Abnormal activation of complement C3 in the spinal dorsal horn is closely associated with progression of neuropathic pain

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Abstract. The aim of the present study was to investigate the role of complement activation in the pathogenesis of neuropathic pain (NPP) induced by peripheral nerve injury. We modified a classical chronic constriction injury (CCI) model (mCCI), and verified its reliability in rats. Furthermore, reverse transcription-PCR and immunohistochemistry were conducted to investigate complement activation in the spinal dorsal horn and the effect of a complement inhibitor, cobra venom factor (CVF), on the behavior of the mCCI model rats. We found that rats in the mCCI group presented a better general condition, without signs of autophagy of the toes. Moreover, mCCI induced a significant increase (+40%) in the expression of *component 3* (*C3*) mRNA in the spinal dorsal horn, which was associated with hyperalgesia. Correlation analysis showed a negative correlation between the mechanical pain threshold and the expression of C3 in the spinal cord. Administration of CVF reduced the occurrence of hyperalgesia in mCCI rats and nearly reversed the hyperalgesia. In addition, the mCCI rats exhibited significantly less spinal superoxide dismutase activity and significantly greater levels of maleic dialdehyde compared to the sham-operated rats. Transmission electron micrographs revealed mitochondrial swelling, cell membrane damage, and cristae fragmentation in the neurons of the spinal dorsal horn 14 days after mCCI. Mitochondrial swelling was attenuated in mCCI rats receiving CVF. The findings demonstrated that abnormal complement activation occurred in the dorsal horn of the spinal cord in rats with NPP, and C3 in the spinal dorsal horn could play an important role in the cascade reaction of complements that are involved in the development of hyperalgesia.

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Introduction

Nerve injury induced-neuropathic pain (NPP) causes local functional and biochemical changes and it also leads to pathology of the spinal cord and brain (1,2). Currently, no effective treatment is available for NPP, and the mechanisms underlying its occurrence and development are poorly understood. In traditional animal models of chronic constriction injury (CCI) of the sciatic nerve, which are widely used in studying NPP, it is difficult to replicate the level of nerve injury due to variables in surgery and severity of nerve ligation. This often leads to variation among the individual models (such as the percentage of autophagy), making the experiments unreplicable. Therefore, the development of new animal models and understanding the mechanisms underlying NPP are essential (3,4) in order to meet clinical needs in pain management.

The complement system has a vital role in human nonspecific immunity. In the normal physiological condition, components of the complement system (i.e., complements) exist in blood plasma in the form of enzyme precursors. Once they are activated, a cascade chain of enzymatic reactions occurs, leading to the formation of series of activated fragments of the complement proteins and eventually membrane attack complexes (MACs) (5). The activation of complements plays a significant physiological role in clearing apoptotic cells and neuronal fragments. However, improper activation can also cause pathological injury to the host (5,6).

There is increasing research on the association between complement activation and neuropathic pain. Griffin *et al* (7) employed a gene chip technique to screen 216 genes that were detected in three NPP models induced by peripheral nerve injury. Among them, 54 genes were co-expressed in all three models and they are related with immunity, including genes expressing complement proteins. These findings indicate that activation of complements may play a role in the development of NPP. It has also been shown that intrathecal injection of dissolvable soluble complement receptor 1 (sCR1) could inhibit activation of the complement reaction cascade and attenuates pain intensity in diverse rat models of inflammatory, constrictive, and viral infection injuries of the sciatic nerve (8,9). Kleinschnitz *et al* (10) found a reduced pain response and a

decrease in the accumulation and activity of macrophages in the injured nerves of rats with a complement deficiency after CCI. It is well known that there are several significant changes in the spinal dorsal horn in NPP models such as abnormal discharge of neurons and cytokine release of glial cells which contribute to the development of neuropathic pain. However, it is unknown whether the complements in the spinal dorsal horn are abnormally activated and whether complements are associated with development of neuropathic pain.

Among all the components of the complement system in serum, complement component 3 (C3) has the highest percentage (5). It is also the meeting point of the classic, alternative, and mannose-binding lectin (MBL) pathways of complement activation, and has a key role in the defense mechanism of the host (5). It has been demonstrated that neurons and glial cells synthesize the components of the complement cascade reaction (11,12). They can also express complement regulatory proteins and their receptors such as complement component 3a receptor 1 (C3ar1) which is upregulated following inflammatory central nervous system disease (CNS) injuries (11). Increased C3a was found to provoke the production of inflammatory mediators, which in turn further enhanced synthesis of the complement. Thus, a feedback loop is formed between activation of inflammatory cytokines and the complement system, leading to nerve-immune activation (13). Chinese cobra venom factor (CVF), an acidic glycoprotein extracted from Chinese cobra venom, is the main anti-complement component in the venom (14). In the present study, we hypothesized that C3 is a key mediator in the NPP mechanism. We used a modified CCI (mCCI) rat model to explore the association between the abnormal activation of C3 in the spinal dorsal horn and the occurrence and development of NPP. Moreover, CVF, as a non-specific inhibitor of complement function, could be an effective intervention reagent to identify the roles of abnormally activated complements in NPP.

Materials and methods

Animals and experimental groupings. Healthy adult male Sprague-Dawley rats (250-300 g) were employed in this study, and were provided by the Animal Experiment Center of the Third Military Medical University, with Animal Medical Certificate no. SCXK (Military) 2002008. Rats were housed four to a cage. The cage floor was covered with sawdust at a room temperature of 20±2˚C. The animals were exposed to a strictly alternating light-dark pattern of illumination, each for 12 h, and they were provided with adequate water and food. After a one-week adaptation period, the modified rat models were studied.

Thirty-six rats were divided into three groups (n=12): sham-operated, traditional CCI, and mCCI. Traditional CCI models were prepared as previously described by Bennett and Xie (15), and the mCCI models were treated with slight modifications as introduced later. Behavioral responses consisting of changes in paw withdrawal thermal latency (PWTL) and paw withdrawal threshold to mechanical stimuli (PWMT) were observed at two weeks post-surgery. After these observations, the mCCI model was adopted in subsequent experiments.

Animals were randomly divided into seven groups (designated A to G), including 11 subgroups (n=12 in each group). Group A was the normal controls, rats that did not undergo surgery but that were otherwise treated identically, with the same survival requirement as the other groups. Group B rats were sham-operated, they underwent the same course of surgery as the model rats but without ligation of the sciatic nerve. Subgroups B1, B3 and B7 corresponded to 1, 3 and 7 days post-surgery, respectively. Group C was the mCCI group, rats that underwent the mCCI model operation. Subgroups C1, C3 and C7 corresponded to 1, 3 and 7 days post-surgery, respectively. Group D, or sham + saline, underwent a sham operation with saline intravenous (IV) injection at the same volume as Group F. Group E, or mCCI + saline, underwent mCCI surgery and IV injection of normal saline each day pre- and post-surgery. Group F, or mCCI + CVF, underwent mCCI surgery and received IV CVF (50 μg/kg body weight pre-surgery, 20 μg/kg post-surgery, each day). Group G, mCCI + saline + CVF, underwent mCCI surgery and a single IV CVF (50 μg/kg body weight) 4 days post-surgery, and saline injection as for Group E. All the experiments were conducted in the daytime.

The Animal Care and Use Committee at the Third Military Medical University in the P.R. China approved the experimental protocols and all protocols were in accordance with the guidelines for animal study set by the International Association for the Study of Pain. Every effort was made to minimize both animal suffering and the number of animals used.

Experiment preparations. Chinese CVF was produced by Beijie Biotechnology Co., Ltd. (Kunming, China). A solution (0.5 mg/ml) was prepared by dissolving 1 mg of CVF powder in 2 ml of 0.01 M phosphate-buffered saline (PBS; pH 7.2-7.4). The solution was filtered and sterilized through disposable aseptic filters, sub-packaged into aseptic bottles, and stored at -20˚C.

Goat anti-rat C3 antibody was produced by Cappel (USA) (cat. no. 55713). The superoxide dismutase (SOD) test box (article no. A00) and maleic dialdehyde (MDA) kit were provided by Jiancheng Bioengineering Institute (Nanjing, China). The RNAprep tissue/bacteria kit was manufactured by Tiangen Biotech (Beijing, China) (cat. no. DP401). The cDNA synthesis kit was produced by Toyobo, code no. FSK-100. The reverse transcription (RT)-PCR kit was produced by Toyobo, code no. PCR-400. The C3 assay kit was manufactured by Shanghai XiTang Biotechnology Co., Ltd. (Shanghai, China; batch no. 0610225).

Establishment of the mCCI model and administration of CVF. Modifications were made to the traditional procedures for creating the CCI rat model, described in the following steps (15). After an intraperitoneal (IP) injection of 1% pentobarbitone (40 mg/kg) and adequate anesthesia, the rat was put in the prone position, with four limbs and incisor teeth fixed to the operating table. The sciatic nerve was exposed with the routine method, and the peripheral tissues were liberated. The sciatic nerve was enveloped with porous films ~5 mm from the proximal end of the nerve branching. Four ligation bands were then made with 4/0 chromic suture at an interval of 1 mm, to the degree that the epineurial blood flow was not affected (Fig. 1). The main difference between the mCCI and traditional models was that the sciatic nerve was

enveloped with porous films in the mCCI rats which makes the strength of ligation more symmetrical and protects the nerve from acute crush and incised injuries. CVF was administered via tail vein injection 10 min before surgery (50 μg/kg body weight) and each day after surgery (20 μg/kg) in rats in Group F (mCCI + CVF). An equivalent volume of IV normal saline was injected daily in rats in Group D (sham + saline) and Group E (mCCI + saline) before and after surgery. Rats in Group G (mCCI + saline + CVF) were injected with CVF (50 μg/kg) 4 days after surgery and with normal saline at other time-points similar to Group F.

Observation of behavioral changes. Observations were made of the gait and posture of the operated limb for spontaneous pain behaviors as well as for the presence of autophagy, and were scored according to Attal *et al* (16). Behaviors of the operated hind limb were scored as: 0, the limb rests on the floor normally; 1, the limb rests partially on the floor, with toes in the ventral flexion; 2, only the medial part of foot rests on the floor; 3, only the heel rests on the floor, with toes lifted up; 4, the whole posterior foot is lifted up away from the floor; 5, the animal licks the foot of the operated limb. Behaviors were observed for 5 min each time at 10 a.m. every day for two weeks after surgery. The score of the behavior with the longest duration was used to represent the rat's level of spontaneous pain.

The PWMT was determined according to the method introduced by Dixon (17). The rat was placed onto an elevated metal mesh that was then covered with a transparent plexiglass cover. After 20 min of adaptation, von Frey filaments (range of measurement power 0.2-20 g) were used to perpendicularly stimulate the skin of the sole of the foot between the 2nd and 3rd toes, with the pressure increasing gradually. The stimulation persisted for 6-8 sec. A positive reaction was recorded if prompt paw withdrawal occurred during the stimulation period, or upon the removal of the von Frey filaments. Paw withdrawal resulting from body movement was not deemed a positive reaction. Ten stimuli were applied around the threshold, with an interval of at least 15 sec, waiting for the disappearance of the response to the previous stimulus. The 50% withdrawal threshold was calculated according to the median calculation method and the pressure was recorded as the PWMT.

The PWTL was determined according to the method described by Rokyta *et al* (18). An RYT-1 thermal stimulator (made in the Fourth Military Medical University, Xian, China) for pain threshold was used. The PWTL of each rat was tested in the morning. Following adaptation for 5 min on the glass bedplate, the rat was irradiated with a fixed intensity of light (the first level of 3) for <30 sec. The test was repeated three times for each rat with a resting interval between tests of 5 min. The mean time of paw withdrawal for the three tests was recorded as the PWTL.

Biochemical measurements of C3, total SOD (T-SOD), and MDA. Immunoturbidimetry was adopted to determine the concentration of C3 in spinal cord homogenate. The spinal cord (segments L4-6) was exposed at different time-points (3, 5 or 7 days) after surgery. The dorsal corner on the operated side was cut rapidly and preserved in liquid nitrogen. After

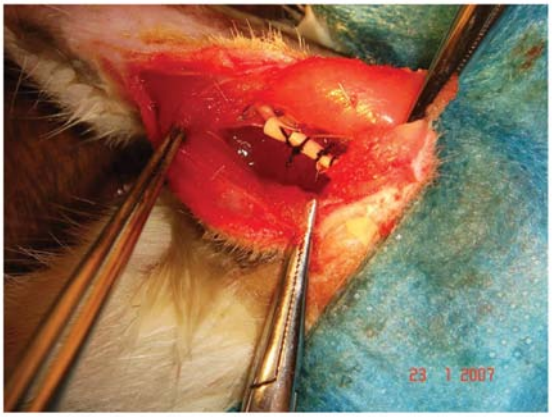

Figure 1. Image showing surgical induction of the mCCI model. The sciatic nerve was enveloped with porous films ~5 mm from the proximal end of the nerve branching. Four ligation bands were constructed with 4/0 chromic suture at intervals of 1 mm.

weighing, the spinal cord samples were added into a blender, added to iced salt water, one part tissue to nine parts liquid to prepare a 10% spinal cord homogenate. The homogenate was then centrifuged for 10 min at 3,000 rpm at 4˚C. The clear supernatant was diluted with normal saline (1:11), and blended for 30 sec with a vortex mixer; the mixture was added to anti-C3 serum (100 μl/ml). After adequate mixing, this was incubated in a thermostatic water bath at 37˚C for 15 min. A UV spectrophotometer was set at a wavelength of 340 nm, and normal saline was used to determine the zero adjustment. The absorbance of each tube was measured, and a standard preparation was diluted in the proportion given by the kit's instructions and measured as value A.

Based on the concentration of the standard preparations given by the kit and the value A obtained in this experiment, we drew a standard curve and then obtained the linear equation $Y = aX + b$, where Y is value A, and X is the concentration of C3 of the standard preparation at different dilution ratios, a and b are coefficients obtained from the kit's information. Using this equation, we calculated the concentration of C3 of each sample (19).

A spectrophotometric method was employed to determine T-SOD activity and the MDA content of the spinal cord tissues. Xanthine oxidase was used to measure SOD activity and an optical density (OD) value for the test tube was read at a wavelength of 550 nm. The thiobarbituric acid (TBA) method was used to assess the concentration of MDA, and the OD value of each tube was read at a wavelength of 532 nm (20).

RT-PCR analysis of C3 mRNA expression. Animals were euthanized with an overdose of pentobarbital at different time-points. We adopted RT-PCR to determine the level of *C3* mRNA in the spinal cord segments L4-6, on Days 1, 3 and 7 after surgery. The dorsal corner of the ipsilateral spinal cord segments (L4-6) was dissected, and preserved in liquid nitrogen. The methods of extraction, identification, and RT of RNA were the same as previously described (21). The two-step approach for RT-PCR analysis was employed to evaluate the expression of *C3* mRNA in the spinal cord tissues. For the primer sequences, we looked up the corresponding RNA sequence in GenBank, and designed the primer with software

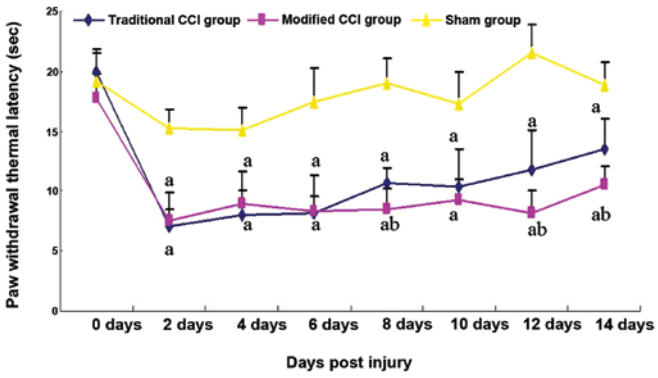

Figure 2. Curve chart of PWTL in 3 groups. a, P<0.05, compared with sham group; b, P<0.05, compared with traditional CCI group. PWTL decreased significantly by Day 2 post-surgery in both the traditional CCI and mCCI groups, and the change in the latter lasted longer.

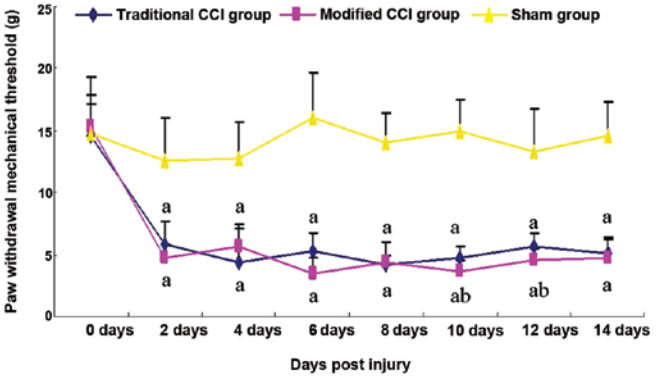

Figure 3. Curve chart of PWMT in three groups. a, P<0.05, compared with sham group; b, P<0.05, compared with traditional CCI group. PWMT decreased significantly by day 2 post-surgery in both the traditional CCI and mCCI groups, and the change in the latter lasted longer.

WinStar 5.08 (WinStar Bio. Co.). The primer sequences were: for Actb, forward, 5'-CGTAAAGACCTCTATGCCAACA-3' and reverse, 5'-CGTAAAGACCTCTATGCCAACA-3'; for *C3*, forward, 5'-GCTGTGCCTTATGTCATTGTCC-3' and reverse, 5'-ATTTCTCCCACTGTTCGGTCTG-3'. The results were analyzed with the help of the software Quantity One E4.0.

Expression of C3 protein by immunohistochemistry. The immunoreactivity of C3 in the spinal cord segments L4-6 was tested. On post-surgical days 1, 3 and 7, 1% pentobarbitone (IP, 40 mg/kg) was administered to the sham-operated and mCCI rats. The chest was then opened and the heart was exposed. After infusing normal saline and paraformaldehyde through the left ventricle and aortic cannula, segments L4-6 of the spinal cord were dissected, preserved and fixed for 24 h, followed by dehydration, infiltration with supporting paraffin, and paraffin embedment. Serial sections (thickness, 4 μm) were cut. Immunohistochemical staining was performed. The primary antibody was goat anti-rat C3 (50 μl, 1:200) and the second antibody was biotin-labeled rabbit anti-goat IgG (50 μl). Following incubation in enzyme-labeled horseradish peroxidase anti-biotin fluid (50 μl) at 37˚C for 20 min, diaminobenzidine coloration was performed, then after staining with hematoxylin, sections were cleared and mounted, and observed under an Olympus light microscope. With a clear background, we were able to clearly visualize blue nucleus staining, and the brownish yellow intra-cytoplasmic particles which were identified as cells testing positive for C3 (22).

Pathomorphological observation. The structures of neurons in the dorsal part of the spinal cord were observed under a transmission electron microscope (TEM). The spinal cord samples were fixed by immersion in 2.5% glutaraldehyde for 4 h, rinsed in PBS for 2 h, and fixed in osmic acid for 2 h. Following dehydration through an alcohol series, samples were stained through saturation with uranyl acetate for 30 min, and embedded in epoxide resin overnight at 37˚C. The embedded lump was made into the form of a pyramid with four smooth sides and a trapezoid on the top, from which we obtained 60-nm sections using an ultramicrotome. The sections were observed under a TEM (Tecnai 10; Philips, The Netherlands) after citric acid staining for 10 min, PBS rinsing three times, and air drying (23).

Statistical analysis. Statistical analysis was performed with SPSS 10.0 software. Repeated-measures ANOVA and the t-test were employed for group comparisons, and the correlation between the complement protein content and the pain threshold was analyzed using Pearson's correlation analysis. All data are presented as mean ± standard error (SE). P<0.05 was considered to indicate a statistically significant difference.

Results

Rats with mCCI present no autophagy but have greater thermal hyperalgesia than rats with traditional CCI. The mCCI rats had no autophagy and maintained thermal hyperalgesia for a longer period than did the traditional CCI rats (3/12 rats with the traditional CCI showed signs of autophagy of the posterior toes on the injured side). In regard to spontaneous pain, the rats in the sham-operated group all scored 0 points in posture and walking, while scores in the traditional and mCCI groups ranged from 2 to 4. The pain sensitivity of the mCCI rats lasted >14 days, while the pain sensitivity in the traditional group returned back to the level approximately similar to that in the sham group on Day 14 after surgery (Fig. 2).

No statistically significant difference was found in the preoperative thermalgesia threshold among the three groups. In the sham-operated group, the thermalgesia threshold exhibited a transitional and small decrease on post-surgical day 2; it then returned to normal. In the traditional and mCCI groups, however, hind PWMTs in response to thermal stimuli on the ligated side significantly dropped by 53.6 and 51.0%, respectively, two days after surgery, relative to the sham-operated rats. Six days after surgery, the PWMTs dropped to the lowest level by 53.1 and 52.0%, in the traditional and mCCI groups, respectively. Although the PWMTs gradually increased afterwards until two weeks after surgery, they were still lower than the preoperative level, 28.0 and 44.4% lower than the sham-operated group, respectively (P<0.01) (Fig. 2).

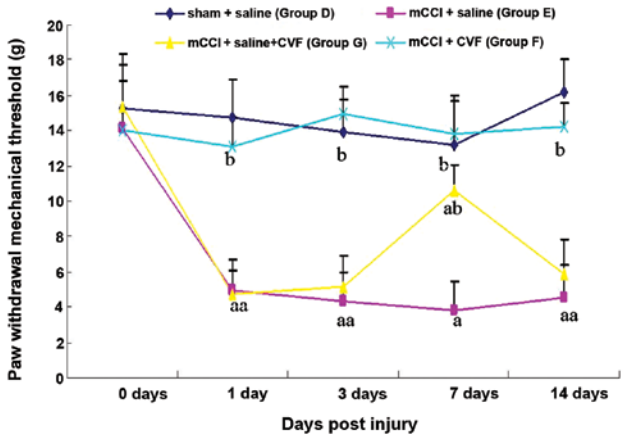

Figure 4. Effects of CVF on pain threshold. aa, P<0.01 and a, P<0.05, compared with sham + saline group; b, P<0.05, compared with mCCI + saline group. Group D showed no changes in PWMT two weeks post-surgery. Group E showed a significant decrease in PWMT. Group F had almost the same PWMT trend as Group D. Group G exhibited a CVF injection-related effect on PWMT stabilization.

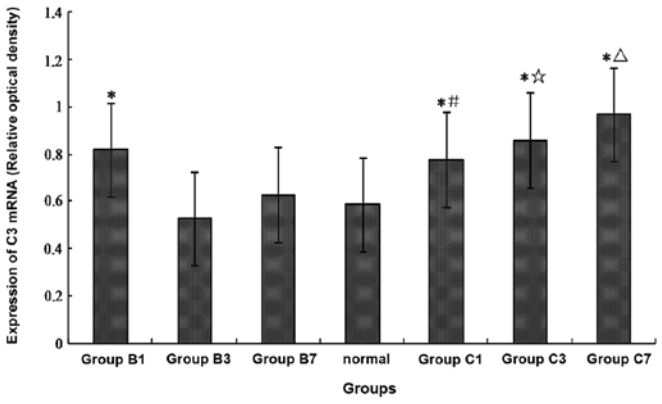

Figure 5. Expression of *C3* mRNA in spinal cords of different groups. Analysis of RT-PCR products was based on relative OD. *P<0.05, compared with the 'normal' Group A; #P<0.05, compared with Group B1; ☆P<0.05, compared with Group B3; △P<0.05, compared with Group B7.

mCCI rats show greater mechanical hyperalgesia than traditional CCI rats. To assess different pain responses to mechanical stimuli in the rats with the mCCI and traditional CCI, the PWMTs in the mCCI and traditional CCI rats were compared. The pain sensitivity of mCCI rats remained unchanged over the 14 days post-surgery, while that in the traditional group began to return to the level similar to that in the sham group beginning on Day 8 (Fig. 3). Prior to surgery, there were no significant differences in the PWMT among the three groups. The sham-operated rats exhibited no evident change in response to the von Frey hair stimulus, but relative to these the pain thresholds of the traditional CCI and mCCI groups were reduced by 53.2 and 61.9%, respectively, 2 days after surgery. Six days after the operation, the thresholds further declined by 67.0 and 78.1%, respectively, their lowest value. Although the thresholds then gradually increased until two weeks after surgery, they had significantly decreased by 65.1 and 67.8%, respectively, compared to the sham-operated group prior to surgery (P<0.01) (Fig. 3).

CVF reverses the mechanical hyperalgesia induced by mCCI. To investigate if the complement cascade reaction plays a significant role in the formation of NPP hyperalgesia, the complement activity inhibitor CVF was administered by IV 50 μg/kg prior to surgery and 20 μg/kg each day post-surgery, or by bolus IV of 50 μg/kg on Day 4 after surgery. Continuous tail vein injection of CVF completely reversed the mechanical hyperalgesia induced by mCCI (Fig. 4); bolus IV CVF showed such effects for about a week. Administration of normal saline instead of CVF in mCCI rats did not produce significant changes, when compared to the sham operation rats in mechanical hyperalgesia (Fig. 4). These results indicated that tail vein injection of CVF could reverse mechanical hyperalgesia in rats with mCCI.

The expression of C3 mRNA increases in the spinal cord of mCCI rats. One day after surgery, in the sham-operated and mCCI groups, the expressions of spinal *C3* mRNA were higher (increased by almost 40%) than those in the normal control group (Fig. 5) (P<0.01). Three and seven days after surgery, there were no statistically significant differences in the expression of spinal *C3* mRNA in the sham-operated rats when compared with the normal controls. However, in the mCCI groups, the expressions of spinal *C3* mRNA were significantly higher 3 and 7 days post-surgery, compared to either the normal controls or the corresponding 3- and 7-day sham-operated rats (Fig. 5) (P<0.01).

The number of C3-immunoreactive cells increases on the ipsilateral spinal cord in mCCI rats and the increase is reversed by CVF. To examine the effect of CVF on the expression of C3 after mCCI, we observed the morphology and number of C3-positive cells under the microscope following immunohistochemistry. In the dorsal horn of the spinal cord, segments L4-6, of the sham-operated rats there were only a few C3-positive cells, appearing as brownish yellow particles in the cytoplasm and a clear blue nuclear stain (Figs. 6 and 7). However, their numbers were almost equivalent on both the ipsilateral and contralateral sides on Days 1, 3 and 7 (Fig. 6A, B and C, respectively). By contrast, the ipsilateral side of mCCI rats had more than a 3-fold increase in C3-positive cells compared to the contralateral one day after surgery. Furthermore, in the mCCI rats, a greater number of C3-positive cells (1.5- to 2-fold) was found in the 3- and 7-day groups than in the 1-day group (Fig. 6D, E and F for Group C at 1, 3 and 7 days, respectively). However, the number of C3-positive cells in the spinal dorsal horn markedly decreased by 20% at 14 days in the rats with mCCI + CVF (Group F) (Fig. 7C), compared with those without CVF administration (Fig. 7B), and with the sham-operated rats (Fig. 7A).

We noted that C3 immunoreactivity was also observed in the cells which were similar in morphology to neurons, and we considered that C3 could be expressed by glial cells as well as by neurons.

C3 concentration increases significantly in the spinal cord homogenate of mCCI rats, and CVF inhibits the increase. Immunoturbidimetry measurement of the concentration of C3 in spinal cord homogenate showed that the content of C3

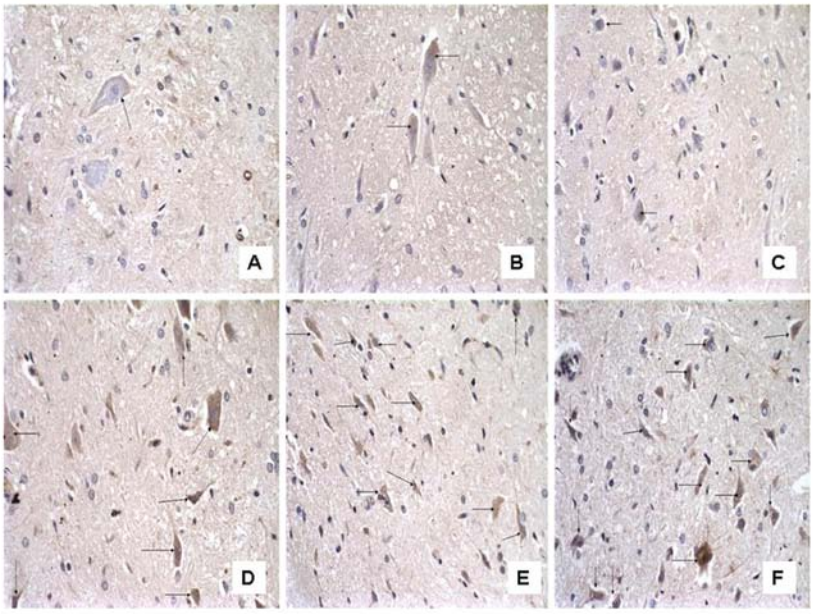

Figure 6. C3 expression observed after surgery, via immunohistochemistry, as brown granule-like substances in the cytoplasm of cells in the spinal dorsal horn (arrows). (A-C) Sham-operated rats, 1, 3 and 7 days post-surgery, respectively. Only a small amount of C3 was observed. (D-F) mCCI rats, 1, 3 and 7 days post-surgery, respectively. Significantly more C3 was found in cells of the mCCI rats compared to the sham-operated rats (x400).

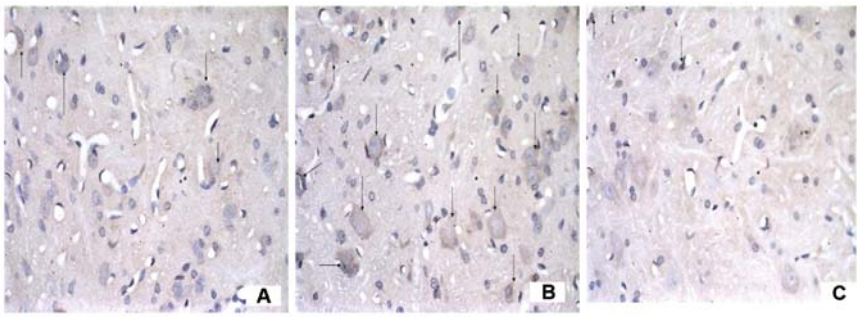

Figure 7. C3 expression (arrows) in cells of the spinal dorsal horn observed 14 days after surgery via immunohistochemistry. (A) A small quantity of C3 in sham + saline rats (Group D). (B) A large quantity of C3 in mCCI + saline rats (Group E). (C) Only a few C3-positive cells were seen in mCCI rats receiving CVF (Group F; x400).

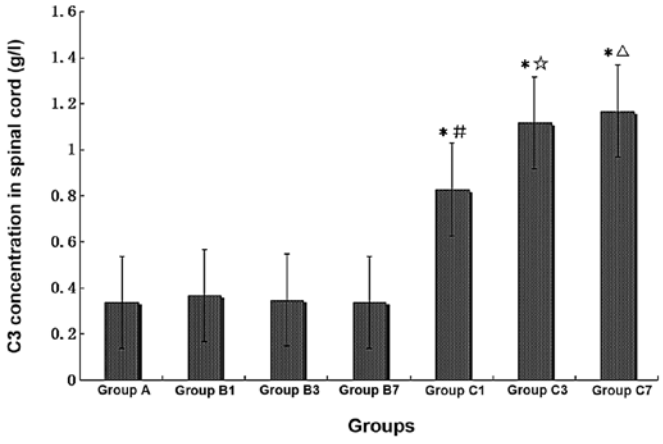

Figure 8. Comparison of C3 concentrations in rat spinal cords of different treatment groups (n=6, each group). A, normal, non-operated; B1, B3 and B7, sham-operated, 1, 3 and 7 days post-surgery, respectively; C1, C3 and C7, mCCI, 1, 3 and 7 days post-surgery, respectively. *P<0.01, compared with Group A; #P<0.05, compared with Group B1; ☆P<0.01, compared with Group B3; △P<0.01, compared with Group B7.

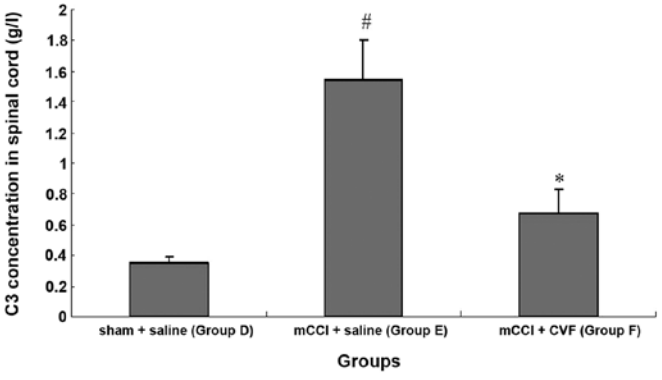

Figure 9. Effect of CVF on C3 expression in the dorsal horn of the spinal cord. #P<0.01, compared with sham + saline group; *P<0.01, compared with mCCI + saline group. The sham-operated and saline-treated rats (Group D) had a low C3 content in the spinal cord. The mCCI + saline-treated rats (Group E) presented a significantly higher C3 content in the spinal cord than the sham + saline group (P<0.01). In the mCCI + CVF-treated group (Group F), C3 was significantly lower than in the mCCI + saline group (Group E, P<0.01).

Table I. Comparison of SOD and MDA in different groups (mean ± SE).

Groups	Sham + saline (Group D)	mCCI + saline (Group E)	mCCI + CVF (Group F)
SOD (U/mgprot)	150.84±10.17	109.58±8.10[a]	121.89±9.37[a,b]
MDA (nmol/mgprot)	1.64±0.27	9.19±0.67[a]	4.71±0.52[a,b]

Compared with the sham + saline group, [a]P<0.01; compared with the mCCI + saline group, [b]P<0.01. SOD, superoxide dismutase; MDA, maleic dialdehyde.

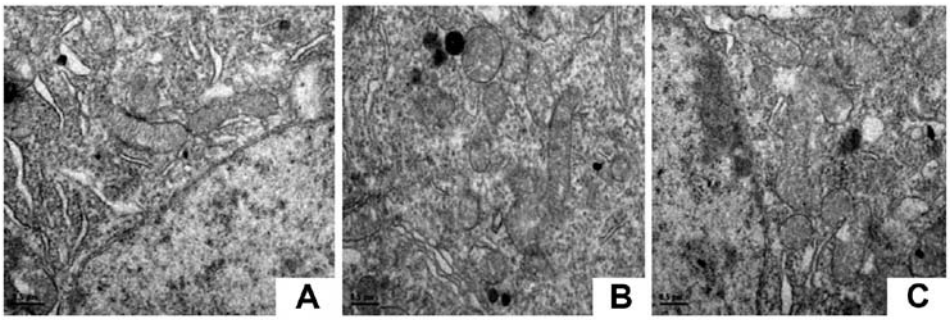

Figure 10. TEM observations (x15,000). (A) Normal morphology of mitochondria with clear outline of the spinal dorsal horn neurons in sham + saline rats (Group D). (B) Micrograph shows swelling of mitochondria, cristae breakage, obscure outline and nuclear membrane damage of the spinal dorsal horn neurons in mCCI + saline rats (Group E). (C) Mild swelling of the mitochondria and clear outline of the spinal dorsal horn neurons in mCCI + CVF rats (Group F).

was significantly elevated in spinal cord homogenate prepared from mCCI rats. When compared with the normal controls, the C3 content of the sham-operated rats was greater on Day 1 post-surgery, but comparable on Days 3 and 7. However, for the 1-, 3- and 7-day mCCI groups, the C3 content was ~2.5- to 3.5-fold higher than that of the normal control or sham-operated groups of the corresponding period (Fig. 8) (P<0.01). Furthermore, the increase in C3 in the spinal cord induced by mCCI was markedly inhibited 14 days after daily tail vein injections of CVF administered prior to and following surgery (Fig. 9) (P<0.01).

The activity of SOD decreases and the content of MDA increases in the spinal cord of mCCI rats, and CVF reverses these changes. The activity of SOD in the spinal cord decreased significantly by ~45%, while the MDA content increased 5.5-fold in the 14-day mCCI rats (Group E), when compared with the sham + saline group (Group D, P<0.01) (Table I). However, the decreased SOD activity and increased MDA level were significantly inhibited by ~50% after the mCCI rats were treated with CVF (Group F, P<0.01) (Table I).

The neuronal mitochondria are damaged in the spinal dorsal horn in the mCCI rats, and CVF attenuates the injuries. Electron micrographs showed that neuronal mitochondria and other subcellular structures in the spinal dorsal horn were intact in sham-operated rats (Group D). However, in the mCCI rats 14 days post-surgery, the neuronal mitochondria had become swollen, the cell membrane was damaged and the cristae fragmentized (Group E). Fourteen days after administration of CVF, the swelling of the neuronal mitochondria had attenuated in mCCI rats (Group F), as illustrated in Fig. 10.

PWMT is negatively correlated with C3 content in the spinal cord. The correlation between the PWMT and C3 content in the spinal cord was explored through Pearson's product moment correlation analysis. The result showed that PWMT and C3 content were well correlated, with a coefficient correlation of r=-0.899 and P<0.0001, suggesting that the pain threshold was negatively correlated with C3 content in the spinal cord, i.e., the higher the content of spinal cord C3, the greater the pain sensitivity.

Discussion

Using a model of CCI is the standard method for studying NPP (24,25). In the traditional models, it is difficult to control the level of nerve injury due to surgical variables and differences in severity of nerve ligation. These often lead to marked differences among individuals and unreliability (26,27). Additionally, autophagy is often observed in experimental animals, which not only contributes to infection in local tissues, but also augments character differences between the model and chronic NPP patients in the clinic. In the present study, we modified the construction of the CCI models by using porous films to envelop the sciatic nerve, thereby avoiding acute nerve injuries, including acute crush and incised injuries. The porosity of the film ensures material exchange between the ligated nerve and the peripheral tissue space, and thus the ligated nerves are provided with enough nutrition. The results of the present study demonstrate that the mCCI model is an ideal peripheral nerve injury-induced NPP model, as it is more stable, longer-lasting, with less autophagy and longer pain sensitivity, and it is more suitable for long-term animal observation and research.

Research on the mechanisms underlying NPP has demonstrated that it may result from the synergistic effects of central and peripheral nerve sensitization (28-30). The complement system is an essential component in human nonspecific immunity, and its activation plays a significant physiological role in clearing apoptotic cells and neuronal fragments. However, abnormal activation of complements could also cause pathological injuries to the host, and is involved in the formation and aggravation of several CNS diseases. Bonifati and Kishore (31) reported that the activation of the complement system is an important cofactor in the mechanisms of numerous CNS diseases including Alzheimer's and Parkinson's disease (31). Nevertheless, there have been few reports on the association between the complement system and the occurrence of NPP. C3 is at the crossing point of the classic, alternative, and MBL pathways of complement activation, and among the humoral components of the complement system it has the highest percentage (5). Its activating correlative fragments such as C3a, C5a and their protein complexes such as C4b2a3b and C3bBb enable its multifunctional characteristics including important roles in immunoprotection and immunoregulation (11,13). For this reason, in this study, C3 content was considered an indicator for activation of the complement system.

In the present study, mCCI-induced abnormal activation of the complements in the spinal cord dorsal horn was detected in the NPP model rats one day after surgery, and was accentuated on Days 3 and 7. The results of RT-PCR analysis also revealed that the levels of *C3* mRNA in the spinal cord dorsal horn of mCCI rats progressively increased 1, 3 and 7 days after surgery. These results were consistent with the induction and development of hyperalgesia in the mCCI rats. Moreover, Pearson's correlation analysis also proved that the expression of C3 in the spinal dorsal horn was negatively correlated with the pain threshold in these rats.

The present study also demonstrated that a bolus tail vein injection of CVF could temporarily reverse the thresholds of thermalgesia and mechanical hyperalgesia, perhaps by intervening in the expression of C3 in the spinal dorsal horn. Continuous tail vein injection of CVF was clearly able to continually inhibit hyperalgesia in mCCI rats; however, when CVF was administered only on post-surgical day 4 the effect was maintained for no more than a few days. It is possible that its inhibitory effect on the pain threshold is gradually reduced and, consequently, hyperalgesia returns when the effect of CVF administration is diminished.

Collectively, the results of the current study indicate that the complement cascade reaction takes place in the spinal cord dorsal horn after CCI of the sciatic nerve, and that complement activation plays a significant role in the formation of NPP hyperalgesia, which is consistent with previous studies. Kleinschnitz *et al* (10) developed a model of sciatic nerve constriction injury using experimental animals with complement depletion, and found a reduction of pain and a decrease in the accumulation and activity of macrophages. Moreover, Twining *et al* (9) showed that intrathecal injection of sCR1 in nerve injury rat models could inhibit activation of the complement cascade reaction, consequently attenuating pain intensity.

Currently, little is known regarding initiating factors for abnormal activation of the complements in the spinal cord and blood of NPP animal models. In physiological conditions, the concentration of C3 in serum is 300-fold higher than that in the cerebrospinal fluid (CSF). If inflammatory factors and activated complement components are able to slightly change the integrity of the blood-brain/spinal cord barrier, blood-borne complements may penetrate into the CSF and markedly elevate its concentration of complements (32). In the NPP model with ligated spinal nerve, the content of glial fibrillary acidic protein, an activated astrocyte marker, is increased in the rat spinal cord; additionally, a microgliocyte marker, OX-42, is accentuated when pain occurs (33). This indicates that astrocytes and microglial cells are activated in the pain model. These activated glial cells could synthesize and release complements, and the released inflammatory mediators can also activate the complement cascade reaction from the cells in the spinal cord (33,34). We hypothesize that an increase in complements could provoke generation of inflammatory mediators, while the inflammatory mediators in return further activate or upregulate the synthesis of complements. Thus, a positive feedback loop is formed between inflammatory mediator release and complement activation leading to inflammatory factor accumulation, which may contribute to immuno-neuropathic injury (15).

Our study showed that there was a positive correlation between a significant increase in *C3* mRNA expression in the spinal dorsal horn induced by mCCI and the occurrence of hyperalgesia. This correlation was confirmed by the inhibition of hyperalgesia affected by administration of CVF. These results suggest that C3 in the spinal dorsal horn could play an important role in the cascade reaction of complements, which may be involved in the formation of NPP.

However, it remains unclear how complement activation affects neuronal functions. We speculate that certain mechanisms related to C3 could affect cellular functions. Once complement is activated, cascade reaction occurs rapidly leading to producing end products of terminal complement C5b-9 complex, i.e. C5b-8 complex and MACs, making a pinhole in target cell membrane (35). With continual extension of the hole by multitude of C9 fragments enter the target cells, the cell membrane ruptures, and consequently, the cells are dissolved (36). It has been shown that neurons are generally not damaged by the MAC (37), therefore, we assume that there are two possible outcomes for complements released by the activated glial cells: one is that complements act on neurons in which glial cells regulate the transmission of peripheral nociceptive signals released by the neurons; the other one is to act on the neighboring glial cells and to increase their activations. Being activated by the complements, the neurons are further activated and release ATP, glutamatic acid, fractalkine, and other substances which in turn act on the glial cells and induce them to release more inflammatory factors (e.g., IL-1, IL-6 and TNF) and complements (38-40). The interaction between glial cells and neurons further boosts the activation of glial cells and the excitability of the neurons, and eventually the nociceptive signals are amplified, leading to hyperalgesia, or even allodynia.

In this study, spinal SOD activity was significantly lower but MDA levels were markedly higher in the mCCI rats, compared to those in the sham-operated rat spinal cord. Moreover, TEM revealed mitochondrial swelling, cell membrane damage, and cristae fragmentation in the neurons of the spinal dorsal horn

14 days following mCCI surgery. These results suggest that inflammatory cytokines play important roles in the abnormal activation of complements of neurons and glial cells. We also showed that tail vein injection of CVF enhanced the activity of spinal SOD but decreased the quantity of MDA, and relieved the swelling of mitochondria in the neurons of the spinal dorsal horn in mCCI rats. The results suggest that CVF inhibits or even stops the effects of the complement cascade reaction on neurons, maintains the integrity of neuronal structure and protects neuronal function, so as to regulate the sensation of pain.

As indicated by Mika (41), clinically safe and reliable intervention agents which target glial cells and the proteins they produce should be the direction of research and development in the treatment of NPP. Based on previous studies and our findings, we strongly believe that NPP can be managed by means of blocking the reaction chain of the complement activation or by improving resistance of the neuronal cell membrane to activation of the complement.

In conclusion, the mCCI animal model is more suitable for the long-term observation and research of peripheral nerve injury-induced NPP. In this study, mCCI induced a significant increase in expression of *C3* mRNA in the spinal dorsal horn, and was consistent with the occurrence of hyperalgesia. Administration of CVF reversed the hyperalgesia induced by mCCI. These results demonstrated that abnormal complement activation occurs in the dorsal horn of the spinal cord in rats with NPP, and that C3 in the spinal dorsal horn could play an important role in the cascade reaction of complements which is involved in the formation of hyperalgesia.

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