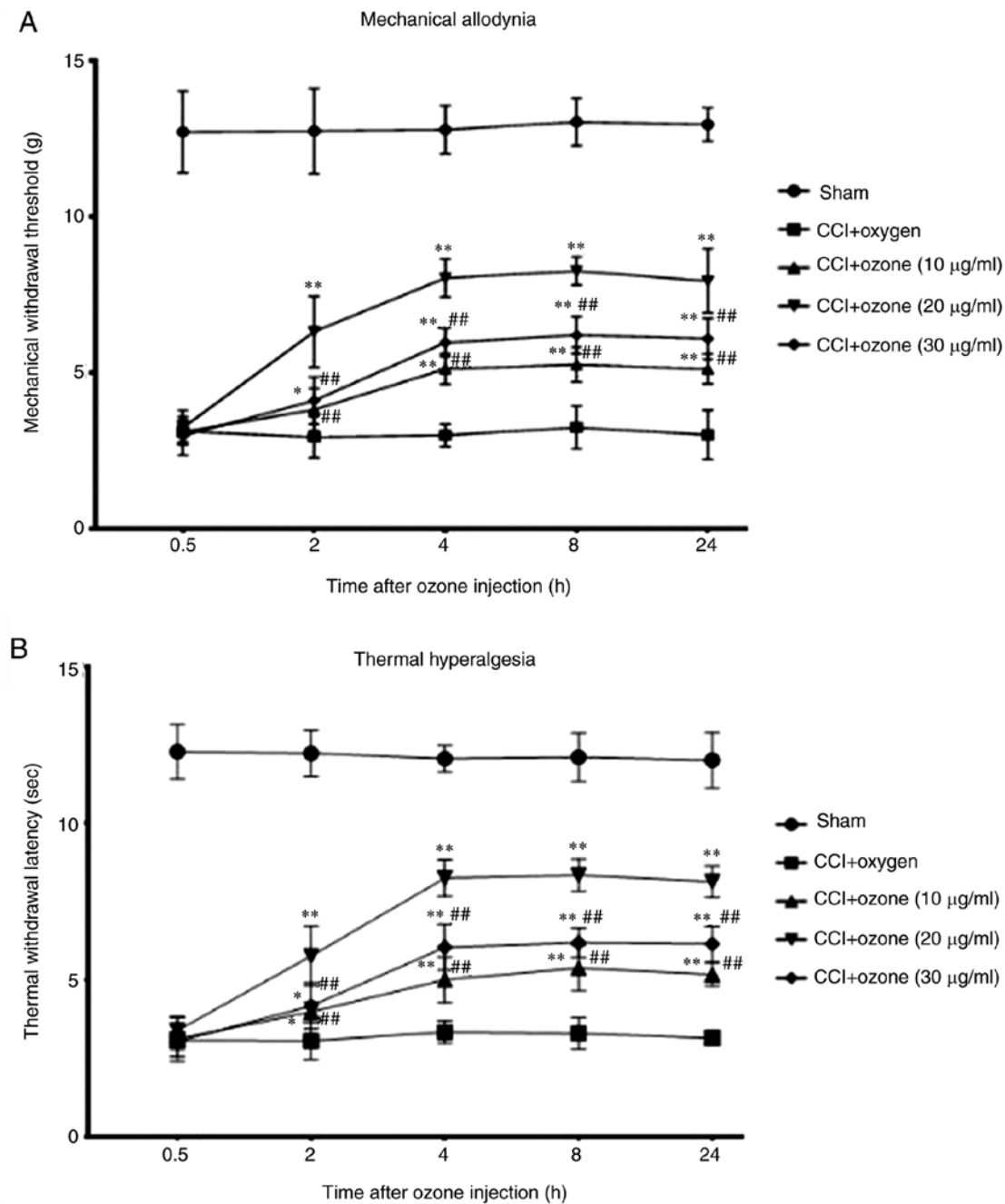


Figure 6. Intrathecal injection of ozone attenuated CCI-induced neuropathic pain behaviours in rats on day 7 after CCI. (A) The alterations of pain scores of mechanical allodynia in rats at different time points after ozone injection in the indicated groups. (B) The alterations of pain scores of thermal hyperalgesia in rats at different time points after ozone injection in the indicated groups. * $P < 0.05$ and ** $P < 0.01$ vs. CCI + oxygen group; ## $P < 0.01$ vs. CCI + ozone (20 μ g/ml) group ($n = 6$ in each group). CCI, chronic constriction injury.

intrathecal treatment with low-concentration ozone could alleviate mechanical allodynia and thermal hyperalgesia and downregulate the expression of GluR6, IL-1 β , IL-6, TNF- α and NF- κ B/p65 in the spinal cord of CCI rats. In addition, ozone at 20 μ g/ml had the most evident effect. Therefore, it was hypothesized that the analgesic effects of ozone might be mediated, at least in part, through the GluR6-NF- κ B/p65 pathway. However, further research is warranted to determine whether there is an association between GluR6 and PDE2A or cGMP/cAMP.

In conclusion, the present study demonstrated that GluR6 served a crucial role in the neuropathic pain induced

by CCI, which might serve as a potential new analgesic target in the development of new therapies for neuropathic pain. Furthermore, intrathecal injection of low-concentration ozone could attenuate neuropathic pain and might downregulate the GluR6-NF- κ B/p65 pathway in CCI rats.

The present study had a number of limitations. The lack of immunohistochemistry, the sample size for behavioral assessment and the lack of determination of direct evidence that GluR6 upregulation is associated with alleviation of neuropathic pain induced by ozone are limitations of the study, which will be addressed in the future.

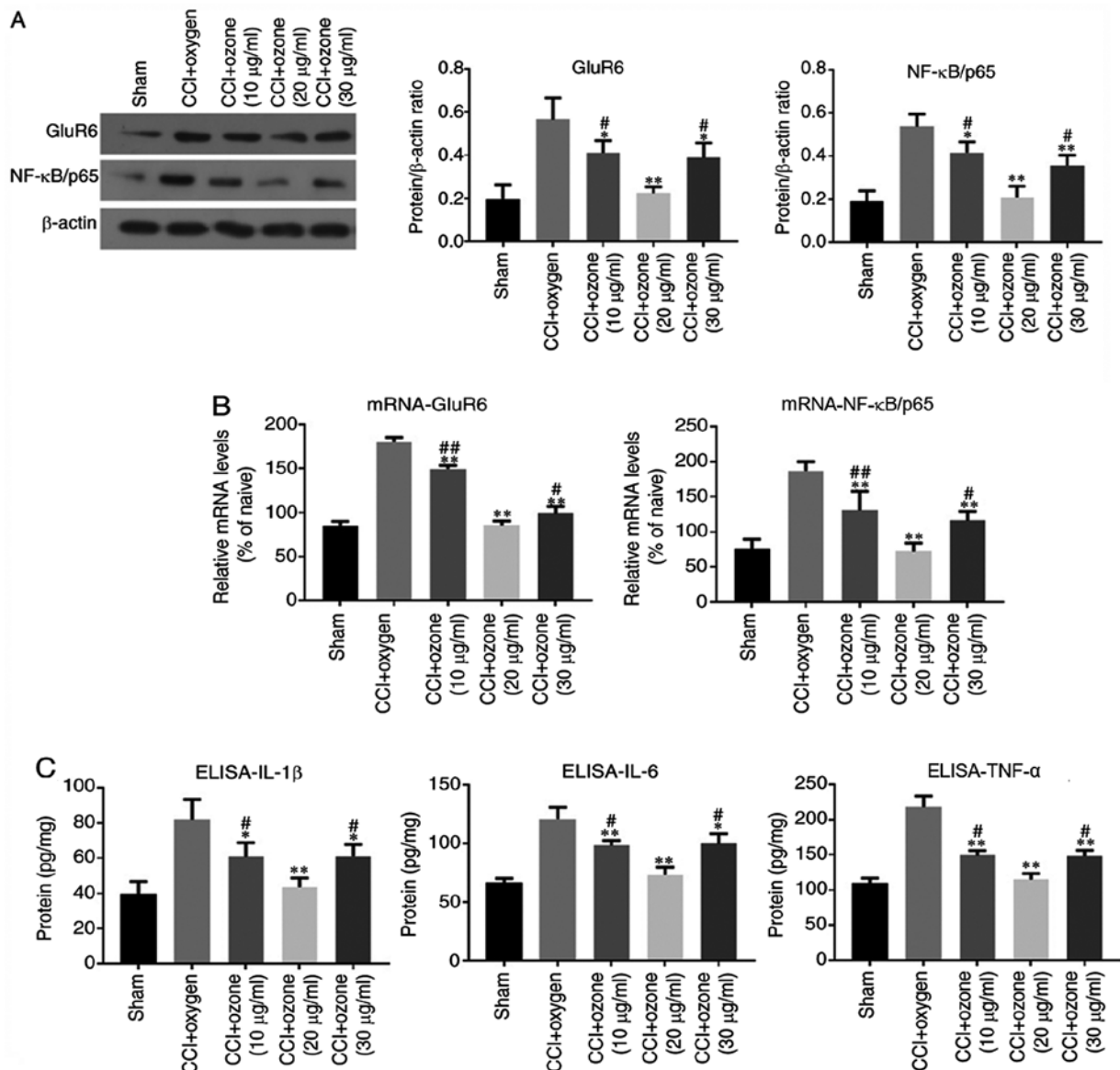


Figure 7. Intrathecal injection of ozone decreased spinal GluR6, NF-κB/p65, IL-1β, IL-6 and TNF-α expression levels in rats on day 7 after CCI. (A) The expression of spinal GluR6 and NF-κB/p65 protein in rats at 24 h after ozone intrathecal injection in the indicated groups. (B) The expression of spinal GluR6 and NF-κB/p65 mRNA in rats at 24 h after ozone intrathecal injection in the indicated groups. (C) The expression of spinal IL-1β, IL-6 and TNF-α protein in rats at 24 h after ozone intrathecal injection in the indicated groups. *P<0.05 and **P<0.01 vs. CCI + oxygen group; #P<0.05 and ##P<0.01 vs. CCI + ozone (20 μg/ml) group (n=6 in each group). GluR6, glutamate receptor 6; CCI, chronic constriction injury.

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Availability of data and materials

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

Authors' contributions

WZ, LZ, TS and ZF designed the study. WZ and FW performed the experiments and analyzed the data. WZ wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Shandong Provincial Hospital is affiliated to Shandong University. The experiments in the present study were

carried out in the laboratory at Shandong University. All animal procedures were approved by the Ethics Committee of Clinical Medical College at Shandong University and performed in compliance with the guidelines set by the Ethics Committee for the Care and Use of Laboratory Animals.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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