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Processed aconite root and its active ingredient neoline may alleviate oxaliplatin-induced peripheral neuropathic pain

Toshiaki Suzuki [a,c], Keisuke Miyamoto [b], Naomi Yokoyama [a], Mayuko Sugi [a], Akina Kagioka [a], Yuka Kitao [b], Takumi Adachi [a], Masahiro Ohsawa [b], Hajime Mizukami [a,d], Toshiaki Makino [a,*]

[a] Department of Pharmacognosy, Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabe-Dori, Mizuho-ku, Nagoya 467-8603, Japan
[b] Department of Neuropharmacology, Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabe-Dori, Mizuho-ku, Nagoya 467-8603, Japan
[c] Department of Cellular Pharmacology, Graduate School of Pharmaceutical Sciences, Nagoya University, Nagoya 464-8601, Japan
[d] The Kochi Prefectural Makino Botanical Garden, Kochi 781-8125, Japan

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ABSTRACT

Ethnopharmacological relevance: Processed aconite root (PA, the root of *Aconitum carmichaeli*, Ranunculaceae) is a crude drug used in traditional Chinese or Japanese kampo medicine to generate heat in the body and to treat pain associated with coldness. Oxaliplatin (L-OHP) is a platinum-based anticancer drug that frequently causes acute and chronic peripheral neuropathies, including cold and mechanical hyperalgesia.
Aim of the study: We investigated the effects of PA on L-OHP-induced peripheral neuropathies and identified the active ingredient within PA extract.
Materials and methods: L-OHP was intraperitoneally injected into mice, and PA boiled water extract was orally administered. Cold and mechanical hyperalgesia were evaluated using the acetone test and the von Frey filament method, respectively. Dorsal root ganglion (DRG) neurons were isolated from normal mice and cultured with L-OHP with or without PA extract. Cell viability and neurite elongation were evaluated.
Results: PA extract significantly attenuated cold and mechanical hyperalgesia induced by L-OHP in mice. In cultured DRG neurons, L-OHP reduced cell viability and neurite elongation in a dose-dependent manner. Treatment with PA extract significantly alleviated the L-OHP-induced reduction of neurite elongation, while the cytotoxicity of L-OHP was not affected. Using activity-guided fractionation, we isolated neoline from PA extract as the active ingredient. Neoline significantly alleviated L-OHP-induced reduction of neurite elongation in cultured DRG neurons in a concentration-dependent manner. Moreover, subcutaneous injection of neoline attenuated cold and mechanical hyperalgesia in L-OHP-treated mice. PA extract and neoline did not show sedation and motor impairment.
Conclusions: The present study indicates that PA and its active ingredient neoline are promising agents to alleviate L-OHP-induced neuropathic pain.

1. Introduction

Oxaliplatin (L-OHP), a key drug in the FOLFOX regimen (Grothey and Goldberg, 2004), is commonly used to treat colorectal cancer. L-OHP frequently induces acute and chronic peripheral neuropathy, with this side effect potentially being dose limiting, leading to discontinuation of chemotherapy (Pasetto et al., 2006). Patients treated with L-OHP acutely exhibit muscle fasciculation (Hill et al., 2010), sensory paresthesia, or occasional dysesthesia (Raymond et al., 1998), which are triggered by mild cold sensation (Cassidy and Misset, 2002). Although the detailed mechanism of L-OHP-induced neurotoxicities remains unknown, several treatments to relieve neuropathic pain in patients treated with L-OHP have been evaluated. The effects of intravenous injection of calcium and magnesium and the administration of the anti-epileptic agents, including gabapentin and pregabalin, to reduce L-OHP-induced neuropathic pain have been investigated in clinical trials; however, no sufficient effects have been reported for this indication (Saif et al., 2010; Loprinzi et al., 2014).

Recently, in Japan, there has been increased interest in the clinical effectiveness of traditional Japanese kampo medicine to treat intractable chronic diseases that are not treated effectively by

* Corresponding author.
E-mail address: makino@phar.nagoya-cu.ac.jp (T. Makino).

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modern medicines (Uezono et al., 2012). Goshajinkigan, a kampo formula composed of 10 crude drugs, is used to relieve the following symptoms in patients who are easily fatigued and suffer from coldness in the extremities: leg pain, lower back pain, numbness, pruritus, dysuria, frequent urination, and edema (The Japan Society for Oriental Medicine, 2005). A randomized double-blind clinical trial showed that goshajinkigan prevented L-OHP-induced neuropathy in patients with advanced or recurrent colorectal cancer who were treated with the standard FOLFOX regimen (Kono et al., 2013). In animal experiments, cold and mechanical hyperalgesias in L-OHP-treated rats were attenuated by oral administration of goshajinkigan, without affecting anti-tumor activity (Ushio et al., 2012). It has been suggested that the preventive effects of goshajinkigan are mediated by suppressing functional alteration of TRPA1 and TRPM8 in dorsal root ganglion (DRG) neurons (Kato et al., 2014; Mizuno et al., 2014). In another animal experiment, keishikajutsubuto, a kampo formula composed of 7 crude drugs, significantly alleviated L-OHP-induced neuropathy in rats (Ahn et al., 2014).

The root of *Aconitum carmichaeli* (Ranunculaceae) is a common component of both goshajinkigan and keishikajutsubuto kampo formulae and is a well-known crude drug that potently stimulates heat generation within the body and overcomes coldness sensation. It is widely used to relieve pain related to cold symptoms (Reid, 1987). The raw root of *A. carmichaeli* contains toxic alkaloids, such as aconitine, mesaconitine, and hypaconitine, and has a reported LD_{50} value of 0.5–1.8 g/kg for oral administration in mice (Hikino et al., 1977). Therefore, various processing methods to reduce the toxicity of the raw root have been developed. The sixteenth edition of the Japanese Pharmacopoeia (JPXVI) registers processed aconite root (PA) as the dried material of the heat-treated or autoclaved root of *A. carmichaeli* (The Society of Japanese Pharmacopoeia, 2012). Highly toxic aconitine-type esterified diterpene alkaloids are degraded into less toxic de-esterified diterpene alkaloids (*e.g.,* benzoylmesaconine) by heating or autoclaving (Hikino et al., 1977). PA is considered to be an effective herbal agent to relieve pain. It has been previously reported that PA produces antinociception effects in repeated cold stress and adjuvant articular inflammation in rats and mice (Oyama et al., 1994), an effect that is mediated by the activation of noradrenergic and serotonergic neuronal signaling (Isono et al., 1994). A sub-analgesic dose of PA has been shown to attenuate the development of morphine tolerance (Shu et al., 2006). Moreover, treatment with PA reduces mechanical and thermal hyperalgesia in a chronic constriction injury model through effects on the spinal κ-opioid receptor (Xu et al., 2006). Recently, treatment with PA was shown to relieve neuropathic pain through inhibition of astrocytes in the spinal cord (Shibata et al., 2011). These reports raise the possibility that PA could be the primary active component of goshajinkigan and keishikajutsubuto formulae.

In this study, we evaluated the effects of PA on cold and mechanical hyperalgesia in mice treated with L-OHP. Furthermore, we were able to identify neoline, one of the alkaloids contained in PA, as the active ingredient responsible for these anti-hyperalgesia effects.

2. Materials and methods

2.1. Processed aconite root and its fractionation

Processed aconite root (PA) (JPXVI grade; lot number 8511606), the autoclaved root of *A. carmichaeli* (Ranunculaceae), was purchased from Uchida Wakanyaku (Tokyo, Japan). According to the manufacture's information, the alkaloid contents of the PA was 0.5–1.4% for total alkaloids, 50 μg/g for aconitine, and 150 μg/g for mesaconitine. The PA was supplied as small pieces by cutting the whole crude drug into 2–4 mm blocks. Powdered PA was prepared in our laboratory using a mill and desiccated for storage.

To prepare the boiled water extract of PA, powdered PA (100 g) was boiled in 2 l of H_2O for 30 min, and the filtrated decoction (about 1 l) was lyophilized. The extract yield was 30 g. The PA extract was suspended in H_2O at 100 mg/ml and stored at –20 °C until use.

For the fractionation of alkaloids (AL) from PA extract, 15 g of the PA extract was suspended in 0.75 l of 10% NH_3 and partitioned with 0.3 l of ether three times. The ether layer and 10% NH_3 layer were dried under reduced pressure conditions. The weights of the ether layer (AL fraction) and the 10% NH_3 layer (non-AL fraction) were 280 mg and 14.7 g, respectively. Twenty-eight mg of the AL fraction were subjected to silica gel preparative thin layer chromatography (TLC) and expanded with solvent (ethyl acetate: ethanol:25% NH_3=20:5:1). The yields in order of low to high hydrophobicity were 6.6 mg, 3.5 mg, 3.9 mg, 5.8 mg, and 3.5 mg for fractions 1–5, respectively. Since fraction 4 was the most active, fraction 4 (5 mg) was subjected to the same TLC process, and pure compound **1** (1.5 mg) was yielded. Using ^{1}H-and ^{13}C-nuclear magnetic resonance (NMR) and electron ionization-mass spectrometry (EI-MS) analysis, and following data presented in a previous article (Hanuman and Katz, 1994), compound **1** was identified as neoline.

In order to isolate neoline from PA for the animal experiments, 1.0 kg of PA was immersed in 0.5 l of boiling water, 2.5 l of methanol were added, and the mixture was stirred at 60 °C for 1 h, then filtrated. The residue was further extracted with 2 l of methanol, and this process was repeated twice. Total methanol solutions were evaporated at 60 °C under reduced pressure condition, and yielded 80 g of extract. The extract was suspended in 0.7 l of 10% NH_3 and partitioned with 0.3 l of ether three times to yield the ether layer (5.2 g). This ether layer was subjected to silica gel open column chromatography (5.6 cm *i.d.* × 35 cm) loaded with solvent (ethyl acetate: ethanol:28% NH_3=40:3:2) to yield crude neoline (1.5 g). Crude neoline was further subjected to silica gel open column chromatography (3.6 cm *i.d.* × 43 cm) loaded with solvent (chloroform:methanol=9:1) to yield pure neoline (333 mg).

2.2 Quantification of neoline in PA

Boiled water extract of PA was diluted to 4.0 mg/ml in methanol, and neoline was dissolved in methanol at the concentrations of 1.0×10^2, 10, and 1.0 μM. Then, 100 μl of these solutions were mixed with 100 μl MeOH containing 10 μM of *p*-hydroxybenzoic acid butyl ester (PHB; Wako Pure Chemicals, Osaka, Japan) as an internal standard. The neoline concentration was determined using a liquid chromatography (LC)-MS/MS system (Waters Quattro Premier XE, Milford, MA, USA) with an electrospray ionization source in the positive ion mode and multiple reaction monitoring. High-performance LC separation was performed under the following conditions: column, Inertosil ODS-3 (2.0 × 75 mm; GL Science, Tokyo, Japan); mobile phase, a linear gradient elution system, 0.05% AcOH in H_2O (solvent A): 0.05% AcOH in acetonitrile (solvent B) (A/B)=95/5–95/5 for 0–1 min; 95/5–10/90 for 1–3 min; 10/90–95/5 for 3–4.6 min at a flow rate of 200 μl/min. The injection volume of the sample was 10 μl. Both quadrupoles were maintained at the unit resolution and the transitions (precursor to daughter) monitored were 438.32→420.29 *m/z* for neoline (retention time, 3.0 min) and *m/z* 195.2→139.0 *m/z* for PHB (4.3 min). Linear regression of the concentration range of neoline was calibrated by the peak area ratio of these compounds to PHB using the least-squares method ($r^2 > 0.999$).