

Randomized Trial

Tanshinone IIA Attenuates Chronic Pancreatitis-Induced Pain in Rats via Downregulation of HMGB1 and TLR4 Expression in the Spinal Cord

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Background: Chronic pancreatitis (CP) is a long-standing inflammation of the exocrine pancreas, which typically results in severe and constant abdominal pain. Previous studies on the mechanisms underlying CP-induced pain have primarily focused on the peripheral nociceptive system. A role for a central mechanism in the mediation or modulation of abdominal pain is largely unknown. Tanshinone IIA (TSN IIA), an active component of the traditional Chinese medicine Danshen, exhibits anti-inflammatory properties via downregulation of the expression of high-mobility group protein B1 (HMGB1), a late proinflammatory cytokine. HMGB1 binds and activates toll-like receptor 4 (TLR4) to induce spinal astrocyte activation and proinflammatory cytokine release in neuropathic pain.

Objective: In this study, we investigated the effect of TSN IIA on pain responses in rats with trinitrobenzene sulfonic acid (TNBS)-induced CP. The roles of central mechanisms in the mediation or modulation of CP were also investigated.

Study Design: A randomized, double-blind, placebo-controlled animal trial.

Methods: CP was induced in rats by intrapancreatic infusion of trinitrobenzene sulfonic acid (TNBS). Pancreatic histopathological changes were characterized with semi-quantitative scores. The abdomen nociceptive behaviors were assessed with von Frey filaments. The effects of intraperitoneally administered TSN IIA on CP-induced mechanical allodynia were tested. The spinal protein expression of HMGB1 was determined by western blot. The spinal mRNA and protein expression of proinflammatory cytokines IL-1 β , TNF- α , and IL-6 were determined by RT-PCR and western blot, respectively. The spinal expression of the HMGB1 receptor TLR4 and the astrocyte activation marker glial fibrillary acidic protein (GFAP) were determined by western blot or immunohistological staining after intraperitoneal injection of TSN IIA or intrathecal administration of a neutralizing anti-HMGB1 antibody.

Results: TNBS infusion resulted in pancreatic histopathological changes of chronic pancreatitis and mechanical allodynia in rats. TSN IIA significantly attenuated TNBS-induced mechanical allodynia in a dose-dependent manner. TNBS significantly increased the spinal expression of HMGB1 and proinflammatory cytokines IL-1 β , TNF- α , and IL-6. These TNBS-induced changes were significantly inhibited by TSN IIA in a dose-dependent manner. Furthermore, TSN IIA, but not the neutralizing anti-HMGB1 antibody, significantly inhibited TNBS-induced spinal TLR4 and GFAP expression.

Limitations: In addition to TLR4, HMGB1 can also bind to toll-like receptor-2 (TLR2) and the receptor for advanced glycation end products (RAGE). Additional studies are warranted to ascertain whether HMGB1 contributes to CP-induced pain through activation of these receptors.

Conclusions: Our results suggest that spinal HMGB1 contributes to the development of CP-induced pain and can potentially be a therapeutic target. TSN IIA attenuates CP-induced pain via downregulation of spinal HMGB1 and TLR4 expression. Therefore, TSN IIA may be a potential anti-nociceptive drug for the treatment of CP-induced pain.

Key words: Chronic pancreatitis, HMGB1, proinflammatory cytokine, Tanshinone IIA, spinal cord, astrocyte, TLR4

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Chronic pancreatitis (CP) is a long-standing inflammation of the pancreas, which leads to progressive pancreatic damage and fibrosis. Patients with CP usually present with persistent abdominal pain or steatorrhea resulting from malabsorption caused by pancreatic insufficiency (1). The most common and prominent clinical symptom of CP is recurrent upper abdominal pain, which is usually intense and long-lasting. Currently, studies on the mechanisms underlying CP-induced pain have primarily focused on peripheral effects of chronic pancreatitis, including increased pressure in pancreatic tissue, ischemia, fibrosis, pseudocyst formation, inflammation, and pancreatic nerve damages (2-6). However, recent studies have demonstrated that the nociceptive processing in the central nervous system (CNS) contributes to CP-induced pain (7-9). Specifically, activation of the spinal cord microglia and astrocytes was detected in CP-induced pain, similar to that seen in neuropathic pain induced by peripheral nerve damages (8,10). Spinal astrocytes, once activated in neuropathic pain, express the protein marker glial fibrillary acidic protein (GFAP) and release inflammatory cytokines, including interleukin beta (IL-1 β) and tumor necrosis factor alpha (TNF- α), promoting inflammation and pain (11,12).

High-mobility group protein B1 (HMGB1) is an important chromatin protein widely expressed in most eukaryotic cells. In the nucleus, HMGB1 interacts with nucleosomes, transcription factors, and histones, regulating target gene transcription. In addition, HMGB1 can be secreted as a cytokine mediator of inflammation by binding to toll-like receptors (TLRs) TLR2 and TLR4 and RAGE (receptor for advanced glycan end products). Interaction of HMGB1 and TLR4 results in upregulation of NF- κ B, which leads to increased production and release of inflammatory cytokines IL-1 β and TNF- α (13-15). HMGB1 is an important inflammatory mediator in intracerebral hemorrhage (16), spinal cord ischemic injury (17), and cancer-induced bone pain (18). However, the precise role of spinal HMGB1 in the mediation or modulation of CP-induced pain remains unclear.

Since HMGB1 is a late mediator of inflammation, targeting HMGB1 may provide a relatively wide therapeutic window for HMGB1-driven pathogenesis (15,19). Significant attempts have been made to exploit the potential analgesic effects of HMGB1 antagonists; however, studies have found that anti-inflammatory steroids (e.g., dexamethasone) and nonsteroidal anti-inflammatory drugs (NSAIDs) (e.g., aspirin and ibupro-

fen) fail to inhibit endotoxin-induced release of HMGB1 (20).

Tanshinone IIA (TSN IIA) is a steroid-like pigment derived from the Chinese herb Danshen (*Salvia miltiorrhiza*), a cardiovascular medicine widely used throughout China (21,22). Previous studies have demonstrated that TSN IIA inhibits HMGB1 release in cell cultures *in vitro* (20,23,24) and attenuates ischemia-induced HMGB1 expression in rat brains *in vivo* (25). However, whether TSN IIA affects spinal HMGB1 expression in rats with CP is largely unknown.

TLR4 is a potent HMGB1 receptor. In the CNS, TLR4 is expressed mostly on the surface of astrocytes (26,27), with lower expression levels found in neurons and microglia (28). Previously, Liu et al (26) showed that activation of TLR4 is required for the activation of spinal astrocytes and microglia and the release of cytokines which promote chronic pain, suggesting that the HMGB1-induced TLR4 activation in spinal astrocytes and microglia contributes to the development of neuropathic pain (29). However, the exact role of the spinal HMGB1-TLR4 pathway in CP-induced pain is unclear.

In this study, therefore, we investigated the role of spinal HMGB1 in CP-induced pain in a rat model. Additionally, the effects of TSN IIA on the spinal HMGB1-TLR4 pathway and CP-induced pain behaviors were examined. The results from this investigation may assist in the development of novel analgesics for CP-induced pain in the future.

METHODS

Animals

Adult male Sprague-Dawley rats (180 – 220 g) were obtained from Tianjin Medical University (Tianjin, P.R. China). Rats were housed in cages (3 in each cage) under a 12:12 hour light/dark cycle (lights on at 06:00 am) with free access to food and to water. All animal studies were approved by the Committee of Animal Care and Use for Research and Education at Tianjin Medical University (Tianjin, China).

Induction of Chronic Pancreatitis

Chronic pancreatitis was induced following previously described techniques (9,30). Briefly, the common bile duct was temporarily occluded near the liver with a small vascular clamp. A blunt 28-gauge needle connected to PE-10 tubing was inserted into the duodenum, guided through the papilla into the duct, and secured with suture. Phosphate buffered saline containing 2%

TNBS and 10% ethanol (0.5 mL, pH 7.4) was infused into the pancreatic duct over 2 – 5 minutes under 50 mmHg pressure. After 30 minutes, needle and tubing were removed. The opening in the duodenum was sutured and the vascular clamp was removed to restore the bile flow. Sham-operated animals were infused with the same volume of saline instead of TNBS.

Pancreas Histology

Rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and euthanized. The pancreas was extracted and fixed in 4% paraformaldehyde in phosphate buffered (PB, pH 7.4) at 4°C overnight. The pancreas was embedded in paraffin after progressive xylene washes. Sections (5- μ m) were cut from the paraffin blocks with a microtome, stained with hematoxylin and eosin (H&E), and examined by a pathologist blinded to the treatment conditions. The severity of CP was morphologically assessed by semi-quantitative scores according to previous studies: graded glandular atrophy (0 – 3); intralobular, interlobular, and periductal fibrosis (0 – 3); inflammatory cell infiltration (0 – 3) (31,32).

Animal Treatment

To study the time-dependent antinociceptive effects of TSN IIA (Shanghai number 1 Biochemical Pharmaceutical Company, China), 24 rats were randomly divided into 4 groups (N = 6 for each group) as follows: sham + vehicle, sham + TSN IIA (20 mg/kg), TNBS + vehicle, TNBS + TSN IIA (20 mg/kg). TSN IIA or vehicle alone was intraperitoneally injected from the fourth week to the fifth week after TNBS infusion. Mechanical allodynia was measured with a 40.7 mN von Frey filament before the induction of pancreatitis and once weekly for up to 5 weeks after the induction.

To study the effects of TSN IIA on force-dependent response, 24 rats were randomly divided into 4 groups (N = 6 for each group) as follows: sham + vehicle, sham + TSN IIA (20 mg/kg), TNBS + vehicle, and TNBS + TSN IIA (20 mg/kg). TSN IIA was intraperitoneally injected from weeks 4 to 5 after TNBS infusion. Mechanical allodynia was tested with von Frey filaments in increasing order of force (2.29, 2.75, 6.76, 16.6, 40.7, and 69.2 mN) one hour after TSN IIA injection.

To study the dose-dependent antinociceptive effects of TSN IIA, 18 rats were randomly divided into 3 groups (N = 6 for each group) as follows: TNBS + TSN IIA (10 mg/kg), TNBS + TSN IIA (20 mg/kg), and TNBS + TSN IIA (50 mg/kg). TSN IIA was intraperitoneally injected

from weeks 4 to 5 after TNBS infusion. Mechanical allodynia was tested one hour after TSN IIA injection.

To study the effects of TSN IIA on spinal HMGB1 expression and cytokine production, 42 rats were randomly divided into 7 groups (N = 6 for each group) as follows: naïve, sham + vehicle, sham + TSN IIA (50 mg/kg), TNBS + vehicle, TNBS + TSN IIA (10 mg/kg), TNBS + TSN IIA (20 mg/kg), and TNBS + TSN IIA (50 mg/kg). TSN IIA was intraperitoneally injected from weeks 4 to 5 after TNBS infusion. One hour after TSN IIA injection, the spinal HMGB1 protein expression was determined by western blot. The spinal TNF- α , IL-1 β , and IL-6 mRNA and protein expressions were examined by real time PCR (RT-PCR) and western blot, respectively.

To investigate the mechanisms underlying the antinociceptive effects of TSN IIA, 24 rats were randomly divided into 4 groups (N = 6 for each group) as follows: sham + saline, sham + TSN IIA (20 mg/kg), TNBS + saline, TNBS + TSN IIA (20 mg/kg). TSN IIA was intraperitoneally injected from weeks 4 to 5 after TNBS infusion. One hour after TSN IIA injection, the spinal TLR4 and GFAP protein expressions were examined by immunohistochemical staining and/or western blot.

To investigate the effects of neutralizing anti-HMGB1 antibody on spinal TLR4 expression and astrocyte activation, 24 rats were randomly divided into 4 groups (N = 6 for each group) as follows: sham + vehicle, sham + neutralizing anti-HMGB1 (50 μ g), TNBS + vehicle, and TNBS + neutralizing anti-HMGB1 (50 μ g). Neutralizing anti-HMGB1 was intrathecally administered from weeks 4 to 5 after TNBS infusion. One hour after anti-HMGB1 administration, the spinal TLR4 and GFAP expressions were examined by immunohistochemical staining and western blot.

Mechanical Allodynia

Mechanical allodynia in the rats was tested as in previously conducted experiments (30,33). Briefly, the belly skin of the rats was shaved, and the rats were placed in inverted plastic boxes (30 \times 30 \times 50 cm³) on an elevated mesh floor and allowed to acclimate for 30 minutes. The von Frey filaments (Stoelting, USA) were applied in increasing order of force onto the abdominal area. Each filament was applied 10 times for 1 – 2 seconds each with a 15 second interval between applications. A positive response was recorded when the rat lifted its belly (withdrawal response). Data was expressed as percentages of positive responses of each rat with each filament.

Western Blot

The thoracic (T) 10 spinal dorsal horn was rapidly extracted, homogenized, and centrifuged at 4°C for 15 minutes at 1,000 g. The supernatants were collected and the protein concentrations were determined. Samples (50 µg) were separated on 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, USA). After blocking in Tris-buffered saline containing 0.02% Tween (TBS-T) and 3% non-fat dry milk for one hour, the membranes were incubated overnight at 4°C with rabbit anti-HMGB1 (1:1000; BD Pharmingen, USA), rabbit anti-TLR4 (1:500; Abcam, UK), or goat anti-GAPDH (1:1000; Santa-Cruz Biotechnology, USA). After washing, the membranes were incubated with anti-rabbit or anti-goat horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000; Amersham Pharmacia Biotech Inc., USA). Protein bands were visualized using the enhanced chemiluminescence detection kit (Amersham). Data were normalized to GAPDH and expressed as fold changes relative to the sham-control group.

Real-time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and euthanized. The thoracic (T) 10 spinal dorsal horn was immediately extracted. Total RNA was extracted with Trizol (GIBCO/BRL Life Technologies Inc., USA). Single stranded complementary DNA (cDNA) was synthesized with oligo (dT) 12 – 18 using Superscript™ Reverse Transcriptase for real-time PCR (RT-PCR) (Invitrogen, USA). The primers used in the PCR are shown in Table 1. GAPDH served as the internal control. Equal amounts of RNA (1 µR) were used to prepare cDNA using the SYBR® Premix Ex Taq™ (Takara, Japan) and analyzed by real-time PCR in a detection system (Applied Biosystems, USA). The amplification

protocol included: 3 minutes at 95 °C, followed by 45 cycles of 10 seconds at 95 °C for denaturation, and 45 seconds at 60 °C for annealing and extension. All tests were performed in 2 independent experiments. The PCR reactions were carried out in triplicate in each experiment. The threshold cycle number (Ct) of amplification was obtained using the Sequence Detection System software (Applied Biosystems).

Intrathecal Administration of Neutralizing Anti-HMGB1

An intrathecal catheter for the administration of neutralizing anti-HMGB1 was implanted as described in our previous report (34). Briefly, the rats were anesthetized with sodium pentobarbital (45 mg/kg, i.p.), and a midline 3-cm incision was made at the thoracic vertebrae levels of 2 – 4. A PE-10 tube (I.D. 0.28 mm and O.D. 0.61 mm) (Clay Adams, USA) was inserted at the T8-T10 level of the spinal cord, and 2 cm of the free end was left exposed in the upper thoracic region. The rats were allowed to recover for 3 – 5 days. Lidocaine (2%, 10 µl) was administered through the intrathecal catheter. Only the animals judged neurologically normal and that showed complete paralysis of the tail and bilateral hind legs were used in subsequent experiments. Neutralizing antibodies against HMGB1 B box (10 or 50 µg, IgY subclass; Shino-Test Corporation, Japan) or nonimmune control IgY (vehicle control) were applied.

Polyclonal neutralizing antibodies against HMGB1 B box were raised in rabbits and affinity-purified using cyanogen bromide-activated Sepharose beads following standard procedures. Neutralizing activity of the antibodies was assessed by measuring the inhibition of TNF-α release in HMGB1-stimulated macrophage cultures (18,35). An antibody that showed > 80% inhibition of TNF-α release was used as the neutralizing anti-HMGB1 antibody in the animal experiments. The

Table 1. Primers sequence for the rat genes characterized in this experiment.

Genes	Primers		Accession number
TNF-α	Forward primer	5'-TGATCGGTCCCAACAAGG A-3'	AY427675
	Reverse primer	5'-TGCTTGGTGGTTTGTACGA-3'	
IL-1β	Forward primer	5'-TGCTGATGTACCAGTTGGGG-3'	NM031512
	Reverse primer	5'-CTCCATGAGCTTTGTACAAG-3'	
IL-6	Forward primer	5'-GCCCTTCAGGAACAGCTATG-3'	NM012589
	Reverse primer	5'-CAGAATTGCCATTGCACAAC-3'	
GAPDH	Forward primer	5'-CCCCAATGTATCCGTTGTG-3'	NM01008
	Reverse primer	5'-TAGCCCAGGATGCCCTTTAGT-3'	

antibody (50 µg) was administered through the intrathecal catheter. Nonimmunized rabbit IgG (Sigma, USA) was used as control.

Immunohistochemistry

At the fifth week after TNBS infusion, the rats were perfused through the ascending aorta with 100 mL of normal saline followed by 500 mL of 0.1 M PB (pH 7.4) containing 4% paraformaldehyde and 2% picric acid. Immediately after the perfusion, the spinal segment T10 was removed and postfixed in the same fixative for 2–4 hours and then stored for 24 hours at 4°C in 0.1 M PB containing 30% sucrose for cryoprotection. The segment was cut serially into 30 µm-thick frontal sections on a frozen microtome (Kryostat 1720; Leitz, Germany). The sections were placed in 2 dishes, with each dish containing a complete set of serial sections. Sections in the first dish were rinsed in 0.01 M phosphate-buffered saline (PBS, pH 7.3) 3 times for 10 minutes each, blocked in 0.01 M PBS containing 2% goat serum and 0.3% Triton X-100 for one hour at room temperature, and incubated overnight at 4°C with mouse anti-GFAP (1:5000; Chemicon, USA). The sections were then washed 3 times in 0.01 M PBS for 10 minutes each, incubated with biotinylated horse anti-mouse IgG (1:200; Vector, USA) diluted in PBS-NDS for 4 hours, and then incubated with the avidin-peroxidase complex (1:100 dilution; Vector, USA) in PBS for one hour. The signals were visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Dojin, Japan) as the substrate. Sections in the second dish were processed following the same procedures except the incubation with the anti-GFAP antibody and served as control. All sections were analyzed under a VANOX microscope (Olympus, Japan).

Statistical Analysis

All data are presented as means ± standard error of the mean (means ± S.E.M.). All data were obtained by researchers blinded to treatment regimes. Repeated measures analysis of variances (ANOVA) with Bonferroni confidence interval adjustment was used in the interpretation of pancreatic histopathology data. Two-way analysis of variance (ANOVA) was used in the analysis of behavioral as well as western blot and RT-PCR data. Dunnett's C post hoc test that does not assume equal variances was employed when appropriate. Differences with a $P < 0.05$ were considered statistically significant. All statistical analyses were performed using SPSS version 16.0 software (SPSS Inc., USA).

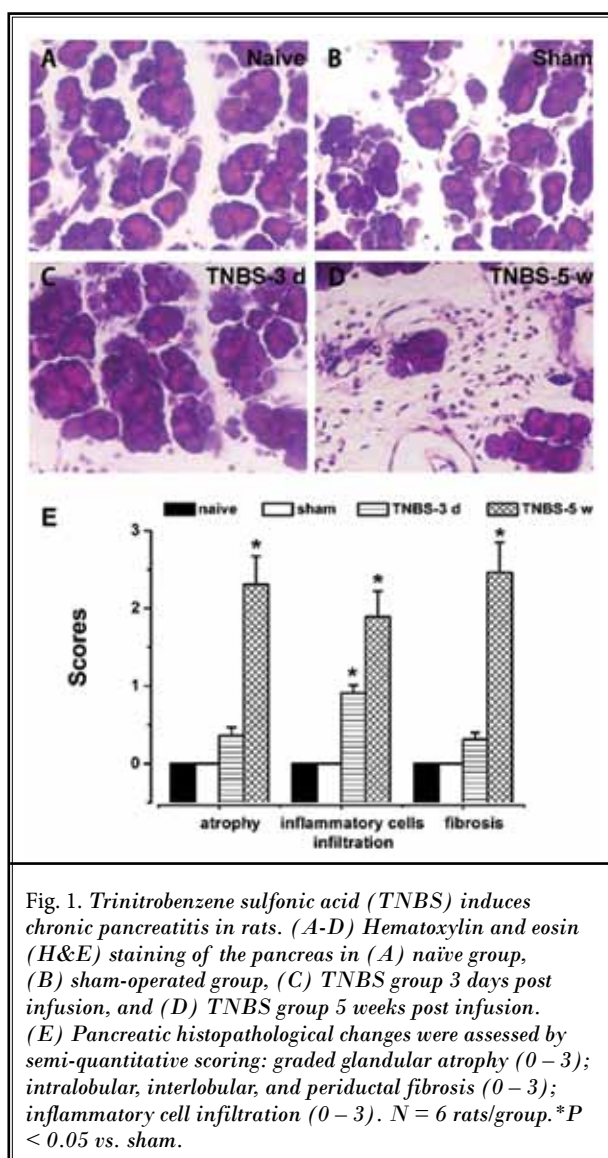
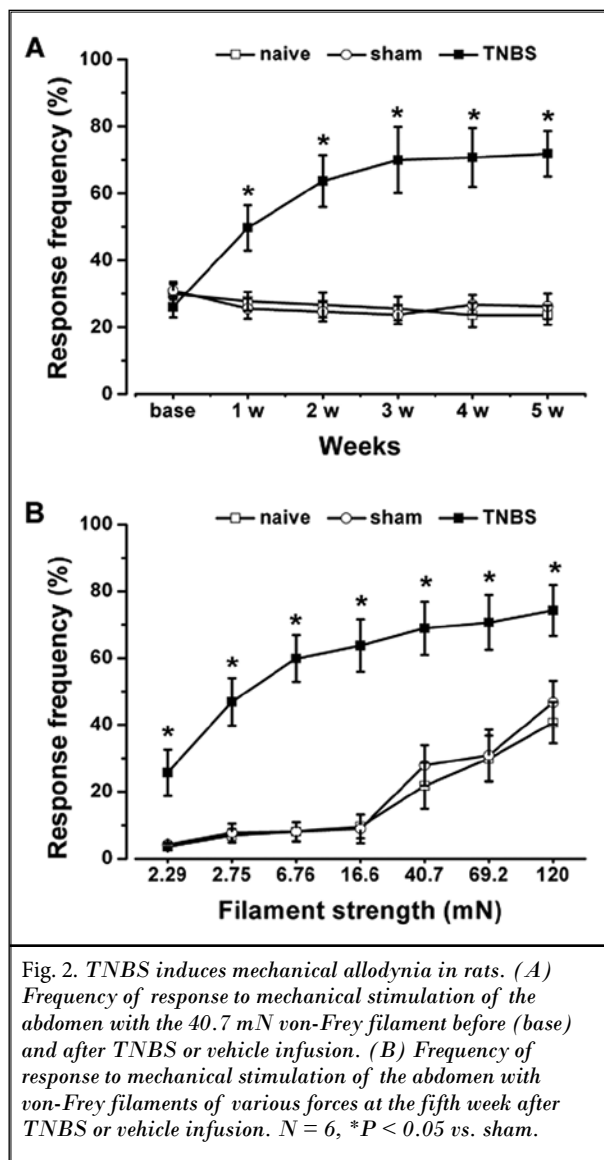


Fig. 1. Trinitrobenzene sulfonic acid (TNBS) induces chronic pancreatitis in rats. (A-D) Hematoxylin and eosin (H&E) staining of the pancreas in (A) naive group, (B) sham-operated group, (C) TNBS group 3 days post infusion, and (D) TNBS group 5 weeks post infusion. (E) Pancreatic histopathological changes were assessed by semi-quantitative scoring: graded glandular atrophy (0–3); intralobular, interlobular, and periductular fibrosis (0–3); inflammatory cell infiltration (0–3). $N = 6$ rats/group. * $P < 0.05$ vs. sham.

RESULTS

TNBS Induces Chronic Pancreatitis in Rats

TNBS infusion-induced histopathological changes in the pancreas were examined using H&E staining. The severity of pancreatitis was evaluated based on the histological scores of gland atrophy, presence of inflammatory infiltrates, and periductular and intralobular fibrosis (8,30). TNBS effects on CP in the rat are summarized in Fig. 1. Moderate infiltration of inflammatory cells was observed 3 days following the TNBS infusion. Five weeks after the infusion, the pancreas showed



histological changes consistent with chronic pancreatitis including acinar atrophy, inflammatory infiltration, and periductular and intralobular fibrosis (Fig. 1). The pancreas of the naïve and sham rats showed no signs of abnormality.

TNBS Induces Mechanical Allodynia

CP-induced pain has been previously characterized by increased abdomen response frequencies (RFs) (7,33,36). TNBS effects on mechanical allodynia are summarized in Fig. 2. TNBS-infused rats displayed significantly increased RFs starting from the first week (RF of $49.8 \pm 6.8\%$ for the TNBS group vs. $27.8 \pm 2.8\%$

for the sham group, $P < 0.05$, Fig. 2A) up to the fifth week (RF of $70.2 \pm 5.9\%$ for the TNBS group vs. $23.4 \pm 2.9\%$ for the sham group, $P < 0.05$, Fig. 2A) after the TNBS infusion. No significant differences were observed between the sham and naïve groups. When the rats were stimulated with von-Frey filaments in increasing order of force from 2.29 to 120 mN during the fifth week after CP induction, TNBS-infused rats displayed significantly higher RFs with all filaments as compared to the sham or naïve rats ($P < 0.05$, Fig. 2B). Therefore, these results showed that TNBS significantly induces CP and causes mechanical allodynia in rats.

TSN IIA Attenuates TNBS-induced Mechanical Allodynia

The effects of TSN IIA on CP-induced pain were examined and these results are summarized in Fig. 3. TSN IIA was intraperitoneally injected from weeks 4 to 5 after TNBS infusion, and the rats were evaluated in the mechanical allodynia test one hour after TSN IIA administration. Compared with the vehicle control group, TSN IIA-treated rats (20 mg/kg) showed significantly attenuated RFs at the filament force of 40.7 mN ($43.1 \pm 8.9\%$ vs. $70.6 \pm 9.1\%$, $P < 0.05$, Fig. 3A). In addition, TSN IIA (20 mg/kg) significantly attenuated TNBS-induced mechanical allodynia at all filament forces tested except 120 mN (Fig. 3B). Moreover, TSN IIA significantly decreased TNBS-induced mechanical allodynia in a dose-dependent manner (Fig. 3C). However, TSN IIA did not change the RFs of sham-operated rats ($P > 0.05$) at any given time point or any filament force.

TSN IIA Inhibits TNBS-induced Spinal HMGB1 Expression

The role of spinal HMGB1 in mediating or modulating CP was evaluated and these results are summarized in Fig. 4. Spinal HMGB1 expression was determined before and after TNBS infusion. The HMGB1 protein level in the thoracic spinal dorsal horn was significantly increased in the first week after TNBS infusion and maintained at high levels up to the fifth week. Specifically, the spinal HMGB1 protein level of TNBS-infused rats was 1.99, 2.66, 2.95, 3.71, and 3.38-fold of that of the sham-operated rats at weeks 1, 2, 3, 4, and 5, respectively, after the TNBS infusion (Fig. 4A and C, $P < 0.05$). These data indicated that TNBS-induced chronic pancreatitis was accompanied by significantly increased spinal HMGB1 expression, suggesting that HMGB1 may contribute to CP-induced inflammation and pain.

Subsequently, investigation of the effects of TSN

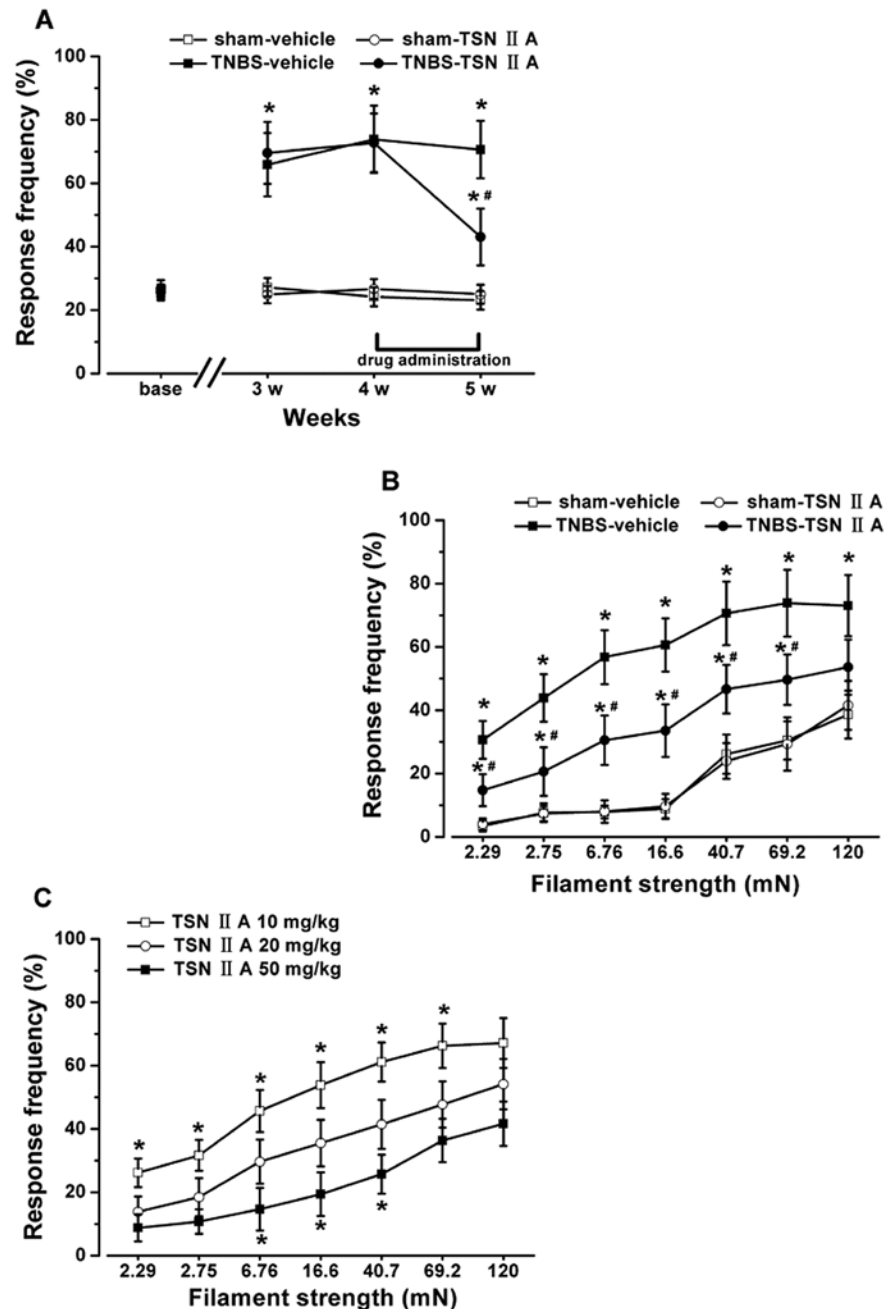


Fig. 3. Tanshinone IIA (TSN IIA) significantly ameliorates TNBS-induced mechanical allodynia in rats. TSN IIA or saline was intraperitoneally administered from the fourth to the fifth week after TNBS or vehicle infusion. (A) Frequency of response to mechanical stimulation of the abdomen with the 40.7 mN von-Frey filament. The behavioral experiments were performed before (base) and at 3 weeks, 4 weeks, and 5 weeks after TNBS or vehicle infusion. The behavioral experiments at the fifth week were performed one hour after TSN IIA administration (20 mg/kg). $N = 6$, $*P < 0.05$ vs. sham-vehicle, $\#P < 0.05$ vs. TNBS-vehicle. (B) Frequency of response to mechanical stimulation of the abdomen with von-Frey filaments of various forces at the fifth week after TNBS or vehicle infusion. The behavioral experiments were performed one hour after TSN IIA administration (20 mg/kg). $N = 6$, $*P < 0.05$ vs. sham-vehicle, $\#P < 0.05$ vs. TNBS-vehicle. (C) Frequency of response to mechanical stimulation of the abdomen with the 40.7 mN von-Frey filament at the fifth week after TNBS or vehicle infusion. The behavioral experiments were performed one hour after TSN IIA administration at 10, 20, and 50 mg/kg. $N = 6$, $*P < 0.05$ vs. TSN IIA 20 mg/kg.

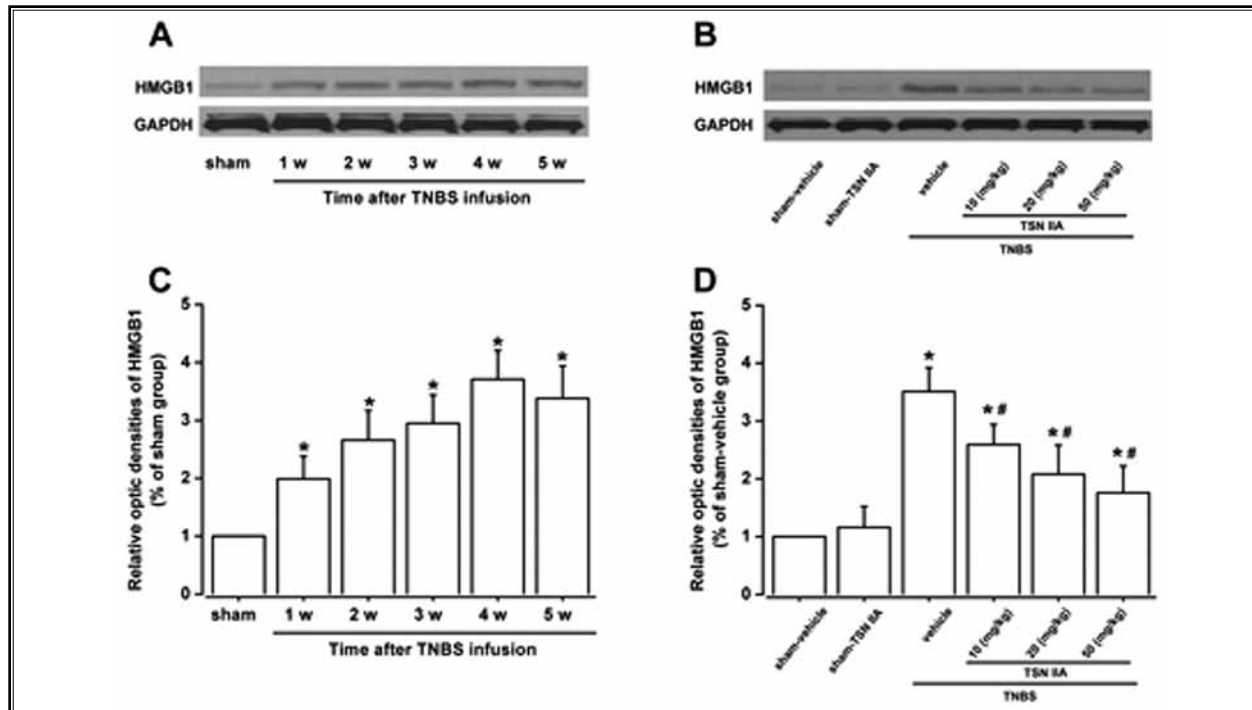


Fig. 4. TSN IIA significantly attenuates TNBS-induced increases in spinal HMGB1 expression. HMGB1 protein expression in the thoracic (T) 10 spinal dorsal horn was determined by western blot. (A, C) Spinal HMGB1 protein expression in sham-operated rats and TNBS-infused rats at 1 week, 2 weeks, 3 weeks, 4 weeks, and 5 weeks after infusion. Data were normalized to sham. $N = 6$, $*P < 0.05$ vs. sham. (B, D) Spinal HMGB1 protein expression at the fifth week after TNBS or vehicle infusion. TSN IIA was intraperitoneally injected at the indicated doses from the fourth week to the fifth week after TNBS or vehicle infusion. Data were normalized to sham-vehicle. $N = 6$, $*P < 0.05$ vs. sham-vehicle, $\#P < 0.05$ vs. TNBS-vehicle.

IIA on CP-induced spinal HMGB1 expression was evaluated and TSN IIA was intraperitoneally injected at 20 mg/kg dose from weeks 4 to 5 after TNBS infusion. The spinal HMGB1 expression was evaluated one hour after TSN IIA administration. Our results showed that TNBS-induced increases in spinal HMGB1 as protein levels were significantly suppressed by TSN IIA treatment (Fig. 4B, D). However, TSN IIA did not change the spinal HMGB1 expression in the sham-operated rats.

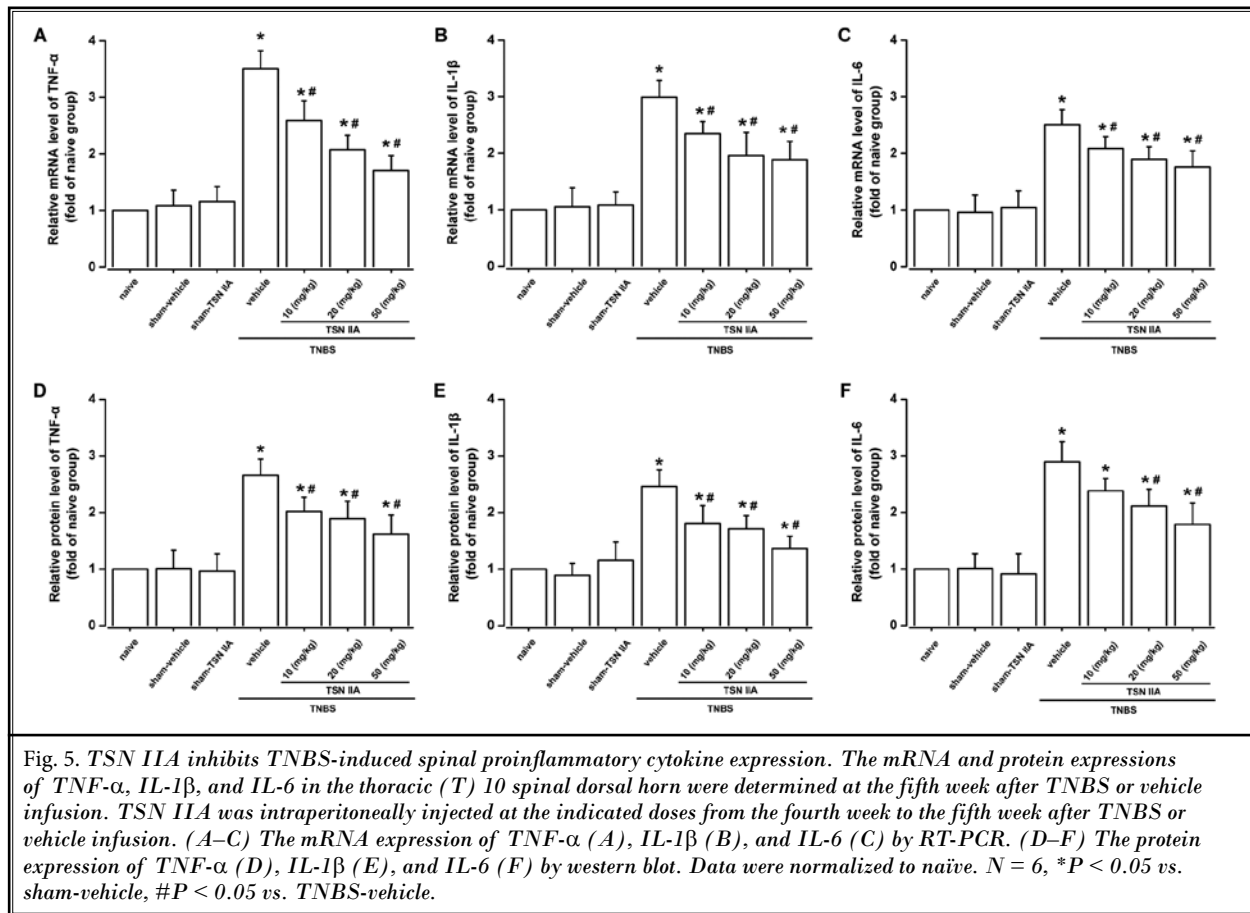
TSN IIA Inhibits TNBS-induced Spinal Cytokine Expression

It is well established that the activation of the HMGB1-TRL4 pathway leads to upregulation of pro-inflammatory cytokines such as TNF- α , IL-1 β , and IL-6. Having shown that TSN IIA inhibits TNBS-induced spinal HMGB1 expression, we further investigated the effects of TSN IIA on the spinal expression of TNF- α , IL-1 β , and IL-6 and these data are summarized in Fig. 5. Consistent with increased HMGB1 expression, significantly higher mRNA and protein levels of TNF- α , IL-1 β , and IL-6 were

detected in the thoracic spinal dorsal horn of the TNBS-treated rats compared with the sham-operated rats. Intraperitoneal injection of TSN IIA at 20 mg/kg but not vehicle significantly reduced the spinal mRNA and protein expression of TNF- α , IL-1 β , and IL-6 in TNBS-treated rats (Fig. 5). TSN IIA at 50 mg/kg resulted in even greater inhibitory effects on these cytokines (Fig. 5D-F). These inhibitory effects of TSN IIA on TNBS-induced spinal inflammatory cytokine expression were most likely mediated through HMGB1 downregulation.

TSN IIA but Not Neutralizing Anti-HMGB1 Inhibits TNBS-induced Spinal TLR4 Expression

TRL4 is the HMGB1 receptor that mediates HMGB1-dependent activation of proinflammatory cytokine release. The effects of TSN IIA on spinal TRL4 protein expression were evaluated using western blot and are summarized in Fig. 6. We found that TNBS infusion significantly increased the spinal TRL4 protein expression, and TSN IIA treatment (20 mg/kg) significantly attenuated TNBS-induced increases in spinal TRL4 expression



(Fig. 6B, D). However, TSN IIA did not change the spinal TLR4 expression of sham-operated rats. These results suggested that TSN IIA ameliorates TNBS-induced spinal inflammation and pain behaviors through inhibition of the expression of both HMGB1 and its receptor TLR4 in the spinal cord.

The effects of intrathecal injection of a neutralizing anti-HMGB1 antibody on spinal TLR4 expression were also tested but no significant effects were identified in our investigation (Fig. 6A, C), suggesting that TSN IIA inhibits the expression of spinal TLR4 via mechanisms other than decreasing HMGB1 activity.

TSN IIA but Not Neutralizing Anti-HMGB1 Inhibits TNBS-induced Spinal Astrocyte Activation

Previous studies have shown increased activation of spinal astrocytes in CP (8,30). Since the activation of TLR4 is required for the activation of astrocytes (26), we speculated that TSN IIA would inhibit spinal astrocyte

activation in TNBS-infused rats through downregulation of HMGB1 and TLR4. The results of our present investigation are summarized in Fig. 7. Compared with sham-operated rats, significantly increased spinal GFAP (specific marker for astrocyte activation) expression was detected in TNBS-infused rats as revealed by our immunohistochemical and western blot studies (data not shown). Intraperitoneal administration of TSN IIA (20 mg/kg) significantly inhibited the spinal GFAP expression in TNBS-infused rats (Fig. 7E–H). Collectively, these results suggest that the analgesic effects of TSN IIA in TNBS-infused rats are mediated by downregulation of the HMGB1-TLR4 pathway and subsequent inhibition of spinal astrocyte activation and cytokine release.

Interestingly, we found that the neutralizing anti-HMGB1 antibody had no inhibitory effects on TNBS-induced spinal astrocyte activation (Fig. 7A–D). These results suggested that TSN IIA suppresses TNBS-induced spinal astrocyte activation mainly through inhibition of spinal TLR4 rather than HMGB1 expression.

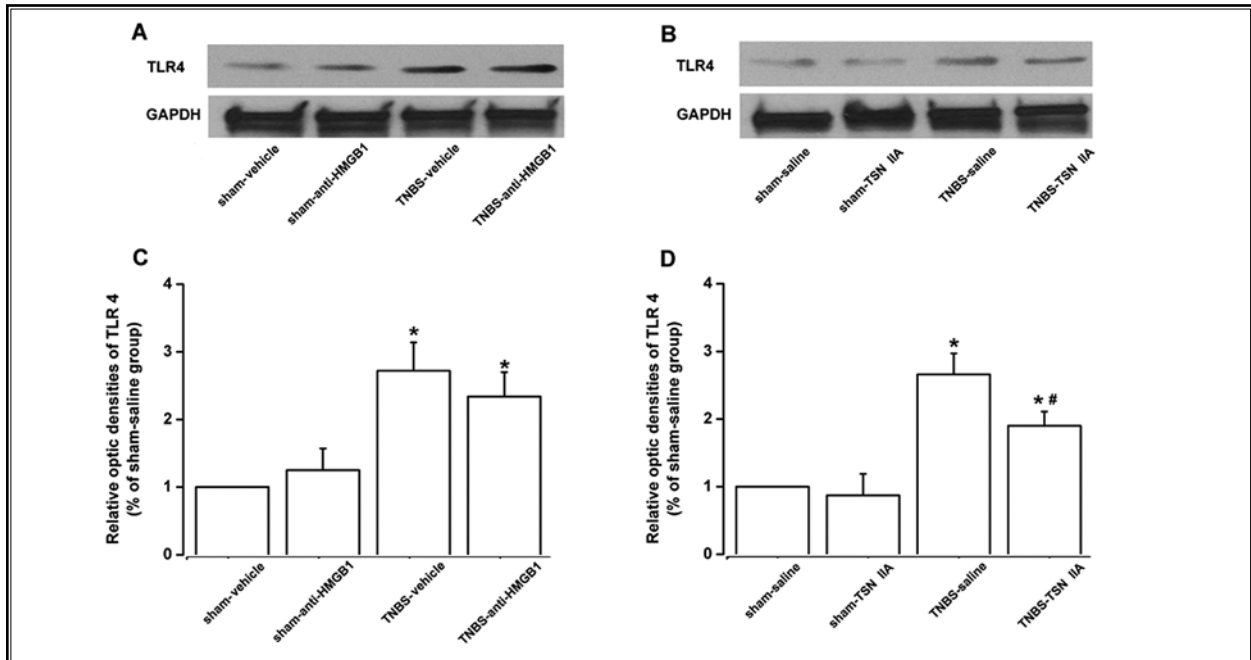


Fig. 6. TSN IIA but not neutralizing anti-HMGB1 significantly inhibits TNBS-induced spinal TLR4 expression. The TLR4 protein expression in the thoracic (T) 10 spinal dorsal horn was determined by western blot at the fifth week after TNBS or vehicle infusion. Neutralizing anti-HMGB1 (50 μ g) (A, C) or TSN IIA (20 mg/kg) (B, D) was administered from the fourth week to the fifth week after TNBS or vehicle infusion. Data were normalized to sham-control. N = 6, *P < 0.05 vs. sham-anti-HMGB1 (C) or sham-TSN IIA (D). #P < 0.05 vs. TNBS-saline.

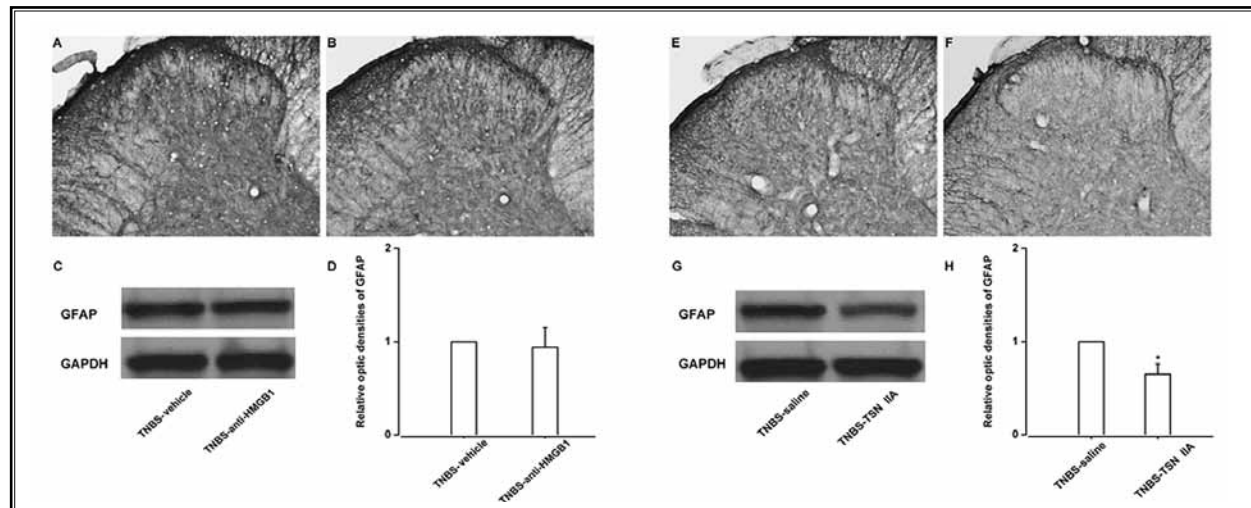


Fig. 7. TSN IIA but not neutralizing anti-HMGB1 significantly inhibits TNBS-induced spinal GFAP expression. The GFAP protein expression in the thoracic (T) 10 spinal dorsal horn was determined by immunohistochemical staining (A, B, E, F) and western blot (C, D, G, H) at the fifth week after TNBS infusion. Neutralizing anti-HMGB1 (50 μ g) (B) or TSN IIA (20 mg/kg) (D) was administered from the fourth week to the fifth week after TNBS infusion. Data were normalized to TNBS-vehicle (D) or TNBS-saline (H). N = 6, *P < 0.05 vs. TNBS-saline.

Discussion

The present investigation demonstrates that TNBS-induced pancreatic histopathological changes of CP and persistent mechanical allodynia in rats by upregulation of the spinal HMGB1-TRL4 pathway leads to spinal astrocyte activation and increased production of the proinflammatory cytokines TNF- α , IL-1 β and IL-6. TSN IIA significantly inhibited TNBS-induced spinal HMGB1-TRL4 pathway activation, astrocyte activation, and proinflammatory cytokine production and attenuated TNBS-induced mechanical allodynia. The inhibitory effects of TSN IIA on the spinal HMGB1-TRL4 pathway were attributed to downregulation of HMGB1 and its receptor TRL4. Therefore, the results of our study suggest that TSN IIA may be a potential anti-nociceptive agent for the treatment of CP-induced pain.

Previous studies on the mechanisms of CP-induced pain have mostly focused on histopathological changes in the pancreas (37). Recently, several studies have reported that the CNS may additionally contribute to CP-induced pain (38,39). Inflammation of a viscus can sensitize the local nociceptive system and activate the release of neuroactive substances to peripheral terminals of sensory neurons (40). The pain signal is transmitted to the spinal cord leading to the sensitization of nociceptive neurons in the dorsal horn (41,42). This results in the release of cytokines that activate spinal astrocytes (43). The activated astrocytes subsequently secrete a variety of signaling molecules including proinflammatory cytokines, which further enhance neuronal activity and pain processing (44).

The signaling molecules involved in spinal pain processing include early proinflammatory cytokines such as TNF- α , IL-1 β , and IL-6 and late proinflammatory cytokines such as HMGB1. The early proinflammatory cytokines are rapidly induced and released, usually within hours after stimulation (1). In the present study, significantly elevated TNF- α , IL-1 β , and IL-6 levels were detected in the spinal cord of TNBS-infused rats with mechanical allodynia, suggesting that these early proinflammatory cytokines are involved in CP-induced pain. However, the rapid induction of early proinflammatory cytokines makes it difficult to target them clinically because of their narrow therapeutic windows (45).

In comparison, the late proinflammatory cytokine HMGB1 can be released by neurons and astrocytes following stimulation (46). Previous studies have revealed that spinal astrocytes are activated in the spinal dorsal horn of rats suffering from CP (8,30), suggesting that HMGB1 may contribute to CP-induced pain. Further-

more, a few recent studies have reported significantly increased serum HMGB1 levels in patients (47) and animals (48,49) with severe acute pancreatitis (SAP), and the HMGB1 levels were correlated with disease severity. In the present study, elevated HMGB1 levels were detected in the thoracic spinal cord one week after TNBS infusion and remained high throughout the study. Elevated HMGB1 levels were correlated with mechanical allodynia observed in TNBS-infused rats, suggesting that spinal HMGB1 acts as a proinflammatory mediator in CP-induced pain. Given that HMGB1 is a late proinflammatory cytokine with relatively slow induction and long duration, HMGB1 inhibitors may have a relatively wide therapeutic window in the treatment of CP-induced pain. The HMGB1 inhibitors investigated in previous studies have primarily been neutralizing antibodies targeted against HMGB1 (18,50). These antibodies are still in preclinical development and their efficacy and safety in humans are largely unknown. In this regard, a number of HMGB1 receptor antagonists have been investigated (51,52). However, HMGB1 inhibitors that target HMGB1 expression have rarely been described in the literature. Tanshinone IIA (TSN IIA), an active component of Danshen, has recently been reported to inhibit endotoxin-induced HMGB1 release in macrophage/monocyte cultures and rescue mice from lethal sepsis by preventing systemic accumulation of HMGB1 (20). TSN IIA has also been reported to inhibit the expression of HMGB1 and its receptor TLR4 in the ischemic brain (25) and spinal dorsal cord of rats with spinal nerve ligation-induced neuropathic pain (53). In the present study, TSN IIA dose-dependently inhibited spinal HMGB1 and TRL4 expression and mechanical allodynia in TNBS-infused rats. Moreover, TSN IIA inhibited TNBS-induced spinal expression of TNF- α , IL- β , and IL-6 and the activation of spinal astrocytes, which are events downstream of the HMGB1-TRL4 pathway.

Collectively, these data indicated that the analgesic effects of TSN IIA in TNBS-infused rats were mediated by inhibition of the HMGB1-TRL4 pathway and downstream signaling. Interestingly, a neutralizing anti-HMGB1 antibody failed to inhibit spinal TRL4 expression and spinal astrocyte activation in TNBS-infused rats. Therefore, TSN IIA, which acts through downregulation of both HMGB1 and its receptor TRL4, might be more effective than neutralizing anti-HMGB1 antibodies in treating CP-induced pain.

Clinical pharmacokinetic studies have shown that TSN IIA can be rapidly absorbed and has no serious adverse effects in human (54), furthering the clinical

development of TSN IIA as a potential therapeutic drug for CP-induced pain. In addition to TRL4, HMGB1 can bind to other receptors such as TLR2 and RAGE. Further investigations are warranted to find out whether HMGB1 contributes to CP-induced pain through activation of these receptors.

CONCLUSION

Our results support the concept that spinal HMGB1 contributes to the development of CP-induced pain. TSN IIA attenuates CP-induced pain through down-regulation of the expression of HMGB1 and its receptor TRL4 in the spinal cord, which leads to inhibition of spinal astrocyte activation and proinflammatory cytokine production. This study provides evidence that TSN IIA is a potential novel therapy for CP-induced pain in the future.

Competing Interest Statement

Dr. Ye-song Wang, Li-hua Wang, and Yuan-yuan Li contributed equally to this work. There was no financial relationship with any organization that might have an interest in the submitted work during the previous 3 years, and there are no other relationships or activities that could appear to have influenced the submitted work. Kun Wang, Holly C. Long, and Lei Chen are equal contributors of this work.

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