Anti-nociceptive effects of Gentiopicroside on neuropathic pain induced by chronic constriction injury in mice: A behavioral and electrophysiological study

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Abstract

Gentiopicroside (Gent) is promising as an important protective secoiridoid compound against pain. The present study was designed to investigate whether administration of Gentiopicroside would alleviate the expression of nociceptive behaviors and whether it would cause the relevant electrophysiological changes in a chronic constriction injury (CCI) model of neuropathic pain in mice. Gentiopicroside was administered from the 7th day after surgery for consecutive 8 days. Behavioral parameters and sciatic functional index were assessed immediately before surgery and on days 7, 8, 10, 12 and 14 post-CCI, and electrophysiological activities of sciatic nerve were recorded immediately after the behavioral test on the last day. Present study has shown that administration of Gentiopicroside (at a dose of 50 and 100 mg/kg.) increased behavioral parameters from the day 8 compared to the CCI-NS group. Electrophysiological data indicated that CCI caused a significant reduction in nerve conduction velocities in the sciatic nerves and the amplitudes of compound action potential, while Gent at a dose of 50 or 100 mg/kg caused a significant recovery of electrophysiological changes induced by CCI. Our data indicated that Gentiopicroside has anti-nociceptive effects on neuropathic pain induced by CCI.

Keywords:

Gentiopicroside, Neuropathic pain, Analgesia, Behavior, Electrophysiology

1. Introduction

Neuropathic pain (NP) is defined by the International Association for the Study of Pain (IASP) as “Pain caused by a lesion or disease of the somatosensory nervous system”(Haanpää et al. 2011). Neuropathic pain results from damage to the peripheral nerve, the dorsal root ganglion or dorsal root or the central nervous system which, with peripheral nerve injury. It is characterized by the sensory abnormalities such as unpleasant abnormal sensation (dysesthesia), an increased response to noxious stimulus (hyperalgesia) and pain in response to a stimulus that does not normally provoke pain (allodynia) (Woolf and Mannion 1999). Neuropathic pain is a common condition with an overall prevalence between 0.9% and 8.0%, and it is a major chronic pain condition that remains difficult to treat.(Carlton et al. 1999; O’ Connor et al. 2009; Toth et al. 2010). Previous studies suggested that individuals with NP were known to experience more severe pain compared to non-NP sufferers (O’ Connor et al. 2009). Neuropathic pain severely affected the quality of life, reduced individual productivity and increased patient and healthcare resource expenditure (Navarro et al. 2011).

Gentiopicroside (Gent) is a secoiridoid compound isolated from *Gentiana lutea* (Leguminosae), one of the most common herbal medicines used in China. Animal experiments have revealed its choleric, antihepatotoxic, antioxidant, and anti-inflammatory activities (Hase et al. 1997; Ozturk et al. 2006; Senol et al. 2012). Studies have also shown that Gent has an analgesic effect (Chen et al. 2008). However, little is known about its analgesic potential on neuropathic pain. Therefore, the present study were undertaken to explore the antinociceptive effects of Gent on neuropathic pain.

To investigate the possible parts of its antinociceptive effect, such as Gent’s effects on the function of sciatic nerve of neuropathic pain in mice, we also used electrophysiological technology to test sensory nerve conduction velocity (SNCV) and the sensory action potential (SNAP) amplitudes.

To study the mechanisms of neuropathic pain, a large number of animal nerve injury models have been developed (Colleoni et al. 1988; De Leo et al. 1994;

Decosterd et al. 2000; Fox et al. 2003; Kim et al. 1992; Seltzer et al. 1990). But chronic constriction injury (CCI) model was a widely employed for induction of neuropathic pain in experimental animals (Bennett and Xie 1988; Wang et al. 2003) due to its similarity of neuropathic pain in human. Therefore, we examined whether starting Gent treatment when symptomatology is already established would be effective against the neuropathic pain in the mice CCI model. In addition, the novel compounds pregabalin (Lyrica), which is a selective Cav2.2 ($\alpha 2$ -subunit) channel blocker and has approved clinical efficacy in neuropathic pain (Dworkin et al. 2003; Farrar et al. 2001), served as positive control in this study. The goals of the present study were to examine the anti-nociceptive effects of Gent and to investigate the possible involvement of the functional recovery of injured sciatic nerve subsequent to Gent treatment in neuropathic pain model.

2. Materials and Methods

2.1 Experiment animals

Male ICR mice weighed 18–22g were obtained from the Experimental Animal Center of Ningxia Medical University (Certificate number was SYXK Ningxia 2005-0001). The animal house was controlled at 22–24°C and relatively humidity of 45–65% under a 12h light and dark cycles. The experimental protocol was duly approved by the institutional animal ethics committee of Ningxia Medical University, Yinchuan city, Ningxia. This study complied with the internationally accredited guidelines and ethical regulations on animal research.

2.2 Compounds

Gentiopicroside (Nanjing Jingzhu Biological Science and Technology Co., Ltd. Nanjing Jiangsu) with purity 98%, sodium pentobarbital (Sigma-Aldrich, Steinheim, Germany) and pregabalin (Pfizer Manufacturing Deutschland GmbH, Betriebsstatte Freiburg) were dissolved in saline solution (0.9% NaCl) and injected intraperitoneally (i.p.) in an application volume of 0.1 ml/10 g body weight. Gentiopicroside was administered i.p. over 15 min each day for consecutive seven days in Gentiopicroside 25, 50 and 100 mg/kg groups, starting from the 8th day. Normal saline of 0.9% was administered in the same way in mice in sham operated

and CCI groups.

2.3 CCI model surgery

Neuropathic pain was induced by the chronic constriction of the sciatic nerve (CCI), which was employed according to the methods described by Bennett and Xie 's method((Bennett and Xie 1988). Briefly, mice were anesthetized by an intraperitoneal (i.p.) injection of sodium pentobarbital (0.8%). The biceps femoris and the gluteus superficialis were separated by blunt dissection, and the right sciatic nerve was exposed. Close to the bifurcation, a segment of about 7mm of the nerve was freed, and then the ligatures (4-0 silk) were tied loosely around the nerve with 1mm spacing, until a brief twitch was elicited in the respective hindlimb. This ensures an appropriate ligation was applied and a special care was taken to preserve epineural circulation. After performing nerve ligation, the muscular and skin layer was immediately sutured with thread and a topical antibiotic was applied at once. In sham-operated controls, an identical surgical procedure was performed, except that the sciatic nerve was not ligated (Hervera et al. 2010). All surgical procedures were performed under normal sterile conditions by the same person.

2.4 Experimental groups

Sixty male mice were divided into six equal groups as follows (n=10 in each group): Sham+NS; CCI+NS; CCI+ Gent (25 mg/kg); CCI+Gent (50 mg/kg); CCI+Gent (100 mg/kg) and CCI+pregabalin(10 mg/kg). CCI+NS or Gent injections were started on day 7 post-CCI and continued daily up to day 14. Behavioral parameter/observation and Sciatic functional index were assessed immediately before surgery (day 0) and on days 7, 8, 10, 12 and 14 post-CCI. Immediately after the last behavioral test, electrophysiological measurements were performed in all groups.

A total of another forty mice were used for the rota-rod test and spontaneous locomotor activity, 40 animals for each test were divided into 4 groups: CCI+NS; CCI+Gent (25 mg/kg); CCI+ Gent (50 mg/kg) and CCI+Gent (100 mg/kg). The motor coordination and spontaneous locomotor activity tests were performed on the 14th day after surgery, 15 min after the injection of agents.

2.5 Behavioral tests

All behavioral tests (mechanical, cold and thermal pain testing) and sciatic functional index were performed between the hours of 10:00 and 14:00, immediately before surgery (day 0), and on days 7, 8, 10, 12 and 14 post-CCI.

2.5.1 Mechanical allodynia (von-Frey filament testing)

In brief, mice were placed in a Plexiglas (Chengdu Technology & Market Co., Ltd, Sichuan, China) box with a wire mesh grid that allows their paws access to the von Frey filaments (North Coast Medical, Inc., San Jose, CA). Bending forces ranging from 0.008 to 3.5 g were applied using a modified version of the up-down paradigm, as previously reported by Chaplan's method (Chaplan et al. 1994). Mice were allowed to habituate themselves to the environment until exploratory behavior ceased. Beginning with the 0.4 g force, the filament was vertically stimulated between the third and fourth metatarsus or lateral plantar until it bowed slightly. The 4.0 g filament was used as a cut-off. A clear paw withdrawal, shaking or licking of the paw were considered nociceptive-like responses. Each filament was tested five times, at an interval of at least 30s. The nociceptive behavior responses appearing three or more times out of 5 tests were recorded as a positive reaction. Then, the strength of the next filament was decreased or increased according to the response (Hervera et al. 2010). The baseline values were between 1.3 and 1.5 g.

2.5.2 Cold allodynia

Cold allodynia of the hind paw was assessed by using the cold plate, previously described by Jasmin's method (Jasmin et al. 1998). The mice were placed on a metal plate (20 cm in length, 10 cm in width), the temperature of which was maintained at $4\pm 0.5^{\circ}\text{C}$, allowing access to the hind paws. Cold allodynia was reflected by the reaction of either paw withdrawal. The total numbers of observation of hind paw withdrawal, licking or shocking on the operated side were recorded during the period of 5 min.

2.5.3 Thermal hyperalgesia

For thermal hyperalgesia, paw withdrawal latency to a thermal nociceptive stimulus was assessed as described elsewhere (Hargreaves et al. 1988). The mice were placed in a PL-200 Plantar Analgesia Tester (Chengdu Technology & Market CO., LTD,

Sichuan, China) positioned on a glass surface, and were allowed to adapt to the apparatus for at least 10 min before measurements every time. The radiant heat lamp source was positioned under the plantar surface of the hind paw and was adjusted vertically to project a light spot of 5 mm in diameter onto the glass plate. The mean paw withdrawal latencies from the operated side hind paws were determined from the average of three separate trials, taken at 1 min intervals to prevent thermal sensitization and behavioral disturbances (Hervera et al. 2010). To avoid possible tissue injury, a cutoff time of 12 s was applied.

2.6 Sciatic Nerve Function—Walking test

Sciatic functional index (SFI) (de Medinaceli et al. 1982), an index of the functional condition of mice sciatic nerve based on the measurements made from walking tracks (Vrinten et al. 2003 and Yamamoto et al. 2011). To assess the degree of the nerve function, the 3 variables measured on each side were entered into the following formula(de Medinaceli et al. 1982):

$$SFI = -38.3(EPL - NPL)/NPL + 109.5(ETS - NTS)/NTS + 13.3(EIT - NIT)/NIT - 8.8$$
, where N is normal, E is experimental, PL is print length, TS is total toe spreading, and IT is the distance between intermediary toes. SFI=0, no sciatic nerve injury; SFI=-100, sciatic nerve was completely broken off.

2.7 Electrophysiological measurements

We used a BL-420F biological function experimental system(Chengdu Technology & Market CO., LTD, Sichuan, China) to record and analyze the electrophysiological data.

2.7.1 Sensory Nerve Conduction Velocities

The sensory nerve conduction velocity (SNCV) was determined in the sciatic nerve of each animal as previously described in several experimental paradigm(Dai et al. 2012; Zangiabadi et al. 2014). In a temperature-controlled environment, electrophysiological indexes were recorded in the operating sciatic nerve of test mice, under sodium pentobarbital(0.8%, 0.1ml/10g) anesthesia by intraperitoneal injection. The rectal temperature was maintained at 37°C to decrease the animal's stress due to

anesthesia. A small incision was made in the sciatic notch and ankle of the experimental side. The sciatic-sural nerve was stimulated proximally at the sciatic notch via a bipolar stimulating electrode with an interpolar distance of about 10 mm with supramaximal stimuli (1V) using a 0.2-ms rectangular pulse from BL-420F biological function determination system. After stimulation, the compound action potentials were recorded by a bipolar recording electrode, which was placed behind and below the navicular tubercle on the medial side of foot over the abductor hallucis muscle.

The sensory nerve conductive velocities (SNCV) were measured using two bipolar recording electrodes. The distance between stimulating and recording electrodes was from 10 to 14 mm. The SNCV (in meters by seconds) were calculated as the ratio of the distance between the two bipolar recording electrodes and the time difference between the two latencies ($V=S/\Delta t$). The electrophysiology studies lasted less than 30 min in each mouse (Gould et al. 2014).

2.7.2 The Sensory Nerve Action potentials Amplitudes

The sensory nerve action potentials (SNAP) amplitudes were measured from peak to peak by method of 2.7.1.

2.8 Sedative and Hypnotic effects

2.8.1 Motor coordination test

Motor coordination was measured using the YLS-4C accelerating rota-rod apparatus (Shandong Academy of Medical Science Device Station, Jinan, China) which consists of a base platform and a rotating horizontal rod (7 cm in diameter, 50 cm in length) with a nonskid surface. The rod is divided by four disks into five sections of equal length in which five mice can be tested simultaneously. Under each drum section, 26 cm below the rod was a platform. The animals were acclimatized to the revolving drum by a training run 30 min before drug testing. The rod was set to accelerate from 5 to 40 rpm in a 90 s period. The time required for the mice to fall from the drum onto the plate was recorded, with a maximum cut-off of 300 s (Kayser et al. 2003).

2.8.2 Spontaneous locomotor (exploratory) activity test

The locomotor activity test has been used to determine the side effects of medicine on the exploratory behavior of animals for 5 min (Kayser et al. 2003). In this study, locomotor activity in the open field was determined as distance covered due to horizontal movements. Spontaneous locomotor activity was measured with a computerized video tracking system in boxes with a size of 15×15 cm and 10 cm high nontransparent walls. Five mice could be measured simultaneously in five different boxes.

2.9 Statistical analysis

The analysis was performed using SPSS 16.0 software (Chicago, IL). All the results were expressed as mean±standard; *n* refers to the numbers of experimental animals. Parametric values were analyzed by one-way analysis of variance (ANOVA) followed by the LSD post hoc test. For analyzing difference between two groups where equal variances of the data were not assumed, Tamhane's T2 test was used. In all statistical analyses, a value of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1 Gent alleviated mechanical allodynia

As shown in Figure1, one day before surgery, the paw withdrawal threshold (PWT) value between groups showed no significant variation ($P > 0.05$). Seven days after surgery, mice subjected to CCI had significant mechanical allodynia as compared to the sham group ($P < 0.01$). Compared with the CCI-NS group, administration of Gent resulted in a dose-dependent reversal of PWT decrease induced by ICC. Except the lowest dose (25 mg/kg, $P > 0.05$), this effect is significant ($P < 0.05$) or highly significant ($P < 0.01$) at 50 mg/kg and 100 mg/kg, respectively. It is worthwhile to notice that pregabalin (10mg/kg) also significantly attenuated CCI-induced decrease in PWT ($P < 0.01$), while effect of Gent at 100 mg/kg is comparable to the active control on days 12 and 14.

3.2 Gent alleviated cold allodynia

The effects of Gent on cold allodynia were shown in Figure 2. As expected, the

counts of paw withdrawal value among all groups showed no significant difference ($P>0.05$) on the day before (on the day prior to) surgery. Seven days after surgery, the significant rising on the counts of paw withdrawal for mice subjected to CCI were observed ($P<0.01$) from all groups. While the counts remained constantly high over the time in the CCI-NS group, the number of counts was decreased over the time in all other CCI groups and in comparison with CCI-NS group, the significant differences were observed on days 10, 12 and 14. The results indicate that the cold allodynia induced by CCI was significantly attenuated by administration of Gent (25 mg/kg, 50 mg/kg and 100mg/kg) ($P<0.01$) and treatment of pregabalin over the time post-surgery.

3.3 Gent alleviated thermal hyperalgesia

The effects of Gent on thermal hyperalgesia in mice were shown in Figure 3. Paw withdrawal latency (PWL) were similar among groups ($P>0.05$) one day before surgery. Seven days after surgery, mice subjected to CCI showed significant reduction in latency time ($P<0.01$) compared to the sham group. Compared with the CCI-NS group, a partial, but significant or highly significant, reveal of the reduced latency time in comparison with the CCI-SN group was seen from all groups with administration of Gent ($P<0.01$ for 100 mg/kg, $P<0.05$ for 50 mg/kg groups) and positive control group treated with pregabalin.

3.4 Gent restored Sciatic Nerve Function

The recoveries of Gent on paw print's and sciatic functional index (SFI) changes in mice were shown in Figure 4 and 5. One day before surgery, there was no significant difference on sciatic functional index (SFI) among groups ($P>0.05$). Seven days after surgery, SFI was significantly reduced in the mice subjected to CCI ($P<0.01$) compared to the sham group. In contrast with the CCI-NS group, administration of Gent and pregabalin caused a recovery of SFI from the CCI-induced reduction Except Gent 25 mg/kg group, in comparison with the CCI-NS group, differences in SFI from other treatment groups were significant ($P<0.01$ for 100 mg/kg group, $P<0.05$ for 50 mg/kg group).

3.5 Gent restored sensory nerve conduction velocities

The recoveries electrophysiological parameters by Gent in mice were shown in Figure 6. The recoveries of sensory nerve conduction velocities after CCI in mice were shown in Fig.7. After the 14th day of surgery, when compared with the sham group, sensory nerve conduction velocities (SNCV) ($P<0.01$) were significantly slower in the CCI groups. Compared with the CCI-NS group, administration of Gent (50 mg/kg, 100mg/kg) and pregabalin(10mg/kg) caused remarkably recovery of the SNCV ($P<0.01$). This effect is statistically significant in Gent 50, 100 mg/kg groups and in the pregabalin group, while there was no significant variation after giving Gent (25 mg/kg) ($P>0.05$).

3.6 Gent restored sensory nerve action potentials amplitudes

The recoveries of Gent on electrophysiological changes in mice were shown in Figure 8. After the 14th day of surgery, when compared with the sham group mice of the CCI group generated significant decreased in sensory nerve action potentials (SNAP) amplitudes ($P<0.01$). Compared with the CCI group, administration of Gent (50 mg/kg, 100mg/kg) and pregabalin(10mg/kg) significantly recovered CCI induced lower in SNAP amplitudes ($P<0.01$), while there was no significant variation after giving Gent (25 mg/kg) ($P>0.05$).

3.7 Gent effects on motor coordination

The effects of Gent on motor coordination were assessed as the performance time on the rod measured from the start of acceleration until the mice fell from the drum onto the counter-trip plate. Compared with that in the CCI-NS group, the Gent (25–100 mg/kg) did not alter the rota-rod performance time in sciatic nerve-ligated mice ($P>0.05$)(Figure 9).

3.8 Gent effects on spontaneous locomotor (exploratory) activity

The effects of Gent on spontaneous locomotor activity were evaluated by the number of movements within five observation periods, where the mice were observed in a closed square-field arena. Compared with that in the CCI-NS group, the Gent (25–100 mg/kg) had no influence on spontaneous locomotor activity ($P>0.05$) (Figure10).

4. Discussion

The present investigation was carried out to evaluate the possible mechanism of analgesic activity of Gentiopicroside, the main secoiridoid compound isolated from *G. lutea*. Our data clearly demonstrated that the repeated treatment with Gent, starting intermediately (7 days after the model induction) during full establishment of neuropathy and continued for 8 days, effectively mediated a dose-dependent reversal of pain behaviors and electrophysiological responses in one of the well characterized models of neuropathic pain, the CCI model. We found that Gent at doses of 50 or 100 mg/kg is effective against CCI-induced hyperalgesia and allodynia.

An important and new aspect of our study is that Gent promotes the recovery of tactile allodynia and thermal hypersensitivity impairments with long-term treatment even when symptomatology is already established. Recent study results, including evidence of beneficial effects of Gent on acute and chronic models of pain are consistent with the present study (Chen et al. 2008; Liu et al. 2014). There was research showed that Gent inhibited expression of TNF- α and NF- κ B (Yamada et al. 2014). Many findings provide evidence that Gent has function of antioxidant defense (Yamada et al. 2014; Liu et al. 2014). Our previous study results indicate that expression of NF- κ B increased significantly in the spinal cord and oxidative effect produced by CCI mice (Xu et al. 2014). In the present study, protective effect of Gent in neuropathic pain might be related with these reasons.

Following CCI in rats, there was a marked reduction in the number of large myelinated fibers distal to the ligature, a significant loss of small myelinated fibers, ultrastructural evidence of damage to unmyelinated fibers, and a significant reduction in the size of the unmyelinated fibers (Nuytten et al. 1992). The injury of the sciatic nerve in CCI and other nerve injury models typically target nerves that were bad for both sensory and motor functions. NF- κ B stimulated TNF- α , IL-1 β , vascular endothelial growth factor (VEGF) production increased, vascular basement membrane thicker and new blood vessels to form. These led to the luminal stenosis and vascular occlusion, nerve endoneurium blood flow decreased and ischemia and hypoxia of nerve. So as to damage neurons and schwann cells to slow nerve conduction velocity

down(Lu et al. 2008). Eventually these made peripheral nerves degeneration (Suzuki et al. 2004). Evaluation of functional recovery after peripheral nerve injury mainly depend on the degree of axonotmesis and demyelination, which are commonly assessed by electromyography. Meanwhile, slower NCV is related with reflected demyelination on nerve fiber, and the lower action potential amplitudes can reflect the density of normal nerve fiber and the number of active axons (Wang et al. 2005; Lehning et al. 2000).

The reduction of SNCV and sensory nerve action potential (SNAP) amplitude ($p<0.05$) observed in our CCI mice are in agreement with the works of previous studies (Daemen et al. 1998; Wei et al. 2011; Jou et al. 2004) . Intraperitoneal injection of Gent (50, 100 mg/kg) significantly accelerated the functional recovery of sensory nerve as measured by SNCV, SNAP amplitude and SFI. The rise of significant protection from CCI-induced neuropathic pain in SNCV , SNAP amplitude of sciatic-sural nerve and SFI with 50 and 100 mg/kg doses of Gent suggested the possibility that Gent may have functional recovery effect of peripheral nerve($P<0.05$). These results of electrophysiological test have lead us to think that gentiopicroside might caused functional recovery of the injured sciatic nerve. Gent has the removal of oxygen free radicals, antioxidation, the calcium ion flow blocked and to improve the function of the nerve blood vessels. Thus it cloud protect the structure and function of myelin sheath and axon and stimulate regeneration of axons to rescue the sciatic nerve(Wang et al. 2012; Lv. 2008). At the same time, Gent can inhibit the expression of NF- κ B, TNF $-\alpha$ and other factors (Yamada et al. 2014). The experimental results were in conformity with the reports, but the exact mechanism still need further research.

We inferred that the potential analgesic drugs might be based on the experimental animal muscle relaxation effect or damaged to the animal's motor coordination function to lead that experimental animals can't make the corresponding avoidance reaction to pain (Plummer et al. 1991; Dunham and Miya 1957). Thus, we used the Rota - rod experiments to rule out false positive results of the painful behavior due to the inhibition of drugs on animal motor function. Animals' spontaneous locomotor reacted the function and status of central nervous system, activity increased when

excited and activity decreased when inhibitory. Research had shown that high doses of Gent produced the central nervous system inhibitory effect (Tu et al. 2002). Therefore, this study conducted spontaneous locomotor activity test to observe whether the drug doses in this study had central inhibitory or excited effect. This study of the experimental results show that compared with model group, Gent did not affect in rot time and the spontaneous numbers($P>0.05$). Therefore, this drug administration doses of this study, Gent did not have central inhibitory or exciting effect in mice and did not impact on its motor coordination function.

Interestingly, we found that the Gent had obvious protective effect on sensory nerve of CCI-induced mice by electrophysiology experiment. However, through the motor coordination and locomotor activity test only to find it did not have an impact on motor function. Therefore, we guessed that Gent couldn't impact on the motor nerve in neuropathic mice. These might be related to that different nerve fibers on the basis of different neurotransmitters can't form functional connectivity with target organ resulting in specific regeneration (Weiss et al. 1945; Gutmann 1945; Martini et al. 1992; Colognato et al. 2002). After nerve damaged, proliferation Schwann cells had different guiding role in regeneration of axons (Sulaiman et al. 2002).

Pregabalin is a kind of new type calcium channel regulator and have anti-anxiety, anticonvulsants and analgesic effect as $\alpha 2-\delta$ ligands. The ligand is a kind of auxiliary proteins of voltage-gated channel. It can participate in calcium influx of presynaptic voltage sensitive channels, result in nerve endings within the calcium ion flow reduced. Therefore, Pregabalin can reduce substance P, norepinephrine, glutamate and other excitatory neurotransmitter release, thus pain controlled effectively (Dong 2014; Ye et al. 2014). The nerve protective effect of Pregabalin in the past experiment were confirmed. It can prevent the myelin sheaths nerve fiber degeneration and demyelination and improve on the symptoms of peripheral neuropathy to increase the nerve conduction velocity.(Huang et al. Zhang et al. 2015). So, we choose pregabalin as the positive control drug in the present study.

In summary, previous studies have shown that Gent was effective against inflammatory pain only when started immediately after nerve injury. However,

behavioral test in the present study demonstrated that continuous daily administration of Gent when and after symptomatology of neuropathic pain (NP) has already established, can also be effective against NP. This effect may be related to its protective effect of sciatic-sensory nerve as demonstrated from the results of electrophysiological and walking test. Further studies are required to investigate the mechanisms underlying the neuroprotective effects of Gent.

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References

- Bennett G.J., and Xie Y.K. 1988. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain*, 33:87-107.
- Carlton, S.M., Du, J., Tan, H.Y., Nesic, O., Hargett, G.L., et al. 2009 . Peripheral and central sensitization in remote spinal cord regions contribute to central neuropathic pain after spinal cord injury. *Pain* , 147 : 265-276.
- Chaplan S.R., Bach F.W., Pogrel J.W., Chung, J.M., and Yaksh, T.L. 1994. Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods*, 53:55–63.
- Chen L., Liu J.C., Zhang., X.N., Guo Y.Y., Xu Z.H., et al. 2008. Down-regulation of NR2B receptors partially contributes to analgesic effects of gentiopicoside in persistent inflammatory pain. *Neuropharmacology*, 54(8):1175–1181.
- Colleoni, M., and Sacerdote, P. 2010. Murine models of human neuropathic pain. *Biochim. Biophys. Acta*, 1802:924-933.
- Colognato, H., and Yurchenco, P.D. 2002. Form and function: The laminin family of Heterotrimers . *Dev Dyn*, 18:213-234

- Daemen, M., Kurvers, H., Bullens, P., Slaaf, D., Freling, G., et al. 1998. Motor denervation induces altered muscle fibre type densities and atrophy in a rat model of neuropathic pain. *Neurosci Lett*, 247:204-208.
- Dai, C.S., Li, J.C., Lin, W., Li, G.X., Sun, M.C., et al. 2012. Electrophysiology and ultrastructural changes in mouse sciatic nerve associated with colistin sulfate exposure. *Toxicology Mechanisms and Methods*. 22(8): 592-596.
- Decosterd, I., and Woolf, C.J. 2008. Spared nerve injury: an animal model of persistent peripheral neuropathic pain. *Pain*, 87(2) :149–158.
- de Medinaceli, L., Freed, W.J., and Wyatt, R.J. 1982. An index of the functional condition of rat sciatic nerve based on measurements made from walking tracks. *Exp Neurol*, 77: 634–643.
- De Leo, J.A., Coombs, D.W., Willenbring, S., Colburn, R.W., Fromm, C., et al. 1994. Characterization of a neuropathic pain model: sciatic cryoneurolysis in the rat. *Pain*, 56 (1): 9-16.
- Dunham, N.W., and Miya, T.S. 1957. A note on a simple apparatus for detecting neurological deficit in rats and mice. *J Am Pharm Assoc Am Pharm Assoc (Baltim)*, 46(3): 208-209.
- Dworkin, R.H., Corbin, A.E., Yung, J.P., Sharma, U., La Moreaux, L., et al. 2003. Pregabalin for the treatment of postherpetic neuralgia: a randomized, placebo-controlled trial. *Neurology*, 60(8): 1274–1283.
- Farrar J.T., Young J.P., La Moreaux L., Werth J.L., and Poole, R.M. 2001. Clinical importance of changes in chronic pain intensity measured on an 11-point numerical pain rating scale. *Pain*, 94(2):149-58.
- Fox, A., Gentry, C., Patel, S., Kesingland, A., and Bevan, S. 2003. Comparative activity of the anti-convulsants oxcarbazepine, carbamazepine, lamotrigine and gabapentin in a model of neuropathic pain in the rat and guinea-pig. *Pain*, 105 (1-2): 355-362.

- Gould, H.J., Soignier, R.D., Hernandez, C., Diamond, I., and Paul, D. 2014. Ranolazine Attenuates Mechanical Allodynia Associated with Demyelination Injury Pain Medicine, 15: 1771–1780
- Gutmann, E. 1945. The reinnervation of muscle by sensory nerve fibers. J Anat, 79:1-10.
- Haanpää, M., Attal, N., Backonja, M., Baron, R., Bennett M., et al. 2011. NeuPSIG guidelines on neuropathic pain assessment. Pain. 152(1):14-27. doi: 10.1016/j.pain.2010.07.031.
- Hargreaves, K., Dubner, R., Brown, F., Flores, C. and Joris, J. 1998. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain, 32 (1) : 77-88.
- Hase, K., Li, J., Basne, P., Xiong, Q., Takamura, S., et al. 1997. Hepatoprotective principles of Swertia japonica Makino on D-galactosamine/lipopolysaccharide - induced liver injury in mice. Chem. Pharmaceut. Bull (Tokyo), 45(11):1823–1827.
- Hervera A, Negrete R, Lea´nez S, Martín-Campos, J. and Pol, O. 2010. The role of nitric oxide in the local antiallodynic and antihyperalgesic effects and expression of δ -opioid and cannabinoid-2 receptors during neuropathic pain in mice. J. Pharmacol Exp. Ther. 334:887–896.
- Jasmin, L., Kohan, L., Franssen, M., Janni, G., Goff, J.R. 1998. The cold plate as a test of nociceptive behaviors: Description and application to the study of chronic neuropathic and inflammatory pain models. Pain, 75:367–82.
- Jou, I., Chang, P., Wu, H., Wang, P., Chu, K., et al. 2004. A longterm analysis of the spinal somatosensory evoked potential of rats with sciatic constriction injury. Acta Anaesthesiol Taiwan, 42:69–76.
- Kayser, V., Farre, A., Hamona, M., and Bourgoin, S. 2003. Effects of the novel analgesic, cizolirtine, in a rat model of neuropathic pain. Pain, 104:169–177.
- Kim, S.H., and Chung, J.M. 1992. An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. Pain, 50 (3): 355-363.

- Lehning, E.J., Jortner, B.S., Fox, J.H., Arezzo, J.C., and Kitano T. 2000. gamma-diketone peripheral neuropathy. I. Quality morphometric analyses of axonal atrophy and swelling. *Toxicol Appl Pharmacol*, 165:127-140.
- Liu, L., Wong, T.P., Pozza, M.F., Lingenhoehl, K., Wang, Y., et al. 2004. Role of NMDA receptor subtypes in governing the direction of hippocampal synaptic plasticity. *Science* 2004, 304(5673):1021–1024.
- Liu, S.B., Zhang, N., Guo, Y.Y., Zhao, R., Shi, T.Y., et al. 2012. G-protein-coupled receptor 30 mediates rapid neuroprotective effects of estrogen via depression of NR2B-containing NMDA receptors. *J. Neurosci*, 32(14):4887–4900.
- Liu, S.B., Zhao, R., Li, X.S., Guo, H.J., Tian, Z., et al. 2014. Attenuation of Reserpine-Induced Pain/Depression Dyad by Gentiopicroside Through Downregulation of GluN2B Receptors in the Amygdala of Mice. *Neuromol Med*, 16(2): 350-359.
- Lu, B.C., Zhao, M., Wang, Y.T., and Di, H.J. 2008. Expression of NF- κ B of sciatic nerve in diabetic rats and its relationship with nerve conduction velocity. *Chin J Diabetes*, 16(1): 42-44
- Lv, Q.X. 2008. The research status of *G. macrophylla* Pall. and *G. straminea* Maxim. *Chinese. J. Clin. Med*, 18(8):1203-1204.
- Martini, R., Xin, Y., Schmitz, B., and Schachner, M. 1992. The L2/HNK-1 carbohydrate epitope is involved in the preferential outgrowth of motor neurons on ventral roots and motor nerves. *Eur. J. Neurosci*, 4:628-639.
- Navarro, A., Saldana, M.T., Perez, C., Torrades, S., and Rejas J. et al. 2011. A cost – consequences analysis of the effect of pregabalin in the treatment of peripheral neuropathic pain in routine medical practice in primary care settings. *BMC Neurol*, 11:7.
- Nuytten, D., Kupers, R., Lammens, M., Dom, R., Hees, J., et al. 1992. Further evidence for myelinated as well as unmyelinated fibre damage in a rat model of neuropathic pain. *Exp Brain Res*, 91:73-78.

- O' Connor, A.B. 2009. Neuropathic pain quality of life impact, costs and cost effectiveness of therapy. *PharmacoEconomics*, 27 (2) : 95-112.
- Ozturk, N., Korkmaz, S., Ozturk, Y., and Baser, K. H. 2006. Effects of gentiopicroside, sweroside and swertiamarine, secoiridoids from gentian (*Gentiana lutea* ssp. *symphyandra*), on cultured chicken embryonic fibroblasts. *Planta Medica*, 72(4): 289–294.
- Peng, P., Huang, Y., Zou, man., Rao, J., and Yu, S.Y. 2012. Protective effect of pregabalin on taxol-induced neuropathic pain in rats. *Chin. J. Cancer Prev. Treat*, 19(16): 1201-1204.
- Plummer J.L., Cmielewski, P.L., Gourlay, G.K., Owen, H. and Cousins M.J. 1991. Assessment of antinociceptive drug effects in the presence of impaired motor performance. *J Pharmacol Methods*, 26(1): 79-87.
- Rodriguez, M.J., Diaz, S., Vera-Llonch, M., Dukes, E., and Rejas, J. 2007. Cost effectiveness analysis of pregabalin versus gabapentin in the management of neuropathic pain due to diabetic polyneuropathy or post herpetic neuralgia. *Curr. Med. Res. Opin*, 23 (10): 2585-2596.
- Seltzer, Z., Dubner, R., and Shir, Y. 1990. A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. *Pain*, 43 (2):205-218.
- Senol, F. S., Tuzun, C.Y., Toker, G., and Orhan, I. E. 2012 . An in vitro perspective to cholinesterase inhibitory and antioxidant activity of five *Gentiana* species and *Gentianella caucasea*. *International Journal of Food Sciences and Nutrition*, 63(7):802–812.
- Stanika, R.I., Pivovarova, N.B., Brantner, C.A., Watts, C.A., Winters, C.A., et al. 2009. Coupling diverse routes of calcium entry to mitochondrial dysfunction and glutamate excitotoxicity. *Proc. Natl. Acad. Sci. U.S.A.* 106:9854–9859.
- Sulaiman, O.A., Midha, R., Munro, C.A., Matsuyama, T., Al-Majed, A., et al. 2002. Chronic Schwann cell denervation and the presence of a sensory nerve reduce motor axonal regeneration. *Exp Neuro*, 176(2):342-354.

- Suzuki, T., Sekido, H., Kato, N., Nakayama, Y., and Yabe-Nishimura, C. 2004. Neurotrophin-3-induced production of nerve growth factor is suppressed in Schwann cells exposed to high glucose: involvement of the polyol pathway. *J Neurochem*, 91 :1430-1438.
- Toth, C. 2011. Substitution of Gabapentin therapy with pregabalin therapy in neuropathic pain due to peripheral neuropathy. *Pain Med* , 11: 456-465.
- Tu, R.S., Zhang G.X., and Sun, B.H. 2002 . Research on independent activity patterns of mice. *Chinese Pharmacological Bulletin*, 18(4):464-465.
- Vrinten, D.H., and Hamers, F.F. 2003. ‘CatWalk’ automated quantitative gait analysis as a novel method to assess mechanical allodynia in the rat; a comparison with von Frey testing. *Pain*, 102: 203–209.
- Wang, H., Sorenson, E., Spinner, R., and Windebank, A. 2008 . Electrophysiologic findings and grip strength after nerve injuries in the rat forelimb. *Muscle Nerve*, 38:1254-1265.
- Wang, L., and Wang, Z. 2003. Animal and cellular models of chronic pain. *Adv Drug Deliv Rev*, 55: 949-965.
- Wang, Q.S., Xie, K.Q., Zhang, C.L., Zhu, Y.J., Zhang, L.P., et al. 2005. Allyl Chloride-induced time dependent changes of lipid peroxidation in rat nerve tissue. *Neurochem Res*, 30:1387–1395.
- Wang, Y., Zeng, W.X., Song, X.L., Chen, Y.F., and Zhang, A.L., et al. 2012. Pharmaceutical research development and clinical application compatibility of Gentiopicroside. *Northwest Pharmaceutical Journal*, 27(5):502-505.
- Wei, S.M., Zhao, Z.H., Chen, J., and Wang, X.L. 2011. Effect of anti-inflammation analgesic injection combined with extract from rabbit skin inflamed by vaccinia virus on sciatic nerve injury in rats. *Chin J Anesthesiol*, 31:50-54.
- Weiss, P., and Edds, M. 1945. Sensory-motor nerve crosses in the rat. *J Neurophysiol*, 8:173-193.

- Woolf, C.J., and Mannion, R.J. 1999 . Neuropathic pain: Aetiology, symptoms, mechanisms, and management. *Lancet* , 353:1959–1964.
- Xu, Y.Q., Jin, S.J., Liu N, Li Y.X., Yu J.Q., et al. 2014. Alopentine attenuated neuropathic pain induced by chronic constriction injury via anti-oxidation activity and suppression of the nuclear factor kappa B pathway. *Biochem Biophys Res Commun*, 451(4):568-73.
- Yamada, H., Kikuchi, S., Inui, T., Takahashi, H., and Kenichi K. 2014. Gentiolactone, a Secoiridoid Dilactone from *Gentiana triflora*, Inhibits TNF- α , iNOS and Cox-2 mRNA Expression and Blocks NF- κ B Promoter Activity in Murine Macrophages. *PloS One*, 9(11):e113834. doi: 10.1371
- Yamamoto, M., Okui, N., Tatebe, M., Shinohara, T., and Hirata, H. 2011. Regeneration of the perineurium after microsurgical resection examined with immunolabeling for tenascin-C and alpha smooth muscle actin. *J Anat*, 218: 413–425.
- Ye, M., Zhang, M., and Yu, J. 2014. Therapeutic effect of pregabalin and alpha lipoic acid on diabetes mellitus with painful neuropathy. *chin J Diffic and Compl Cas*, 12(8) : 820-823.
- Zhang, Y., and Shen, J. 2015. Pregabalin joint lipoic acid injection in the treatment of painful diabetic neuropathy: efficacy and safety analysis. *J. Clin. Exp. Med*, 14(20): 1704—1706.
- Zangiabadi, N., Mohtashami, H., Hojatipour, M., Jafari, M., Asadi-Shekaari, M., et al. 2014. The Effect of Angipars on Diabetic Neuropathy in STZ-Induced Diabetic Male Rats: A Study on Behavioral, Electrophysiological, Sciatic Histological and Ultrastructural Indices. *Scientific World Journal*. 2014:721547. doi: 10.1155/2014/721547.

Legends for illustrations

Figure 1. Effects of Gentiopicroside on mechanical allodynia in von Frey test. Fifteen minutes after administration of Gentiopicroside (25, 50 and 100 mg/kg), the paw withdrawal threshold (g) to von Frey filaments were measured at different days (days 8, 10, 12, 14). Data were obtained seven days after surgery, and the mean $\pm$ SD is shown, n=10 per group. * P <0.05 compared with the sham+NS group; # P <0.05 and ## P <0.01 versus the CCI+NS group.

Figure 2. Effects of Gentiopicroside on cold allodynia in the cold-plate test. Fifteen minutes after administration of Gentiopicroside (25, 50 and 100 mg/kg), the numbers of paw lifting from the cold plate were measured at different days. Data were obtained from the day prior to (the same day as) surgery and days 7, 8, 10, 12, 14 post-surgery, and the mean $\pm$ SD is shown, n=10 per group. * P <0.05 compared with the sham+NS group and # P <0.05 and ## P <0.01 versus the CCI+NS group.

Figure 3. Effects of Gentiopicroside on thermal hyperalgesia in the Plantar test. Fifteen minutes after administration of Gentiopicroside (25, 50 and 100 mg/kg), the paw withdrawal latencies to radiant heat were measured at different days (days 8, 10, 12, 14). Data were expressed as the mean $\pm$ SD, n=10 per group. * P <0.05 compared with the sham+NS group and # P <0.05 and ## P <0.01 versus the CCI+NS group.

Figure 4. Fifteen minutes after administration of Gentiopicroside (25, 50 and 100 mg/kg), the walking pawprint were measured at last time (day 14). (A) Sham+NS group. (B) CCI+NS group. (C) Positive control (pregabalin) group. (D), (E), (F) administration of Gentiopicroside (25, 50 and 100 mg/kg) group.

Figure 5. Effects of Gentiopicroside on sciatic functional index in walking test. Fifteen minutes after administration of Gentiopicroside (25, 50 and 100 mg/kg), the sciatic functional index to walking pawprint were measured at different days (days 8, 10, 12, 14). the mean $\pm$ SD is shown, n=10 per group. * P <0.05 compared with the sham+NS group and # P <0.05 and ## P <0.01 versus the CCI+NS group.

Figure 6. Representative traces of compound action potential (CAP) of sciatic-sural nerve in mice at 14 days after administration of Gentiopicroside at a dosage of 25, 50, 100mg/kg for 7 days. (A) CAP trace of the sham+NS. (B) CAP trace of the CCI+NS.

(C) CAP trace of the positive control (pregabalin). (D), (E), (F) Traces of CAP in different dosages of Gent (25, 50, 100mg/kg), respectively.

Figure 7. Effects of the sciatic nerve chronic constriction injury (CCI) and Gentiopicroside on the sensory nerves conduction velocity (SNCV) examined on day 14 after the surgery. Gentiopicroside (25, 50 and 100mg/kg, i.p.) was injected daily on days 7~14 post-surgery. Data were obtained on day 14 after surgery, and the mean $\pm$ SD is shown, n=10 per group. * P <0.05 compared with the sham+NS group and $^{\#}P$ <0.05 and $^{\#\#}P$ <0.01 versus the CCI+NS group.

Figure 8 . Effects of the sciatic nerve chronic constriction injury (CCI) and Gentiopicroside on the sensory nerve action potential (SNAP) amplitudes examined on day 14 after the surgery in CCI mice. Gentiopicroside (25, 50 and 100mg/kg, i.p.) was injected daily on days 7~14 post-surgery. Data were obtained on day 14 after surgery, and the mean $\pm$ SD is shown, n=10 per group. * P <0.05 compared with the sham+NS group and $^{\#}P$ <0.05 and $^{\#\#}P$ <0.01 versus the CCI+NS group.

Figure 9. Effects of Gentiopicroside on motor coordination test. Effects of Gentiopicroside at different dosages on motor coordination test after chronic constriction of sciatic nerve, compared with the CCI+NS group. Data are expressed as mean $\pm$ SD, n=10 per group.

Figure 10. Effects of Gentiopicroside on the spontaneous locomotor activity test. Effects of Gentiopicroside at different dosages on spontaneous locomotor activity test after chronic constriction of sciatic nerve compared with the CCI+NS group. Data are expressed as mean $\pm$ SD, n=10 per group.

Figure 1.
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