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Skin penetration of topically applied white mustard extract and its effects on epidermal Langerhans cells and cytokines

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ABSTRACT

White mustard (*Sinapis alba* L.), a traditional Chinese medicine, is widely used in China for clinical prevention and treatment of the common winter diseases of asthma and bronchitis by percutaneous administration in the summer. The present study is to investigate the skin penetration behavior of white mustard extract to elucidate the possible mechanism underlying its immune regulation activity. The principle active compound of the extract, sinapine thiocyanate (ST), was used as a marker. The skin penetration of ST in white mustard extract was examined in vitro and in vivo. In vitro study on excised guinea pig hairless skin using Franz diffusion cell revealed ST can permeate through the skin and also accumulate in the skin. In vivo study was carried out on the guinea pig hairless skin for 24 h, and then skin was excised for frozen section, ST from the sections were extracted to quantify the amount of drug in different skin layers. The detailed distribution of ST showed that it accumulated in the epidermis, especially in the stratum corneum. After treatment with white mustard extract for 24 h, the skin was stained with ATPase, and the morphometric parameters of epidermal LCs were compared to the untreated control through image-analysis system. A statistically significant reduction in LC density and increase in shape factor were observed. Cytokines related to LCs migration including interleukin 1β (IL-1β) and tumor necrosis factor α (TNF-α) were also measured after white mustard extract treated at different time points. Compared to the untreated group, white mustard extract significantly enhanced the release of IL-1β and TNFα. The morphometric changes of LCs and the local cytokine release after topical white mustard treatment may explain the activity of the white mustard extract against asthma and bronchitis.

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1. Introduction

White mustard (*Sinapis alba* L.) has been used as a traditional Chinese medicine in China and a spice in Spain, India and elsewhere (Manda et al., 2010), and its essential oil is now developed to a food preservative in the food industry (Ekanayake et al., 2012). Being a traditional Chinese medicine, white mustard is widely used in China as an alternative medicine for clinical prevention of the common winter diseases of asthma, bronchitis, and a variety of other health conditions through percutaneous administration in the summer (Fang et al., 2010; Li et al., 2012; Wen et al., 2012), and the medication is suitable for widely application in community due to its practical convenience and fewer side effects. Additionally, the extract from white mustard has been reported to exhibit anticancer activity against sporadic and obesity-associated colon cancer (Eskin et al., 2007), and the persistent supplementation with mustard seeds suppresses hyperproliferation of colonic epithelial cells, formation of aberrant crypt foci, and tumors in azoxymethane-exposed mice, which may be the results of its antioxidative and immunosupportive activity (Yuan et al., 2011; Zhu et al., 2012). Clinical studies of percutaneous administration of white mustard for reducing exacerbation frequency of chronic lung diseases in humans have been registered in the Chinese Clinical Trial Registry (Peng et al., 2012). Topical percutaneous application of white mustard is thought to have systemic effect and regulates the immunity function, which likely underlies white mustard's ability to prevent asthma and bronchitis (Fan et al., 2003). However, the exact mechanism of action of the white mustard remains unclear, and the local responses at or close to the application site on the skin are still unknown.

Skin is much more than a passive physical barrier between the external environment and internal tissues, a major role of the skin is to provide immune function at this crucial interface between

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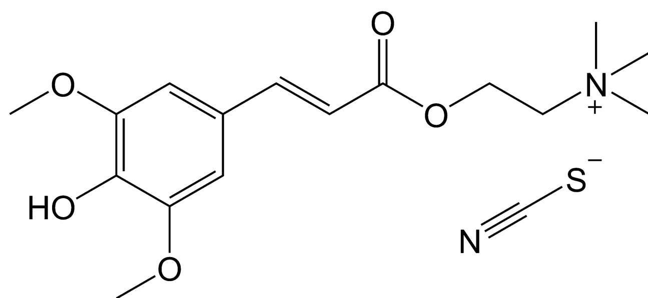

Fig. 1. Chemical structure of sinapine thiocyanate (ST).

inside and outside (Williams and Kupper, 1996). The cutaneous immune network is the most accessible immune compartment of the body, which makes the skin an attractive interface for the administration of vaccines and immunomodulators. Recently, the skin has emerged as an alternative route for non-invasive delivery of vaccines, termed as transcutaneous immunization (TCI), because of increased understanding of the skin immune system and technical innovations in transcutaneous delivery systems (Bal et al., 2010; Seid et al., 2012; Vyas et al., 2005; Cui and Sloat, 2006). The immune protection conferred by TCI is not restricted to the site of immunization but can be observed at distant sites such as the lung and gut-associated mucosa (Karande and Mitragotri, 2010). Similar to lung and gut mucosal barriers, the skin exploits the immune surveillance versatility of a well-coordinated system of epithelial and immune cell (Meglio et al., 2011). It is equipped with a unique set of immunocompetent cells (i.e., keratinocytes, and Langerhans cells (LCs)), strategically located lymph nodes, and subsets of T-lymphocytes that constitute the skin-associated lymphoid tissue (Partidos et al., 2003). LCs in the epidermis not only act as the professional antigen-presenting cells (APCs) to induce antigen specific T cells for adaptive immune responses, but also initiate a cascade of innate immune responses by sensing external stimuli (Ishii et al., 2008). Additionally, upon activation by various stimuli, the keratinocytes secrete a wide range of cytokines (Uchi et al., 2000). These cytokines, especially IL-1β and TNF-α, then facilitate the disassociation of LCs from keratinocytes, the migration of LCs, and the homing of the LCs toward the lymph nodes and subsequently initiating T cell-mediated protective immunity (Cumberbatch et al., 1997; Shornick et al., 2001; Wang et al., 1999).

Sinapine thiocyanate (ST, Fig. 1) is of choline ester of sinapic acid. It is one of the major active compounds of white mustard, and serves as the quality control standard of white mustard listed in the Pharmacopeia of the People's Republic of China. It was reported that ST has anti-inflammatory activities in xylene-induced swelling of mouse ear, can inhibit blood capillary permeability of skin induced by histamine in rats (Zhang et al., 1996), and relieve asthma through relaxing airway smooth muscle (Wang et al., 2011).

Giving the role of topical delivery of white mustard extract for the prevention of asthma and bronchitis, the present study focused on evaluating in vitro and in vivo skin penetration of white mustard extract and its effects on LCs and cytokine production in the skin application sites. ST was used as a standard to study white mustard extract penetration through the skin in vitro as well as the local site-specific disposition in vivo. The influence of white mustard extract on LCs was investigated by ATPase staining, and the secretion of cytokines of TNF-α and IL-1β was examined by ELISA.

2. Materials and methods

2.1. Chemicals

White mustard was obtained from a local drugstore (Guangzhou, China). One kilogram of white mustard was extracted with 10 L of 80% ethanol at boiling temperature for 2 h each time and extracted for three times. After every extracting, the filtration was carried out and the filtrate was collected and mixed as the extraction solution. The extraction solution was concentrated at 60 °C under vacuum until the relative density of the extract was 1.30. The solid content of the extract was 0.65 g/ml and the content of ST in the extract was not less than 1.86% (w/w) as determined by high performance liquid chromatography (HPLC). ST standard was obtained from the National Institute for the Control of Pharmaceutical and Biological Product of China (batch number: 111702-200501). Acetonitrile and potassium phosphate monobasic (KH_2HPO_4) were from Thermo Fisher Scientific Co. (PA, USA). Disodium adenosine triphosphate, Tris, maleic, ethylenediaminetetraacetic acid (EDTA) and sodium cacodylate were from Qiyun Biological technology Corporation Ltd. (Guangzhou, China). Lead nitrate, ammonium sulfide, picric acid, xylene, formaldehyde and absolute ethyl alcohol were from Guangzhou Chemical Reagent Factory (Guangzhou, China). Neutral balsam was from Jia Aaron Biological Technology Corporation Ltd. (Shanghai, China). Guinea Pig IL-1β and TNF-α kits were from Shanghai LangKa Trade Corporation Ltd. (Shanghai, China). All other reagents used in the experiments were of analytical grade.

2.2. Animals

Guinea pigs (male, 275–325 g) were from the Research Center of Laboratory Animals, Guangzhou University of Traditional Chinese Medicine (license number: SCXK (YUE) 2008-0020). Animal experiments were performed in accordance to the Principles of Laboratory Animal Care and Use in Research published by the Chinese Ministry of Health. The animal protocol was approved by the Institutional Animal Care and Use Committee at Sun Yat-sen University. Guinea Pigs were kept in well-spaced ventilated cages and provided standard diets prior to the studies for 24 h. The cages were housed under climate-controlled room (temperature of 25 ± 2 °C, humidity of 45 ± 5%, light/dark cycle of 12/12 h).

2.3. In vitro penetration studies

Guinea pigs were euthanized with carbon dioxide asphyxiation. Hair on the dorsal of the guinea pigs was carefully trimmed. The dorsal skin was excised. After the removal of subcutaneous fat and other tissues, the skin was then rinsed with normal saline (NS) solution. Skin specimens (n = 6) were sandwiched securely between the donor and receptor compartments of Franz diffusion cell (Kaikai Technology, Ltd., Shanghai, China) with the stratum corneum (SC) side facing the donor compartment. The receptor compartment was filled with 8 ml of 10% (v/v) ethanol in NS solution (pH value was adjusted to 5.5 with dilute hydrochloric acid). The solution in the receptor compartment was continuously stirred at 300 rpm with a magnetic bar and maintained at 37 ± 0.2 °C with a circulating water bath. White mustard extract of 210 mg was carefully spread onto the skin surface, and the donor cell was then occluded with parafilm (Chicago, USA). One milliliter of the receiving solution was withdrawn at 0.5, 1, 2, 4, 6, 8, 12, 24 h, and the same volume of fresh receiving solution was added into the receptor compartment after each sampling.

At the end of the study (i.e., after 24 h), the skin was removed from Franz diffusion cell, thoroughly washed, dried with paper, minced manually with scissors, and then added to a tube with 3 ml of 10% (v/v) ethanol in NS solution (pH 5.5) for homogenization with ultrasonic processor (Qsonica, USA). The homogenate was centrifuged at a speed of 3000 rpm for 10 min. The supernatant was then filtered through a microporous membrane of 0.22 μm. All samples were analyzed with HPLC to determine the concentration of ST.

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