



Oral exposure to diisodecyl phthalate aggravates allergic dermatitis by oxidative stress and enhancement of thymic stromal lymphopoietin



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ABSTRACT

Diisodecyl phthalate (DIDP) is extensively used as an environmentally friendly plasticizer. However, little is known about the adverse effects and the underlying mechanisms of DIDP exposure on immunological diseases. We aimed to determine the role and mechanisms of DIDP exposure in allergic contact dermatitis-like skin lesions. We show that oral DIDP exposure can aggravate allergic dermatitis in mice. Moreover, an increase of ROS, total serum IgE and IL-4 levels were concomitant with this deterioration. We detected the expression of Thymic stromal lymphopoietin (TSLP) and the activation of STATs and NF-κB signal pathways. The data indicated that DIDP in combination with FITC triggers TSLP production. Our results also suggested that DIDP exacerbated the activation of NF-κB signal pathways, with an enhancement in TSLP expression, which potentiated the activation of STATs and the degranulation of mast cells in the skin, and finally exacerbated allergic dermatitis. The study also suggested that melatonin enhanced the expression of Nrf2, up-regulated the antioxidant genes HO-1 and NQO1, reduced the levels of oxidative stress and TSLP, and alleviated allergic dermatitis. The results demonstrated that DIDP exacerbated allergic dermatitis through oxidative stress and enhanced TSLP production.

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1. Introduction

Diisodecyl phthalate (DIDP) is widely used in food packaging, home furnishings, medical equipment and other consumer products (Gimeno et al., 2014; Kasper-Sonnenberg et al., 2012). Diet is one of the major sources of human exposure to DIDP (Sakhi et al., 2014). Some metabolites of DIDP have been detected in human urine (Koch et al., 2013; Saravanabhavan and Murray, 2012). Young children mouthing PVC toys and ingesting house dust may be at a higher risk of exposure to DIDP than adults who are exposed from food, dust, and indoor air (Stringer et al., 2000). Until recently, DIDP was considered to be a low toxic phthalate. It showed no effect on reproduction or development (Chen et al., 2014; Patyna et al., 2006), nor did it show carcinogenic potential in a two-year carcinogenicity study using F344 rats (Cho et al., 2008). However,

studies did show that oral exposure to DIDP can cause liver enlargement in rats. Of particular interest is that there is evidence that phthalates, including DIDP, have an impact on immune and allergic responses (Kimber and Dearman, 2010). Surveys have shown that current asthma was modestly associated with urinary concentrations of DIDP metabolites in Norwegian children (Bertelsen et al., 2013). Positive patch test reaction to DIDP was found in some workers (Lensen et al., 2012). However the association between allergic dermatitis and exposure to DIDP has not been fully elucidated and the mechanism behind DIDP related allergic dermatitis is still unclear.

Among the various types of allergic dermatitis, one of the most common skin allergies in western countries is allergic contact dermatitis (ACD), also referred to as contact hypersensitivity (CHS). ACD is induced by an allergic response to a multitude of chemical substances which are the result of environmental contamination (Vocanson et al., 2009). Epidemiological studies suggest that phthalate exposure may be related to the risk of allergy in children and workers in the plastics industries (Bornehag and Nanberg, 2010; Kimber and Dearman, 2010). The prevalence of ACD has increased with the increase in industrial pollution, suggesting that certain environmental toxins, such as DIDP, may play an important

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role in the enhancement of allergic diseases by increasing the potency of allergens.

Contact hypersensitivity (CHS) is induced by epicutaneous contact with an allergen (sensitization) leading to the development of immunologic memory and enhanced inflammatory skin responses at sites of re-exposure to the allergen (Watanabe et al., 2002). It is believed that CHS is accompanied by a switch in the local cytokine profile, from Th1 to Th2 with enhanced serum immunoglobulin E (IgE) levels (Shigeno et al., 2009). Studies suggest that Th2 conditions can induce pruritus and barrier dysfunctions (Kabashima, 2013). Reactive oxygen species (ROS)-induced oxidative stress is considered to be one of the important pathogenic mechanisms involved in various inflammatory skin diseases, including ACD (Bowler and Crapo, 2002; Fuchs et al., 2001; Luís et al., 2014; Tsukahara et al., 2003). It has been suggested that the skin sensitizer 1-fluoro-2,4-dinitrobenzene (DNFB) leads to a ROS-dependent activation of ER stress in dendritic cells (DC) (Luís et al., 2014). ROS is also found to be involved in mast cell activation. In acquired immunity, the cross-linking of receptor-bound IgE can activate mast cells, after which some mediators involved in the allergic inflammatory response are released (Otsuka and Kabashima, 2015; Swindle and Metcalfe, 2007).

The transcription factor Nrf2, which is a powerful redox sensor, can be activated in response to oxidative stress (Hu et al., 2010). Various antioxidant genes, such as heme oxygenase-1 (HO-1) and NADPH quinone oxidoreductase 1 (NQO1), can be regulated by Nrf2 (Kobayashi and Yamamoto, 2005). HO-1 is considered to be the main antioxidant gene among the Nrf2 target genes (Rushworth and MacEwan, 2008).

Melatonin (MT) is produced by the pineal gland, especially at night. It is a kind of hormone (*N*-acetyl-5-methoxytryptamine) that plays an important role in the human body. Melatonin is an effective antioxidant with anti-inflammatory functions that can protect organisms from oxidative stress (Reiter et al., 2003). Both physiological and pharmacological concentrations of melatonin are able to protect against free radical destruction, which has been documented by *in vitro* and *in vivo* studies (Rao and Chhunchha, 2010; Tan et al., 2002). In addition, Melatonin, as a natural constituent of certain foods, is widely used as a nutritional food additive (Hardeland et al., 2006).

The cytokine thymic stromal lymphopoietin (TSLP) is mainly produced by epithelial cells, and plays an essential role in the pathogenesis of allergic dermatitis and asthma (Larson et al., 2010; Leyva-Castillo and Li, 2014; Nakajima et al., 2012). Signaling between epithelial cells and innate immune cells via TSLP is considered to drive AD as well as the atopic march. It was reported that epithelial cells can promote itching by directly communicating with cutaneous sensory neurons via TSLP (Wilson et al., 2013). Studies have shown that the expression of TSLP is induced by various environmental stimuli, and is also related to numerous gene mutations. TSLP has been implicated in the development and progression of allergic inflammation in both humans and mice, and has been shown to promote a Th2-type response (Larson et al., 2010; Moniaga et al., 2013; Siracusa et al., 2011). It was reported that TSLP expression was elevated on Th2 instead of Th1 or Th17 cells in rodents (Kitajima et al., 2011). In another study it was reported that the transcription factors NF- κ B regulate human TSLP gene expression (Takai, 2012). TSLP production can also be induced or enhanced by proinflammatory cytokines, Th2-related cytokines and IgE (Takai, 2012). The signal transducers and activators of transcription STAT5 is critical in dendritic cells (DC) for development of Th2-dependent immunity and was required for TSLP-dependent DC activation (Bell et al., 2013). In the human body, studies have shown that, in addition to STAT5, TSLP stimulation also activated STAT 1, 3, 4, 5, and 6 (Arima et al., 2010).

Fluorescein isothiocyanate (FITC) is used as a hapten in studies, to build a contact hypersensitivity (CHS) model. Interestingly, FITC-induced CHS is an acute model which is mainly regulated by Th2 rather than by Th1 cells (Shigeno et al., 2009).

In this study, the deleterious effect of DIDP on allergic dermatitis in an FITC-induced allergic dermatitis model is presented, and the levels of inflammatory factors, oxidative stress, TSLP expression, STATs and the NF- κ B signal pathway in skin lesions of model mice are also investigated. We then evaluate the protective effect of melatonin on ACD and investigate its mechanism as an antioxidant.

2. Materials and methods

2.1. Ethics statement

All protocols used in these studies were approved by the Office of Scientific Research Management of Central China Normal University, with the certification on the Application for the Use of Animals dated 5 December 2013 (approval ID: CCNU-SKY-2011-008). All experiments in this study were performed in accordance with the approved guidelines and regulations.

2.2. Experimental animals

Specific Pathogen Free Balb/c mice (Male; about 6–7 weeks; 22–23 g) were bought from the Hubei Experimental Animal Center (Wuhan, China), and all mice were housed under SPF raising conditions, and were provided a commercial diet and filtered water *ad libitum*. All mice were fed for 7 days before the study. Seven mice were used in each group in order to minimize the number of experimental animals needed and still ensure statistical validity.

2.3. Main reagents and kits

Tween-80 was obtained from Amresco (Solon, OH, USA). DIDP (99%), DBP (99%), FITC and pentobarbital sodium were obtained from Sigma–Aldrich (St. Louis, MO, USA). The ROS and GSH test kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). The protein test kit was provided by Beijing Dingguo Changsheng Biotechnology Co. LTD (Beijing, China). Mouse enzyme-linked immunosorbent assay (ELISA) kits for total IgE were obtained from Biolegend (San Diego, CA, USA). An ELISA kits for IL-4 and interferon (IFN)- γ were obtained from eBioscience (San Diego, CA, USA). All other chemicals were of analytical grade. All the operations were performed according to manufacturer's instructions.

2.4. Exposure and immunization

The mice were divided randomly into seven groups of seven mice each. The groups were treated as follows: (1) Saline oral exposure combined with a saline-treated group (Saline); (2) 200 mg/(kg.d) DIDP oral exposure combined with a saline-treated group (DIDP200); (3) Saline oral exposure combined with a 0.5% FITC sensitized group (FITC); (4) 2 mg/(kg.d) DIDP oral exposure combined with a 0.5% FITC sensitized group (FITC + DIDP2); (5) 20 mg/(kg.d) DIDP oral exposure combined with a 0.5% FITC sensitized group (FITC + DIDP20); (6) 200 mg/(kg.d) DIDP oral exposure combined with a 0.5% FITC sensitized group (FITC + DIDP200); (7) 200 mg/(kg.d) DIDP and 10 mg/(kg.d) MT oral exposure combined with a 0.5% FITC sensitized group (FITC + DIDP200 + MT). The particular protocols are shown in Fig. 1. Groups (1)–(7) were given a daily gavage of saline or one of the three different concentrations of DIDP, while group (7) was also given a gavage of MT once a day for a continuous 21-day period.

Groups (1) and (2) were sensitized with saline, while groups (3)–(7) were sensitized with 0.5% FITC, on days 22, 23, and 28.

2.5. Analyses of IgE

All mice were anesthetized with 100 mg/kg pentobarbital sodium by i.p. injection. On day 29, serum samples were collected after centrifugation (3000 rpm, 25 °C, 15 min) and stored at –70 °C before analysis. The levels of serum total IgE (T-IgE) were measured using an ELISA kit with a sensitivity of 15 pg/mL.

2.6. Ear swelling and bilateral ear weight measurement

Mice were killed by cervical dislocation. Ear swelling was measured using a vernier caliper at the beginning of sensitization (day 21) and again on day 29. The ears were then cut and punched along the edge of the middle ear by a corneal trephine, 8 mm in diameter, and this section was weighed immediately. The difference in bilateral ear weight was then calculated.

2.7. Histological examination

The right ears of the mice were collected and fixed overnight in 10% formalin, and then sectioned and stained with hematoxylin and eosin. The numbers of inflamed cells in each sample were counted using Image-Pro Plus software (Image-Pro Plus 6.0, Media Cybernetics). A non-stained region was selected and used as the background. All tissue sections were examined qualitatively by two experienced pathologists, in a blinded fashion.

2.8. Immunohistochemistry

The right ears of mice were collected and fixed overnight in 4% paraformaldehyde. The ear sections were cut and processed for paraffin sections of 3 µm thickness. The sections were deparaffinized, rehydrated and subjected to antigen retrieval, then incubated with 0.3% hydrogen peroxide and blocked by appropriate normal serum. Immunohistochemical detection of Nrf2, HO-1, NQO-1, TSLP, phospho-STAT3, phospho-STAT5, phospho-STAT6, NF-κB p65, and Tryptase was performed using primary antibodies: rabbit anti-Nrf2 (Proteintech, Chicago, USA), rabbit anti-HO-1 (Proteintech, Chicago, USA), rabbit anti-NQO-1 (Proteintech, Chicago, USA), rabbit anti-TSLP (GeneTex, CA, USA), rabbit anti-phospho-STAT3 (Y705) (Abcam, MA, USA), rabbit anti-phospho-STAT5A(Y694) (Abclonal, MD, USA), rabbit anti-phospho-STAT6(Y641) (Abclonal, MD, USA), rabbit anti-phospho-p65 (S536) (Abcam, MA, USA) and mouse anti-mast cell Tryptase (Abcam, MA, USA), respectively. After incubation in primary antibodies, the sections were sequentially incubated in an appropriate biotinylated immunoglobulins and avidin-biotin peroxidase complex. The reaction product was visualized using hydrogen peroxide (3%) and diaminobenzidine tetrahydrochloride (5 mg/10 ml) as the chromogen. The immunohistochemical control was obtained by omitting the primary antibody. All sections were finally counterstained with hematoxylin, dehydrated and mounted in DPX (Sigma-Aldrich).

2.9. Analysis of IL-4 and IFN-γ content

The right ears of mice were homogenized with ice-cold phosphate-buffered saline (PBS; pH 7.5) to make a 10% tissue homogenate. The levels of IL-4 and IFN-γ in the ear homogenate were determined by ELISA kits following a previously described method (Zeng et al., 2013). The sensitivities of IL-4 and IFN-γ kits were both 15 pg/mL. Concentrations were determined in duplicate for each sample.

2.10. Detection of ROS

ROS levels were monitored using DCFH-DA using the method previously described in Min et al. (2014). Briefly, the supernatants were diluted 100-fold in PBS (pH = 7.5). 100 µL of this diluted solution was removed to a 96-well microplate, and 100 µL of 10 µmol/L DCFH-DA was added. The reaction mixture was kept in the dark for 30 min at 37 °C. Fluorescence intensity was then measured at an excitation wavelength of 488 nm and an emission wavelength of 525 nm by a fluorescence reader (FLx 800; BioTek Instruments, Winooski, VT, USA).

2.11. GSH depletion assay

DTNB and glutathione (GSH) react to generate a yellow compound, 2-nitro5-thiobenzoic acid (TNB). Once the proteins have been precipitated, the pH needs to be adjusted to 7.5 to enable the color change reaction with DTNB (60 µg/mL). Samples were analyzed using a microplate reader to measure absorbance at 412 nm. Based on the standard curve, the GSH concentration was determined.

2.12. Statistical analysis

Data are presented as mean ± SEM. Statistical graphs were generated using GraphPad Prism 5.0 (San Diego, CA, USA). A one-way ANOVA combined with Tukey's multiple comparison tests between groups were used to determine the significance of differences. $p < 0.05$ was considered significant and $p < 0.01$ was considered extremely significant. Data analyses were carried out using SPSS ver13 (SPSS, Chicago, IL, USA).

3. Results

3.1. DIDP exacerbating CHS induced by FITC

To explore DIDP-induced effects regulating the sensitization potency of FITC, the mice were gavaged with sterile saline or DIDP (2, 20 and 200 mg/kg/d) for 3 weeks before being sensitized with or without FITC.

As shown by HE staining, saline (Fig. 2 A1) or DIDP exposure groups (Fig. 2 A2) did not experience significant pathological alterations, while the FITC group showed inflammatory cell infiltration when compared with the saline group (Fig. 2 A3, B: $p < 0.01$). However, compared with the FITC group, the number of inflammatory cells infiltrating was increased in the FITC + DIDP groups, especially in the FITC + DIDP200 group (Fig. 2 A6, B: $p < 0.05$). In addition, the pathological alterations in the FITC + DIDP200 + MT exposure group was alleviated with fewer inflammatory cells infiltrating compared to the FITC + DIDP200 group.

24 h after the last challenge, ear swelling and bilateral ear weight of the mice were investigated (Fig. 2C and D). It was shown that the DIDP200 group did not show significant changes when compared with the saline group, while the FITC, FITC + DIDP2, FITC + DIDP20, FITC + DIDP200, FITC + DIDP200 + MT groups demonstrated very significant changes ($p < 0.01$). The FITC + DIDP200 group presented more significant ear swelling and enhanced bilateral ear weight when compared with FITC group ($p < 0.01$). Compared with the FITC + DIDP200 group, ear swelling in the FITC + DIDP200 + MT group was significantly attenuated ($p < 0.01$).

These results indicate that exposure to DIDP does not cause allergic dermatitis but could exacerbate the allergic dermatitis-like effects induced by FITC.

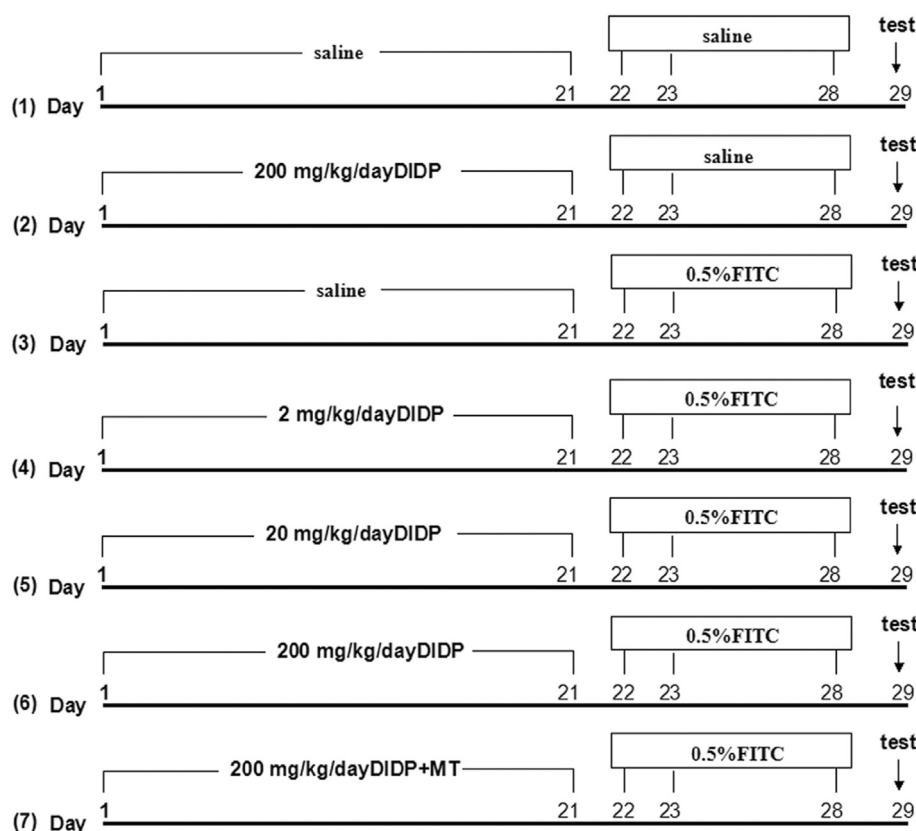


Fig. 1. Exposure and sensitization protocol. For long-term oral exposure, male Balb/c mice were gavaged with saline or DIDP (2, 20 and 200 mg/(kg·d) from day 1–21, and then sensitized with 120 μ l of saline or 0.5% FITC (in 1:1 acetone/DBP) on days 22 and 23, on their shaven backs. On day 28, the mice were challenged with 20 μ l of saline or 0.5% FITC to the right ear, and saline or vehicle (1:1 DBP/acetone) to the left ear. Then, baseline ear thickness was measured with calipers. $n = 7$ mice in each group. (1) Saline, (2) DIDP200, (3) FITC, (4) FITC + DIDP2, (5) FITC + DIDP20, (6) FITC + DIDP200, (7) FITC + DIDP200 + MT.

3.2. DIDP synergistic enhancement of total serum IgE and degranulation of mast cells

To further determine the effects of DIDP on ACD and explore the

inducing mechanism, we investigated the serum IgE of mice from all experimental groups (Fig. 3A). When compared with the saline group, the DIDP200 group did not demonstrate a marked change in serum T-IgE, while the level of T-IgE in the FITC group increased

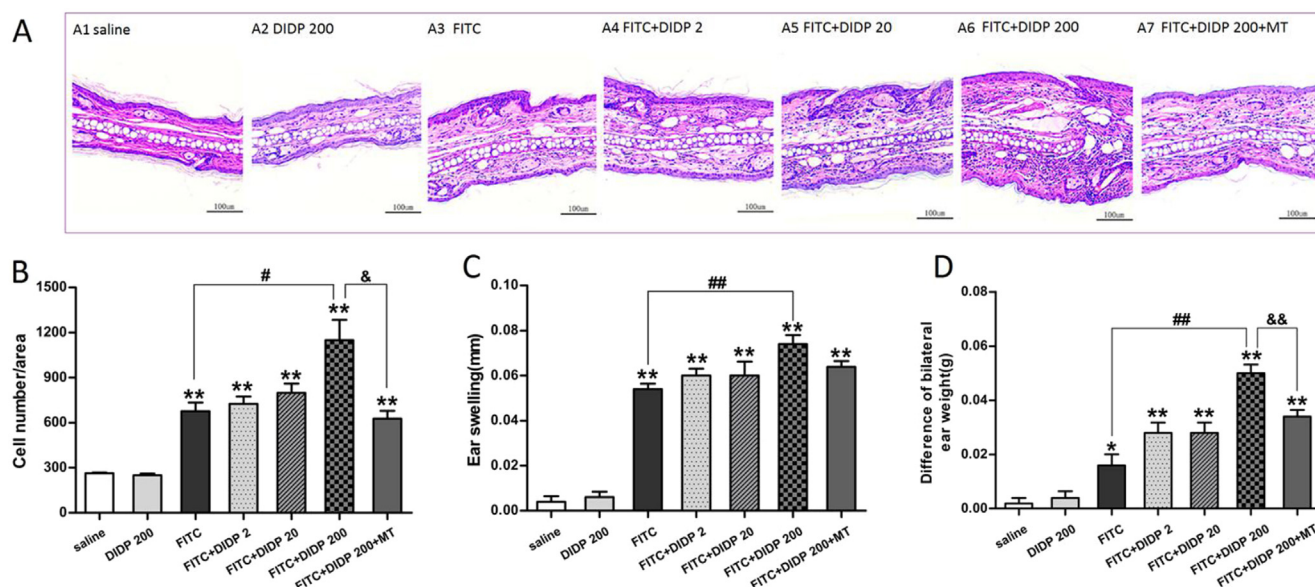


Fig. 2. Effects of oral DIDP exposure on skin lesions induced by FITC. (A) Stained with hematoxylin & eosin (H&E). A1–A7 represent different exposure groups (saline, DIDP200, FITC, FITC + DIDP2, FITC + DIDP20, FITC + DIDP200, FITC + DIDP200 + MT). (B) Number of inflammatory cells infiltrating. ** $p < 0.01$, compared with saline group; # $p < 0.05$, compared with FITC group; & $p < 0.05$, compared with FITC + DIDP200 group ($n = 7$). (C) Measurement results of ear swelling. (D) Measurement results of differences in bilateral ear weight. * $p < 0.05$, ** $p < 0.01$, compared with saline group; ## $p < 0.01$, compared with FITC group; && $p < 0.01$, compared with FITC + DIDP200 group ($n = 7$).

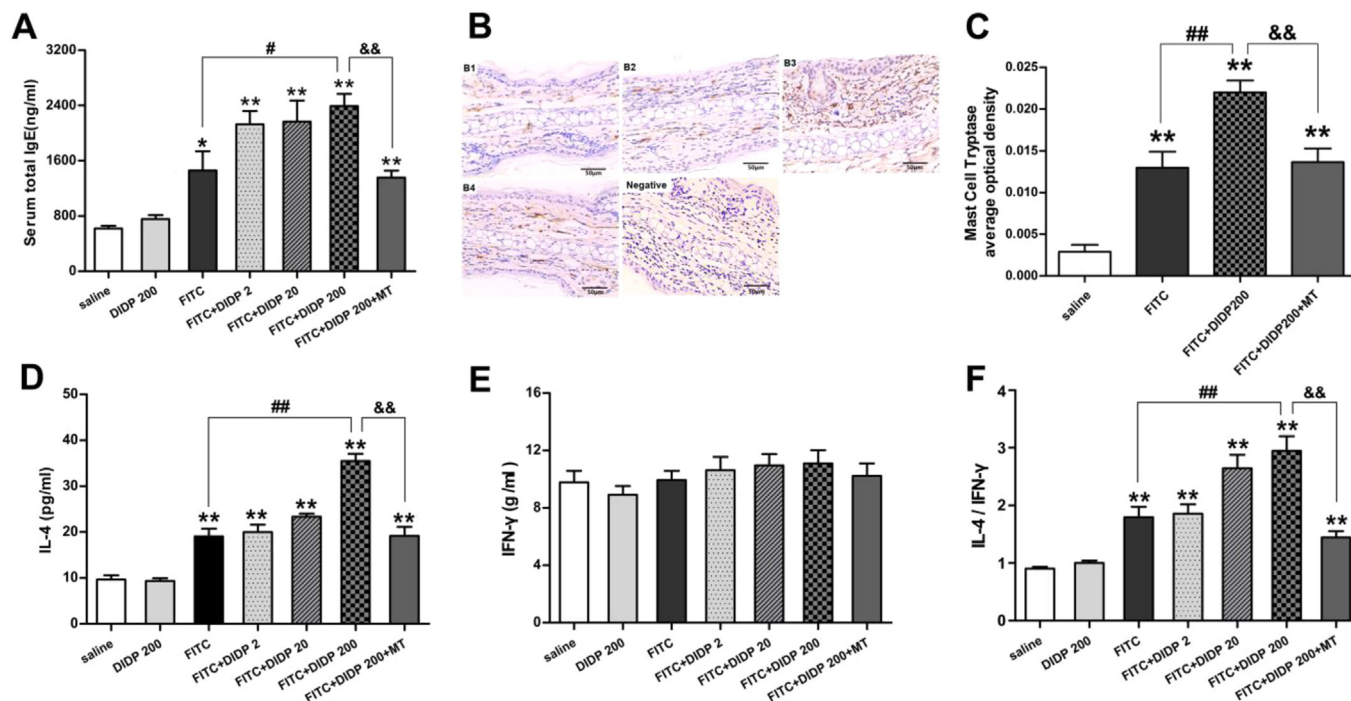


Fig. 3. DIDP exacerbation of Th2 biased hypersensitivity (A) Total IgE concentrations (ng/ml). (B) Immunohistochemistry with Tryptase and H&E staining was performed by using ears of mice. B1–B4 and negative represent different exposure groups (saline, FITC, FITC + DIDP200, FITC + DIDP200 + MT, negative control). Scale bars = 50 μ m. (C) The scores of degranulation were calculated from the expression levels of tryptase. (D) IL-4 concentrations. (E) IFN- γ concentrations. (F) The ratio of IL-4 and IFN- γ . * p < 0.05, ** p < 0.01, compared with saline group; # p < 0.05, ## p < 0.01, compared FITC + DIDP200 group with FITC group; && p < 0.01, compared FITC + DIDP200 group with FITC + DIDP200 + MT group (n = 7).

significantly (p < 0.05), and in other FITC-immunized groups the T-IgE levels increased very significantly (p < 0.01). The level of T-IgE in the FITC + DIDP200 group was enhanced compared with the FITC group (p < 0.05). In addition, the T-IgE levels of the FITC + DIDP200 + MT group decreased significantly compared with the FITC + DIDP200 group (p < 0.01). These results are in accordance with the results of AD-like effects tested in ears.

The levels of tryptase, a specific marker for mast cell degranulation, were investigated by Immunohistochemical detection (Fig. 3B). It was found that more mast cells appeared in the FITC sensitization group and there were more potent sensitizers when FITC was applied in combination with DIDP. The DIDP synergistic enhancement of antigen-mediated degranulation was seen in mouse mast cells according to differences in the tryptase-positive mast cell counts (Fig. 3B and C). These results indicate that the trigger and aggravation of CHS induced by FITC and DIDP are IgE-dependent mast cell activation.

3.3. DIDP associated Th2 cytokine expression

The typical Th1 cytokine (IFN- γ) and the Th2 cytokine (IL-4) content and the ratio of IL-4 and IFN- γ in the ear homogenate were measured in order to evaluate the nature of the lymphocyte cytokine response to DIDP with or without FITC (Fig. 3D, E and 3F). When compared with the saline group, the DIDP200 group did not show any change in IL-4, nor in the ratio of IL-4 and IFN- γ while both of these values increased very significantly (p < 0.01) in all of the FITC-immunized groups. There was a significant enhancement of IL-4 and resulting skew in its ratio to IFN- γ in the FITC + DIDP20 group, and a very significant enhancement in the FITC + DIDP200 group (p < 0.05) when compared with the FITC group. Due to the administration of MT, both IL-4 and its ratio to IFN- γ decreased significantly (p < 0.01). However, there was no significant change in

IFN- γ among the experimental groups.

3.4. DIDP aggravating oxidative stress

The ROS and glutathione (GSH) content in mice ears were measured to evaluate the role of oxidative stress in DIDP-associated effects, with or without 0.5% FITC. A significant increase in ROS and decrease in GSH levels induced by DIDP were observed when comparing the DIDP200 group with the saline group (Fig. 4A and B). All the FITC-immunized groups showed very significant changes (p < 0.01) with regard to ROS and GSH levels. There is a significant increase of ROS and decrease of GSH levels in the FITC + DIDP200 group (p < 0.01) compared with the FITC group. It was found that the strength of the increase in ROS and decrease in GSH levels was attenuated in the presence of MT by comparing the FITC + DIDP200 and FITC + DIDP200 + MT groups (p < 0.01).

3.5. MT promoting the expression of Nrf2 and antioxidant genes HO-1 and NQO1

The expression of Nrf2 and the antioxidant genes HO-1 and NQO1 were detected using immunohistochemistry in order to investigate whether MT inhibits oxidative stress (Fig. 5). It was observed that the expression of transcription factor Nrf2 increased when MT was applied (Fig. 5A and D). The expression of antioxidant genes HO-1 (Fig. 5B and E) and NQO1 (Fig. 5C and F) were enhanced when comparing the MT group with the other groups.

3.6. TSLP expression is induced by DIDP in the epidermal cells of ears

To further explore the underlying mechanisms of ACD associated with DIDP, the expression of TSLP in the ears of mice was

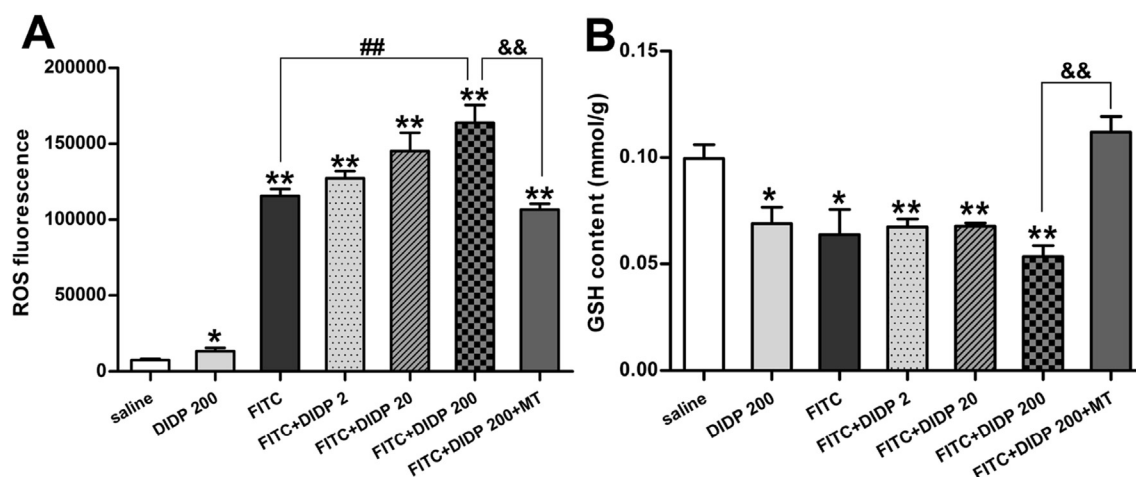


Fig. 4. DIDP aggravating oxidative stress (A) ROS in ear tissue. (B) GSH concentrations in ear tissue. * $p < 0.05$, ** $p < 0.01$, compared with saline group; ## $p < 0.01$, compared with FITC group; && $p < 0.01$, compared with FITC + DIDP200 group ($n = 7$).

investigated. As is shown in Fig. 6A and F, FITC sensitization induced the expression of TSLP in the epidermal cells of ears. This expression was enhanced when applied in combination with DIDP, while the potency of enhancement was inhibited when MT was applied.

3.7. DIDP exacerbating activation of STATs and NF- κ B signal pathways

As is shown in Fig. 6B and G, FITC sensitization induced the activation of an NF- κ B signal pathway in hypodermal cells and epidermal cells of ears. This sensitization also activated STAT3 (Fig. 6C and H), STAT5 (Fig. 6D and I) and STAT6 (Fig. 6E and J). This activation was exacerbated when applied in combination with DIDP, and this exacerbation was inhibited when applied with MT (Fig. 6).

4. Discussion

DIDP as a longer chain phthalate is increasingly being used to replace di(2-ethylhexyl) phthalate (DEHP). It is used as an environmentally friendly plasticizer and is thought to be a less toxic phthalate. Previous toxicity studies indicated that DIDP has limited toxicity when applied orally, dermally or via inhalation routes (Cho et al., 2008). However, its use is still controversial due to a lack of sufficient scientific evidence regarding its toxicity. In particular, the potential molecular mechanisms relating to the impact of oral DIDP exposure on ACD remain incompletely understood.

The European Union's risk assessment report (EU RAR) estimated the exposure to DIDP in infants was 226.5 μ g/kg/d (Saravanabhavan and Murray, 2012). This exposure level approaches the tolerable daily intake (TDI) values for DIDP (0.25 mg/kg/d) obtained by the Scientific Committee on Toxicity, Ecotoxicity and the Environment (Yen et al., 2011). According to the EU RAR, the no-observed-adverse-effect level (NOAEL) of DIDP in rats was identified as 60–150 mg/kg bw/day based on increased liver weights from a 13-week toxicity study. On the basis of this limit, and taking into account the differences in drug tolerance between humans and rodents, the mice were treated with a daily oral exposure of 0, 2, 20, or 200 mg/kg/d DIDP solution according to their group. The high DIDP dose was slightly above the NOAEL while the other doses (2 and 20 mg/kg/d) of DIDP were far below the NOAEL. This study demonstrated that exposure to a low dose of 2 or 20 mg/kg/d DIDP slightly, but not significantly, exacerbated skin swelling, an increase

in T-IgE and IL-4 levels and enhancement of oxidative stress, in the presence of FITC. However, it was suggested that long-term oral exposure of 200 mg/kg/d DIDP could markedly exacerbate allergic dermatitis-like skin lesions in mice. Increased total serum IgE and skin levels of Th2 cytokines such as IL-4 were concomitant with this deterioration. Furthermore, 200 mg/kg/d DIDP enhanced the accumulation of inflammatory cells and the degranulation of mast cells in the skin in the ACD model. In addition, the levels of oxidative stress in the skin were elevated after DIDP exposure in this ACD model via an increased production of ROS and a reduction in the production of GSH. Interestingly, melatonin could reduce the levels of oxidative stress and alleviate FITC-induced CHS.

Although the toxicity of DIDP is limited, it is still unknown whether long-term oral exposure to DIDP could exacerbate the Th2 biased hypersensitivity. In this study, the deleterious effect of DIDP was assessed using two groups of pivotal biomarkers: "immune response" and "ACD pathology". We concluded that exposure to DIDP alone could not cause obvious histological and physiological changes in mice. Nevertheless, after 21 days of oral exposure, the 200 mg/kg/d DIDP + FITC exposure groups exhibited aggravated levels of IgE and IL-4 in the skin; histological changes such as enhancement of inflammatory cells and degranulation of mast cells in the skin; and physiological changes such as ear swelling when compared with the FITC group. These results show that oral exposure to DIDP could exacerbate FITC-induced ACD-like symptoms.

It is believed that oxidative stress plays a significant role in allergic dermatitis pathogenesis. ROS especially has been proven to be associated with the cellular signaling cascades (Luis et al., 2014; Zuo et al., 2013). In this study, the levels of ROS were seen to be significantly increased in the FITC sensitized group compared with the control group, which demonstrated that oxidative stress was related to this ACD model. The ROS level in skin was further enhanced when the FITC sensitized mice were also exposed to DIDP.

ROS may act as a signal to induce the production of inflammatory factors such as IL-4. IL-4 is considered to be a typical Th2 cytokine and IFN- γ is considered to be a Th1 cytokine. In this study we saw that the oxidative stress induced by the FITC challenge was aggravated with DIDP co-exposure. Meanwhile, there was a significant increase of IL-4 that skewed its ratio to IFN- γ with FITC and DIDP co-exposure. The IFN- γ levels of all experimental groups showed no significant change. These results suggest that DIDP is

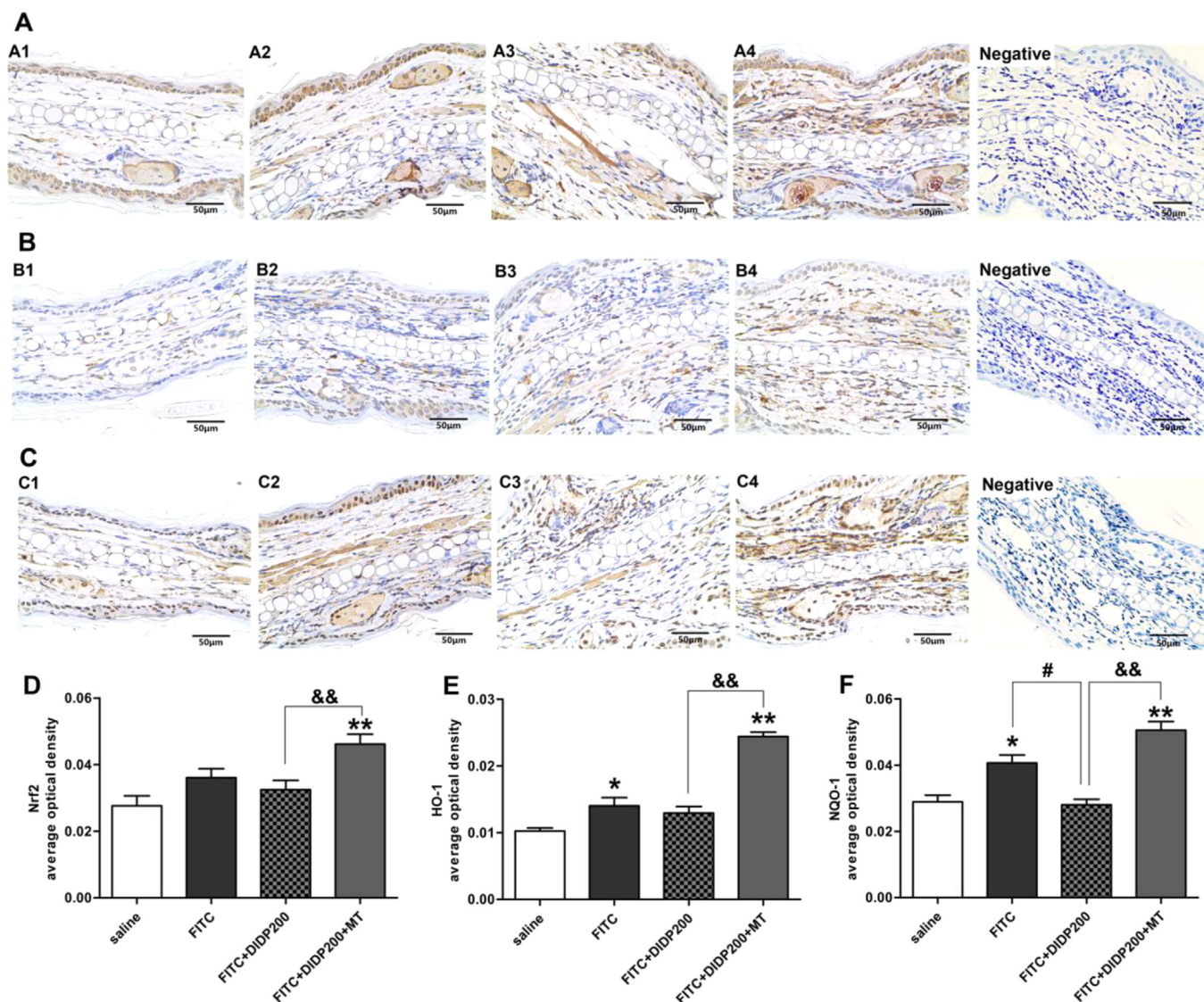


Fig. 5. Expression of Nrf2 and antioxidant genes HO-1 and NQO1. (A) Immunohistochemistry for Nrf2. (B) Immunohistochemistry for HO-1. (C) Immunohistochemistry for NQO1. Immunohistochemistry and H&E staining was performed by using ears of mice. A1–A4, B1–B4, C1–C4 and negative represent different exposure groups (saline, FITC, FITC + DIDP200, FITC + DIDP200 + MT, negative control). (D) The expression scores of Nrf2. (E) The expression scores of HO-1. (F) The expression scores of NQO1. The expression scores were calculated by statistical analysis of optical density of immunohistochemistry. * $p < 0.05$, ** $p < 0.01$, compared with saline group; # $p < 0.05$, compared FITC + DIDP200 group with FITC group; && $p < 0.01$, compared FITC + DIDP200 group with FITC + DIDP200 + MT group ($n = 7$).

related to Th2 cytokine expression in FITC-induced ACD model.

Many situations in which ROS is present involve mast cell activation. Mast cells are activated by cross-linking of receptor-bound IgE, which causes the release of mediators involved in the allergic inflammatory response. In this study, when FITC co-exposure with DIDP resulted in the enhancement of ROS levels, the total serum IgE level were also synergistically increased. Histopathological changes in the mice were also observed to increase in severity. These changes mainly included an increase in inflammatory cells, more mast cells appearing, enhancement of antigen-mediated degranulation of mast cells in the skin, and skin swelling, all of which are related to ACD in humans.

Interestingly, these ACD symptoms and oxidative stress in mice subjected to FITC challenge and DIDP exposure can be effectively alleviated by pretreatment with MT. Actually, all endpoint effects including high levels of total-IgE, IL-4, and different bilateral ear weights were counteracted. MT scavenged free radicals induced by co-exposure with DIDP and FITC, leading to a considerably lower

level of intracellular ROS as well as to a relative drop in the depletion of intracellular GSH levels. These results also show that oxidative stress plays an important role in the DIDP-adjuvant effect. It is believed that Nrf2 is an effective redox sensor to oxidative stress (Hu et al., 2010), and is involved in the regulation of various antioxidant genes (Kobayashi and Yamamoto, 2005). Therefore, we posit that MT could enhance the expression of Nrf2, and promote the up-regulation of the expression of HO-1 and NQO1 in order to inhibit the accumulation of ROS. These results illuminate why MT plays an antioxidant role and attenuates the effect of ACD.

The cytokine TSLP, mainly expressed by epithelial cells has been regarded as a key player in the pathogenesis of various human diseases, such as allergy, autoimmunity and cancer (Leyva-Castillo and Li, 2014). It is reportedly highly expressed in the epidermis in skin lesions of atopic dermatitis patients (Alysandratos et al., 2010; Soumelis et al., 2002). It was illustrated that keratinocytic TSLP was essential not only for generating skin allergic inflammation and allergen-induced Th2 responses, but also for developing

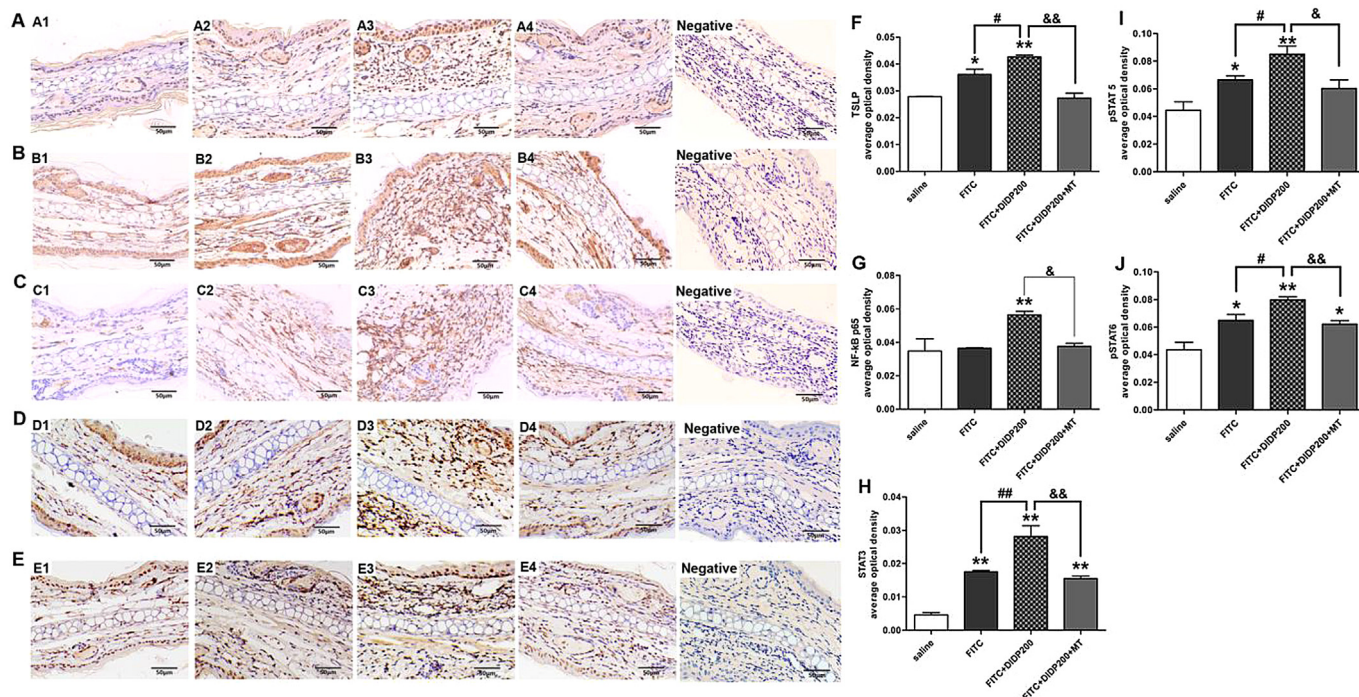


Fig. 6. Expression of TSLP and the activation of NF-κB and STATs. (A) Immunohistochemistry for TSLP. (B) Immunohistochemistry for NF-κB p65 (phospho S536). (C) Immunohistochemistry for STAT3 (phospho Y705). (D) Immunohistochemistry for STAT5 (phospho Y694). (E) Immunohistochemistry for STAT6 (phospho Y641) were performed by using ears of mice. A1–A4, B1–B4, C1–C4 and negative represent different exposure groups (saline, FITC, FITC + DIDP200, FITC + DIDP200 + MT, negative control). (F) The expression scores of TSLP. (G) The activation scores of NF-κB p65 (phospho S536). (H) The activation scores of STAT3 (phospho Y705). (I) The activation scores of STAT5 (phospho Y694). (J) The activation scores of STAT6 (phospho Y641). The expression or activation scores were calculated by statistical analysis of optical density of Immunohistochemistry. * $p < 0.05$, ** $p < 0.01$, compared with saline group; # $p < 0.05$, ## $p < 0.01$, compared FITC + DIDP200 group with FITC group; & $p < 0.05$, && $p < 0.01$, compared FITC + DIDP200 group with FITC + DIDP200 + MT group ($n = 7$).

sensitization to the allergen (Leyva-Castillo et al., 2013). It was subsequently found that TSLP is a critical element linking responses at interfaces between the body and the environment to Th2 responses. Studies have shown that many environmental stimuli could induce the expression of TSLP (Leyva-Castillo and Li, 2014). Here we posit that FITC sensitization induced the expression of TSLP in the epidermis, and this expression was exacerbated in the epithelial cells of ears when applied in combination with DIDP. The potency of this enhancement is inhibited when treated with MT.

Cytokines can induce, enhance, or suppress TSLP production. In primary human small airway epithelial cells, a combination of IL-1β and TNF-α caused the release of TSLP (Allakhverdi et al., 2007). In a human bronchial epithelial cell line, IL-4 enhanced and IFN-γ inhibited the release of TSLP (Kouzaki et al., 2009). Here we propose that DIDP exacerbated TSLP expression by elevating the levels of IgE and IL-4, which induces the activation of mast cells and skews the Th1/Th2 balance.

It was demonstrated that the activation of NF-κB is necessary for the inducible human and mouse TSLP gene expression (Lee and Ziegler, 2007). The human TSLP gene promoter contained binding sites for the transcription factors NF-κB. Our results suggested that DIDP exacerbated activation of NF-κB signal pathways, which enhanced the expression of TSLP. The enhancement of TSLP expression further exacerbated ACD, including an increase in inflammatory cells, the appearance of more mast cells, enhancement of antigen-mediated degranulation of mast cells in the skin, and skin swelling. STAT5 is critical for development of Th2-dependent immunity and was required for TSLP-dependent activation (Bell et al., 2013). In this study, it was found that, in addition to STAT5, FITC sensitization and DIDP also induced the activation of STAT3 and STAT6.

Thus, a possible mechanism is that DIDP, in combination with FITC, triggers TSLP production by ROS, leading to the initiation of the sensitizing process and the deterioration of ACD. Proinflammatory cytokines, Th2-related cytokines such as IL-4, and IgE promote TSLP production, which is thought to be an amplification cycle for the Th2 response. TSLP production can be positively regulated via transcription factors NF-κB and be concomitant with activation of STAT3, STAT5 and STAT6.

This study is the first report to show that long-term DIDP oral exposure can exacerbate allergic dermatitis in mice. It provides new explanations for the increased prevalence of allergic diseases. That is, long-term oral exposure to a kind of environmental toxin such as phthalates may endow an allergic dermatitis in humans or animals. In addition, the high levels of oxidative stress in the animal model suggested that oxidative stress might play an important role in the aggravation effect. This result could help to provide effective prevention strategies against allergic dermatitis and understand the molecular mechanisms driving the aggravation effect of DIDP in the future.

5. Conclusion

The data demonstrated that long-term DIDP oral exposure can exacerbate allergic dermatitis in mice. This deterioration was mediated by oxidative stress and was concomitant with exacerbating the activation of STATs and NF-κB signal pathways, with an enhancement in TSLP expression. These findings significantly improve our understanding of the molecular mechanisms driving the aggravation effect of DIDP and provides new explanations for the increased prevalence of allergic diseases.

Conflict of interest

The authors declare no competing financial interests.

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Transparency document

Transparency document related to this article can be found online at <http://dx.doi.org/10.1016/j.fct.2016.11.016>.

Appendix A. Supplementary data

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