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Effect of gestational and lactational nonylphenol exposure on airway inflammation in ovalbumin-induced asthmatic rat pups

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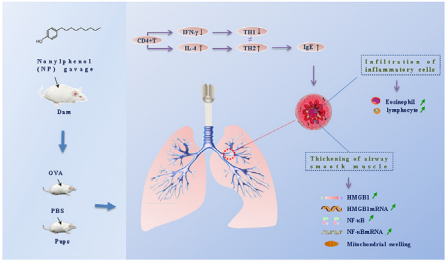
HIGHLIGHTS

- Perinatal NP exposure in dams exacerbated airway inflammation in asthmatic pups.
- There was an interactive effect between NP and OVA on airway inflammation in rats.
- Perinatal NP exposure increased HMGB1 and NF-κB expressions in the lung of pups.

GRAPHICAL ABSTRACT



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ABSTRACT

To investigate the effect of gestational and lactational nonylphenol (NP) exposure on airway inflammation in ovalbumin (OVA)-induced asthmatic pups. Dams were gavaged with NP at dose levels of 25 mg/kg/day (low dose), 50 mg/kg/day (middle dose), 100 mg/kg/day (high dose) and groundnut oil alone (vehicle control) respectively from gestational day 7 to postnatal day 21. The results showed that the NP content in the lung tissues of pups in the 100 mg/kg NP group was significantly higher than that of the control group ($P = 0.004$). In the 100 mg/kg NP group, the infiltration of lymphocytes and eosinophils with thicken smooth muscle layer and inflammatory cells in the lumen were observed in the lung tissues of pups. Osmiophilic lamellar bodies were found in the cytoplasm of type II epithelial cells; mitochondria were clearly swollen. Compared with the control group, the levels of interleukin-4 (IL-4) in BALF ($P = 0.042$) and ovalbumin-specific serum immunoglobulin E (OVA-sIgE) ($P = 0.005$) in the OVA group were significantly higher. 25 mg/kg NP-OVA co-exposure synergistically decreased nuclear factor-κB (NF-κB) mRNA expression in the lung tissues of pups; Exposure to 50 mg/kg NP combined with OVA antagonized the increased expression of high mobility group box 1 (HMGB1) mRNA in the lung tissue. The combined exposure to 50 mg/kg NP and OVA synergistically increased HMGB1 protein expression in the lung tissues. 25 mg/kg NP-OVA co-exposure antagonized the increased nuclear factor-κB (NF-κB) protein expression in the lung tissues. There was a positive correlation between NP content and HMGB1 protein expression in the lung tissue of asthmatic pups ($r = 0.602$, $P < 0.001$). In conclusion, gestational and lactational exposure to 100 mg/kg NP in maternal rats exacerbated airway inflammation in OVA-induced asthmatic pups, and there is an interactive effect between NP and OVA. When the perinatal rats were exposed to 100 mg/kg NP, the levels of HMGB1 and NF-κB in the lung tissues of OVA-induced asthmatic pups were increased.

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1. Introduction

There has been a worldwide increase in the prevalence and mortality of bronchial asthma. The Global Initiative for Asthma (GINA) 2018 Report states that asthma affects 1%–18% of people in different countries around the world (GINA. 2018), and China is currently one of those with the highest rates of asthma deaths. In our previous study, we determined that the prevalence of adult asthma and related symptoms in Zunyi, Guizhou Province reached 13.1% (Jie et al., 2013). Asthma is a complex disease caused by genetic and environmental effects (Méndez-Enríquez and Hallgren, 2019). Among the many factors that affect asthma, scientists highly doubt the role of environmental factors, especially xenoestrogens (environmental estrogens that are endocrine disruptors). Nonylphenol (NP) is a typical representative of an estrogen-like chemical found in the environment as a result of pollution (Colerangle and Roy. 1996). Because NP possesses both hydrophilic and hydrophobic properties, it is widely used in many industries as an emulsifier for non-polar substances such as oil and grease to manufacture various items such as plastics, detergents, personal hygiene products, paints, pesticides, non-ionic surfactants, and stabilizers (Gülgün et al. 2003; Kristine et al., 2005; Xu et al., 2013). It can be discharged into water in the environment via sewage, and environmental degradation is difficult because of its high lipid solubility and bioaccumulation (Willeke et al., 2019). NP mainly enters organisms when they ingest contaminated water and food, and can be detected in blood, milk, and fat (Lu and Gan, 2014; Jacobson et al., 2016; Sise and Uguz, 2017). Current studies on NP focus on its effects on reproduction (Tanaka et al., 2019), the endocrine system (Jie et al., 2019c; Jie et al., 2018), immunity (Jie et al., 2010), the nervous system (Jie et al., 2019a, 2019b), and other aspects. However, there have been no published reports on the accumulation of NP in the lung, nor clear reports describing the effects of NP on bronchial asthma.

Our previous study proved that NP is an immunotoxin because exposure to NP during pregnancy had an inhibitory effect on the immune function of male pups (Jie et al., 2010). This result laid a foundation for the current study to further explore the association between NP exposure and asthma attack, as well as a possible mechanism. Pregnancy and lactation are critical periods for the development of the immune and respiratory systems (Sunyer et al., 2010). Bisphenol A (BPA), an environmental estrogen, has been proven to increase the degree of asthma attack in postnatal rats after oral exposure during the maternal perinatal period (Xu et al., 2016). Koike et al. found that diisononyl phthalate promoted the degranulation of mastocytes and the inflammatory response of eosinophils, and determined that the developing immune system might be the target organ for diisononyl phthalate (Koike et al., 2010). In addition, high-mobility group box 1 (HMGB1), nuclear factor-κB (NF-κB), and estrogen receptor β (ER-β) levels increased in airway inflammation in animals (Imbalzano et al., 2017), as well as airway hyperresponsiveness and TGF-β1 level (Zhang et al., 2018). However, the effect of perinatal exposure to NP on allergic asthma attacks is still unknown. Therefore, this study further investigated the intensity of asthma attacks in rat pups exposed to allergens, and the possible mechanism of NP influence on asthma attacks in children.

Allergic asthma is characterized by airway hyperresponsiveness, inflammation, and cell infiltration dominated by eosinophils (Wills-Karp, 1999). Based on our previous study, here, we established an asthma model in rat pups through ovalbumin (OVA) sensitization, and investigated the effect of perinatal exposure to NP on airway inflammation in OVA-induced asthmatic pups. Does NP accumulate in the lung tissues of the asthmatic rat pups? Does NP reduce or aggravate the occurrence of asthma in pups? Is its toxic effect caused by the estrogen-like effect of NP (ER-β)? This study is of great scientific significance for the prevention and control of NP pollution and health protection for those who are sensitive.

2. Materials and methods

2.1. Reagents

Nonylphenol (purity ≥ 98%) was purchased from the Shandong Xiya Chemical Industry co. LTD (Shandong, China). Estradiol (E_2) (purity ≥ 96.3%) was purchased from National Institutes For Food and Drug Control (Beijing, China). Ovalbumin (OVA, grade V) was purchased from Sigma (St. Louis, MO, USA). Rat IL-4 ELISA Kit, rat IFN-γ ELISA Kit, rat OVA-sIgG1 ELISA Kit and rat OVA-sIgE ELISA Kit were all purchased from Shanghai Jianglai Biotechnology Co., Ltd. (Shanghai, China). All the antibodies used in Western blot were all obtained from Abcam (Cambridge, MA, USA). All other chemicals were commercially available.

2.2. Animals

Sixty female and 15 male Sprague-Dawley (SD) rats aged 8 weeks (clean grade) were obtained from Animal Center of the Third Military Medical University (License No : SCXK2012-0005, Chongqing, China). The animals were raised at 22 ± 3 °C with humidity of 50–60% under artificial illumination (12 h light/12 h dark). They were fed with standard feed, and water was freely allowed. All methods were performed in accordance with guidelines and regulations of the Zunyi Medical University (No: 2015-2-011).

2.3. Groupings and perinatal exposure

After one week of acclimation, the SD rats were mated. On the next day, a vaginal smear was examined under an optical microscope to determine whether the rats were pregnant. The day that sperm were observed was recorded as day 0.

After the 1-week mating experiment, the pregnant dams were housed individually and were randomly assigned to an exposure condition. The 50 dams were assigned to five groups; there were 8–10 pregnant female rats in each group. Only the male pups were used for all the tests. The first group was orally administered with corn oil, served as a negative control; the second, third and fourth groups received gavage with NP at dose levels of 25 mg/kg/day (low dose), 50 mg/kg/day (middle dose), 100 mg/kg/day (high dose); the fifth group were gavaged with E_2 at dose level of 30 μg/kg E_2, served as a positive control, respectively. During the perinatal period (from gestational day 7 to postnatal day 21), the pregnant rats received drugs by gavage once per day at the same time, at 5 mL/kg/day according to the weight of the pregnant rats.

2.4. Dose setting and reagent preparation

In our early study, exposure of pregnant rats to NP (80–200 mg/kg/day) affected the development of the immune organs in pups (Jie et al., 2010). Combined with 1/20–1/5 half lethal dose (LD_{50}: 1231 mg/kg), i.e., the high NP dose given to female rats was 100 mg/kg, and the minimum interval between dose groups was not less than 2 times (Wang, 2012), the female rats in this experiment received NP doses of 25 mg/kg, 50 mg/kg, or 100 mg/kg. Five groups were set in this experiment: negative control group (corn oil), 25 mg/kg NP group, 50 mg/kg NP group, 100 mg/kg NP group, and positive control group (30 μg/kg E_2). The NP purity was 98%, and that of E_2 was 96.3%. For stock preparation, 1.276 g, 2.551 g, and

5.102 g NP and 1.558 mg E_2 were accurately weighed, added to corn oil (Luhua, Shandong, China), and mixed in a 250- mL volumetric flask to prepare NP stock solutions with concentrations of 5 mg/mL, 10 mg/mL and 20 mg/mL, and an E_2 stock solution with a concentration of 6 μg/mL. All solutions were stored in a refrigerator at 4 °C for future use.

2.5. Establishment of the asthma model

Pups were divided into 6 groups on PND 22: control group (group C), OVA group (model group), 25 mg/kg NP + OVA, 50 mg/kg NP + OVA, 100 mg/kg NP + OVA, and 30 μg/kg E_2+OVA. There were 8−10 male pups in each group that were intraperitoneally injected with 1 mL of sensitizer (1 mg OVA and 2 mg alumina hydroxide mixed with 1 mL phosphate-buffered saline (PBS)) on PND 22, PND 29, and PND 36. On PND 43, the sensitized pups were stimulated by inhalation of 2% OVA (Gong, 2009) for 30 min in an atomization chamber, which was repeated 10 times, 1 time for every 3 days. The control group (group C) was sensitized and stimulated by PBS (Fig. 1).

After the last time of atomized stimulation (on PND 73), the pups fasted overnight and were anesthetized through administering 20% ethyl carbamate (0.5 mL/100 g) via intraperitoneal injection. 5 mL blood was obtained from the abdominal aorta of each pup, then it was centrifuged at 3000 r/minute for 10 min and meanwhile the supernatant was collected and stored at −80 °C for subsequent analysis (Jie et al., 2019a, 2019b).

2.6. Body weight, general appearance, and observation of the lung tissue

The pups were regularly weighed. After atomization, the rats were fasted for 24 h, and then intraperitoneally injected with 20% ethyl carbamate (0.5 mL/100 g) for anesthesia so that their general appearance could be photographed. The thoracic cavity was opened and photographed to obtain a macroscopic view of the lung tissue (Jie et al., 2019, 2019b).

2.7. Quantitation of NP by high-performance liquid chromatography (HPLC)

NP concentration in lung tissue was detected using the HPLC technique described by Jie et al. (2017). The peak time of standard sample of NP is determined as 3.8 min (1) Processing before injection: 0.05 g lung tissue was removed from the middle and lower lobe of the right lung, placed into a stoppered glass tube containing 2 mL of a mixture of n-hexane and ether (v-hexane: v-ether = 7:3), centrifuged to collect the supernatant in the glass tube, dried in a water bath at 40 °C, dissolved in 0.5 mL acetonitrile, filtered with a 0.22-μm organic filter, and injected for detection.

(2) Chromatographic conditions: column (ECLIPSE XD8-C18, 150 mm × 460 mm, 5 μm, Agilent, USA), mobile phase (mixture of acetonitrile and 0.1% glacial acetic acid, volume ratio = 85:15), temperature 40 °C, flow rate 1 mL/min, injection volume 10 μL, excitation wavelength 275 nm, emission wavelength 312 nm, fluorescence detector (FLD) (Jie et al., 2017).

2.8. Histopathological changes in the lung

2.8.1. Histological observation

The right upper lobe of the lung was fixed for 24 h in an Eppendorf tube filled with 10% formaldehyde solution. Then, the tissue was dehydrated, cleared, immersed in wax, embedded, sliced, pasted, dried, dewaxed with xylene, infiltrated with gradient alcohol (100% , 95% , 80% , 70%), stained with hematoxylin, washed with water, differentiated with alcohol containing 1% HCl, rinsed, washed with water, dyed with 0.5% eosin, dehydrated with gradient alcohol (70%, 80%, 95%, 100%), cleared with xylene, and sealed with neutral gum. The morphological changes in the lung tissue were observed under an optical microscope (Niklas et al., 2019).

2.8.2. Observation of ultrastructure

Approximately 1 mm^3 of tissue was quickly cut off from the tip of the right lung with a blade, fixed in 2.5% neutral glutaraldehyde,

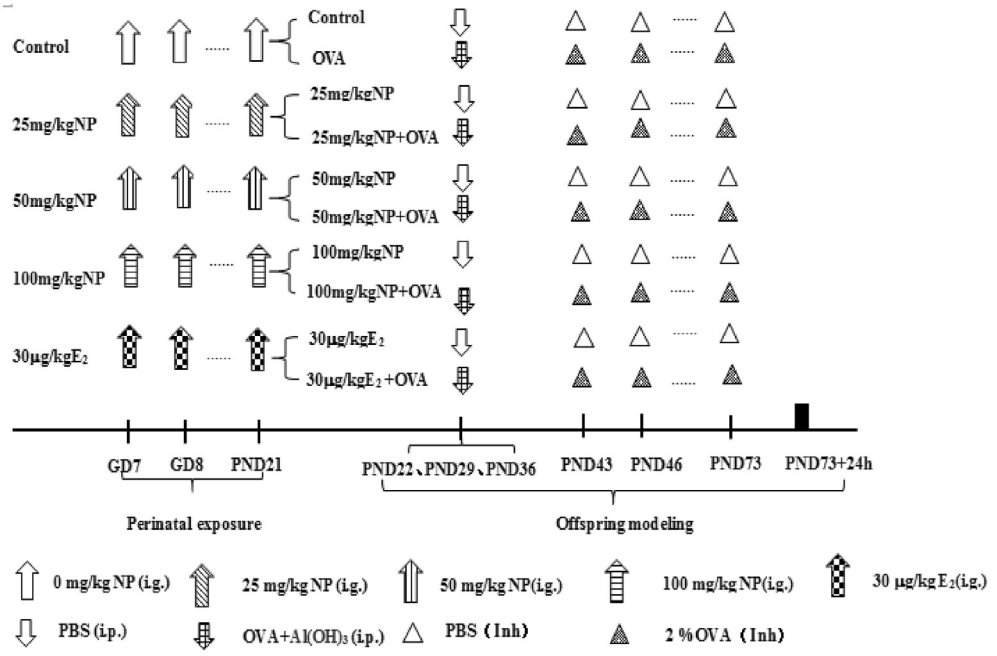

Fig. 1. Flow chart of the influence of perinatal exposure to NP on asthma attack in male pups. i.p.: Intraperitoneal injection. i.g.: Intragastric gavage. Inh: Atomized inhalation. PND: Postnatal day. GD: Gestational day. h: Hour.

rinsed with 0.1 mL PBS, fixed with 1% osmium, and rinsed with 0.1 mL PBS.

Ethanol (30% , 50% , 70% , 90% , 100%) gradient dehydration consisted of starting with a 100% ethanol:100% acetone (1:1) solution to bathe the tissue samples and finishing with 100% acetone (molecular sieve), followed by resin penetration, embedding, fixation, semi-thin section location, ultra-thin sectioning, uranium-lead double staining, and then transmission electron microscopy observation (Sirasha et al., 2019).

2.9. Cell counting in broncho-alveolar lavage fluid (BALF)

2.9.1. Cell counting

A gavage needle was inserted into the trachea through the oral cavity, and the trachea and the right lung were ligated. The left lung was injected with 3 mL normal saline, rinsed for approximately 1 min, and the lavage solution was recovered (7.2−8.5 mL BALF from each rat, with a recovery rate of 80%). Next, BALF mixed solution was used to count the total number of white blood cells on the Neubauer hemocytometer (Jie et al., 2019a, 2019b). Total number of white blood cells = (number of white blood cells in 4 squares/20) × 10^9/L. Subsequently, the recovered BALF was centrifuged at 1500 rpm for 10 min at 4 °C to separate the supernatant and precipitate. The precipitate was collected and used to perform smears and cell counting after staining. The supernatant was stored at −80 °C for subsequent analysis.

2.9.2. Distribution of inflammatory cells (monocytes, lymphocytes, neutrophils and eosinophils) in a BALF smear

A BALF smear was prepared, dried, placed on a horizontal operating table, and Liu A Solution and Liu B Solution were added to the stain (Yu-Tzu et al., 2018). After washing and drying, the smear composed of inflammatory cells was observed with an optical microscope.

2.10. Measurement of cytokines in BALF and OVA-specific serum IgE and IgG1 with ELISA

The levels of interleukin-4 (IL-4) and γ-interferon (IFN-γ) in the BALF supernatant were detected by respective Rat Enzyme Linked Immunosorbent Assay (ELISA) Kits. Ovalbumin-specific serum immunoglobulin G1 (OVA-sIgG1) and immunoglobulin E (OVA-sIgE) antibodies were measured with a mouse OVA-IgE ELISA kit and a mouse OVA-IgG1 ELISA kit, respectively. The experimental procedures were performed strictly according to the instruction manuals. iMark microplate reader (BioRad, Hercules, California, USA) was set to 450 nm wavelength, and sequentially detected the absorbance OD value, as previously described (Shilovskiy et al., 2019).

2.11. Detection of the expression levels of inflammatory genes in lung tissues by RT-PCR

A real-time reverse transcriptase−polymerase chain reaction (RT-PCR) was performed in a thermo cycler (c1000 Thermal Cycler, Bio-Rad, Hercules, California, USA) to detect the expression levels of HMGB1, NF-κB and TGF-β1 mRNA in lung tissue samples. In brief, RNA was extracted from lung tissue using the TRIzol method. The RNA concentration of 1 μl RNA was measured using a Nano-Photometer, and RNA with OD260/OD280 of 1.8−2.0 was taken as a qualified sample. RNA was reverse-transcribed into cDNA, using GAPDH as an internal reference. The mRNA expression of each gene was calculated by a relative quantitative method (Wang et al., 2013), and the sequence of the primer is shown in Table 1.

Table 1
Primer sequences.

ID	Gene name	Sequences (5′−3′)		ProdSize
25459	HMGB1	Forward	ACAACACTGCTGCGGATGACAAG	177
		Reverse	CCTCCTCGTCGTCTTCCTCTTCC	
81736	NF-κB	Forward	GAAACCCTTTCTCTACTACCCC	262
		Reverse	GATGCCTGTGTTGGATTTAGTG	
59086	TGF-β1	Forward	CTTCAATACGTCAGACATTCGG	91
		Reverse	CACAGTTGACTTGAATCTCTGC	
24383	GAPDH	Forward	GACATGCCGCCTGGAGAAAC	92
		Reverse	AGCCCAGGATGCCCTTTAGT	

2.12. Detection of the expression levels of related inflammatory proteins (HMGB1, NF-κBp65) and estrogen receptor β protein (ER β) in lung tissue by Western blot

Western blot was performed according to standard procedures. Briefly, for tissue preparation, 50 mg lung tissue was cut into tiny pieces with iris scissors and homogenized on ice. The protein concentration was measured by the bicinchoninic acid (BCA) method at 562 nm using a microplate reader. After protein denaturation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel preparation, electrophoresis, electric transfer to membranes, sealing, incubation with primary antibody and then secondary antibody, and exposure were performed. The experiments were repeated three times. The average gray value of each band was calculated with ImageJ software, and the relative protein expression levels were determined (Luo et al., 2017).

2.13. Statistical methods

The measurement data are expressed as the mean ± standard deviation (SD). The total count of white blood cells and distribution of inflammatory cells in BALF is expressed as the mean ± standard error (SE). SPSS 18.0 software was used for data analysis. Univariate analysis of variance (ANOVA) was used for inter-group comparison, and least significant difference (LSD) was used for pairwise comparison. Spearman correlation analysis was performed with a significance level of $\alpha = 0.05$.

3. Results

3.1. Changes in the general appearance and body weight of asthmatic pups

3.1.1. General appearance

With the increase in perinatal exposure to NP, the body size of the asthmatic pups gradually increased, and that of the 25 mg/kg NP group was significantly smaller than that of the OVA group (Fig. 2).

3.1.2. Weight changes

At the end of the intraperitoneal OVA injection period (on PND43), there was a significant difference in body weight among the treatment groups ($F = 35.327$, $P < 0.001$). The body weight of the 25 mg/kg NP group was significantly lower than that of the control ($P < 0.001$), OVA group ($P < 0.001$), 50 mg/kg NP + OVA ($P < 0.001$) and 100 mg/kg NP group ($P = 0.007$). After the completion of all OVA atomization treatments (on PND 73), there was a significant difference in body weight among the treatment groups ($F = 2.882$, $P = 0.022$). The body weight of the 25 mg/kg NP group was significantly lower than that of the 100 mg/kg NP group ($P = 0.039$, Fig. 3).

Intraperitoneal injection of 100 mg/kg NP + OVA had an

Fig. 2. Changes in the general appearance of perinatal NP-exposed pups.

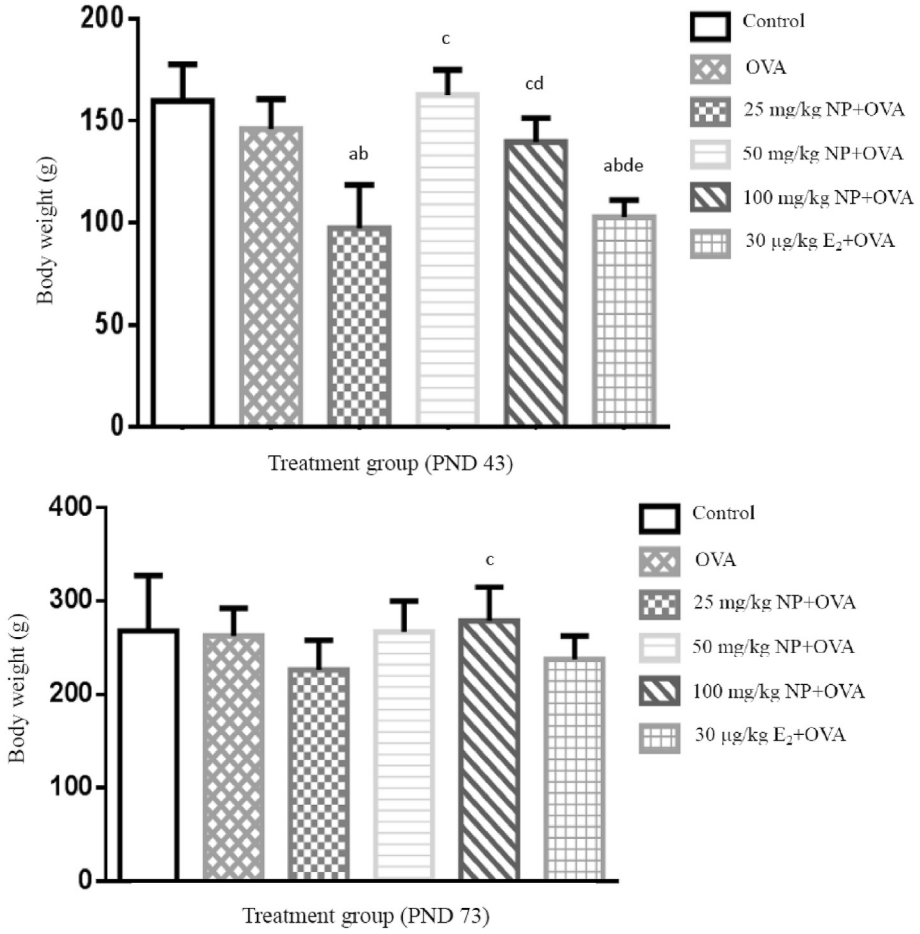

Fig. 3. Influence of perinatal exposure to NP on the weight of the pups (mean ± SD, n = 8). [a] Compared with the control group, $P < 0.05$; [b] Compared with the OVA group, $P < 0.05$; [c] Compared with the 25 mg/kg NP + OVA group, $P < 0.05$; [d] Compared with the 50 mg/kg NP + OVA group, $P < 0.05$; [e] Compared with the 100 mg/kg NP + OVA group, $P < 0.05$.

interactive effect on the body weight of PND 43 pups ($F = 12.675$, $P = 0.001$), and the 100 mg/kg NP + OVA exposure antagonized the weight gain on PND 43 pups. There was an interaction between the effects of 25 mg/kg NP combined with atomized OVA exposure on the body weight of PND 73 pups ($F = 5.497$, $P = 0.025$), and 25 mg/kg NP + OVA exposure was associated with their weight loss.

3.2. Behavior of asthmatic pups

After being sensitized by OVA, the behavior of the control group was normal. The respiratory rhythm of the OVA group was deepened and accelerated, and those of the 25 mg/kg NP group, 50 mg/kg NP group, and 30 μg/kg E_2 group were slightly deepened, with occasional scratching of the ears and cheeks. The respiratory rhythm of the 100 m/kg NP group was deepened and accelerated, and the breathing sound of the lungs could be heard, with non-stop scratching of the ears and cheeks. The head and upper body were rubbed around in the glass atomization box with significantly high frequency.

3.3. Observation of the lungs of asthmatic pups

The lung color of the control group was not abnormal, showing pink color with a smooth profile. The OVA group exhibited lungs with obvious edema with a rough surface. The lungs of the 25 mg/kg NP group were darker with a smoother surface. The lungs of the 50 mg/kg NP group were darker in color with hemorrhagic points on the surface. The lungs of the 100 mg/kg NP group were significantly loose with edema, and some of the edges were translucent. In the 30 μg/kg E_2 + OVA group, the color was darker and the bottom was slightly edematous (Fig. 4).

3.4. NP content in the lung tissue of asthmatic pups

There was a significant difference in the content of NP in the lung tissues among asthmatic pups ($F = 124.66$, $P < 0.001$). The NP content in the lung tissues of the three NP treatment groups was significantly higher than that in the control group ($P < 0.001$). The content in the lung tissues of the 50 mg/kg NP group ($P < 0.001$) and the 100 mg/kg NP group ($P = 0.004$) was significantly higher than that of the 25 mg/kg NP group (Fig. 5).

It was observed that 100 mg/kg NP and OVA exposure antagonized the increase in NP content in the lung tissues of the pups ($F = 15.576$, $P < 0.001$).

3.5. Histopathological changes of the lungs in asthmatic pups

In the control group, there was no obvious infiltration of inflammatory cells into the lung tissue. The thickness of the smooth muscle layer was normal, and no detached epithelial cells in the lumen were observed. In the OVA group, infiltration of focal lymphocytes, monocytes, and few eosinophils was observed in and around the bronchial wall of the lung tissue. No significant inflammatory cell infiltration was found in the 25 mg/kg NP group. In the 50 mg/kg NP group, infiltration of a small number of monocytes, lymphocytes, and eosinophils was observed in the lung tissue, and a small number of epithelial cells were detached in the lumen. Infiltration of lymphocytes and a small amount of eosinophils were observed in the lung tissues of the 100 mg/kg NP group. The thickness of the smooth muscle layer was significantly increased, inflammatory cells were exuded in the lumen, and a small amount of epithelial cells were detached. In the 30 μg/kg E_2+OVA group, focal lymphocytes were observed in the lung tissue, and the smooth muscle layer was slightly thickened (Fig. 6).

3.6. Observation of the ultrastructure of lung tissue

No abnormalities were observed in type I epithelial cells of the lung tissue in the control group. Osmiophilic lamellar bodies were observed in the cytoplasm of type II epithelial cells, and no obvious abnormalities were observed in the mitochondria. Osmiophilic lamellar bodies were found in the cytoplasm of type II epithelial cells in the lungs of the OVA group. Most lamellar bodies were emptied, and the number of microvilli-like protuberances on the cell surface was reduced. In the 100 mg/kg NP group, type I epithelial cells were swollen. Osmiophilic lamellar bodies were found in the cytoplasm of type II epithelial cells. Most lamellar bodies were emptied, mitochondria were clearly swollen, and some microvilli-like protuberances were found on the cell surface. Osmiophilic lamellar bodies were observed in the cytoplasm of type II epithelial cells in the lung tissues of the 30 μg/kg E_2 group, and the number of microvilli-like protuberances on the cell surface was reduced (Fig. 7).

3.7. Distribution of inflammatory cells in BALF

The perinatal exposure to NP and E_2 resulted in significant differences in the total number of white blood cells ($F = 7.172$, $P < 0.001$), monocytes ($F = 9.905$, $P < 0.001$), lymphocytes ($F = 6.277$, $P < 0.001$), neutrophils ($F = 3.765$, $P = 0.007$), and eosinophils ($F = 4.341$, $P = 0.003$) in BALF of OVA-induced pups. The total counts of white blood cells in the OVA group ($P = 0.020$), 50 mg/kg NP group ($P = 0.025$), 100 mg/kg NP group ($P = 0.036$), and 30 μg/kg E_2 group ($P = 0.034$) were significantly higher than those in the control group. The counts of monocytes in the OVA group ($P = 0.010$), 25 mg/kg NP group ($P = 0.008$), and 50 mg/kg NP group ($P = 0.004$) were significantly higher than those in the control group. There were significantly more lymphocytes in the OVA group ($P = 0.017$) and the 30 μg/kg E_2 group ($P = 0.024$) than in the control group. The counts of eosinophils in the 25 mg/kg NP group ($P = 0.010$), 50 mg/kg NP group ($P = 0.010$), and 100 mg/kg NP group ($P = 0.001$) were significantly lower than those in the OVA group (Fig. 8).

The exposure to the combination of 25 mg/kg NP and OVA had an interactive effect on the total counts of white blood cells ($F = 14.216$, $P = 0.001$), monocytes ($F = 27.950$, $P < 0.001$), lymphocytes ($F = 11.638$, $P = 0.002$), neutrophils ($F = 5.797$, $P = 0.023$), eosinophils ($F = 5.227$, $P = 0.030$), and basophils ($F = 8.063$, $P = 0.008$) in BALF. It was observed that 25 mg/kg NP + OVA exposure antagonized the increase in white blood cell count, monocytes, lymphocytes, neutrophils, and eosinophils in BALF; 25 mg/kg NP + OVA exposure antagonized the decrease in basophils in BALF. Additionally, 100 mg/kg NP + OVA exposure had an interactive effect on monocytes ($F = 7.251$, $P = 0.012$) and eosinophils ($F = 9.504$, $P = 0.005$) in BALF of pups, but 100 mg/kg NP + OVA exposure antagonized the increase in monocytes and eosinophils in BALF.

3.8. Detection of levels of OVA-sIgG1 and OVA-sIgE in serum, and levels of IL-4 and IFN-γ in the supernatant of BALF

The levels of IL-4 in BALF ($P = 0.042$) and OVA-sIgE ($P = 0.005$) in serum of the OVA group were significantly higher than those in the control group. The level of IFN-γ in BALF of the OVA group was significantly lower than that in the control group ($P < 0.001$). There was no significant difference in serum OVA-sIgG1 between the OVA group and the control group (Table 2).

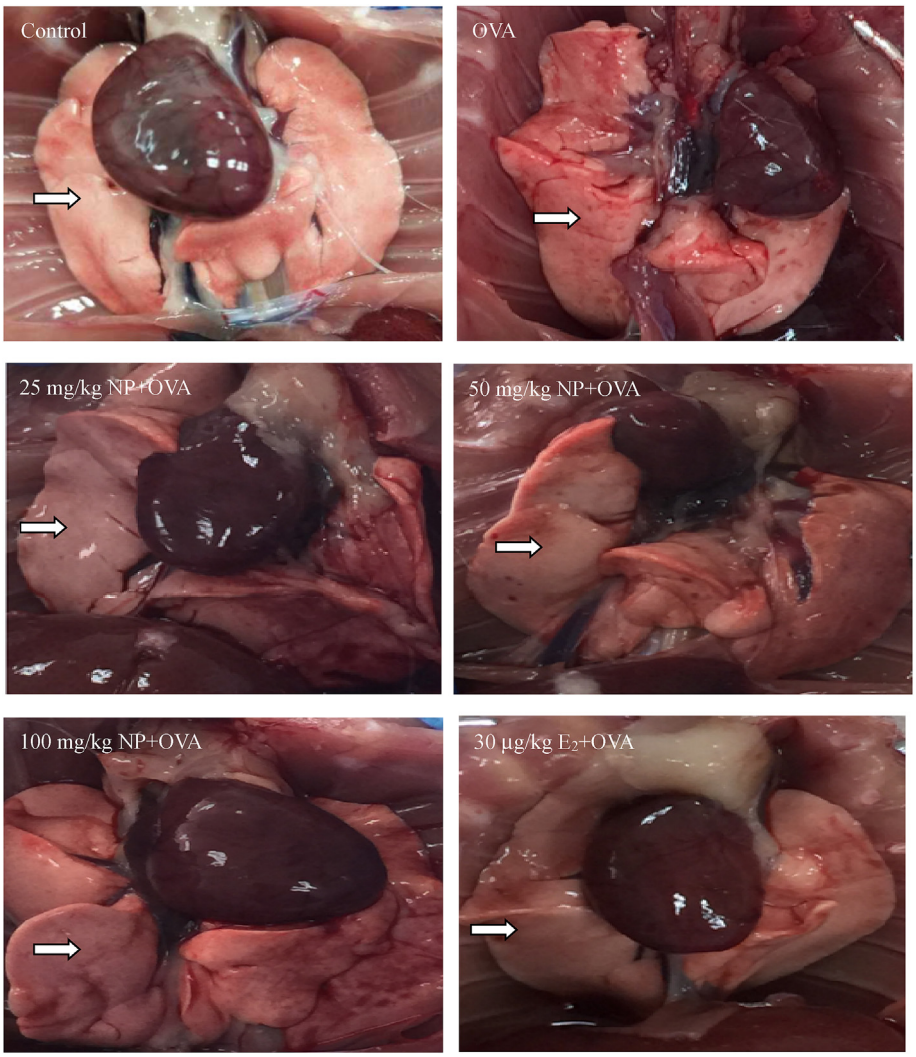

Fig. 4. Changes in the lung tissues of perinatal NP-exposed pups.: Lung.

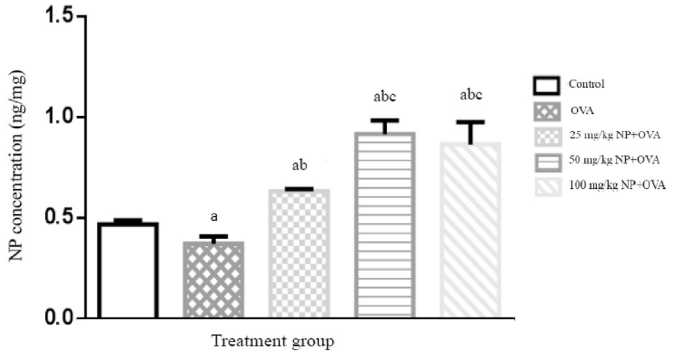

Fig. 5. Effect of perinatal NP exposure on NP content in the lung tissue of pups (mean ± SD, n = 8).[a] Compared with the control group, $P < 0.05$; [b] Compared with the OVA group, $P < 0.05$; [c] Compared with the 25 mg/kg NP + OVA group, $P < 0.05$.

3.9. Expression of the inflammatory factor gene (HMGB1 and NF-κB mRNA) in lung tissue

There were significant differences in the relative expression of HMGB1 mRNA (F = 3.350, P = 0.018) and NF-κB mRNA (F = 11.637, P < 0.001) in the lung tissue of OVA-induced pups, but no significant difference in TGF-β1 mRNA (F = 2.226, P = 0.082). The relative expression of HMGB1 mRNA in the lung tissue of the 100 mg/kg NP group was significantly higher than that of the 30 μg/kg E_2+OVA group (P = 0.012). The relative expression of NF-κB mRNA in the lung tissue of the 100 mg/kg NP group (P < 0.001) was significantly higher than that of 25 mg/kg NP and 50 mg/kg NP groups (Fig. 9).

There was an interaction between 25 mg/kg NP + OVA exposure and NF-κB mRNA expression in the lung tissues of pups (F = 6.111, P = 0.020), and 25 mg/kg NP-OVA co-exposure synergistically decreased NF-κB mRNA expression in the lung tissues. A similar relationship was observed between 50 mg/kg NP + OVA exposure and the expression of HMGB1 mRNA in the lung tissue (F = 4.748, P = 0.037), with 50 mg/kg NP + OVA exposure antagonizing the increased expression of HMGB1 mRNA in the lung tissue.

3.10. Expression of relevant inflammatory proteins and ER-β protein in the lung tissue

The expression of HMGB1 (F = 6.560, P < 0.001), NF-κBp65 (F = 3.131, P = 0.022), and ER-β protein (F = 6.870, P < 0.001) in the OVA-induced pups was significantly different when perinatal rats were exposed to NP and E_2. The relative expression of HMGB1

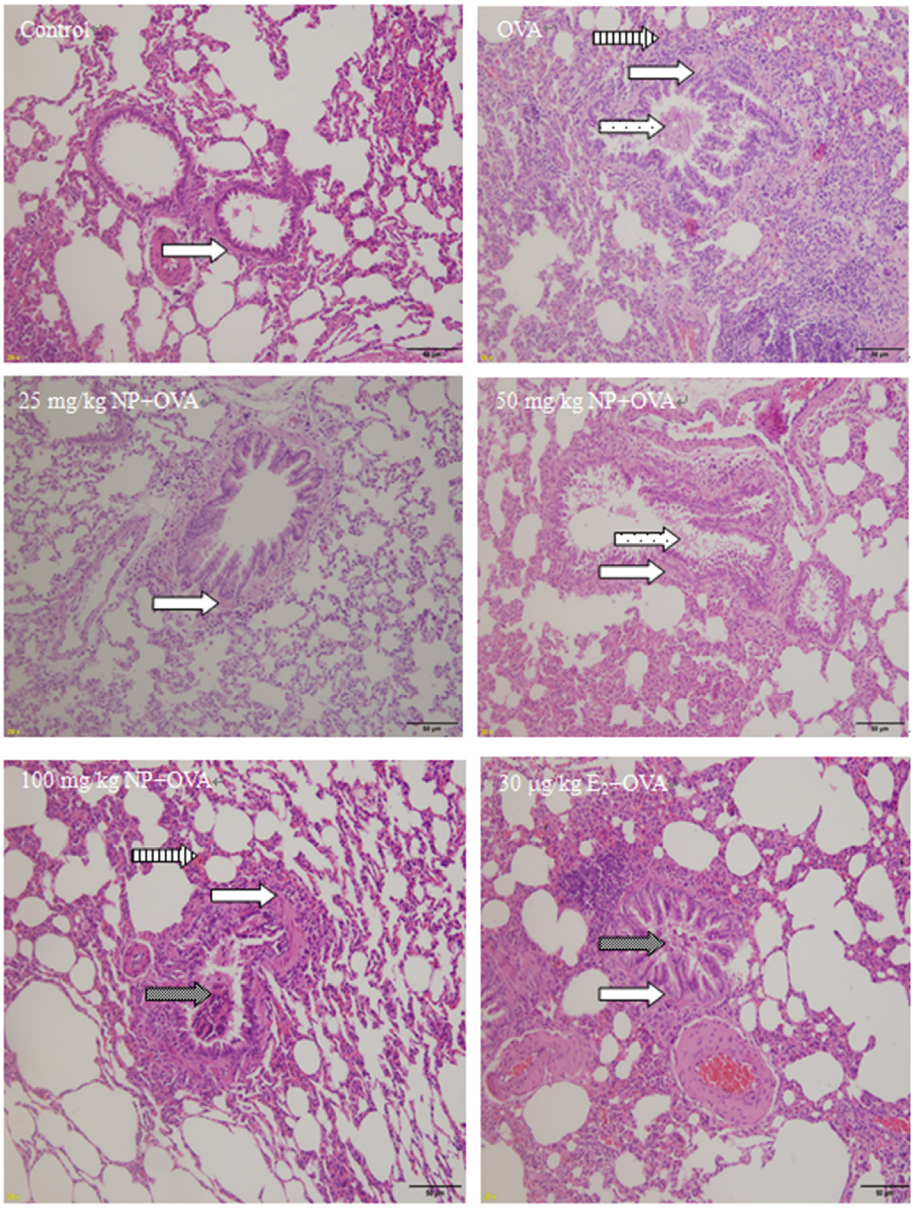

Fig. 6. Effects of perinatal exposure to NP on the pathological changes in the lungs of pups (Scale bar, 50 μm).: Smooth muscle ; : Contents were exuded in the lumen;: Inflammatory cells were exuded in the lumen;: Eosinophils.

protein in the OVA group (P = 0.046), 50 mg/kg NP group (P = 0.004), and 100 mg/kg NP group (P = 0.024) was higher than that in the control group. The expression of NF-κBp65 protein in the OVA group (P = 0.048), 100 mg/kg NP group (P = 0.007), and 30 μg/kg E_2 group (P = 0.002) was higher than that in the control group. The expression of ER-β protein in the 30 μg/kg E_2+OVA group was significantly higher than that in the control group (P < 0.001), OVA group (P < 0.001), 25 mg/kg NP + OVA group (P < 0.001), 50 mg/kg NP + OVA group (P < 0.001), 100 mg/kg NP + OVA group (P = 0.001, Fig. 10).

There was an interaction between the influence of 25 mg/kg NP + OVA exposure and NF-κB protein expression in the lung tissues of the pups (F = 4.488, P = 0.044), and 25 mg/kg NP + OVA exposure antagonized the increased NF-κB protein expression in the lung tissues. There was an interaction between 50 mg/kg NP + OVA exposure and HMGB1 protein expression in the lung tissues of pups (F = 14.238, P = 0.001), and exposure to 50 mg/kg NP combined with OVA antagonized the increased expression of HMGB1 mRNA in the lung tissue.

3.11. Correlation between content of NP and expression of relevant inflammatory genes and proteins in the lung tissues

There was a positive correlation between NP content and HMGB1 relative protein expression in the lung tissue of asthmatic pups ($r = 0.602$, P < 0.001), but no correlations were found between NP content and NF-κBp65 ($r = 0.264$, P = 0.158) and ER-β relative protein ($r = 0.059$, P = 0.759) expressions in the lung tissue. No correlations were also revealed between NP content and HMGB1 mRNA ($r = 0.235$, P = 0.133), NF-κB mRNA ($r = 0.089$, P = 0.574), and TGF-β1 mRNA ($r = 0.059$, P = 0.759) relative expressions (Fig. 11).

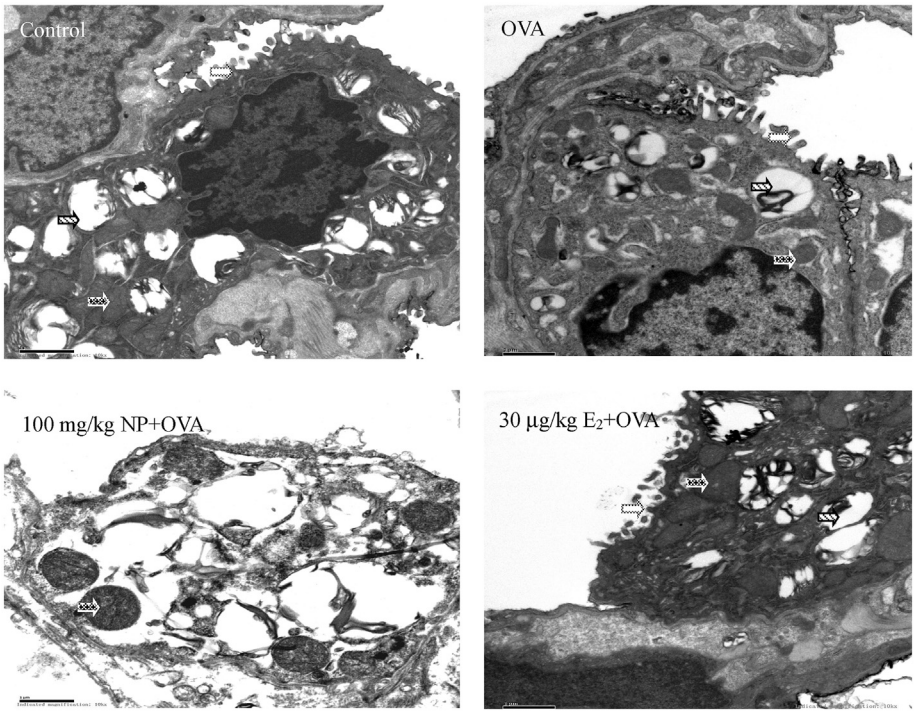

Fig. 7. Effects of perinatal exposure to NP on the ultrastructure of lung in pups (Scale bar, 1 μm).: Osmiophilic lamellar bodies.: Mitochondria.: Microvillus.

4. Discussion

In this study, OVA was used to establish an allergic bronchial asthma model, and the results are as follows. (1) Behavioral performance of the asthma model group (OVA) was basically consistent with typical asthma attack symptoms (Jie et al., 2019c). (2) There were significantly more lymphocytes, neutrophils, and eosinophils in the bronchoalveolar lavage fluid of the OVA groups as compared to those in the control group. (3) Levels of OVA-sIgE in the model group were significantly higher than those in the control group, indicating that OVA caused hypersensitivity as an allergen. Serum total IgE has been used as a marker for asthma diagnosis (Hatcher et al., 2013), and the involvement of immunoglobulin IgE is an important pathogenic factor in the pathogenesis of asthma (Hayden, 2007). The process of mastocytes releasing IgE in response to antigens is considered to be a major event in immediate hypersensitivity reactions, such as generalized anaphylaxis (Donald, 2012). Elevated IgE is associated with the development of asthma (Froidure et al., 2016). (4) The level of IL-4 in the model group was significantly higher than that in the control group, and the level of IFN-γ in the model group was significantly lower than that in the control group, which was consistent with Th1/Th2 imbalance, a classic pathogenesis process of asthma (Wisniewski and Borish, 2011). (5) Histology showed that the smooth muscle layer in the OVA group was slightly thicker than that in the control group, with greater lumen contents. The ultrastructure showed that most lamellar bodies were emptied in the OVA group as compared with the control group. The results of this study indicated that OVA, as an allergen, had been successfully used to establish a model of allergic bronchial asthma, providing a theoretical basis for exploring the effect of perinatal exposure to NP on airway inflammation in asthmatic rat pups induced by OVA.

In the current study, focal lymphocytes and monocytes with very little eosinophil infiltration were observed in and around the bronchial wall in the OVA group. In the 100 mg/kg NP + OVA group, the thickness of smooth muscle layer was significantly increased, inflammatory cells were exuded in the lumen. Some remote mediators act directly on the airway smooth muscle (ASM) through blood circulation, or indirectly regulate the level of choline activation of ASM through the nervous system. In addition to the paracrine regulation mode, ASM is also affected by an endocrine regulation mode (Bossé, 2014). Proliferation of ASM cells driven by persistent inflammation is a signature of asthma remodeling (Nilesh et al., 2018). It indicates that exposure to 100 mg/kg NP during the perinatal period can thicken the smooth muscle and increase the inflammatory exudation in the asthmatic pups, which aggravates the incidence of asthma in the pups. In the current study, asthma was mainly manifested by lymphocytes, which are different from conventional eosinophils, and might be related to factors such as long modeling time and the large individual differences in pups.

Osmiophilic lamellar bodies were observed in the cytoplasm of type II epithelial cells in the lung tissues of the pups in the OVA group. Compared with the control group, most lamellar bodies were emptied, and there were less microvilli on the cell surface. Type I epithelial cells in the 100 mg/kg NP + OVA group were swollen. Osmiophilic lamellar bodies were observed in the cytoplasm of type II epithelial cells. Most of the lamellar bodies were emptied, the mitochondria were slightly swollen, and there were significantly less microvilli-like protrusions on the epithelial cell surface. A previous study indicated that asthma is related to abnormal mitochondrial metabolism, and mitochondrial dysfunction in airway epithelial cells may trigger airway remodeling to induce asthma-like features or increase asthma severity (Bhatraju and Agrawal, 2017). Osmiophilic lamellar bodies could be observed in the cytoplasm of type II epithelial cells in the lung tissues of pups sensitized by OVA, and most of the lamellar bodies were emptied, indicating that OVA could reduce the surface tension of alveoli and damage lung function through sensitization. Perinatal exposure to 100 mg/kg NP in perinatal rats aggravated the

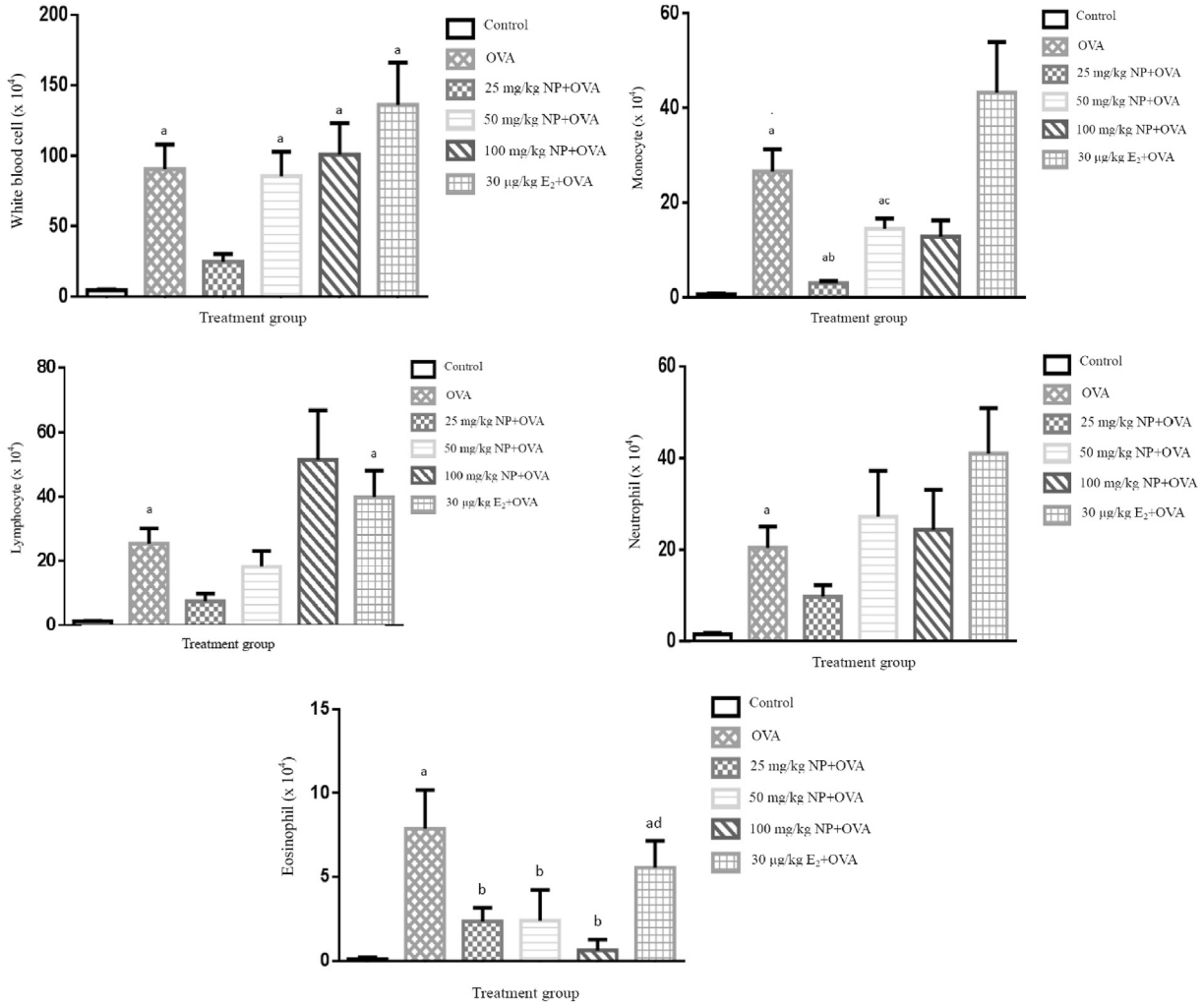

Fig. 8. Effects of perinatal exposure to NP on the total count of white blood cells and distribution of inflammatory cells in lung BALF of pups (mean ± SD, n = 8). [a] Compared with the control group, $P < 0.05$; [b] Compared with the OVA group, $P < 0.05$; [c] Compared with the 25 mg/kg NP + OVA group, $P < 0.05$; [d] Compared with the 100 mg/kg NP + OVA group $P < 0.05$.

Table 2
Comparison of serum OVA-sIgG1, OVA-sIgE, and IL-4 and IFN-γ in BALF between the OVA and control group (mean ± SD, n = 8).

Group	OVA-sIgG1 (ng/mL)	OVA-sIgE (ng/mL)	IL-4 (pg/mL)	IFN-γ (pg/mL)
Control	84.37 ± 25.30	0.34 ± 0.11	27.43 ± 3.76	445.55 ± 37.70
OVA	84.60 ± 11.47	2.44 ± 1.45	31.48 ± 3.47	360.74 ± 25.86
t value	−0.023	−4.086	−2.236	5.247
P value	0.982	0.005*	0.042*	<0.001*

Note: *Compared with the control group $P < 0.05$.

incidence of asthma in asthmatic pups by slightly swelling of mitochondria in the lung tissue.

The above results suggest that exposing perinatal rats to 100 mg/kg NP can aggravate asthma, and therefore, it was conjectured what effect 25 mg/kg NP exposure would have on asthma. HMGB1 regulates airway inflammation in asthma (Li et al., 2018), which is upregulated in the asthmatic airway, and enhances airway smooth muscle contraction through TLR4 (Leonarda et al., 2017). Contraction of the smooth muscle is associated with airway hyperresponsiveness. The expression of the HMGB1 gene and protein in the lung tissues of OVA-induced pups with maternal perinatal exposure to 100 mg/kg NP was the highest. Perinatal exposure to 25 mg/kg NP led to the lowest expression of the HMGB1 gene and protein in the lung tissues of OVA-induced pups, while exposure to 30 g/kg E_2 resulted in the highest expression of the NF-κB gene and protein in the lung tissues. The expression of the NF-κB gene and protein was the highest in the 100 mg/kg NP + OVA group. There was no significant difference in the expression of the TGF-β1 gene between two NP groups, but the expression of the TGF-β1 gene in the 100 mg/kg NP group and the 30 μg/kg E_2 group was significantly higher than that in the other groups. This indicated that perinatal exposure to 100 mg/kg NP aggravated airway inflammation in OVA-induced asthmatic pups and increased HMGB1 level in the lung tissues. The expression of ER-β protein was the highest in the lung tissues of the OVA-induced pups, which was not significantly different from that of the NP

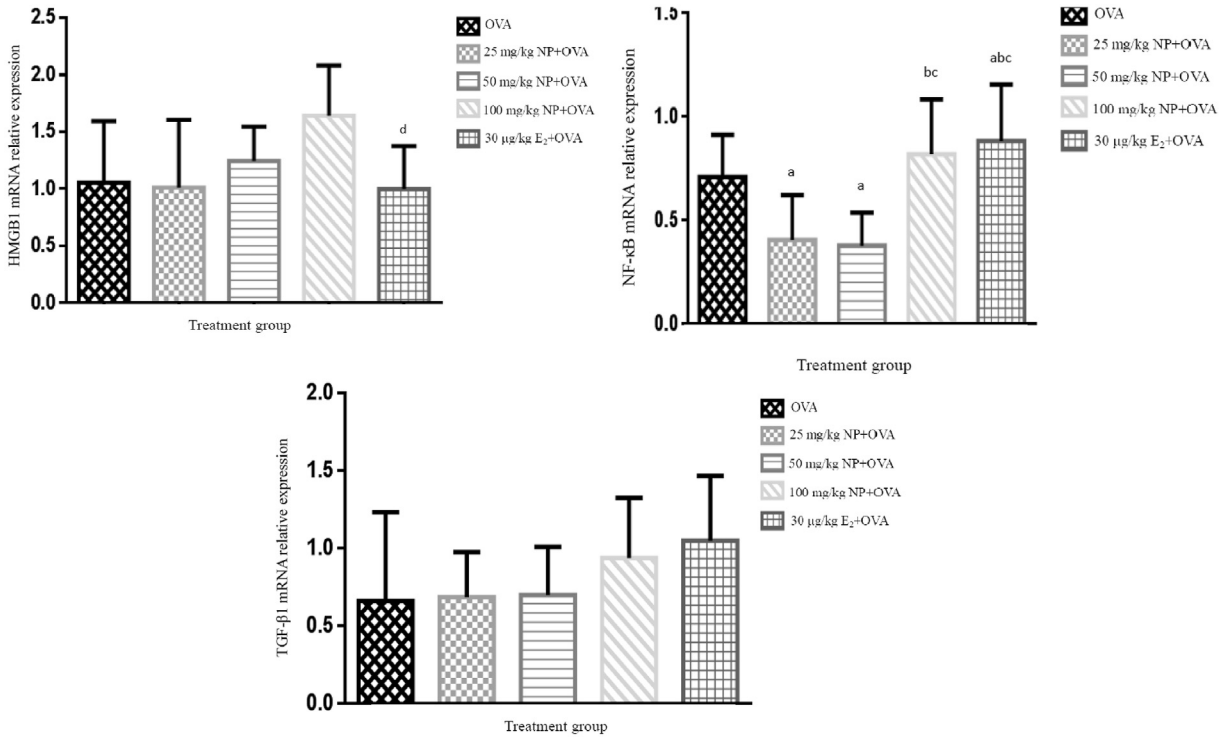

Fig. 9. Expression level of relevant inflammatory factor genes in the lung tissue after perinatal exposure to NP (mean ± SD, n = 8). [a] Compared with the OVA group, $P < 0.05$; [b] Compared with the 25 mg/kg NP + OVA group, $P < 0.05$; [c] Compared with the 50 mg/kg NP + OVA group $P < 0.05$; [d] Compared with the 100 mg/kg NP + OVA group, $P < 0.05$.

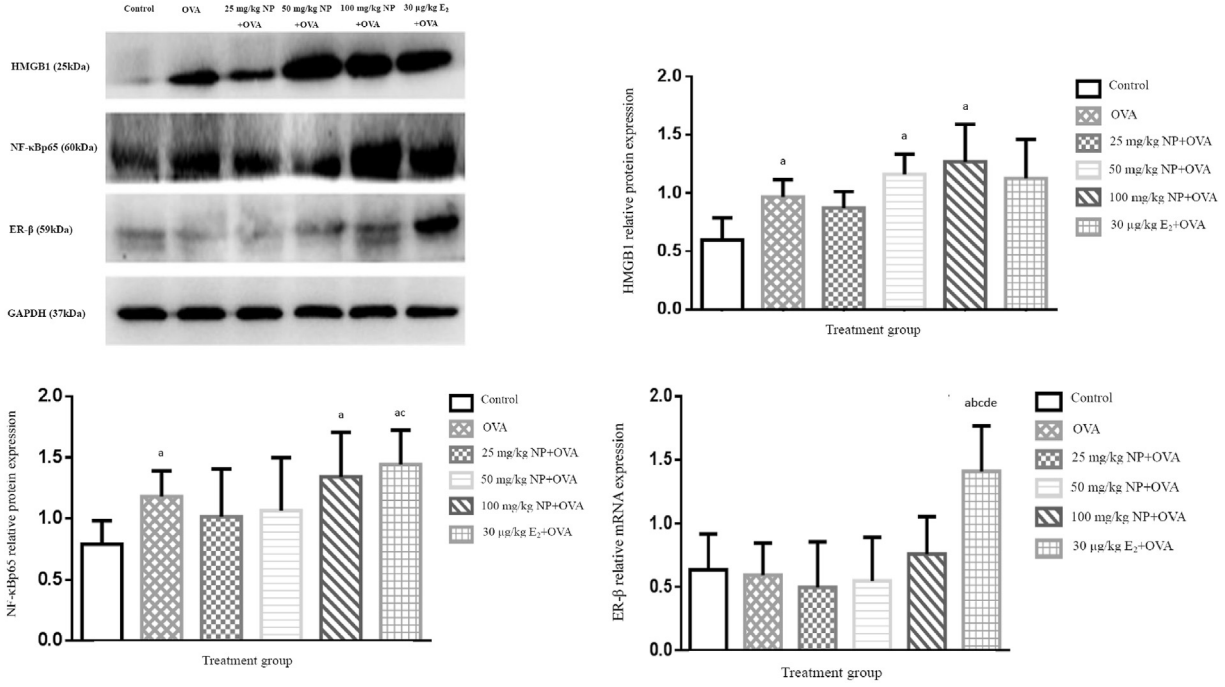

Fig. 10. Expression levels of relevant inflammatory and ER-β proteins in the lung tissues of perinatal NP-exposed pups (mean ± SD, n = 6). [a] Compared with the control group, $P < 0.05$; [b] Compared with the OVA group, $P < 0.05$; [c] Compared with the 25 mg/kg NP + OVA group, $P < 0.05$; [d] Compared with the 50 mg/kg NP + OVA group, $P < 0.05$; [e] Compared with the 100 mg/kg NP + OVA group, $P < 0.05$.

group. The activation of ER-β can reduce airway remodeling in human ASM by inhibiting the pro-proliferation signal transduction pathway (Ambhore et al., 2018), indicating that airway remodeling in the 30 μg/kg E_2 group may be inhibited.

Compared with the control group and the OVA group, the dose of NP in the lung tissues of asthmatic rat pups gradually increased with increasing exposure doses of NP administered to the perinatal mother. However, the NP in the lung tissues of the OVA group was

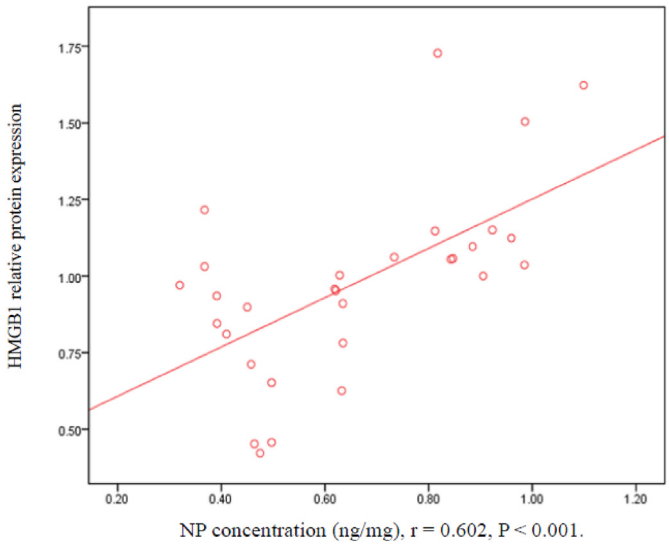

Fig. 11. Correlation between NP concentration and HMGB1 relative protein expression in the lung tissue of pups.

lower than that in the control group, suggesting that the NP content in the pups might be consumed after asthma. The content of NP in the lung tissues of asthmatic pups was positively correlated with the expression of HMGB1 protein in the lung tissues. Perinatal BPA exposure has been reported to protect male pups from airway sensitization. BPA can downregulate neutrophils and lead to alleviated pulmonary inflammation [11], which is consistent with the result that exposure to 25 mg/kg NP in perinatal maternal rats could alleviate airway inflammation in OVA-induced asthmatic pups. In contrast, 100 mg/kg NP aggravated the OVA-induced airway inflammation and increased the levels of the HMGB1 and NF-κB gene and protein in the lung tissue of the asthmatic rats, but it is uncertain whether NP affects the occurrence of asthma through estrogen-like effects.

5. Conclusions

Perinatal exposure to 25 mg/kg NP in maternal rats alleviated airway inflammation in OVA-induced asthmatic pups, while 100 mg/kg NP exacerbated the airway inflammation, there is interactive effect between NP and OVA on airway inflammation in pups. When the perinatal rats were exposed to 100 mg/kg NP, the levels of HMGB1 and NF-κB in the lung tissues of OVA-induced asthmatic pups were increased.

Availability of data and materials

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Ethics statement

Animal experimental procedures were approved by Zunyi Medical University Ethics Committee (2015-2-011). All methods were performed in accordance with guidelines and regulations of the Zunyi Medical University.

Credit author statement

Jie Xu and Jie Yu designed the study. FangXu Tuo, Jie Yu, Jie Xu and Ya Luo analyzed and interpreted the data. FangXu Tuo conducted the laboratory work. FangXu Tuo participated in the sample collection. Jie Yu wrote the manuscript, Jie Xu revised the manuscript. All the authors read and approved this paper.

Declaration of competing interest

The authors declare that they have no competing interests.

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