



Personalised Medicine

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 1 β (IL-1 β) 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-1 β and TNF α . 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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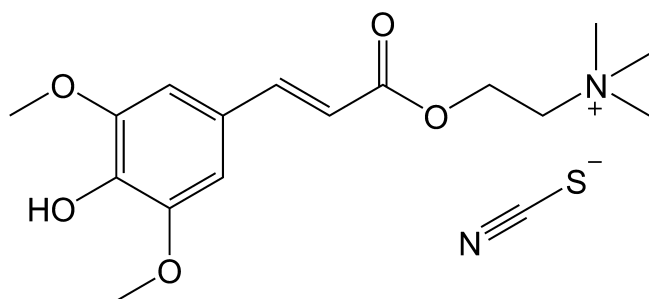


Fig. 1. Chemical structure of sinapine thiocyanate (ST).

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-1 β 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-1 β 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 (KH₂PO₄) 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-1 β 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 $\pm$ 2 °C, humidity of 45 $\pm$ 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 $\pm$ 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 μ m. All samples were analyzed with HPLC to determine the concentration of ST.

2.4. In vivo penetration studies

Twelve guinea pigs were divided into 2 groups, 6 animals per group. Guinea pigs were anesthetized with 20% (m/v) Urethane. Hair on the dorsal of the guinea pigs was carefully trimmed. The trimmed skin was cleaned with an alcohol swab, air-dried, and a 1 cm × 1 cm area was marked on the skin surface. White mustard extract of 210 mg was carefully applied onto the marked area, and the skin was then covered with a piece of self-adhesive Tegaderm® patch from 3 M (St. Paul, MN, USA) for better skin adherence. In the untreated control group, the skin area was only covered with a patch without applying the extract. The patch and the remaining extract were removed 24 h later. The animals were sacrificed, and the application area of the skin was harvested and embedded in optimal cutting temperature (OCT) compound. Section of the skin was cut horizontally on an LKB Ultratome III (LKB, Bromma, Sweden) from outside to inside and the cuts of each skin layer were summarized to one sample (Schmalfu et al., 1997; Volpato et al., 1998). Based on the preliminary histological H&E staining of skin specimen (data not shown), the thickness of each skin layer was as follows: stratum corneum (SC) 25 μm, viable epidermis (VE) 100 μm, dermis (D) 1000 μm, and the remaining for hypodermis (HD). ST was extracted with 10% (v/v) ethanol in NS solution (pH 5.5) as mentioned in above. The samples were stored at −20 °C prior to analysis.

2.5. HPLC

HPLC was employed to determine ST content in all samples. HPLC was performed with a Shimadzu LC-15C system, consisting two pumps (LC-15c), a column thermostat (CO-1000), a sample processor (7725i) and a UV detector (SPD-15c). Compounds were separated on a C-18 reverse column (5 μm, 250 mm × 4.6 mm, Welch). The mobile phase was a mixture of phosphate buffer (0.08 M KH₂PO₄) and acetonitrile (10:90, v/v). The flow rate was 1.0 ml/min. The column temperature was 25 °C. The detector was set at 326 nm and the injection was 10 μl. The ST peak in samples was identified by comparison of the retention time with the ST standard analyzed under the same condition.

2.6. Membrane-associated ATPase staining

The ATPase staining technique is routinely used for human and mice skin LCs. Guinea pigs (*n*=21, 3 for each time point) were treated with white mustard extract as in the in vivo penetration experiment. The animals were sacrificed at 0, 1, 2, 4, 8, 12, 24 h after

the initiation of percutaneous dosing. The treated skin was excised and incubated for 4 h at 37 °C in a 0.76% (w/v) Na₂EDTA solution, and the epidermal sheet was separated from the dermis. The epidermal sheet was washed 3 times with 0.2 M Tris-maleate buffer (pH 7.2) for 10 min, fixed for 20 min at 4 °C in 0.1 M cacodylate buffer (pH 7.3) and then washed again. The washed sheet was incubated at 37 °C for 30 min with an ATPase solution containing 8 mg of ATP, 1 ml of 2% PbNO₃, 1 ml of 0.1 M MgSO₄, 5 ml of 0.2 M Tris-maleate buffer (pH 7.2) and 4 ml of distilled water. The incubated sheet was washed, developed for 5–10 min at room temperature with 1% (w/v) ammonium sulfide, then washed with water and dehydrated in an ethanol gradient. Subsequently, the stained epidermal sheet was mounted on the glass slide with neutral balsam and observed with a Nikon EclipseTs10-F light microscope (Tokyo, Japan).

2.7. Morphometric analysis of LCs

Morphometric parameters of LCs, assessed following ATPase staining, were measured with an image-analysis system: Image-Pro Plus (EOS Utility Version 2.8.1.0). The software analyzes randomly selected images by sorting them according to the distribution of gray levels. Experiments were carried out in duplicate. One hundred cells were analyzed in each plate. The cell morphological characteristics studied were the average perimeter (μm), the average surface (μm²), the shape factor (SF), and density of the cells. The SF is a morphological criterion without unit, which varies between 0 and 1 and reflects the cell shape. Its mathematical formula is: $SF = 4 \times \pi \times (\text{cell image area}) / (\text{cell perimeter})^2$ (Seíguier et al., 2000). Thus the shape factor is closer to 1 if the cell is round, and closer to 0 if it is elongated. The more dendritic the cell, the closer SF is to zero (Touitou et al., 2002). The density is the number of LCs per square millimeter of epidermis. In our study we expressed the density and SF results as percent change compared to data obtained from the control samples: %change = (value after treatment – control value)/control value × 100%.

2.8. In vivo cytokines release and assay

Guinea pigs (*n*=27) were divided into 9 groups with 3 guinea pigs in each group, 6 groups for white mustard treated groups. Controls were comprised of 2 positive groups that had received intradermal injection of IL-1β (640 pg/ml, 30 μl) and TNF-α (240 pg/ml, 30 μl), respectively. Guinea pigs in the negative control group were left untreated. The white mustard extract was applied topically on guinea pigs as mentioned above, and animals were sacrificed 1, 2, 4, 8, 12, 24 h later. The skin samples were excised and washed with normal saline. The skin samples in guinea pigs in

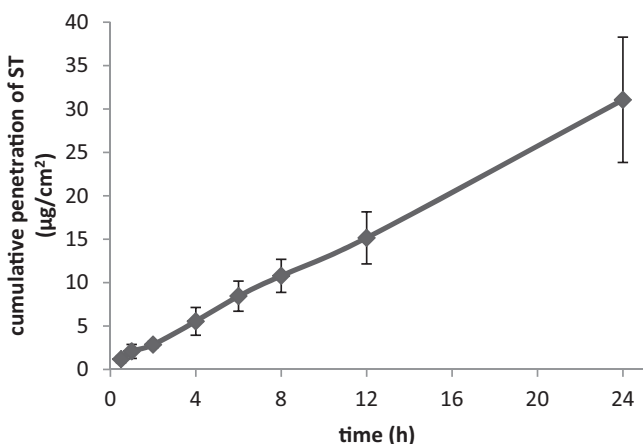


Fig. 2. In vitro permeation of ST through guinea pig skin. Each point represented mean ± SD from 6 determinations.

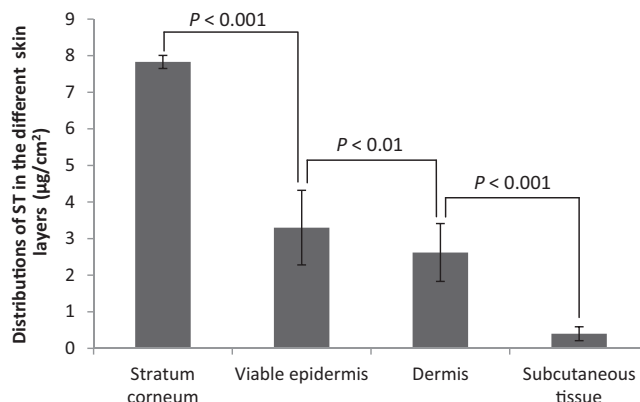


Fig. 3. Comparison of the ST accumulation in the skin strata.

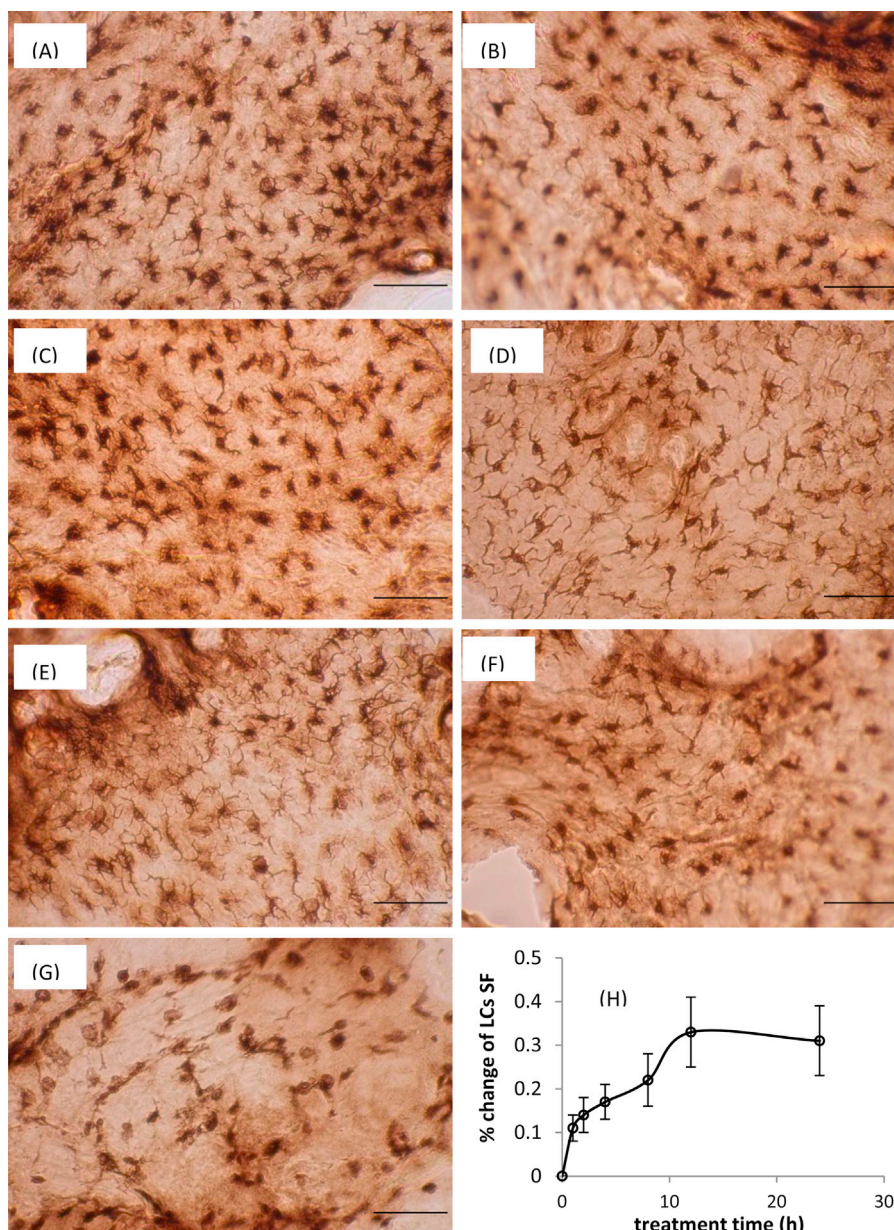


Fig. 4. LC morphology as seen under the light microscope after treatment with white mustard extract.

the positive groups were collected 24 h after the injection. Every skin sample was mixed with 1 ml of normal saline and homogenized. The homogenate was centrifuged at a speed of 3000 rpm for 10 min to collect the supernatant. The concentrations of IL-1 β and TNF- α in the supernatant were measured with commercially available ELISA kits according to the manufacturer's instructions. The ELISA kits contain specific antibodies, peroxidase-conjugated secondary antibody, and tetramethylbenzidine (TMB) substrate. The intensity of the colored product was read at 450 nm (BioTek® Synergy HT, USA). Data (in pg/ml) were extrapolated from absorbance values using standard curves generated by linear regression analysis.

In order to observe the sustained release of cytokines, another Guinea pigs ($n=12$) were divided into 4 groups with 3 guinea pigs in each group. The white mustard extract was applied topically on guinea pigs for 24 h as mentioned above, and then removed. Animals were sacrificed 12, 24, 48 and 72 h after the termination of treatment. The skin samples were prepared and the cytokines were measured as mentioned above.

2.9. Statistical analysis

All measurements were performed in sextuplicate or triplicate. Comparisons between two groups were analyzed by Student's *t*-test using SPSS 13.0 software (SPSS Inc., Chicago, IL USA). Results are expressed as the mean $\pm$ SD. A difference of $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Penetration of ST through guinea pig skin in vitro

The penetration of ST through guinea pig skin in vitro was carried out to evaluate if the active compound(s) in white mustard extract can be released from the extract and penetrate into the stratum corneum and the other skin layers. Guinea pig is often used to establish animal models of asthma; its skin is easily available (Canning and Chou, 2008), and thus is a suitable substitute for human skin in the in vitro permeation experiment. The dose of

Table 1

The morphologic parameters of LCs in guinea pig skin samples treated or untreated with mustard extract for 24 h. Data are mean $\pm$ SD ($n = 6$).

Group	Dendricity	Cell area (μm^2)	Cell perimeter (μm)	SF	Density (cells/ mm^2)
Untreated	5.9 ± 1.1 (4–7)	142 ± 28	128 ± 21	0.11 ± 0.02	1428 ± 176
White mustard extract	$2.3 \pm 0.7^*$ (1–3)	$79 \pm 12^*$	$54 \pm 8^*$	$0.36 \pm 0.09^*$	$1155 \pm 183^*$

* $P < 0.05$ vs. untreated.

the white mustard extract used in the guinea pig studies was fixed at 210 mg based on dose conversion from the clinical application of white mustard to human. For in vitro drug penetration study, the receiving solution was maintained with the sink condition in order to avoid artificial saturation effects. Because ST is a water and ethanol soluble thiocyanate of sinapine; the sink condition can be provided with a normal saline solution or 10% (v/v) ethanol in NS solution. However, at the end of the penetration study (i.e., after 24 h), ST was extracted from the skin for measuring the retention, the extracting ratio in 10% (v/v) ethanol in NS solution is higher than that in NS without ethanol. In order to compare ST content through the skin with that in the skin, the 10% (v/v) ethanol in NS solution was chosen to be the receiving solution as well as the extract solvent. Additionally, ST is a choline ester of sinapic acid, and the ester of ST can be easily hydrolyzed into sinapic acid and choline in the neutral and alkaline condition (Li and Rassi, 2002). In our preliminary experiment, the 10% (v/v) ethanol in NS solution was adjusted to different pH values with dilute hydrochloric acid, and ST stability in these solutions was investigated. Finally, the solution with pH adjusted to 5.5 was used as the receiving solution because no apparent hydrolysis of ST was detected in this solution within 48 h at 37°C , and the solubility of ST in this solution is 2.83 ± 0.21 mg/ml.

The cumulative amounts of ST permeated across guinea pig skin ($\mu\text{g}/\text{cm}^2$) were calculated and plotted as a function of time (Fig. 2). The steady-state flux was determined as the slope of the linear portion of the plot. It was observed that ST penetrated through the skin in accordance with an almost linear increasing with the time. The correlation between the cumulative amount of ST and the time was $Q_t = 1.263 \times t + 0.5528$, $r = 0.9995$, indicating near zero-order penetration kinetics. The cumulative permeated amount after 24 h and the steady-state flux were 31.05 ± 7.22 $\mu\text{g}/\text{cm}^2$ and 1.263 $\mu\text{g}/\text{cm}^2$ h, respectively. The amount of ST remained in the skin at 24 h was 19.51 ± 3.94 $\mu\text{g}/\text{cm}^2$. Taken together, these results revealed that ST could release from the white mustard extract and penetrate through the skin. The relative higher retention of ST in the skin implied that the skin is a reservoir for ST.

3.2. Distribution of ST in guinea pig skin in vivo

To evaluate the local distribution of ST after topically percutaneous application of white mustard, the concentrations of ST in the skin were determined following the in vivo experiment, and the amount of ST in each section was determined, respectively. In order to investigate the distribution of ST in the different skin strata, based on the preliminary histological H&E staining of skin specimen, frozen sectioning, instead of the conventional tape-stripping method which is often used to separate the stratum corneum from the skin, was applied to obtain the exact different layers of skin (Djabri et al., 2012; Manconi et al., 2011). The mean content of ST in different sections of skin was 7.83 ± 0.179 $\mu\text{g}/\text{cm}^2$ in SC, 3.30 ± 1.02 $\mu\text{g}/\text{cm}^2$ in VE, 2.62 ± 0.79 $\mu\text{g}/\text{cm}^2$ in D, and 0.40 ± 0.19 $\mu\text{g}/\text{cm}^2$ in HD (Fig. 3). The content expressed as percentage of skin absorbed dose is 55.34% of SC, 23.32% of VE, 18.52% of D, and 2.83% of HD. The distribution of ST in different sections of the skin was $\text{SC} > \text{VE} > \text{D} > \text{S}$. The total retention of ST was 14.15 $\mu\text{g}/\text{cm}^2$ (100%) in the skin, with 11.13 $\mu\text{g}/\text{cm}^2$ (78.66%) in the epidermis (i.e., SC and VE). It is obvious that most ST was accumulated in the epidermis, especially in the stratum corneum when white mustard

extract was applied for 24 h, suggesting that a higher drug concentration would be provided by site-specific delivery to local skin after percutaneous administration of white mustard extract.

3.3. Changes in LCs following topical treatment with white mustard extract

Langerhans cells are epidermal dendritic cells which function as professional antigen presenting cells (APCs) to process exogenous antigen and to present it in an immunogenic form to T Lymphocytes for initiating the immune responses (Banchereau and Steinman, 1998). Upon sensing stimulating signals, LCs may be activated and exhibit rapid and profound changes in morphology, gene expression profile, surface phenotype, and function (Nishibu et al., 2006), which lead to the maturation and migration of LCs via the afferent lymphatics to the draining lymph nodes. Therefore, we evaluated the effect of percutaneously applied white mustard extract on the morphology of the LCs in guinea pig skin. The results of a representative ATPase staining experiment are shown as Fig. 4. Fig. 4A shows the morphology of LCs from untreated skin. Dendritic cells were clearly seen uniformly distributed. The cell body was big and circular, and the dendrites were thick and long, which are characteristics of dendritic cell morphology and are in agreement with the

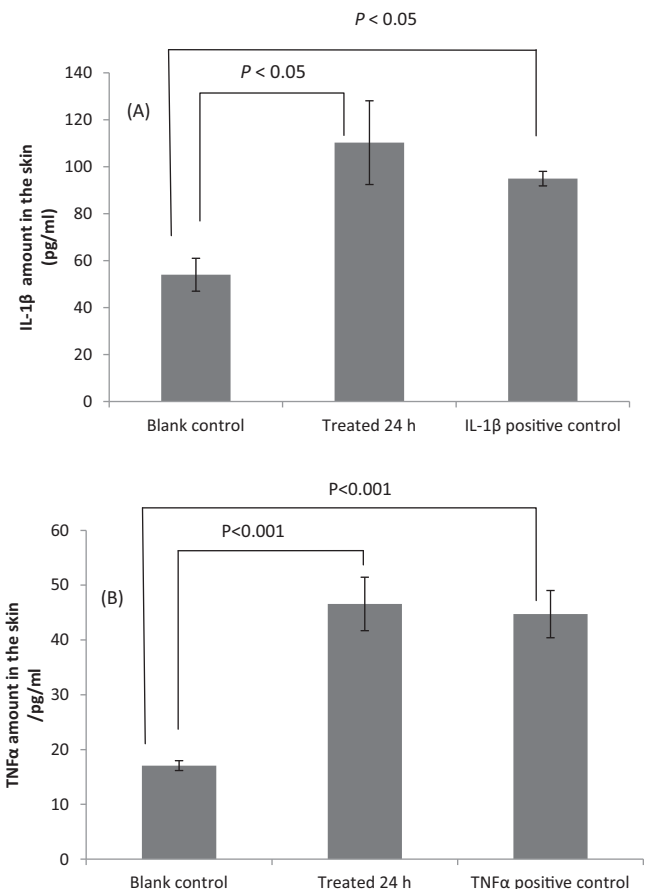


Fig. 5. Cytokines (IL-1 β and TNF α) release after treated with white mustard extract.

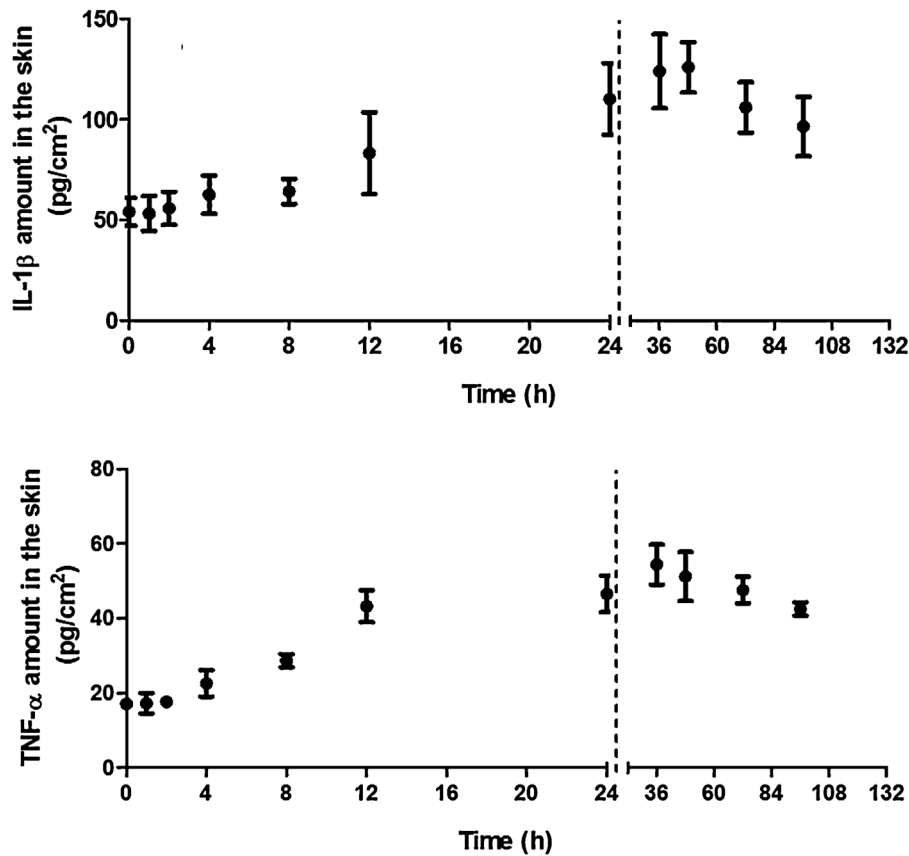


Fig. 6. Cytokines (IL-1 β and TNF α) release after treated with mustard extract for different time.

reports by others (Aiba and Katz, 1990; Figueroa and Caorsi, 1980). However, after the treatment with white mustard extract, the morphology of LCs changed significantly (Fig. 4B–G). The cell body was smaller and round, and the dendrites were short and small or even disappeared (Mummert et al., 2003), the percentage of change of LCs SF with the treatment time was shown in Fig. 4H. It might implicate that the treatment with white mustard extract induced LCs maturation and disassociation from the surrounding keratinocytes and migration from the epidermis to draining lymph nodes. The dendricity, average area, perimeter, SF values and density of LCs measured from skins treated and untreated with white mustard extract for 24 h are shown in Table 1. White mustard extract application led to a large decrease in cell density (-19.12% , $P < 0.05$) and a significant increase in dendritic shape (227.27% , $P < 0.01$), showing that LCs numbers in skin samples treated with white mustard extract were significantly reduced as compared to untreated samples, and the dendritic shape was also dramatically affected. These changes in LCs may be the result of cell migration out of the skin and toward the lymph nodes and then initiating T cell-mediated protective immunity.

3.4. Enhancement of cytokines Release in the skin by white mustard extract

The maturation and migration of LC require the interplay of a complex series of events involving cytokines, chemokines, adhesion molecules and matrix metalloproteinases (Jakob et al., 2001). The migration of LCs and the availability of signals that induce this response are integral to the initiation of cutaneous immunity. Of the cytokines involved in the migration of LCs, IL-1 β and TNF- α are necessary and provide the mandatory signals for the migration of LCs from the epidermis to draining lymph nodes (Cumberbatch

et al., 1997, 2000). In order to clarify the possible role of white mustard extract on the secretion of IL-1 β and TNF- α by epidermal cells, the contents of IL-1 β and TNF- α in the treated and untreated skin were analyzed by ELISA (Shankar et al., 1996). As shown in Fig. 5A and B, compared to the untreated group, white mustard extract significantly enhanced the contents of IL-1 β ($P < 0.05$) and TNF- α ($P < 0.001$) in the skin, even higher than those in the positive injection groups, but there is no significant difference between them, IL-1 β ($P = 0.434$) and TNF- α ($P = 1.00$). Both IL-1 β and TNF- α were increased as a function of the application time (Fig. 6A and B), and they remained detectable even 72 h after the extract was removed (Figs. 6A and B). Hence it appears that topical treatment with white mustard extract induced the sustained secretion of these two cytokines in local skin, which likely plays an important role in LC maturation and migration and the resultant immune response.

4. Conclusions

In the present study, the penetration of white mustard extract through the guinea pig hairless skin was carried out with its effective compound ST as a marker. ST was demonstrated clearly to permeate across the skin with a zero kinetics and deposit in the skin in vitro. The deposition of ST in the skin was further conducted in vivo and that most ST was accumulated in the epidermis, especially in the stratum corneum. As expected, the penetration results contributed a conclusion that the active compound of white mustard extract could be delivered percutaneously and produced a high local drug concentration for site-specific sustaining stimuli. The morphometric changes of Langerhans cells were analyzed in comparison to the untreated group, which implied that, encountering active compounds of white mustard extract, LCs disassociate

from epidermis in preparation for migration into draining lymph nodes for induction of cutaneous immune responses. In addition, the enhancement of release of IL-1 β and TNF α after the treatment of white mustard extract were also observed and it can be inferred that the enhanced release of the two cytokines would lead to the changes of LCs. Overall, We have shown that the active compounds of white mustard extract could penetrate across the skin and accumulate in the skin, especially in the epidermis. Topical application of white mustard extract also induced morphological change in LCs in the skin and stimulated the secretion of IL1b and TNF α in the skin, suggesting the activation of immunity responses, which may be the rationale underlying the clinical prevention and treatment of asthma and bronchitis with white mustard extract through percutaneous administration. Nevertheless, further work needs to be done in order to have a complete elucidation of the immunity protective mechanism of white mustard extract through percutaneous administration.

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