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Effects of inhalable microparticle of flower of *Lonicera japonica* in a mouse model of COPD

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ABSTRACT

Ethnopharmacological relevance: Flower of *Lonicera japonica* (FLJ) is a traditional herbal medicine widely used in East Asia as an anti-inflammatory and anti-oxidative agent. The purpose of this study is to develop an inhalable powder formulation of FLJ and to evaluate its biological effects in a mouse model of chronic obstructive pulmonary disease (COPD).

Methods: Inhalable dry powder containing FLJ was produced by spray-drying with leucine as an excipient. Its aerodynamic properties and anti-inflammatory activities were evaluated using the Anderson cascade impactor (ACI) and a mouse model of COPD, respectively.

Results: FLJ microparticle (FLJmp) had a hollow spherical shape in electron microscopy and showed aerodynamic properties suitable for inhalation (fine particle fraction of 54.0 ± 4.68% and mass median aerodynamic diameter of 4.6 ± 0.34 μm). FLJmp decreased TNF-α and IL-6 expression in RAW264.7 cells activated by lipopolysaccharide (LPS). In mice challenged with LPS and cigarette smoke solution (CSS) to develop COPD, FLJmp decreased the levels of TNF-α and IL-6 in bronchoalveolar fluidas well as the number of inflammatory cells including neutrophils in peripheral blood. In addition, FLJmp induced recovery of elastin and collagen distribution, reduction of caspase-3 expression in lung tissues of COPD mice.

Conclusions: Inhalational delivery of FLJ using a microparticle system is a promising strategy for the treatment of COPD.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is a major pulmonary disease characterized by lung inflammation and increasing difficulty in breathing, ranked as one of the top four high mortality diseases worldwide (Rabe et al., 2007; World Health Organization, 2000). The number of patients has continuously grown, soon to make COPD the third leading cause of death (Lopez and Murray, 1998). Medical expenditure for COPD treatment and related costs for the morbidity bring about enormous economic and social burden for societies and for public and private payers (Chapman et al., 2006). Currently there are no direct therapies to save lung functions of COPD patients. Pharmacological approaches are limited to relieving symptoms and attenuating lung exacerbation (Rabe et al., 2007). Bronchodilators, which constitute main medicines for allopathy, have a relatively high risk of side effects to COPD patients, who are mostly elderly and likely to have other associated diseases. Corticosteroids are also used for temporary treatment of acute exacerbation and for continuous treatment of severe stage- or frequent exacerbation. However, they can induce skin bruising, reduction of bone density, myopathy, which contributes to muscle weakness, decreased functionality, and respiratory failure in subjects with advanced COPD (Decramer et al., 1996; The Lung Health Study Research Group, 2000). The lack of ultimate treatment and potential side effects of current therapies impose a significant challenge to long-term

Abbreviations: ACI, Anderson cascade impactor; BALF, bronchoalveolar fluid; COPD, chronic obstructive pulmonary disease; CSS, cigarette smoke solution; FBS, fetal bovine serum; FPF, fine particle fraction; FLJ, flower of *Lonicera japonica*; FLJmp, FLJ microparticle; H&E, hematoxylin–eosin; HPLC, high pressure liquid chromatography; LPS, lipopolysaccharide; MMAD, mass median aerodynamic diameter; M-T, Masson-trichrome; MMPs, matrix metalloproteinases; PAS, periodic acid-Schiff; SD, standard deviation

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management of COPD. For sustainable management of COPD, a safe and effective alternative therapy has long been anticipated.

The flower of *Lonicera japonica* (FLJ), also called Geum-eun-hwa (Jin-yin-hua, 金銀花), is an herbal medicine which has been clinically used for the treatment of inflammatory diseases in East Asian countries (Kim, 1996). Ithas been prescribed to treat respiratory tract infections, exopathogenic wind-heat, epidemic febrile diseases, sores, carbuncles and some infectious diseases (Ma et al., 2002; Shang et al., 2011). Due to the effects on fever and edema, 'Shen Nong Ben Cao Jing' has listed FLJ as one of the 'top grade' herbal medicines (Gu, 2007). In addition, 'Ben Cao Gang Mu', a famous classical book of Chinese materia medica, proposed that FLJ could be used to remove the heat-evil, and treat the edema and dysentery (Li, 1979). Recently, an increasing number of studies have shown that FLJ can inhibit various inflammatory reactions, such as COX-2, TNF-α, IL-6 and ERK phosphorylation (Kang et al., 2004; Park et al., 2012; Xu et al., 2007). Especially FLJ reduced the influx of neutrophils and total cells, decreased TNF-α and IL-6 levels in mice with lipopolysaccharide (LPS)-induced COPD (Lee, 2009). Given the strong evidence of anti-inflammatory effects, there is a well-justified expectation that FLJ would be a promising alternative medicine for COPD management.

Despite the therapeutic potential of FLJ, its oral administration (like many other herbal medicines) has been quite challenging due to the large volume (>100 mL of liquid extract) and/or negative organoleptic effects (Choi et al., 2004). It is even more challenging to introduce FLJ to western patients, who may not be used to the consumption of herbal extracts. We hypothesize that an optimally engineered inhalable form of FLJ can reduce the dose requirement by limiting the effect to the site of action (lower airways) and improve the patient acceptance. Therefore, successful development of inhalable FLJ formulation will provide a safe and sustainable complementary option for long-term management of COPD.

With this goal in mind, we aimed to develop an inhalable formulation of FLJ in the form of dry powder. The objective of this study is to produce an inhalable microparticle form of FLJ (FLJmp) and evaluate its aerodynamic properties and bioactivity in an animal model of COPD. The results of our study show that FLJ can be successfully formulated as an inhalable microparticle, which can reduce lung injury in mice with COPD.

2. Methods

2.1. Materials

FLJ was obtained from Humanherb Co. (Kyeongbuk, Korea). RAW264.7 cell lines were purchased from ATCC (Manassas, VA, USA). All media and their components were purchased from Invitrogen (Carlsbad, CA, USA). Leucine (L-form, 99%) was purchased from Alfa Aesar (Ward Hill, MA, USA). Chlorogenic acid, isochlorogenic acid A and B were purchased from KOCbio (Daejeon, Korea).HPLC-grade reagents, acetonitrile and water were obtained from J.T. Baker (Phillipsburg, NJ, USA). All other chemicals were of reagent grade.

2.2. Preparation of FLJ extract

Flower of *Lonicera japonica* (lot No. K1312061, cultivated at Henan province in China in 2011) was obtained from Humanherb Co. (Kyeongbuk, Korea), a licensed herb company, and identified by Professor G.H. Jeong (Oriental Medical College, Daejeon University, Daejeon, Korea). A voucher specimen (No. 2012-014) was deposited in the herbarium stock room at the Department of Pharmacognosy, Oriental Medical College, Daejeon University. FLJ dry herb was suspended in 70% ethanol and extracted at 60–70 °C for 3 h by reflux extraction. The ethanolic extract was evaporated at 45 °C and lyophilized subsequently. The extraction yield was 20.6%.

2.3. Production of spray dried particles

Inhalable microparticle forms of FLJ extract were prepared by spray-drying using the LabPlant SD-05 spray dryer (Lab-Plant Ltd., Huddersfield, UK). Lyophilized FLJ extract (80 wt%) and an excipient (20 wt%),which were leucine, DPPC, or a 1:1 mixture of leucine and DPPC, were dissolved in 70% ethanol. Typically the solution was introduced to the spray dryer at 17–20 mL/min and atomized through a 1-mm nozzle using compressed air with an inlet temperature of 150 °C.

2.4. HPLC analysis of FLJ and its microparticles

The 100 mg of FLJ extract and microparticles of FLJ (FLJmp) was accurately weighed and dissolved in 10 mL of 50% methanol aqueous solution. The solution was centrifuged at $12{,}000 \times g$ for 10 min and filtered with a 0.45 μm microporous membrane. The filtered sample was analyzed by reverse phase-high performance liquid chromatography (HPLC) using Waters Alliance 2695 system (Waters Co., Milford, MA, USA), coupled with a 2996 photodiode array detector. Phenomenex Luna C18 column (250 mm × 4.6 mm; particle size 5 μm, Phenomenex, Torrance, CA, USA) was used as a stationary phase. The mobile phase was composed of 0.1% (v/v) trifluoroacetic aqueous solution (A) and acetonitrile (B). The elution conditions were as follows: at 0 min the mobile phase consisted 95% A/5% B and was held for 10 min. From 10 to 60 min a gradient was applied to 70% A/30% B, followed by a wash with 100% B for 8 min and a 12 min equilibration period at 95% A/5% B. The separation temperature was maintained at 40 °C throughout the analysis, with a flow rate of 1.0 mL/min and injection volume of 10 μL.

Peaks were detected at 254 nm and identified by comparing their retention times with those of commercial standards (Chlorogenic acid, isochlorogenic acid A and B). For quantitation of each peak, a mixture of standard compounds of known concentrations was analyzed in duplicate before and after the batch of samples, and the peak areas were used to calculate the compound contents in each sample. Calibration curves of the standards ranging from 12.5 to 400 μg/mL (6 levels) revealed good linearity with R^2 values exceeding 0.99 (peak areas vs. concentration).

2.5. Physical evaluation of FLJ microparticles

The potential ability of microparticles to deposit in the lower airways was estimated in vitro using the eight-stage Mark II Anderson Cascade Impactor (ACI) as we previously reported (Ibrahim et al., 2010; Yang et al., 2009, 2010). Dry particles (10 mg) were manually loaded in a gelatin capsule (size 3), put into a Rotahaler, and split open to release the particles. Each set of dry powders was drawn through the induction port into ACI operated at a flow rate of 28.3 L/min for 8.5 s. The amount of particles deposited at each impaction stage was determined by measuring the difference in weight of the collection plate (for the filter stage, glass filter with pore size <1 μm, ThermoFisher). The effective cutoff aerodynamic diameters for each stage were Stage 0, 9 μm; Stage 1, 5.8 μm; Stage 2, 4.7 μm; Stage 3, 3.3 μm; Stage 4, 2.1 μm; Stage 5, 1.1 μm; Stage 6, 0.65 μm; Stage 7, 0.43 μm. The fine particle fraction (FPF) was defined as the amount of powder with an aerodynamic size <4.7 μm (particles deposited at stage 3 and lower) divided by the initial total powder loaded in the Rotahaler (10 mg, nominal dose). The cumulative mass of powder less than effective cutoff diameter as percent of total mass recovered in the ACI was plotted against the effective cutoff