

Warming acupuncture affects TTX-R sodium current of DRG and PGE2 of KOA rats.

Yong-Liang Zhang^{1,2}, Jia-Hong Gang¹, Hua-Min Wang¹, Yi-Qun Mi^{1*}

¹Department of Acupuncture and Massage, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Shanghai, PR China

²Second Hospital of Hebei Medical University, Shijiazhuang, PR China

Abstract

Background: This study aimed to elucidate mechanism of analgesic effect of warming acupuncture on Knee Osteoarthritis (KOA).

Methods: A total of 40 SD rats were randomly divided into control group, KOA group, Pharmacotherapy (PT) group and Warming Acupuncture (WA) group. Rats in PT and WA groups received intra-articular injection of papain into bilateral knees. In PT group, rats were intragastrically treated with meloxicam; in WA group, rats received warming acupuncture. Then, Tetrodotoxin Resistance (TTX-R) sodium channel current of Dorsal Root Ganglion (DRG) and serum PGE2 were measured.

Results: TTX-R sodium channel current was significantly different among groups ($P < 0.01$). When compared with control group, TTX-R sodium channel current increased significantly in KOA group ($P < 0.01$). When compared with KOA group, TTX-R sodium channel current reduced markedly in PT group and WA group ($P < 0.01$). It was suggested that WA and PT were able to reduce TTX-R sodium channel current to exert certain analgesic effects. In addition, WA and PT also reduced peripheral serum PGE2 to a certain extent in rats with KOA.

Conclusions: Warming acupuncture may reduce TTX-R sodium channel current of DRG and decrease inflammatory PGE2 to improve inflammatory hyperalgesia, exerting analgesic effect.

Keywords: Warm puncture, Osteoarthritis, Tetrodotoxin-insensitive sodium channel current, Rat dorsal root ganglion.

Accepted on January 2, 2016

Introduction

Knee Osteoarthritis (KOA) is a degenerative disease characterized by knee cartilage degradation, reactive sclerosis and hyperplasia of the subchondral bone. Clinically, symptoms of KOA include joint pain, stiffness, swelling and limitation of joint motion. Severe KOA may cause joint deformity and even loss of joint function. Traditionally, KOA is treated with non-steroidal anti-inflammatory drugs. Dorsal Root Ganglion (DRG) is a primary sensory neuron. On the basis that skin is innervated by spinal nerves L2-4, it was speculated that DRG of spinal nerves L2-4 were associated with pathogenesis of KOA. Studies have confirmed that acupuncture may exert analgesic effects on KOA in clinical practice, but the specific mechanism is still poorly understood. This study was undertaken to investigate potential mechanism underlying analgesic effects of acupuncture on rats with early KOA.

Knee osteoarthritis belongs to the category of "heumatism" of "polioplus" in traditional Chinese medicine. The etiology and pathogenesis of polioplus is described in Neijing, indicating that the basic of the disease is liver and kidney deficiency, and

its reality is that wind, cold, and wet occurs together. Therefore therapy about reversing the insufficiency and decreasing the wind, cold, and wet showed good curative effect [1].

Warming acupuncture belongs to moxibustion, it is the embodiment of the "if cold, warm it". When adding a moxibustion on needle, the heat reaches the disease part along the needle while not act directly on the skin, which makes the thermal penetrates acupoints and joint to promote blood circulation, remove blood stasis, warm menstruation and relief pain. A study has proved that the effect of warm acupuncture is better than simple acupuncture [1]. Modern researches [1-5] have confirmed that warming acupuncture can repair function of articular cartilage. Such repair effect of warming acupuncture is associated with related growth factors, such as transforming growth factor β , bone protection factor, basic fibroblast growth factor, insulin-like growth factor 1, etc. All these factors have similar effect to promote cartilage cell proliferation and repair cartilage. A number of studies have demonstrated that warming acupuncture can increase the growth factor expression level [6-8], therefore plays an important role in promoting the repair of articular cartilage.

Material and Methods

Animals and grouping

This study has been approved by the Ethic Committee of The Sixth People's Hospital, School of Medicine, Shanghai Jiaotong University (2014-10-2J). And international guidelines for animal welfare were followed. A total of 40 male SD rats (specific pathogen free) weighing 250 ± 50 g and aging 3 months were randomly divided into control group, KOA group, WA group and PT group (n=10 per group).

Establishment of KOA model in rats

The KOA model was established by intra-articular injection of 1.6% papain into bilateral knee joint cavities according to a previously report [9] at three time points (1, 4 and 7 days, 0.1 ml per joint). On the basis that super fatigue may cause KOA, rats were allowed to run on a roller for 30 min daily for 2 weeks. Then, stable KOA was observed in rats.

Treatments

Different treatments were administered in 4 groups: 1) control group: rats were intragastrically injected with normal saline as equal volume as in PT group and fixed on a table without other treatments. 2) KOA group: rats were fixed on a table, but did not receive any treatment; 3) WA group: warming acupuncture was done at bilateral anterior knee acupoints. Localization of anterior knee acupoint was anterior knee of hind limb. Acupuncture of anterior knee acupoint was able to exert analgesic effects and regulate hind limb functions. 4) PT group: meloxicam (7.5 mg/tablet; Shanghai Boehringer Ingelheim Pharmaceutical Co., Ltd. H20020217) was grounded and dissolved in normal saline into a 0.5 mg/ml solution. Rats were intragastrically injected with 3.75 mg/kg meloxicam once a day and also fixed as in WA group. After treatment for 20 days, rats were sacrificed, serum and DRG were collected, and ELISA was performed to detect *PGE2* with rat *PGE2* ELISA kit (RD company). The whole-cell patch-clamp technique was employed for detection of TTX-R sodium channel current.

Detection of serum PGE2 by ELISA

The serum was thawed to room temperature and ELISA kits were allowed to warm to room temperature. There were 14 standard wells and 2 non-specific wells, and samples were added to the remaining wells. First, 150 μ l of reagent or sample was added to each well, followed by addition 50 μ l of primary antibody solution (except for non-specific wells). The plates were sealed and incubated with constant shaking at 500 ± 50 rpm for 1 h. Then, 50 μ l of *PGE2* binding solution was added to each well, and the plate was sealed, followed by incubation with a constant shaking for 2 h. After washing with wash buffer for 4 times, 200 μ l of substrate solution was added to each well, followed by incubation in dark for 30 min. Then, 100 μ l of stop solution was added to each well, and absorbance was measured within 30 min at 450 nm. Wavelength at 540-570 nm was used if wavelength adjustment was possible;

measurement was done at 450 nm, 540 nm and 570 nm if wavelength adjustment was impossible, and the results of absorbance at 450 nm minus that at 540 nm or 570 nm was used as a final absorbance.

Patch-clamp technique

The neurons were clamped at -70 mV and stimulated with step voltage at -70~40 mV (step=10 mV; duration=30 ms). TEA at 2 mM and CdCl₂ at 100 μ M were added independently to block potassium current and inward calcium current, and the total sodium current was measured. Then, TTX at 250 nM was added to block TTX-S sodium current, and TTX-R sodium current was measured. The peak of sodium current at depolarization was subtracted and final TTX-R sodium current was expressed as current/membrane capacitance (pA/pF). Origin 8.0 software was used to delineate I-V curve of TTX-R sodium current, current at corresponding voltage was recorded, and averages were obtained. The difference in I-V curve was compared.

Statistical analysis

SPSS version 18.0 was employed for statistical analysis, and data were expressed as means \pm standard deviation ($\bar{x} \pm s$). Comparisons of means among groups were done with Student-Newman-Keuls or least significant difference t. A value of $P < 0.05$ was considered statistically significant.

Results

Comparisons of serum PGE2 in rats

Rats were intraperitoneally anesthetized with 5 mg/ml at 4 mg/100 g body weight, and blood (5-10 ml) was collected into a centrifuge tube, followed by centrifugation at 3000 r/min for 3 min. Serum was harvested for detection of *PGE2*. Results of normality test and homogeneity of variance test showed that data displayed nearly normal distribution and had homogeneity of variance. Thus, t-test was employed. Results showed that peripheral serum *PGE2* was significantly different among groups ($P < 0.01$). Compared to KOA group, serum *PGE2* reduced significantly in the other groups ($P < 0.01$), but there was no significant difference between Warming Acupuncture (WA) group and Pharmacotherapy (PT) group ($P > 0.05$). In addition, significant difference in serum *PGE2* was also observed between WA group and control group ($P < 0.01$).

TTX-R sodium channel current

Neurons were clamped at -70 mV, and TTX-R sodium channel current of DRG at whole-cell mode was measured in control group, KOA group, WA group and PT group (Figures 1A-1D). Results of data analysis showed that there were significant differences of TTX-R sodium channel current among four groups. Current density was calculated according to the peak of TTX-R sodium channel current. Results showed that current density displayed in normal distribution, with heterogeneity of variance. Thus, non-parametric Kruskal-Wallis H test and

Mann-Whitney U test were employed. Results showed there were significant differences in TTX-R sodium channel current among groups ($P < 0.01$). When compared with control group, density of TTX-R sodium channel current in KOA group increased significantly ($P < 0.01$); when compared with KOA group, current density in WA group and PT group reduced significantly ($P < 0.01$). There was no significant difference in current density between WA group and PT group ($P > 0.05$).

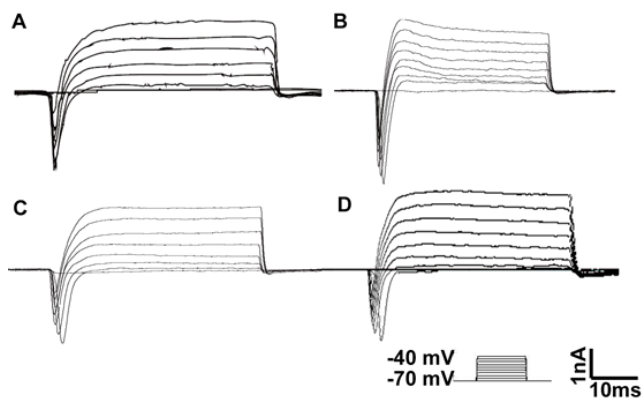


Figure 1. A: TTX-R sodium current in blank control group. B: Amplitude of TTX-R sodium current in KOA group increased significantly when compared with control group ($P < 0.05$); C: TTX-R sodium current density in WA reduced markedly as compared to KOA group ($P < 0.05$); D: TTX-R sodium current density in PT reduced markedly as compared to KOA group ($P < 0.05$).

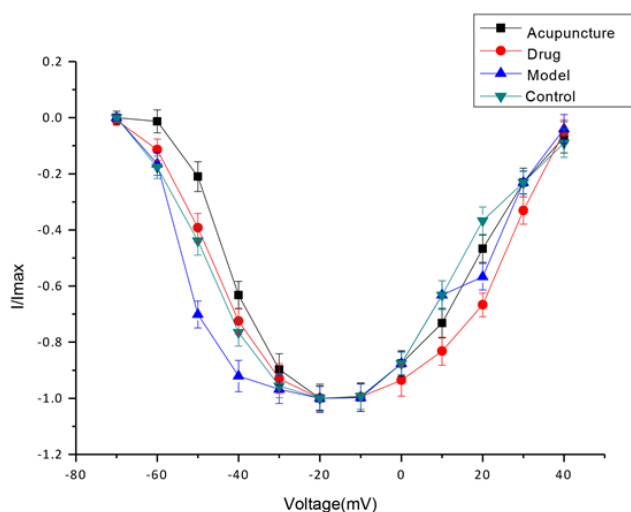


Figure 2. The I-V curves of TTX-R sodium current were similar in 4 groups. Current was first measured at -70 mV and peaked at -20 mV ($P > 0.05$).

On the basis of voltage-dependence of TTX-R sodium current, the I-V curve was delineated. A current at a specific voltage was recorded, and averages were obtained. A I-V curve of TTX-R sodium current was V-shaped, a TTX-R sodium current was gradually activated and peaked at an amplitude of -20 mV. Thereafter, current amplitude reduced gradually, and TTX-R sodium current became inactivated. These suggested voltage-dependence of TTX-R sodium current (Figure 2). Bothman equation was employed to fit a curve according to current and voltage (Figure 3). There was no significant difference in $V_{1/2}$

among 4 groups (Figure 1C). The slope factor K (2.123) in WA group was comparable to those in PT group (2.078) and control group (2.32). Taken together, current-voltage dependence of TTX-R sodium current remained unchanged.

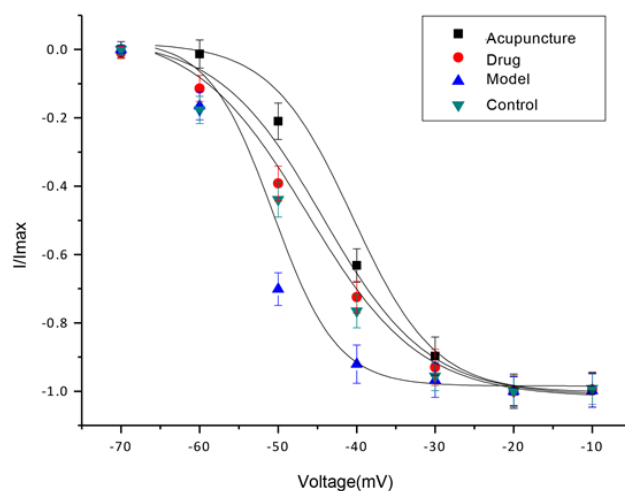


Figure 3. Bothman equation was used to fit an equation according to the standardized current and depolarizing voltage. Significant difference was not observed in the slope factor $V_{1/2}$ among 4 groups ($P > 0.05$).

Discussion

Mechanism of pain is complex, which generally divides into nerve pathological pain and inflammatory pain. Knee osteoarthritis pain belongs to the inflammatory pain. TTX-R sodium channel current testing on the DRG of electrophysiology was used to determine the presence of inflammatory pain. And inflammatory pain occurs with a variety of inflammatory cytokines. *PGE2*, among many inflammatory cytokines, can be used as clinical biomarker of early detection of joint damage. So the combination of those two was tested in the present study.

Prostaglandins are metabolites produced by arachidonic acid cyclooxygenase and their production undergoes a series of enzyme-mediated catalyses. *PGE2* acts via activating a group of G protein-coupled receptors including EP1, EP2, EP3 and EP4 [10]. *PGE2* has been found to play an important role in acute inflammation and may act on peripheral circulation system to induce congestion and oedema. In EP knockout mice, results showed prostaglandins including *PGE2* may exert both pro-inflammatory and anti-inflammatory effects, which are dependent on the regulation of corresponding genes [11]. *PGE2* may not only promote osteogenesis, but stimulate bone absorption. *PGE2* at a low concentration induces osteogenesis, but *PGE2* at a high concentration may cause bone absorption [12]. After warming acupuncture in rats with KOA, ELISA was performed to detect nitric oxide (NO) and *PGE2* in knee synovial fluid and serum, and results showed that NO and *PGE2* increased significantly in rats with KOA when compared with control rats; and warming acupuncture could reduce elevated NO and *PGE2* in the knee synovial fluid and serum when compared to untreated KOA rats. This suggested

that the increase of *PGE2* was closely related to pathogenesis of KOA and might be an important participant in pathogenesis and pathology of KOA; and warming acupuncture might exert therapeutic effects [13].

TTX-R sodium ion channel current is mainly produced by NaV1.8 and NaV1.9 [14]. This current is formed due to inward sodium fluxing into cells. TTX-R sodium ion channel current is involved in depolarization activation of action potential and maintenance of cell excitation [15]. In addition, it also plays an important role in peripheral pain transmission [16]. The abnormal TTX-R sodium ion channel current may cause hyperalgesia, and trigger neuropathic and inflammatory pains [17,18]. DRG has both TTX-S and TTX-R sodium ion channel currents which play crucial roles in pathophysiology of different pains. TTX-R is mainly produced in sensory neurons and involved in pain transmission [19,20]. Especially, TTX-R plays important roles in the pathogenesis of chronic inflammatory and neuropathic pains [21]. DRG is a primary neuron in pain transmission and usually does not produce action potential spontaneously. With presence of inflammation, sodium channel is opened, and depolarizing sodium current is produced to form action potential, which causes frequent discharge, reduces threshold of mild pain and delays progression of inflammatory hyperalgesia [22,23]. The reduced threshold of sodium channel activation in neurons may cause frequent formation of action potential, signal transmission increase and cell excitation elevation, which further deteriorate cell swelling and cause cell apoptosis [24-26].

Although available studies reported that warming acupuncture might exert favourable analgesic effects on inflammatory pain, the specific mechanism is still unclear. In the present study, whole-cell patch clamp technique was employed to detect TTX-R sodium channel current of DRG in control group, KOA group, WA group and PT group. Results of this study showed that warming acupuncture could significantly reduce density of TTX-R sodium channel current. This indicated that warming acupuncture reduced number of opened sodium channels and decreased inward sodium flux; depolarization compensation of action potential reduced; action potential might not be induced; refractory period of neurons prolongs and frequency of signal transmission reduced. Moreover, threshold of TTX-R sodium channel current activation reduced which increased activation potentials of NaV1.8 and NaV1.9, and then decreased discharge frequency of DRG. The reduced density of TTX-R sodium channel current may also reduce discharge frequency of neurons and improve hyperexcitability of DRG [27,28], exerting protective effects on neurons. Reflex arc of pain transmission center in sensor was blocked, which reduced stimulation of central nervous system by pain signals, and thus pain was alleviated.

PGE-2 plays an important part in the pathogenesis of knee osteoarthritis. In this study, warm acupuncture treatment affected the concentration of *PGE-2* in serum and joint fluid and reduced the high excited state DRG ionization sodium channel, promoting new ion channels and nerve fiber hyperplasia and transforming the high sensitivity of the

peripheral pain, thus decreasing the central nervous sensitization and playing a role in analgesia. By the in-depth study about the link of warm acupuncture affecting *PGE-2* concentration in serum and joint fluid and DRG TTX-R channel current density change in rats with knee osteoarthritis, we provided reliable experimental basis for acupuncture treatment of knee osteoarthritis and guidance of the clinical curative effect. Besides, we explained the relationship between DRG TTX-R Na current with the mechanism of pain. Pain exists in knee osteoarthritis, and it is inflammatory pain. The generation mechanism of inflammatory pain is related to DRG TTX-R Na current. The detection of DRG TTX-R Na current determined the degree of inflammatory pain. The TTX-R Na current data showed that the inflammatory pain was relieved, which further confirmed the curative effect for knee osteoarthritis, and at the same time further inferred that the key for the treatment of knee osteoarthritis may be through blocking the DRG TTX-R Na current.

In WA group, TTX-R sodium channel current was still voltage dependent, suggesting that physiological voltage dependence of sodium channel remained unchanged. In the present study, density of TTX-R sodium channel current reduced in both WA group and PT group, but there was no significant difference between them. It is suggested that both warming acupuncture and pharmacotherapy exerted favourable therapeutic effects on KOA. And when reducing the number of open sodium channels, flow in the sodium ions is decreased, depolarization compensation of action potential is reduced, and the action potential cannot be triggered, refractory period of neurons cannot be extended, and signal conduction frequency is slowed. Meanwhile, the no longer decrease of TTX-R sodium channel current activation threshold causes NaV1.8 and NaV1.9 channel activation potential increase, thereby reducing the dorsal root ganglion discharge frequency. Overall TTX-R sodium channel current density is reduced, and neuron discharge frequency is reduced at the same time, which improves the high excitability of dorsal root ganglion neurons [27] and shows protective effect on neurons. Receptors of nociceptive transmission backbone of reflex arc are impeded, thus reducing the stimulation of pain signals in the central nervous system. The corresponding clinical symptoms are expressed as pain relief. However, combination use of warming acupuncture and pharmacotherapy required further studies.

Conclusions

In the present study, results indicated that TTX-R sodium channel played an important role in pathogenesis of pain secondary to KOA, and warming acupuncture was able to inhibit TTX-R sodium channel current to exert analgesic effects. In addition, blocking TTX-R sodium channel may become one of the directions in future studies on KOA.

Acknowledgements

The study was supported by Three-year Program of Traditional Chinese Medicine for Special Talents of Shanghai Health and

Family Planning Committee (ZY3-PCPY-3-1018) and Traditional Chinese Medicine Fund of Traditional Chinese Medicine (2012J012A).

References

1. Xiong GP, Huang Q, Dong YF, Chen P, Luo T. Thirty cases of knee osteoarthritis treated by acupuncture and warming needle moxibustion. *Chin Acup Moxi* 2011; 31: 551-552.
2. Zhang Q, Yue J, Lu Y. Acupuncture treatment for chronic knee pain: study by Hinman under estimates acupuncture efficacy. *Acupunct Med* 2015; 33: 170.
3. Zhang Q, Yue J, Sun Z, Lu Y. Acupuncture for chronic knee pain: a protocol for an updated systematic review. *BMJ Open* 2016; 6: e008027.
4. Witt C, Brinkhaus B, Jena S, Linde K, Streng A. Acupuncture in patients with osteoarthritis of the knee: a randomised trial. *Lancet* 2005; 366: 136-143.
5. Liu SL, Jiang HX. Electroacupuncture treatment for Knee Osteoarthritis: a case report. *Eur J Bio Med Res* 2015; 1: 44-45.
6. Shimomura K, Kanamoto T, Kita K, Akamine Y, Nakamura N, Mae T, Yoshikawa H, Nakata K. Cyclic compressive loading on 3D tissue of human synovial fibroblasts upregulates prostaglandin E2 via COX-2 production without IL-1beta and TNF-alpha. *Bone Joint Res* 2014; 3: 280-288.
7. Mastbergen SC, Lafeber FP, Bijlsma JW. Selective COX-2 inhibition prevents proinflammatory cytokine-induced cartilage damage. *Rheumatology (Oxford)* 2002; 41: 801-808.
8. Luo QF, Wang X. The role of PGE2 in bone formation and bone reconstruction. *J Moder Stomatol* 2005; 19: 518-521.
9. Sun LN, Zhao YH, Huang GC, Xia JL. Relationship between synovium pathological change and injection timing in rabbits with papain-induced knee osteoarthritis. *J Clin Rehab Tissue Engineer Res* 2011; 15: 9311-9313.
10. Loffredo FS, Pancoast JR, Cai L, Vannelli T, Dong JZ. Targeted delivery to cartilage is critical for in vivo efficacy of insulin-like growth factor 1 in a rat model of osteoarthritis. *Arthritis Rheumatol* 2014; 66: 1247-1255.
11. Lin WZ, Wang P. Experimental acupuncture. Shanghai Sci Technol Press 1999.
12. Hua XB, Zhou HL. The development of rat acupoint Atlas. *Exp Animals Animal Exp* 1991; 1: 1-5.
13. Tjalsma H, Boleij A, Marchesi JR, Dutilh BE. A bacterial driver-passenger model for colorectal cancer: beyond the usual suspects. *Nat Rev Microbiol* 2012; 10: 575-582.
14. Dib-Hajj SD, Binshtok AM, Cummins TR, Jarvis MF, Samad T. Voltage-gated sodium channels in pain states: role in pathophysiology and targets for treatment. *Brain Res Rev* 2009; 60: 65-83.
15. Momin A, Wood JN. Sensory neuron voltage-gated sodium channels as analgesic drug targets. *Curr Opin Neurobiol* 2008; 18: 383-388.
16. Su YY, Ye M, Li L, Liu C, Pan J, Liu WW, Jiang Y, Jiang XY, Zhang X, Shu Y, Bao L. KIF5B promotes the forward transport and axonal function of the voltage-gated sodium channel Nav1.8. *J Neurosci* 2013; 33: 17884-17896.
17. Dib-Hajj SD, Cummins TR, Black JA, Waxman SG. Sodium channels in normal and pathological pain. *Annu Rev Neurosci* 2010; 33: 325-347.
18. Ekberg J, Jayamanne A, Vaughan CW, Aslan S, Thomas L, Mould J, Drinkwater R, Baker MD, Abrahamsen B, Wood JN, Adams DJ, Christie MJ, Lewis RJ. muO-conotoxin MrVIB selectively blocks Nav1.8 sensory neuron specific sodium channels and chronic pain behavior without motor deficits. *Proc Natl Acad Sci USA* 2006; 103: 17030-17035.
19. Rugiero F, Mistry M, Sage D, Black JA, Waxman SG, Crest M, Clerc N, Delmas P, Gola M. Selective expression of a persistent tetrodotoxin-resistant Na⁺ current and NaV1.9 subunit in myenteric sensory neurons. *J Neurosci* 2003; 23: 2715-2725.
20. Tan ZY, Piekarczyk AD, Priest BT, Knopp KL, Krajewski JL, McDermott JS, Nisenbaum ES, Cummins TR. Tetrodotoxin-resistant sodium channels in sensory neurons generate slow resurgent currents that are enhanced by inflammatory mediators. *J Neurosci* 2014; 34: 7190-7197.
21. Joshi SK, Honore P, Hernandez G, Schmidt R, Gomtsyan A. Additive antinociceptive effects of the selective Nav1.8 blocker A-803467 and selective TRPV1 antagonists in rat inflammatory and neuropathic pain models. *J Pain* 2009; 10: 306-315.
22. Lai J, Gold MS, Kim CS, Bian D, Ossipov MH. Inhibition of neuropathic pain by decreased expression of the tetrodotoxin-resistant sodium channel, Nav1.8. *Pain* 2002; 95: 143-152.
23. Galbavy W, Safaie E, Rebecchi MJ, Puopolo M. Inhibition of tetrodotoxin-resistant sodium current in dorsal root ganglia neurons mediated by D1/D5 dopamine receptors. *Mol Pain* 2013; 9: 60.
24. Zheng Q, Fang D, Cai J, Wan Y, Han JS, Xing GG. Enhanced excitability of small dorsal root ganglion neurons in rats with bone cancer pain. *Mol Pain* 2012; 8: 24.
25. Chen J, Gong ZH, Yan H, Qiao Z, Qin BY. Neuroplastic alteration of TTX-resistant sodium channel with visceral pain and morphine-induced hyperalgesia. *J Pain Res* 2012; 5: 491-502.
26. Ross GR, Gade AR, Dewey WL, Akbarali HI. Opioid-induced hypernociception is associated with hyperexcitability and altered tetrodotoxin-resistant Na⁺ channel function of dorsal root ganglia. *Am J Physiol Cell Physiol* 2012; 302: C1152-1161.
27. Oda A, Iida H, Tanahashi S, Osawa Y, Yamaguchi S, Dohi S. Effects of alpha2-adrenoceptor agonists on tetrodotoxin-resistant Na⁺ channels in rat dorsal root ganglion neurons. *Eur J Anaesthesiol* 2007; 24: 934-941.
28. Kharatmal SB, Singh JN, Sharma SS. Rufinamide improves functional and behavioral deficits via blockade of tetrodotoxin-resistant sodium channels in diabetic neuropathy. *Curr Neurovasc Res* 2015; 12: 262-268.

***Correspondence to**

Yi-Qun Mi

Department of Acupuncture and Massage

Shanghai Jiao Tong University Affiliated Sixth People's
Hospital

PR China