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### Adjuvant effect of di-(2-ethylhexyl) phthalate on asthma-like pathological changes in ovalbumin-immunised rats

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## Adjuvant effect of di-(2-ethylhexyl) phthalate on asthma-like pathological changes in ovalbumin-immunised rats

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Pollution of phthalates, including di-(2-ethylhexyl)phthalate (DEHP), is widespread because of their use in plastics and other daily consumer products. Epidemiological studies have shown that the largest source of general population exposure to DEHP is on dietary. Studies have suggested an association between exposure to phthalate plasticisers, and increased prevalence of asthma. To investigate the adjuvant effect of DEHP on asthmatic pathological changes in ovalbumin-immunised rat model. Wistar rats were randomly divided into five groups (eight rats of each group): (1) saline; (2) ovalbumin (OVA); (3) OVA + DEHP  $0.7\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ ; (4) OVA + DEHP  $70\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ ; and (5) DEHP  $70\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ . DEHP treatment groups which were administered to  $0.7\text{mg}$  and  $70\text{mg}$  DEHP/kg/d by gastric gavage for 30d before and during the process of OVA immunisation or saline treatment. The *in vivo* pulmonary function analysis, bronchoalveolar lavage (BAL) fluid collection and section histological observation were conducted. DEHP exposure could significantly increase airway hyperresponsiveness, airway remodeling, and eosinophil infiltration in the OVA-immunised rats. But the DEHP exposure alone group does not show significant airway structural change and inflammatory cell infiltration, compared with control group. DEHP administered by gastric gavage play an adjuvant effect on respiratory systems in ovalbumin-immunised rat model.

**Keywords:** di-(2-ethylhexyl)phthalate; asthma; ovalbumin-immunised rat; adjuvant effect

### Introduction

The prevalence of bronchial asthma has increased over the last few decades. The World Health Organization (WHO, 2004) reported that asthma affects about 150 million people world wide, and is a major incidence causing hospitalisations for chronic diseases in children in the western world. The conventional factor (i.e. atopy) is not sufficient to explain the increased prevalence of asthma (Platts-Mills & Woodfolk, 1997). There is a general acknowledgement that changes in lifestyle, diet and the environment, either singly or in concert, better diagnosis and reporting of asthma are likely to have driven the observed increases in disease prevalence (Devereux & Seaton, 2005; Kimber, 1998; Ring, 1997; Strachan, 1996). Against this background it has been proposed that some kinds of man-made chemicals may play an important pathogenetic role in predisposition

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to allergy and asthma (Delfino, 2002; Ebtekar, 2006). Moreover, as well as man-made chemicals, recently some other risk factors have been suggested as causes for the increase in asthma, these include: (a) higher humidities leading to increased mites and moulds (Cho et al., 2006); and (b) increased immunisations leading to fewer early life infectious diseases leading to an allergy phenotype (Ring, Krämer, & Behrendt, 2005). Unfortunately, the exact molecular mechanism for the increase in asthma is still far from elucidated.

Phthalate esters (PAEs) are used for industrial chemicals which serve as important additives to impart flexibility to plastics. They are a key component in a wide range of products, including flexible polyvinyl chloride (PVC) plastics, vinyl tile, food packaging, insecticides, pharmaceuticals, and personal care products (ATSDR, 2001, 2002; NTP-CERHR, 2003; Schettler, 2006). Because they are not chemically bound to the plastics, phthalates can be released from the products that are used and discarded (Bornehag et al., 2005; Oie, Hersoug, & Madsen, 1997). Consequently they are widely distributed in the ecosystem and have been described as being among the most abundant man-made environmental pollutants. In particular, di-(2-ethylhexyl)phthalate (DEHP) is the most commonly used plasticiser. Globally, more than 18 billion pounds of phthalates are used each year and well above two million tons of DEHP alone are produced annually worldwide (Lorz et al., 2002).

Epidemiological studies have suggested that DEHP has been associated with the development of wheezing, rhinitis, asthma or asthma symptoms (Bornehag et al., 2004; Jaakkola et al., 1999, Jaakkola, Verkasalo, & Jaakkola, 2000). However, animal study has shown that DEHP was not allergen. There was no significant effect on levels of serum IgE and IL-4 after exposures on DEHP (Butala et al., 2004). This has led to a conception that DEHP may cause asthma via a non-IgE-mediated mechanism. In this study, we propose to establish the animal models of DEHP-induced asthma to further examine the effect of DEHP on the respiratory systems. As regards, the airway hyperresponsiveness (AHR) testing, lung histology and cytokines profiles were studied.

## Materials and methods

### *Experimental animals*

Wistar rats (male, 5–6 weeks old and 70–80 g) were purchased from the Hubei experimental animal centre (Wuhan, China) and maintained in pathogen-free room on environmental conditions that temperature was kept at 20–25°C and relative humidity at 50–70%.

### *The exposure protocol*

The Wistar rats were randomly divided into five groups of eight animals. The saline group (group A) was administered with a hypodermal injection (i.p.) of 1 ml saline at day 19 and 27. During days 31–37, animals placed in the glass chamber were challenged with an aerosol of saline for 30 min per day using an ultrasonic neutraliser (Yuyue 402AI type, China); the OVA-immunised group (group B) was treated with a hypodermal injection of 100 mg OVA (Sigma, Grade) and 100 mg aluminum hydroxide fine powder was suspended in 1 ml of saline at day 19 and 27. From day 31 to 37, animals were exposed to an aerosol of 1% OVA for 30 min per day; the DEHP groups (group C, D and E). For preparation,

DEHP (Sigma) was dissolved in Tween-80 (Amresco) in the ratio 1:1 and diluted by sterile water to the final concentration designed in advance, the rats were oral administration (ad) by gavage dosing and was initiated with 0.7 mg and 70 mg DEHP/kg/d, respectively, and immunised and challenged with OVA or saline following the protocol, which is shown in Figure 1.

### Airway hyperresponsiveness (AHR) testing

For each group, AHR was assessed 24hs after the final aerosol using the AniRes2005 lung function system (Bestlab, AniRes2005, version 2.0, China). Animals were anaesthetised intraperitoneally with pentobarbital sodium (Urchen) (95 mg/kg), and then instrumented with a tracheal cannula connected to a tap for monitoring tracheal pressure and mechanically ventilated with the lung function system. The respiratory rate was preset as 75/min and the time ratio of expiration/inspiration as 1.5:1. After that, an injector needle ( $\Phi = 0.45$  mm) was inserted into the jugular vein connected to a saline-filled catheter. Methacholine (MCH) (O-Acetyl- $\beta$ -methylcholine chloride, Sigma, USA) was administered orderly in 5 min with the dosage of 0.025, 0.05, 0.1, 0.2 mg/kg, and AHR was examined through the change of lung function by a computerised recording of expiratory resistance (Rr), inspiratory resistance (Ri), and dynamic lung compliance (Cldyn). The relative area (R-area) (i.e. the area under the peak curve of Rr or Ri and beyond the baseline during the timeframe of 250 s) and the peak value

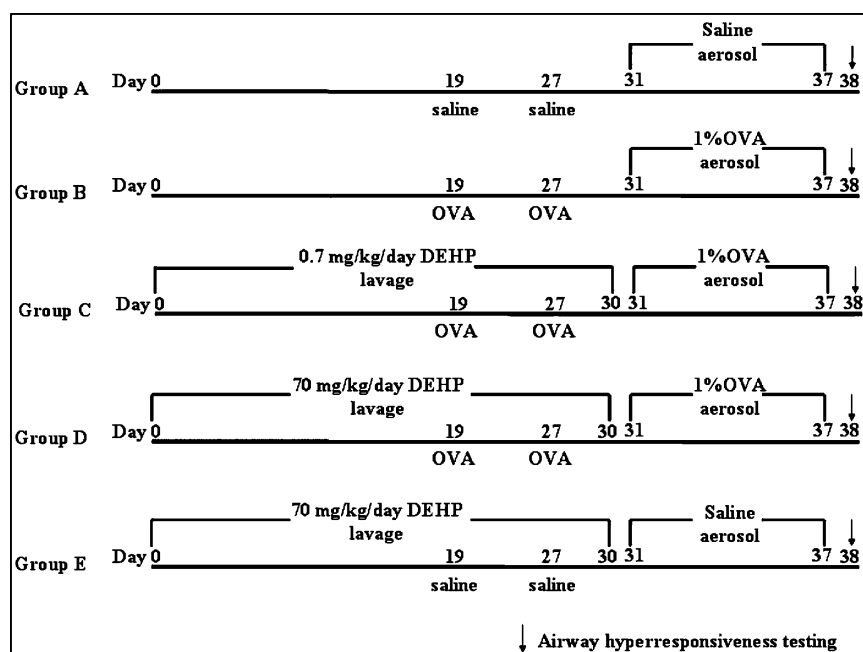


Figure 1. The establishment of DEHP-induced asthma model in rats. (Group A: saline control; Group B: OVA-immunised group; Group C: 0.7 mg DEHP/kg/d + OVA group; Group D: 70 mg DEHP/kg/d + OVA group; Group E: 70 mg DEHP/kg/d alone group.)

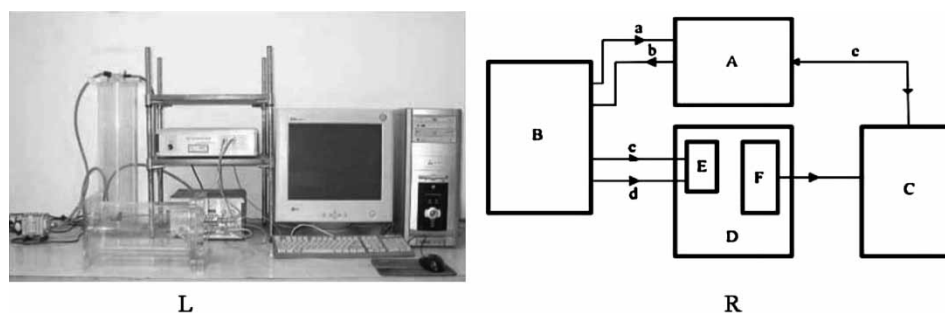


Figure 2. The airway hyperresponsiveness was assessed by the AniRes2005 lung function monitoring system. (L: the setup of AniRes2005 lung function system; R: the illustration of AniRes2005 lung function system. After being anaesthetised and intubated with a tracheal cannula, the experimental animal was placed into B, a chamber with the tracheal cannula connected to A, the respirator for mechanical ventilation. Two tubes were contained, one a was the expiration channel and the other b was for the inspiration. Meanwhile, a distributor c was combined to measure the tracheal pressure using a sensor E that could also monitor the lung volume by indirect determining the volume change of the chamber B via another tube d connected to the animal chamber B. By the signal transistor D, signals of the two indexes monitored were collected by a USB memory card F and switched to that recognised by Anires 2005 software C. In parallel, the respirator A was also controlled by the computer C via the circuit e.)

(Min) of dynamic lung compliance ( $C_{ldyn}$ ) were adopted as the quantitative indicators of AHR.

### ***Cell counts in the bronchoalveolar lavage fluid (BALF)***

After MCH testing, the alveolus of the animal was immediately lavaged via the trachea cannula rinsed with three washes of 3 ml saline to prepare the bronchoalveolar lavage fluid (BALF). The cell samples in BALF were concentrated by centrifugation (1000 rpm for 10 min at 4°C) and re-suspended in 800 µl saline. Two hundred µl of the cell suspension was used to do the total cell counting using the blood cells analysis system (Sysmex KX-21, Japan), and another 400 µl of specimen was used for eosinophils (EOS) counting with a further cell survival treatment. These cells were centrifuged and re-suspended in 400 µl of dilution solution containing 20 µl of Eosin Y sodium solution (Amresco, High Purity Grade, USA) (2%), 20 µl of acetone solutions and 360 µl of deionised water, in which only EOS are viable. Then the number of EOS was assessed using a hemocytometer (Sigma) under a microscope (Olympus).

### ***Lung histology observation***

Following the lung lavage, the left lungs of the rats were isolated and aerated to achieve a physiological state *in vivo*. After that, the sample was soaked into the fixing solution (saturated trinitrotoluene (TNT): formalin: glacial acetic acid = 15:5:1) at room temperature for 24 hs and then stained with hematoxylin and eosin (H&E). Finally, the tissues were embedded in paraffin and cut into 10-µm sections. Asthma remodelling,

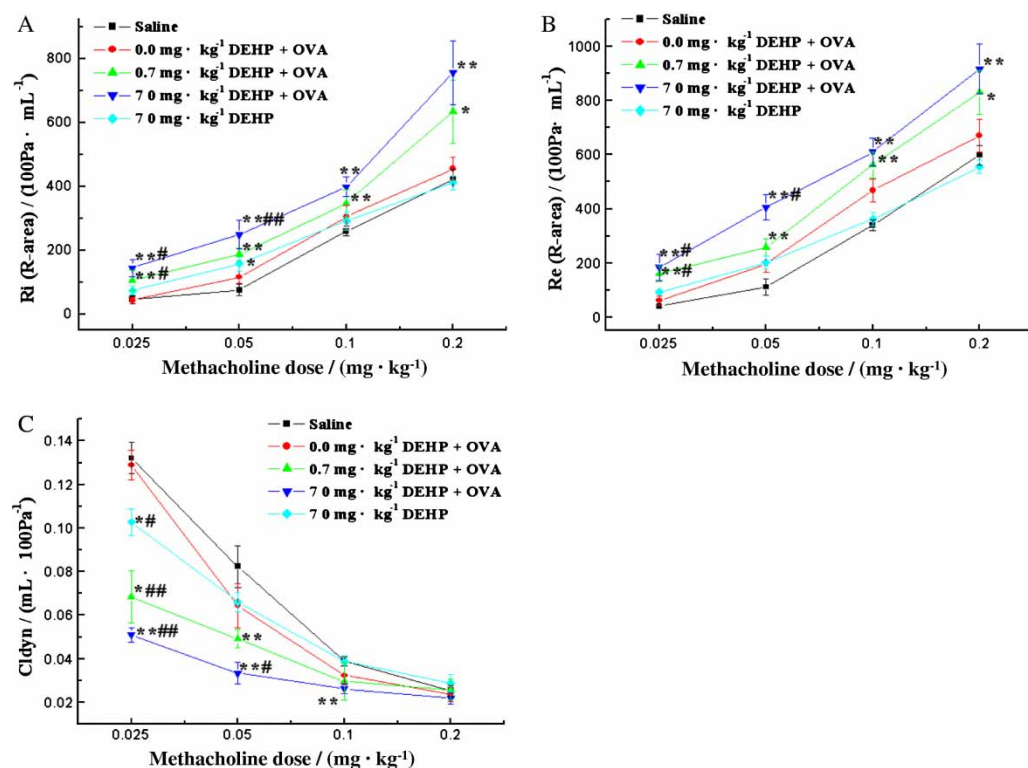


Figure 3. Airway hyperresponsiveness was assessed 24 h after the final OVA aerosol. (A: R-area of Re with different dose of MCH; B: R-area of Ri with different dose of MCH; C: the peak value of Cldyn with different dose of MCH. ■: the saline group; ●: the OVA-immunised group; ▲: 0.7 mg DEHP/kg/d + OVA group; ▼: 70 mg DEHP/kg/d + OVA group. ◆: 70 mg DEHP/kg/d DEHP alone group \* $p < 0.05$ , \*\* $p < 0.01$ , compared with negative control; # $p < 0.05$ , ## $p < 0.01$ , compared with OVA-immunised group.  $n = 8$ .)

including mucus secretion, airway wall thickening and inflammatory cell infiltration, was examined under a microscope (Leica DM 4000B, Germany).

### Statistical analysis

The results were expressed as mean  $\pm$  SE, and two population  $t$ -test was applied in statistical analysis.  $P$  value  $< 0.05$  was considered as the significant difference. The dose-effect relationships were plotted with Origin 6.1 software.

## Results

### Assessment of airway hyperresponsiveness (AHR)

The airway responsiveness was assessed in the lung function measurement and indicated by indexes of R-area of respiratory resistance (Rrs, including Re and Ri) and the peak value of Cldyn (Figure 2). As shown in Figure 3, the OVA-induced asthma group exhibited

greater airway responsiveness to MCH. DEHP exposure could significantly enhance the degree of AHR of OVA-immunised rats in a dose-dependent manner. For the DEHP exposed non-immunised rats, AHR was slight higher than the negative control but lower than the DEHP exposed OVA groups.

### ***Lung histological study***

Lung histology results demonstrated the airway remodelling in OVA-immunised group, including higher levels of mucus secretion, increased inflammatory cells infiltration and airway wall thickness which, in humans, are associated with asthma. DEHP exposure was manifested to aggravate such asthma-like syndromes in immunised rats, and the most serious alter of the airway structure was shown in 70 mg DEHP/kg/d group (see Figure 4). On the other hand, we observed no significant pulmonary abnormalities in the rats exposed to DEHP only.

### ***Cell infiltrations in the Bronchoalveolar lavage fluid (BALF)***

A significant increase ( $p < 0.05$ ) in EOS counts in OVA-immunised/challenged rats was observed compared with the saline group (see Figure 5 A&B). For the DEHP-treated groups, both the total cells and EOS in BALF were dramatically increased ( $p < 0.01$ , compared with the OVA group and saline group), with a dose-dependent pattern. However, the result of 70 mg DEHP/kg/d only group showed no significant differences versus controls.

## **Discussions**

Epidemiological studies have shown that the largest source of general population exposure to DEHP is on dietary, followed by indoor air. Dietary exposures result both from DEHP accumulating in certain foods and from leaching of DEHP during processing, packaging, and storing. Some food surveys indicated a range of DEHP content, but fatty foods, including dairy, fish, meat, and oils, contain the most (Huber, Grasl-Kraupp, & Schulte-Hermann, 1996; Meek & Chan, 1994). In this study, we, for the first time investigate the potential role of DEHP in the pathogenesis of asthma by exposure to oral ad.

The dose of DEHP administered in this study is greater than dose estimated for an average person (Koch, Drexler, & Angerer, 2003; Koch, Rossbach, Drexler, & Angerer, 2003; Kohn et al., 2000). The uptake rang has been estimated to be 3–30  $\mu\text{g/kg/d}$  (NTP-CERHR, 2003; Shea, 2003). Preventive limit values, such as reference dose (RfD) of the US Environmental Protection Agency (EPA) and tolerable daily intake (TDI) of the European Union are 20  $\mu\text{g/kg/d}$  and 37  $\mu\text{g/kg/d}$ , respectively (Koch, Drexler et al., 2003; Koch, Rossbach et al., 2003). However, higher exposures occur occupationally (up to about 700  $\mu\text{g/kg/d}$ , based on current workplace standards) and in the medical settings through use of plastic tubing, IV bags may receive up to 8.5 mg/kg/day (adults) and 22.6 mg/kg/day (neonates) of DEHP (Doull et al., 1999; FDA, 2001; Rockville, 2001). Median urinary concentrations of mono-2-ethylhexyl phthalate (MEHP), which is a metabolite of DEHP in infants, in neonatal intensive care units (NICUs) were between 30 (Green et al., 2005) and 50 times (Calafat, Needham, Silva, & Lambert, 2004) higher than the median concentration of MEHP in the general adult US population (Silva et al., 2004a) suggesting

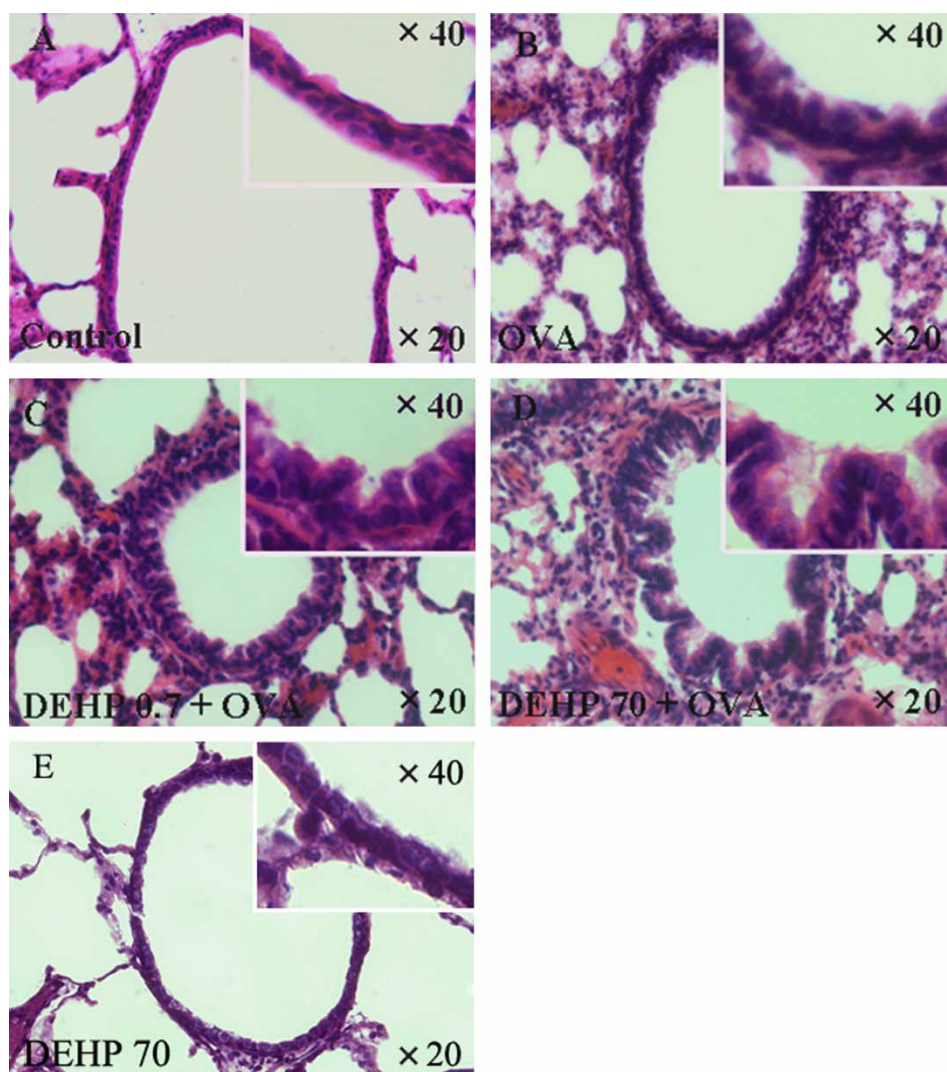


Figure 4. Histological examination of lung tissue airway remodelling. (A) the saline group; (B) the OVA-immunised group; (C) 0.7 mg DEHP/kg/d + OVA group; (D) 70 mg DEHP/kg/d + OVA group; (E) 70 mg DEHP/kg/d alone group. Lung tissue was fixed, stained with H&E, and section in 10- $\mu$ m slices.

that these patients may be exposed to DEHP at levels closer to the doses used in the present study.

#### ***Establishment of DEHP-OVA-asthma rat model***

The frequently limited information on the underlying mechanisms of chemical-induced asthma is probably reflecting a lack of good animal models. Two main factors, DEHP exposure and allergen ad, were concerned in the establishment of DEHP-induced asthma

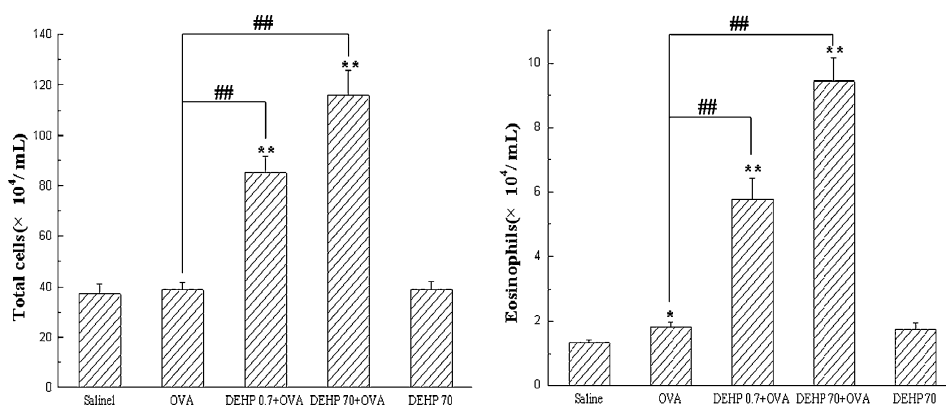


Figure 5. Cell counts in the bronchoalveolar lavage fluid (BALF). (A: total cell counts in BALF among experimental groups; B: EOS numbers in BALF of different groups. \* $p < 0.05$ , \*\* $p < 0.01$ , compared with negative control; ## $p < 0.01$ , compared with OVA-immunised group.  $n = 8$ .)

model. Herein, the traditional OVA (as allergen) model was utilised in our studies, even if which is a relatively poor model for common allergens associated with asthma. Therefore, better models such as cockroach allergen (e.g. Blag2) (Chen, Zhou, & Kang, 2001) or house dust mite allergen (Ahn et al., 2007) may be used in the further study of ours.

In this work, we want to quantitatively assess the physiological parameters of OVA-asthma rat model and take advantage of it for analysing the potential roles of suspected asthmagens. For the AHR testing, it is closely related to the severity of asthma and the left-shift of the dose-response curves indicates a lower threshold of the airway response to a specific airway stimulus in the asthma (Gillis & Lutchen, 1999; Leigh et al., 2002). To document the phenotype, MCH is utilised as the non-specific airway stimulant due to its high safety, short response-time and low side effects.

Compared with techniques that requiring the removal of the lungs or airways from the animal, the approach in our study has obvious advantages: the mechanical ventilator can control the breathing pattern; the lungs are analysed in situ, securing near normal conditions during the systemically assessments (Drazen, Finn, & De Sanctis, 1999). Concerning the measurement of AHR levels, the R-area of the respiratory resistance (RL, including  $R_e$  and  $R_i$ ) and peak values (Min) of Cldyn have been adopted as the sensitive indexes. The underlying physiological meanings of the parameters of RL and Cldyn are based on two points: RL explains the state of the large airways, whereas changes in Cldyn reflect variety in small airways or the parenchyma (Drazen et al., 1999; Karol, 1994).

By H&E staining on lung sections, the airway can be observed in chronic respiratory conditions like asthma (Boulet & Sterk, 2007). A series of events are involved in airway remodelling, including airway wall thickening, inflammatory cell infiltration, sub-epithelial fibrosis, mucus metaplasia, hypertrophy and hyperplasia of smooth muscle and goblet cells (Yamauchi, 2006). It is believed that airway remodeling is a critical factor in the development of asthma syndromes such as AHR and airflow limitation, and closely related to the aggravation of this respiratory disorder (Elias, 2000; Leigh et al., 2002; Yamauchi, 2006).

### ***Adjuvant effect of DEHP on respiratory systems***

In the present study, from AHR testing results (Figure 3), the airway reaction curves increases in a dose-dependent manner with DEHP exposure. The highest AHR level emerges when 70 mg DEHP/kg/d was administrated followed with OVA immunisation. In this case, even at the lowest dose of MCH (0.025mg/kg), significantly higher RL and lower Cldyn is observed comparing with the other groups. This result is further testified in the lung histological study with H&E staining. Compared with those without DEHP exposure, remarkable airway structural changes is observed in 70 mg DEHP/kg/d exposed OVA-immunised rats, including thickening of the respiratory tract, enhancement of cells infiltration and mucus metaplasia (see Figure 4), showing the aggravate asthma symptoms in DEHP exposure OVA-immunised rats.

To elucidate whether airway inflammation played a role, another indicator of asthma EOS, which is considered to be an important effector cells in the pathogenesis of asthma and closely associate with the aggravation of this disease (Boulet & Sterk, 2007) is investigated in BALF. Our data shows that the numbers of EOSs and total cells are increased in OVA-immunised rats with DEHP exposure, significantly elevated number even in the exposure group to 0.7 mg DEHP/kg/d compares with the OVA-immunised rats and the control. The results are in line with Larsen's research, numbers of EOSs increases with increasing concentrations of DEHP, giving rise to a significantly elevated number after exposure to 13 mg DEHP/m<sup>3</sup> compares with the OVA control group (Larsen et al., 2007). For further study our group would explore more information on the relation between the adjuvant effect of DEHP and the levels of cytokines such as IL4, INF $\gamma$ , TNF $\alpha$ , IL1 $\beta$  and/or IL-6.

Interestingly, no notable difference is observed in DEHP alone group when compared with the saline control, neither in the airway structure exhibits in the lung H&E stained sections (see Figure 5) nor in the total cell and EOS count in BALF. There is slight increased AHR at the lowest dose of MCH (0.025mg/kg), however, no significant elevated AHR at high doses of MCH in the rats exposed to DEHP only.

Taken together, we can conclude that DEHP processes an adjuvant effect on respiratory systems in rats. But the pathogenesis of asthma in vivo is a very complicated interaction, involving lots of inflammatory cells and cytokines. Previous studies have shown that DEHP affected OVA-specific IgG1 production in a concentration-dependent manner, whereas little or no effect is seen on the OVA-specific IgE and IgG2a responses on BALB/c mice regardless of the route of ad (inhalation versus subcutaneous) (Larsen et al., 2001, 2007), IgG1 antibody production in the mouse is often used as a surrogate for IgE antibody responses as IgG1 antibody is subject to similar cytokine regulation as in IgE antibody (Snapper, Finkelman, & Paul, 1988). What is the relevance of IgG1, if translated to the rat system? The further research needs to test them to explore the mechanism of DEHP-induced asthma on rat.

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