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Original article

New insights into the effects and mechanism of a classic traditional Chinese medicinal formula on influenza prevention

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ABSTRACT

Background: KangBingDu (KBD) is a classic traditional Chinese medicinal formula widely used to treat influenza. However, little information is available from controlled studies regarding the anti-influenza pharmacological activities of KBD and its underlying mechanisms, at least partly due to the lack of appropriate study models.

Purpose: We hypothesized that KBD might provide a protection against influenza infection by reducing the host's susceptibility to viruses. To prove it, mouse restraint stress model was employed.

Methods: Mice were restricted and infected with influenza virus. KBD (13 and 26 mg/kg/d) was orally administrated to mice from the first day of restraint stress and lasted for 7 days (twice a day). Mice were monitored daily for morbidity, symptom severity, and mortality for 21 days. The histopathologic changes were examined. For the study of mechanisms of action, we investigated whether KBD could promote mitochondria antiviral signaling protein (MAVS)-mediated antiviral signal and inhibit nuclear factor-kappa B (NF-κB)-mediated inflammation response.

Results: KBD significantly decreased the susceptibility of restraint mice to influenza virus, as evidenced by lowered mortality, attenuated inflammation and reduced viral replications in lungs. Further results revealed that KBD elevated the protein expression of MAVS, which subsequently increased the IFN-β and IFITM3 protein levels, thereby helping to fight viral infections. Finally, we identified that (*R,S*)-goitrin, mangiferin, forsythin and forsythoside A were effective components in KBD against influenza viral infections.

Conclusion: KBD can reduce the susceptibility to influenza virus via mitochondrial antiviral signaling.

Introduction

KangBingDu (KBD) oral liquid, a classic traditional Chinese medicinal formula which is modified based on the traditional Chinese medicine (TCM) formulations of "BaiHutang" and "QingWenBaiDuYin", is widely used for clinical treatment of viral infections, especially influenza virus. KBD has been used for the treatment of epidemic encephalitis B, mild childhood hand-foot-mouth disease, acute upper respiratory infection and pneumonia in clinic with few side effects (Qiao et al., 2012). Despite the use and development of KBD in anti-influenza therapy, the pharmacological activities of many herbal formulae still lack evidence-based investigation and mechanism study.

Chinese herbal medicines are guided by TCM theory in increasing the host's resistance to pathogens, which is different from the theory of western medicines aiming at "setting a thief to catch a thief". The three distinct host strategies that are used to deal with the same infection are resistant host, tolerant host, and susceptible host (Iwasaki and Pillai, 2014). Therefore, increasing resistance/tolerance or reducing the susceptibility of the host are key strategies for prevention of influenza. Stress has been well-known to perturb the homeostatic state of the body (Jaggi et al., 2011), making people vulnerable to diseases. Our previous studies indicated that the restraint stress could increase the susceptibility to influenza virus in mice and provide a useful model basis for evaluating the effectiveness of the herbal medicinal product and natural products (Cao et al., 2012; He et al., 2011; Tang et al., 2014). Hence,

Abbreviations: KBD, KangBingDu oral liquid; NP, nucleoprotein; NF-κB, nuclear factor-kappa B; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor α; IFITM3, interferon inducible transmembrane 3; IFN-β, interferon-β; MAVS, mitochondrial antiviral signaling; TCM, traditional Chinese medicines; RT-PCR, reverse transcription polymerase chain reaction.

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in the present study, we employed this model to evaluate the anti-influenza activities of KBD and investigate its related mechanisms.

Material and methods

Drugs and reagents

KBD (Batch No. 201408062), forsythoside I, forsythoside H, pinoresinol-4-O-glucoside and β-asarone were provided by Guangzhou Xiangxue Pharmaceutical Co., Ltd. (Guangzhou, China). (*R,S*)-goitrin, mangiferin, forsythoside A, forsythin, α-pinene, β-pinene, borneol, methyleugenol, patchouli alcohol and α-asarone were purchased from National Institutes for Food and Drug Control (Beijing, China). Terpinen-4-ol was provided by Dr. Ehrenstorfer GmbH (Augsburg, Germany) and α-terpineol was procured from Sigma-Aldrich (St. Louis, MO, USA). The purity of all reference compounds was >98.0%.

KBD is composed of Radix isatidis (Banlangen; the radix of *Isatis indigotica* Fortune ex Lindl. (a synonym of the accepted name *Isatis tinctoria* L. according to theplantlist.org), of family Brassicaceae), Rhizoma phragmitis (Lugen; the rhizome of *Phragmites communis* Trin. of family Poaceae), Radix rehmanniae (Dihuang; the radix of *Rehmannia glutinosa* (Gaertn.) DC. of family Scrophulariaceae), Radix curcumae (Yujin; the radix of *Curcuma wenyujin* Y.H.Chen et C.Ling. (a synonim of the accepted name *Curcuma aromatica* Salisb. according to theplantlist.org), of family Zingiberaceae), Rhizoma anemarrhenae (Zhimu; the rhizome of *Anemarrhena asphodeloides*. Bunge of family Asparagaceae), Rhizoma acori tatarinowii (Shichangpu; the rhizome of *Acorus tatarinowii* Schott. (a synonym of the accepted name *Acorus calamus* L.) of family Acoraceae), Herba pogostemonis (Guanghuoxiang; the caulis of *Pogostemon cablin* (Blanco) Benth. of family Lamiaceae), Fructus forsythiae (Lianqiao; the fructus of *Forsythia suspensa* (Thunb.) Vahl. of family Oleaceae) and Gypsum fibrosum (Shigao; one mineral with hydro calcium sulfate fibriform crystallized polymeric) in a dry weight of 129 g, 61 g, 32 g, 25 g, 25 g, 25 g, 29 g, 46 g and 57 g, respectively. Radix isatidis, Rhizoma phragmitis, Radix rehmanniae and Gypsum fibrosum were identified by Xiupai Zeng, Xiaoying Lin, Miaochan Cai and Bixi Li, respectively. Rhizoma anemarrhenae, Herba pogostemonis and Fructus forsythiae were identified by Longcheng Ye. Radix curcumae and Rhizoma acori tatarinowii were identified by Shijie Mo. These people are experts in authenticating Chinese medicines. Guangzhou Xiangxue Pharmaceutical Co., Ltd. prepared the sample. These herbs were mixed and soaked in water and heated for 3 h. The volatile was collected with hydroxypropyl-β-cyclodextrin and the water solution was filtered. Then, the filter residue was again soaked in water and heated for 1.3 h to collect filtrate. The two filtrates were combined, concentrated and then added the 85% ethanol until the ethanol content was 70%. Subsequently, the solution was filtered and the filtrate was concentrated under reduced pressure. The ethanol solution and volatile were mixed and water was added to 1000 ml. The final KBD test sample was sterilized and stored at 4 °C. The ratio of dry herb to dry extract was 100: 23.

Oseltamivir was purchased from Yichang Changjiang Pharmaceutical Co., Ltd. (Wuhan, China). CORT was obtained from Sigma (MO, USA). Antibodies against mitochondrial antiviral signaling protein (MAVS) (#3993), transcription factor nuclear factor-kappa B p65 (NF-κB p65) (#3033), phosphorylation interferon regulatory factor 3 (p-IRF3) (#4947) and tumor necrosis factor α (TNF-α) (#3707) were provided by Cell Signaling Technology Inc. (Boston, MA, USA). Antibody against interferon-β (IFN-β) (TA306437) was bought from OriGene Technology (Rockville, MD, USA). Antibodies against interferon inducible transmembrane 3 (IFITM3) (ab15592), influenza virus nucleoprotein (NP) (ab20343), and interleukin-1β (IL-1β) (ab9722) were purchased from Abcam (Cambridge, UK).

Virus

The influenza virus A/FM/1/47 (H1N1) was donated by Prof. Jianxin Chen in College of Veterinary Medicine, South China Agricultural University (Guangzhou, China). A double LD50 dose was used for viral challenges in all animal experiments. All influenza virus related experiments were performed in a Biosafety Level 2 Laboratory in Jinan University.

HPLC and GC analysis method

KBD was analyzed by HPLC with UV detection at 236 nm. Waters Corp. (Milford, MA, USA) CORTECS C18 column (150 mm × 4.6 mm, 2.7 μm) was used to separate components of KBD and the oven temperature was 30 °C. The compounds were eluted (eluent A, 0.1% phosphoric acid in water; eluent B, methyl alcohol; eluent C, acetonitrile) at a flow rate of 1 ml/min using a gradient program. The calibration curves were established by plotting the peak area *versus* the concentration of the standard solution prepared with the reference compounds in the seven different concentration ranges (Table 1). For GC elution and separation an HP-INNOWAX column (30 m × 0.32 mm, CA, USA) with a film thickness of 0.25 μm was used. Detection was carried out using a hydrogen flame ionization detector. The column temperature was programmed as follows: the initial temperature was 80 °C and increased to 100 °C at 1 °C/min, ramped to 145 °C at 4 °C /min, increased to 165 °C at 1 °C /min and held for 20 min, then increased to 230 °C at 40 °C /min and held for 5 min. The injection volume was 1 μl in split mode (5:1). Nitrogen was the carrier gas with an initial flow rate of 0.6 ml/min, holding for 34 min, increasing to 1 ml/min at 2 ml/min^2, holding for 25 min, and increasing to 2 ml/min at 2 ml/min^2. N-heptadecane (National Institutes for Food and Drug Control, Beijing, China) was used as an internal standard for quantitative analysis by GC. The internal standard calibration curves were established by plotting the ratio of the analyte response to the internal standard response against the ratio of the analyte amount to the internal standard amount (Table 1).

Animals and treatments

Male Kunming mice (13–15 g) were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, China). All mice were acclimatized in a pathogen-free animal room with a controlled temperature at 23 ± 2 °C, 12 h light/dark cycles. All animal care and experimental procedures were approved by the Laboratory Animal Ethics Committee of Jinan University (20131011017) and were in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals (the 7th edition, Washington, DC, USA).

The first experiment was conducted to evaluate the anti-influenza virus effects of KBD in normal mice. Animals were randomly divided into 5 groups with 10 mice in each group: Control, Virus, Oseltamivir (virus + 31 mg/kg/d oseltamivir), KBD-L (virus + 13 mg/kg/d KBD, and KBD-H (virus + 26 mg/kg/d KBD). Mice were inoculated intranasally with virus (35 μl) under anesthetization by diethyl ether vapor. Control mice were inoculated with PBS. Oseltamivir and KBD were administered to mice for 7 days (twice a day) while other groups received saline only. The survival and several typical symptoms of illness were recorded for 21 days or until death.

The second experiment was performed to evaluate the anti-influenza virus effects of KBD in mice loaded with restraint stress. Mice were randomly divided into 6 groups with 10 mice in each group: Control, Virus, "Restraint stress+Virus", Oseltamivir (restraint stress + virus + 31 mg/kg/d oseltamivir), KBD-L (restraint stress + virus + 13 mg/kg/d KBD) and KBD-H (restraint