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Acute and subchronic oral toxicity assessment of the herbal formula Kai-Xin-San

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ABSTRACT

Ethnopharmacological relevance: *Kai-Xin-San* (KXS) is a famous traditional Chinese medicine (TCM) formula. It has been used in the treatment of diseases including neurasthenia, Alzheimer's disease and neurosis.
Aim of the study: To provide information on the potential toxicity of KXS, we evaluated the acute and subchronic toxicity in rodents.
Materials and methods: In acute study, a single administration of KXS was given orally to mice at doses ranging from 19.67 to 60.04 g/kg. In the sub-chronic oral toxicity study, KXS was administered to rats at 0, 1, 3 and 9 g/kg for 13 weeks. Moreover, 30 days of post treatment (withdrawal study) was conducted. Mortalities, clinical signs, body weight changes, food and water consumption, haematological and biochemical parameters, gross findings and organ weights were monitored during the study period.
Results: In the sub-chronic study in rats, daily oral administration of KXS at the dose of 9 g/kg/day result in significant increase in WBC, lymphocyte, alkaline phosphatase, blood sugar and significant decrease in bodyweight, serum Cre, CK and CHO at the last week of treatment. Recovery except for the body weight was observed after 30 days of post treatment.
Conclusions: KXS is relatively safe for oral medication. The LD_{50} of KXS was over 32.59 g/kg for mice. The no-observed-adverse-effect-level (NOAEL) was considered to be 19.67 g/kg/day for rats.

1. Introduction

Medicinal herbs are used for the prevention and treatment of diseases, and have a long history. However, the most commonly used herbal formulae have no indications of quality, safety and efficacy (Firenzuoli and Gori, 2007; Wang et al., 2009). Kai-Xin-San (KXS) is a famous traditional Chinese medicine (TCM) formula, initially recorded in the Chinese ancient book Bei-Ji-Qian-Jin-Yao-Fang (means precious formulas for emergence) compiled by Sun Si-Mao in 652 AD. It was reported to possess various pharmacological effects such as enhancing learning and memory, anti-oxidation, and anti-depression (Huang et al., 1999; Bian et al., 2000; Shang et al., 2003; Wang et al., 2007), and has been clinically used for the treatment of neurasthenia, Alzheimer's disease and neurosis. The formula consists of four herbs, namely ginseng (Ren-Shen, *Panax ginseng*), hoelen (Fu-Ling, *Wolfiporia cocos*), polygala (Yuan-Zhi, *Polygala tenuifolia*) and *Acorus gramineus* (Shi-Chang-Pu). As a principal component of KXS, ginseng has been extensively demonstrated to improve learning and memory in animals (Petkov and Mosharrof, 1987; Zhao and McDaniel, 1998; Kennedy and Scholey, 2003). The pharmacologically active ingredients in ginseng have been elucidated to be saponins and the most abundant saponins are ginsenosides Rg1, Rb1 and Re (Rg_1, Rb_1 and Re). The effects of the three compounds on learning and memory have also been well demonstrated (Yamaguchi et al., 1996; Mook et al., 2001), however, there is very little information on its safety. As a part of a safety evaluation of KXS, acute and sub-chronic oral dose toxicity studies were conducted to investigate the potential toxicity after single or 13-week repeated oral dosing of KXS in Sprague-Dawley rats.

2. Materials and methods

2.1. Preparation of Kai-Xin-San (KXS) extract

An aliquot of 1.5 kg ginseng (Panax ginseng), 1.5 kg hoelen (Wolfiporia cocos), 1 kg polygala (Polygala tenuifolia) and 1 kg Acorus gramineus were soaked together in 50 L water and extracted

Abbreviations: WBC, white blood cell; ALT, alanine amino transferase; AST, aspartate amino transferase; URE, blood urea nitrogen; ALP, alkaline phosphatase; Bil, total bilirubin; TP, total protein; GLU, glucose; Alb, albumin; Cre, creatinine; CHO, total cholesterol; CK, creatine kinase; TG, triglyceride; RBC, red blood cell; PLT, platelet counts; HCT, hematocrit; HGB, haemoglobin; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration.

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3 times using a circumfluence extraction method. The water extracts were filtered and evaporated under reduced pressure to obtain concentrates, which were freeze-dried to yield powder. 1 g yield powder contains 7.14 g total original herbs. Powders of KXS extract were stored at 4 °C. The contents of biochemical ingredients including ginsenoside Rg1, ginsenoside Re and triterpene saponin in KXS were analyzed in prior study (Liu et al., 2005; Hou, 2008).

2.2. Analysis of chemical contents of KXS by HPLC

The chemicals used for the identification and quantification of compounds in the KXS extract included the following: tenuifoliside A, 1-o-(E)-Benzoyl -[3-o-(E)-Alphatolluyl]-β-D-fructofuranosy-(2 → 1)-[β-D-glucopyranosyl-(1 → 2)]-α-D-glucopyranoside and ginsenoside Rb_1 which are the components respectively.

KXS extract (28 mg) was dissolved in 1 mL of methanol and filtered through 0.45 μm syringe filter before injection into the HPLC system. The HPLC system was composed of L-2200 Autosampler (Hitachi, Japan), L-2130 pump (Hitachi, Japan), ELSD 2000ES Detector (Alltech, America) and Agilent HC C_{18} (4.6 mm × 250 mm) column. Agradient solvent system of acetonitrile (A) and 0.65% ammonium acetate in water (B) was used as follows: 5%A/95%B (start), 15% A/85%B (8 min), 20%A/80%B (35 min), 28%A/72%B (60 min), 35%A/65%B (70 min), 100%A (74 min) and 5%A/95%B (75 min), at a flow rate of 1.0 mL/min (Fig. 1).

In order to make standard calibration curves, tenuifoliside A (11–220 μg/mL), 1-o-(E)-Benzoyl-[3-o-(E)-Alphatolluyl]-β-D-fructofuranosy-(2 → 1)-[β-D-glucopyranosyl-(1 → 2)]-α-D-glucopyranosid (13–260 μg/mL) and ginsenoside Rb_1 (13–260 μg/mL) were diluted in methanol and injected into the HPLC to give individual chromatograms. The calibration curves were plotted by calculating the peak area ratio, which has a relatively smaller error than the peak height ratio. The fitting equations of each calibration curve were as follows: tenuifoliside A, $y = 12{,}366x + 277{,}479$ ($r^2 = 0.9996$);1-o-(E)-Benzoyl-[3-o-(E)-Alphatolluyl]-β-D-fructofuranosy-(2 → 1)-[β-D-glucopyranosyl-(1 → 2)]-α-D-glucopyranoside, $y = 11{,}862x - 144{,}955$ ($r^2 = 0.9992$); and ginsenoside Rb_1, $y = 2169.9x - 10{,}933$ ($r^2 = 0.9999$). The chemical contents in KXS extract were determined to be the following: tenuifoliside A 9.99 ± 0.12 mg/g, 1-o-(E)-Benzoyl-[3-o-(E)-Alphatolluyl]-β-D-fructofuranosy-(2 → 1)-[β-D-glucopyranosyl-(1 → 2)]-α-D-glucopyranoside 16.71 ± 0.16 mg/g, and ginsenoside Rb_1 5.79 ± 0.04 mg/g.

2.3. Animals and housing condition

The acute toxicity test was carried out on 1.5 months old KM mice of either sex weighing between 19 and 30 g. Wistar rats of both sexes aged 2 months and weighing 140–180 g were used for the subchronic toxicity assessment.

All animals were obtained from the experimental animal center of the Academy of Military Medical Sciences. Animals were housed in colony cages (5 rats or mice per cage), under standard laboratory conditions (ventilated room, 24 °C, 75% humidity, 12 h light/dark cycle) and had free access to standard commercial diet and tap water. All animal experiments were conducted in accordance with the internationally accepted principles for laboratory animal use and care as described in the European Community guidelines (Official Journal of European Union L197 vol. 50, July 2007) and were approved by the Animal Ethics Committee of our hospital (0999 and 09100).

2.4. Acute oral toxicity study in mice

Mice were randomly assigned to each of six groups of 20 mice (10 females and 10 males). They were fasted overnight (12 h) with free access to water prior to administration of single doses (60.04, 48.03, 38.42, 30.74, 24.59 and 19.67 g/kg) of the extract dissolved in distilled water. Treatment was given twice by gavage of 0.8 mL/mice/time with the interval of 8 h. The general behaviour of the mice was continuously monitored for 4 h after the treatment, intermittently during a 24-h period (Twaij et al., 1983), and thereafter daily up to 7 days. The LD_{50} was determined as previously described (Molle, 1986).

2.5. Sub-chronic oral toxicity study in rats

Animals were randomly divided into 4 groups (I–IV) of 30 each (15 females and 15 males). The extract, dissolved in distilled water, was administered by daily gavage for 91 days, to groups I–IV (doses of 0, 1, 3 and 9 g/kg, respectively). The animals were observed for signs of toxicity and mortality throughout the experimental period. The BW, water and food consumption were recorded weekly.

At the end of the 91-day experiment, 20 rats of each group (10 rats left for the withdrawal study) were sacrificed by decapitation under anaesthesia (thiopental 50 mg/kg). Blood was collected with and without anticoagulant (EDTA) for haematological and biochemical studies respectively. The organs (brain, thymus, heart, lung, liver, spleen, kidneys, adrenal glands, testicles, epididymis, ovaries, uterus) were weighted and the relative organ weight (weight of organ as proportion of the total body weight of each rat) was calculated and compared with the value of control. Organ samples (kidney, pancreas, lung and liver) were fixed in 10% formalin for histopathological examination.

In 30-day of post treatment, the animals were withdrawed of KXS extract and had free access to standard commercial diet and tap water. At the end of withdrawal study, rats were treated according to the 91-day experiment.

2.6. Measurement of haematological and biochemical parameters in rats

Whole blood cell (WBC), red blood cell (RBC) and platelet counts (PLT), hematocrit (HCT), haemoglobin (HGB), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC), were determined using a haematology analyzer MEK-6318K (Nihon Kohden Co. Ltd.).

For biochemical analysis, blood without additive was centrifuged at 3000 × g at 4 °C for 10 min. Serum was separated and stored at −20 °C until determination of alanine amino transferase (ALT), aspartate amino transferase (AST), blood urea nitrogen (URE), alkaline phosphatase (ALP), total bilirubin (Bil), total protein (TP), glucose (GLU), albumin (Alb), creatinine (Cre), total cholesterol(CHO), creatine kinase (CK) and triglyceride (TG) using an automatic biochemistry analyzer (Italy SABA 18 Co. Ltd.).

2.7. Statistical analysis

The data, expressed as mean ± SD, were subjected to Kruskal–Wallis one way analysis of variance (ANOVA). Inter group comparisons were made by Mann–Whitney-*U*-test (two-tailed) for only those responses which yielded significant treatment effects in the ANOV test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Acute oral toxicity of KXS in mice

The effects of oral administration of single doses of KXS in mice are summarized in Table 1. There was a regular dose-dependent increase in mortality and adverse effects in both sexes. The mortality rate (0% at 19.67 g/kg) progressively rose to 100% at the