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Dibutyl phthalate induced oxidative stress does not lead to a significant adjuvant effect on a mouse asthma model

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Abstract

The prevalence of allergic diseases around the world has been increasing dramatically in recent years. Epidemiological and experimental studies have suggested the involvement of phthalate esters (PAEs) to this increase in allergic diseases. It has been previously reported that PAEs may act as adjuvants thus contributing to the increase of these diseases. In this study we focus on whether dibutyl phthalate (DBP) exhibits an adjuvant effect via an oxidative stress mechanism in a murine asthma model. The DBP was applied through a daily gavage exposure route. Mice were immunized with ovalbumin (OVA) to initiate immune responses, and melatonin (MT) was used as an antioxidant. However, we did not see a significant difference in the level of oxidative stress which suggested that the possible adjuvant effect of DBP is not via an oxidative stress mechanism. Additionally, the level of immunoglobulin E (IgE) in serum, cytokines and inflammation cells in bronchoalveolar lavage fluid (BALF) showed no significance between the OVA positive control group and the DBP and OVA combined exposure group. The significant difference that was observed in the expiratory resistance of airway hyper responsiveness (AHR) may be attributed to changes in the three dimensional structure of the airway wall and the slight shrinkage of the airway. The administration of MT significantly reversed all these effects. Taken together with our data, these results suggest that DBP has little or no adjuvant effect in a murine mouse model and is not mediated through an oxidative stress mechanism.

Keywords

adjuvant effect, dibutyl phthalate, oxidative stress, asthma model, melatonin, mice

Introduction

Asthma is a chronic airway disorder characterized by airway inflammation, mucus hypersecretion, immunoglobulin E (IgE) production and airway hyper responsiveness (AHR),[1] and is one of the most notable childhood diseases, causing substantial morbidity. The pathogenesis of asthma is complicated.[2] At the cellular level, allergens are internalized by antigen-presenting cells which activate the cluster of differentiation 4 (CD4) T-helper (Th2) cells, causing the release of the Th2 associated cytokine. This action results in the synthesis of IgE antibodies, followed by mast cell degranulation and airway mucosa infiltration, all of which induce airway remodeling and AHR.[3, 4] Over the past three decades, asthma has become an increasingly prevalent disease worldwide and the rapid rise in asthma is attributed to numerous diverse factors, including increased awareness of the disease, altered lifestyle and activity patterns and other nonspecific "adjuvant" factors which include the phthalate esters (PAEs) found in the indoor environment.[5-7]

PAEs are common chemicals used to impart flexibility to polyvinyl plastics such as intravenous bags, pharmaceuticals, cosmetics, personal care products, and plastic food wrap.[8] The association between exposure to PAEs, and respiratory symptoms including asthma, was primarily found from epidemiological studies carried out in developed countries.[9-11] These studies also indicated that PAEs may act as adjuvants thus contributing to the increase of respiratory diseases. A separate epidemiological investigation pointed to a possible correlation between the prevalence of PAEs in house dust, and asthma and other allergic symptoms in children.[12] Di (2-ethylhexyl) phthalate (DEHP) in particular, has been associated with the development of wheezing and allergic airway diseases.[13] As the homologue of DEHP, dibutyl phthalate (DBP) is analogous to DEHP in chemical structure and is used in latex adhesives, in nail polish and other cosmetic products. Due to different relative distributions between the gas and liquid phases, coupled with different toxicologic and pharmacokinetic behaviors, DBP and DEHP may contribute to the different human health outcomes.[12] DBP is known to be a development and reproductive toxicant, and it has been shown that subchronic oral exposure to DBP causes peroxisome proliferation and hepatomegaly in rats.[14, 15] A recent study showed that high levels of DBP in house dust of Japan were related to bronchial asthma and atopic dermatitis.[16] What's more, the metabolites of DBP (monobenzyl phthalate, MBP) were positively associated with allergic symptoms and sensitization in adults.[17] Most studies[18,19] illustrated the adjuvant effect of DBP using DBP as a solvent for

fluorescein isothiocyanate (FITC) to induce contact hypersensitivity (CHS) which is another Th2-type immune disease and there have been no reports focusing on the associations between DBP and asthma on a mouse model. DBP only showed an adjuvant effect with immunoglobulin G1 (IgG1) after a second booster with ovalbumin (OVA).[20] Since there is no other direct evidence of investigations into the link between DBP and asthma, the relationship between DBP and asthma needs further study.

A well-accepted mechanism in the pathogenesis of asthma is oxidative stress,[21] which occurs not only as a result of environmental exposure, but also because of inflammation, as both airway and intravascular inflamed cells generate reactive oxygen species (ROS).[22] Oxidative stress aggravates airway inflammation by inducing diverse proinflammatory mediators, enhancing bronchial hyperresponsiveness, stimulating bronchospasm, and increasing mucin secretion.[23] Accumulating evidence clearly indicates that oxidative stress resulting from an oxidant/antioxidant imbalance, an excess of oxidants, or a depletion of antioxidants such as glutathione (GSH), plays a critical role in asthma.[24] At low to moderate concentrations, ROS function in physiological cell processes, but at high concentrations ROS produce adverse modifications to cell components, such as lipids, proteins, and DNA, which contribute to many pathological conditions, including cancer, neurological disorders, atherosclerosis, diabetes and asthma.[25] Our previous studies showed that exposure to DBP with OVA- sensitized induced a markly increased level of oxidative stress compared with saline groups and the degree of depression was higher than in non-DBP groups.[26] Furthermore, DBP caused oxidative injuries in a variety of organs like brain, lung, liver, and ovaries, which are most sensitive to the toxicity, indicating the major toxicity of DBP.[27] But if the oxidative stress could induce or not the significant adjuvant effect on the mouse asthma model is still unknown. In order to block the effect of oxidative stress, exogenous melatonin (MT) has typically been used as an antioxidant to scavenge excess ROS. MT is a lipid-soluble compound, which is twice as active as vitamin E, and is believed to be the most effective lipophilic antioxidant.[28] Many previous studies have also indicated that MT is very effective in detoxifying the highly reactive •OH, in increasing the mRNA levels for antioxidant enzymes, for stimulating the activity of GPx, and also in directly neutralizing singlet oxygen.[29, 30] In addition, a considerable amount of evidence has shown that MT enhances the immune function, both at central and at peripheral levels, and also regulates

natural killer cell cytotoxicity.[31]

In the experiment described in this paper, we used an experimental model of allergic asthma to evaluate the adjuvant effect of DBP via an oxidative stress dependent mechanism. Exogenous MT was used as a potent antioxidant to block the effect of oxidative stress and inhibit allergic airway inflammation. Our data indicated that DBP had a less pronounced adjuvant effect and was not mediated through an oxidative stress mechanism.

Material and methods

Ethics Statement

The followed experimental procedures were approved by the Office of Scientific Research Management of Central China Normal University, and the approval ID was CCNU-SKY-2011-008. According to the ethics requirements only 5-7 mice were used in each group, and to ensure the validity of the experimental data all samples were assayed in triplicate.

Animals

Specific-pathogen-free male BALB/c mice, 6-8 weeks of age, were purchased from Hubei experimental animal center (Wuhan, China). The mice were housed in a 12-h photoperiod (8:00 am-20:00 pm) at temperatures between 20-25 ^{o}C and humidity of 50-70%. OVA-free food and tap water were available *ad libitum*. The mice were kept in conventional animal housing facilities for at least 1 week before use.

Sensitization and Airway Challenge

The mice received one of the following treatments: (1) sensitization plus challenge with saline (saline group); (2) sensitization plus challenge with saline and DBP (10 mg/kg/day, >99.0%, sigma, DBP : Tween 80 [CAS No. 9005-65-6, analytical reagent, Sinopharm Chemical Reagent Co, Ltd. China] is 1:1 in v/v) exposure (DBP group); (3) sensitization plus challenge with OVA (CAS No. 9006-59-1, A5253-500G, GradeⅡ, Sigma, OVA group); (4) sensitization plus challenge with OVA and DBP (10 mg/kg/day) exposure (DBP+OVA group); (5) sensitization plus challenge with OVA and DBP (10 mg/kg/day) exposure along with melatonin (CAS No. 73-31-4, >99.0% [TLC], Sigma, DBP+OVA+MT group). Briefly, mice were sensitized using both intraperitoneal (*i.p.*) and subcutaneous (*s.c.*) injection of either saline or 83.33 μg OVA and 2.92 mg aluminum hydroxide (CAS No. 21645-51-2, Strem Chemicals Inc., USA) in 500 μL saline, on day 6, 13, 20,

27. Each mouse was given saline or DBP to gavage calculated by body mass every day. MT was used as an antioxidant in this study. MT-treated mice received the same sensitization and challenge routine with DBP+OVA. MT was given subcutaneously at 10 mg/kg body weight half an hour before each sensitization of OVA. All mice were exposed to an aerosol of 1% w/v OVA or saline on seven successive days (days 28-34) for 30 min using an ultrasonic nebulizer (Yuyue 402A type Ⅰ, China). Mice were sacrificed 24 h after the last challenge. A schematic diagram of this treatment schedule is showed in Fig. 1.

Measurement of ROS in Lung Homogenates

ROS were measured by the DCFH-DA fluorescent assay method as previously described.[32] After 24 h of challenge, the mice were sacrificed and the lung tissue was weighed and homogenized with ice-cold PBS (pH 7.4, lung: PBS is 1: 9 w/v). 10% of the homogenate was centrifuged (10, 000 g, 10 min, 4 ^{o}C) and the supernatant was used immediately for the assays of ROS, GSH and malondialdehyde (MDA). Supernatants were diluted 200 fold in cold-PBS. 100 μL of the diluted supernatant was mixed with 100 μL DCFH-DA (D6883/50, Sigma, 20 μM, diluted 100 fold from dimethylsulfoxide [DMSO, CAS No. 67-68-5, HPLC grade, 99.9%, Sigma-Aldrich]-dissolved-stock solution) and placed into each well of a microplate and incubated at 37 ^{o}C for 5 min in complete darkness. After incubation, the ROS level was detected by an FLx800 fluorescence reader (Bio-Tek, USA) with 485 nm excitation and 520 nm emission wavelengths.

Determination of GSH in Lung Homogenates

GSH content was measured by assay kit (Nanjing Jiancheng Bioengineering Institute, A006-2, China) and all experiments were conducted in strict accordance with the manufacturer's instructions.

Measurement of MDA in Lung Homogenates

MDA, as the marker of lipid peroxidation, was determined by the thiobarbituric acid (TBA, CAS No. 504-17-6, Sinopharm Chemical Reagent Co., Ltd. China) method.[33] In brief, 2 mL 0.6% TBA solution (0.6 g solid TBA dissolved in 100 mL 10% trichloroacetic acid [CAS No.76-03-9, analytical reagent, Sinopharm Chemical Reagent Co., Ltd. China]) was added to 0.5 mL 10% homogenate. The MDA reacts with TBA to form a pink chromogen during a 15 minute bath in boiling water. After that, all samples were rapidly cooled in tap water and centrifuged at 10, 000

rpm for 10 minutes. The pink supernatants were determined spectrophotometrically at 532, 450, 600 nm, respectively. The quantity of MDA was calculated using the standard curve equation by reference: C_{MDA} (μmol/L) = 6.45(OD_{532}-OD_{600})-0.56 OD_{450}.[34]

Inflamed Cell Count in bronchoalveolar lavage fluid (BALF)

All mice were sacrificed by administering pentobarbital sodium (P3761-5G, Sigma) intraperitoneally 24 h after the last aerosol challenge. The lungs were then lavaged three times with 0.5 mL, 0.6 mL and 0.7 mL ice-cold saline via a tracheal cannula. The volume of qualified samples was between 1.3-1.5 mL. The BALF was then centrifuged (250 g, 4 ^{o}C, 10 min) and the supernatant collected and stored at -70 ^{o}C for cytokine determination. The pellets were resuspended with 500 μL saline and an automatic hematology analyzer (Matenu, MTN-21, China) was used to count cells. The total number of each cell type (total cells, eosinophils, neutrophils) was calculated in this manner.

ELISA Measurements of Cytokines in BALF

The BALF supernatants were thawed at room temperature before use. The levels of interlukin-4 (IL-4) and interferon-γ (IFN-γ) were detected using ELISA kits (eBioscience, USA) according to the manufacturer's instructions, and compared with known standards. Both lowest detection limits were 15 pg•mL^{-1}.

Assay for Serum Total IgE

Blood was obtained by heart puncture on the 35th day. The blood was kept at room temperature for 30 min and then centrifuged (25 ^{o}C, 2, 500 g,10 min). The resulting supernatant was stored at -70 ^{o}C before use. The total IgE (T-IgE) was measured using specific mouse IgE ELISA kits (Biolegend, cat. No. 432401, USA) in strict accordance with the manufacturer's instructions. The lowest detection limit was 0.156 ng•mL^{-1}.

Lung Function Determination

The AHR measurement was conducted 24 h after the last aerosol challenge. The AniRes 2005 lung function system (Bestlab, version 2.0, China) was used to evaluate the lung function of mice and it performed according to the manufacturer's instructions. Mice were anesthetized with pentobarbital sodium (CAS No. 57-33-0, Sigma-Aldrich, USA) at a dose of 100 mg/kg by *i.p.* injection. The trachea was intubated with a cannula and connected to a computer-controlled small ventilator, with the respiratory rate controlled at 75 times/min and the ratio of expiration/inspiration preset at

1.5:1. The AniRes 2005 program was then started up and methacholine (O-Acetyl-β-methylchololine chloride, MCH, Sigma-Aldrich, USA) injected into the jugular vein a few minutes later with dosages of 0.025, 0.05, 0.1 and 0.2 mg•kg^{-1} delivered successively every 5 min. After injection, the inspiratory and expiratory resistance R-area (Ri and Re), and the lung dynamic compliance (Cldyn), were recorded by the software. The R-area (100Pa•mL^{-1}) is the area between the peak curve of Ri or Re and the baseline level during the 250s period from when the MCH entered the vein. The valley values of Cldyn (mL•1000Pa^{-1}) were recorded to quantitatively assess lung compliance.[35]

Histopathology Examination

Lungs were fixed in 4% neutral buffered polyformaldehyde (20120320, Chemically Pure, Sinopharm Chemical Reagent Co., Ltd. China), paraffinized and cut into slices. The slices were stained with hematoxylin and eosin (H&E, Sigma-Alorich, USA) to examine cell infiltration, Periodic acid-Schiff (PAS) for mucus production and Masson's trichrome for collagen deposition, respectively.

Statistical Analysis

Data are presented as the mean ± standard error of the mean (SEM). One-way analysis of variance (one-way ANOVA) and the Least Significant Difference (LSD) test were used to determine the levels of difference between groups. A p-value less than 0.05 was considered to be a significant difference.

Results

Sequential Changes in the Degree of Oxidative Stress in Lungs

The changes in the levels of ROS is shown in Fig. 2A. Compared with the control group (saline group), the intracellular ROS levels are significantly higher ($p < 0.01$) in both the OVA with or without DEHP exposure groups (OVA group and DBP+OVA group). However, injection of MT significantly inhibited ($p < 0.01$) the increase of ROS content in the lung, which indicated that MT could scavenge excessive ROS caused by DBP exposure and OVA sensitization.

To determine the redox status of the lung, GSH was measured in the lung homogenate. As shown in Fig. 2B, the GSH level decreased significantly ($p < 0.01$) in both the OVA with or without DBP exposure groups. On the other hand, MT administration clearly restored the GSH

activities in the lung ($p < 0.05$) and efficiently elevated the anti-oxidant level of the lung.

In order to further evaluate the level of oxidative stress, MDA, the product of lipid peroxidation, was measured in the lung homogenates. When compared to the control group, the MDA level was significantly increased ($p < 0.01$) in OVA with or without DBP groups. Pretreatment with MT significantly ($p < 0.01$) attenuated DBP-OVA-induced lipid peroxidation and revealed its capability as an anti-oxidant. The MDA changes are shown in Fig. 2C.

Cell Counts in BALF

The number of total cells, eosinophils and neutrophils in BALF were detected and are shown in Fig. 3. Our protocol gave rise to a marked lung inflammation dominated by a high number of total cells and eosinophils in both the OVA and DBP+OVA groups when compared with the control group ($p < 0.01$). Furthermore, the application of MT had a significant inhibitory effect on the numbers of total cells and eosinophils ($p < 0.05$). The neutrophils showed a similar change trend among all groups, unlike other inflammatory cells in BALF, with only the DBP+OVA group showing a significant increase when compared to the control group ($p < 0.05$). There were no significant differences between the OVA group and the control group ($p > 0.05$). Moreover, the MT-pretreated mice attenuated the number of neutrophils to some extent but not significantly ($p > 0.05$).

Cytokine Production in BALF

The levels of IFN-γ and IL-4, which represent the Th1 and Th2 cell response respectively, were assayed by ELISA in BALF. The levels of IFN-γ in different groups showed no significant difference ($p > 0.05$), and all OVA-sensitized and challenged groups (OVA, DBP+OVA, DBP+OVA+MT) had a little lower IFN-γ than the control group (Fig. 4A). The IL-4 levels in the OVA-sensitized and challenged groups were higher than the control ($p < 0.05$) and MT-pretreated mice attenuated the production of IL-4 and had a significant difference ($p < 0.05$) when compared with the DBP+OVA group (Fig. 4B). In addition, the ratio of IFN-γ/IL-4 showed an opposite trend to the IL-4 level (Fig. 4C), and the application of MT elevated the ratio remarkably ($p < 0.01$), demonstrating the domination of the Th2 response in our protocol.

Serum Total IgE levels

The concentrations of total IgE in serum were measured. OVA sensitization and challenge with or without DBP exposure elevated the levels of total IgE remarkably (both $p < 0.01$, Fig. 5).

Intraperitoneal injection of MT before OVA sensitization significantly lowered the levels of total IgE ($p < 0.05$).

Determination of AHR

As shown in Fig. 6A and Fig. 6B, as the dosages of MCH gradually increase, the expiratory and inspiratory resistance in all OVA with or without DBP exposure groups increased remarkably when compared with the control group (both $p < 0.01$), while the Cldyn (Fig. 6C) decreased significantly ($p < 0.01$) in OVA and DBP+OVA groups. When pre-administrated with MT, the expiratory and inspiratory resistance was distinctly weakened (Fig. 6A-B). On the contrary, MT-treated mice restored the Cldyn slightly (Fig. 6C), and at high MCH dosages (0.1 and 0.2 mg/kg) showed a very slight significant difference ($p < 0.01$ and $p < 0.05$).

Histopathology Examination of the Lung

Evidence of inflammatory cell infiltration was further investigated by looking at lung sections stained with H&E (Fig. 7A1-A4). An extensive eosinophil and mononuclear cell infiltration around the airways was seen in the lungs of the OVA-sensitized and challenged with or without DBP exposure groups compared with the saline control group. Furthermore, a marked hyperplasia in goblet cells and shrinkage of the airway lumen was observed in OVA-treated mice. Treatment of sensitized mice with MT (Fig. 7A5) significantly reduced the infiltration of inflammation cells and reduced the damage to the airway epithelium of OVA-treated mice. Subepithelial collagen deposition could be seen in lung sections stained with Masson's trichrome. When compared to the saline control group (Fig. 7B1), the collagen layers under the lamina propria in the OVA-treated group (Fig.7B3-B4) were obviously thickened. In contrast, MT treatment (Fig. 7B5) reduced collagen deposition to levels that appeared similar to those in the saline control or DBP exposure group (Fig. 7B2). To evaluate the mucus hypersecretion which is considered an important characteristic of airway remodeling, lung sections were stained with PAS. In the OVA and DBP+OVA groups (Fig. 7C3-C4), overproduction of mucus was distinctly observed as a violet color in the bronchial airways. On the contrary, the PAS-positive cells in MT-pretreated mice were markedly diminished (Fig. 7C5) versus the DBP+OVA group.

Discussion

Asthma is a complex and heterogeneous syndrome,[36] and oxidative stress is believed to play an important role in the development of asthma.[37] Oxidative stress can occur as a result of either excess ROS generation and/or impaired antioxidant capacity.[33] Losing control of the generation of ROS will induce immune disorders such as asthma. In the current investigations, the significant increase in the ROS levels in all OVA-treated groups demonstrated that exposure to DBP and OVA sensitization/challenge increased the level of oxidative stress in the mouse asthma model. Meanwhile, pre-treatment with MT ameliorated ROS due to its antioxidant effect and ability to scavenge free radicals that had been engendered in the lung. The presence of MDA, an end-product of lipid peroxidation and caused by redundant intracellular ROS, was observed to markedly increase in our studies and indicated that the excess ROS generated by inflammatory cells gave rise to oxidation damage in pulmonary cells and inflammation induced by our protocol. In fact, ROS toxicology is controlled by the antioxidant defense system that works at the molecular level. GSH, a major reductant in cells, can reduce a wide variety of disulfides by trans-hydrogenation, and is an important ROS scavenger.[38] The data from this study showed a remarkable depletion of GSH in the OVA-treated mice which indicated the imbalanced redox state of intracellular environment. The application of MT could restore the balance of the oxidant/antioxidant states. Through comprehensive analysis of our data, we dramatically found that DBP exposure could not significant augment the level of oxidative stress (ROS, MDA, GSH) and it suggested that the adverse effect of DBP exposure was may not mediated via oxidative stress mechanism, and the MT-treated mice can significantly inhibit the adverse effects may reflects the nature of OVA-induce asthma model itself.

Asthma is a complicated pathophysiological event, involving the interaction of many cell types and many cytokines. An unbalanced Th1 and Th2 immune response is an important pathological basis for allergy and allergic asthma.[39] Th1 cells secrete interferon-γ (IFN-γ) while Th2 cells secrete the interleukins IL-4, IL-5 and IL-13. In particular, IL-4 and IFN-γ are used as markers for Th cell differentiation.[9] Besides, the BALF exhibits the cellular and biochemical alterations of inflammation and lung injury in response to various toxic agents and offers a faithful reflection of the asthmatic condition.[40] In this study, we found that IL-4 was markedly increased in the OVA sensitization/challenge group whereas the secretion of IFN-γ showed no significant differences

among all groups. However, a slight downward trend of IFN-γ could be seen from the groups exposed to OVA with or without DBP. Meanwhile, the ratio of IFN-γ/IL-4 in OVA sensitization/challenge group indicated the predominant Th2-type cytokine profile on the mouse asthma model. OVA sensitized with or without DBP exposure groups exhibited a decreased IFN-γ/IL-4 ratio with no statistical significance with each other indicated that DBP exposure combined with OVA could not induced significant adverse effect in the present study. Eosinophil is the principal effector cell for the pathogenesis of allergic inflammation.[41] The cell count of total cells, eosinophils and neutrophils in BALF of aerosolized OVA groups reflected a clear inflammation cell infiltration, which was also proved by the lung sections stained with H&E, into the airways in lung. It has been shown that IL-4 could activate B cells to produce IgE, and elevated serum IgE is a hallmark of a Th2 immune response.[41, 42] As expected, our data showed that the serum levels of total IgE was substantially increased in both of the aerosolized OVA groups, but there was no statistically significant difference between the OVA sensitization/challenge groups with or without DBP exposure. The application of MT weakened the Th2 immune response and reestablished the balance of Th1/Th2 immune response.

Airway remodeling has been documented in all degrees of asthma severity, and in both large and small airways.[43] The histopathologic features of airway remodeling include the loss of epithelial integrity, subepithelial fibrosis (thickening of basement membrane), inflammatory cell infiltration, hyperplasia, hypertrophy of the bronchial smooth muscle and mucus hypersecretion.[43, 44] Our findings demonstrated that a dramatic augmentation of collagen deposition and mucous secretion were observed by Masson's trichrome and Periodic acid-Schiff stains in the OVA sensitization/challenge with or without DBP exposure groups. The collagen deposition in the perivascular regions of the lung was obviously thickened in the aerosolized OVA groups which created a stiffer lung. Goblet cell hyperplasia was observed in the OVA-treated mice but not in the control animals and a marked increase in mucus secretion in the airway was observed in the OVA-treated mice as compared with the control animals. A previous study proved that IL-4 played an important role in the maintenance of mucus glycoprotein hypersecretion in allergic airways, and ROS has been shown to strongly evoke bronchial hyperreactivity and to enhance mucous secretion.[45, 46] This was coincident with our present data. As expected, the degree of airway remodeling observed in the OVA sensitization/challenge with or without DBP exposure mice was

significantly reduced by MT, which blocked the imbalance between the oxidant and antioxidant states.

The duration of asthma has been associated with reduced lung function, increased AHR and other asthma symptoms.[43] AHR is considered to be a hallmark of inflammation in asthma, reflecting the severity of the disease, and is recognized as a clinical endpoint for therapeutic intervention.[47] We found that with a gradually increased dosage of MCH, the expiratory and inspiratory resistance increased dramatically and the cldyn decreased in the OVA-treated mice with or without DBP exposure. The presence of AHR indicates a fundamental change in the function of airway smooth muscle (ASM) that is partially due to the changes in ASM structure and its relationship with the surrounding airway wall structures.[48] This is the reason why the airway remodeling process has been proposed to explain these features.[43] With an understanding of the relationship between oxidative stress and asthma, we know that oxidative stress resulted in a deterioration of asthma via a number of means, and one of these being that ROS can contribute to enhanced AHR by increasing vagal tone and damaging oxidant-sensitive β-adrenergic receptors.[47] Although many factors linked to AHR have been confirmed, the mechanism of the formation of AHR is still unclear. A comprehensive analysis of AHR data of Ri uncovered a dramatic significant difference in the OVA sensitization/challenge with or without DBP exposure groups. This may be explained by the change in the three-dimensional structure of the airway wall that strongly influences lung function, and the slight shrinkage of the airway lumen might cause tremendous resistance to pulmonary ventilation.

An adjuvant effect is known to promote the immunological responses from both the uptake of antigens into dendritic cells, and the production of antibodies. Consequently the adjuvant effect of PAEs has attracted close attention of many researchers.[49, 50] Previous studies have demonstrated that DEHP has an adjuvant effect,[51-53] but this adjuvant property is not inherent to all phthalates, rather being highly dependent on the chemical structure of the compound. An optimum adjuvant effect was seen in phthalates containing eight to nine carbon atoms in the side chains, whereas PAEs with longer or shorter chains showed a less pronounced, or no adjuvant effect.[20] In this study we used an experimental model of allergic asthma to evaluate the adjuvant effect of DBP via an oxidative stress dependent mechanism. To our knowledge, this is the first time it has been shown that DBP resulted in a less pronounced adjuvant effect via an oxidative stress dependent

mechanism on a murine asthma model. Our data has shown that exposure to DBP did not induced a significant change of the asthma-like symptoms in OVA-sensitized mice. However, the trend of increase of some symptoms, such as IgE production, eosinophilia infiltration, mucus production and fibroblast proliferation, demonstrated that exposure to DBP could enhance the adverse effect induced by environmental contaminants. In addition, DBP exposure could augment the level of oxidative stress to some extent, causing further adverse effects to mice. Since the side chains of DBP contain only four carbon atoms, this may explain why DBP has a weak adjuvant effect.

Conclusion

In summary, this study indicated that exposure to DBP could not induce a significant alteration of oxidative status and the asthma-like symptoms. However, the increase trend of some symptoms in OVA-sensitized mice induced by DBP exposure suggested that the special chains side chains of DBP cause it to have a less pronounced adjuvant effect, and that this weak adjuvant effect is not mediated through an oxidative stress mechanism on a murine asthma model (Fig. 8). Furthermore, exposure to DBP has been shown to increase the severity of asthma, as evidenced by the small increasing trend of some asthma-like symptoms, and this should be seriously considered.

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Conflict of interest statement

None of the authors have any conflicting interests.

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Figure legend

Fig. 1

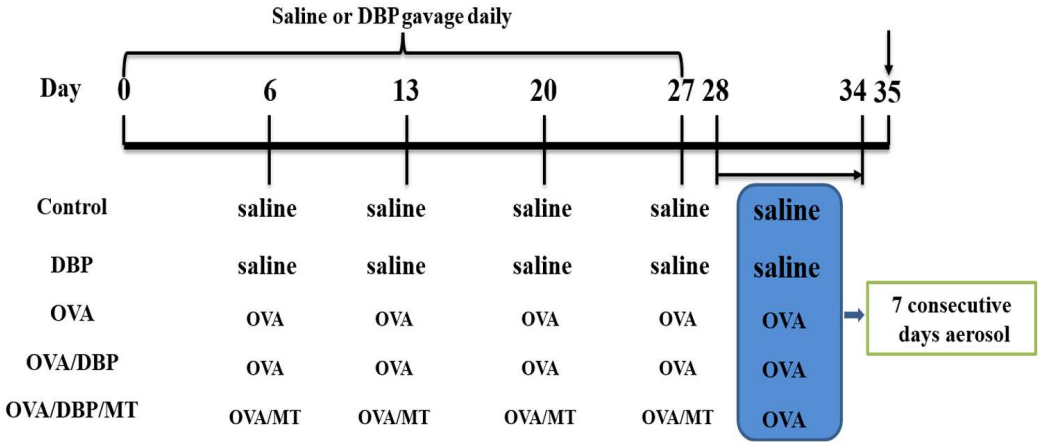

Fig. 1 Exposure and immunization schedule. Mice were sensitized by four *i.p.* and *s.c.* injections of OVA on days 6, 13, 20 and 27, and then received 7 consecutive days of aerosolized OVA or saline challenge for 30 min on days 28 to 34. MT was given by *s.c.* at 10 mg/kg body weight at half an hour before each sensitization of OVA. Each group was given saline or DBP to gavage calculated by body mass daily. On day 35, mice were sacrificed.

Fig. 2

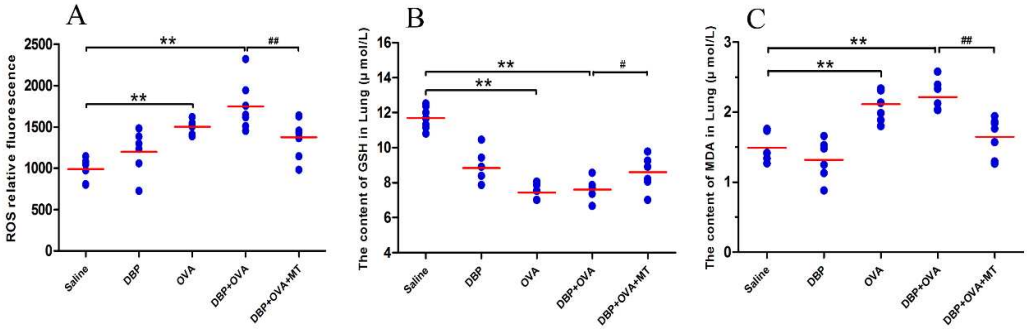

Fig. 2 Determination of the level of oxidative stress in lungs. (A) The ROS relative fluorescence in lung homogenate. (B) The content of GSH in lung homogenate. (C) The content of MDA in lung homogenate. The red lines present the mean of each group (n = 6 or 7). **, $p < 0.01$, significant difference compared with the control, *, $p < 0.05$, significant difference compared with the control, and ##, $p < 0.01$, significant difference compared between OVA-treated with DBP exposure group and MT-treated mice.

Fig. 3

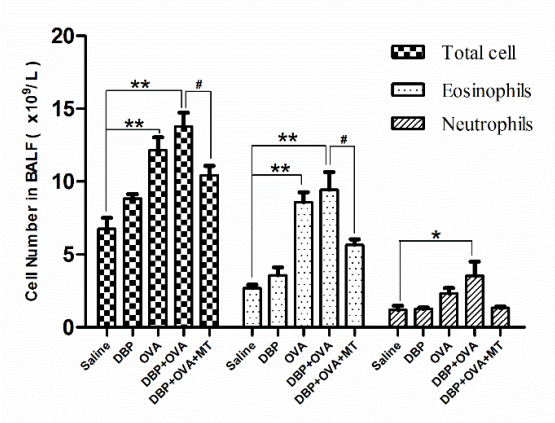

Fig. 3 Inflammatory cell counts in BALF. Mice were sacrificed 24 h after the last aerosol challenge, and the lungs were lavaged three times with 0.5, 0.6 and 0.7 mL ice-cold saline via a tracheal cannula. The BALF was then centrifuged and the pellets were re-suspended with 500 μL and used for cell counting. Group data are expressed as means ± SEM for six mice per group. **, $p < 0.01$, significant difference compared with the control, *, $p < 0.05$, significant difference compared with the control, and #, $p < 0.05$, significant difference compared between OVA-treated with DBP exposure group and MT-treated mice.

Fig. 4

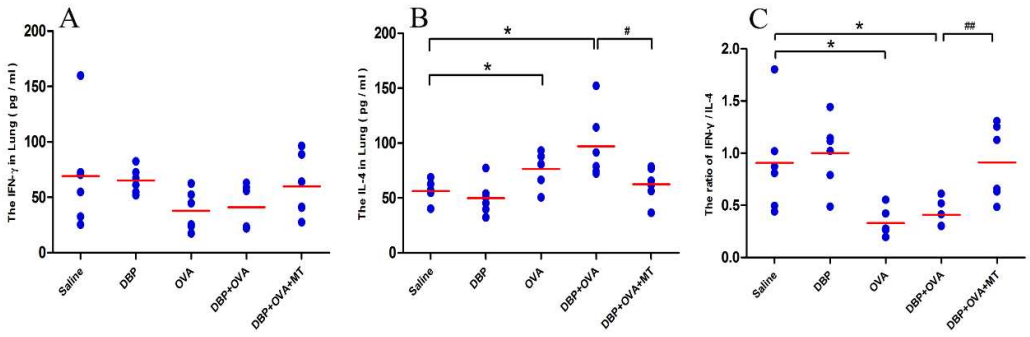

Fig. 4 Levels of IL-4 and IFN-γ in BALF. Mice (n = 6) were sacrificed 24 h after the last aerosol challenge, and the lungs were lavaged three times with 0.5, 0.6 and 0.7 mL ice-cold saline via a tracheal cannula. The BALF was then centrifuged and the supernatants were collected for cytokines determination. (A) The level of IFN-γ in BALF. (B) The level of IL-4 in BALF. (C) The ratio of IFN-γ/IL-4. *, $p < 0.05$, significant difference compared with the control, #, $p < 0.05$ and ##, $p < 0.01$, significant difference compared between OVA-treated with DBP exposure group and MT-treated mice.

Fig. 5

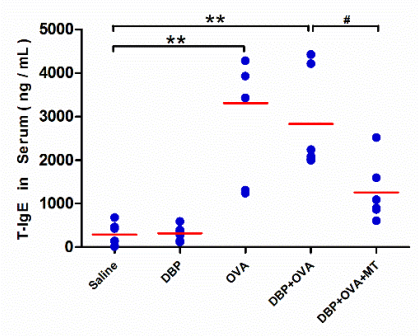

Fig. 5 Total IgE in serum. Blood was obtained by heart puncture on the final day. It was placed at room temperature for 30 min and then centrifuged. The resulting serum (supernatant) was collected for IgE detection (n = 5). **, $p < 0.01$, significant difference compared with the control, and #, $p < 0.05$, significant difference compared between OVA-treated with DBP exposure group and MT-pretreated mice.

Fig. 6

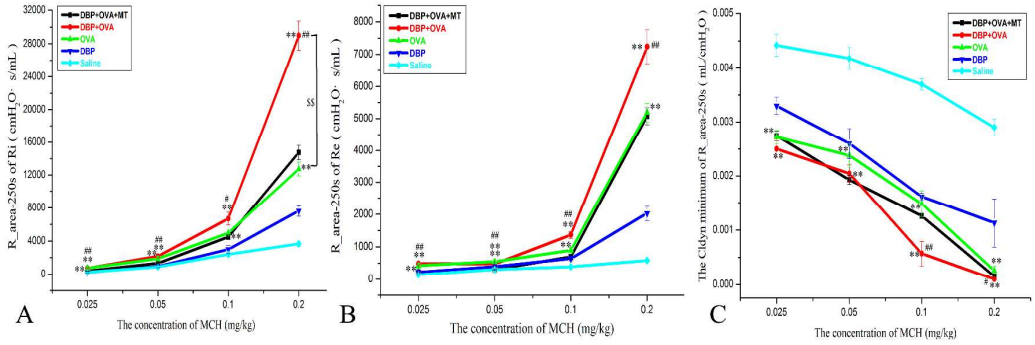

Fig. 6 AHR measurements in mice. Mice were anesthetized, the trachea was intubated with a cannula and connected to a computer-controlled small ventilator, with the respiratory rate controlled at 75 breaths/min and the ratio of expiration to inspiration preset at 1.5:1. Run the software of AniRes 2005, injected methacholine into the jugular vein a few minutes later and the dosages of 0.025, 0.05, 0.1 and 0.2 mg/kg, given successively every 5 min. AHR is expressed as the area between the peak curve of (A) inspiratory resistance (Ri) and (B) expiratory resistance or the valley value of (C) lung dynamic compliance (Cldyn) and the baseline level during the 250s from the time MCH entered the vein. Group data are expressed as means ± SEM for six or seven mice per group. *, $p < 0.05$ and **, $p < 0.01$, significant difference compared with the control, #, $p < 0.05$ and ##, $p < 0.01$, significant difference compared between OVA-treated with DBP exposure group and MT-treated mice; $$, $p < 0.01$, significant difference compared between OVA sensitized with or without DBP exposure groups.

Fig. 7

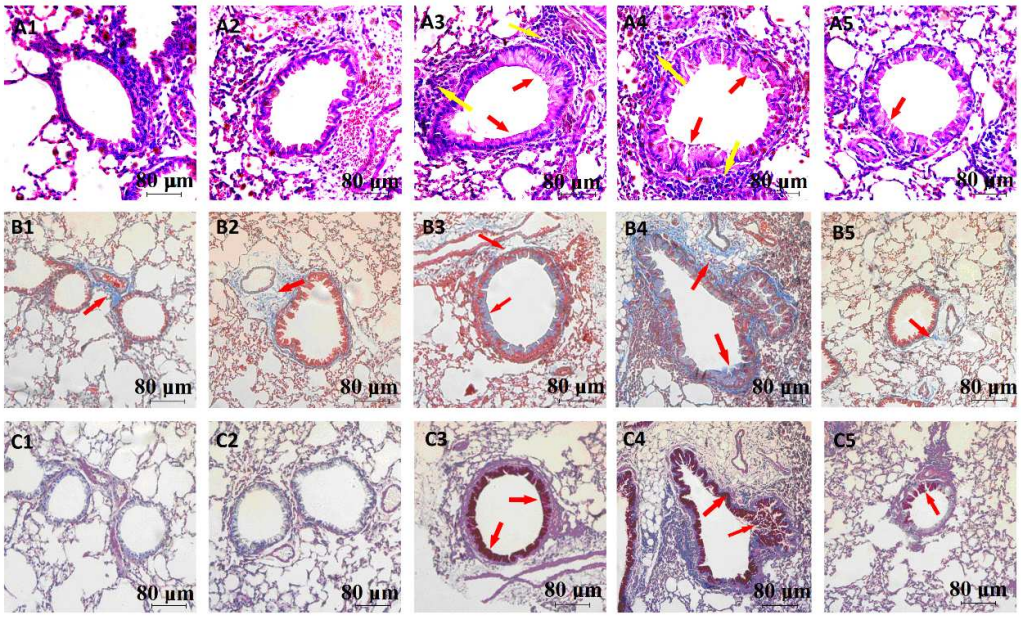

Fig. 7 Histopathology Examination in the Lung. A1-C1: control groups; A2-B2: DBP groups; A3-B3: OVA-sensitized groups; A4-B4: OVA-sensitized with DBP exposure groups; A5-B5: MT treated groups. Lungs obtained from sensitized mice 24 hours after the last saline aerosol or OVA aerosol challenge were fixed in 4% neutral buffered polyformaldehyde, paraffinized and cut into slices. The slices were stained with hematoxylin and eosin (H&E) (A1-A5) to examine cell infiltration (yellow arrows) and hypertrophy of structural cells (red arrows), Masson's trichrome (B1-B5) for collagen deposition (red arrows) and Periodic acid-Schiff (PAS) (C1-C5) to examine mucus production (red arrows).

Fig. 8

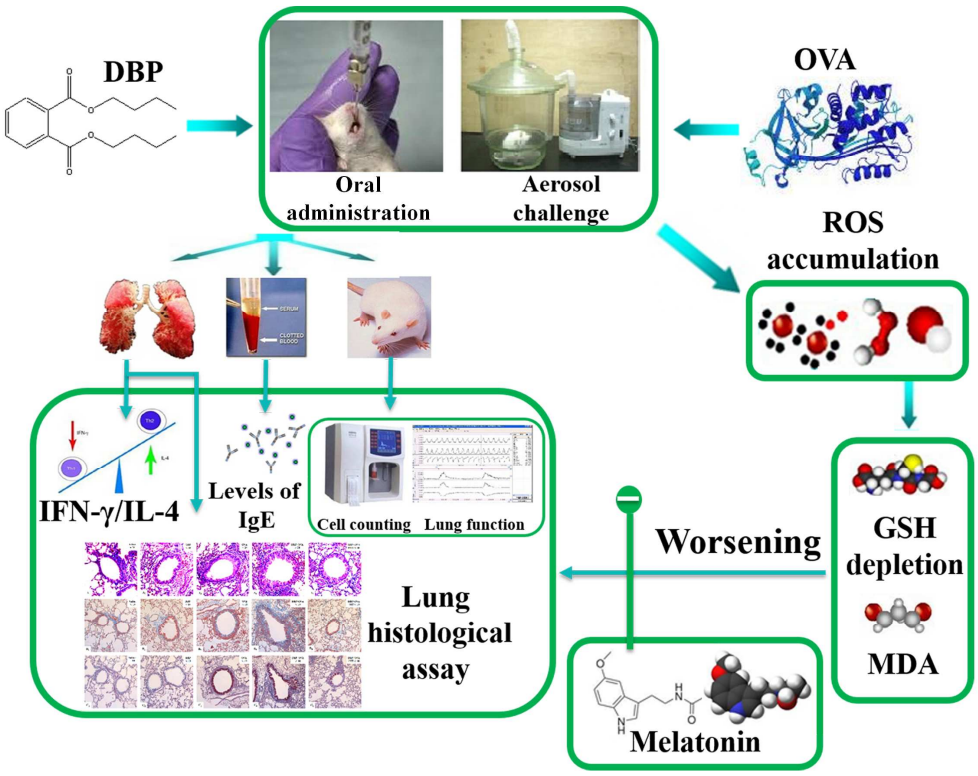

Fig. 8 Potential mechanism of DBP-induced toxicity in mice. The exposure of DBP may cause adverse effects in the mice lung occurs through ROS generation thus contributing to the deterioration of asthma-like multiple markers. The applied of melatonin reversed all these effects caused by DBP through its antioxidant properties.