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Original Article

Yiqi Huayu recipe relieves nerve root constriction induced radicular neuralgia by down-regulating TRPV4 expression in dorsal root ganglion

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Abstract: The aim of this study was to observe the effects of Yiqi Huayu recipe on TRPV4 expression in radicular neuralgia model induced by chronic constriction to the rat lumber nerve root. Healthy male SD rats were divided into 3 groups for radicular neuralgia (RN) model construction: the sham operation group, model groups (day 3, 7, 14 and 28), and medication groups (day 3, 7, 14 and 28). Von-Frey hairs test was performed to detect the 50% with drawal threshold (50% TPW) for rats of each group. The expression of TRPV4 in dorsal root ganglion was detected at both mRNA and protein level. Rats from all model groups displayed hyperalgesia with significantly reduced 50% TPW values compared with sham-operation group (P<0.01); Yiqi Huayu recipe medication groups showed higher 50% TPW than model group since 7 days post medication (P<0.01); the medication groups showed decreased TRPV4 expression than that of model groups (P<0.01). In conclusion, Yiqi Huayu recipe alleviates nerve root constriction induced radicular neuralgia by repressing TRPV4 expression in dorsal root ganglion.

Keywords: Dorsal root ganglion, hyperalgesia, TRPV4

Introduction

Frequently caused by noxious stimuli including mechanical pressure and inflammatory stimulus on dorsal root ganglion (DRG), radicular neuralgia (RN) is the common symptom of spinal diseases [1]. Clinically speaking, RN could be originated from DRG lesions caused by lumbar disc protrusion, spinal canal stenosis, lumbar spinal instability, hypertrophy of ligamentum flavum, zygapophyseal joints hyperplasia, tumors in vertebral canal, or other spinal space occupying diseases [2-4].

Among numerous ion channels in DRG neuron membrane, transient receptor potential vanilloid channel 4 (TRPV4) plays important roles in RN caused by DRG lesions [5, 6]. TRPV4 is a non-selective cation channel with medium permeability to Ca^{2+} and the sensory transducer of harmful stimulus caused by hypotonic stimulation [7]. The happening of Ca^{2+} influx through TRPV4 occurs upon the perception of ambient pressure. The elevated cellular Ca^{2+} level not only elites series of physiological responses, including releasing of pain transmitters and activation of PKA/PKC signaling pathway [8], but also activates Ca^{2+}/calmodulin dependent protein kinases that produce chains of pathological reactions [9]. TRPV4 is widely expressed in various tissues. Immunohistochemistry evidence showed that TRPV4 is localized in membrane, cytoplasmic vesicles, and nucleus of DRG neurons in rat [5, 10].

Following the dialectical theory of Shi's traumatology (yi qi wei zhu, yi xue wei xian) in spinal degenerative diseases, professor Qi She, from Shanghai University of Traditional Chinese Medicine, proposed the basic rule of Yiqi Huayu for the treatment of secondary RN caused by spinal degenerative diseases, and originally created the Yiqi Huayu recipe [11]. The preliminary study showed that Yiqi Huayu recipe composing *Astragalus mongholicus* and *Ligusticum wallichii* could effectively reduce inflammatory responses of RN, inhibit hypernomic permeability of capillary hyperplasia, abate content of

Table 1. 50% TPW values post mechanistic stimuli for each group ($\bar{x}$ ± s)

Groups	Basal	Day 3	Day 7	Day 14	Day 28
Sham	17.7±0.82	17.2±1.22	18.0±1.31	16.5±0.98	17.1±0.82
Model	17.5±0.79	8.7±1.16$^{\Delta\Delta}$	4.7±0.67$^{\Delta\Delta}$	8.0±0.94$^{\Delta\Delta}$	11.2±0.79$^{\Delta\Delta}$
Medication	17.1±0.97	8.1±0.88$^{\Delta\Delta}$	13.4±0.70$^{\Delta\Delta,**}$	15.7±0.82**	16.8±0.63**

Note: For comparisons with sham group, $^{\Delta}$ and $^{\Delta\Delta}$ represent P<0.05 and P<0.01, respectively; for comparisons between corresponding model and medication group, * and ** represent P<0.05 and P<0.01, respectively.

inflammatory mediators (PGE2 and PLA2), and improve microcirculation [11], while the underlying molecular mechanism is largely unknown. In this study, we generated the rat RN model via DRG constriction. Based on the model, we observed the TRPV4 expression changes and therapeutic effects of Yiqi Huayu recipe at different time points of constriction on DRG.

Materials and methods

Animals

Ninety male rats (SPF, 3 months, weight 250-300 g, certificate number: 2007000542991) were purchased from Shanghai SLAC experimental animal, LLC. These rats were randomly divided into 3 groups: 10 for sham-operated group, 40 for model group, and 40 for medication group. The model and medication groups were further evenly divided into day 3, 7, 14 and 28 groups.

Waist DRG constriction model and medication

This model was constructed based on the previously described constriction model with silica gel sheet. Silica gel sheets (2×2×1 mm, 10±1.5 mg) were disinfected in 75% ethanol for 2 hours and stored in bromo-geramine. The rats were anaesthetized by intraperitoneal injection of etamine (0.1 ml/100 g). After fur shaving around waist and hip, rats were regularly disinfected and covered with aseptic towel. Incision was made to the middle right of L4-L5 spinous process. After consecutive exposure of skin, lumbodorsal fascia, lumbar longissimus muscle, multifidus, and musculi semispinalis dorsi, blunt dissection was made to expose processus transversus. The premade silica gel sheet was horizontally inserted into the foramen of L4-L5 to exert pressure on DRG. After sutures and analepsia, the rats were raised in cage for observation. The sham group underwent the same operation procedures except for the insertion of silica gel sheet. The Yiqi Huayu recipe was composed of 15 g *Astragalus mongholicus*, 12 g *Codonopsis pilosula*, 9 g *Ligusticum wallichii*, 9 g *Salvia miltiorrhiza*, and 0.03 g muscone. Rats from day 3, 7, 14 or 28 medication groups were subjected to 28 days' intragastric administration of water decoction of the recipe 3, 7, 14 or 28 post-surgery days, respectively. Medication was performed twice a day.

$$\text{Daily dose (per rat)} = \frac{\text{Daily dose (per human adult)} \times \text{Body surface of rat}}{\text{Body surface of human}}$$

DRG sampling

The rapid DRG isolation was performed according to the previous method [12]. The rats were deeply anaesthetized by injection of 2% pentobarbital sodium (50 mg/kg). After decapitation, the lumbar spine was peeled and DRG was isolated and stored in liquid nitrogen.

Threshold testing of 50% paw withdraw (50% TPW)

The sham and experimental groups were subjected to 50% TPW testing 3, 7, 14, and 28 post-surgery days. The up-down method developed by Dixon was used for 50% TPW measurement [13]. Testing was started at 2.0 g stimulus with Von Fry fiber pen to the middle of rear paws for 6-8 s. In the absence of a paw withdrawal response (negative, labeled as '○'), a stronger stimulus was exerted, while in the event of paw withdrawal (positive, labeled as '×'), a weaker stimulus was chosen. The paw withdrawal during or right after a stimulus was defined as positive response. The testing should be done while the rat was still. After the first positive response, 5 more tests were carried out with 5 s interval between two consecutive tests.

$$5\%\ \text{TPW} = \frac{10^{[xf + \kappa\delta]}}{1000}$$

Where X_f = value (in log units) of final von Frey hair used, κ = tabular value for the pattern of positive/negative responses, and δ = mean difference (in log units) between stimuli.

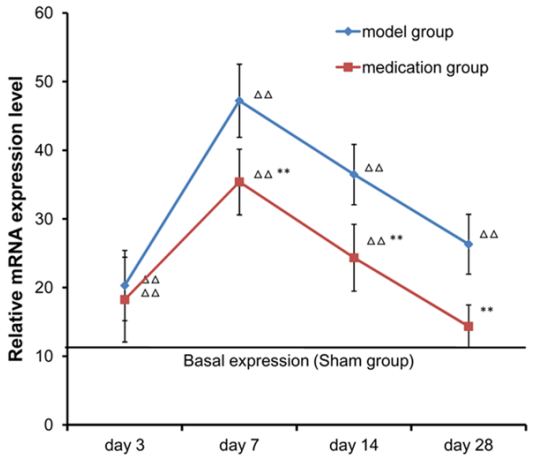

Figure 1. *TRPV4* mRNA expression for each group. Basal line indicates the *TRPV4* mRNA expression level of sham group. For comparisons with sham group, $^{\Delta}$ and $^{\Delta\Delta}$ represent P<0.05 and P<0.01, respectively; for comparisons between corresponding model and medication group, * and ** represent P<0.05 and P<0.01, respectively.

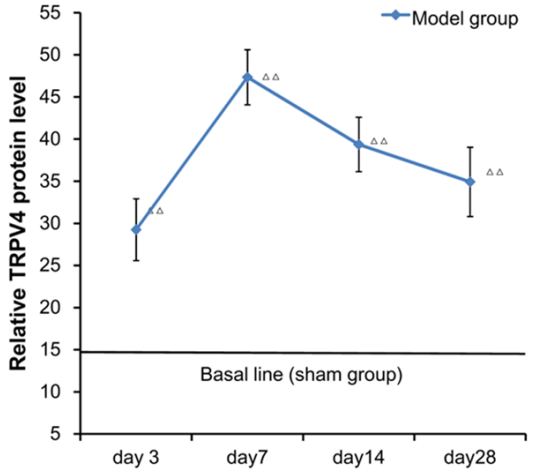

Figure 2. Expression changes of TRPV4 protein in each group's DRG neuron. Basal line indicates the TRPV4 protein level of sham group. For comparisons with sham group, $^{\Delta}$ and $^{\Delta\Delta}$ represent P<0.05 and P<0.01, respectively.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA for each group was extracted with DRN from each group. For each sample, 2 μg total RNA was used for cDNA synthesis (Promega, U.S.A.). SYBR master mix (Shanghai Jingmei Biological Engineering, Co., Ltd.) was used for qRT-PCR (Lightcycler 2.0, Roche, Switzerland). Primers for each gene were purchased from Life technologies, U.S.A. TRPV4 forward: 5'-AAGTGGCGTAAGTTCGGG-3'; TRPV4 reverse: 5'-TAAGGGTAGGGTGGCGTG-3'; β-actin forward: 5'-AGACCTTCAACACCCCAG-3'; β-actin reverse: 5'-CACGTTTCCCTCTCAGC-3'.

Western blotting

Total protein was extracted with DRN samples from each group. The protein samples were quantified with Bradford method, separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and electro-transferred onto polyvinylidenedifluoride (PVDF) membrane. After blocking, the membrane was incubated with respective primary antibody (TRPV4 antibody: Alomone, Israel; β-actin antibody: Abcam, U.K.) and horseradish peroxidase conjugated secondary antibody consecutively. The protein bands were visualized with Flurochem 9900-50 gel imaging system (Alpha Innotech, U.S.A.).

Statistical analysis

SPSS10.0 was used for all the statistical analysis. Comparison between groups was performed with pairwise T-test, whereas comparisons among several groups were carried out with analysis of variance.

Results

Yiqi Huayu recipe relieves RN caused by constant mechanical constriction

After surgery, rats from all the model groups displayed significantly reduced 50% TPW values than sham-operation group (P<0.01) (**Table 1**). The 50% TPW value of model group declined to trough at 7 days post surgery. Below the basal line of sham group, the 50% TPW of model group raised up slowly since 7 days post-surgery. Three days post recipe intervention, both model and medication groups showed lower 50% TPW values than sham group (P<0.01), while no 50% TPW difference between model and medication group was observed (P>0.05) (**Table 1**). After medication, rats from day 7, 14 and 28 groups had higher 50% TPW values when compared with responsive model groups (P<0.01). And after more than 14 days' medication, the pain sensitivity recovered to the level of sham-operation group (P>0.05).

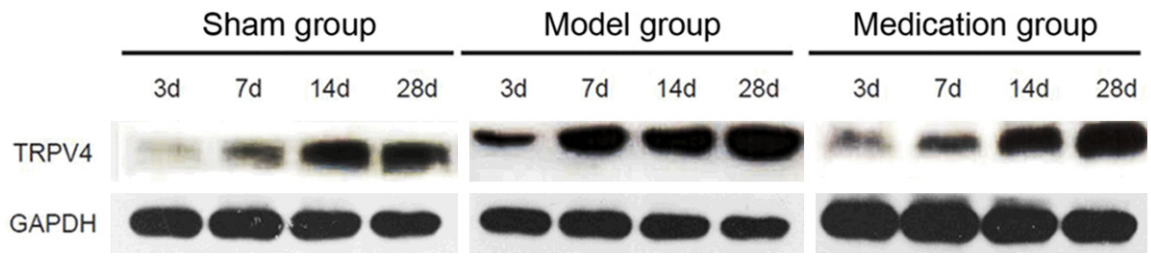

Figure 3. Expression of TRPV4 protein for all groups. GAPDH was served as internal reference.

Yiqi Huayu recipe down-regulates RN induced TRPV4 expression

Constant mechanistic constriction up-regulated TRPV4 expression at both mRNA (**Figure 1**) and protein levels (**Figures 2** and **3**). Compared with sham group, enhanced TRPV4 expression was observed in all the model groups at both mRNA and protein level ($P<0.01$). Day 3, 7 and 14 medication groups still had higher level of *TRPV4* mRNA compared with sham group ($P<0.01$), while no significant difference of *TRPV4* mRNA level was observed between day 28 medication group and sham group ($P>0.05$). Three days post medication, no expressional difference of *TRPV4* mRNA was developed between model and medication group ($P>0.05$), whereas day 7, 14 and 28 medication group had lower *TRPV4* mRNA expression than day 7, 14 and 28 model group, respectively ($P<0.01$).

Discussion

Previous reports showed that constant constriction on rat's DRG significantly induced the expression of TRPV4, abated both mechanical and hyperthermal pain threshold values, increased the ratio of neurons responsive to hypotonic solution and 4α-PDD in DRG, and elevated cytoplasmic Ca^{2+} concentration, while TRPV4 knockdown partially rescued the abnormal algesia [14, 15]. Our results showed that 50% TPW value and TRPV4 expression were positively correlated with the constriction time length, further confirming the importance of TRPV4 in RN by DRG constriction. Following Yiqi Huayu recipe intervention, the down-regulated expression of TRPV4 and relief of RG indicate that Yiqi Huayu could alleviate RN by down-regulating TRPV4 expression.

The classical route of drug development is to develop selective ligand that acts on single disease target to obtain highly effective and safe drug with low side effects, while there is a decline in the number of new drugs launched into the clinical field in the past decades [16]. The main explanation to this phenomenon is the fact that the pathogenesis of most diseases is multi-factorial rather than single target involved. The complexity of Chinese traditional medicine makes it possible to act on multiple targets of a certain disease simultaneously [17]. The inhibitory effect of Yiqi Huayu recipe on the transcription of *TRPV4* is the functional output of many active ingredients within the recipe. The transcription factors (TFs) binding to *TRPV4* promoter are the potential targets of Yiqi Hueyu recipe. By analyzing consensus motifs within *TRPV4* promoter and utilizing chromatin immuno-precipitation technologies, TFs directly regulating *TRPV4* transcription could be identified and studied. Further investigations could be made to check the responses of the above TFs upon the treatment of Yiqi Huayu recipe.

In conclusion, our results indicate that Yiqi Huayu recipe could alleviate RN caused by constant constriction on DRG through repressing TRPV4 expression in rat model. Our findings are helpful for better understanding to molecular mechanism of RN, and informative for the development of effective therapeutic methods against RN.

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Disclosure of conflict of interest

None.

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