



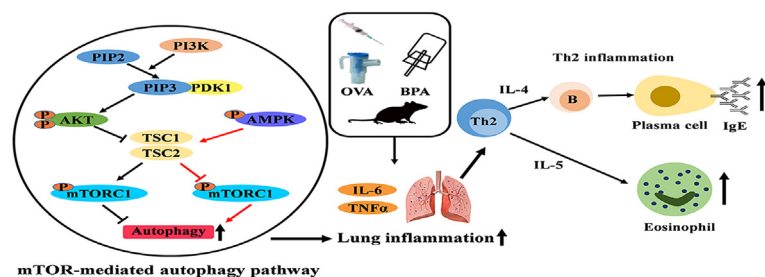
Lung inflammation induced by exposure to Bisphenol-A is associated with mTOR-mediated autophagy in adolescent mice

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GRAPHICAL ABSTRACT



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ABSTRACT

Epidemiologic studies show that there is a link between Bisphenol A (BPA) exposure and lung inflammation. Despite this, the molecular mechanisms are not entirely known. This study sought to determine whether exposure to BPA affected the development of ovalbumin (OVA) induced lung inflammation in adolescent female mice and whether the mechanism was related to mTOR-mediated autophagy pathway. Female 4-week-old C57BL/6 mice after one week of domestication were randomly divided into five groups (8/group): control group, OVA group, 0.1 $\mu\text{g mL}^{-1}$ BPA + OVA group, 0.2 $\mu\text{g mL}^{-1}$ BPA + OVA group and 0.4 $\mu\text{g mL}^{-1}$ BPA + OVA group. BPA exacerbated airway hyperresponsiveness (AHR), induced the pathological changes in the lung, which also enhanced inflammatory cells and cytokine levels. In addition, BPA exposure affected expression of autophagy associated proteins and genes. This research results indicated that BPA aggravated OVA-induced lung inflammation and induced abnormal immune function in mice, and its mechanism was related to the activation of autophagy pathway by down-regulation expression of mTOR. These findings suggest that therapeutic strategies to target autophagy may offer a new approach for severe asthma therapy.

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1. Introduction

Lung inflammation is a shared element for many lung diseases and asthma is one of these diseases (Abdelhaffez et al., 2017). Asthma is a chronic disease of the airways characterized by

recurrent attacks of breathlessness and wheezing (Kumar et al., 2016). Asthma is an immune response adjusted by helper T cell 2 (Th2) to general environmental allergens and identified by airway inflammation with increased eosinophilic granulocyte account, airway hyperresponsiveness (AHR), and raised levels of serum immunoglobulin E (IgE) (Petzold et al., 2014). In developed countries, the prevalence of asthma is approximately 10% in adults and the prevalence of asthma in children is higher, however, in the developing world, the morbidity rate is lower but growing quickly (He et al., 2016). Asthma is the most common chronic disease of childhood, which affects about 6.5 million children in the United States (approximately 9% prevalence) (Zahran et al., 2018). In recent years, the prevalence of asthma rises year by year in China (Zou et al., 2018). Data released in 2013 showed that the prevalence of asthma in children aged 0–14 was 3.0% (Zhou and Hong, 2018). In addition to genetic susceptibility, it has been reported that environmental factors have also been an important cause of increased allergic diseases in the past few decades (Petzold et al., 2014). Studies have shown that endocrine disruptor bisphenol A (BPA) may be linked to asthma and allergies (Wang et al., 2016; Xu et al., 2016; Lin et al., 2018).

BPA is the composition part of polycarbonate plastics and epoxy resins and is widely applied in food, drink containers and receipt-like thermal papers (Wang et al., 2016). The main way of exposure to BPA is by oral administration, but it can also be contacted through the skin or pulmonary routes (Lin et al., 2018). Humans are more likely to be exposed to BPA and around 95% of Americans have tested BPA concentrations in their urine (Youssef et al., 2018). Exposure to BPA is identified as a risk factor for asthma, cancer, obesity, liver disease and neurodegenerative diseases (Youssef et al., ; Landolfi et al., 2017). The increasing of prevalence of global asthma and the widespread use of industrial chemicals occurred at the same time, such as BPA (Liu et al., 2017a,b). However, the relationship between BPA exposure and increased rates of allergies and asthma is no definite agreements. Studies have shown that BPA can influence the development of asthma by facilitating allergic immune responses (Bonds and Midoro-Horiuti, 2013; Lee et al., 2017). A few human epidemiological studies have reported positive correlation with BPA exposure and severe allergic asthma (Vaidya and Kulkarni, 2012). Donohue et al. (2013) reported a relationship between urinary BPA levels after birth and children asthma attack. Spanier et al. (2012) pointed out the relation between prenatal urinary BPA levels and asthmatic infant. Several animal studies have shown that BPA-treated adult mice can cause the production of IgE and inflammatory cytokines (Bauer et al., 2012; Lee et al., 2012; Kendzierski et al., 2012; Nakajima et al., 2012). Low-dose BPA exposure may exacerbate allergic airway inflammation by increasing Th2 responses (Koike et al., 2018). However, a recent study showed that low-dose BPA exposure was irrelevant to airway allergic reaction in adulthood (Bauer et al., 2012). Therefore, the relation between BPA and asthma, and its mechanism deserve researching deeply.

Autophagy is a lysosomal-dependent protein degradation pathway in eukaryotic cells, which can transport damaged proteins and aging organelles to lysosomes for degradation (Gomes et al., 2017). It is known that there are three distinct categories of autophagy, namely macroautophagy, microautophagy and chaperone-mediated autophagy (Gomes et al., 2017). Macroautophagy is the main way of autophagy (Zhao et al., 2018). When autophagy levels are too strong or too weak for some reason, the intracellular environment may be disturbed, leading to excessive proliferation or degeneration of cells and death (Zhang et al., 2013). Therefore, appropriate levels of autophagy are necessary for cells to perform normal physiological functions. Autophagy is participated in physiological processes such as anti-aging, differentiation,

development and immunity. In addition, autophagy is also involved in regulating inflammatory responses in different diseases (Deretic and Levine, 2018).

In the initiation phase of autophagy, the mammalian target of rapamycin (mTOR) plays a central regulatory role, which is a serine/threonine protein kinase. One of the main complexes of mTOR is mTOR complex 1 (mTORC1) (Rabanal-Ruiz et al., 2017), which is a key regulator of negative regulation of autophagy. Autophagy may be controlled by mTORC1-centered signaling pathway (Noda, 2017). While, the two important upstream pathways to regulate mTORC1 are PI3K/Akt and AMPK signaling pathway (Wang et al., 2017; El-Kott et al., 2019).

The purpose of this study was to confirm whether exposure to BPA affects the development of OVA induced lung inflammation in adolescent female mice and whether the mechanism is related to mTOR-mediated autophagy pathway. To our knowledge, the role of autophagy in BPA aggravating OVA-induced lung inflammation has not been reported.

2. Materials and methods

2.1. Experimental animals

Female 4-week-old C57BL/6 mice were purchased from the animal center of China Medical University (license number, SYXK (Liao) 2013–0007). All animals were processed according to the Guidelines for Animal Experimentation issued by the Chinese Association for Laboratory Animal Science. Mice were housed at 24 ± 1 °C temperature, $45 \pm 5\%$ humidity and a 12-h light/12-h dark cycle, and allowed free access to food and water. To minimize the pollution of BPA in the breeding environment, we used animal cages made of polymethylpentene and water bottles made of polypropylene during the experiment. All mice began the experiment after a week of adaptive feeding.

2.2. Treatment protocols

The animals were divided into 5 groups (8/group). The control group and the ovalbumin (OVA; Sigma, St. Louis, MO, USA) group were given water containing 1% ethanol, the concentration used as vehicle for BPA solution. The remaining three groups were exposed to BPA (Sigma-Aldrich, St. Louis, Missouri) by administration in the drinking water at levels of $0.1 \mu\text{g mL}^{-1}$ (L-BPA), $0.2 \mu\text{g mL}^{-1}$ (M-BPA) and $0.4 \mu\text{g mL}^{-1}$ (H-BPA). Exposure to BPA or normal water in mice started one month before the first allergen injection and were kept up until the end of the asthma induction protocol. Mice in OVA group and the other three BPA dosing groups were sensitized with intraperitoneal injections (200 μL /mouse) on days 31–33 and 44 with 50 μg OVA in normal saline with 20 μL Imject Alum (Thermo, Rockford, IL, USA). On days 44, 47, 51, 54, and 57, mice were challenged with inhalations of 2% OVA for 30 min, delivered with an ultrasonic nebulizer (DeVilbiss Pulmo-Aide 5650D, USA). Meanwhile, the control group was sensitized and atomized with normal saline under the above conditions (Fig. 1A).

2.3. The activity of mice

After sensitization and during the process of atomization, the mice were observed for wheezing, breathing difficulties (rapid reaction) and urine and feces incontinence.

2.4. Measurement of AHR

Airway hyperresponsiveness to methacholine was measured in conscious, spontaneously breathing animals by DSI's Buxco®

OVA sensitization model

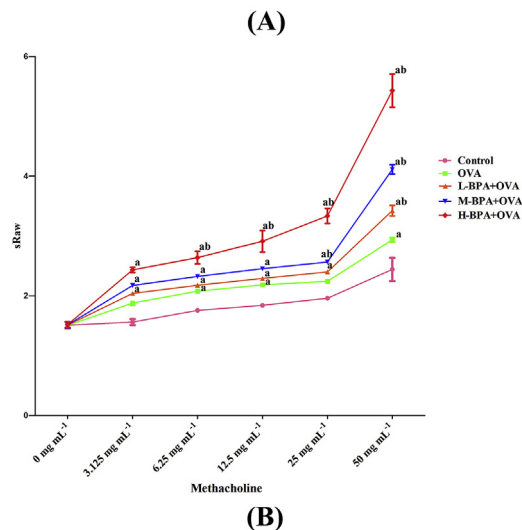
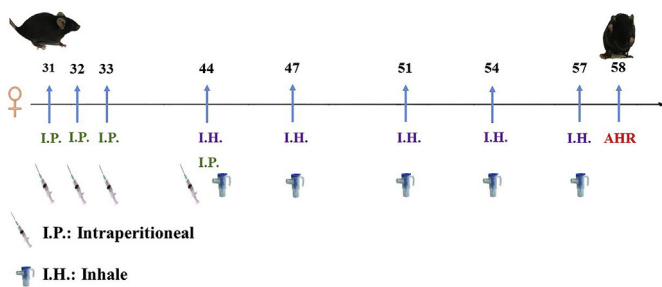


Fig. 1. Flow chart of asthma modeling and the effect of BPA on allergen-induced AHR in mice. (A) On days 31–33 and 44, mice were sensitized with OVA by intraperitoneal injection (IP) and challenged with 2% OVA inhalation (IH) on days 44, 47, 51, 54 and 57. One month before OVA challenge, mice were treated with water containing 1% ethanol and BPA ($n = 8$ mice/group). (B) AHR was measured as the sRaw value. Five independent experiments were analyzed, and data were presented as means $\pm$ SD, $n = 6$ /group. ^a $p < 0.05$ vs Cont.; ^b $p < 0.05$ vs OVA.

FinePointe Non-Invasive Airway Mechanics (NAM) instrument. The chambers were also calibrated before every data collection. Specific airway resistance (sRaw) is measured by determining how much the nasal flow signal is delayed with respect to the thoracic flow signal, which is proportional to the airway resistance and the thoracic gas volume. The FinePointe software computes sRaw with all other ventilator parameters derived by the NAM analyzer. Mice were exposed to nebulized PBS (for the baseline measurement) or methacholine (3.125, 6.25, 12.5, 25 and 50 mg mL⁻¹) for 1 min and readings were taken and averaged for 3 min after each nebulization. Data obtained were expressed by sRaw and atomized methacholine concentration (mg mL⁻¹).

2.5. Histological analysis of lung tissue

One lobe of right lung fixed in formaldehyde was dehydrated by a series of increasingly concentrated ethanol solutions and embedded in paraffin wax. Sections of 3 μ m thickness were prepared and dewaxed. After hydration, tissues were stained with hematoxylin and eosin (HE) or periodic acid-Schiff (PAS). Lung inflammation was assessed by pathologists who were unaware of the treatment group. For perivascular and peribronchial inflammation, the semi-quantitative lung scores are as follows: 1 = normally, there are few inflammatory cells around the arteries

or airways; 2 = inflammatory cells scattered around the arteries or airways can be as deep as two rings; 3 = the cell cuff or inflammatory cells cluster around the artery or airway to a depth of three or more rings. Goblet cell hyperplasia in PAS-stained sections was assessed using the following scoring system: 0 = normal; 1 = minimal, hyperplasia of goblet cell in large bronchioles; 2 = mild, hyperplasia of goblet cell in large and medium bronchioles; 3 = moderate, hyperplasia of goblet cell in large, medium, and some small bronchioles; and 4 = marked, hyperplasia of goblet cell in all airways.

2.6. Bronchoalveolar lavage fluid (BALF)

Mice were intubated with indwelling needle and the left lung was washed with PBS. The lungs were lavage twice, 0.35 mL each time. The average recovery is 80% of the injection volume. Fluids from the two lavages were mixed, cooled to 4 °C, and centrifuged for 10 min at 1500 rpm. The supernatants would assay cytokine levels. We used the Procyte Dx analyzer (IDEXX Laboratories, Inc. Westbrook, Maine, USA) to confirm white blood cell count (WBC).

2.7. Quantitation of cytokines in BALF

The cytokine levels in the BALF were determined by enzyme-linked immunosorbent assays (ELISA). IL-4, IL-5, IL-6 and TNF- α were measured using ELISA kits from R & D Systems Inc. (Minneapolis, USA). The absorption at 450 nm (sub-wave length, 540 nm) for IL-4, IL-5, IL-6 and TNF- α were measured using a microplate reader (Spectrafluor, Tecan, Salzburg, Austria).

2.8. OVA-specific IgE assay

OVA-specific IgE was measured using a Mouse OVA Specific IgE Pre-coated ELISA kit from BioLegend (USA). The absorption at 450 nm (sub-wave length, 570 nm) for OVA-specific IgE was measured using a microplate reader (Spectrafluor, Tecan, Salzburg, Austria).

2.9. Flow cytometry

Splenocytes were separated and suspended in cell culture medium (RPMI 1640 (Gibco) containing 10% fetal bovine serum (FBS, Tianhang Biological Technology Co. China)). The cells were stained with True-Nuclear Transcription Factor Buffer Set (424,401, Biolegend), FITC-anti-CD4 (100,509, Biolegend) and PE-anti-CD25 antibodies (102,007, Biolegend) for 15–20 min. The cells were then resuscitated in Fixation Buffer (420,801, Biolegend), and stained with APC-anti-Foxp3 antibody (320,013, Biolegend) for the intracellular antigen.

2.10. Immunohistochemistry

Immunohistochemistry for autophagosomal LC3 (Cell Signaling, Danvers, MA, USA) was performed on the hydrated slices. The glass slides were dried in a 60 °C thermostat for 40 min, dewaxed in xylene, and dehydrated in an ethanol series. The slices were microwaved in 0.3% citrate buffer, treated with 3% H₂O₂ deionized water, and then LC3 primary antibody was incubated in 4 °C overnight. The slides were then incubated with secondary antibody (Invitrogen, Carlsbad, CA) at 37 °C for 30 min, washed twice with PBS and incubated with diaminobenzidine (DAB; Invitrogen) for immunolabeling. After dyeing with hematoxylin and dehydrating with ethanol, the slides were sealed with neutral rubber cover and observed under optical microscope.

2.11. Western blotting assay

Preparation of protein sample and Western blot analysis were conducted according to standard procedures. The concentration of the protein extracted from the right lung was quantitated with a BCA protein assay kit (Beijing Dingguo Changsheng Biotechnology, Beijing, China) prior to mixing with loading buffer and heating at 100 °C for 5 min. The extracted protein (50 µg) was separated by SDS-PAGE and then electrophoretically transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The primary antibodies included: rabbit anti-AMPK, anti-PI3K, rabbit anti-p-PI3K, rabbit anti-AKT, rabbit anti-p-AKT, rabbit anti-TSC1, rabbit anti-TSC2, rabbit anti-mTOR and rabbit anti-p-mTOR, rabbit anti-ULK1, rabbit anti-p-ULK1, rabbit anti-Beclin1, rabbit anti-p62, rabbit anti-LC3 (Cell Signaling Technology, Danvers, MA, USA). Blots were incubated overnight at 4 °C in primary antibody in 5% BSA followed by HRP-conjugated anti-rabbit IgG antibody (Absin Bioscience, Shanghai, China). Specific bands were visualized by ECL detection and quantified by Image J software (Bio-Rad, Hercules, CA, USA).

2.12. Quantitative PCR (qPCR) analysis

Total RNA was isolated from the right lung using TRIZOL reagent (Life Technologies, USA), following the manufacturer's instructions. The cDNA was synthesized from the total RNA by using the PrimeScript RT reagent kit (Takara, Japan). Then using cDNA as template, SYBR® Premix Ex Taq™ II (Takara, Japan) and QuantStudio 6 Flex Real-Time PCR System (Life Technologies) were used for real-time PCR amplification. RNA abundance was expressed as $2^{-\Delta\Delta Ct}$ for the target gene normalized against the β -actin/Actb gene (as the reference gene), and presented as fold change versus control sample. The sequences of the primer pairs are shown in Table S1.

2.13. Statistical analysis

Data were expressed as mean $\pm$ standard deviations (SD). Data analysis of AHR were performed with GraphPad Prism 5 (GraphPad software, SanDiego, CA) and were carried out using two way ANOVA repeated measures with Bonferroni post-test. Other analyses were used by the SPSS 21.0 software (SPSS, Inc., Chicago, IL, USA). Differences between groups were analyzed by one-way analysis of variance (ANOVA) and were determined as statistically significant at a level of $p < 0.05$.

3. Results

3.1. The performance of mice after sensitization and after atomization stimulation

There was no obvious abnormality after sensitization in control group. In the asthma model group, crouching motionless, arching back, shortness of breath, urine or fecal incontinence, itchy head and face and feeling of uneasiness occurred 15 min after sensitization. There were no significant difference between the asthma model groups.

3.2. The effect of BPA on allergen-induced AHR in mice

Airway function was evaluated as the AHR, and the sRaw values were calculated to make sure the AHR. We found a significant dose-dependent increase in sRaw values after inhalation of methacholine. The sRaw values in OVA group were significantly higher than that in control group. Meanwhile, with the increase of BPA

exposure dose, sRaw values were gradually increased under the stimulation of various concentrations of methacholine ($p < 0.05$). At 50 mg mL⁻¹ of inhaled methacholine, BPA-treated asthmatic mice showed significantly higher sRaw values compared to OVA group. Hence, BPA could significantly exacerbate AHR in asthmatic mice (Fig. 1B).

3.3. The effect of BPA on inflammatory cell infiltration and lung goblet cell proliferation

In order to study the effect of BPA on OVA-induced airway inflammation and goblet cell proliferation, the lung pathology of the mice was studied (Fig. 2A–D). No pathological changes were observed in the lungs of the control group. Treatment with OVA alone caused inflammatory cell infiltrate and slight goblet cell proliferation in the bronchial epithelium. The combined action of BPA and OVA apparently increased the proliferation of airway goblet cells in the airway and caused eosinophils and lymphocytes to swell significantly in the connective tissues of the airways.

3.4. Effects of BPA on inflammatory cells in BALF

We counted different types of inflammatory cells to assess whether BPA increased the inflammatory response in asthmatic mice by increasing the number of inflammatory cells in BALF. Compared with control group, the total number of leukocytes and the number of eosinophils, neutrophils, monocytes and lymphocytes in OVA group and three BPA dose groups were significantly increased ($p < 0.05$). In addition, the administration of OVA combined with three BPA dose groups resulted in significant increases in the total number of leukocytes and the number of eosinophils, neutrophils, monocytes and lymphocytes compared with OVA group ($p < 0.05$). Furthermore, the total number of leukocytes and the number of eosinophils, neutrophils, monocytes and lymphocytes were increased gradually in M-BPA + OVA and H-BPA + OVA groups compared with L-BPA + OVA group ($p < 0.05$). Compared with M-BPA + OVA group, the total number of leukocytes and the number of eosinophils, neutrophils and monocytes in H-BPA + OVA group were significantly increased ($p < 0.05$) (Fig. 3A and B).

3.5. BPA modulated cytokine levels in BALF

We detected the cytokine levels in the BALF, such as IL-4, IL-5, IL-6 and TNF- α . Compared with control group, the levels of IL-4, IL-5, IL-6 and TNF- α in OVA group and three BPA dose groups were significantly increased ($p < 0.05$). In addition, the administration of OVA combined with three BPA dose groups resulted in significant increases in the levels of IL-4, IL-5, IL-6 and TNF- α compared with OVA group ($p < 0.05$). Furthermore, the levels of IL-4, IL-5, IL-6 and TNF- α were increased gradually in M-BPA + OVA and H-BPA + OVA groups compared with L-BPA + OVA group ($p < 0.05$). Compared with M-BPA + OVA group, the levels of IL-4, IL-5, IL-6 and TNF- α in H-BPA + OVA group were significantly increased ($p < 0.05$) (Fig. 4A–D).

3.6. BPA enhanced the level of OVA-specific IgE in the serum

We detected the level of OVA-specific IgE in the serum. Compared with control group, the level of OVA-specific IgE in OVA group and three BPA dose groups was significantly increased ($p < 0.05$). In addition, the administration of OVA combined with three BPA dose groups resulted in a significant increase in the level of OVA-specific IgE compared with OVA group ($p < 0.05$). Furthermore, the level of OVA-specific IgE was increased gradually in the

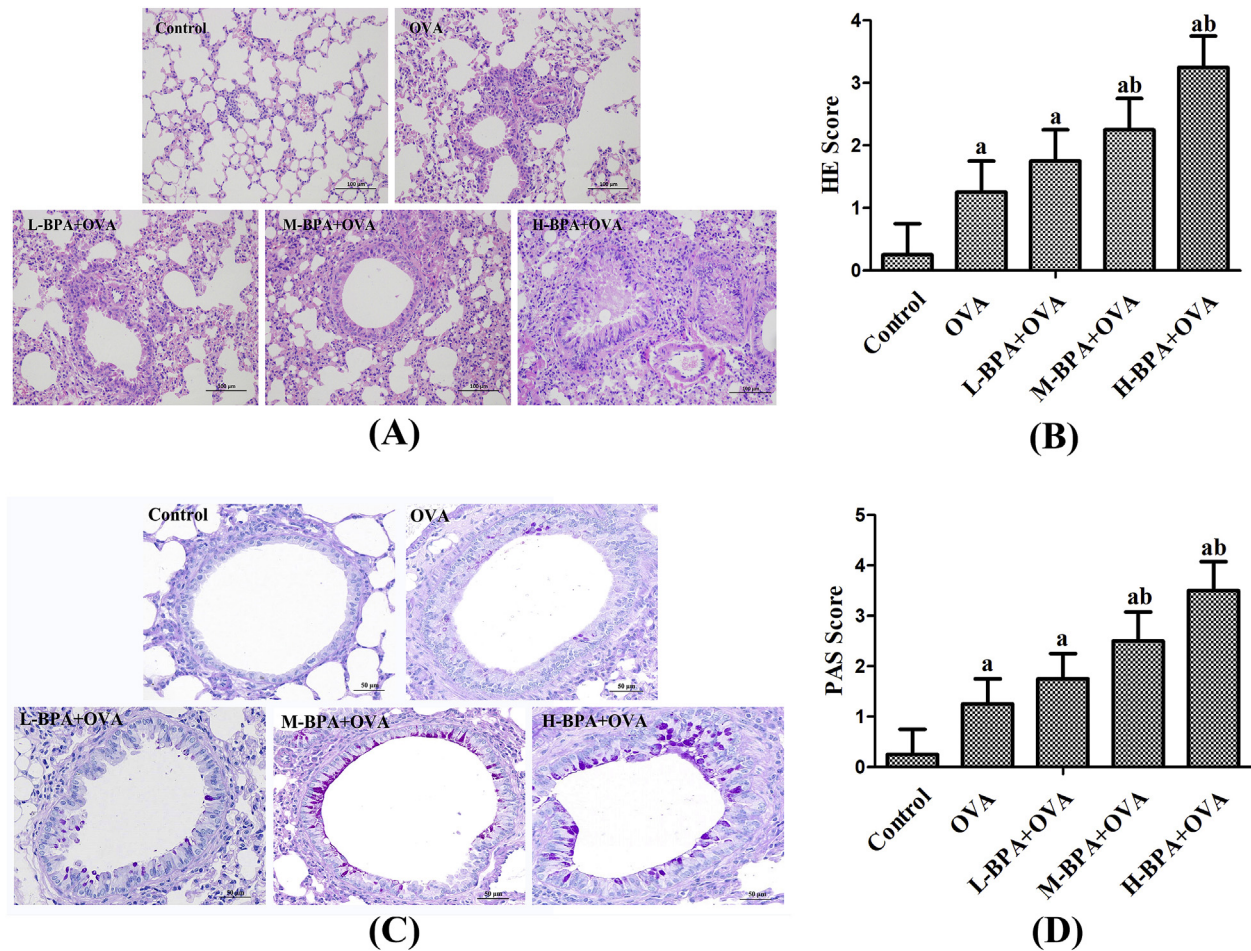


Fig. 2. HE and PAS staining of lung tissues from five studied groups. Histological sections of lung tissues from control and OVA-stimulated mice with or without BPA treatment. (A) BPA increased inflammatory cell infiltration (HE stain: 200 $\times$ magnification) (B) Scoring of inflammation in pathological evaluation of inflammatory cell infiltration in lung sections. (C) PAS stained lung sections demonstrated goblet cell hyperplasia. (PAS stain: 200 $\times$ magnification). (D) Scoring of goblet cell proliferation in lung sections. Five independent experiments were analyzed, and data were presented as means $\pm$ SD, $n = 4$ /group. ^a $p < 0.05$ vs Cont.; ^b $p < 0.05$ vs OVA.

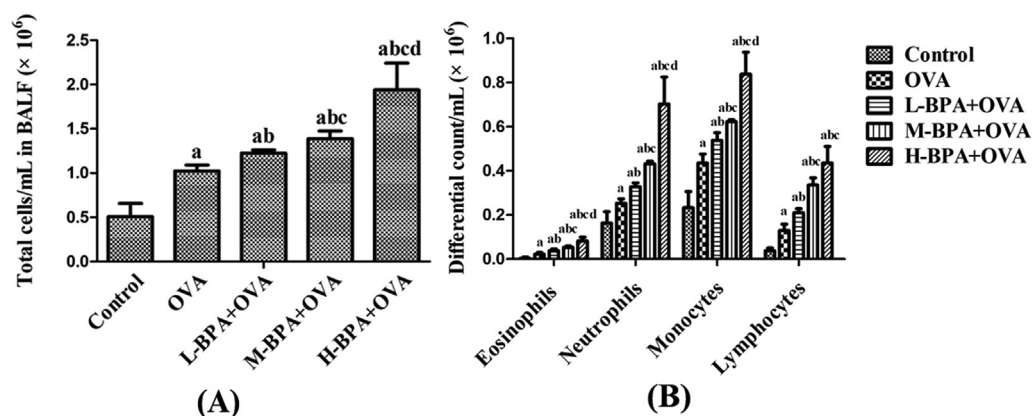


Fig. 3. The effect of BPA on inflammatory cells in BALF. (A) Numbers of total cells in BALF with the increasing BPA doses. (B) Numbers of inflammatory cells in BALF with the increasing BPA doses. Five independent experiments were analyzed, and data were presented as means $\pm$ SD, $n = 6$ /group. ^a $p < 0.05$ vs Cont.; ^b $p < 0.05$ vs OVA; ^c $p < 0.05$ vs L-BPA + OVA; ^d $p < 0.05$ vs M-BPA + OVA.

M-BPA + OVA and H-BPA + OVA groups compared with L-BPA + OVA group ($p < 0.05$). Compared with M-BPA + OVA group, the level of OVA-specific IgE in H-BPA + OVA group was significantly increased ($p < 0.05$) (Fig. 4E).

3.7. The effect of exposure to BPA on the percentage of Treg cells in splenocytes from mice

Foxp3⁺ regulatory T (Treg) cells play an important role in regulating Th2-related inflammatory responses. In the current

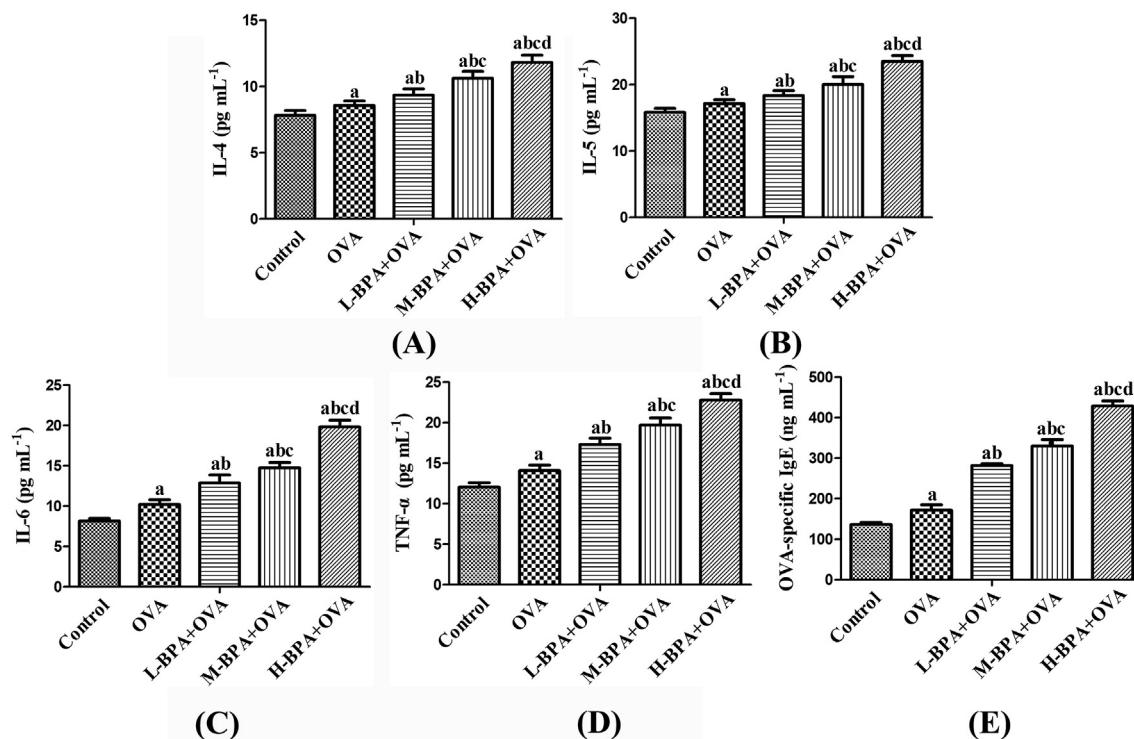


Fig. 4. The effect of BPA on cytokine levels in BALF and OVA-specific IgE levels in serum. The concentrations of (A) IL-4, (B) IL-5, (C) IL-6 and (D) TNF- α were measured by enzyme-linked immunosorbent assay in BALF from control and OVA mice, without or with BPA treatment ($n = 7/\text{group}$). (E) The serum level of OVA-IgE were shown from control and OVA-stimulated mice, without or with BPA treatment ($n = 8/\text{group}$). Five independent experiments were analyzed, and data were presented as means $\pm$ SD. ^a $p < 0.05$ vs Cont.; ^b $p < 0.05$ vs OVA; ^c $p < 0.05$ vs L-BPA + OVA; ^d $p < 0.05$ vs M-BPA + OVA.

study, the percentage of Treg cells in splenocytes from mice was measured by flow cytometry. We found that the proportion of Treg cells was gradually decreased as the increase of BPA dose compared with control group ($p < 0.05$). In addition, the administration of OVA combined with three BPA dose groups resulted in a significant decrease in the proportion of Treg cells compared with OVA group ($p < 0.05$). Furthermore, the proportion of Treg cells was decreased gradually in M-BPA + OVA and H-BPA + OVA groups compared with L-BPA + OVA group ($p < 0.05$). Compared with M-BPA + OVA group, the proportion of Treg cells in H-BPA + OVA group was significantly decreased ($p < 0.05$). Here, we demonstrate that BPA can reduce CD4⁺CD25⁺Foxp3⁺ Treg to aggravate the symptoms associated with lung inflammation (Fig. 5A and B).

3.8. The changes of LC3 expression in the lung of mice

We detected the expression level of LC3 in the lung of mice. Compared with control group, the expression level of LC3 in OVA group and three BPA dose groups was significantly increased ($p < 0.05$). In addition, the administration of OVA combined with three BPA dose groups resulted in a significant increase in the expression level of LC3 compared with OVA group ($p < 0.05$). Furthermore, the expression level of LC3 was increased gradually in the M-BPA + OVA and H-BPA + OVA groups compared with L-BPA + OVA group ($p < 0.05$). Compared with M-BPA + OVA group, the expression level of LC3 in H-BPA + OVA group was significantly increased ($p < 0.05$) (Fig. 6A and B).

3.9. The changes of autophagy-related protein expression

We detected the protein expression levels of mTOR, p-mTOR, ULK1, p-ULK1, Beclin1, p62 and LC3 in the lung of mice. The

expression levels of mTOR and ULK1 were no significant difference among the groups. The expression levels of p-ULK1, Beclin1 and LC3 only exposed to OVA were significantly higher ($p < 0.05$), while the levels of p-mTOR and p62 were significantly lower than that of control group ($p < 0.05$). Compared with OVA group, the expression levels of p-ULK1, Beclin1 and LC3 in BPA dose groups were significantly increased ($p < 0.05$) except p-ULK1 in H-BPA + OVA group. However, the expression levels of p62 and p-mTOR in BPA dose groups were significantly decreased ($p < 0.05$). Furthermore, the expression level of p-mTOR was decreased gradually in the M-BPA + OVA and H-BPA + OVA groups compared with L-BPA + OVA group ($p < 0.05$). The expression levels of p-ULK1, Beclin1 and LC3 in M-BPA + OVA group were significantly higher than the other two BPA dose groups ($p < 0.05$). The expression level of p62 in both M-BPA + OVA and H-BPA + OVA group was significantly lower than L-BPA + OVA group ($p < 0.05$), but there is no significant difference between the two groups (Fig. 7A and B).

3.10. The effect of exposure to BPA on the regulation of mTOR by PI3K/Akt and AMPK autophagy related signaling pathways

We detected the protein levels of PI3K, p-PI3K, Akt, p-Akt, TSC1, TSC2 and AMPK to verify if decreased mTOR protein expression induced by BPA was associated with PI3K/Akt and AMPK signaling pathway. The expression levels of PI3K and Akt were no significant difference among the groups. The expression levels of TSC1, TSC2 and AMPK in the lung of mice only exposed to OVA were significantly higher ($p < 0.05$), while the levels of p-PI3K and p-Akt (Ser473 and Thr308) were significantly lower than that of control group ($p < 0.05$). Compared with OVA group, the expression levels of TSC1 and TSC2 in BPA dose groups were significantly increased ($p < 0.05$). However, the expression levels of p-PI3K and p-Akt

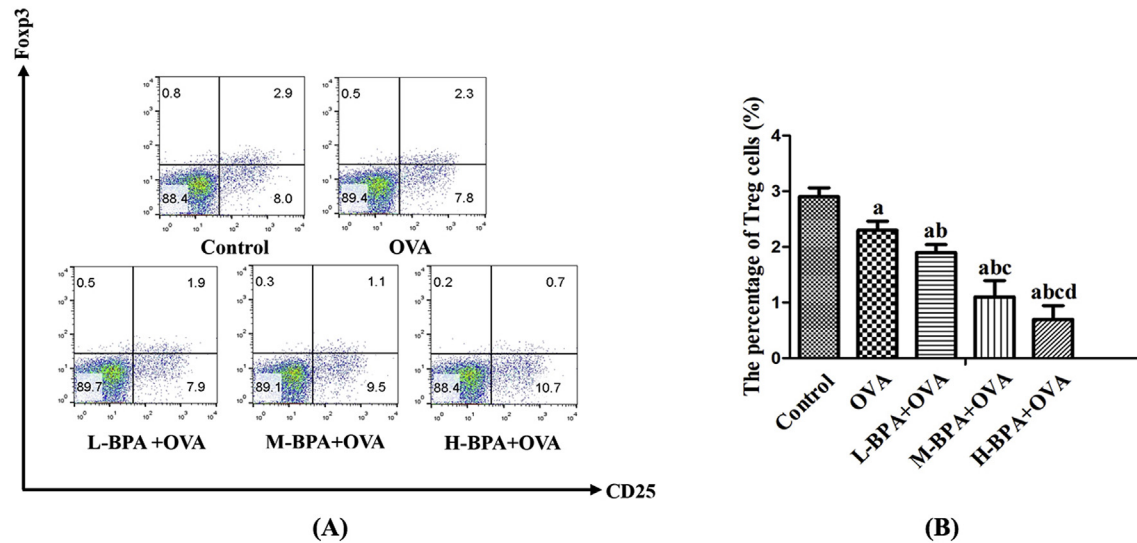


Fig. 5. The effect of BPA on the percentage of Treg cells in splenocytes from mice. (A, B) CD4⁺ CD25⁺ Foxp3⁺ T cells in CD4⁺ T cells. All data were presented as means $\pm$ SD, n = 4/group. ^a $p < 0.05$ vs Cont.; ^b $p < 0.05$ vs OVA; ^c $p < 0.05$ vs L-BPA + OVA; ^d $p < 0.05$ vs M-BPA + OVA.

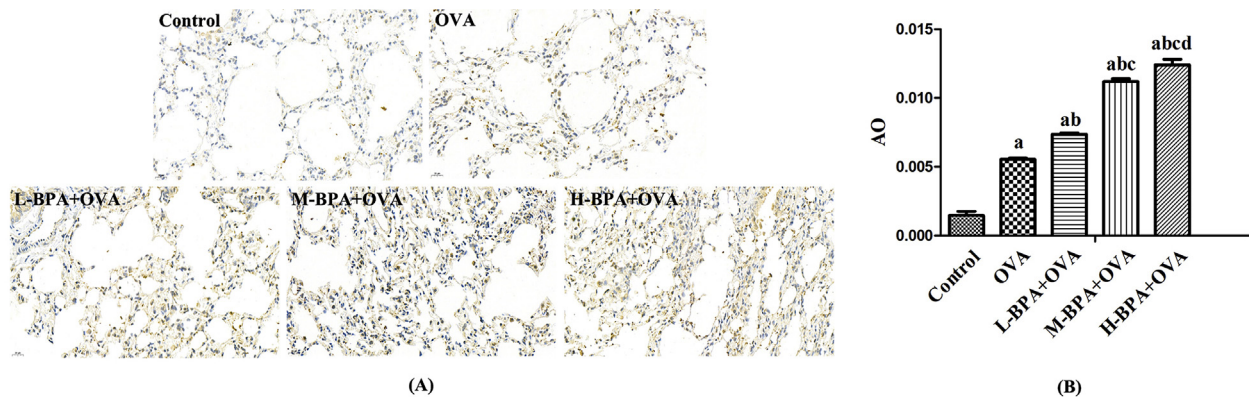


Fig. 6. Immunohistochemical expression of LC3. Staining of LC3 of lung tissues from five studied groups. Histological sections of lung tissues from control and OVA-stimulated mice with or without BPA treatment. (400 $\times$ magnification) (A) Yellowish brown reaction product refers to LC3-positive signal distribution. (B) The detection of LC3 average optical (AO value) in five groups. All data were presented as means $\pm$ SD, n = 3/group. ^a $p < 0.05$ vs Cont.; ^b $p < 0.05$ vs OVA; ^c $p < 0.05$ vs L-BPA + OVA; ^d $p < 0.05$ vs M-BPA + OVA.

(Ser473 and Thr308) in BPA dose groups were significantly decreased ($p < 0.05$) except p-PI3K in H-BPA + OVA group. Furthermore, the expression level of AMPK was increased gradually in the M-BPA + OVA and H-BPA + OVA groups compared with L-BPA + OVA group ($p < 0.05$). The expression levels of TSC1 and TSC2 in M-BPA + OVA group were significantly higher ($p < 0.05$), while the levels of p-PI3K and p-Akt (Ser473 and Thr308) were significantly lower than that of the other two BPA dose groups ($p < 0.05$) (Fig. 7C–F).

3.11. The effect of exposure to BPA on mRNA expression in the lung of mice

We detected the mRNA levels of *Pik3r1*, *Akt1*, *Prkaa2*, *Mtor*, *Ulk1*, *Becn1*, *Map1lc3b* and *p62/Sqstm1*. The expression levels of *Prkaa2*, *Ulk1*, *Becn1* and *Map1lc3b* only exposed to OVA were significantly higher ($p < 0.05$), while the levels of *Pik3r1*, *Akt1*, *Mtor* and *p62/Sqstm1* were significantly lower than that of control group ($p < 0.05$). Compared with OVA group, the mRNA expression levels of *Pik3r1*, *Akt1*, *Mtor* and *p62/Sqstm1* in BPA dose groups were significantly decreased ($p < 0.05$). However, the mRNA expression

levels of *Prkaa2*, *Ulk1*, *Becn1* and *Map1lc3b* in BPA dose groups were significantly increased ($p < 0.05$). Furthermore, the mRNA expression levels of *Prkaa2*, *Ulk1*, *Becn1* and *Map1lc3b* were increased gradually in the M-BPA + OVA and H-BPA + OVA groups compared with L-BPA + OVA group ($p < 0.05$), while the levels of *Pik3r1*, *Akt1*, *Mtor* and *p62/Sqstm1* were gradually decreased ($p < 0.05$). Compared with M-BPA + OVA group, the mRNA expression levels of *Pik3r1*, *Akt1*, *Mtor* and *p62/Sqstm1* in H-BPA + OVA group were significantly decreased ($p < 0.05$), while the levels of *Prkaa2*, *Ulk1*, *Becn1* and *Map1lc3b* were significantly increased ($p < 0.05$) (Fig. 7G).

4. Discussion

Past research have shown the relation between xenoestrogen exposure and development of allergic disease (Bonds and Midoro-Horiuti, 2013). In the past few years, BPA, one of the most familiar endocrine disrupting chemicals, has received increasing attention due to its harmful effects on health. Exposure to BPA is a dangerous factor for asthma, cancer, obesity, liver diseases and neurodegenerative diseases (Landolfi et al., 2017; Liu et al., 2017a,b; Rezg et al.,

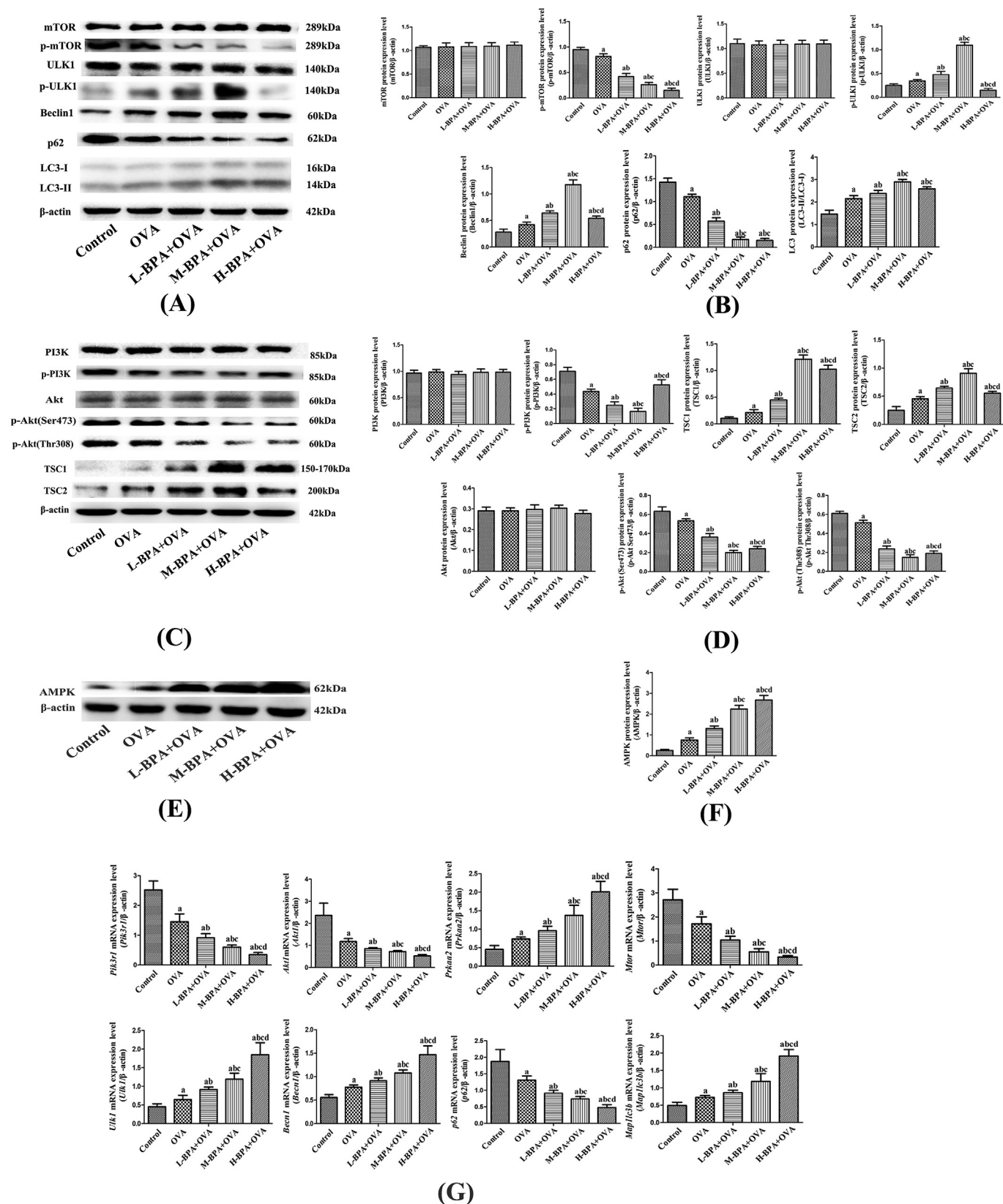


Fig. 7. The changes in expression of autophagy associated proteins and autophagy related signaling pathways in different groups of lung tissues. (A, C, E) The protein expression of mTOR, p-mTOR, ULK1, p-ULK1, Beclin1, p62, LC3, PI3K, p-PI3K, Akt, p-Akt (Ser473 and Thr308), TSC1, TSC2 and AMPK in lung tissue from control and OVA mice with or without BPA treatment. (B, D, F) The fold-change in the expression of mTOR, p-mTOR, ULK1, p-ULK1, Beclin1, p62, LC3, PI3K, p-PI3K, Akt, p-Akt (Ser473 and Thr308), TSC1, TSC2 and AMPK was measured relative to the expression of β -actin. (G) Gene expression (qPCR) of *Pik3r1*, *Akt1*, *Prkaa2*, *Mtor*, *Ulk1*, *Becn1*, *Map1lc3b* and *Sqstm1/p62* in different groups of lung tissues. All data were presented as means $\pm$ SD, n = 4/group. ^a $p < 0.05$ vs Cont; ^b $p < 0.05$ vs OVA; ^c $p < 0.05$ vs L-BPA + OVA; ^d $p < 0.05$ vs M-BPA + OVA.

2014). Asthma is a chronic disease affecting children and adults and has been the emphasis of clinical and public health interventions in recent years (Galant et al., 2017). The ultimate goal of this study was to investigate whether BPA exposure aggravates asthma induced by OVA and its possible mechanism of BPA action. In this research, the exposure dose of BPA was selected according to up and down of the lowest observable adverse reaction level (LOAEL) estimated by the US Environmental Protection Agency (EPA), which is $50 \mu\text{g kg}^{-1} \text{BW d}^{-1}$ (Shi et al., 2017). Because in our study, the average amount of drink water was about 5 mL d^{-1} in mice and the average weight during puberty was about 20 g. We selected $0.1 \mu\text{g mL}^{-1}$, $0.2 \mu\text{g mL}^{-1}$ and $0.4 \mu\text{g mL}^{-1}$ BPA to represent low, medium and high doses of BPA exposure, respectively. The average doses of BPA exposure in the three groups were 25, 50 and $100 \mu\text{g kg}^{-1} \text{BW d}^{-1}$ respectively. It is worth noting that at the lowest dose of BPA, the secretion of pro-inflammatory mediators increased significantly, which is much lower than humans' average exposure level, indicating the need for continued BPA studies, especially at low doses (O'Brien et al., 2014).

When designing experiments to deal with specific aspects of asthma, it is important to select the adequate mouse strain. C57BL/6 and BALB/c mouse strains are the two most common animal models of asthma (Gueders et al., 2009). Although peribronchial inflammation caused by allergens is some resemblance between the two strains, however, the eosinophilic infiltration of bronchial wall was more evident in C57BL/6 mice (Gueders et al., 2009). Bauer et al. (2012) found among adult C57BL/6 mice exposed to BPA during perinatal period, only female mice had increased airway lymphatic and lung inflammation, while male mice did not. Thus, we used female C57BL/6 mice.

Many researches have shown that inflammation played a vital role in the development of asthma (Al-Shami et al., 2005; Jember et al., 2001). The endpoints most commonly assessed in asthma model are AHR test and the number of inflammatory cells in BALF, especially eosinophils. Increases in these responses are characteristic of asthma (Selgrade et al., 2013). Eosinophils are the main inflammatory cells that cause asthma. The infiltration of eosinophils led to AHR and airway remodeling (Qian et al., 2017). However, there is an increase in neutrophil counts in the sputum of adults with persistent asthma (Jatakanon et al., 1999), and in that of children with acute asthma exacerbations (Norzila et al., 2000). Therefore, increased neutrophils in the airway are associated with asthma severity. In our study, neutrophils were much greater than eosinophils in BALF of mice exposed to BPA and OVA, and has a dose-effect relationship. This implied that BPA exposure could aggravate OVA-induced inflammatory response in the lungs.

AHR is an important characteristic of asthma. Allergens may cause acute asthma attacks, which are characterized by bronchoconstriction and severe shortness of breath (Huang et al., 2017). In clinic, children and adults with asthma can be diagnosed by measuring AHR, evaluating airflow, and discussing the pathologic feature of asthma (Fatani, 2014; Tong et al., 2015). Nygaard et al. (2015) commented that despite early exposure to BPA caused a mild allergic reaction, it was only seen at high doses. Two other studies pointed out that maternal exposure to BPA had subtle effects on respiratory allergic reactions in offspring, which varied according to gender and the way to sensitize animals. This suggests that early life exposure to BPA does not intensify allergic inflammation later in life (Becher et al., 2015) and may even inhibit the development of airway responses (Petzold et al., 2014). Meanwhile, asthma is an immune response to general environmental allergens regulated by Th2 cells (Robinson et al., 2017). Then, Th2 cells predominantly secrete cytokines such as IL-4 and IL-5, which play a central role in the pathophysiology of asthma (Robinson et al., 2017). We found that co-exposure to BPA and OVA led to

decreased lung function, increased accumulation of lymphocytes, monocytes, neutrophils and eosinophils in BALF, inflammatory cell infiltration and lung goblet cell proliferation, which affected pathologically similar to human asthma. In addition, the levels of IL-4, IL-5, IL-6 and TNF- α in BALF were also increased significantly. The level of OVA specific IgE in serum was gradually increased. The increased level of OVA specific IgE might be associated with heightened levels of cytokines and pulmonary eosinophilia. Therefore, BPA aggravated specific IgE reactions, which enhanced the risk of anaphylaxis.

Treg cells have a potential inhibitory effect on adverse immunity, which have potent anti-inflammatory effects (Ji et al., 2019). Treg cells are defined by combined expression of cell surface markers CD4 and CD25. Foxp3, a member of foxhead box transcription factor has been proved to be a specific marker and determinant of the development and function of Treg cells (Lu et al., 2015). It has also been reckoned that maternal exposure to BPA affects the function of Treg cells (Ohshima et al., 2007). We found that BPA can reduce the expression of $\text{CD4}^+\text{CD25}^+\text{Foxp3}^+$ Treg cells to aggravate the symptoms associated with lung inflammation. Similarly, the decrease of Treg amount in the spleen of rats exposed to BPA had reduced levels of Treg in their spleens in other study (Ménard et al., 2014a) was in contrast with the results reported by Ménard et al. (2014b) that perinatal exposure to BPA had no influence on Treg amount. The difference effect of BPA exposure on Treg amount is related to dose, time, route, physiological state and sex etc.

For the past few years, in vivo and in vitro experiments have shown that autophagy is an important mediator of immunity and inflammation (Shibutani et al., 2015), which is involved in regulating, maintaining and controlling inflammatory responses and, importantly, preventing excessive inflammatory responses (Cadwell, 2016; Liu et al., 2017a,b). Autophagy is completed by a group of specialized genes, which are called autophagy related genes (Atg) (Bestebroer et al., 2013). In the initiation phase of autophagy, mTOR plays a central regulatory role. When mTORC1 is inhibited, it can release the inhibition of its downstream UNC-51-like kinase (ULK) complex and induce phagocytic formation (Liu et al., 2016). Beclin1 is an Atg 6 homologue, which is an important regulator in the initiation stage of autophagy. It can combine with Class III PI3K to form a compound, recruit other autophagic regulatory proteins in the cytoplasm, and form a protein complex to promote the formation of autophagosomes (Liu et al., 2016). Microtubule-associated protein 1 light chain 3 (LC3) is a homologue of yeast Atg 8 gene in mammalian cells, which took part in the formation of autophagosomes (Wu et al., 2019). When autophagy occurs, LC3 is first converted to LC3-I by Atg 4 modification, and is covalently bound to phosphatidylethanolamine on the autophagic membrane by ubiquitination to form esterified LC3-II, which interactions regulate to promote phagocytic extension and closure to form autophagosomes. At this time, only LC3-II is present on the autophagosome membrane, and its content is proportional to the number of autophagosomes, and is thought of as a marker molecule of autophagy (Wu et al., 2019). The autophagosome membrane protein p62 forms a compound with LC3-II and is degraded in autophagosomes. Its content is inversely proportional to the number of autophagic vacuoles, reflecting the level of autophagy and autophagic flow (Yoshii and Mizushima, 2017).

Our results showed that except for H-BPA + OVA group, with the increase of BPA exposure dose, co-exposure of BPA and OVA led to the gradual down-regulation of p62 protein level, while p-ULK1, Beclin1 and LC3 were gradually up-regulated. The protein level of p-mTOR was gradually down-regulated with the increase of BPA exposure dose. Meanwhile, the mRNA expression levels of *Ulk1*,

Becn1 and *Map1lc3b* were increased gradually, while *p62/Sqstm1* was gradually up-regulated. Our immunohistochemical results showed that alveolar epithelial cells had brown-yellow positive products. LC3 is mainly expressed in the cytosol, especially in alveolar septal epithelial cells. With the increase of BPA exposure dose, co-exposure of BPA and OVA led to the gradual up-regulation of LC3 expression.

Autophagy serves as a basic procedure and interconnects multiple cell survival pathways (Chaabane et al., 2013). The most representative pathways include the PI3K/Akt and the AMPK signaling pathway. The PI3K/Akt signaling pathway is the main negative regulatory signal of autophagy (Luo et al., 2003). During the process of autophagy, activated Class I PI3K can phosphorylate the substrate phosphatidylinositol 4,5-bisphosphate (PIP2) to generate phosphatidylinositol 3,4,5-triphosphate (PIP3), and PIP3 as a second messenger can bind to Akt and phosphoinositide-dependent protein kinase 1 (PDK1) containing PH domain in the cell, promoting Akt phosphorylation at threonine 308 and serine 473 to activate (Luo et al., 2003). p-Akt stimulates growth by phosphorylating the tuberous sclerosis complex 2 (TSC2) and suppressing formation of a TSC1:TSC2 complex. This complex inhibits an activator of translation p70 ribosomal protein S6 kinase 1 and activates an inhibitor of translational initiation 4E binding protein 1 (4E-BP1) (Luo et al., 2003). These functions of TSC1 and TSC2 are mediated through inhibition of mTOR (Luo et al., 2003).

Our results showed that except for H-BPA + OVA group, with the increase of BPA exposure dose, the co-exposure of BPA and OVA led to the gradual down-regulation of p-PI3K and p-Akt protein levels, while TSC1 and TSC2 were gradually up-regulated. Meanwhile, with the increase of BPA exposure dose, the mRNA expression levels of *Pik3r1*, *Akt1* and *Mtor* were decreased gradually.

In the above results, the protein levels of TSC2, p-ULK1 and Beclin1 were lower, while the level of p-PI3K was higher in the H-BPA + OVA group than those in the M-BPA + OVA group, which implied that the protein levels of p-PI3K, TSC2, p-ULK1 and Beclin1 did not show a dose-dependent manner with BPA doses. Because the exacerbation of asthmatic symptoms mainly depends on inflammation. In our study, the levels of IL-4, IL-5, IL-6 and TNF- α in BALF were the highest in H-BPA + OVA group, which showed a dose-dependent manner with BPA doses. Thus, the inconsistent protein expression of p-PI3K, TSC2, p-ULK1 and Beclin1 between H-BPA + OVA group and M-BPA + OVA group implied that the initiation phase of autophagy was inhibited in the H-BPA + OVA group. Some studies have also reported that the adverse effect of BPA on immunologic function presents a “nonlinear” distribution (Kuo et al., 2012; Özyaydin et al., 2018). However, its specific mechanism needs to be further studied.

AMP-activated protein kinase (AMPK) signaling pathway is a positive regulation signal of autophagy (Agarwal et al., 2015). In contrast to the role of Akt, AMPK activates the TSC1:TSC2 complex to inhibit activation of mTORC1 and initiates autophagy (Agarwal et al., 2015). The up-regulation of AMPK resulted in decreased phosphorylation of mTORC1 and also ULK1 (Ser-757), which was increased after BPA treatment (Agarwal et al., 2015). Our experimental results indicated that with the increase of BPA exposure dose, the co-exposure of BPA and OVA led to the gradual up-regulation of AMPK protein and *Prkaa2* mRNA expression level. This suggests that mTOR participates in the autophagy process as a downstream effector module of the AMPK pathway (Agarwal et al., 2015).

Our results showed that there were up- or down-regulation of autophagy related mRNA levels, which were basically consistent with the expression levels of their protein, except that the levels of p-ULK1 protein and *Ulk1* mRNA expression were inconsistent in H-

BPA + OVA group. Because the expression of *Ulk1* mRNA increased, but its protein expression decreased in H-BPA + OVA group compared with control group. This may be related to the epigenetic modification of ULK1 protein, and its mechanism needs to be further explored in the future.

At present, the role of autophagy in airway inflammation remains unclear (Sachdeva et al., 2019). At baseline, autophagy is critical for inhibiting spontaneous lung inflammation, such as autophagy deficient mice (Atg5^{-/-} and Atg 7^{-/-}) developed spontaneous sterile lung inflammation (Abdel Fattah et al., 2015). However, the deficiency of Atg5 in airway epithelial cells resulted in an increased airway inflammation (Wang et al., 2018), and the disruption or deletion of autophagy in airway epithelial cells resulted in AHR (Inoue et al., 2011). The inhibition of autophagy in murine model has been shown to attenuate airway inflammation, and subsequently lead to a reduced airway remodeling (Sachdeva et al., 2019). Meanwhile, autophagy also potentially amplifies inflammation through delivery of cytosolic or phagocytosed microbially derived components to endosomes for innate immune recognition and to endolysosomal compartments for antigen presentation (Lee et al., 2007; Schmid et al., 2007; Mintern et al., 2015). Therefore, although autophagy appears to be a protective mechanism, autophagy may also exacerbate airway inflammation.

In our study, co-exposure of BPA and OVA in mice resulted in the activation of autophagy and inflammatory response. Thus, we speculate that the activation of autophagy may exacerbate OVA-induced allergic lung inflammation. The reason is if the role of autophagy is a protective effect in this study, the levels of allergic lung inflammation should be decreased accordingly. However, this is just a speculation, and its mechanism should be further verified with inhibitors of autophagy, such as chloroquine.

5. Conclusion

The study results suggest that BPA exposure may aggravate OVA-induced lung inflammation in adolescent female mice, and its mechanism may be related to mTOR-mediated autophagy activation. While, the down-regulation of mTOR induced by BPA is associated with the down-regulation of PI3K/Akt and the up-regulation of AMPK signaling pathway. Our study further supports that BPA exposure may increase the risk of asthma in childhood, and provides a reference for further revise the exposure threshold of BPA in population. Therapeutic strategies to target autophagy may provide a new idea for the treatment of severe asthma.

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Ethics approval and consent to participate

This study was approved by the Animal Research Committee at China Medical University (IACUC Issue No. CMU2019187).

Declaration of competing interest

The authors declare that they have no competing interests.

CRediT authorship contribution statement

Simeng Wang: Data curation, Methodology, Formal analysis, Writing - original draft, Writing - review & editing. **Yilong Yang:** Formal analysis, Funding acquisition. **Dan Luo:** Data curation, Investigation. **Dan Wu:** Data curation, Investigation. **Hezuo Liu:** Data curation, Investigation, Software. **Mengqi Li:** Data curation, Investigation. **Qi Sun:** Data curation, Investigation. **Lihong Jia:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.chemosphere.2020.126035>.

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Table 1S The primer sequences for the target genes

Genes	Accession number	Primer sequences (5'-3')
<i>Pik3r1</i>	NM_001024955	Forward: -CTGGGAGAGAGCAGGCAAATTA Reverse: -TTCCTTGGCTTTGCTCGGTT
<i>Akt1</i>	NM_009652.3	Forward: -TAGGCCCAGTCGCCCCG Reverse: -GCCCTCCTTCACAATGGCTA
<i>Prkaa2</i>	NM_178143.2	Forward: -ATTGACAGGCCATAAAGTGGCA Reverse: -CTCTTCAACCCGCCCCATGT
<i>Mtor</i>	NM_020009.2	Forward: -CTGTTACCTCACCCGTCCCT Reverse: -AGTTTCAGCATCGTGGGGTC
<i>Ulk1</i>	NM_009469.3	Forward: -CAGGTGCACAATTACCAGCG Reverse: -GGGGAGAAGGTGTGTAGGGA
<i>Becn1</i>	NM_019584.4	Forward: -ATCCAGGAACTCACAGCTCCA Reverse: -TGTAGACATCATCCTGGCTGGG
<i>Map1lc3b</i>	NM_026160.4	Forward: -TTATAGAGCGATACAAGGGGGAG Reverse: -CGCCGTCTGATTATCTTGATGAG
<i>p62/Sqstm1</i>	NM_011018.3	Forward: -TACCCACATCTCCCACCAGA Reverse: -ACAATGGTGGAGGGTGCTTC
<i>β-actin/Actb</i>	NM_007393	Forward: -AATCGTGCGTGACATCAAAGAG Reverse: -CAAGAAGGAAGGCTGGAAAAG