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Antispasmodic effect of shakuyakukanzoto extract on experimental muscle cramps *in vivo*: Role of the active constituents of Glycyrrhizae radix

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ABSTRACT

Ethnopharmacological relevance: Shakuyakukanzoto (SKT) composed of Glycyrrhizae radix (G. radix) and Paeoniae radix (P. radix) has been traditionally used in Japan, Korea and China as an antispasmodic drug for the treatment of skeletal muscle cramps and intestinal cramps.

Aim of this study: To evaluate the antispasmodic activity of SKT and its two components, as well as to identify the key constituents of the components which mediate this effect in skeletal muscles *in vivo*.

Materials and methods: An experimental cramp model was constructed to evaluate the effects of peripherally-acting muscle relaxants on electrically-induced cramps under physiological conditions. This was accomplished by surgically isolating the motor supply to the gastrocnemius muscle in an anesthetized rat and delivering electrical stimuli to an isolated tibial nerve to induce tetanic contractions. We first tested dantrolene, a well-known peripherally-acting relaxant, to determine the sensitivity and reliability of our experimental model. We then evaluated the effects of SKT, P. radix, G. radix, and the eight active constituents of G. radix against tetanic contractions.

Results: We found that dantrolene (10 and 30 mg/kg, i.d.) rapidly and significantly inhibited tetanic contractions ($P<0.01$) irrespective of dose. SKT (0.5, 1.0, and 2.0 g/kg, i.d.) and G. radix (0.5 and 1.0 g/kg, i.d.) also significantly inhibited tetanic contractions ($P<0.01$) but in a dose-dependent manner owing to the actions of six of the eight active constituents in G. radix (liquiritin apioside, liquiritigenin, isoliquiritin apioside, isoliquiritigenin, glycycoumarin, and glycyrrhetinic acid, 20 μmol/kg, i.v.). These constituents, which include flavonoids, a triterpenoid, and a courmarin derivative, demonstrated temporal variations in their inhibitory activity. In contrast, P. radix (0.5 and 1.0 g/kg, i.d.) did not show a statistically significant antispasmodic effect in our study; however, we previously found that it had a significant antinociceptive effect.

Conclusions: Our findings show that SKT inhibits tetanic contractions *in vivo* and that G. radix is the main antispasmodic component due to the actions of its active constituents, thus supporting the traditional use of SKT. We further propose that SKT containing the antispasmodic G. radix and antinociceptive P. radix is a pharmaceutically elegant option for muscle cramps as treatment requires a two-pronged approach, *i.e.*, inhibition of hyperexcitable skeletal tissues and modulation of the pain accompanying cramps.

1. Introduction

Muscle cramps are a painful physiological phenomenon of the skeletal muscles that often affect the triceps surae muscle, yet their etiology and management have not been well established (Katzberg et al., 2010). Some of the precipitating factors include strenuous exercise, metabolic disorders, electrolyte disturbance, pregnancy, and iatrogenic causes such statins, diuretics, and immunosuppressants (Miller and Layzer, 2005). In Japan pharmacotherapy for muscle cramps includes muscle relaxants, anticonvulsants, vitamin and mineral supplements, and traditional Japanese herbal medicines (Kampo) such as shakuyakukanzoto (SKT) (Hinoshita et al., 2003; Hyodo et al., 2006). Most muscle relaxants and anticonvulsants, however, have untoward adverse effects of hepatotoxicity and central nervous system (CNS) depression, and those that directly target the skeletal muscles while sparing the CNS are scare (Richards et al., 2012).

Abbreviations: SKT, shakuyakukanzoto; G. radix, Glycyrrhizae radix; P. radix, Paeoniae radix; CNS, central nervous system; CICR, calcium-induced calcium release; HPLC, high-performance liquid chromatography

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Shakuyakukanzoto (pronounced in Chinese as "Shaoyaogancao-tang" and in Korean as "Jackyakamcho-tang") is one of the 148 Kampo extract formulations for prescription approved by the Japanese government which are listed on the "National Health Insurance Drug Tariff". SKT is an extract powder containing Glycyrrhizae radix (G. radix) and Paeoniae radix (P. radix), which is manufactured according to the standards described in the Japanese Pharmacopoeia (Sixteenth Edition, JP16), thus its quality and authenticity are assured. The daily extract dose of SKT in human is 2.5 g, which is derived from 6 g of G. radix and 6 g of P. radix. Clinical studies have shown that SKT induces a rapid and clinically significant improvement in muscle cramps arising from different causes, including liver cirrhosis (Kumada et al., 1999), hemodialysis (Hinoshita et al., 2003), and chemotherapy-induced myalgia and arthralgia (Yamamoto et al., 2001; Yoshida et al., 2009). Basic pharmacological studies have shown that SKT promotes intestinal smooth muscle relaxation via significant anticholinergic and phosphodiesterase 3 inhibitory actions (Satoh and Tsuro, 2011), suppresses neuromuscular transmission via intracellular Ca^{2+} mobilization (Dezaki et al., 1995), and alleviates pain associated with diabetic peripheral neuropathy via activation of spinal α_2-adrenoceptors (Omiya et al., 2005; Lee et al., 2011). In addition, a comprehensive pharmacological study of SKT has revealed that a dose of 2 g/kg does not affect behavior nor significantly alter the functions of CNS, respiratory, cardiovascular, gastrointestinal and renal systems in normal animals (Takeda et al., 2003). Individually, P. radix has been shown to have anti-inflammatory, immunomodulatory, and anticoagulant effects (Meselhy and Hattori, 2000), while some of the putative actions of G. radix include antispasmodic, hepatoprotective, and antioxidative effects (Shen et al., 2007; Yim et al., 2007).

The spontaneous nature of cramps and the absence of effective experimental methods to simulate muscle cramps in laboratory animals have hampered efforts to understand their pathophysiology (Caress et al., 2000). Current data indicate a peripheral origin, although the potential for central modification cannot be dismissed (Miller and Layzer, 2005). Among the traditional screening methods for evaluating muscle relaxants, the rotarod test has been found to show the greatest sensitivity to central effects of muscle relaxants at the lowest concentration. While these basic pharmacological studies are useful for assessing adverse effects, they do not adequately evaluate the efficacy of drugs against muscle cramps. Moreover, they do not have the sensitivity to differentiate between central and peripheral drugs and between therapeutic and toxic doses.

The relaxant effects of SKT have been primarily confirmed by *in vitro* studies (Kimura et al., 1984, 1985; Dezaki et al., 1995), whereas *in vivo* studies investigating its effects on skeletal muscle contractile properties are virtually nonexistent. On the basis of extant data, we hypothesized that SKT is a direct-acting muscle relaxant with a unique mechanism of action outside of the CNS. Given the lack of relevant experimental models to study cramps, as well as the paucity of evidence for safe and effective drug therapies, our first objective of the study was to develop a rat *in vivo* cramp model that could evaluate the effects of peripherally-acting muscle relaxants on electrically-induced cramps under physiological conditions. Secondly, we tested dantrolene, a well-known peripherally-acting relaxant, as a control drug to determine the sensitivity and reliability of the experimental model. We then evaluated SKT, G. radix, P. radix, and the active constituents of G. radix to identify the principal constituents with antispasmodic property. This was accomplished by surgically isolating the motor supply to the gastrocnemius muscle in an anesthetized rat and delivering electrical stimuli to an isolated tibial nerve to induce muscle contractions. We considered the use of an *in vivo* assessment particularly useful as it leverages the use of an intact whole muscle to examine directly the contractile properties at body temperature with normal blood circulation. Finally, findings from the electrical stimulation experiment using dantrolene were then compared with dantrolene-induced behavioral changes in a rotarod test, which is considered one of the more sensitive tests for evaluating muscle coordination, to compare the sensitivity of the two experimental models to changes in muscle activity.

2. Materials and methods

2.1. Animals

Four to six male Wistar rats (7–8 weeks of age, 250–300 g in weight) were used for the electrical stimulation experiment *in vivo*. For the rotarod test, 60 male ddy mice in groups of 10 weighing approximately 30 g (28 ± 3 g) were used. All animals were housed in an environmentally controlled facility (12–12 h light–dark cycle, 23 ± 2 °C temperature, 55 ± 10% humidity) and standard laboratory chow and water were provided *ad libitum*. All experimental procedures were approved by the Laboratory Animal Committee of Tsumura & Co. and followed their guidelines for the care and use of laboratory animals. At the end of each experiment, rats were euthanized with urethane overdose.

2.2. Test drugs

SKT is an extract powder containing equal proportions of Glycyrrhizae radix (G. radix) (root and stolon of *Glycyrrhiza uralensis* Fischer, Fabaceae) and Paeoniae radix (P. radix) (root of *Paeonia lactiflora* Pallas, Ranunculaceae). The identification and preparations of SKT, G. radix and P. radix were performed in accordance with the standards outlined in the JP. Briefly, SKT was prepared by decocting G. radix and P. radix in boiling water for 60 min, separating the effluent from the residuals, then spray-drying the effluent to produce the extract powder. The extract powder of SKT, G. radix, and P. radix supplied by the Botanical Raw Materials Research Department of Tsumura & Co. (Ibaraki, Japan) were suspended in distilled water and 5 mL/kg of each extract was separately administered through an i.d. catheter. The powdered extracts of seven of the G. radix constituents (liquiritin, liquiritin apioside, liquiritigenin, isoliquiritin, isoliquiritin apioside, isoliquiritigenin and glycycoumarin) were dissolved in a mixture of ethanol, propylene glycol and saline solution prepared in a ratio of 1:4:5. The powdered extract of glycyrrhetinic acid alone was dissolved in a mixture of ethanol, 25% ammonia water, and saline solution prepared in a ratio of 1:0.05:8.95. Dantrolene sodium purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) was used as a positive control. Urethane and α-chloralose were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Distilled water was used as a negative control.

The doses for all of the study drugs were as follows: dantrolene (10 and 30 mg/kg, i.d.), SKT (0.5, 1.0, and 2.0 g/kg, i.d.), P. radix (0.5 and 1.0 g/kg, i.d.), G. radix (0.5 and 1.0 g/kg, i.d.), five liquiritin derivatives of G. radix (liquiritin, liquiritin apioside, liquiritigenin, isoliquiritin, isoliquiritin apioside, 20 μmol/kg, i.v.), isoliquiritigenin (2 and 20 μmol/kg, i.v.), glycycoumarin (2.7 and 27 μmol/kg, i.v.), and glycyrrhetinic acid (7 and 35 μmol/kg, i.v.). A dose of 20 μmol/kg was used to determine the effects of liquiritin derivatives based on the results of a preliminary study which showed that isoliquiritigenin demonstrated the greatest antispasmodic effect at this dose.