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Evaluating pulmonary toxicity of Shuang–Huang–Lian *in vitro* and *in vivo*

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ABSTRACT

Ethnopharmaocological relevance: Shuang–Huang–Lian (SHL) is a traditional Chinese formula and has been used for the treatment of respiratory tract infections by inhalation. However, the pulmonary toxicity *via* inhalation is largely uninvestigated.

Aim of study: : To evaluate the pulmonary toxicity of SHL following *in vivo* intratracheal spray to rats and *in vitro* exposures to A549 and Calu-3 cells.

Methods: : Calu-3 and A549 cells were exposed to SHL, chlorogenic acid, baicalin and forsythin solutions and *in vitro* cytotoxicity was evaluated using an MTT assay, whilst rats were subjected to intratracheal administration of SHL solutions and *in vivo* toxicity was indicated by assaying the LDH activity and total protein content in bronchoalveolar lavage fluid (BALF) and observing the histopathologic changes of the lungs. Secretion of inflammatory mediators, including IL-6, IL-8 and TNF-α, in cell culture media and BALF was quantified by ELISA.

Results: : The MTT cell viability data revealed the presence of minor toxicity to Calu-3 or A549 cells following exposure to SHL and its major ingredients for 24 h or 48 h. However, the cell cultural media showed no sign of inflammatory responses. The *in vivo* results showed that exposures to SHL at doses of up to 50 mg/kg did not significantly increase the total protein content, the LDH activity and the concentrations of IL-6, IL-8 and TNF-α in BALF. However, although intratracheal sprayed SHL at doses of up to 6 mg/kg for histopathologic study and up to 25 mg/kg for cell counts showed no sign of adverse effects, inhaled SHL at elevated doses appeared to induce alveolar fusion in the lung and significant increases in the cell number of monocytes and granulocytes in the BALF.

Conclusion: : The results demonstrated that the pulmonary safety of inhaled SHL was dependent on the administered dose. Inhalation therapy of SHL may be safely used when the inhaled dose was properly controlled.

1. Introduction

Inhalation therapy of herbal medicine has been practiced for thousands of years. The earliest written document about inhalation therapy dated back to about 2000 BC, when Indian used the smoke of *Atropa belladonna* leaves for treatment purpose in India. Ancient traditional Chinese medicine (TCM) practitioners had also used the smoke and vapours of medicinal plants for the treatment of respiratory tract diseases. In recent decades, more than 50 TCM formulas have been reported for the treatment of lung disorders *via* inhalation in the literature (Wang et al., 2007). It is well-known that inhalation therapy for respiratory diseases offers unique advantages including a rapid onset of therapeutic action, a massive reduced dose relative to the oral or other systemic routes and consequently, reduced systemic side effects and other adverse reactions accompanied by injectables (Byron and Patton, 1994; Dalby and Suman, 2003). However, although delivery of TCM to the respiratory tract has been widely utilized in clinic in China for the management of various pulmonary diseases and infections, pulmonary toxicity profiles of this medicine have yet been thoroughly established.

Amongst these inhaled formulas, the inhalation delivery of Shuang–Huang–Lian (SHL) has been most extensively studied and numerous clinical papers have documented the therapeutic efficacy of SHL *via* inhalation (Zhou and Wang, 2001; Lu, 2007; Liu et al., 2010). SHL is a traditional Chinese compound formula prepared from honeysuckle flower, radix scutellariae and fructus forsythiae, and has been widely used for treating respiratory tract infection, such as influenza, acute tonsillitis, acute bronchitis and acute faucitis (Guan and Wu, 1995; Li and Wang, 2001; Liu, 2009). Clinically, SHL products are delivered by different routes of administration (e.g. oral, injectable and pulmonary routes) (Sun et al.,

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2006). Although the metered dose inhaler formulations have been approved by the State Food and Drug Administration of China and the nebulization therapy has been clinically applied in hospital settings, the pulmonary safety of SHL has not been documented. Therefore, the aim of this study was to evaluate the potential pulmonary toxicity of inhaled SHL *in vitro* and *in vivo*.

2. Materials and methods

2.1. Materials

SHL freeze-dried injectable powders were obtained from the Second Chinese Medicine Factory, Harbin Pharm Group Co., Ltd. (Harbin, China) and the powders contained 17, 11 and 282 mg of chlorogenic acid, forsythin and baicalin per gram of powders, respectively, as determined by an HPLC assay. Chlorogenic acid, baicalin and forsythin were bought from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). L-Glutamine (200 mM), non essential amino acid, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide), trypsin (0.25%, w/v) and EDTA (0.22%, w/v) solutions were purchased from Sigma–Aldrich. Minimal essential medium (MEM) and Nutrient Mixture F-12 (F-12) were obtained from Invitrogen Corporation (USA), whilst fetal bovine serum (FBS) was from Hyclone (USA). Rats and human interleukin-6 (IL-6), interleukin-8 (IL-8) and tumor necrosis factor-α (TNF-α) kits were bought from Research Diagnostics Inc. (Flanders, USA), whilst LDH and BCA kits were from Nanjing Jiancheng Co., Ltd. (Nanjing, China) and Beijing Saichi Biotechnology Co., Ltd. (Beijing, China), respectively. All other chemicals and reagents were of analytical grade.

2.2. In vitro toxicity evaluation

2.2.1. Cells in culture

The A549 and Calu-3 cell lines were bought from the Cell Culture Center, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, China) and used between passages 30–40 and 22–36, respectively. A549 cells were grown in F-12 medium supplemented with 10% (v/v) FBS, 1% (v/v) L-glutamine solution (200 mM), 100 U/ml of penicillin and 125 U/ml streptomycin, whereas Calu-3 cells were in MEM medium supplemented with 10% (v/v) FBS, 1% (v/v) non-essential amino acid solution (10 mM), 1% (v/v) L-glutamine solution (200 mM), 100 U/ml of penicillin and 125 U/ml streptomycin (Liu et al., 2008). Cell cultures were maintained using 15 cm^2 flasks in a humidified atmosphere under 5% CO_2 at 37 °C. Medium was exchanged every other day and cells were subcultured weekly. Fresh medium was replaced the evening before the experiment.

For the *in vitro* study, tests for mitochondrial activity (MTT assay) and cytokine production (IL-6, IL-8 and TNF-a) were performed and done in triplicate at least.

2.2.2. MTT toxicity assay

The MTT assay was used to evaluate mitochondrial activity (Mosmann, 1983). A549 and Calu-3 cells were seeded into 96-well plates (Corning, New York, USA) in 100 μl of the same medium used for culture in flasks at a density of 5000 and 8000 cells per well, respectively. Cells were allowed to attach overnight at 37 °C in a 5% CO_2 atmosphere before used. To initiate the assay, cell culture medium was replaced by 100 μl of fresh medium containing different concentration of test solutions. After 24 h and 48 h exposure, culture medium was discarded and plates were washed 3 times with PBS because SHL and baicalin could react with MTT. Then, 20 μl of the MTT solution (0.5 mg/ml in PBS, pH 7.4) was added to each well and incubated. After 4 h, medium was removed carefully, and 200 μl DMSO was added to each well to dissolve any formazan crystals generated. Then, plates were shaken for 10 min, and the optical density of each well was measured immediately by microplate reader (lQuant™, BioTek Instruments, Inc., USA) at 570 nm.

2.2.3. IL-6, IL-8 and TNF-α determination in cell culture media

The IL-6, IL-8 and TNF-α concentration in A549 and Calu-3 cell supernatants were measured using a commercially available ELISA kits (human), according to the manufacturer's protocol. Cells were seeded in 96-well plates, exposed to different concentrations of test solutions, and incubated 24 h and all samples were assayed in triplicate. After incubation, the plate was centrifuged at 1900 rpm for 4 min. Then the cell culture supernatants were added to pre-coated monoclonal antibody micro-Elisa wells and analyzed the concentration of pro-inflammatory cytokines using different concentrations of recombinant cytokine solutions as calibration. The optical density of each samples were measured by a microplate reader at 450 nm in 15 min.

2.3. In vivo toxicity study

2.3.1. Animals and drug administration

Animal studies were all performed under "Guide of the care and use of laboratory animals" of Institute of Medicinal Plant Development, Chinese Medical Science Academy & Peking Union Medical College. Male Sprague Dawley rats (180–220 g, 8 weeks old) were purchased from Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences (Beijing, China). The animals were kept at specific pathogen free (SPF) animal house, for at least 7 days prior to the experiments, until weight 220–300 g. All animals were maintained at controlled temperature (22 ± 2 °C), under 12 h light/dark cycles, and free access to diet and water.

The animals divided into 8 groups randomly, each group consisted of at least 5 rats, which were given saline or 1.0 mg/kg to 200 mg/kg SHL solution *via* intratracheal route. Prior to administration, animals were anesthetized by an intraperitoneal injection of pentobarbital sodium (20 mg/kg). Following anesthesia, intratracheal spray was conducted at a volume of 250 μl/kg after the curved balled needle of the micro-sprayer (Model IA-1B, Penn-Century Inc., USA) attached on a syringe was inserted into the trachea under visual guidance. After intratracheal dosing, the delivery devices were removed and the animals were held in an upright position for 1 min to ensure deposition of the dose.

2.3.2. Bronchoalveolar lavage (BAL) and cell counts

Twenty-four hours after drug administration, the animals were anesthetized by pentobarbital sodium (40 mg/kg) and the chests were carefully opened following the sacrifice of rats. Subsequently, a catheter inserted into the trachea and warm saline (37 °C) was instilled with 2 ml for 5 times. The resultant bronchoalveolar lavage fluids (BALF) were combined on ice and centrifuged 10 min (5000 rpm) at 4 °C. The cell-free supernatants were frozen for further analysis. The lungs of rats were picked up for histopathology section. Cells recovered from the BALF were counted using PCE-140 Cell Counter (Erma, Japan) following the dispersion of cell pellets in 2.5 ml saline and total number of white blood cells (WBC) as well as differential counts of lymphocytes, monocytes, and granulocytes were determined.

2.3.3. Total protein assay

The total protein concentration in BALF was measured by BCA assay using bovine serum albumin as standard for calibration. Briefly, an aliquot of 150 μl of each standard or test samples was transferred into a 96-well plate in duplicate. Subsequently, 150 μl of the working reagent, which was prepared by mixing 50 parts of BCA reagent A and 1 parts of reagent B, was added to each well