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ETHANOLIC EXTRACT OF *ACONITI BRACHYPODI* RADIX ATTENUATES NOCICEPTIVE PAIN PROBABLY VIA INHIBITION OF VOLTAGE-DEPENDENT NA^+ CHANNEL

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Abstract

Aconiti Brachypodi Radix, belonging to the genus of *Aconitum* (Family Ranunculaceae), are used clinically as anti-rheumatic, anti-inflammatory and anti-nociceptive in traditional medicine of China. However, its mechanism and influence on nociceptive threshold are unknown and need further investigation. The analgesic effects of ethanolic extract of *Aconiti Brachypodi* Radix (EABR) were thus studied *in vivo* and *in vitro*. Three pain models in mice were used to assess the effect of EABR on nociceptive threshold. *In vitro* study was conducted to clarify the modulation of the extract on the tetrodotoxin-sensitive (TTX-S) sodium currents in rat's dorsal root ganglion (DRG) neurons using whole-cell patch clamp technique. The results showed that EABR (5-20 mg/kg, i.g.) could produce dose-dependent analgesic effect on hot-plate tests as well as writhing response induced by acetic acid. In addition, administration of 2.5-10 mg/kg EABR (i.g.) caused significant decrease in pain responses in the first and second phases of formalin test without altering the PGE_2 production in the hind paw of the mice. Moreover, EABR (10 μg/ml -1 mg/ml) could suppress TTX-S voltage-gated sodium currents in a dose-dependent way, indicating the underlying electrophysiological mechanism of the analgesic effect of the folk plant medicine. Collectively, our results indicated that EABR has analgesic property in three pain models and useful influence on TTX-S sodium currents in DRG neurons, suggesting that the interference with pain messages caused by the modulation of EABR on TTX-S sodium currents in DRG neurones may explain some of its analgesic effect.

Key words: *Aconiti Brachypodi* Radix; analgesic effect; dorsal root ganglion; sodium channel

Introduction

The medical use of Aconitum spans many centuries. For example, *aconite tuber*, the roots of aconite, is traditionally used in China and other countries to therapeutically increase peripheral temperature, relieve rheumatic pain, treat neurological disorders, and improve cardiovascular function (Sato et al., 1979; Hikino et al., 1980). However, the genus *Aconitum* consist of more than 200 species in China, the toxicities, effectiveness, and phytochemistry are diversified by their phylogeny (Xiao et al., 2006). Among these species *Aconiti Brachypodi* Radix (雪上一枝蒿 xuě shàng yī zhī hāo; the dried roots of *Aconitum brachypodum* Diels, Family Ranunculaceae) is well known for its anti-rheumatic and analgesic properties. It is mainly distributed in Yunnan and Sichuan provinces in China. Its Chinese name "snow grass" refers to the fact that it grows in altitudes up to 3000 m. The local people always apply the tubers and roots for various diseases, such as collapse, syncope, rheumatic fever, painful joints, gastroenteritis, diarrhea, oedema, bronchial asthma, various tumors, and some

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endocrinal disorders like irregular menstruation.

However, the analgesic mechanism of this folk plant remains unclear. From the anatomical point of view, dorsal root ganglion (DRG) where the sensory nerve in the abdomen and the visceral afferent sensory fibers gathered is the link between internal and external environment of spinal cord. The DRG neurons change the information regarding injury and pain in the peripheral nerve into nerve impulses which would be transmitted to the nerve centre by primary afferent nerve fibers (Gebhart, 2000).

Accumulation and increased membrane density of Na^+ channels have been detected histochemically in injured DRG axons, at sites proximal to the nerve transsection (England et al., 1996). Since the tetrodotoxin-sensitive (TTX-S) voltage- gated sodium channels in primary sensory neurons play an important role in pain-generating mechanism (Waxman, 1999), the present study was undertaken to describe the analgesic effect of ethanolic extract of *Aconiti Brachypodi* Radix (EABR) and explore the underlying mechanisms. In this study, three algesic models, i.e. hot plate model, acetic acid-induced writhing response and formalin -induced licking response models of mice were established, demonstrating most of the functional characteristics of various painful circumstances including central, peripheral, and inflammatory pain (Du et al., 2007). To examine the analgesic impact and pain-generating mechanisms of EABR, whole-cell patch-clamping technique was used to record TTX-S sodium currents in cultured DRG induced by EABR.

Materials and methods

Extract preparation

The dried roots of *Aconiti Brachypodi* Radix were purchased commercially from Bozhou City, Anhui province in China in Sept. 2010. The plant was authenticated by Dr. Wan Dingrong, Professor in Pharmacognosy at School of Pharmacy, South-central University for Nationalities. The voucher specimen (No. 20101003) was deposited at the Herbarium of South-Central University for Nationalities. The dried roots of *Aconiti brachypodi* Radix (1 Kg) were ground into powder and submerged in 95% ethanol (8 L × 3, each 3 days) and left to macerate for three times. The combined solution was filtered and evaporated to complete dryness using a standard Buchi rotary-evaporator. The extract was dissolved in MeOH and identified by co-TLC (thin-layer chromatography). The result revealed that the extract contained some foregone alkaloids such as bullatine A, aconitine, mesaconine and hypaconitine, whose R_f values were 0.67, 0.18, 0.15 and 0.37, respectively, which coincide with the previous study (Liu, 1983). For animal tests, the extract (EABR) was freshly dissolved in dimethyl sulfoxide (DMSO, Amresco, USA) and diluted by normal saline at the desired concentrations just before use. For whole -cell patch-clamp experiment, the extract was made freshly in the external solution. The final DMSO concentration in the solution was not more than 0.5%.

Animals and administration

One-month-old male or female Wistar rats (100-150 g) as well as female Kunming mice (20–25 g) were obtained from Center of Laboratory Animals of Hubei Province (Wuhan, P.R. China). The animals were maintained in a room with controlled temperature (22 ± 2 °C) for 12 h light/dark cycle with free access to food and water. Twelve hours before each experiment animals received only water, in order to avoid food interference with substances absorption. The animals were handled in accordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of the People's Republic of China on November 14, 1988. The Reference was made to principles of laboratory animal care, and was approval by the local ethical committee at South-Central University for Nationalities (No. yxy20100908). Mice intragastrically (i.g.) received EABR (1-20 mg/kg), control animals intragastrically

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received the same volume of normal saline (20 ml/kg) and positive control animals intragastrically received aspirin (200 mg/kg) or morphine (5 mg/kg).

Chemicals and reagents.

The Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin used in this study were obtained from Hyclone (Logan, Utah, USA). Acetic acid was obtained from Merck (Darmstadt, Germany). Aspirin was obtained from Asilikang Pharmaceutical Group Co. Ltd., China. Morphine was obtained from the Northeast Pharmaceutical Group Co. Ltd., China. Four authentic alkaloids, bullatine A (Product ID: 110859), aconitine (Product ID: 110720), mesaconine (Product ID: 110799) and hypaconitine (Product ID: 110798) were obtained from National Institutes for Food and Drug Control in China with more than 99% purities. External solution used to record TTX-S voltage-gated sodium currents in DRG neurones contained (mmol/L) NaCl 125.0, TEA-Cl 20.0, CsCl 5.0, $CaCl_2$ 1.8, $MgCl_2$ 1.0, HEPES 5.0, D-Glucose 25.0 and TTX 0.001. The external solution was adjusted to pH 7.4 with 1 mol/L TEA-OH and osmolarity was 313 mOsm. Internal solution contained (mmol/L) CsCl 70.0, CsF 70.0, NaCl 5.0, EGTA 2.0, HEPES 5.0 and TTX 0.001. The internal solution was adjusted to pH 7.4 with 1 mol/L CsOH and osmolarity was 267 mOsm. TEA-Cl, CsCl, HEPES, TTX, TEA-OH, CsF, EGTA and CsOH all came from Sigma (USA). All other chemicals were of analytical grade unless otherwise stated.

Hot-plate test

Mice were individually placed on a hot-plate maintained at 55 ± 0.5 ^{o}C and the time of licking of the hind paws or attempt to jump out of the beaker was recorded as the latency period (Esmaeili-Mahani et al. 2010). The cut-off time was 60 s to avoid tissue damage. Before drug administration, baseline latency was examined. Animals showing any latency lower than 5 s or higher than 30 s were rejected. The paw withdrawal latency was tested after 30 min of drug administration. The maximum possible effect (MPE) was calculated as: MPE%= (latency after drug administration−baseline latency)/ (60−baseline latency) ×100.

Writhing response induced by acetic acid

After 30 min of drug administration, 0.6% acetic acid saline solution (10 ml/kg) was injected intraperitoneally. Immediately after challenge, mice were placed in separate boxes, numbers of writhing responses consisting of contraction of the abdominal muscle together with stretching of the hind limbs, were counted over a period of 20 min (Dai et al., 2002).

Hind paw licking response induced by formalin

After 30 min of drug administration, the formalin test was performed as described previously (Hunskaar and Hole, 1987; Matsuda et al., 1997) with slight modification. In brief, 1.4% formalin (0.5% formaldehyde), 25 µl was injected under the surface of the right hind paws using a microsyringe. The amount of time spent licking the injected paw was counted with a chronometer and was considered as an indication of pain. The licking time in the early phase (0-5 min) and late phase (15-30 min) represented the neurogenic and inflammatory pain responses, respectively. Lastly, the mice were sacrificed and two hind paws were cut. After weighed respectively, the tissues were minced and homogenated. The homogenate was centrifuged (2500 rpm, 10 min) and the supernatant was prepared for PGE_2 and protein detection.

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Measurement of PGE_2

The amount of PGE_2 in the tissue of mouse hind paw was measured using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's (R&D, Minneapolis, MI) instructions. Protein concentration was measured using Coomassie blue protein-binding assay using bovine serum albumin (BSA) as a standard.

Whole-cell patch-clamp experiment

Whole-cell patch-clamp experiment was conducted as previously described (Liu et al., 2004; 2006). Briefly, Wistar rats (100 -150 g) were knocked out and decapitated, DRG was taken out. The cell suspension was obtained using enzymatic dissociation. The suspension was filtrated through 200 mesh gauze and transferred into a 35-mm culture dish to keep still. The solution was replaced by the external solution twice after the cells were adhered to the disk. Whole-cell patch clamp recordings were carried out using an EPC-9 amplifier (HEKA, Germany). Voltage-clamp commands were delivered and currents were recorded using the Pulse software (Version 8.5, HEKA, Germany). Current signals were low-pass filtered at 2.9 KHz (Bessel) and digitized at 10 KHz (Bessel). Only large DRG neurons (approximately 30 μm) were used for experiments as this kind of cells usually expressed a high percentage of TTX-S sodium currents (Tate et al., 1998). Patch pipettes were fabricated with boro-silicate glass capillary tubes (inner diameter = 1.4 mm) on a 2-stage puller (PIP-5, HEKA, Germany). Recording pipettes had resistances of 2-5 MΩ when filled with internal solution. The formation of 1±5 GΩ seal between a pipette and DRG membrane was facilitated by applying a weak suction to the interior of the pipette. After gigaseal formation, membrane capacitance was compensated (c-fast). With a gentle suction, the membrane was ruptured and membrane capacitance was compensated (c-slow) again. Thus the whole-cell voltage-clamp configuration was obtained and TTX-S sodium currents were recorded at a holding potential. Drugs were delivered using a solution exchange system (DAD-12, ALA, USA) and the distance from the mouth of tubule to the cell examined was less than 100 μm. Before the next record, the residual drug was washed out with the external solution until the sodium currents recovered completely. All the experiments were performed at 22-25 ^{o}C.

Statistical analysis.

Data were analyzed using Pulse Fit software (Version 8.5, HEKA, Germany) and Igor Pro software (Version 4.09, WaveMetrics, USA). Results were expressed as the mean ± SEM. A t-test was used to determine whether the means of two groups were statistically different from each other. Analysis of variance (ANOVA) was a statistical measure used for determining whether differences existed among more than two groups, with significance established at $P < 0.05$.

Results

Effect of EABR on hot-plate test

Hot-plate data showed that although the anti-nociception was not significantly altered by 2.5 mg/kg EABR, there was a tendency of increase in MPE% in hot-plate response mice. However, EABR (5-20 mg/kg i.g.) could elicit a dose-dependent antinociceptive effect after 30 min of the injection. The MPE% of 20 mg/kg EABR was (32.53±8.95) %, which was similar to that of morphine (30.04±3.21%). Administration of vehicle did not show any antinociceptive response.

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Effect of EABR on writhing response induced by acetic acid

Table 1 showed that lower dose of EABR (2.5 mg/kg) had a tendency of inhibition on the number of writhing for 20 min, but it had no significance compared to the Control ($P > 0.05$). EABR (5 mg/kg) could significantly inhibit the writhing response induced by an intraperitoneal injection of acetic acid in mice. The positive control drug, aspirin (200 mg/kg), also significantly inhibited the writhing response (Table 1). More obvious effect of EABR were observed at doses of 10 and 20 mg/kg. But there was no significant difference between 10 and 20 mg/kg groups ($P > 0.05$). However, administration of vehicle did not show any antinociceptive response.

Table 1: Effect of EABR on acetic acid-induced writhing responses in mice (n=8).

Treatment	Dose (mg/kg)	Number of withering for 20 min	% inhibition
Control	_	46.2±7.8	
Asprin	200	11.9±5.6 **	74.3
EABR	20	12.2±6.4 **	73.6
	10	11.1 ± 4.5 **	80.3
	5	24.5 ± 6.7 *#	47.0
	2.5	40.3±7.2##	12.8

Data are mean ± S.E.M. *$P < 0.05$, ** $P < 0.01$, significantly different versus control group. # $P < 0.05$, ## $P < 0.01$, significantly different versus positive control group.

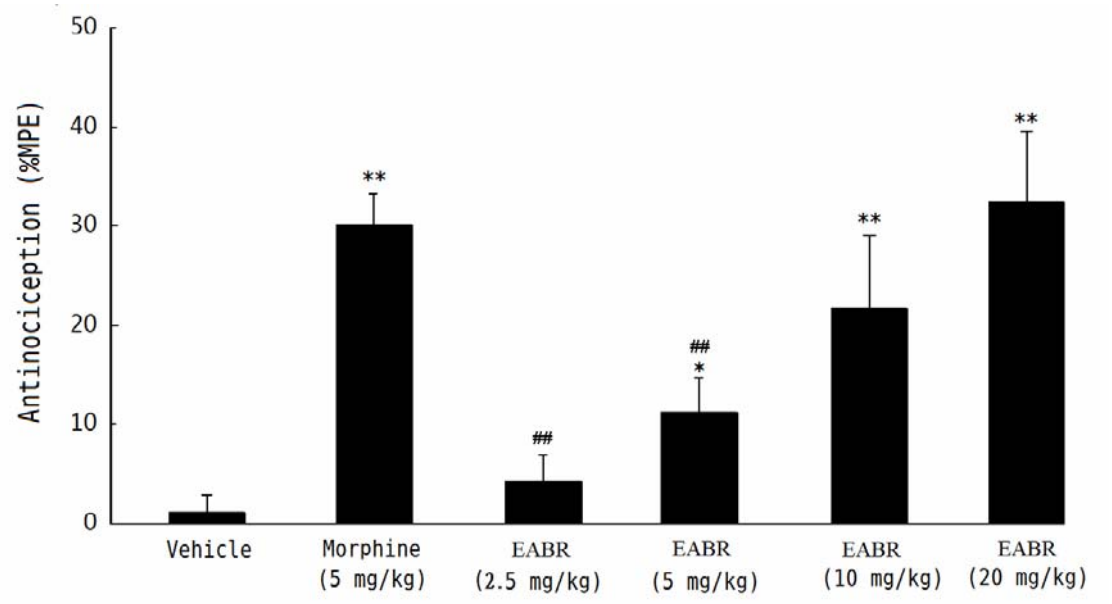

Figure 1: Anti-nociceptive effect of intragastrically (i.g.) EABR on hot-plate test in mice (n=8). Values represent means ± SEM (n = 8). *$P < 0.05$, **$P < 0.01$, significantly different versus vehicle-treated group. ## $P < 0.01$, significantly different versus control group.

Effect of EABR on hind paw licking response and PGE_2 release in hind paw of mice induced by formalin

Results are shown in Table 2. EABR (2.5-10 mg/kg) produced a dose-dependent inhibition on formalin-induced biphasic pain responses (neurogenic and inflammatory pain) in mice. The analgesic effects seemed to be more obvious

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against the late phase than the early phase. Meanwhile, the equation weight of left and right foot reduced dose-dependently, indicating an anti-inflammatory effect of EABR. The positive control drug, aspirin (200 mg/kg), also significantly attenuated the pain responses of two phases and attenuated the inflammatory response of mice feet. Administration of vehicle did not show any antinociceptive response. The lower dosage of EABR (1.0 mg/kg) had no obvious effect on the pain response (P >0.05 versus control group).

As shown in Figure 2, the PGE_2 production increased significantly in hind paw of mice induced by formalin compared with control. The positive control drug, aspirin (200 mg/kg), significantly decreased the PGE_2 production. On the other hand, 1 - 10 mg/ml EABR could not obviously inhibit the PGE_2 release.

Table 2: Antinociceptive effect of EABR on the early phase (0-5 min) and the late phase (15-30 min) of the formalin test in mice (n=8).

Treatment	Dose (mg/kg)	Licking time (S)		Equation weight of left and right foot (g)
		Early phase 0-5 min	Late phase 15-30 min	
Control	_	90.5±16.7	97.0±17.5	0.07 ± 0.01
Asprin	200	59.5±16.8**	47.8 ± 9.6**	0.07 ± 0.01*
EABR	10	51.0±17.6**	3.6±7.4**	0.05 ± 0.01**
	5	72.0±27.7*	22.6±5.7**	0.06 ± 0.02*
	2.5	80.5±23.7	42.7±5.1**	0.07 ± 0.01
	1.0	88.6±25.4	78.5±6.4	0.07±0.01

Data are mean ± S.E.M. *P < 0.05, ** P < 0.01, significantly different versus control group.

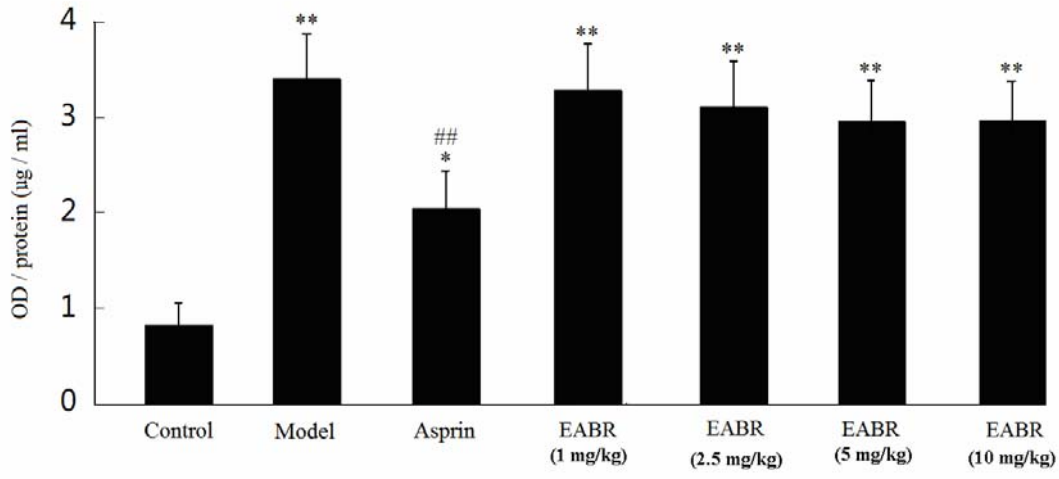

Figure 2: Effect of EABR on PGE_2 production in hind paw of mice induced by formalin (n=8). Data are means ± S.E.M. *P < 0.05, ** P < 0.01, significantly different versus control group. ## P <0.01, significantly different versus model group.

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Effects of EABR on TTX-S sodium peak currents in DRG neurons

At a holding potential of -80 mV, the control TTX-S sodium currents in DRG neurons were recorded. The cell was then exposed to drug for 1 s and the pulse protocol was repeated. The influence of EABR (0.01 – 8 mg/ml) on TTX-S sodium peak current amplitudes were calculated according to the records in the absence and presence of drug. All the results are illustrated by the numerical data in Figures 3 - 4. The result indicated that lower concentrations of EABR (0.01 - 1 mg/ml) could reduce TTX-S sodium peak current amplitudes to some extent. On the contrary, it showed opposite effect at the dosages above 2 mg/ml. 2 - 8 mg/ml EABD dose-dependently increased the TTX-S sodium peak current amplitudes. (Figure 4).

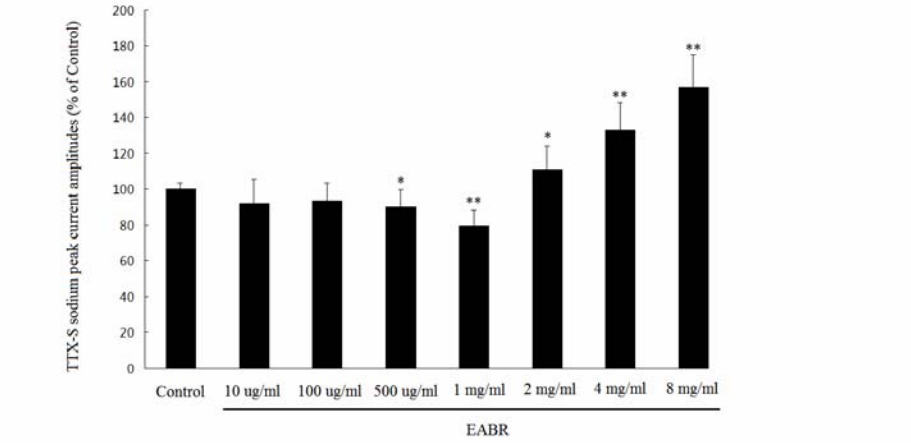

Figure 3: Inhibition rates of EABR on TTX-S sodium peak currents (n=6). Data are means ± S.E.M. *$P < 0.05$, ** $P < 0.01$, significantly different versus control group.

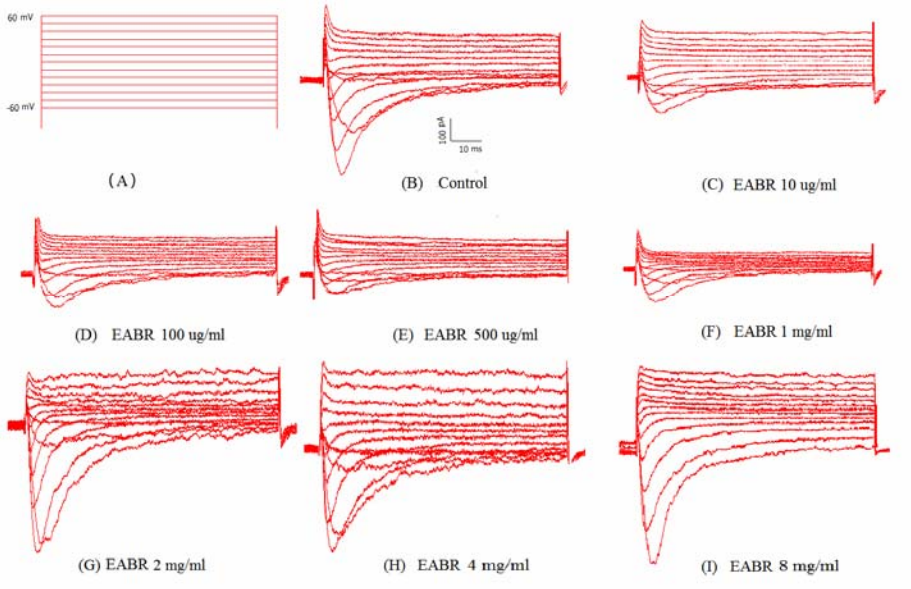

Figure 4: Influence of EABR (10 μg/ml-8 mg/ml) on TTX-S sodium peak currents (n=6).

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Discussion

In this study three experimental models of pain were used to assess the analgesic property of EABR, in such a way that central, peripheral and inflammatory mediated effects were investigated. The hot-plate test revealed central activity, while the acetic acid-induced writhing is a visceral pain model and widely used for detecting both central and peripheral analgesia (Fukawa et al., 1980). Formalin is known to produce biphasic pain behaviors. The formalin test can be divided into two distinct phases, an early phase (neurogenic pain) lasting the first 5 min and a late phase (inflammatory pain) lasting from 20 to 30 min after injection of formalin (Hunskaar and Hole, 1987; Wheeler-Aceto and Cowan, 1991; Tjolsen et al., 1992; Santos and Calixto, 1997). Our results showed that EABR had analgesic property in chemical and thermal nociceptive tests in mice, demonstrating that the extract possessed both central and peripheral antinociceptive effect. It could also be concluded that EABR has a potential anti-inflammatory activity against acute and chronic phases of inflammation not via PGE_2 pathway. Additional mechanisms should be approached and discussed through the other pathways. Since pain is one of the main symptoms of tumor, rheumarthritis, arthrolithiasis, trauma and all kinds of wound, the data also suggested that EABR can be used for the treatment and/or management of painful conditions and supported at least partly the validity of the use of *Aconiti brachypodi* Radix in traditional medicine. Considering the ethnopharmacological uses of the plant, further study on this possibility is needed.

As the primary afferent neurons which contain DRG neurons are the first order neurons in the pain pathway, DRG neurons play an important role in the early stage of sensory processing. The sensory nerve fibers from the abdominal region and the visceral nerve fibers convey pain signals gathered together in DRG neurons. After peripheral nerve injury, the spontaneous discharges are generated from nociceptive neurons which are extremely sensitive to mechanical displacement. The spontaneous afferent discharge generated from the injured site of axon or from the cell body, but not from the terminals, is called ectopic discharge, which is incapable of reflecting the property and strength of stimulation. The previous experiment also proved that a significant amount of ectopic discharges are generated within the DRG neurons besides the injured site of axon (Janicki and Parris, 1997).

Voltage-gated sodium channels are essential for the DRG neurons excitability and the generation and propagation of action potential. TTX-S voltage-gated sodium current in the DRG neuron is a major contributor to the rapid depolarizing phase of the action potential (Blair and Bean, 2002), which plays an important role in action-potential generation. In addition, the resting TTX-S sodium current is large enough to initiate and sustain the ectopic discharge (Lyu et al., 2000). There are more evidences indicating that either the rat peripheral axotomy model of neuropathic pain or the model of inflammatory pain could induce the phenotypic modification and the molecular alteration of sodium channel in DRG neuron (Xiao et al., 2002), so it is obvious that hypersensitivity of the peripheral neuron following injury relates to TTX-S sodium channel in the primary sensory neuron. This experiment was focused on TTX-S sodium current, precisely because of its important action in the nociceptive transmission.

The results of the present study indicated that temperate concentrations of EABR (0.01-1 mg/ml) reduce TTX-S peak sodium current amplitudes in a dose-dependent way, suggesting the modulation of EABR on the TTX-S sodium currents involved in its intervention in the input of nociceptive information. Since the TTX-S sodium channels is of vital importance in the generation and conduction of pain, these results indicated that the analgesic effect of EABR should be caused by the modulation on the TTX-S sodium currents.

However, the cardio- and neurotoxicity of this drug is potentially lethal (Fu et al., 2007; AMERI, 1998), and the improper use of Aconitum in China, India, Japan and some other countries still results in a high risk of severe intoxications

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(Singhuber et al., 2009). Our study also observed that the analgesic effects of EABR were within a rather small therapeutic range in vivo and in vitro. It would display obvious toxicity at the doses above 20 mg/kg *in vivo* and 2 mg/ml *in vitro*. In the preliminary test, some of the animals accepted EABR above the dose of 20 mg/kg showed central depression, body stretching or even death (Data not shown). Compared to the lower dosage group, EABD (2-8 mg/ml) displayed obvious excitability on the TTX-S sodium currents, demonstrating potential toxicity on neuron cells when largely used, suggesting a toxic reaction of higher doses of EABD. So, it's very important to control the therapeutic dosage of the medicine in clinical use. The toxic-effect relationship may be interesting and needs to be further confirmed.

Taken together, the present study demonstrated that EABR has analgesic effects within a small therapeutic range, probably via a mechanism involving Na^+ channels. The findings of this study suggested that EABR has a potential to be developed into an effective drug for the prevention and treatment of various pain syndromes if correctly employed.

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