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Bisphenol A impairs macrophages through inhibiting autophagy via AMPK/mTOR signaling pathway and inducing apoptosis

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ABSTRACT

Bisphenol A (BPA) is a widespread endocrine disruptor that induces the impairment of immune cells, but the mechanism remains unknown. Macrophages are one of the most important immune cells in innate and adaptive immunity. In this study, we aimed to probe the effects of BPA on the damage of RAW264.7 cells and its mechanisms of action, especially focusing on the relationship between autophagy and apoptosis. Cells were pretreated with 10 mg/L LPS, or added autophagy activator RAPA, autophagy inhibitor 3-MA or Bcl-2 inhibitor ABT-737, then treated with BPA (0, 10, 100 and 200 μmol/L) for 12 h. Results have shown that BPA decreased the cell viability and disrupted secretory function by promoting pro-inflammatory cytokines TNF-α and IL-6 and reducing anti-inflammatory cytokines IL-10 TGF-β, as well as phagocytic ability. Moreover, autophagy was inhibited by BPA through decreasing p-AMPK/AMPK and increasing p-mTOR/mTOR, and further down-regulating autophagy proteins ATG6, LC3II/I ratio, and up-regulating autophagy flux protein p62. Additionally, BPA significantly increased Bax/Bcl-2 ratio, Caspase-3 expression and apoptosis rate. We found that RAPA ameliorated the cell viability, Bax/Bcl-2 ratio, and macrophage function damage induced by BPA. Intriguingly, ABT-737 might promote ATG6 expression. In summary, our study demonstrated that the effects of BPA on macrophages seemed to be mediated by inhibiting AMPK/mTOR-dependent autophagy and inducing apoptosis via endogenous mitochondrial pathway. Both Bcl-2 and ATG6 were involved in the regulation of apoptosis and autophagy by BPA. These findings provide a broader perspective for understanding the interaction between autophagy and apoptosis in BPA-induced immune cell injury.

1. Introduction

Bisphenol A [2, 2-bis (4-hydroxyphenyl) propane; BPA] is an organic synthetic plasticizer, which is a plastic ingredient used in the manufacturing of polycarbonate plastics and epoxy resin, including food containers, metal cans, medical equipment and dental sealant composites in surging quantities globally (Wang et al., 2020). The data of biological monitoring study showed that BPA was detected in the urine of 95% of American adults and the content of unmetabolized BPA in blood serum samples was in the range of 0.5–3 ng/mL, raising public health concerns (Ribeiro et al., 2017). As a result, numerous health regulatory agencies have begun to ban the use of BPA in baby plastic bottles in 2009–2012, including Canadian Food Inspection Agency (CFIA), European Union and the Food and Drug Administration of US (FDA), while France abolished the use of BPA in food containers in 2015 (Anand et al., 2020). BPA is categorized as an endocrine disruptor as it interferes with metabolic processes by mimicking the estrogen and affects the dynamic balance of the body. Remarkably, both epidemiological data and laboratory research have revealed that BPA has a negative effect on human diseases including diabetes, obesity, male infertility and immune disorder (Liu et al., 2021). With the aggravation of pollution, people pay more attention to the risk of BPA to immune function. Macrophages are extensive in the body and play a significant role in immune response, which produce cytokines when stimulated (Liu et al., 2014). In response to an external stressor, macrophages are induced to differentiate into different isoforms, including classically activated pro-inflammatory M1 macrophages and alternately activated anti-inflammatory M2 macrophages, which provide an important basis for understanding the

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regulation of the inflammatory process and immune response (Murray et al., 2014). M1-type macrophages are characterized by massive production of pro-inflammatory cytokines, including TNF-α, IL-6 and IL-1β, promoting inflammatory and leading to tumoricidal and microbial state (Ampem et al., 2019). In contrast, M2-type macrophages secrete anti-inflammatory cytokines such as IL-10, TGF-β (Lu et al., 2019). Inflammatory response is a protective stress response of the immune system to invasive pathogens, which is a double-edged sword (Rock et al., 2010). Strong inflammatory response will lead to excessive activation, synthesis and secretion of more inflammatory factors in immune cells. However, the mechanism of M1/M2 unbalance induced by BPA remains unclear.

To maintain the dynamic balance of the immune system, autophagy and apoptosis are usually the two major ways of cell self-degradation. Autophagy plays dual roles in cell survival and death, which maintains intracellular metabolic homeostasis by removing certain intracellular substances for metabolic homeostasis (Vanzo et al., 2020). The autophagy process is regulated and maintained by a large group of autophagy-related proteins and complex signaling pathways. Mammalian target of rapamycin (mTOR) plays a key role in the negative regulation of autophagy (Agarwal et al., 2020). Furthermore, several studies described the complex relationship between apoptosis and autophagy. Apoptosis is triggered mainly via mitochondrial pathway and death receptor pathway. Autophagy is a prerequisite for apoptosis, which could either restrain or advance apoptosis under certain conditions (Gump and Thorburn, 2011). However, the crosstalk of autophagy and apoptosis of macrophages induced by BPA needs to be further elucidated.

Therefore, we chose RAW264.7 cells for relevant studies that it resembles mammalian macrophages (Sakagami et al., 2009). The present work aimed to examine the molecular mechanism of BPA including autophagy and apoptosis in RAW264.7 cells. Furthermore, we investigated the role of AMPK/mTOR in BPA-induced cytotoxicity and impairment of macrophage function. More importantly, a crosstalk between autophagy and apoptosis was explored via Bcl-2 and ATG6. We hope this study could help to uncover the mechanisms of macrophage damage by BPA, and provide new evidence into the prevention of the endocrine disruptor-induced immunotoxicity.

2. Materials and methods

2.1. Chemicals and reagents

Bisphenol A (BPA, 99% pure) was obtained from Tokyo Chemical Industry (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM) and phosphate buffered saline (PBS) were provided by Hyclone (Logan City, USA). Lipopolysaccharide (LPS) and Dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, USA). Epizyme Biopharmaceutical Technology Co., Ltd. (Shanghai, China) provided SDS-PAGE gel, Phenylmethylsulfonyl fluoride (PMSF) and RIPA lysis buffer. Fetal bovine serum (FBS) and penicillin-streptomycin antibiotics were purchased from Biological Industries (Beit Haemek, Israel). Vazyme Biotech Co., Ltd. (Nanjing, China) provided Trizol reagent, the Enhanced Chemiluminescence (ECL) reagent, Bicinchoninic Acid Protein Assay (BCA), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Kit and Annexin V-FITC/PI Detection Kit. Caspase-Glo® 3/7 Assay was obtained from Promega Biological products Co., Ltd. (Madison, USA). Prime Script TM RT Master Mix cDNA Synthesis Kit, SYBR® Premix Ex Tap TM II Kit was purchased from TaKaRa (Tokyo, Japan). Antibodies against AMPK (#10929), p-AMPK (#383462), mTOR (#66888), p-mTOR (#67778), LC3 (#14600), p62 (#18420), ATG6 (#11306), TNF-α (#17590), IL-6 (#66146), IL-10 (#20850), TGF-β (#21898), Bax (#YT0458), Bcl-2(#YT0470), ERα (#24251), β-actin (#20536), FITC-conjugated anti-rabbit IgG, the horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and HRP-conjugated anti-mouse IgG were purchased from Proteintech (Wuhan, China) and Immunoway Biotechnology Company (Beijing, China), respectively. Rapamycin (RAPA), 3-Methyladenine (3-MA) and N-{4-[4-(4′-chloro-biphenyl-2-ylmethyl)-piperazin-1-yl]-benzoyl}-4-(3-dimethylamino-1-phenyl-sulfanylmethyl-propylamino)-3-nitro-benzenesulfonamide (ABT-737) were purchased from Selleck (Texas, USA).

2.2. Cell culture

RAW264.7 cells were obtained from the Shanghai Institute of Cell Biology (Chinese Academy of Sciences, China). Cells were cultured within the DMEM culture medium contained 10% FBS and 1% penicillin-streptomycin antibiotics, followed by incubation within the incubator under 37 °C and 5% CO_2 conditions for 24 h. When the cells were grown to 80–90%, they were sub-cultured. Logarithmic growth phase cells were used for experiments after three sub-cultures. BPA was dissolved in DMEM medium containing 0.1% DMSO. In order to eliminate the interference of irrelevant factors on cells, a control group was set up, that is, cells were treated with DMEM medium containing 0.1% DMSO. According to the pre-experimental results, cells were pretreated with 10 mg/L LPS for 30 min, then treated with BPA (0, 10, 100 and 200 μmol/L) for 12 h to detect the toxic effect of BPA on macrophage. In addition, to further elucidate the underlying mechanisms, RAW264.7 cells were pretreated with 1.25 μg/mL RAPA or 2.5 mmol/mL 3-MA or 2.5 μmol/L ABT-737 for 30 min and followed by the addition of 100 μmol/L BPA for 12 h, respectively.

2.3. Cytotoxicity assessment

The MTT kit was applied to detect the RAW264.7 cell viability. In brief, cells were treated with different concentrations of BPA for 12 h, with 10 mg/L LPS as activator. Then, the MTT solution (10 μL) was added to each well and incubated for 4 h at 37 ℃. Thereafter, 110 μL formazan solution was added to each well, and the microplate reader was used to measure the absorbance (OD) value at 490 nm. Cell viability was expressed as a percentage of living cells against total cell number.

2.4. Annexin V and PI assay for apoptosis

An Annexin V-FITC/PI staining kit was used to detect both early and late cellular apoptosis. RAW264.7 cells were seeded into 6-well plates and allowed to attach overnight and then incubated with (0, 10, 100 and 200 μmol/L) BPA for 12 h in LPS stimulation. Cells were collected by digestion with EDTA-free trypsin, then washed with cold PBS 2 times, and resuspended in Annexin V-binding buffer. Cells were further stained with 5 μL PI and 5 μL Annexin V-FITC for 10 min in the dark. The flow cytometry was used to assess stained cells and the FlowJo-v10 software was employed to analyze the flow cytometric data. The apoptosis rate was expressed by the sum of the percentage of late apoptotic cells in Q2 quadrant and early apoptotic cells in Q3 quadrant.

2.5. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

RAW264.7 cells were treated with various concentrations of BPA for 12 h, with 10 mg/L LPS as activator. Total RNA of RAW264.7 cells were extracted by the Trizol method. Then, total RNAs were reverse-transcribed in 20 μL system according to the instructions of reverse transcription test kit. The qRT-PCR reaction system was 10 μL SYBR, 1.6 μL mixed primer, 2 μL cDNA template, 6 μL DEPC water, to a total volume of 20 μL. The amplification procedure followed: pre-denaturation at 95 ℃ for 30 s; 95 ℃ for 5 s, 60 ℃ for 34 s, 40 cycles. Relative quantitative analysis was performed using GAPDH as internal reference gene, and was calculated using the standard $2^{-\Delta\Delta Ct}$ method. The mRNA name and primer sequences were listed in Supplementary Table S1.

2.6. Western blotting

RAW264.7 cells were treated with different concentrations of BPA for 12 h with 10 mg/L LPS, and then lysed using the precooled RIPA buffer supplemented with protease inhibitors and phosphatase inhibitors. Then the cell lysate was subjected to 20 min centrifugation at 10,000 g and 4 ℃, and protein concentration was determined by the BCA method. 8~12% SDS-PAGE gel electrophoresis, and followed by transfer to the PVDF membranes. Next, 5% nonfat milk dissolved within TBST was used to block the membranes at room temperature for 2 h, followed by incubation with primary antibodies at 4 ℃ overnight and HRP-labeled secondary antibodies on a horizontal shaker at room temperature for 1 h. Bands were visualized using the ECL detection kit and quantified using the ImageJ2x software. Statistical analysis was carried out with β-actin as the reference protein and the ratio of gray value of target protein to gray value of β-actin as relative value of target protein.

2.7. Immunofluorescence staining

After LPS stimulation, cells were treated with BPA for 12 h and fixed with 4% paraformaldehyde for 15 min. Then, the cells were permeabilized with 0.5% Triton X-100 for 20 min and incubated with 5% BSA for 30 min at room temperature. Cells were hatched with primary antibodies LC3, p62 and ATG6 at 4 ℃ overnight followed by incubation with FITC-conjugated anti-IgG secondary antibodies for 1 h. The slides were examined under a fluorescence microscope.

2.8. Detection of Caspase-3/7 activity test

After cells were treated with BPA for 12 h pretreated 10 mg/L LPS, the activity of Caspase-3/7 was detected with the Caspase-Glo®3/7 detection kit. First, the contents of the Caspase-Glo®3/7 buffer bottles were transferred to the amber bottles containing the Caspase-Glo®3/7 substrate when they were equilibrated to room temperature and then mixed by rotating or reversing the content, and the straight end was completely dissolved to form the Caspase-Glo®3/7 reagent. Subsequently, the 96-well plates were added with reagent and wrapped with aluminum platinum paper, followed by incubation at room temperature for at least 3 h. Finally, the culture plate was measured on the luminometer detector according to the instructions.

2.9. Detection of macrophage activity test

Macrophage phagocytic capacity was measured using neutral red solution. In short, cells with 10 mg/L LPS as activator were incubated with different concentrations of BPA for 12 h. After that, 100 μL neutral red solution was added to each well and incubated at 37 ℃ for 2 h. After washing twice with PBS, 150 μL of cell lysates were added to each well and lysed in a shaker for 15 min at room temperature. The phagocytic activity was estimated at a wavelength of 540 nm using a microplate reader and calculated as formula. Phagocytic index = experimental group A540 nm/ control group A540 nm × 100%. Meanwhile, after treatment with a neutral red solution for 2 h, RAW264.7 cells were carefully washed with PBS and observed under an inverted microscope and recorded.

2.10. Statistical analysis

All experiments were performed three times in triplicates and the values were represented as mean ± SD. Statistical analysis was performed by One-way ANOVA followed by LSD or Dunnett's test multiple comparisons using SPSS 20.0 software, $P < 0.05$ was used as a statistically significant difference.

3. Results

3.1. Effects of BPA exposure on the macrophage suivival and function

To evaluate the cytotoxicity of BPA, we detected the cell viability by MTT assay. The results have shown that 10 mg/L LPS significantly increased the viability of RAW264.7 cells compared with the control group. However, BPA inhibited the viability of cells in a dose-dependent manner, especially in the 100 μmol/L and 200 μmol/L BPA groups ($P < 0.05$) when compared with the 0 μmol/L BPA group. Besides, we also detected the viability of cells pretreated by autophagy activator RAPA and autophagy inhibitor 3-MA when exposure to 100 μmol/L BPA. We found that RAPA increased the viability of RAW264.7 cells ($P < 0.05$), while 3-MA strengthened BPA-induced toxicity as compaired with 100 μmol/L BPA group ($P < 0.05$) (Fig. 1A). To further investigate the effects of BPA on macrophage function, we examined the levels of secreted cytokines and the phagocytic capacity. The mRNA expression of TNF-α and TGF-β and the protein levels of IL-10 and TGF-β in 0 μmol/L BPA group was significantly increased compared with the control ($P < 0.05$). Meanwhile, the protein levels of IL-6 and TNF-α were significantly increased in 100 μmol/L and 200 μmol/L BPA groups, while the protein levels of IL-10 and TGF-β in BPA treatment groups were significantly decreased when compared with 0 μmol/L BPA group ($P < 0.05$) (Fig. 1B–D). Furthermore, the phagocytic activity of macrophages is an important functional indicator, and we also observed that 100 μmol/L and 200 μmol/L BPA decreased the uptake of neutral red in macrophages as shown in Fig. 1E and F ($P < 0.05$).

3.2. Alterations in expression levels of autophagy-related genes and proteins

To explore the potential molecular mechanisms for BPA-mediated autophagy in macrophages, related genes and proteins were analyzed. As shown in Fig. 2A, the mRNA expression of LC3II, p62, ATG5 and ATG12 were increased with BPA concentration compared with the 0 μmol/L BPA group, especially in the 100 μmol/L or 200 μmol/L BPA groups ($P < 0.05$). Meanwhile, 100 μmol/L BPA markedly decreased the protein level of ATG6 and LC3II/I ratio and elevated the protein level of p62 compared with the 0 μmol/L BPA group ($P < 0.05$) (Fig. 2B and C). Consistently, immunofluorescence assay showed that the expression of ATG6 and LC3 was significantly decreased in BPA-exposed groups compared with 0 μmol/L BPA group. Additionally, immunofluorescence results also showed that RAPA increased the levels of ATG6 and LC3 inhibited by BPA (Fig. 2D).

3.3. Effects of BPA on autophagy through AMPK/mTOR signaling pathway

In the presence of external stimuli, the AMPK/mTOR signal transduction pathway exerts a vital part in cell autophagy (Agarwal et al., 2020). As shown in Fig. 3A–C, although no significant change was observed in the mRNA expression of AMPK and mTOR, 100 μmol/L BPA markedly down-regulated the ratio of p-AMPK/AMPK and up-regulated the ratio of p-mTOR/mTOR when compared with 0 μmol/L BPA group in RAW264.7 cells ($P < 0.05$). In addition, mTOR inhibitor RAPA was used in the presence or absence of BPA. As shown in Fig. 3, RAPA sharply enhanced p-AMPK/AMPK ratio and the expression of ATG6 and LC3II, whereas declined p-mTOR/mTOR ratio compared with 100 μmol/L BPA ($P < 0.05$). That is, RAPA partially reversed the effects on BPA-mediated autophagy in RAW264.7 cells, which verified that BPA effectively interfered autophagy process by inhibiting AMPK/mTOR pathway.

3.4. Effects of autophagy intervention agents on cytokine secretion and phagocytosis

RAPA was used to explore the role of mTOR in BPA-induced

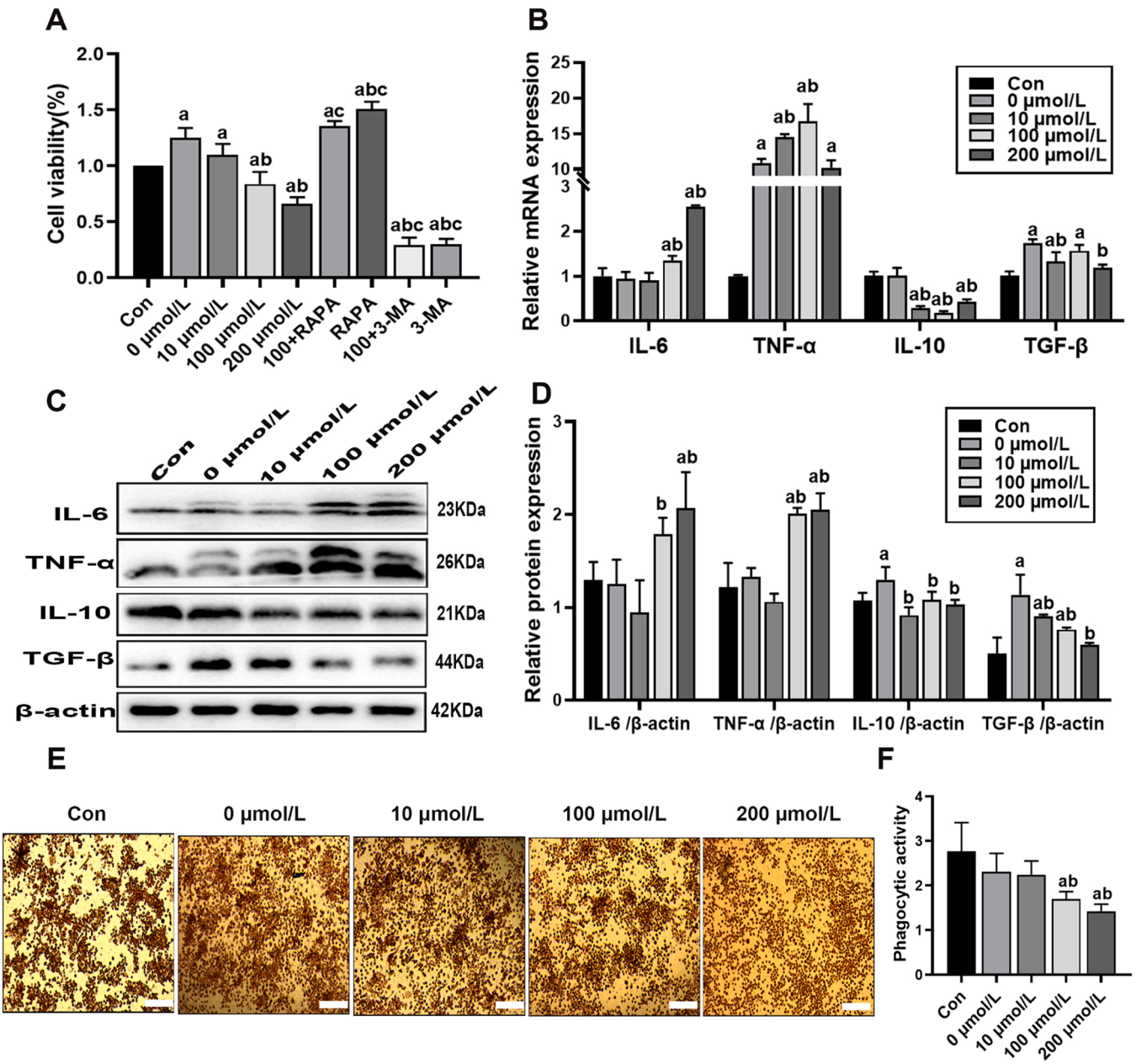

Fig. 1. Effects of BPA exposure on the macrophage suivival and function. Cells were treated with BPA (0, 10, 100 and 200 μmol/L) or 100 μmol/L BPA+ 1.25 μg/mL RAPA or 1.25 μg/mL RAPA or 100 μmol/L BPA+ 2.5 mmol/mL 3-MA or 2.5 mmol/mL 3-MA for 12 h. (A) Cell viability was analyzed by MTT assay. (B) The mRNA expression of cytokines in RAW264.7 cells. (C, D) Western blotting analysis of cytokines proteins. (E) The phagocytic activity of cells was observed by microscopy. Scale bar 100 μm. (F) Quantification of phagocytic activity in (E). Values are described as the mean±SD of three independent examinations (n = 3). $^{a}P < 0.05$, compared with the control group; $^{b}P < 0.05$, compared with 0 μmol/L BPA group; $^{c}P < 0.05$, compared with 100 μmol/L BPA group.

macrophage dysfunction. As shown in Fig. 4A and B, compared with 100 μmol/L BPA group, RAPA significantly attenuated pro-inflammatory factors IL-6 and TNF-α, and increased the anti-inflammatory factors IL-10 and TGF-β in macrophages ($P < 0.05$). Moreover, RAPA improved the uptake of neutral red in macrophages when exposed to BPA, while 3-MA aggravated the damage of phagocytosis induced by BPA ($P < 0.05$) (Fig. 4C and D). These results indicated that autophagy might be participated in impairing function of macrophages induced by BPA.

3.5. Effects of BPA-mediated autophagy on apoptosis

Numerous studies have indicated two sides of autophagy, including cytoprotection and cytotoxicity (Hou et al., 2019). Firstly, we revealed the effect of BPA on apoptosis. The exposure to 100 μmol/L and 200 μmol/L BPA could facilitate the apoptosis of RAW264.7 cells by flow cytometry ($P < 0.05$) (Fig. 5A and B). In addition, we also measured the levels of apoptotic indicators. 10, 100 and 200 μmol/L BPA significantly increased the mRNA expression of Bax, Caspase-3 and Caspase-9 in RAW264.7 cells ($P < 0.05$), but the mRNA expression of Caspase-8 was not statistically distinguishable from that of the 0 μmol/L BPA group (Fig. 5C). Consistently, the ratio of Bax/Bcl-2 and the activity of Caspase-3/7 were significantly increased by 100 μmol/L BPA ($P < 0.05$) (Fig. 5D–F). Then, RAPA and 3-MA were used to further investigate the relationship of BPA-mediated autophagy with the apoptosis in RAW264.7 cells. Intriguingly, RAPA markedly reversed Bax/Bcl-2 ratio compaired with 100 μmol/L BPA group ($P < 0.05$). On the contrary, 3-MA further enhanced Bax/Bcl-2 ratio ($P < 0.05$) (Fig. 5D and E). In addition, both RAPA and 3-MA obviously increased the activity of

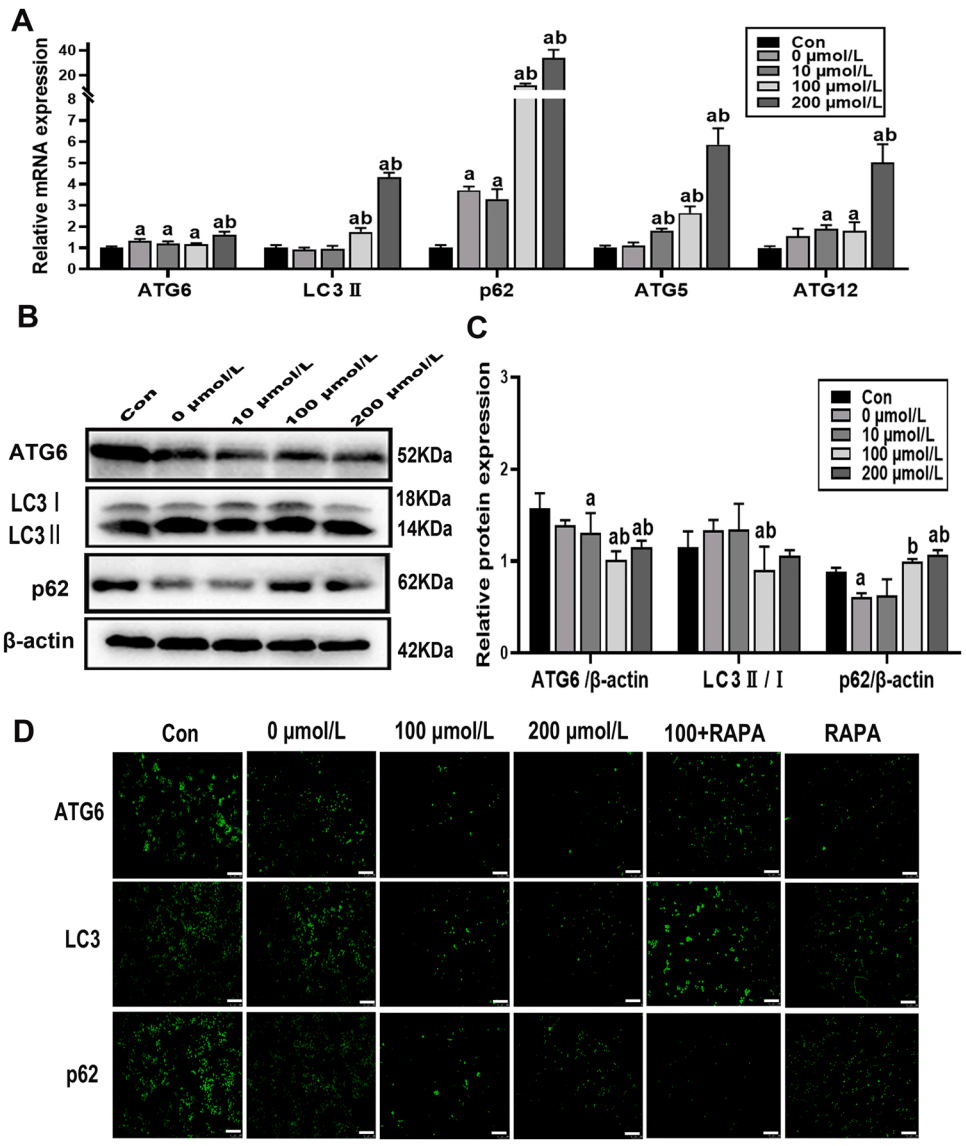

Fig. 2. Alterations in expression levels of autophagy-related genes and proteins. Cells were treated with BPA (0 and 100 μmol/L) or 100 μmol/L BPA+ 1.25 μg/mL RAPA or 1.25 μg/mL RAPA for 12 h. (A) The expression of autophagy-related genes. (B, C) Western blotting analysis of autophagy related proteins. (D) Immunofluorescence staining for ATG6, LC3 and p62 in RAW264.7 cells after BPA treatment. Scale bar 100 μm. Values are described as the mean±SD of three independent examinations (n = 3). $^{a}P < 0.05$, compared with the control group; $^{b}P < 0.05$, compared with 0 μmol/L BPA group; $^{c}P < 0.05$, compared with 100 μmol/L BPA group.

Caspase-3/7 compaired with 100 μmol/L BPA group (Fig. 5F).

3.6. Effects of Bcl-2 inhibitor ABT-737 on ATG6 expression

A recent study reported that Bcl-2 and ATG6 are involved in the regulation of both apoptosis and autophagy(Chen et al., 2019). Therefore, we used ABT-737, an inhibitor of Bcl-2, to observe the effect on ATG6 expression. Firstly, we detected the inhibition of ABT-737 on cell survial by MTT, and the results showed that ABT-737 could significantly reduce the cell viability at 2.5–10 μmol/L ($P < 0.05$). 2.5 μmol/L ABT-737 obviously inhibited cell survial compaired with 100 μmol/L BPA group (Fig. 6A and B). So we chose 2.5 μmol/L for the subsequent experimental studies. As shown in Fig. 6C–E, Bcl-2 inhibitor increased the mRNA and protein levels of ATG6 compaired with 100 μmol/L BPA group.

4. Discussion

Considerable attention has been paid to the adverse effects of BPA on immune system, which include abnormal immune response, immune dysfunction and cell damage (Malaisé et al., 2018). Macrophage is one of the most important immune cells and it is reported that BPA has the significant effects on the growth, differentiation and function of macrophage. However, the underlying molecular mechanisms of BPA on macrophage toxicity remain largely indistinct. Numerous studies suggest that the regulation of autophagy on cell survival has two sides, depending on its degree or magnitude (Luo et al., 2021). Mild autophagy promotes cell survival to a certain extent by protecting cells from pathological and physiological pressure, while excessive autophagy can induce autophagy death, also known as programmed cell death (Zhu and Zhang, 2018). To explore the role of autophagy in macrophage survival, autophagy activator RAPA and autophagy inhibitor 3-MA were used in the present study. Data showed the cell viability was significantly decreased to 81.3%, 70.7% and 55.2% respectively with the rise of BPA concentration. The addition of RAPA could partially reverse the effect, while 3-MA aggravates the toxic effects. Furthermore, the flow cytometry analysis showed that the apoptosis rate was enhanced with the increase of BPA concentration, which was opposite to the decrease of cell survival. Besides, Bcl-2 inhibitor ABT-737 obviously inhibited cell survial. Therefore, our results revealed that autophagy and apoptosis are both involved in regulating macrophage survival of exposure to BPA.

Autophagy is a highly dynamic process, playing a fundamental role in clearing accumulated protein synthesis and organelles biosynthesis (Liu et al., 2021). Interestingly, BPA exerts the positive or negative

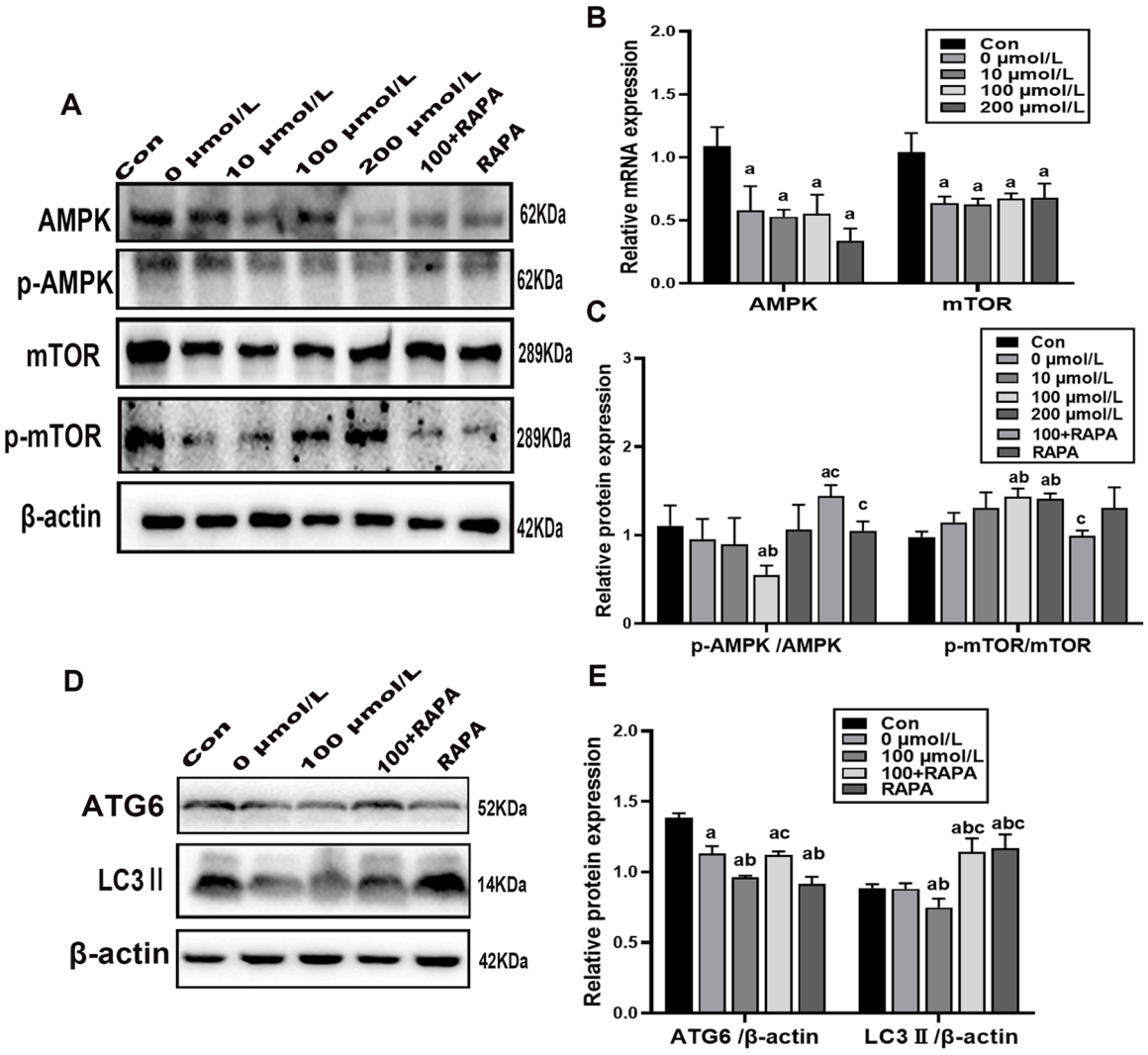

Fig. 3. Effects of BPA on autophagy through AMPK/mTOR signaling pathway. Cells were treated with BPA (0 and 100 μmol/L), 100 μmol/L BPA+ 1.25 μg/mL RAPA or 1.25 μg/mL RAPA for 12 h. (A, C) Western blotting analysis of AMPK/mTOR signal related proteins. (B) The mRNA expression of AMPK and mTOR treated with BPA. (D, E) Western blotting analysis of autophagy related proteins. Values are described as the mean±SD of three independent examinations (n = 3). $^{a}P < 0.05$, compared with the control group; $^{b}P < 0.05$, compared with 0 μmol/L BPA group; $^{c}P < 0.05$, compared with 100 μmol/L BPA group.

regulation on autophagy. There are reports suggesting BPA induced liver lipid deposition by inhibiting autophagosome degradation, or increased inflammation by enhancing autophagy (Song et al., 2019; Wang et al., 2021). In addition, studies have also reported that BPA inhibited the cell viability of osteocytes MLO-Y4 and goat Sertoli Cells (gSCs), and significantly increased the levels of autophagy-regulated proteins in inactive state (Zhang et al., 2017, 2021). Pervious work showed that BPA increased the number of autophagosomes by transfection with single fluorescent GFP-LC3 adenovirus after 5 mg/L LPS stimulation. To better investigate the effect of BPA on autophagy under different activation conditions, 10 mg/L LPS was chosen as activator. The present results displayed that exposure to BPA decreased the levels of the autophagy proteins ATG6 and the ratio of LC3II/I, but up-regulated p62 expression. ATG6 plays a central role by helping recruit autophagy proteins during autophagy initiation. The ratio of LC3II/I reflects the balance between the rate of autophagosome production and degradation in the dynamic pathway, while the main function of p62 is to assist LC3 II binding to degraded target proteins or damaged organelles to regulate the degradation of autophagosomes (Li et al., 2019). Our data suggested that BPA inhibited autophagy through affecting autophagy initiation as well as the number of autophagosomes, further leading to p62 accumulation. All in all, different circumstances containing cell species, cell types, exposure dose as well as activation condition regulate the effect of BPA on autophagy.

AMPK is a positive autophagy regulator, down-regulating the phosphorylation of mTOR to adapt to energy metabolism, which plays a key role in regulating cellular and systemic energy homeostasis. To investigate the role of AMPK/mTOR pathway in BPA-mediated autophagy in macrophages, mTOR inhibitor RAPA was adopted. We observed that p-AMPK/AMPK ratio was down-regulated, while p-mTOR/mTOR ratio was gradually increased. Besides, RAPA improved the inhibition of autophagy induced by BPA through enhancing the levels of ATG6, LC3II in macrophages. Our results were similar with that Aflatoxin B1-induced toxicity in Leydig cells was characterized by reducing cell number via suppressing AMPK/mTOR-mediated autophagy (Chen et al., 2019). Hence, the results suggested that BPA suppresses autophagy by AMPK/mTOR signal pathway.

Macrophages play an extremely important role in inflammation and the innate immune response, which exert the defense function and maintain the stability of internal environment through phenotypic polarization, cytokines release and phagocytosis of foreign particles (Acaroz et al., 2019). To detect the damage of macrophage function caused by BPA, we measured phagocytic ability as well as the changes of secreted cytokines. The results showed that the phagocytosis of

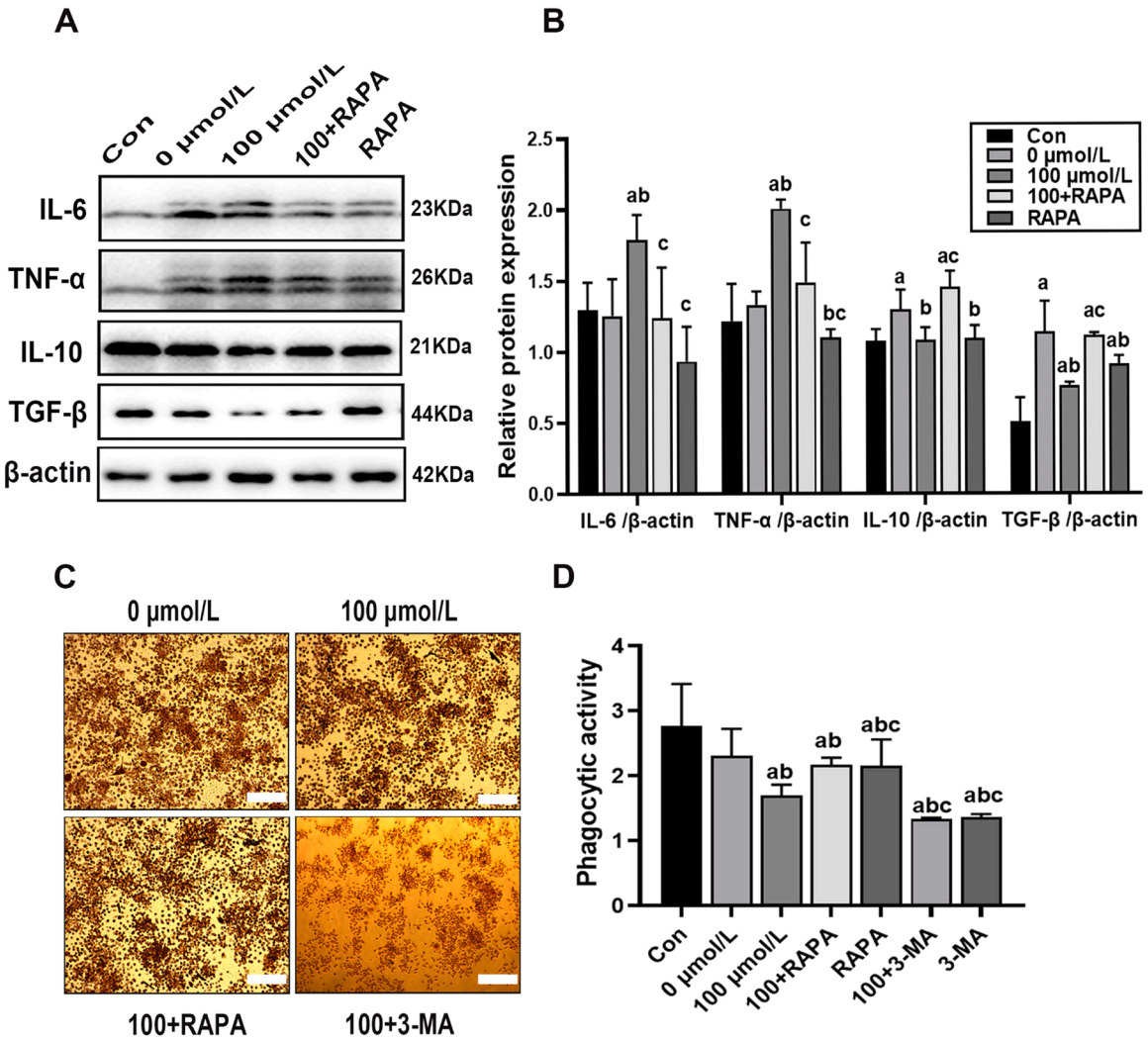

Fig. 4. Effects of RAPA on cytokine secretion and phagocytosis. Cells were treated with BPA (0 and 100 μmol/L), 100 μmol/L BPA+ 1.25 μg/mL RAPA or 1.25 μg/mL RAPA or 100 μmol/L BPA+ 2.5 mmol/mL 3-MA or 2.5 mmol/mL 3-MA for 12 h. (A,B) Western blotting analysis of cytokines. (C, D) The phagocytic activity of cells was observed by microscopy. Scale bar 100 μm. Values are described as the mean ± SD of three independent examinations (n = 3). $^{a}P < 0.05$, compared with the control group; $^{b}P < 0.05$, compared with 0 μmol/L BPA group; $^{c}P < 0.05$, compared with 100 μmol/L BPA group.

macrophages was decreased by BPA, and this effect was aggravated by 3-MA. Furthermore, we found that BPA significantly promoted the polarization of macrophage toward M1-type, shown as high expression of pro-inflammatory cytokines TNF-α and IL-6 which initiate the inflammatory response. Meanwhile, BPA significantly inhibited macrophage polarization toward M2-type characterized by reducing anti-inflammatory cytokines such as IL-10 and TGF-β, which are involved in angiogenesis, debris scavengers, resolved inflammation and the Th2 immune response (Lee et al., 2018). We also examined the expression of the macrophage M1 and M2-type polarization markers CD86 and CD206, respectively (Fig. S1). Data suggested that BPA could induce M1 polarization and inhibit M2 polarization of macrophages. However, the levels of anti-inflammatory cytokines were enhanced and the levels of pro-inflammatory cytokines were down-regulated with RAPA. Our results were in agreement with the previous report (Kuan et al., 2012). It is reported that autophagy deficiency increases the toxic sensitivity of hepatocytes to cytokines IL-1β and TNF-α, and when both coexist, it leads to higher cell mortality (Iracheta-Vellve et al., 2015). Researchers have also noted that IL-10 removes damaged mitochondria by promoting mitochondrial autophagy. Damaged mitochondria are accumulated in macrophages when IL-10 signaling is lacking, promoting inflammatory production. Annexin A1 modulates macrophage polarization via AMPK-mTOR pathway (Xu et al., 2021). Therefore, the results in this study indicated that BPA impairs macrophage function by inhibiting AMPK/mTOR-depentent autophagy, which is characterized by promoting the secretion of M1-type cytokines and reducing M2-type cytokines and phagocytic function.

Apoptosis usually has an inextricable relationship with autophagy in maintaining the counterpoise of certain cell populations within the tissues. Apoptosis will be activated when the external pressure is far more than cells bear and autophagy is unable to save cells (Rahaman et al., 2020). The main signal pathways of apoptosis include the endogenous mitochondrial pathway and the exogenous death receptor pathway (Wong, 2011). In our study, BPA increased the level of Bax and the ratio of Bax/Bcl-2, and up-regulated the gene expression of Caspase-9 and the activity of Caspase3/7, which showed that the endogenous mitochondrial apoptosis pathway was activated. Moreover, there is no significant effect on Caspase-8 expression in macrophages by BPA. Given that, our results suggested that BPA activated the pro-apoptotic protein Bax, initiated Caspase-9 activation, and finally activated the death executor Caspase3/7 to cause cell collapse. Our data was in consistence with the findings that BPA induced mitochondrial apoptosis pathway by increasing Bax and Caspase-3 in prepubertal rat testes (Su et al., 2018). In brief, BPA under this condition may induce apoptosis through

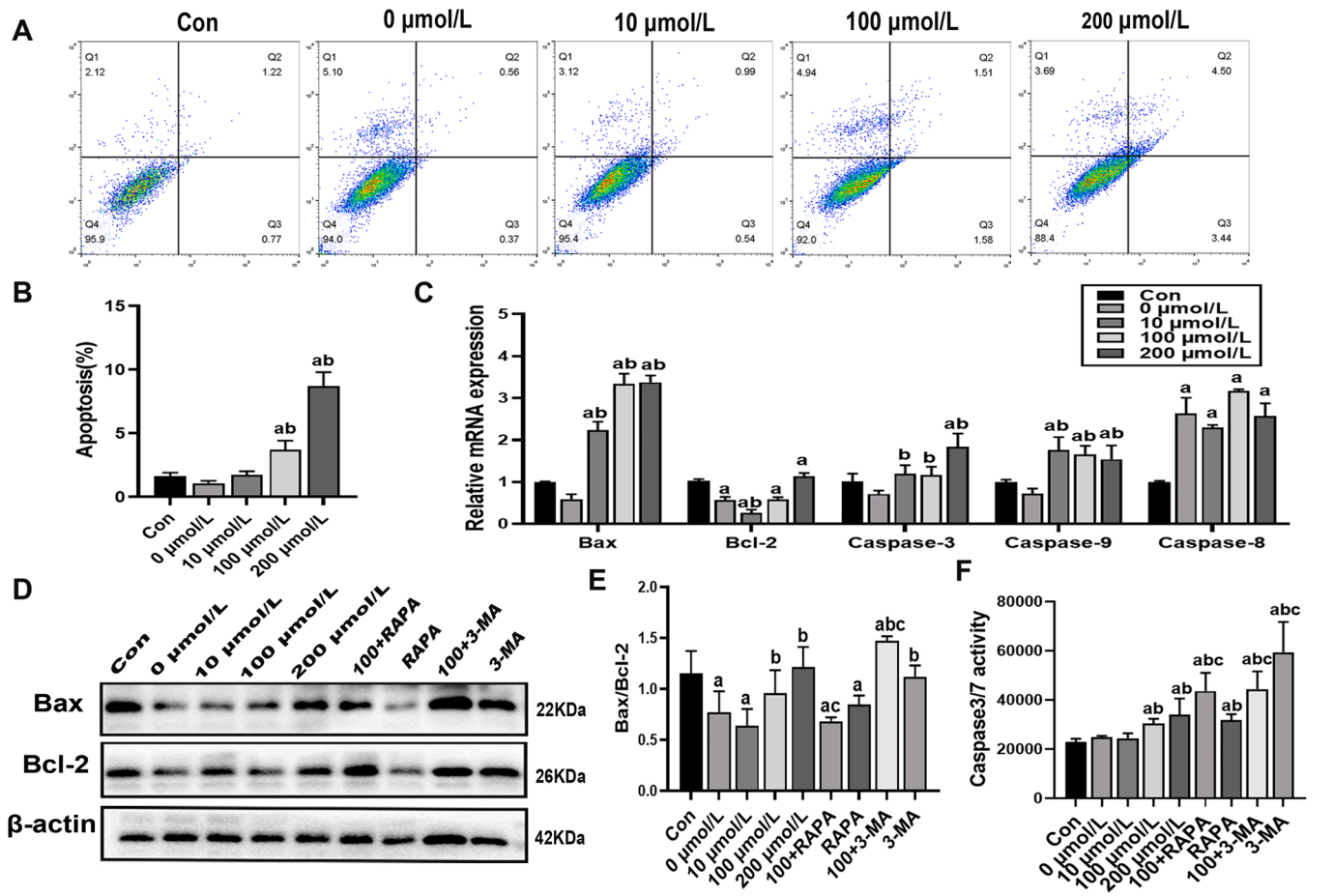

Fig. 5. Effects of BPA-mediated autophagy on apoptosis. Cells were treated with BPA (0, 10, 100 and 200 μmol/L), 100 μmol/L BPA+ 1.25 μg/mL RAPA, 1.25 μg/mL RAPA, 100 μmol/L BPA+ 2.5 mmol/mL 3-MA or 2.5 mmol/mL 3-MA for 12 h. (A) Apoptosis rate of cells was detected by flow cytometry. (B) The percentage of apoptosis rate in each group. (C) The mRNA expression of apoptosis related genes. (D, E) Western blotting analysis of apoptosis related proteins. (F) Detection of Caspase-3/7 activity. Values are described as the mean±SD of three independent examinations (n = 3). $^{a}P < 0.05$, compared with the control group; $^{b}P < 0.05$, compared with 0 μmol/L BPA group; $^{c}P < 0.05$, compared with 100 μmol/L BPA group.

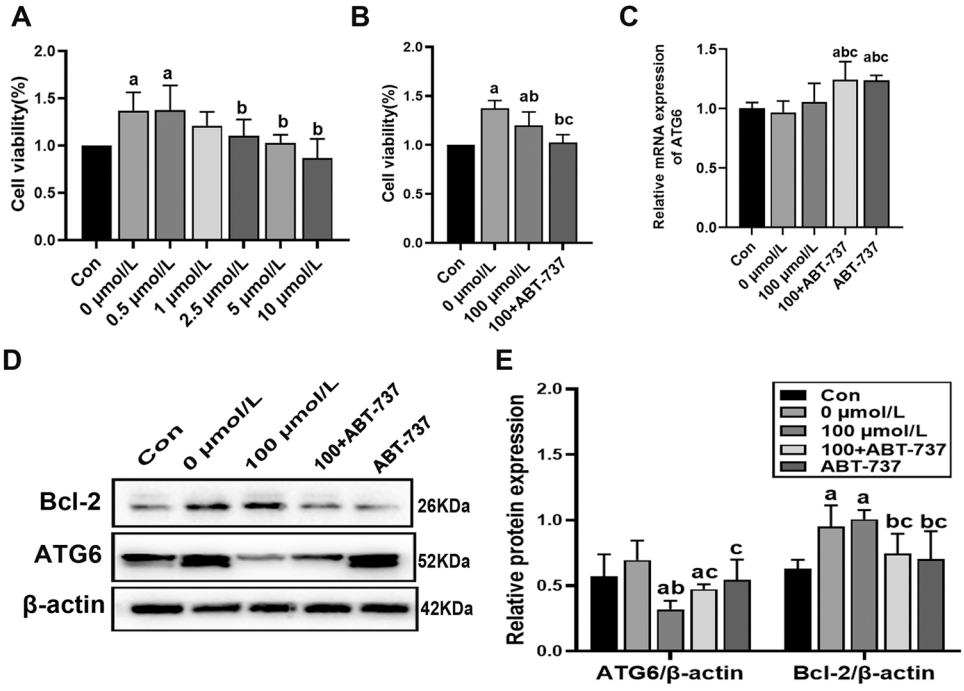

Fig. 6. Effects of Bcl-2 inhibitor ABT-737 on ATG6 expression. (A,B) Cell viability was analyzed by MTT assay. (A) RAW246.7 cells were treated with 0, 0.5, 1, 2.5, 5 and 10 μmol/L ABT-737 for 12 h. (B–E) Cells were treated with BPA (0, 100 μmol/L), 100 μmol/L BPA+ 2.5 μmol/L ABT-737 or 2.5 μmol/L ABT-737 for 12 h. (C) The gene expression of Bcl-2 and ATG6. (D, E) Western blotting analysis of Bcl-2 and ATG6. Values are described as the mean±SD of three independent examinations (n = 3). $^{a}P < 0.05$, compared with the control group; $^{b}P < 0.05$, compared with 0 μmol/L BPA group; $^{c}P < 0.05$, compared with 100 μmol/L BPA group.

endogenous mitochondrial pathway instead of Caspase-8-dependent exogenous death receptor pathway in macrophages.

The mechanisms of immunotoxicity of environmental pollutants mainly focus on autophagy, apoptosis, calcium homeostasis, oxidative damage, genotoxic pathway, inflammatory response, estrogen receptor pathway and so on, especially the interaction between autophagy and apoptosis has received increasing attention. Research has suggested that autophagy and apoptosis may interact with each other through cooperation, antagonism or help under different conditions (Gump and Thorburn, 2011). In general, apoptosis can be activated by autophagy, usually located downstream of autophagy (Hou et al., 2019). For example, the survivin inhibitor YM155 inhibited the growth of prostate cancer cells by inducing autophagy-dependent apoptosis (Yokoyama et al., 2008). On the contrary, autophagy may attenuate Copper-induced apoptosis in RAW264.7 cells (Luo et al., 2021). To further investigate the effect of autophagy on apoptosis, RAPA and 3-MA were researched on the BPA-induced apoptosis. Findings in our study indicated that RAPA treatment inhibited the ratio of Bax to Bcl-2 mediated by BPA, while 3-MA had the opposite effect. Our results were similar with those proposing that moderate induction of autophagy reduced the rate of apoptosis through the application of RAPA (Chen et al., 2019; Luo et al., 2021). Interestingly, we determined that autophagy activation or inhibition increases Caspase-3/7 activity, which discovered that the interaction of autophagy on BPA-induced apoptosis is complex. This phenomenon could be explained by the finding that ATG6 could enhance apoptosis by increasing Caspase-9 activity and then activating Caspase-3 activity (Furuya et al., 2005). Taken together, our results showed that autophagy negatively regulates apoptosis induced by BPA, which may indicate the protective effect of autophagy on apoptosis.

There must be common signal pathways and regulatory proteins in the multiple interactions between apoptosis and autophagy (Zeng et al., 2021). In order to explore the specific action points of apoptosis and autophagy, Bcl-2 inhibitor (ABT-737) was chosen to detect the effect on ATG6. Our results revealed ABT-737 increased the level of ATG6. ATG6 has been proved to be a BH3-only protein, a mammalian autophagy gene homologous to yeast autophagy gene, which can bind to anti-apoptotic protein Bcl-2 to directly regulate autophagy and apoptosis (Chen et al., 2019). On the other hand, autophagy is inhibited by caspase-mediated cleavage of ATG6, namely, some important autophagic proteins including ATG6 are digested when caspase is highly activated, eventually paralyzing the autophagy process and terminating cell self-protection (Mariño et al., 2014). Our results observed that BPA increased the activity of Caspase-3/7 and decreased the protein expression of ATG6, which further confirmed the above report. Some studies have pointed out that the overexpression of ATG5 makes a variety of cells more prone to apoptosis in breast tumor cells (Luo and Rubinsztein, 2007). Researchers also have found that ATG12 promotes apoptosis by binding to Bcl-2 and Mcl-1 (Rubinstein et al., 2011). We detected that the gene expression level of ATG5 and ATG12 were significantly enhanced, meanwhile apoptosis was also increased by BPA. Accordingly, we concluded that autophagy-related protein ATG6 and anti-apoptotic Bcl-2 protein are both involved in the mutual regulation between autophagy and apoptosis, meanwhile Caspase-3, ATG5 and ATG12 may also participate in interaction. But the specific mechanisms need to be further investigated.

It is well known that estrogen receptors including estrogen receptor-alpha (ERα) and -beta (ERβ) are quite important for BPA-induced toxic effect. Several studies pointed out that ERα is the main estrogen nuclear receptor in macrophage biology (Calippe et al., 2010). We observed that BPA increased the expression of ERα (Fig. S2.), which was consistent with the fact that BPA exerts the immune modulatory effect on fish macrophages via ERα in vitro (Yang et al., 2015). Study has shown that the change of estrogen receptor can regulate autophagy through AMPK/mTOR pathway (Gandhi et al., 2021). Research also has reported that the expression level of ERα is closely related to the proliferation and apoptosis of endometrial cancer cells (Biswas et al., 2020). Besides, estrogen receptors were involved in the regulation of the activity of M1 and M2 subtype of human peripheral blood monocyte-derived macrophages (Teixeira et al., 2016). Hence, we speculated that BPA regulates apoptosis and autophagy by ERα, which further induces macrophage toxicity.

5. Conclusion

We provide evidence in this study that autophagy and apoptosis are involved in BPA-induced cytotoxicity and function damage in macrophages. Data support that BPA suppresses AMPK/mTOR-dependent autophagy and induces apoptosis through the endogenous mitochondrial pathway via ERα. And there is a close link between autophagy and apoptosis through the mediation of Bcl-2 and ATG6. Accordingly, these findings provide new information for further study about BPA-induced immunotoxicity.

CRediT authorship contribution statement

Mingfei Wu: Writing – original draft, Formal analysis, Visualization, Validation. **Yan Cong:** Formal analysis, Visualization, Validation. **Kailu Wang:** Formal analysis, Investigation. **Haiyang Yu:** Writing – review & editing. **Xuan Zhang:** Supervision, Writing – review & editing. **Mingyue Ma:** Methodology, Writing – review & editing. **Zhiwen Duan:** Conceptualization, Methodology, Supervision. **Xiucong Pei:** Conceptualization, Project administration, Methodology, Resources.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Availability of data and materials

All data generated during this study are included in this published article.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2022.113395.

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