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A 90-day subchronic toxicity study with sodium formononetin-3′-sulphonate (Sul-F) delivered to dogs *via* intravenous administration

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ABSTRACT

Sodium formononetin-3′-sulphonate (Sul-F) is a water-soluble derivate of formononetin, and an increasing number of studies have shown that Sul-F not only possesses favorable water solubility but also exhibits good lipid-lowering and bioactivities. In the current study, the toxicity of Sul-F was evaluated in dogs after 90-day intravenous infusion. Dogs were treated with Sul-F at dose of 0, 33.3, 100, and 300 mg/kg, and observed for 90-day followed by 28-day recovery period. Weekly measurement of body weight, temperature and food consumption were conducted. Ophthalmoscopy, ECG examination, urinalysis, serum biochemistry and hematology examination were performed at pre-test, on days 45 and 90, and following by 28-day recovery period. Histological examination was performed on day 90 and 28-day recovery period. No mortality, ophthalmic abnormalities or treatment-related findings in body weight, clinical chemistry, hematology, and histopathological examination were detected. However, a white crystal (non-metabolic Sul-F), transient vomiting and recoverable vascular stimulation were observed in 300 mg/kg/day Sul-F treated dogs. Under the conditions, the no-observed-adverse-effect-level (NOAEL) for Sul-F was 100 mg/kg in dogs.

1. Introduction

Trifolium pratense L. (red clover), a species of clover, has been reported to possess a wide range of biological activities, such as vascular relaxation action (Wu et al., 2009), neuroprotective effect (Yu et al., 2005; Liang et al., 2014), anti-apoptosis activity (Huang et al., 2013), and mammary gland proliferation function (Wang et al., 1995). *T. pratense* L. has been applied for various health care industries and medicinal purposes (Geller et al., 2009), and the pharmacological actions is attribute to estrogen-like isoflavonoid. Formononetin ($C_{16}H_{12}O_4$, Fig. 1A), a isoflavonoid of *T. pratense* L., possesses the potent pharmacological activities. Formononetin, meanwhile, is a poorly-water-soluble compound, and is difficult to develop as a drug product. Recently, sodium formononetin-3′-sulphonate (Sul-F, $C_{16}H_{12}O_7SNa$, Fig. 1B.), a derivate of formononetin was synthesized (Chinese patent: ZL200710017326.5), and pharmacological activities have been reported *in vitro* and *in vivo* (Zhang et al., 2011; Zhu et al., 2014). The safety of an novel compound play a vital role for human use, and toxicological evaluation is necessary in experimental animal to predict toxicity and to provide guidelines for selecting a 'safe' dose in humans (Hamid et al., 2008). However, no systematic study about possible toxicity of Sul-F was reported. Our previous acute toxicity of Sul-F have been performed in Sprague–Dawley rats and Beagle dogs (Li et al., 2015). The present 90-day intravenous administration study in beagle dogs was performed to clarify Sul-F subchronic toxicity, and further to obtain information on the safety of Sul-F and provide guidance for selecting a safe dose in human use and exposure to this substance.

2. Materials and methods

2.1. Chemicals

Sodium formononetin-3′-sulphonate (Sul-F, CAS#: 485-72-3. Fig. 1B) with a purity of >99%, was obtained as our previous study (Li et al., 2015).

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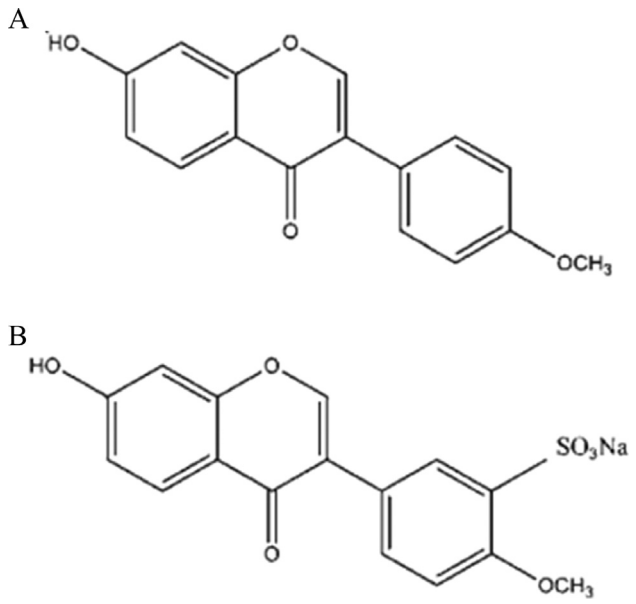

Fig. 1. A) Chemical structure of formononetin Structure of Compound; and B) Chemical structure of Sul-F.

2.2. Animals

The study was conducted with Beagle dogs (4–6 kg), half male and half female, obtained from Nanhai KeJin Limited Company of Experimental Animals (Guangzhou, China). Animals were kept under hygienic conditions in individual cages, and examined for clinical signs of ill health on receipt and observed within 14 days of arrival. Room temperature and humidity were targeted at 23 ± 5 °C and 40–60% relative humidity with natural illumination and was ventilated automatically. The basal diet was obtained from Beijing Ke Ao Xie Li Feed Co., Ltd. (Beijing, China).

2.3. Study design

The study was conducted in accordance with China State Food and Drug Administration ([H] GPT 2-1, 2005), and the OECD Principles of Good Laboratory Practice ENV/MC/CHEM (98)17). 40 animals were randomly divided into a negative control group and three experiment groups (5 dogs/sex/group). Experiment group animals were administered Sul-F at doses of 33.3, 100, and 300 mg/kg *via* the forelimb vein at an infusion rate of 2–3 ml/min (stability testing of Sul-F is OK under our lab condition, and data not shown). The dosing volume was 10 ml/kg in all dogs. The control group animals were administered with the same volume normal saline. Procedures involved in the use of laboratory animals were in accordance with the Guidelines of the Animal Care set by the Association of Laboratory Animal Science and the Center for Laboratory Animal Science.

General symptoms and mortality were observed twice daily. Body weights and temperature were recorded throughout the treatment and recovery periods. Ophthalmoscopy, serum biochemistry (Autolab Analyser, AMS, Italy), hematology (CA-500 counter, AITAIK, Japan), urine (Urine analyzer,FA-100, China), and electrocardiogram(ECG) (Electrocardiograph, ECG-6511, China) were examined on days 0, 45, 90, and following by 28-day recovery period. Gross observation, organ weight, and histopathology examination were determined on 90-day and 28-day of withdrawal, respectively. On days 90, six dogs (three males and three females) of each group were anesthetized with sodium pentobarbital (35 mg/kg) after being fasted overnight, and sacrificed for pathological and bone marrow examinations. The remaining animals were allowed 28-day recovery period to observe the reversibility of toxicity or delayed occurrence of toxic effects if there is the possible toxicity. On days 28 of withdrawal, all animals were then similarly anesthetized, sacrificed, and examined as previous study.

2.4. Ophthalmoscopy, ECG examination and urinalysis

On days 0, 45, 90, and following by 28-day recovery period, ophthalmoscopy, ECG examination and urinalysis were performed. The parameters including (1) ophthalmoscopy: slit lamp examinations, ocular fundus examinations and macroscopic observations; (2) ECG examination: the sclera, conjunctiva, cornea, lens, and iris; and (3) Urinalysis: specific gravity, leukocytes, pH, nitrite, protein, ketones, glucose, urobilinogen, occult blood, bilirubin, and hemoglobin.

2.5. Serum biochemistry and hematology

On days 0, 45, 90, and following by 28-day recovery period, serum biochemical and hematological determinations were detected from samples drawn from the forelimb vein. Serum biochemical analysis included: aspartate aminotransferase (AST), alanine aminotransferase (ALT), glucose (GLU), total protein (TP), creatinine (CRE), albumin (ALB), blood urea nitrogen (BUN), total cholesterol (T.Cho), total bilirubin (T-BILI), alkaline phosphatase (ALP), triglyceride (TG), Gamma-glutamyltranspeptidase (γ-GT), and creatine kinase (CK). All the kits were obtained from Zhongsheng Beikong Bio-technology and Science Inc. (Beijing, China). The serum ion of potassium (K), sodium (Na), and chloride (CL) were analyzed by Easylyte Plus. Hematology samples were collected into ethylene diamine tetraacetic acid (EDTA) treated tubes, and performed with a CA-500 counter. All parameters including: red blood cell (RBC), hematocrit (HCT), hemoglobin concentration (HGB), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), platelet count (PLT), mean corpuscular hemoglobin concentration (MCHC), and white blood cell counts (WBC). Differential counts of leukocytes (Neutrophilic leukocyte; Leukomonocyte; Monocytes) and reticulocytes were counted under a microscope after staining with hematoxylin-eosin (H&E), respectively.

2.6. Gross observation, organ weights and histopathology examination

On days 90 and following by 28-day recovery period, dogs were sacrificed by exsanguination under anesthesia, major organs gross observation and organ weight were examined carefully, such as the heart, lungs, liver, spleen, brain, kidneys, thymus, testes, epididymis, adrenal glands, uterus, and ovaries. In addition, the absolute and relative (organ to body weight ratios) weights of organs and tissues were measured. Then the following tissues and organs were obtained from all dogs: abnormal lesions, mammary gland, pancreas, spleen, nervus opticus, stomach, duodenum, ileum, colon, cecum, mesenteric lymph node, submandibular lymph node, salivary gland, liver, sternum, heart, lung, thymus, trachea, esophagus, aorta, thyroid (and parathyroid), sciatic nerve, brain, spinal cord (cervical, thoracic, and lumbar), kidneys, adrenal glands, pituitary gland, urinary bladder, ovaries, uterus, testes, prostate, epididymis, and injection site. Tissues and organs were fixed with 10% neutral buffered formalin solution, and were routinely processed, embedded in paraffin, and sectioned at 3–5 μm. The sections were stained with H&E stain for microscopic examination.