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The Analgesic Effect of Aconitum Sinomontanum Nakai Pharmacopuncture in Sprague-Dawley Rats

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ABSTRACT

Background: Aconitum sinomontanum Nakai (ASN) has been reported to have analgesic effects. In this study an animal model of pharmacopuncture using ASN (100-500 mg/kg) was examined.
Methods: Sprague-Dawley (SD) rats (n = 40) were randomly assigned to ASN-Low (1 mg/mL, 1.8 mL, ASN-L), ASN-Intermediate (5 mg/mL, 1.8 mL, ASN-M), ASN-High (10 mg/mL, 1.8 mL, ASN-H), negative control (0.2 mL normal saline), and positive control (0.2 mL 0.5% lidocaine) groups. All experiments were administered to the rats' left hind leg. The analgesic response was assessed by monitoring the physical (hot plate, and von Frey test) and chemical (formalin) responses to pain.
Results: All ASN pharmacopuncture groups demonstrated significant differences in pain response to the hot plate test, von Frey test, and formalin test, compared to the control group ($p < 0.05$). The response of the ASN-M group and ASN-H groups to the hot plate, the formalin, and the von Frey tests were significantly different, compared to the lidocaine group ($p < 0.05$).
Conclusion: ASN pharmacopuncture had a significant analgesic effect on SD rats in response to physical and chemical models of pain.

Introduction

Pharmacopuncture is a relatively new acupuncture treatment developed in Korean traditional medicine based on meridian and collateral studies, qi, and flavor studies. Unlike traditional acupuncture, pharmacopuncture uses chemical stimuli a "flavor of herbal medicines" in addition to qi, as a means of treatment [1]. Pharmacopuncture can be used in patients who cannot ingest or absorb herbal medicine. The procedure is simple and the effect is rapid and excellent [2]. Pharmacopuncture has been used to treat conditions such as disc herniation [3], spinal stenosis [4], facial nerve paralysis [5], insomnia [6], and urinary disorder [7].

As the demand for analgesic treatments in Korean medicine increases, focus has been placed on pharmacopuncture [8-10], and in particular, the need to develop pharmacopuncture using herbal medicines with excellent analgesic effect.

Aconitum carmichaeli Debx (Cho O, Cheon Odu, Bu ja) and Aconitum sinomontanum Nakai (ASN), are representative medicinal Aconitum herbs. ASN is the root of a perennial herb mainly grown in central and western regions of China. Its main ingredients are lappaconitine, ranaconitine, lappaconein, 8-O-acetylapaconitine, N-deacetylapaconitine, and excelsine, which are known as diterpenoid alkaloids. ASN acts as an anti-inflammatory agent, antipyretic, and an analgesic. In particular, it is used in China as an analgesic for various diseases, and studies have been conducted on methods of extracting the main ingredients of ASN [11,12].

Based on these previous studies, we hypothesized that ASN would reduce pain. To test this hypothesis ASN extracts were administered during pharmacopuncture into the left hind legs (KI3) of Sprague-Dawley (SD) rats to assess the analgesic effect of ASN pharmacopuncture towards physical, and chemical stimuli.

Materials and Methods

Animals

The animals used in the study were SD male rats aged 10 weeks

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and weighing about 250 g (Samtago, Gyeonggi-do, Republic of Korea). The rats were supplied unlimited sterile negative water and food, and housed in a breeding room with the humidity between 40% and 60%, temperature between 23°C and 24°C with 12 hours of light. The rats were acclimatized to the laboratory environment for more than 1 week prior to experiments. The animal experiments were conducted according to the regulations and policies of the Animal Experimental Ethics Committee of Dong-Eui University (approval no.: A2019-008).

Preparation of ASN extract

ASN (Sichuan, China), about 300 g was thoroughly dried and a fine powder formed using a blender. The ASN powder was diluted to a concentration of 10% in a 90% ETOH solution. Reflux extraction of the deposit was performed twice at 80°C using a heating mantle (DMS637, MTOPS, Seoul, Korea), and the sample was filtered and alkalized to pH > 10 using ammonium hydroxide. The alkaline extract (5.2 L) was dissolved in the same amount of chloroform (5.2 L) and was vacuum-concentrated in a rotary evaporator (Rotavapor R-3, BUCHI, and Switzerland). The residue was dissolved in 9.4 L acetone, and 3 L was extracted by recrystallization with diethyl ether, using rotary evaporation for about 1.5 hours to remove the acetone. The concentrate was placed in a freeze dryer (FD8058, Ilshin, Gyeonggi Province, South Korea) at -60°C and 15 mTorr for 16 days, and 15.58 g of ASN extract (5.19% yield) was obtained.

Rat groups

SD rats were randomly assigned into groups of 8 rats with ASN extracts at concentrations ranging from 100 mg/kg to 500 mg/kg. The ASN pharmacopuncture groups were: ASN-L group with a low concentration (1 mg/mL), ASN-M group with an intermediate concentration (5 mg/mL), and ASN-H group with a high concentration (10 mg/mL). The control groups were normal saline (negative control) and 0.5% lidocaine (positive control).

Pharmacopuncture

Using 1 mL syringes (26 gauge, Jeongrim, North Chungcheong Province, and South Korea) pharmacopuncture using 1.8 mL of ASN-L, ASN-M or ASN-H was administered to the SD rats. The acupoint was selected based on the body's KI3, according to the location of points by bone standards [13]. The control groups of rats were injected at K13 with 0.2 mL of normal saline (negative control), or 0.2 mL of 0.5% lidocaine (positive control).

Hot plate test in SD rats

Assessment of the analgesic effect of ASN in response to a heat stimulus involved the use of a hot plate (RB-200 Intelligent, Seoul, South Korea) set at 55 ± 0.5˚C as previously described by Zhang et al [14]. Latency was defined as the period from the time when the animal was placed on the hot plate surface, to the time the animal licked its back paw or jumped off to avoid thermal pain. The response was measured at 10, 20, 30, 40, 50, and 60 minutes after the administration of pharmacopuncture. To prevent damage to the skin tissue of the SD rats, which is typical resistant to heat pain, the time of contact on the plate was kept to less than 60 seconds (Fig. 1).

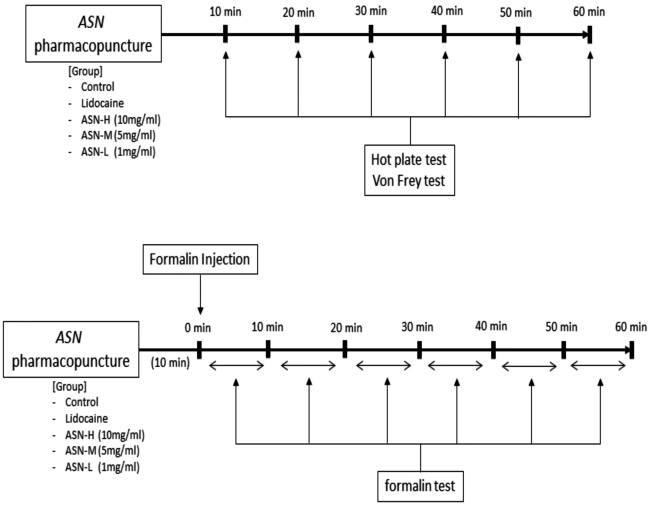

Fig. 1. Scheme showing experimental design.

Formalin test in SD rats

The pain response to chemical stimuli was measured based on a method using formalin as described by Iyengar et al [15]. Ten minutes after pharmacopuncture, the SD rat was placed in an acrylic cage and 50 μL of 0.5% formaldehyde (Sigma-Aldrich, Gyeonggi-do, Korea) diluted with normal saline was injected into the skin tissue of the left foot of the rat using a 26-gauge syringe (Chungrim, North Chungcheong, South Korea). As soon as the formalin was injected, behavioral observations commenced and were maintained for 60 minutes. SD rats are known to show characteristic behaviors such as biting or licking the formalin injection site. These nociceptive responses were recorded by observing the rat between 0-10 minutes, 20-30 minutes, 30-40 minutes, 40-50 minutes, and 50-60 minutes after the injection of formalin (Fig. 1).

Von Frey test in SD rats

The response to physical stimulation was assessed using the von Frey test previously described by Seltzer [16]. Following pharmacopuncture the SD rat was placed on a wire mesh floor in a plastic cage and then stimulated with von Frey filament (Stoelting Company, Illinois, USA) every ten minutes for 1 hour. The stimulus was applied vertically to the skin of the central part of the left hind paw for about 5 seconds at each time point. The absence or presence of a rapid escape reaction initiated by the left hind paw, upon the application of the stimulus was thereafter assessed (Fig. 1).

Statistical analysis

All collected data were verified and the groups were compared using the Student t test ($p < 0.05$). Statistical analysis was performed using SAS Version 9.1.3 (SAS Institute Inc., NC, USA).

Results

Hot plate test in SD rats

The ASN-H group showed a significant delay in response to the heat stimulus 10-60 minutes after pharmacopuncture was performed compared with the control group ($p < 0.01$), and a significant delay in response to heat stimulus 20-60 minutes after pharmacopuncture compared with the lidocaine group ($p < 0.05$). In the ASN-M group, there was a significant delay in response to the heat stimulus 20-60 minutes after pharmacopuncture compared with the control group ($p < 0.05$), and 40-60 minutes after pharmacopuncture compared with the lidocaine group ($p < 0.01$). The ASN-L group had a significant delay in response 30-60 minutes to the heat stimulus compared with the control group ($p < 0.05$), and at 50 minutes compared with the lidocaine group (Table 1).

Formalin test in SD rats

In the ASN-H pharmacopuncture group, the number of characteristic body reactions to formalin was consistently significantly fewer over 60 minutes compared with the control group ($p < 0.01$). In comparison with the lidocaine group, the ASN-H pharmacopuncture group exhibited significantly fewer reactions 10-60 minutes after pharmacopuncture treatment ($p < 0.05$). In the ASN-M pharmacopuncture group, the number of characteristic body reactions to formalin was significantly lower 0-30 minutes compared with the control group ($p < 0.01$), but no significant difference was observed in comparison with the lidocaine group. The ASN-L group showed no significant difference in comparison with the control group or the lidocaine group (Table 2).

Table 1. Latency in Response to the Hot Plate Test at Several Time Points After Treatment.

	Time following treatment (min)					
Treatment	10	20	30	40	50	60
Normal saline control	15.5 ± 1.2	13.1 ± 1.3	15.3 ± 1.0	13.6 ± 1.3	13.1 ± 1.0	14.4 ± 1.4
Lidocaine	39.4 ± 3.4	39.8 ± 2.8	32.7 ± 3.3	16.3 ± 1.9	11.9 ± 0.7	13.5 ± 1.0
ASN-H	55.0 ± 1.9**	58.1 ± 0.7**,#	54.6 ± 1.4**,##	57.5 ± 0.9**,##	56.5 ± 0.8**,##	57.3 ± 1.0**,##
ASN-M	24.9 ± 2.1	33.6 ± 2.9**	34.9 ± 3.2*	43.5 ± 2.8**##	38.9 ± 3.1**,##,†	45.6 ± 2.9**,##
ASN-L	23.4 ± 2.3	23.9 ± 2.3	33.4 ± 2.1**	27.6 ± 2.8**	25.5 ± 1.7*##	22.4 ± 1.9**

Data are presented as mean ± SE. Comparison was performed using the student *t* test.
* $p < 0.05$, ** $p < 0.01$ compared with control group; # $p < 0.05$, ## $p < 0.01$ compared with lidocaine group;
Control: Sprague-Dawley rat group injected with 0.2 mL normal saline.
Lidocaine: Sprague-Dawley rat group injected with 0.2 mL 0.5% lidocaine.
ASN-H: Sprague-Dawley rat group treated with a high dose (1.8 mL of 10 mg/mL) of Aconitum sinomontanum Nakai pharmacopuncture.
ASN-M: Sprague-Dawley rat group treated with an intermediate dose (1.8 mL of 5 mg/mL) of Aconitum sinomontanum Nakai pharmacopuncture.
ASN-L: Sprague-Dawley rat group treated with a low dose (1.8 mL of 1 mg/mL) of Aconitum sinomontanum Nakai pharmacopuncture.

Table 2. Biting, Licking, or Flinching in Response to Formalin at Several Time Points After the Indicated Pharmacopuncture.

	Time following treatment (min)					
Treatment	0–10	10–20	20–30	30–40	40–50	50–60
Normal saline control	34.0 ± 2.0	35.4 ± 1.9	30.9 ± 2.0	22.8 ± 1.6	14.5 ± 1.4	12.1 ± 1.1
Lidocaine	5.6 ± 0.9	5.9 ± 0.9	9.5 ± 1.1	16.1 ± 1.4	13.5 ± 1.7	11.1 ± 1.3
ASN-H	1.9 ± 0.3**	1.4 ± 0.1**,#	1.8 ± 0.2**,##	3.0 ± 0.4**,##	2.0 ± 0.2**,#	2.6 ± 0.4**,#
ASN-M	15.4 ± 1.4**	13.5 ± 1.5**	12.0 ± 1.9**	13.4 ± 1.8	10.5 ± 1.4	7.0 ± 0.8
ASN-L	29.0 ± 1.9	28.4 ± 2.3	25.8 ± 2.0	17.8 ± 1.9	13.4 ± 1.3	12.6 ± 1.6

Data are presented as mean ± SE. Comparison was performed using the student *t* test.
* $p < 0.05$, ** $p < 0.01$ compared with control group; # $p < 0.05$, ## $p < 0.01$ compared with lidocaine group;
Control: Sprague-Dawley rat group injected with 0.2 mL normal saline.
Lidocaine: Sprague-Dawley rat group injected with 0.2 mL 0.5% lidocaine.
ASN-H: Sprague-Dawley rat group treated with a high dose (1.8 mL of 10 mg/mL) of Aconitum sinomontanum Nakai pharmacopuncture.
ASN-M: Sprague-Dawley rat group treated with an intermediate dose (1.8 mL of 5 mg/mL) of Aconitum sinomontanum Nakai pharmacopuncture.
ASN-L: Sprague-Dawley rat group treated with a low dose (1.8 mL of 1 mg/mL) of Aconitum sinomontanum Nakai pharmacopuncture.

Table 3. Paw Withdrawal Threshold Value in Response to von Frey Filaments at Several Time Points After the Indicated Pharmacopuncture.

	Time following treatment (min)					
Treatment	10	20	30	40	50	60
Normal saline control	18.6 ± 1.5	22.0 ± 1.4	17.9 ± 1.3	19.3 ± 1.4	20.1 ± 3.1	22.0 ± 1.4
Lidocaine	75.8 ± 5.2	75.8 ± 5.2	70.0 ± 3.4	43.8 ± 5.0	29.9 ± 5.3	24.9 ± 2.9
ASN-H	80.8 ± 5.2**	85.0 ± 3.8**	85.0 ± 3.8**	80.8 ± 5.2**,##	75.8 ± 5.2**,##	75.8 ± 5.2**,##
ASN-M	66.5 ± 5.7**	60.8 ± 3.6**	66.5 ± 5.7**	61.5 ± 5.1**	66.5 ± 5.7**,#	66.5 ± 5.7**,##
ASN-L	46.6 ± 5.2**	50.3 ± 6.5*	41.0 ± 5.4	36.8 ± 5.3	34.1 ± 5.7	31.1 ± 3.4

Data are presented as mean ± SE. Comparison was performed using the student *t* test.
* $p < 0.05$, ** $p < 0.01$ compared with control group; # $p < 0.05$, ## $p < 0.01$ compared with lidocaine group;
Control: Sprague-Dawley rat group injected with 0.2 mL normal saline.
Lidocaine: Sprague-Dawley rat group injected with 0.2 mL 0.5% lidocaine.
ASN-H: Sprague-Dawley rat group treated with a high dose (1.8 mL of 10 mg/mL) of Aconitum sinomontanum Nakai pharmacopuncture.
ASN-M: Sprague-Dawley rat group treated with an intermediate dose (1.8 mL of 5 mg/mL) of Aconitum sinomontanum Nakai pharmacopuncture.
ASN-L: Sprague-Dawley rat group treated with a low dose (1.8 mL of 1 mg/mL) of Aconitum sinomontanum Nakai pharmacopuncture.

Von Frey test in SD rats

The ASN-H pharmacopuncture group showed a significantly higher pain threshold value over 60 minutes compared with the control ($p < 0.01$), and 40-60 minutes after pharmacopuncture compared with the lidocaine ($p < 0.01$) group. The ASN-M pharmacopuncture group also showed a significantly higher pain threshold value compared with the control group over the 60 minutes ($p < 0.01$), and a significantly higher pain threshold value 50-60 minutes after pharmacopuncture compared with the lidocaine group ($p < 0.05$). The threshold value of pain in the ASN-L group was significantly higher than the control 10-20 minutes after the ASN pharmacopuncture compared with the control group ($p < 0.05$). There was no statistical significance compared with the lidocaine group (Table 3).

Discussion

The genus Aconitum of the Ranunculoideae family is cultivated in various countries including China, Japan, and India, and 76 out of 200 species are used as herbal medicines. The roots of Aconitum herbs mainly have warming effects on the meridian to relieve pain, dissipate cold, and relieve cold pains. However, in their natural forms, Aconitum herbs can be very hot, and extremely poisonous, unless processed appropriately for clinical use [17].

ASN is mainly distributed in Hebei, Shaanxi, Southern Gansu, Sichuan, and Guizhou regions of China. Its main chemical components are lappaconitine, ranaconitine, lycaconitic acid, and monomethyl ester. ASN has anti-inflammatory, analgesic, antipyretic, and local anesthetic effects [18]. In China, lappaconitine extracted from ASN is used clinically in various drug forms such as patches for pain control, and oral preparations [19]. Notably, lappaconitine reportedly had an analgesic effect in a mouse model of leukemia-induced bone pain [20].

In this study, the hot plate, formalin, and von Frey tests were performed to evaluate the analgesic effect of pharmacopuncture using ASN extract and obtained remarkable results. The LD_{50} of ASN could not be assessed because there were no cases of death in the study. Nevertheless, we verified that 0.9 mL of ASN at a concentration of 1 mg/mL provided sufficient analgesic effect in SD rats weighing 250 g compared with the human dose.

Severe toxicity of ASN was not observed in SD rats during this study. To evaluate the effectiveness of ASN pharmacopuncture, administration of a double dose of 0.9 mL of ASN 1 mg/mL was assumed as the low concentration dose, 5 times this double dose was administered to the intermediate-concentration group, and 10 times to the high-concentration group.

This study used the KI3 acupoint, which anatomically controls the sensation of the sole of the foot, the path through which the tibial nerve travels [21]. This acupoint was selected based on its use in a previous SD rat analgesic study [22]. Lidocaine has analgesic, anti-hyperalgesia, and anti-inflammatory effects, and is commonly used as a local anesthesia thus, it was employed as a positive control for ASN pharmacopuncture in this study. The hot plate test is typically used to determine the analgesic effect against acute heat stimulation by analyzing the withdrawal reaction in avoidance of heat stimulation [23]. In contrast, the formalin test, used to assess the animal's behaviors during persistent pain caused by damage and inflammation of peripheral tissues (due to the injection of formalin), was a suitable assay to confirm the continuous analgesic effect of ASN pharmacopuncture.

During the formalin test, reactions occurred in 2 phases; the early phase which lasted for about 10 minutes immediately after the formalin injection, and the late phase that proceeded for about 15 to 20 minutes after the formalin injection, and lasted for about 60 minutes. The early phase is due to the activities of sensory nerves, especially the C-fiber, whereas the late phase is mediated by peripheral inflammation. Nonsteroidal anti-inflammatory analgesics are not effective in reducing the early phase response in the formalin test, but can reduce the late phase response mediated by peripheral inflammation [24,25].

In both the hot plate and the formalin tests, the ASN-H pharmacopuncture group showed a significant delay in pain response time over 60 minutes compared with the control group, and 20-60 minutes after pharmacopuncture compared with the lidocaine group. The ASN-M group showed a significant delay in

pain response at 20-60 minutes after the procedure compared with the control group in the hot plate test, and 40-60 minutes after pharmacopuncture, compared with the lidocaine group. However, in the formalin test, a significantly lower number of characteristic body responses of the ASN-M pharmacopuncture group during the initial 30 minutes was observed only in comparison with the control group, but not with the lidocaine group. The ASN-L group showed a significant delay in pain response time 30-60 minutes after the pharmacopuncture in comparison with the control group only in the hot plate test. Thus, ASN pharmacopuncture demonstrated an analgesic effect in proportion to the dose, indicating that a higher dose more effectively combats acute pain in the early phase due to the activity of C-fiber, as well as providing an anti-inflammatory effect to control the pain in the late phase mediated by inflammation.

Compared with the control group in the von Frey test, the ASN-M group and ASN-H group showed significantly higher threshold values for pain immediately after the procedure, and up to 60 minutes thereafter. In particular, the lidocaine was significantly ineffective after 40 minutes, whereas ASN-H pharmacopuncture group after 40 minutes, showed a significantly higher pain threshold value compared with the lidocaine group.

Lidocaine is a typical analgesic and local anesthetic drug which act on voltage-gated sodium channels which are (an important protein in pain transmission), to cause analgesic effects [26]. Moreover, at low doses, lidocaine suppresses ectopic excitability due to chronic nerve damage, and at high concentrations, it suppresses central sensitization or nerve hyperstimulation, thereby obtaining an overall analgesic effect. The action time of 0.5-1.0% lidocaine is about 30 to 60 minutes in humans, but when the maximum concentration of 300 mg is used, deep vein or cardiovascular collapse may occur [27].

In China, many studies have been conducted on the active substances of ASN. In particular, it has been reported that lappaconitine (the main component), has a remarkable analgesic effect in clinical trials [19]. Hwang et al [19] reported that lappaconitine had a dose-dependent analgesic effect in a threshold study on mechanical and thermal pain in rats. In addition, lappaconitine oral tablets and patches were not only effective in providing analgesic effects in patients with cancer pain, but left no withdrawal symptoms following the discontinuation of the drug after repeated administration, and mild side effects were resolved within a short period. Furthermore, Gong et al [28] reported that intravenous injection of lappaconitine in patients with rectal cancer was effective at controlling postoperative pain.

Thus, many studies on the analgesic effect of lappaconitine have been conducted to determine the mechanisms of lappaconitine actions. However, the exact mechanism has been controversial. For example, Wright [29] argued that although possessing a structure similar to aconitine, lappaconitine binds to sodium channels and acts as a sodium channel blocker, inhibiting the production of action potentials, resulting in analgesic effects. Ono and Satoh [30] compared the analgesic mechanism of lappaconitine with morphine, and diclofenac using the formalin test, and reported that lappaconitine controlled pain in a dose-dependent manner until the early phase related to substance P, and the late phase related to somatostatin.

In this study, a comparison of the analgesic effect of lidocaine, and ASN pharmacopuncture showed the superior analgesic effect of ASN-H 40-60 minutes after pharmacopuncture. The analgesic effect of the ASN-M appeared to have an intermediate effect compared with ASN-L, and ASN-H, suggesting that ASN pharmacopuncture is effective in direct proportion to its concentration up to a concentration of 10 mg/mL and a dose of 1.8 mL. A single dose of 18 mg ASN-H had an analgesic effect in SD rats. Thus, we speculated that the effects of lappaconitine may follow a mechanism similar to that of lidocaine based on the mechanisms revealed in previous studies. However, additional studies are needed to elucidate the exact mechanism of the analgesic effect of ASN pharmacopuncture. Nonetheless, ASN is a herbal medicine with excellent analgesic effects, and ASN pharmacopuncture may be used for various pain-inducing conditions and diseases.

Conflict of Interest

The authors declare that they have no conflicts of interest.

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