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RESEARCH ARTICLE

Long-term dermal exposure to diisononyl phthalate exacerbates atopic dermatitis through oxidative stress in an FITC-induced mouse model

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Abstract **Diisononyl phthalate (DINP), considered to be an environmentally friendly plasticizer, is now widely used. However, the toxic effects of DINP need to be examined, particularly the effects of long-term dermal DINP exposure. Research into the mechanisms underlying these effects is urgently needed. In this study we examined the exacerbation effect of long-term dermal exposure to DINP in fluorescein isothiocyanate (FITC)-induced contact hypersensitivity (CHS) in mice, and sought the potential molecular mechanisms. Forty-nine male Balb/c mice were subjected to a 40-day dermal exposure to saline or one of three concentrations of DINP and then three rounds of sensitization with vehicle or 0.5% FITC. The results of a histopathological examination and measurement of ear swelling as well as immunological and inflammatory biomarkers (total-immunoglobulin (Ig)E and Th cytokines) supported the notion that high doses of DINP may aggravate atopic dermatitis. We also showed that melatonin, an antioxidant, could decrease the levels of oxidative stress and alleviate FITC-induced CHS suggesting that oxidative stress may be one of the molecular mechanisms to explain the exacerbation effect induced by DINP.**

Keywords diisononyl phthalate, contact hypersensitivity, dermal exposure, exacerbation effect, melatonin, oxidative stress

Introduction

Diisononyl phthalates (DINP), a relatively new type of plasticizer, are added to cosmetics as vehicles for fragrance, and to plastics to make them soft and flexible, such as in children's toys and medical devices (Ma et al., 2014). DINP makes up approximately 30 percent of all plasticizers used worldwide. The American Chemistry Council (ACC) figured the annual world production of DINP to be 1.3 million metric tons in 2008 and 1.5 million metric tons in 2013, which works out to be a 2.5% annual growth in production over that period. With the extensive use of DINP in daily life, the potential consequences of human exposure to this kind of plasticizer have drawn increasing public attention.

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Since DINP is used in multiple diverse items, exposure can occur through various routes, including dermal absorption, ingestion and inhalation (Sakhi et al., 2014). Several major metabolites of DINP have been identified in human urine (Shea and the American Academy of Pediatrics Committee on Environmental Health, 2003). Dermal exposure to pollutants is a normal, but often ignored exposure route. Most chemicals are readily absorbed through skin exposure, and this absorption can induce adverse effects and/or contribute to the dose absorbed by inhaling the chemical (Jonak et al., 2009). Recent research has reported that phthalates are added to personal-care products and cosmetics, and that urinary phthalate monoester levels will show an increase if the use of these products is sustained (Duty et al., 2005). Therefore, more attention needs to be paid to the dermal exposure route when assessing the toxicity of DINP.

Atopic dermatitis (AD), which is characterized by chronic eczematous plaques, intense pruritus, and relapsing inflammation, is an allergic inflammatory disease induced by repeated exposure to an antigen. The number of mast cells,

infiltration of eosinophils, and Th2-type immune response are generally elevated in the inflamed skin (Novak et al., 2005). In the last few decades, morbidity and mortality resulting from AD has been increasing, especially in the industrialized countries. Patients with AD often develop asthma and allergic rhinitis, a progression referred to as the "atopic march" (Huang et al., 2003; Bieber, 2008). Epidemiological studies have shown that there is a possible relationship between phthalate exposure and the risk of allergy in workers in the plastics industry as well as in children (Bornehag and Nanberg, 2010; Bekö et al., 2015). Since AD can have a dramatically bad impact on human health, it is pressing and important to study its pathogenesis. Contact hypersensitivity (CHS) is a common type of AD. This pathological reaction dominantly mediated by Th2 cells is mimicked readily in experimental animals by painting fluorescein isothiocyanate (FITC) on the skin (Imai et al., 2006).

Oxidative stress has been implicated in cutaneous damage seen in various inflammatory skin diseases, including AD (Fuchs et al., 2001; Bowler and Crapo, 2002; Tsukaharaet al., 2003). The peripheral blood monocytes in patients with severe atopic dermatitis are primed to secrete superoxide (Bowler and Crapo, 2002). Moreover, enhancement and attenuation of antioxidant defenses have been shown to be associated with the amelioration and exacerbation of AD, respectively (Tsukahara et al., 2003). Antioxidants can slow down or prevent ROS and other free radicals from forming. Melatonin (MT), which is produced by the pineal gland, is a powerful antioxidant, and its antioxidative actions protect organisms from oxidative stress (Tsai et al., 2011; Mukherjee et al., 2014). Previous studies have documented the ability of melatonin to protect against free radical destruction *in vivo* and *in vitro* (Hardeland et al., 2006; Rao and Chhunchha, 2010). Furthermore, clinical use of melatonin has proven highly successful and it has been applied as a protective agent against a variety of processes that damage tissues via ROS mechanisms (Reiteret al., 2000a,2000b).

In a previous study, we showed that long-term dermal exposure to plasticizers could induce Th2 hypersensitivity (Li et al., 2014). However, the molecular mechanism behind this deterioration is still unknown. In this paper, we present the effect of DINP on allergic dermatitis in an FITC-induced allergic dermatitis model and investigate the levels of oxidative stress and inflammatory factors in skin lesions of the model mice. The purpose of this paper is to investigate the role of DINP in allergic dermatitis and elucidate the mechanism involved in the DINP induced deleterious effect. Additionally, we evaluate the effects of Melatonin on the inhibition of AD and explore its mechanism as an antioxidant.

Materials and methods

All protocols used in these studies were approved by the Office of Scientific Research Management of Central China Normal University (Wuhan, China; 8 November 2011; CCNU-SKY-2011-008).

Experimental animals

Male Balb/c mice (5–6 weeks; 20±1.5 g) were purchased from the Hubei Province Experimental Animal Center (Wuhan, China). All mice were housed in pathogen-free cages maintained at 24–26°C with 55%–75% humidity and a 12-h light–dark cycle. They were fed a commercial diet (Hubei Province Experimental Animal Center) and given water *ad libitum*. Mice were quarantined for ⩾7 days before initiation of the study. Seven mice were allocated to each group so as to minimize the number of experimental animals needed while ensuring the statistical validity.

Main reagents and kits

Fluorescein isothiocyanate (FITC), Di-n-butyl phthalate (DBP) (> 99%), paraformaldehyde and pentobarbital sodium were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tween-80 was obtained from Amresco (Solon, OH, USA). All other chemicals were of analytical grade. Mouse enzyme-linked immunosorbent assay (ELISA) kits for total IgE, IL-4, IL-5 and interferon (IFN)-γ was purchased from Biolegend (San Diego, CA, USA). The ROS and GSH test kits were provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The protein test kits were provided by Beijing Dingguo Changsheng Biotechnology Co. LTD (Beijing, China). All operations were performed according to manufacturer's instructions.

Exposure and immunization protocol

A stock solution was prepared by dissolving DINP in Tween 80 (1:1 ratio). DINP was diluted in sterile saline from this stock solution, and this aqueous suspension was used for the long-term treatment with DINP. Skin sensitization and application to ears with 0.5% FITC were performed in acetone-based solvent, in which acetone and DBP (1:1 ratio) were always present. DBP was used in this study as an immune adjuvant, and previous study has shown that acute or long-term skin exposed to DBP alone did not induced AD (Shigeno et al., 2009; Li et al., 2014). Hence, DBP could not disturb the effect of DINP in this study. MT was dissolved in 3% ethanol and diluted with sterile saline to a concentration of 30 mg/kg.

The tolerable daily intake (TDI) for DINP to be 0.15 mg/(kg· d) (Kransler et al., 2012). According to this TDI and the different drug use between humans and mice, the mice in the present study were treated every day with dermal exposure of 0, 1.4, 14, and 140 mg/(kg· d) DINP solution, respectively. Forty daily dermal exposures to DINP rather than a few

immediately-high-dose exposures were administrated before allergen challenge to simulate the real environmental exposure.

Male Balb/c mice were divided randomly into seven groups of 7 mice. Forty-nine male Balb/c mice were divided randomly into seven groups: (i) control group (control); (ii) Melatonin (30mg/(kg·d)) 3 h after saline skin exposure (MT); (iii) 0.5% FITC sensitized group (FITC); (iv) 1.4 mg/(kg· d) DINP skin exposure combined with 0.5% FITC sensitized group (FITC + DINP1.4); (v) 14.0 mg/(kg· d) DINP skin exposure combined with 0.5% FITC sensitized group (FITC + DINP 14); (vi) 140.0 mg/(kg· d) DINP skin exposure combined with 0.5% FITC sensitized group (FITC + DINP 140); (vii) MT (30mg/(kg· d)) 3 h after 140.0 mg/(kg ·d) DINP skin exposure combined with 0.5% FITC sensitized group(FITC + DINP 140.0 + MT). The detailed protocols are shown in Fig. 1.

Serum sample preparation and quantitative analyses of IgE

Twenty-four h after the final challenge (48 d), the mice were anesthetized with pentobarbital sodium (100 mg/kg, i.p.). Heart blood was collected from the mice and serum samples prepared by centrifugation (3000 rpm, 15 min, 24°C) and stored at −70°C for further analysis. Total IgE (T-lgE) was determined using an ELISA kit according to manufacturer protocols. Duplicate tests were performed for each sample.

Measurement of ear swelling and difference in bilateral ear weight

An electronic vernier caliper was used to measure ear swelling. Ear edema was showed as (R±L)∓(R0±L0), where R0 and L0 stand for the thickness of the right and left ear at the first sensitization (40 d), respectively, and R and L represent the thickness as measured on day 48. Next, ears were removed and punched along the edge of the middle ear by a corneal trephine. The difference in bilateral ear weight was calculated by comparing the weight of each ear.

Ear histological assay

Following this, the edema ear (right ears) of mice were collected and fixed overnight in 4% paraformaldehyde at room temperature. Collected ear tissues were then sectioned,

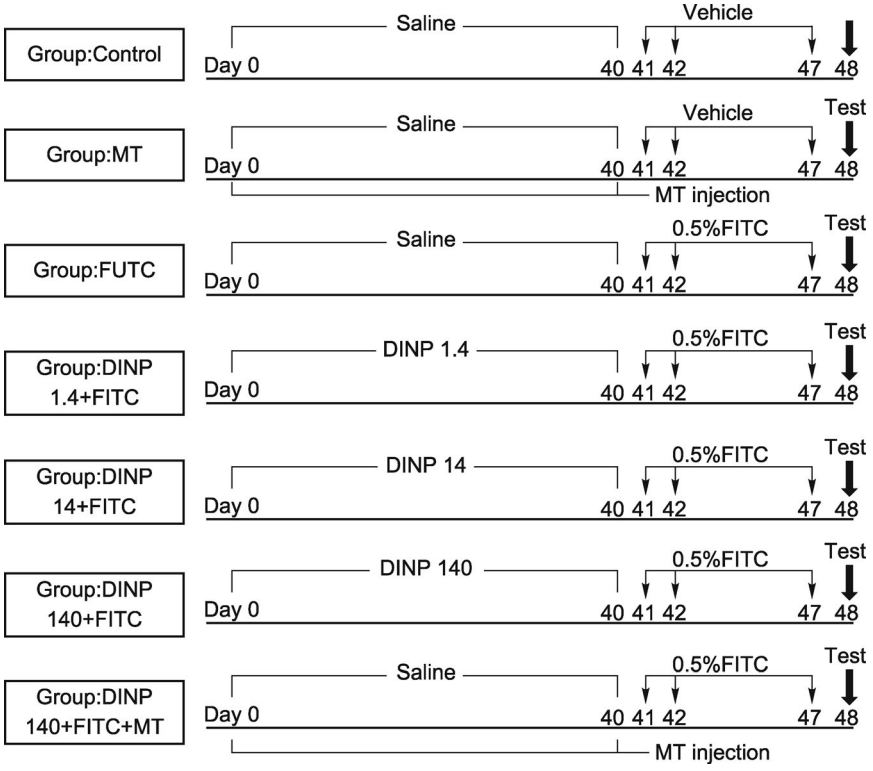

Figure 1 Exposure and immunization schedule. For long-term dermal exposure, male Balb/c mice were smeared with 0.1 mL saline or DINP (1.4, 14 and 140 mg/(kg·d)) from day 0 to 40 (41 times) on their shaven backs, and then sensitized with 120 μL of saline or 0.5% FITC (in 1:1 acetone/DBP) on days 41 and 42, once again on their shaven backs. On day 47, baseline ear thickness was measured with calipers followed by challenge with 20 μL of saline or 0.5% FITC on the right ear, and saline or vehicle (1:1 DBP/acetone) on the left ear. $n = 7$ mice in each group. (1) Control, (2) MT, (3) FITC, (4) DINP 1.4 + FITC, (5) DINP 14 + FITC, (6) DINP 140 + FITC, (7) DINP 140 + FITC + MT.

and stained with hematoxylin and eosin (H&E) as reported previously (Li et al., 2014). Ear sections were observed using a DM 4000B Microscope (Leica, Berlin, Germany) and examined qualitatively by two experienced pathologists in a blinded fashion. The number of cellular infiltrations in each sample were counted using Image-Pro Plus software (Image-Pro Plus 6.0, Media Cybernetics).

Preparation of ear homogenate

Twenty-four h after the final challenge, the right ears of mice were collected, and then homogenized using 10ml/g of ice-cold PBS at pH7.5 to get a tissue homogenate with a concentration of 5%. This homogenate was used to test the levels of IL-4, IL-5, IFN-γ and oxidative stress biomarkers.

Measurement of the levels of Th cytokines

Lung protein levels of IL-4, IL-5 and IFN-γ were measured using ELISA kits according to manufacturer protocols. Concentrations were determined in duplicate for each sample.

Estimation of oxidative stress

ROS assay Reactive oxygen species (ROS) concentrations were determined based on the reactions between ROS and 2',7'-dichlorofluorescein diacetate (DCFH-DA). Supernatant of lung tissue homogenate was diluted 10-fold in PBS (12μL tissue homogenate and 108 μL PBS), and then 100 μL of diluted supernatant was mixed with 100 μL DCFH-DA (diluted 1000-fold with 10 μM dimethylsulfoxide, DMSO) and placed in the wells of a 96-well microplate. The reaction mixture was kept in complete darkness at 37°C for 5min and the ROS contents then measured using a fluorescence reader at wavelengths of 485 and 520nm for excitation and emission, respectively.

GSH assay In accordance with the manufacturer's instructions, GSH content was measured by assay kit (Nanjing Jiancheng Bioengineering Institute, A006-2, China).

MDA assay Malondialdehyde (MDA) concentration was assayed by the thiobarbituric acid (TBA) method as previously described (Li et al., 2014).

Statistical analyses

The data are presented as the mean±standard error of the mean and the statistical graphs were generated using GraphPad Prism 5.0 (San Diego, CA, USA). Results were evaluated statistically using a one-way analysis of variance (ANOVA) combined with a *t*-test. $p < 0.05$ was considered to be a significant difference and $p < 0.01$ to be an extremely significant difference. Data analyses were carried out using SPSS ver13 (SPSS, Chicago, IL, USA).

Results

Effect of dermal exposure to DINP on skin lesions

To evaluate the effect of DINP in AD induced by FITC, histological changes, ear swelling and bilateral ear weight of mice were evaluated. 24 h after the final challenge, the right ears of mice were stained with hematoxylin and eosin (Fig. 2A). Exposure to saline (Fig. 2 A1) or MT (Fig. 2 A2) did not result in significant pathological alterations, while the FITC group showed inflammatory cell infiltration into the skin when compared with the control group (Fig. 2 A3, B: $p < 0.01$). Combined treatment of FITC with high dose DINP (FITC + DINP 140) increased the number of infiltrating inflammatory cells when compared with the FITC group (Fig. 2 A6, C: $p < 0.05$). Furthermore, the pathological alterations and the number of infiltrating inflammatory cells were alleviated in the FITC + DINP 140 + MT group as compared with the FITC + DINP 140 group.

Ear swelling and bilateral ear weight of the mice exposed to DINP was measured 24 h after the final challenge (Fig. 2C, 2D). The group exposed to only MT did not exhibit a significant difference from the control group. However, all FITC-immunized groups (FITC, FITC + DINP1.4, FITC + DINP14, FITC + DINP140, FITC + DINP140 + MT) demonstrated very significant changes with regard to ear swelling as well as in the bilateral ear weight test (Fig. 2C, D, $p < 0.01$). It is interesting that DINP significantly aggravated ear swelling and bilateral ear weight when compared to the group exposed to FITC only, and this exacerbating effect was enhanced with increasing DINP exposure (Fig. 2 C, D). When DINP was applied in combination with MT, ear swelling and the bilateral ear weight were attenuated, which were shown by comparing the FITC + DINP 140 group with the FITC + DINP 140 + MT group (Fig. 2C, D, $p < 0.01$).

DINP synergistic enhancement of serum T-IgE

Twenty-four h after the final challenge, we measured T-IgE in the serum to evaluate the exacerbation effect of DINP skin exposure. All FITC-sensitized groups showed an increase (Fig. 3, $p < 0.01$) in T-IgE concentration as compared with the control group. High doses of DINP (140 mg/kg) exposure significantly elevated the quantity of T-IgE (Fig. 3, $p < 0.05$) in the serum compared with the FITC-sensitization only group. When compared with the FITC + DINP 140 group, the T-IgE levels of the FITC + DINP 140 + MT group decreased significantly ($p < 0.01$).

Th cytokine expression associated with dermal exposure to DINP

To evaluate the association between dermal exposure to DINP and Th cytokine expression, we measured the typical Th1

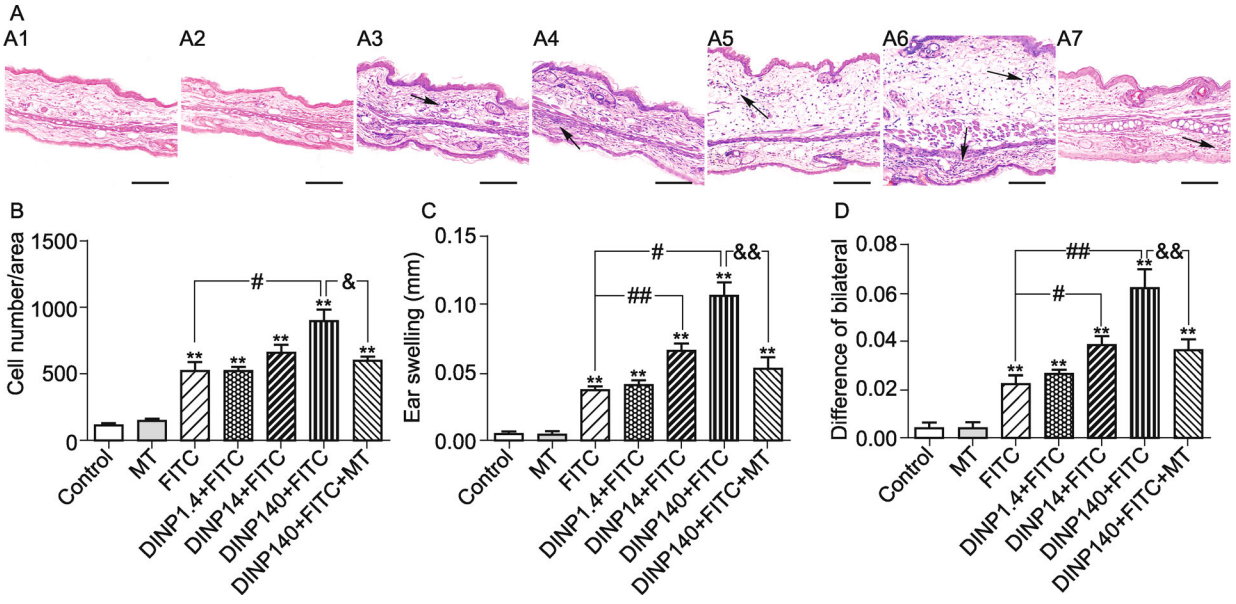

Figure 2 Effects of dermal DINP exposure on skin lesions induced by FITC. (A) Stained with hematoxylin and eosin (H&E). A1–A7 are from the different exposure groups (control, MT, FITC, DINP 1.4 + FITC, DINP 14 + FITC, DINP 140 + FITC, DINP 140 + FITC + MT); black arrows: infiltrating inflammatory cells; scale bars = 100 μm. (B) Number of infiltrating inflammatory cells. $n = 4$ mice per group. ** $p < 0.01$, compared with the control group; # $p < 0.05$, compared with the FITC group; and $p < 0.05$, compared with the DINP 140 + FITC group. (C) Measurement results of ear swelling, $n = 7$ mice per group. (D) Measurement results of differences in bilateral ear weight, $n = 7$ mice per group. ** $p < 0.01$, compared with the control group; ## $p < 0.01$, compared with the FITC group; && $p < 0.01$, compared with the FITC + DINP 140 group.

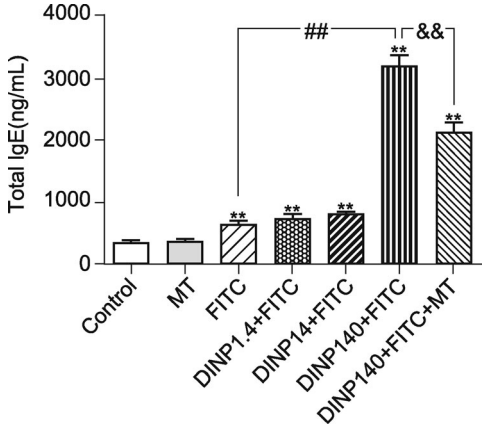

Figure 3 Serum IgE levels. $n = 7$ mice per group. **: $p < 0.01$, compared with the control group; ##: $p < 0.01$, compared with the FITC group; &&: $p < 0.01$ compared with the FITC + DINP 140 group.

cytokine (IFN-γ) and Th2 cytokine (IL-4 and IL-5) concentrations and the ratio of IL-4 to IFN-γ in the ear tissue (Fig. 4). Dermal exposure to MT alone did not result in a change in IFN-γ, IL-4, IL-5 nor in the ratio of IL-4 to IFN-γ compared with the control group. However these values were all found to be significantly higher (Fig. 4, $p < 0.01$) in the FITC-immunized groups. Compared with the FITC group, there was a significant increase of IL-4, IL-5 and a resulting skew in the ratio of IL-4 to IFN-γ in the FITC + DINP groups, and with an increasing concentration of DINP, the exacerbation effect was stronger (Fig. 4 A, C and D, $p < 0.05$). Co-exposure with MT resulted in IL-4, IL-5 and the ratio of IL-4 to IFN-γ decreasing significantly when compared to the FITC + DINP 140 group (Fig. 4, $p < 0.01$). These results suggest that DINP is associated with Th2 cytokine expression by FITC-mediated allergic inflammation.

Dermal exposure to DINP aggravated oxidative stress in the swollen ear, and the elimination effect of MT

The ROS, GSH and MDA content of the ear homogenate were detected to assess the role of oxidative stress in DINP-associated effects with 0.5% FITC. The ROS level in the 140 mg/kg DINP exposure group showed that there was an extremely significant difference ($p < 0.01$) when compared to the control group, and a significant difference ($p < 0.05$) when compared to the FITC group (Fig. 5A). An MT injection, significantly inhibited the increase in ROS content (Fig. 5A. $p < 0.01$).

As is shown in Figs. 5B, compared with the control group, all the FITC-immunized groups (FITC, FITC + DINP1.4, FITC + DINP14, FITC + DINP140, FITC + DINP140 + MT) showed significant changes ($p < 0.01$) with regard to GSH levels. The synergistic DINP enhancement of the FITC-mediated decrease in GSH levels could be seen by comparing the FITC group with the FITC + DINP14 and FITC + DINP140 groups ($p < 0.05$). It is worth mentioning that the size of the decrease in GSH levels was attenuated in the presence of MT, which is seen when comparing the FITC + DINP140 and FITC + DINP140 + MT groups ($p < 0.01$).

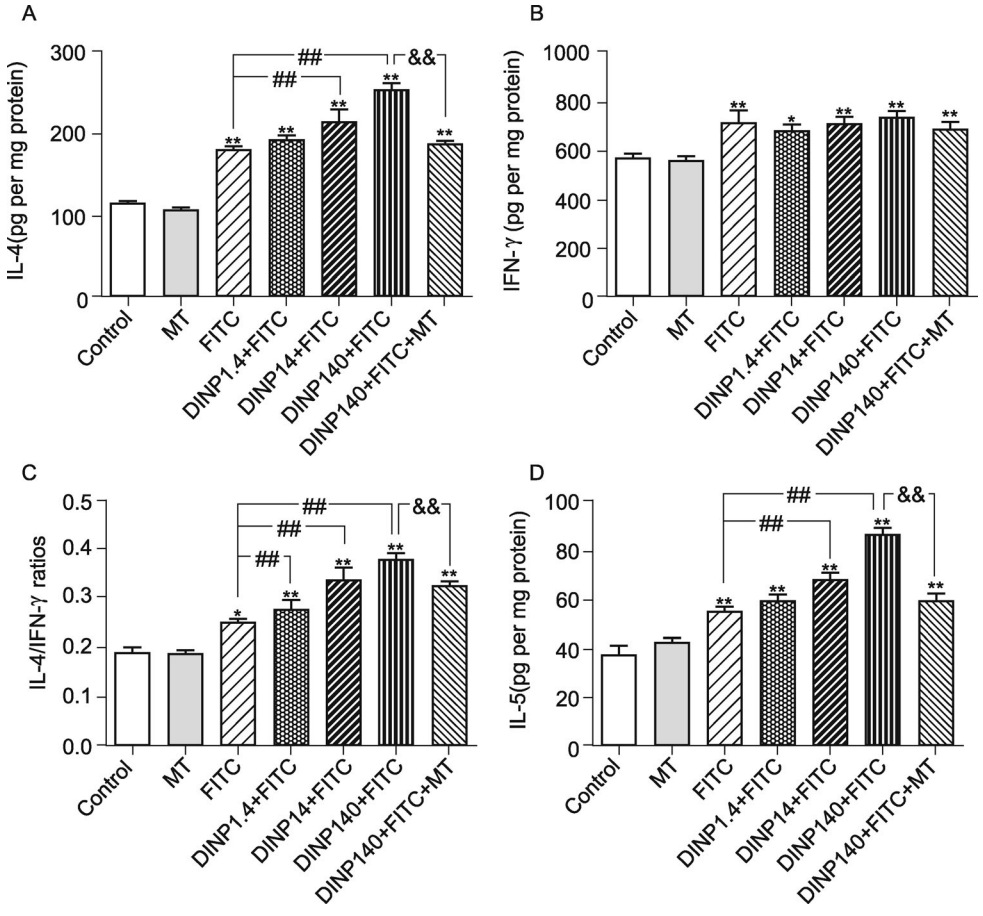

Figure 4 Effects of skin exposure to DINP on the protein expression of Th cytokines in the ear. (A) IL-4 concentrations. (B) IFN-γ concentrations. (C) The ratio of IL-4 to IFN-γ. (D) The ratio of IL-5. $n = 7$ mice per group. *: $p < 0.05$, **: $p < 0.01$, compared with the control group; ##: $p < 0.01$, compared with the FITC group; &&: $p < 0.01$, compared with the FITC + DINP 140 group.

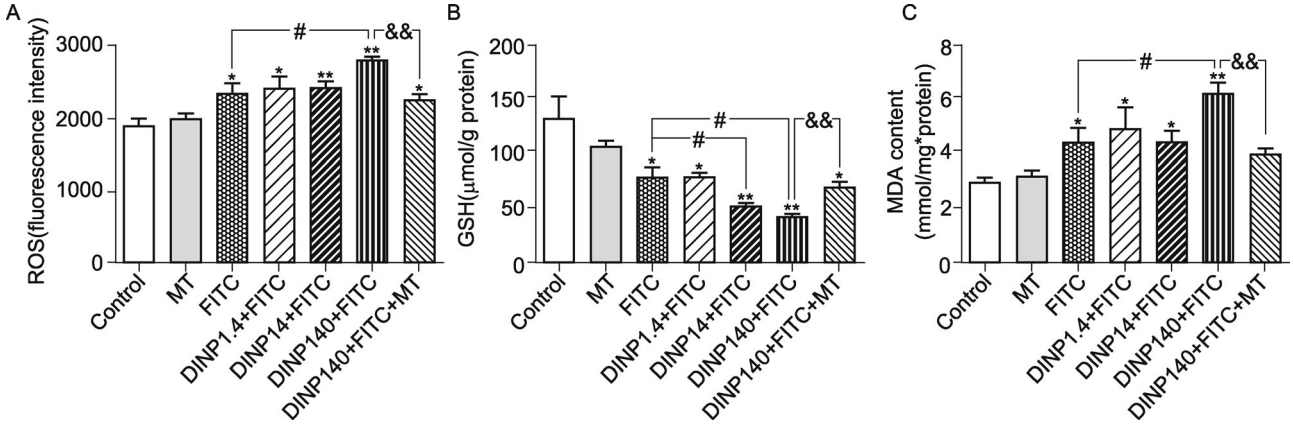

Figure 5 Effect of skin exposure to DINP on oxidative stress in the ear. (A) ROS fluorescence in ear tissue. (B) GSH concentrations in ear tissue. (C) MDA concentrations in ear tissue. $n = 7$ mice per group. *: $p < 0.05$, **: $p < 0.01$, compared with the control group; #: $p < 0.05$, compared with the FITC group; &&: $p < 0.01$, compared with the FITC + DINP 140 group.

Figure 5C shows the difference in MDA content due to the distinct treatments. As can be seen from the results, the MDA content of the swollen ear for the 140 mg/kg DINP exposure group was significantly higher than the saline group ($p < 0.01$) and the FITC group ($p < 0.05$). However, compared with the 140 mg/kg DINP exposure group, administration of MT significantly decreased the MDA content ($p < 0.01$), suggesting that MT could be used as an antioxidant to inhibit the effect of the oxidative damage.

Discussion

This study showed that dermal exposure of 140 mg/(kg· d) DINP could aggravate AD-like skin lesions related to FITC-induced CHS in mice. This deterioration was concomitant with increased total serum IgE, and skin levels of Th2 cytokines such as IL-4 and IL-5. Moreover, 140 mg/(kg· d) DINP potentiated the accumulation of cellular infiltration in the skin in the presence of the allergen. Furthermore, DINP could promote the levels of oxidative stress in the skin with increased production of ROS and MDA, and decreased production of GSH. Melatonin, as an antioxidant, was shown to decrease the levels of oxidative stress and alleviate FITC-induced CHS.

Recently, DINP has been increasingly used to replace DEHP in some plastics (Ma et al., 2013). DINP was thought to be a less toxic phthalate than DEHP, and was used as an environmentally friendly plasticizer. With the widespread use of DINP, its potential toxicity is receiving more attention. In our previous study, DINP exposure was proved to cause damage to the liver and kidney tissues of mice (Ma et al., 2014). It is however still controversial since there is not sufficient scientific evidence on the toxic effects of DINP. In particular, the effect of long-term dermal exposure to DINP remains incompletely understood.

CHS in mice induced by FITC has been characterized as an animal model of allergic contact dermatitis mediated by a Th2-dominant immune system: a high concentration of IL-4 was detected in the inflamed ear, and total serum IgE levels were enhanced (Imai et al., 2006). Dibutyl phthalate (DBP) components have been reported to act as indispensable adjuvants for FITC-induced CHS. In our previous study, we showed that long-term dermal exposure to DBP exacerbates the animal model of allergic contact dermatitis mediated by FITC (Li et al., 2014). However, it is unclear if long-term dermal exposure to DINP demonstrates this adjuvant effect as well, but if it does, the underlying molecular mechanism is still unknown.

"AD pathology" and "Immune response" are two types of important biomarkers to evaluate the exacerbation effect of DINP. The former includes histological and physiologic changes in the skin, and the latter consists of total IgE, IL-4, IL-5 and IFN-γ in the skin. We observed that, after 40 days of dermal exposure, compared with the FITC group, the 14 and 140 mg/(kg·d) DINP + FITC exposure groups showed aggravated histological changes such as cell infiltration in the ear and physiologic changes such as ear swelling, and increased levels of total IgE, IL-4 and IL-5 in the skin. All of these results suggest that long-term exposure to DINP combined with FITC-induced allergic contact dermatitis could exacerbate AD-like symptoms.

Oxidative stress is the result of an imbalance in the production of antioxidants and free radicals. It is thought to play an important part in the pathogenesis of various types of inflammation (Zuo et al., 2013; Rosaet al., 2014;Yang et al., 2014). It is noteworthy that oxidative stress is believed to play an important role in the pathogenesis of atopic dermatitis. ROS in particular has a proven role in the cellular signaling cascades. Excessive ROS leads to oxidative stress, which can cause cell dysfunction and even cell death. GSH, a major reductant in cells, can reduce a wide variety of disulfides by transhydrogenation, and is an important ROS scavenger (Matsue et al., 2003). Malondialdehyde (MDA) is a metabolite of the lipid peroxidation of membranes and usually signifies ROS damage to lipids. In this study, significantly higher levels of ROS and MDA were seen in the FITC sensitized group than in the control group showing that oxidative stress in ear tissue was present in this mouse model of atopic dermatitis. When the FITC sensitized mice were also exposed to DINP (140 mg/kg), the ROS and MDA content in ear tissues was further upregulated, while the GSH level decreased. In this study, the changes in the concentration of ROS, MDA and GSH have provided evidence for a shift in the redox equilibrium toward oxidation, which is a switch to trigger inflammation downstream. The intracellular oxidative stress was first caused by FITC, which was then followed by an upset to the Th1/Th2 balance. A series of events that were connected with the dermal inflammation then took place. Specifically, the depletion of the GSH level inhibited the Th1-associated cytokine production and/or favored the Th2 associated responses (Luft et al., 2008). In this study we showed that DINP co-exposure aggravated the oxidative stress in the swollen ear induced by FITC. With this aggravation of oxidative stress, there was a significant enhancement of IL-4 that skewed its ratio to IFN-γ when treated with FITC in combination with DINP. For IFN-γ, however, there were no significant changes among all the FITC groups. These results indicate that DINP is associated with Th2 cytokine expression in FITC-mediated allergic inflammation. When FITC was applied in combination with DINP, we saw that with the enhancement of ROS levels, the total serum IgE was also synergistically enhanced.

Interestingly, administering MT effectively alleviated the atopic dermatitis-like symptoms in mice subjected to FITC co-exposure with DINP. MT, with its antioxidant effects, scavenged free radicals induced by FITC and DINP, leading to a comparatively lower level of ROS and MDA, as well as a relative drop in the depletion of GSH. Notably, there was a reduction in GSH depletion when MT was administered, in spite of the mice being treated simultaneously with DINP and FITC. This result also lends support to the role of oxidative stress in the DINP-exacerbation effect. In summary, in this mouse model, MT treatment was shown to play a role in a series of results including amelioration of the ear pathology and lower total IgE concentrations and IL-4 and IL-5 concentrations. These can all be explained as resulting from a lower oxidative stress level, which was directly demonstrated by lower levels of ROS, MDA and higher levels of

GSH, and a corresponding decrease in Th2 polarization and inflammatory cytokine release. Furthermore, the elevation in the oxidative stress levels of the FITC sensitized mice by DINP exposure was reduced with pretreatment of MT. This observation prompted us to draw the conclusion that DINP-induced oxidative stress might be responsible for its adjuvant effect (Fig. 6).

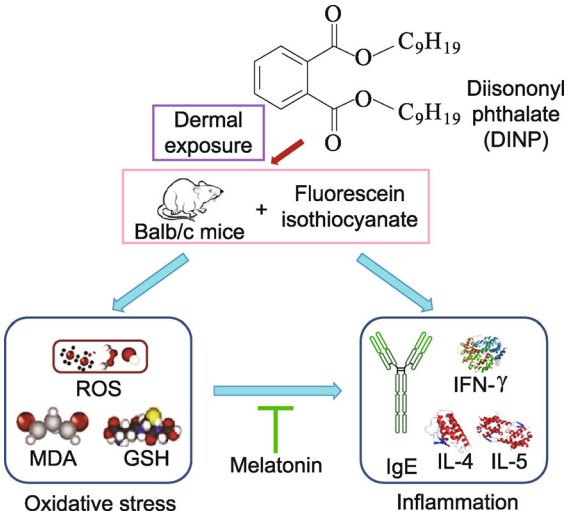

Figure 6 Graphical summary of the study.

Conclusion

The present study demonstrates that long-term dermal exposure to DINP can aggravate allergic contact dermatitis via an oxidative stress pathway in mice. The evidence supports new explanations for the acquisition of an atopic predisposition and the increased morbidity of allergic diseases in society. In other words, long-term skin exposure to types of environmental toxins such as phthalates may endow an atopic predisposition in animals or humans. Moreover, the high levels of markers of oxidative stress in the mouse model demonstrated that oxidative stress might explain the molecular mechanisms in the exacerbation effect. This study can help to provide effective prevention strategies against atopic diseases such as AD. Meanwhile, further studies are needed to determine the treatment effect of MT in AD patient in order to confirm the molecular mechanisms in the aggravate effect of DINP.

Compliance with ethics guidelines

The authors declare that they have no conflicts of interests.

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