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Bisphenol A induces lysosomal-dependent cell death in hepatocytes via mitochondrial ROS-mediated lysosomal membrane permeabilization

Yuelin Chen ^{a,1}, Lingxi Lang ^{a,1}, Jinping Hu ^c, Huanhuan Peng ^a, Jing Lu ^a, Shuang Guan ^{a,b*}^a College of Food science and Engineering, Jilin University, Changchun, Jilin 130062, China^b State Key Laboratory for Diagnosis and Treatment of Severe Zoonotic Infectious Diseases, Key Laboratory for Zoonosis Research of the Ministry of Education, Institute of Zoonosis, and College of Veterinary Medicine, Jilin University, Changchun 130062, China^c College of Animal Science, Jilin University, Changchun, Jilin 130062, China

ABSTRACT: Bisphenol A (BPA), an organic compound widely found in food packaging materials, is inevitably released into the food and beverages. BPA has been confirmed to be hepatotoxic, however, the precise mechanism remains unclear. In this study, we demonstrated that lysosomal membrane permeabilization (LMP) mediates BPA-induced liver injury. Further mechanistic investigation revealed that BPA resulted in cathepsin B (CTSB) release and iron efflux by triggering LMP, ultimately inducing lysosomal-dependent pyroptosis and ferroptosis. Furthermore, mitochondrial reactive oxygen species (mtROS) were identified as the trigger for BPA-induced LMP. BPA elicited mtROS overproduction by disrupting mitochondrial quality control, thereby inducing LMP. In conclusion, we demonstrated that BPA caused CTSB release and lysosomal iron efflux via the mtROS-mediated LMP, which ultimately resulted in pyroptosis and ferroptosis. Our study revealed that the mtROS/LMP axis may be a common pathway for lysosomal-dependent pyroptosis and ferroptosis, providing novel mechanistic insights into BPA-induced hepatotoxicity.

Keywords: BPA, liver, pyroptosis, ferroptosis, lysosomal membrane permeabilization, mtROS

1. Introduction

Bisphenol A (BPA) is a synthetic chemical compound employed as a monomer in the production of polycarbonate plastics, which are extensively utilized in food and beverage containers, medical devices, thermal paper, and dental materials packaging ^[1]. Industrial emissions and product leaching contribute to the release of BPA into the environment, resulting in contamination of food, beverages, air, and soil ^[2,3]. Research has indicated that BPA can leach from plastic containers into food or beverages ^[4], particularly under conditions of elevated temperature or acidity, thereby contributing to food contamination ^[5,6]. The widespread presence of BPA in human biofluids, including urine and blood, has been consistently confirmed by biomonitoring studies ^[7]. As the primary metabolic organ in the body, the liver is vulnerable to BPA-induced damage ^[8]. Chronic exposure to BPA has been documented to cause hepatic injury and metabolic dysfunction ^[9]. Nevertheless, the precise mechanisms underlying BPA-induced liver injury remain to be fully elucidated.

¹ These authors contributed equally to this work.

*Corresponding author

guan_shuang22@jlu.edu.cn

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The liver exhibits a high dependence on the lysosomal system to maintain homeostasis^[10]. Lysosomal dysfunction directly drives hepatic injury by triggering programmed cell death and amplifying inflammation^[11]. As early as 1959, Duve proposed the "Suicide Bag Hypothesis," positing that cellular destruction and death occur when lysosomal hydrolases are released into the cytosol due to instability of the lysosomal membrane^[12]. Subsequent research has revealed that the underlying mechanisms extend far beyond simple "suicide", instead constituting a precisely regulated programmed cell death event^[13]. Loss of lysosomal membrane integrity induces LMP, leading to the leakage of lysosomal contents into the cytosol and the activation of death signaling pathways. This process is defined as Lysosome-dependent cell death (LDCD)^[14]. Previous studies have demonstrated that amelioration of lysosomal function alleviates BPA-induced cell death in C2C12 cells^[15]. Furthermore, BPA has been shown to induce lysosomal dysfunction in HepG2 cells^[16], which prompted our investigation into the potential role of LDCD in BPA-induced liver injury. While the initiation is strictly dependent on LMP, LDCD can adopt apoptotic, ferroptotic, or pyroptotic-like features based on the cellular context and effector molecules^[17].

Cathepsins serve as the primary effector molecules in LDCD^[18]. CTSB can activate the NLRP3 inflammasome. Activated NLRP3 recruits caspase-1, leading to the maturation of IL-1 β family cytokines and gasdermin-D (GSDMD), ultimately triggering pyroptosis. Inhibition of CTSB expression or activity can suppress pyroptosis^[19]. However, cathepsins are not the sole effector molecules in LDCD. Due to the substantial storage of iron ions within lysosomes, LMP results in iron efflux, thereby inducing ferroptosis^[20]. Notably, BPA exposure has been reported to significantly increase hepatic iron content^[21]. Meanwhile, Das et al. reported that pharmacological inhibition of NLRP3 alleviates, but does not completely eliminate, BPA-induced liver injury^[22]. This suggests that BPA may induce both pyroptosis and ferroptosis through LMP, synergistically driving hepatic injury.

Reactive oxygen species (ROS) constitute a core factor responsible for inducing LMP. The iron-rich environment within lysosomes renders the lysosomal membrane particularly susceptible to ROS. ROS can diffuse into lysosomes and undergo the Fenton reaction in the presence of Fe²⁺, generating a substantial quantity of highly reactive free radicals. These radicals attack the lysosomal membrane, ultimately leading to LMP^[23]. Mitochondria serve as the central hub for cellular redox homeostasis. Mitochondrial ROS (mtROS) generated by damaged mitochondria represent the primary intracellular source of ROS^[23]. Although BPA has been reported to disrupt mitochondrial function, inducing excessive mtROS production, whether BPA induces LMP via mitochondria-lysosome crosstalk remains unclear. In this study, we sought to determine whether BPA-induced liver injury is associated with LDCD and to investigate its underlying mechanisms. Our findings revealed that BPA triggered mtROS-mediated LMP by disrupting mitochondrial function, which led to CTSB leakage and iron efflux, ultimately triggering lysosome-dependent pyroptosis and ferroptosis.

2. Materials and methods

2.1 Reagents

BPA (Cat. no.: B108652) was purchased from Aladdin (Shanghai, China). Mitochondria-targeted antioxidant agent (Mito-TEMPO) (Cat. no.: HY-W001187), N-acetylcysteine (NAC, Cat. no.: HY-B0215), CA074-methyl ester (CA074-ME, Cat. no.: HY-100350), Deferoxamine (DFO, Cat. no.: HY-B1625), and mannose (Cat. no.: HY-N0379) were bought from Med Chem Express (Shanghai, China).

2.2. Animal studies

The mice used in this study were C57BL/6 mice (male, aged 6-8 weeks), obtained from Liaoning Changsheng Biotechnology Co., Ltd. (Liaoning, China). All animal experiments and protocols were approved by the Ethical Committee for Animal Experiments of Jilin University (Approval no. SY202405012). Mice were given 50 and 100 $\mu\text{g/kg}$ bw/day by gavage. The concentrations used in mice were based on previous studies [24,25]. The 50 $\mu\text{g/kg}$ bw/day used in mice can be converted from body surface area to a human equivalent dose of approximately 4 $\mu\text{g/kg}$ bw/day, which is the provisional acceptable reference dose for BPA proposed by the European Food Safety Authority in 2015. According to a study in Taiwan^[26], the dietary concentrations of BPA were 419.26 $\mu\text{g/kg}$ (beef), 6.15 $\mu\text{g/kg}$ (vegetables), 5.10 $\mu\text{g/kg}$ (fruits), 9.78 $\mu\text{g/kg}$ (eggs), and 4.49 $\mu\text{g/kg}$ (milk), which, based on the weight of an adult of 60 kg, would result in daily exposure to a dose higher than 4 $\mu\text{g/bw/kg}$. The mice were assigned to nine groups at random ($n = 6$): control group, low-dose BPA (50 $\mu\text{g/kg}$), high-dose BPA (100 $\mu\text{g/kg}$), BPA (100 $\mu\text{g/kg}$) + MCC950 (10 mg/kg), BPA (100 $\mu\text{g/kg}$) + Mito-TEMPO (1 mg/kg), BPA (100 $\mu\text{g/kg}$) + NAC (100 mg/kg), BPA (100 $\mu\text{g/kg}$) + mannose (5 mg/kg), BPA (100 $\mu\text{g/kg}$) + DFO (100 mg/kg), and BPA (100 $\mu\text{g/kg}$) + CA074-ME (10 mg/kg). The control group was administered saline for a period of 30 consecutive days via gavage, while the administered group was treated with BPA for the same duration. Additionally, Mito-TEMPO, NAC, mannose, DFO, and CA074-ME were administered intraperitoneally two weeks prior to the conclusion of the experiment, followed by anesthesia and liver removal.

2.3. Cell Culture

AML12 cells were obtained from the Shanghai Institute of Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12, Thermo Fisher, USA) supplemented with 10% fetal bovine serum (Clark Bioscience, USA), 1% insulin-transferrin-selenium media supplement (Thermo Fisher, USA), 1% penicillin/streptomycin (Thermo Fisher, USA) and 40 ng/mL dexamethasone (Sigma-Aldrich, USA), at conditions of 37 °C and 5% CO₂. 114.15 mg BPA was dissolved in 1 mL DMSO to form a 5 mol/L stock solution. AML12 cells were pretreated with Mito-TEMPO (50 $\mu\text{mol/L}$), NAC (10 $\mu\text{mol/L}$), mannose (10 mmol/L), CA074-ME (10 $\mu\text{mol/L}$) or DFO (10 $\mu\text{mol/L}$) for 1 h, followed by treatment with 0–400 $\mu\text{mol/L}$ BPA for 24 h.

2.4. HE Staining of Liver

HE slices were made according to our previous study^[27]. Briefly, the liver tissue samples were fixed with 4% formaldehyde and then embedded in paraffin wax. The tissue blocks subsequently were cut into slices,

which were dried and stained with hematoxylin-eosin. Finally, the samples were observed using an AE31 microscope (Olympus, Tokyo, Japan). Quantitative analysis was conducted on liver sections using ImageJ software, with five randomly selected high-power fields analyzed per mouse (six mice per group).

2.5. Liver function analysis

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were quantified in mouse serum samples using the AST kit (Cat. no.: C010-2-1) and the ALT kit (Cat. no.: C009-2-1), respectively, supplied by the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The determination of AST and ALT quantification followed manufacturer protocols. Using a microplate reader (BioTek, Winooski, Vermont, USA) set to 510 nm, the optical density (OD) of every well was determined.

2.6. Assay for GSH, MDA, and Iron Ions Content

As previously mentioned, BPA and inhibitors were administered to mice and AML12 cells. GSH (Cat. no.: A006-2-1) and MDA (Cat. no.: A003-1-2) kits from the Nanjing Jiancheng Bioengineering Institute were utilized, and the contents were analyzed following the guidelines included in the kits. Following the instructions of the kit, the iron ion content was measured (Applygen, China; Cat. no.: E1042).

2.7. Cell viability assay

Methyl Thiazolyl Tetrazolium (MTT) assay. AML12 cells (1×10^4 cells per well) were cultivated in a 96-well plate and exposed to 0–800 $\mu\text{mol/L}$ BPA for 24 h. After that, the wells received 20 μL of MTT (0.5 mg/mL) and the wells were cultured for another 4 h. After removing the supernatant, after adding 150 μL of DMSO, and absorbance readings at 490 nm were obtained via microplate spectrophotometry.

Lactate dehydrogenase (LDH) assay. The LDH efflux was quantified via ELISA kit (from China Mei Mian, model MM-43732M2). In 96-well plates, AML12 cells (1×10^4 cells per well) were cultivated and exposed to BPA over 24 h at concentrations ranging from 0–800 $\mu\text{mol/L}$. The cell supernatant was obtained using centrifugation (4 °C, 2500 g, 15 min). After that, LDH release was measured according to the manufacturer instructions.

2.8. Western blotting

RIPA lysis buffer (Beyotime, Shanghai, China) containing PMSF, protease and phosphatase inhibitor was used to lyse all of the samples. A bicinchoninic acid kit was used to measure the amount of protein (Beyotime, Shanghai, P0009). To perform immunoblotting, equal volumes of protein lysates (20–40 μg) were separated via SDS-PAGE and put onto polyvinylidene fluoride membranes. Following that, the membranes were occluded for 2 h using 5% bovine albumin. Primary antibodies were incubated for the entire night at 4 °C. Secondary antibodies were incubated for 2 h at room temperature following three TBST washes. The membranes were washed again three times with TBST. Immunocomplexes were detected using ECL plus (Azure c300).

Antibodies against NLRP3 (1:2000, Proteintech, 68102-1-Ig), GSDMD (1:6000, Proteintech, 20770-1-AP), IL-18 (1:8000, Proteintech, 10663-1-AP), and IL-1 β (1:4000, Proteintech, 16806-1-AP), caspase 1 (1:6000, Proteintech, 22915-1-AP), ACSL4 ((1:1000, Affinity, DF12141), GPX4 (1:6000, Abcam, ab125066), LAMP1 (1:1000, Affinity, DF4806), LAMP2 (1:1000, Affinity, DF6719), CTSB (1:1000, Affinity, AF5189), VDAC1 (1:1000, Affinity, AF5478), Parkin (1:2000, Affinity, AF0235), PINK1 (1:1500, Affinity, DF7742), LC3 (1:4000, Proteintech, 14600-1-AP), p62 (1:8000, Abmart, T55546F), Fis1 (1:6000, Proteintech, 10956-1-AP), Mfn1 (1:6000, Proteintech, 13798-1-AP). HRP-conjugated anti-mouse and anti-rabbit secondary antibodies were sourced from Proteintech Group in Wuhan, China (1:5000).

2.9. Lipid ROS, mtROS, and ROS detection

AML12 cells were planted onto 24-well plates and preprocessed with the compounds shown above. After that, AML12 cells were stained for 30 min at 37 °C in the dark using BODIPY TM 581/591 C11 (5 μ mol/L, Thermo Fisher, USA), MitoSOX (3 μ mol/L, Thermo Fisher, USA), or CM-H2DCFDA (5 μ mol/L, Thermo Fisher, USA). The FV31S-SW fluorescent microscope (Olympus, Tokyo, Japan) was used for examination after three rounds of washing with 1 $\times$ PBS.

2.10. JC-1 Staining

As previously shown, AML12 cells were cultivated in 24-well plates and subjected to pretreatment with the designated compounds. The cells were then incubated at 37 °C for 30 min. without light using 5 μ g/mL JC-1 (Beyotime, Shanghai, China). Following the incubation, cells were examined utilizing a fluorescence microscope AE31 (Olympus, Tokyo, Japan) after being washed three times with 1 $\times$ PBS.

2.11. Acridine Orange (AO) Staining

As previously shown, AML12 cells were planted with the designated substances on a laser confocal dish and preconditioned. Subsequently, AO (10 μ g/mL) solution (Solarbio, Beijing, China) was added into cells and incubated for 30 min at room temperature (RT) out of the light. After three washings with PBS, A fluorescence microscope AE31 (Olympus, Tokyo, Japan) was used to examine the cells.

2.12. LysoTracker Red Staining

As previously shown, AML12 cells were planted pretreatment with the specified substances on a laser confocal dish and underwent. The cells next were incubated with 50 nmol/L LysoTracker red (Beyotime, Shanghai) for 30 min at 37 °C out of the light. After being a thoroughly cleaned, A fluorescence microscope FV31S-SW (Olympus, Tokyo, Japan) was used to examined the cells.

2.13. Double Immunofluorescence Staining

AML12 cells and tissue samples were pretreated as described above.

The colocalization of iron ions and lysosomes: with 1 $\mu\text{mol/L}$ FerroOrange (MKBio, Shanghai, China) and 50 nmol/L LysoTracker green, AML12 cells were co-incubated (Beyotime, Shanghai, China) at 37 °C for 30 min out of the light.

The colocalization of CTSB and lysosomes: the cells firstly were incubated with 50 nmol/L LysoTracker green (Beyotime, Shanghai, China) for 30 min at 37 °C out of the light. After washing, cells were fixed for 30 min at RT using 4% paraformaldehyde. 0.5% Triton X-100 was then used to perforate the cells for 20 min at RT. Subsequently, blocking was performed using 10% goat serum on samples for 1 h at RT. Primary antibody CTSB (1:200) was incubated with cells for a whole night at 4 °C. After washing, cells were incubated with fluorescent secondary antibody for 1 h at RT.

The colocalization of CTSB and NLRP3: the cells and tissue sections were fixed for 30 min at RT using 4% paraformaldehyde. 0.5% Triton X-100 was then used to perforate samples for 20 min at RT. Subsequently, the samples were blocked with 10% goat serum for 1 h at RT, following by co-incubation with primary antibodies CTSB (1:200) and NLRP3 (1:150) overnight at 4 °C. After washing, samples were incubated with anti-rabbit and anti-mouse fluorescent secondary antibodies for 1 h at RT.

The co-localization of LC3 and Mito-Tracker Red: AML12 cells were cultivated in specialized confocal dishes and subjected to the aforementioned treatment regimen. The cells were washed thrice with Phosphate Buffered Saline (PBS) and 1 $\mu\text{mol/L}$ Mito-Tracker Red was co-cultured with the cells for 30 minutes at 37 °C under conditions that provided light protection. Subsequently, the cells were subjected to the same treatment regimen as previously described. Subsequently, the cells were subjected to an overnight incubation with an LC3 antibody (1:150) at 4 °C. Following the washing step, the cells were subjected to an incubation with a fluorescent secondary antibody at RT for a period of one hour. Subsequently, the nuclei were labeled with 10 $\mu\text{g/mL}$ DAPI for a duration of 20 minutes.

Following a thorough washing, a laser confocal microscope (Olympus, Tokyo, Japan) was used to examine each sample.

2.14 Lysosomal Protein Isolation

After the corresponding processing was carried out, AML12 cells were collected and extracted according to the following steps. Briefly, cells were harvested and washed twice with ice-cold phosphate-buffered saline (PBS), followed by resuspension in lysosome isolation buffer containing protease inhibitor cocktail. The cell suspension was homogenized on ice using a Dounce homogenizer until approximately 80%–90% of cells were disrupted, as monitored by microscopy.

The homogenate was first centrifuged at 1,000 r/min for 10 min at 4 °C to remove nuclei and cell debris. The resulting supernatant was then centrifuged at 20,000 r/min for 20 min at 4 °C to obtain the crude lysosomal fraction. The pellet containing lysosomes was resuspended in lysosome extraction buffer to collect the lysosomal protein fraction, while the supernatant represented the cytosolic protein fraction.

Protein concentrations were determined using a BCA protein assay kit. Equal amounts of proteins from lysosomal and cytosolic fractions were subjected to Western blot analysis to detect CTSB distribution. Using V-ATPase subunit B as the internal control [28].

2.15. Transmission Electron Microscopy (TEM)

AML12 cells were collected after the indicated treatments and fixed in 2.5% glutaraldehyde at 4 °C overnight. The samples were post-fixed with 1% osmium tetroxide, dehydrated through a graded ethanol series, and embedded in epoxy resin. Ultrathin sections (60 nm -80 nm) were prepared using an ultramicrotome and stained with uranyl acetate and lead citrate. The sections were observed under a transmission electron microscope. Ultrastructural changes related to pyroptosis and ferroptosis, particularly mitochondrial morphology and plasma membrane integrity, were examined.

2.16. Statistical analysis

The data obtained were shown as mean $\pm$ standard deviation (SD), and the trials were carried out three times or more. The statistical analysis was carried out utilizing the Graph Pad Prism V9.5.0 software (San Diego, CA). The comparisons were analyzed using either One-way ANOVA or t-test, with a significance level of $P < 0.05$ considered indicative of a statistical difference.

3. Results

3.1 BPA caused liver injury in mice

In order to examine the impact of BPA on the liver of mice, mice were treated with BPA (50 and 100 $\mu\text{g/kg}$) for 30 days (Fig. 1A). BPA increased the liver ratio to body weight in mice (Fig. 1B). AST and ALT are low in the blood and more abundant in the liver cells. Damage to the liver cells results in AST and ALT flowing into the blood, suggesting liver damage. As shown in Fig. 1C and D, the mice treated with BPA showed markedly higher serum AST and ALT levels. HE staining of the liver is a commonly used test for liver histopathology. The mice liver in the BPA-treated group showed irregularities in hepatic lobules, inflammatory infiltrates, and nuclear shrinkage (Fig. 1E). Quantitative analysis further demonstrated that BPA exposure significantly increased the vacuole area ratio and inflammatory infiltration area ratio compared with the control group (Fig. 1F). These results indicated that BPA induced liver injury in mice.

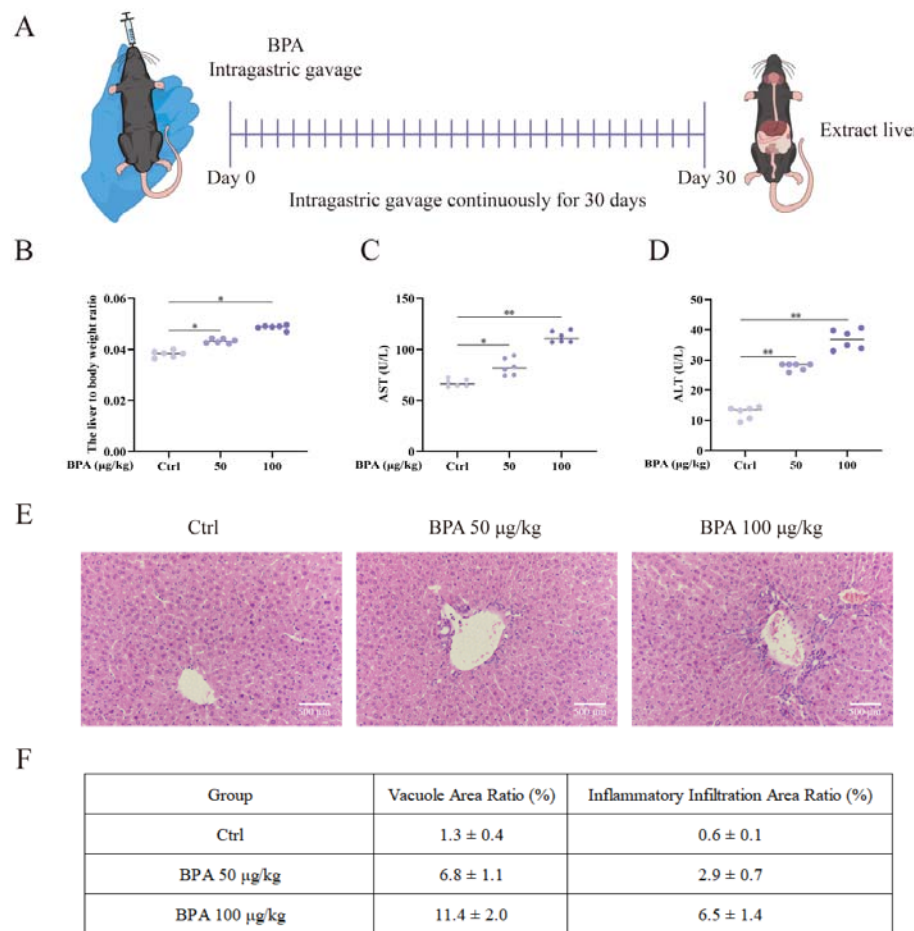


Fig. 1. BPA caused liver injury in mice. (A) Schematic timeline of C57BL/6 mice receiving daily gavage for 30 days. (B) Index of liver to body weight. (C, D) AST and ALT contents were measured by kits. (E, F) HE staining of liver tissues and corresponding quantitative analysis (scale bar, 500 µm). * $P < 0.05$, ** $P < 0.01$.

3.2. LMP mediated BPA-driven liver injury

To assess the potential of BPA to induce lysosomal damage, we conducted a detection of lysosomal membrane protein LAMP1, LAMP2, and lysosomal CTSB. Our findings revealed that BPA exposure significantly diminished LAMP1 and LAMP2 protein levels while concurrently increasing CTSB expression *in vivo* (Fig. 2A). AML12 cells were treated with BPA for 24 h, and cytotoxicity was quantified through the MTT assay. The findings indicated a decline in cell viability with an increase in the dosage of BPA employed (Fig. 2B). BPA was observed to induce a significant level of cell death at a concentration of 100 µmol/L. LDH assay was performed to further assess the cytotoxicity of BPA. Similar to the MTT results, BPA at concentrations above 100 µmol/L decreased cell viability (Fig. 2C). Consequently, the subsequent experiments were conducted using a range of BPA concentrations between 0 and 400 µmol/L (0, 100, 200, 400 µmol/L). Consistent with *in vivo* results, BPA markedly decreased the protein levels of LAMP1 and LAMP2 and increased CTSB expression (Fig. 2D).

Furthermore, we utilized AO dye, a lysosome-specific metachromatic fluorescent probe capable of traversing living cell membranes, to further evaluate lysosomal function. In healthy lysosomes, AO emits a distinctive red fluorescence. However, upon the enhancement of lysosomal membrane permeability, AO permeates into the cytoplasm, manifesting as green fluorescence. We observed a gradual attenuation of red

fluorescence intensity of AO with increasing BPA dose (Fig. 2E). Next, we employed LysoTracker Red, a fluorescent marker specific to lysosomes, for staining AML12 cells. As shown in Fig. 2F, AML12 cells exhibited a noticeably weakened red fluorescence after BPA treatment in a dose-dependent manner. The data indicated that BPA induced LMP. To further determine whether BPA induced true LMP rather than general lysosomal dysfunction, the distribution of CTSB in lysosomal and cytosolic fractions was examined. As shown in Fig. 2G and H, BPA treatment markedly altered the intracellular localization of CTSB in AML12 cells. Specifically, CTSB levels in the lysosomal fraction were significantly decreased, whereas CTSB expression in the cytosolic fraction was markedly increased in a dose-dependent manner following BPA exposure. These results indicated that BPA promoted the release of CTSB from lysosomes into the cytoplasm, providing direct evidence for LMP rather than merely lysosomal dysfunction.

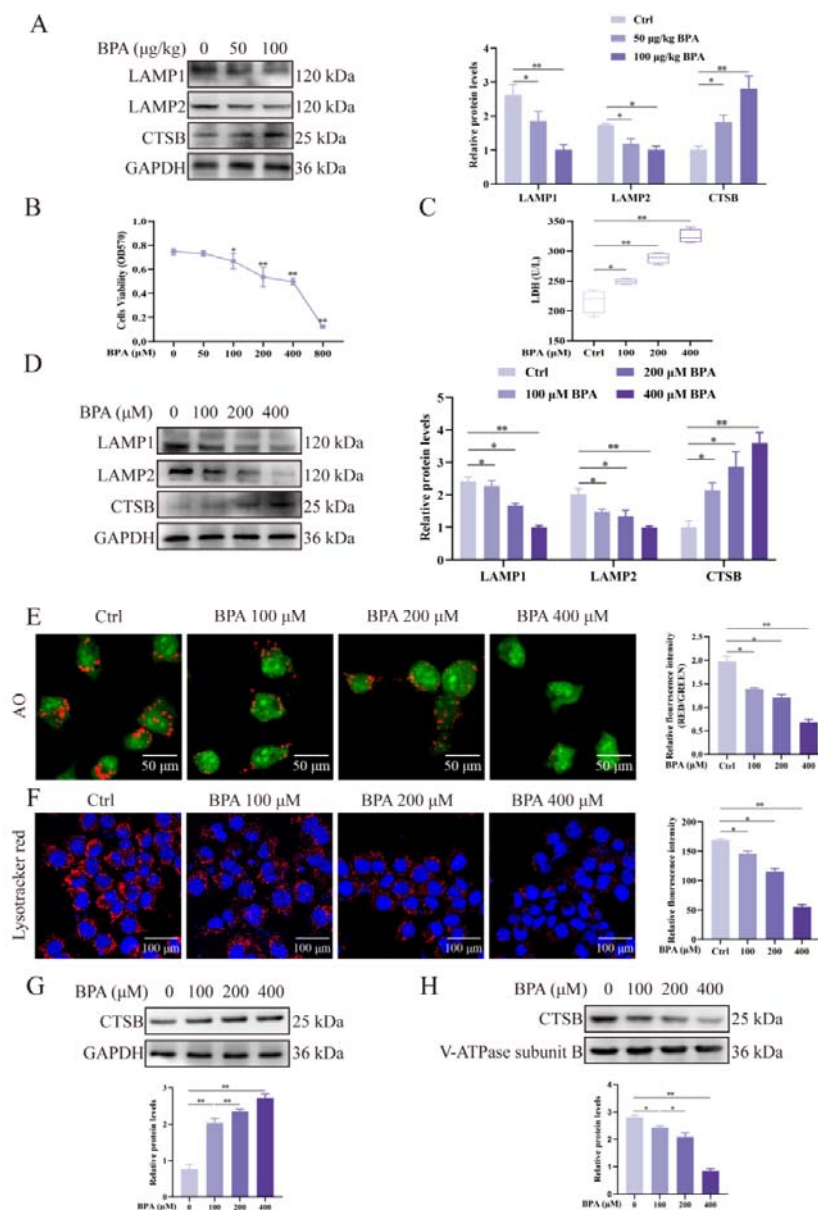


Fig. 2. BPA induced LMP. (A, D) Quantitative study and protein expression profile for LAMP1, LAMP2 and CTSB. (B, C) MTT and LDH assay for cell viability. (E) AO was used to dye AML12 cells (scale bar, 50 µm). (F) LysoTracker red was utilized to stain AML12 cells (scale bar, 100 µm). (G, H) Western blot analysis of CTSB expression in the cytosolic fraction and lysosomal fraction of AML12 cells treated with BPA. * $P < 0.05$, ** $P < 0.01$.

We subsequently investigated the role of LMP in BPA-driven liver injury. Mannose, a lysosomal membrane stabilizer, is phosphorylated intracellularly into mannose-6-phosphate, which promotes the glycosylation of lysosomal membrane proteins, thereby enhancing lysosomal membrane stability^[29]. To evaluate the potential effect of mannose itself, a mannose-only treatment group was included in the animal experiments. As shown in Fig. 3A, BPA exposure markedly altered the expression of lysosomal-associated proteins, as evidenced by decreased LAMP1 and LAMP2 levels and increased CTSB expression. Treatment with the lysosomal membrane stabilizer mannose effectively restored the aberrant expression of these proteins induced by BPA, indicating attenuation of BPA-triggered LMP. Notably, the mannose-only group showed comparable protein expression levels to those of the control group. Consistently, BPA treatment significantly increased the liver index and serum ALT and AST levels, whereas mannose administration markedly suppressed these elevations (Fig. 3B-D). Importantly, no significant differences were observed between the control and mannose-only groups. Histopathological examination further revealed that BPA exposure caused evident hepatic cord disarrangement and inflammatory cell infiltration, whereas mannose treatment alleviated these pathological changes (Fig. 3E). The liver morphology of the mannose-only group was comparable to that of the control group. Mannose treatment significantly reduced both the vacuole area ratio and inflammatory infiltration area ratio induced by BPA (Fig. 3F). Collectively, these results indicated that LMP was a critical mediator of BPA-induced liver injury, while mannose exerted a protective effect by stabilizing lysosomal membranes.

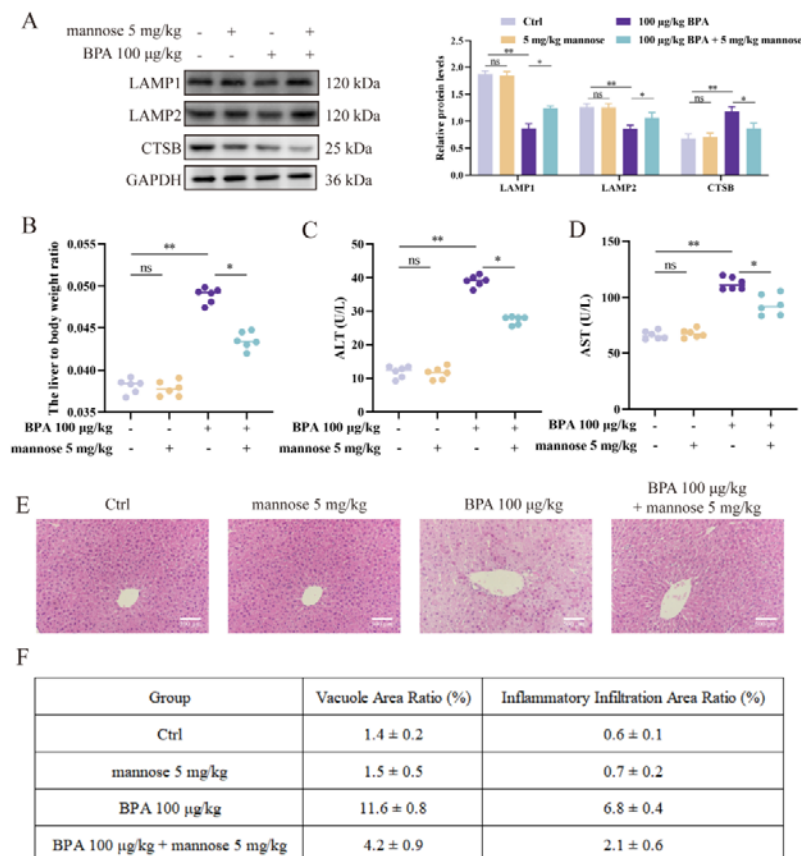
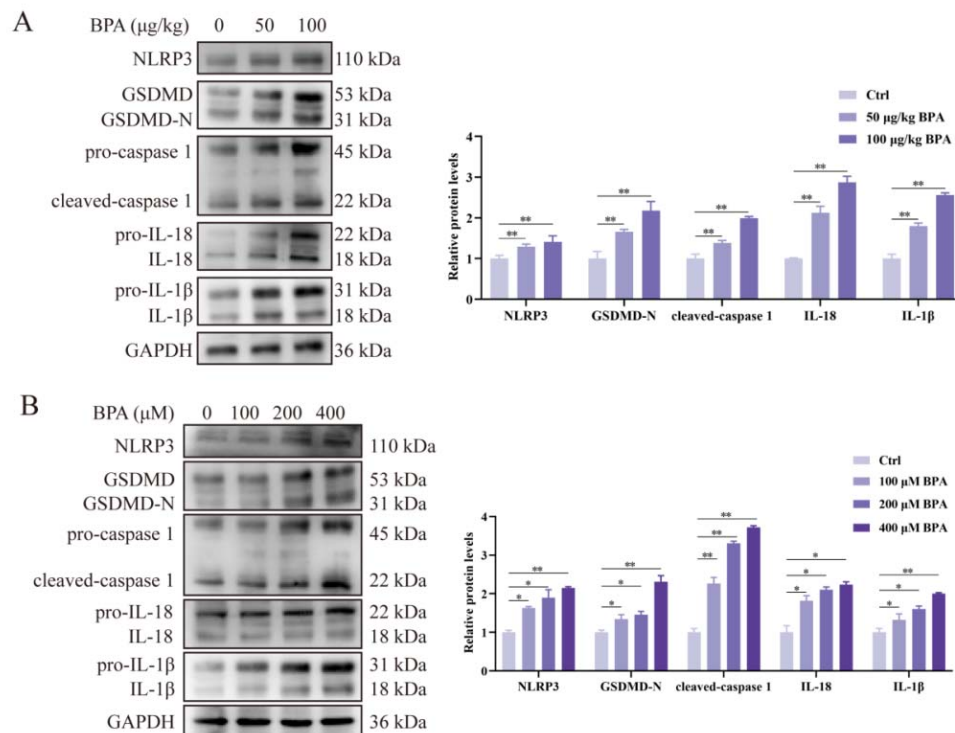


Fig. 3. LMP mediated BPA-driven liver injury. (A) Western blot analysis of LAMP1, LAMP2 and CTSB protein expression. (B) Index of liver to body weight. (C, D) AST and ALT contents were measured by kits. (E, F) HE staining of liver tissues and corresponding quantitative analysis (scale bar, 500 µm). * $P < 0.05$, ** $P < 0.01$.

3.3. BPA induced NLRP3-dependent pyroptosis in mice liver and AML12 cells

NLRP3-dependent pyroptosis is a key downstream death pathway triggered by LMP. Thus, we next investigated the effects of BPA on pyroptosis in hepatocytes. Compared to the control group, BPA caused a notable rise in the levels of protein expression of NLRP3, GSDMD-N, caspase 1, IL-18 and IL-1 β in mice liver (Fig. 4A). The pyroptosis-related proteins were also examined in BPA-treated AML12 cells. In line with *in vivo* findings, BPA significantly increased the expression levels of pyroptosis-related proteins (Fig. 4B). Activated NLRP3 inflammasome is the upstream signal for the classical pathway of pyroptosis. We found that BPA resulted in stronger immunofluorescence of NLRP3 in liver sections and AML12 cells (Fig. 4C, D and Fig.S1A, B). To further examine the ultrastructural changes associated with pyroptosis, TEM was performed. As shown in Fig. 4E, hepatocytes in the control group displayed intact cellular morphology with continuous plasma membranes and normal organelle structures. In contrast, hepatocytes from the BPA-treated group exhibited evident cell swelling and pore formation. These morphological alterations were consistent with the characteristic ultrastructural features of pyroptosis. Concurrently, the NLRP3 inhibitor MCC950 was utilized, and the experimental findings demonstrated that MCC950 significantly curtailed the BPA-induced increase in NLRP3, caspase-1, GSDMD, IL-1 β , and IL-18 protein levels (Fig. 5A). Consistent with the outcomes of the *in vivo* experiments, the *in vitro* experiments yielded analogous results (Fig. 5B). These results suggested that BPA-induced activation of NLRP3 mediated pyroptosis. The results demonstrated that BPA induced hepatocytes NLRP3-dependent pyroptosis *in vivo* and *in vitro*.



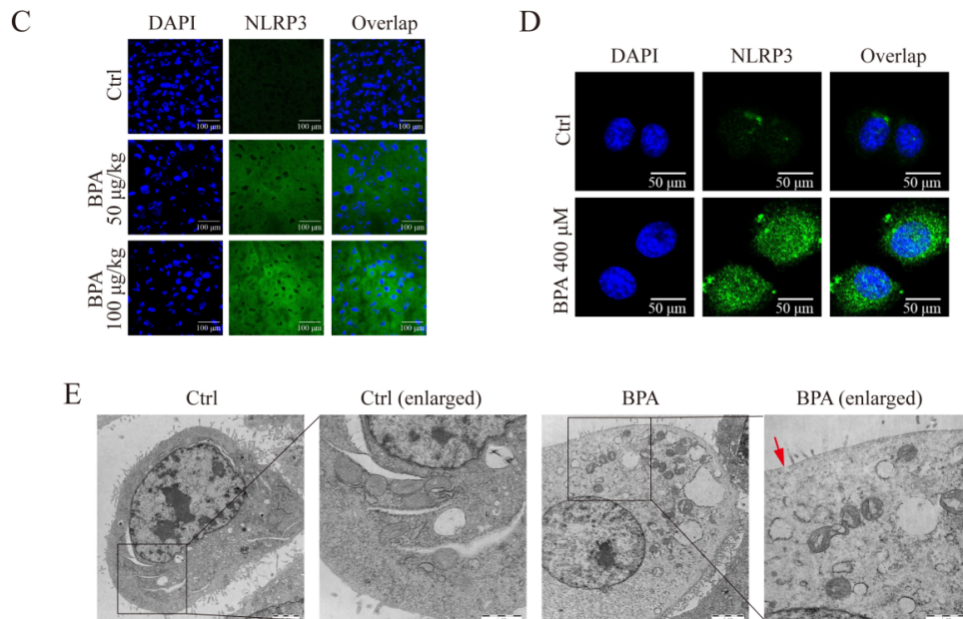


Fig. 4. BPA induced hepatocytes pyroptosis *in vivo* and *in vitro*. (A, B) Western blot analysis of NLRP3, GSDMD-N, cleaved-caspase 1, IL-1 β , and IL-18 protein expression. (C, D) Immunofluorescence staining for NLRP3 in mice and cells (E) TEM was used to observe ultrastructural features of pyroptosis, red arrows indicate plasma membrane rupture. * $P < 0.05$, ** $P < 0.01$.

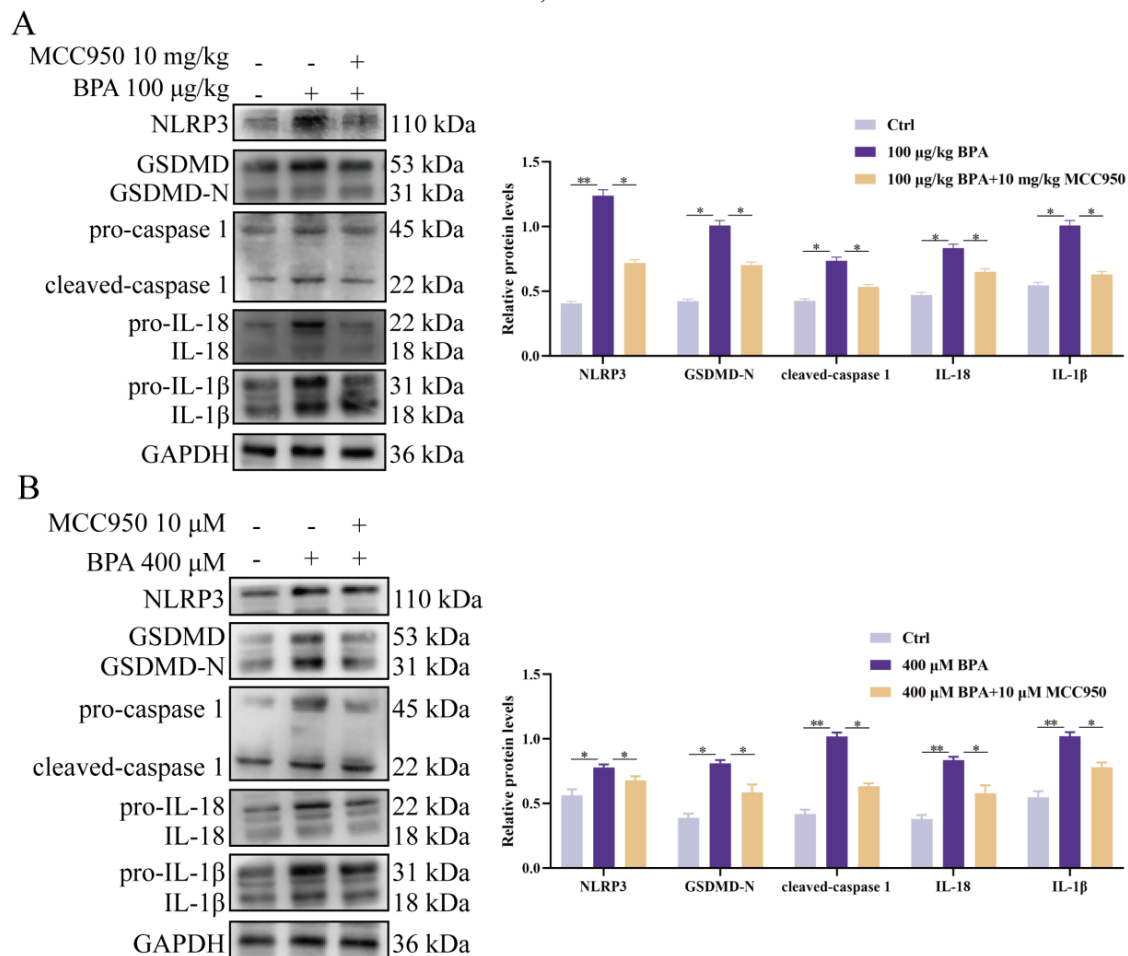


Fig. 5. BPA induced hepatocytes NLRP3-dependent pyroptosis *in vivo* and *in vitro*. (A, B) Western blot analysis of NLRP3, GSDMD-N, cleaved-caspase 1, IL-1 β , and IL-18 protein expression. * $P < 0.05$, ** $P < 0.01$.

3.4. LMP-mediated CTSSB leakage was involved in BPA-induced pyroptosis

To investigate the role of LMP in BPA-induced pyroptosis, we evaluated pyroptosis after administration of mannose. We found that mannose significantly inhibited the upregulation of NLRP3, GSDMD-N, caspase-1, IL-18 and IL-1 β proteins compared with BPA group *in vivo* and *in vitro* (Fig. 6A and 7A). CTSB is a major upstream signal for activation of the NLRP3 inflammasome. Thus, we next explored the efficacy of CTSB on BPA-induced pyroptosis. According to Fig. 6B, CTSB inhibitor CA074-ME markedly ameliorated the BPA-induced increase in pyroptosis-related proteins. We obtained similar results in our *in vitro* experiments (Fig. 7B). Furthermore, immunofluorescence colocalization of CTSB and NLRP3 showed that BPA increased the colocalization of CTSB and NLRP3 and the expression levels of NLRP3. Notably, similar inhibitory effects on these results were observed for mannose and CA074-ME (Fig. 6C and 7C), which suggested that LMP-mediated CTSB leakage activated NLRP3 inflammasome. Our results demonstrated that BPA induced NLRP3-dependent pyroptosis through LMP-mediated CTSB leakage.

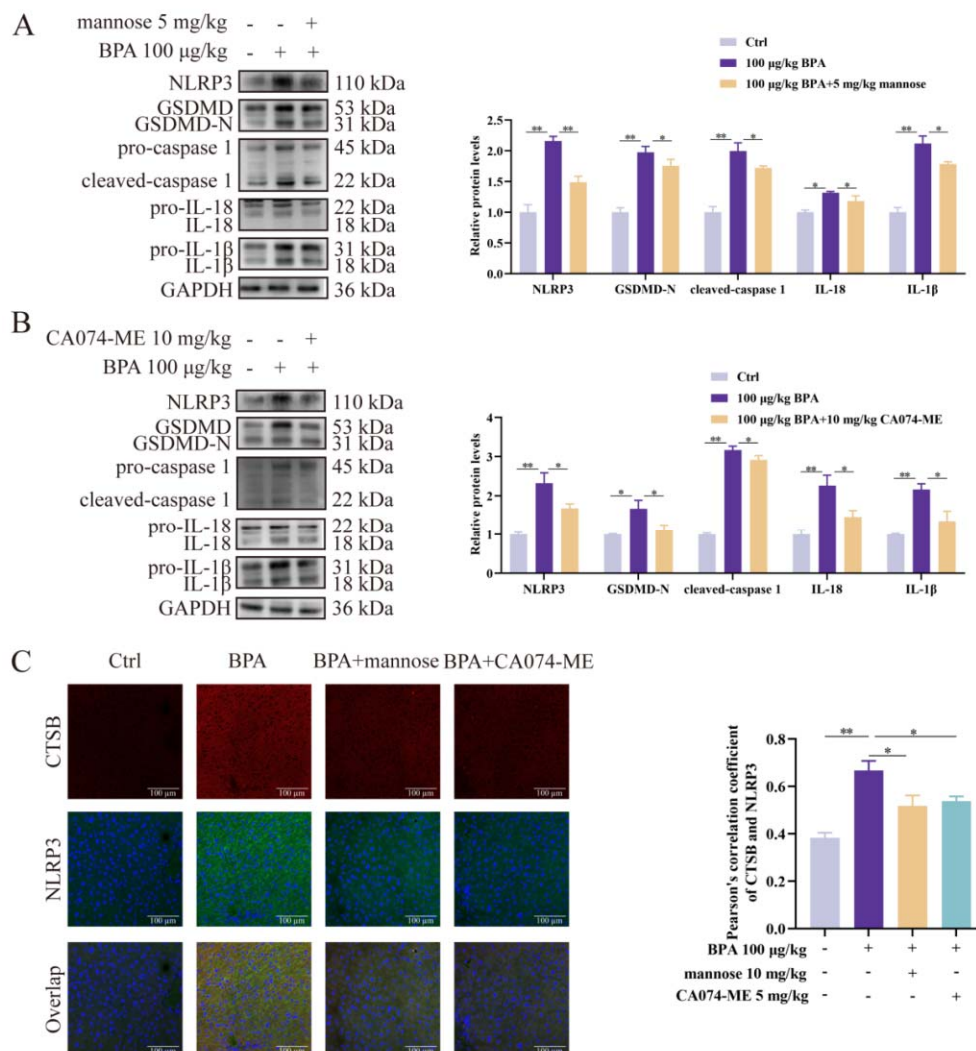


Fig. 6. LMP-mediated CTSB leakage was involved in BPA-induced pyroptosis in mice. (A-B) Quantitative study and protein expression profile for NLRP3, GSDMD, cleaved-caspase 1, IL-1 β , and IL-18. (C) CTSB and NLRP3 colocalization was seen. * $P < 0.05$, ** $P < 0.01$.

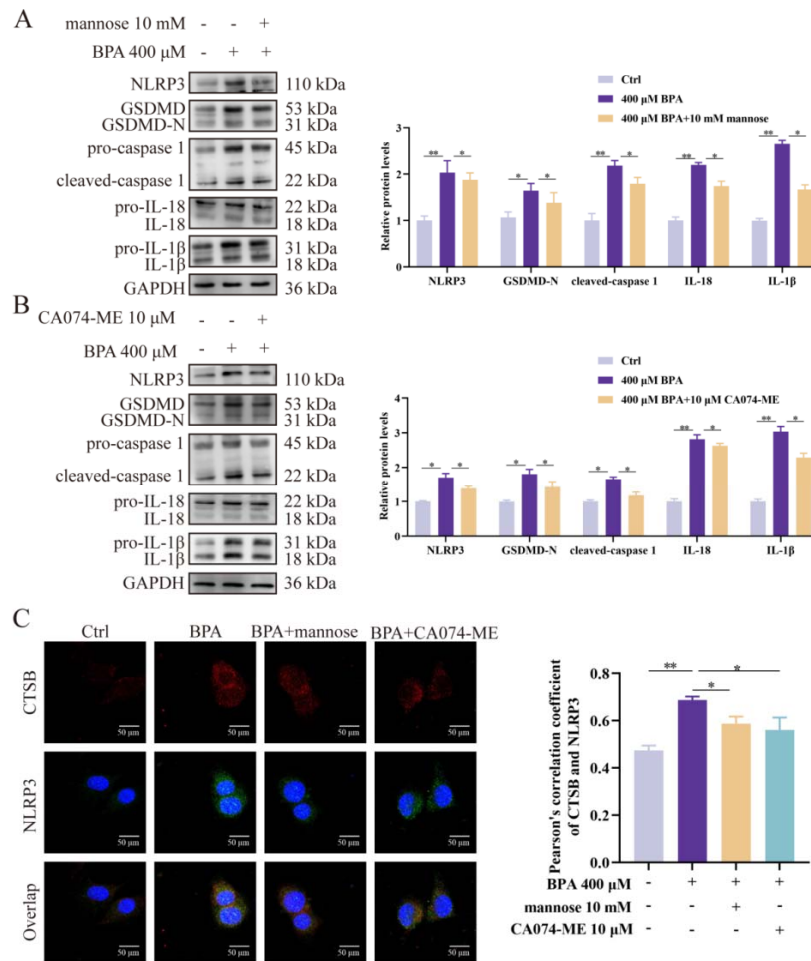


Fig. 7. LMP-mediated CTSSB leakage was involved in BPA-induced pyroptosis in AML12 cells. (A-B) Quantitative study and protein expression profile for NLRP3, GSDMD, cleaved-caspase 1, IL-1β, and IL-18. (C) CTSSB and NLRP3 colocalization was seen. * $P < 0.05$, ** $P < 0.01$.

3.5. BPA induced hepatocytes ferroptosis in mice and AML12 cells

Subsequently, we investigated whether BPA triggered ferroptosis in hepatocytes. Crucially, two essential upstream regulators of ferroptosis are GPX4 and ACSL4. Our findings demonstrated that BPA exerted a dose-dependent inhibitory effect on the expression of the GPX4 protein, while concurrently encouraging the expression of ACSL4 protein (Fig. 8A). In comparison to the control group, a decrease in liver GSH levels was observed in BPA group, concomitant with a marked elevation in MDA and iron ion contents (Fig. 8B-D). These findings collectively suggested that BPA caused ferroptosis in the livers of mice. We next assessed the potential of BPA to induce ferroptosis in AML12 cells. In line with our *in vivo* findings, we found that BPA reduced the expression of GPX4 and elevated the expression of ACSL4 (Fig. 8E). Meanwhile, BPA treatment led to a dose-dependent decrease in GSH levels and an increase in MDA levels and intracellular iron concentrations (Fig. 8F-H). As shown in the lipid ROS staining images (Fig. 8I), there was an excessive production of lipid ROS in AML12 cells after BPA treatment. To determine whether BPA exposure also induced ferroptosis, ultrastructural changes in mitochondria were further analyzed by TEM. As shown in Fig. 8J, mitochondria in hepatocytes from the control group exhibited normal morphology with well-defined cristae and intact membranes. However, BPA-treated hepatocytes displayed pronounced mitochondrial

abnormalities, including mitochondrial shrinkage, increased membrane density, and reduced or vanished cristae. These mitochondrial alterations were widely recognized as the hallmark ultrastructural features of ferroptosis. The above results indicated that BPA caused mice and AML12 cells to undergo ferroptosis.

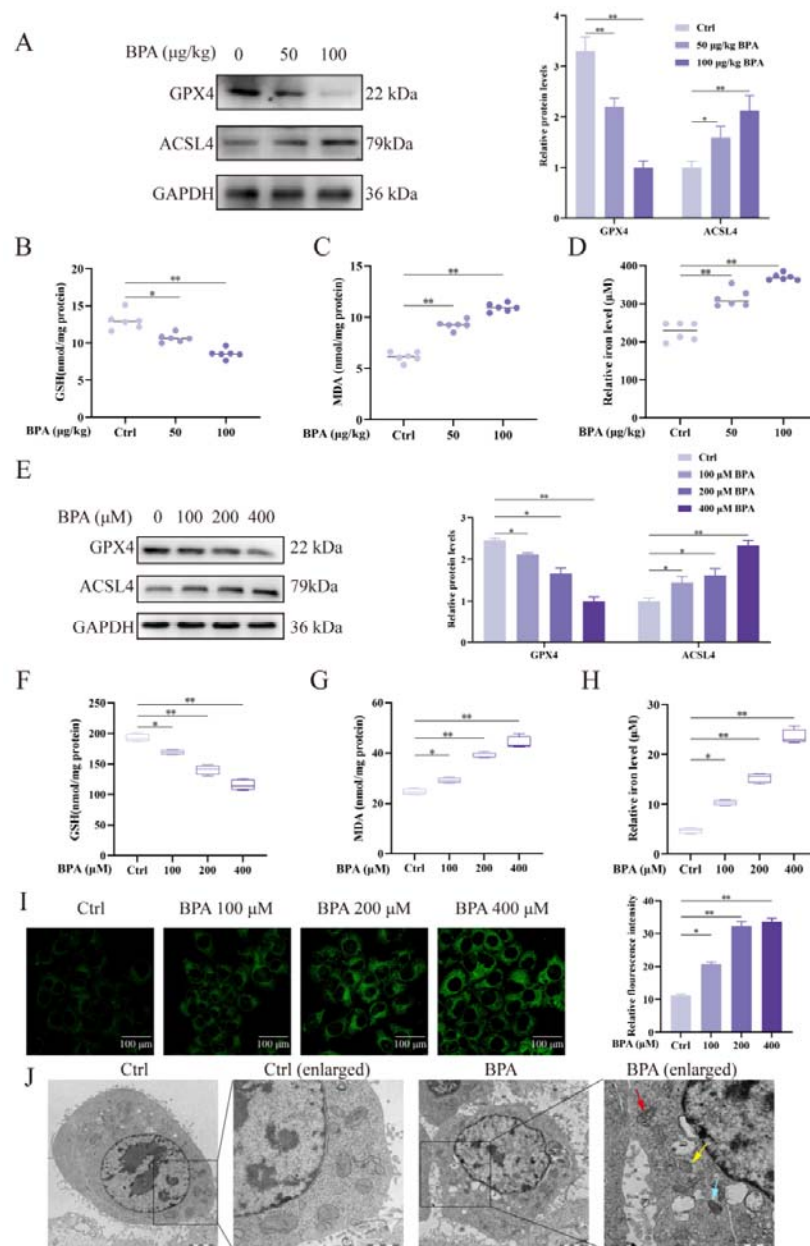


Fig. 8. BPA induced hepatocytes ferroptosis *in vivo* and *in vitro*. (A) Quantitative study and protein expression profiles for GPX4 and ACSL4. (B-D) GSH, MDA and liver iron ion amount contained were calculated using kits. (E) Quantitative study and protein expression profiles for GPX4 and ACSL4. (F-H) GSH, MDA and AML12 cells iron ion amount contained were calculated using kits. (I) Lipid ROS can be detected with BODIPY TM 581/591 C11. (J) TEM was used to observe ultrastructural features of ferroptosis, red arrows indicate mitochondrial shrinkage, yellow arrows indicate reduced or vanished cristae, and blue arrows indicate increased mitochondrial membrane density. * $P < 0.05$, ** $P < 0.01$.

3.6. LMP-mediated iron ion efflux was involved in BPA-induced ferroptosis

To investigate whether LMP was involved in BPA-induced ferroptosis, we administered mannose in mice and AML12 cells. We found that mannose significantly inhibited lysosomal iron efflux compared with BPA group in AML12 cells (Fig. 9A). Mannose markedly ameliorated the decreased GPX4 protein level and increased ACSL4 expression caused by BPA in mice liver (Fig. 9B). Meanwhile, mannose significantly

mitigated the BPA-induced alterations, including decreased GSH levels, elevated MDA levels, increased intracellular iron ion concentration (Fig. 9C-E). We also observed similar results *in vitro* (Fig. 9F-I). Furthermore, the excessive production of lipid ROS caused by BPA also was mitigated by mannose in AML12 cells (Fig. 9J). These data suggested that lysosomal iron efflux may contribute to BPA-induced ferroptosis. To further verify the role of lysosomal iron efflux on BPA-induced ferroptosis, we examined ferroptosis after administration of iron chelator DFO. As expected, DFO obviously alleviated abnormal changes in BPA-induced ferroptosis-related indicators both *in vivo* and *in vitro* (Fig. 9B-J). Collectively, our results demonstrated that BPA induced ferroptosis through LMP-mediated lysosomal iron efflux.

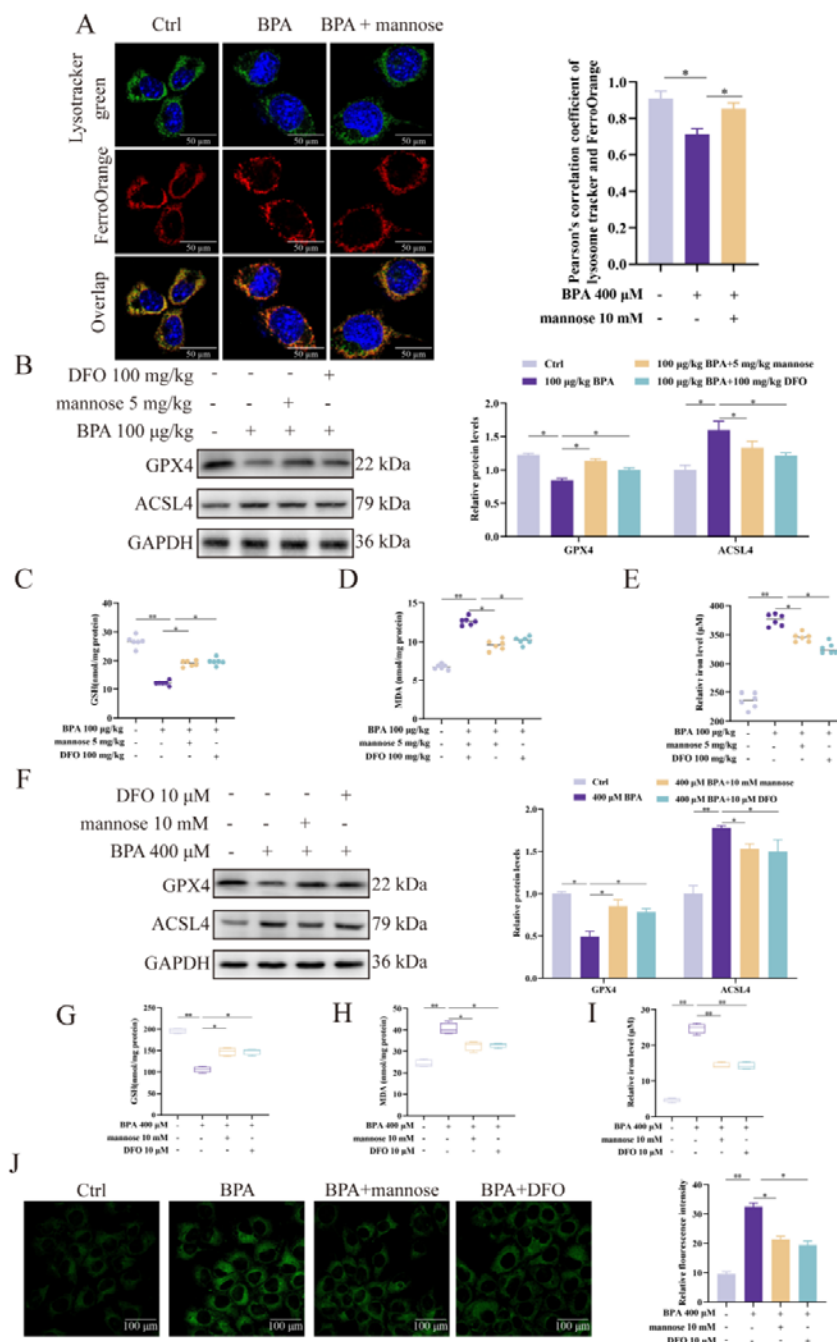
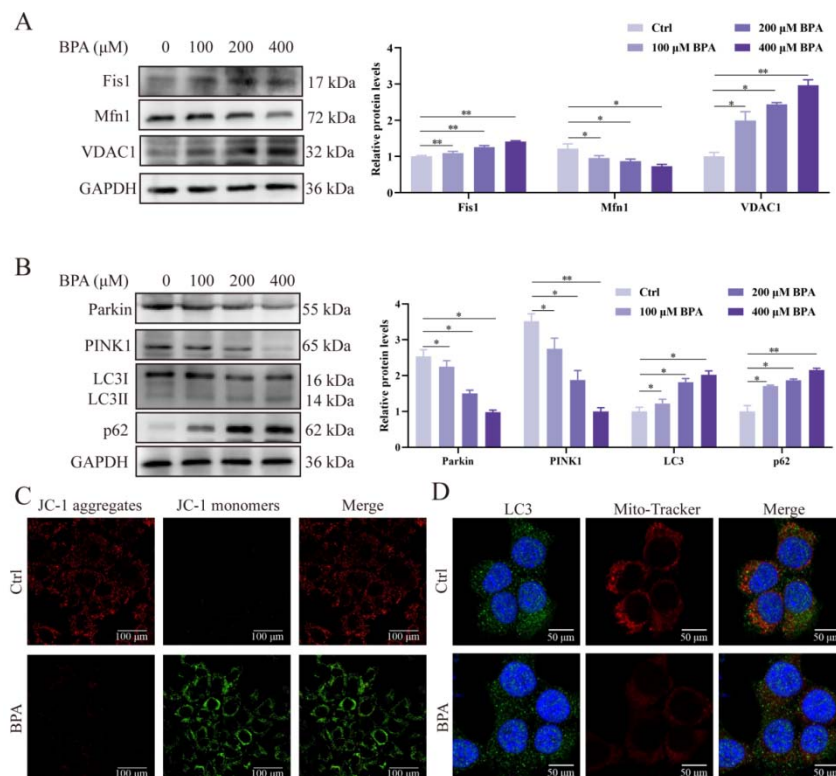


Fig. 9. LMP-mediated iron ion efflux was involved in BPA-induced ferroptosis. (A) LysoTracker green and FerroOrange colocalization staining. (B) Quantitative study and protein expression profile for GPX4, ACSL4. (C-E) GSH, MDA and liver iron ion amount contained were calculated using kits. (F) Quantitative study and protein expression profile for GPX4, ACSL4. (G-I) GSH, MDA and AML12 cells iron ion amount contained were calculated using kits. (J) Lipid ROS was detected with BODIPY TM 581/591 C11. * $P < 0.05$, ** $P < 0.01$.

3.7. BPA caused mitochondrial damage and mtROS overproduction

Mitochondrial quality control is of paramount importance in sustaining mitochondrial homeostasis and functionality. Hence, we conducted an assessment of mitochondrial function subsequent to the administration of BPA. Mitochondrial fission/fusion dynamics and mitophagy play a pivotal role in mitochondrial quality control^[30]. We explored the changes of mitochondrial fission/fusion-related proteins Mfn1 and Fis1, mitophagy-related proteins PINK1, Parkin, LC3 and p62 and mitochondrial functional protein VDAC1 both *in vivo* and *in vitro*. The results showed that BPA dose-dependently increased the expression levels of LC3, p62, Mfn1 and VDAC1, and decreased the protein levels of Fis1, PINK1, and Parkin (Fig. 10A, B and Fig. S1A, B). Utilizing JC-1 staining, we observed reduced red-green fluorescence ratios in BPA-treated AML12 cells (Fig. 10C and Fig. S2C), suggesting that BPA caused a reduction in mitochondrial membrane potential (MMP). To further verify the effect of BPA on mitophagy, the colocalization of LC3 with mitochondria was analyzed by immunofluorescence using LC3 staining and Mito-tracker labeling. As shown in Fig. 10D and Fig. S2D, a certain degree of LC3-mitochondria colocalization was observed in the control group, while BPA treatment significantly decreased the colocalization signals, suggesting reduced recruitment of LC3 to damaged mitochondria and impaired mitophagy. Mitochondrial dysfunction leads to mtROS overproduction^[31]. We found that BPA treatment induced an excessive generation of mtROS and ROS in AML12 cells, and mtROS scavenger Mito-TEMPO and ROS scavenger NAC attenuated these effects (Fig. 10E and F). Our results demonstrated that BPA caused mitochondrial damage and mtROS overproduction through disrupting mitochondrial fission/fusion dynamics and inhibiting mitophagy.



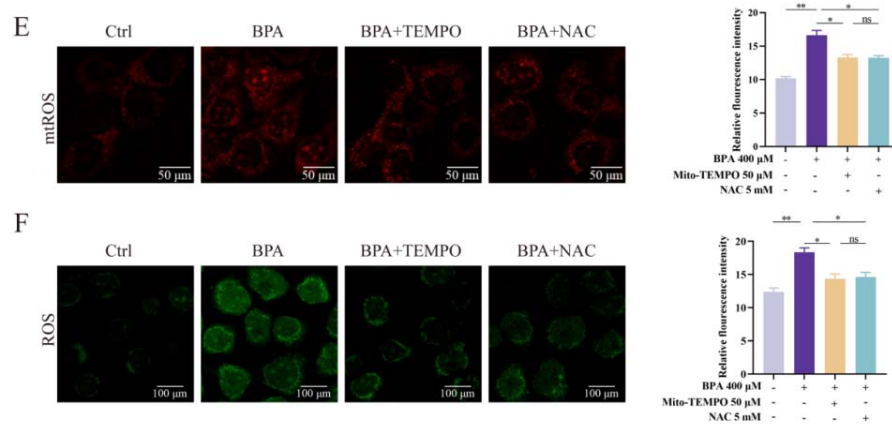


Fig. 10. BPA caused mitochondrial damage and mtROS overproduction. (A) Quantitative study and protein expression profile for Fis1, Mfn1, and VDAC1. (B) Quantitative study and protein expression profile for Parkin, PINK1, LC3 and p62. (C) JC-1 was utilized to monitor the MMP (scale bar, 100 μ m). (D) LC3 and Mito-tracker colocalization was seen (scale bar, 50 μ m). (E) MitoSOX was used to detect mtROS (scale bar, 100 μ m). (F) CM-H2DCFDA was utilized to detect ROS (scale bar, 100 μ m). * $P < 0.05$, ** $P < 0.01$.

3.8. mtROS mediated BPA-induced LMP and subsequent CTSS leakage and iron efflux

Next, we examined the role of mtROS in BPA-induced LMP. Our results showed that Mito-TEMPO and NAC reduced the BPA-caused decline in LAMP1 and LAMP2 protein levels, as well as the elevation in CTSS protein levels both *in vivo* and *in vitro* (Fig. 11A and B). Concurrently, Mito-TEMPO and NAC treatments reduced the BPA-induced reduction in the red-green fluorescence ratio of AO staining (Fig. 11C and Fig. S3A) and enhanced the fluorescence intensity of LysoTracker Red (Fig. 11D and Fig. S3B). These observations firmly demonstrated that mtROS mediated BPA-induced LMP. To ascertain whether the BPA-induced LMP led to CTSS leakage, we conducted CTSS and LysoTracker green colocalization in AML12 cells. The findings demonstrated that BPA dramatically decreased the colocalization of CTSS and lysosomes (Fig. 11E, and Fig. S3C), indicating that BPA caused CTSS leakage from lysosomes. Meanwhile, the iron ion probe FerroOrange and LysoTracker green colocalization was conducted to examine the iron efflux from lysosomes. As shown in Fig. 11F and Fig. S3D, BPA diminished the colocalization of iron and lysosomes, while simultaneously augmenting the fluorescence intensity of FerroOrange, suggesting that iron efflux from lysosomes to the cytoplasm in the course of BPA-induced LMP. Mito-TEMPO and NAC effectively mitigated BPA-induced CTSS leakage and iron efflux (Fig. 11E and F). Taken together, our findings suggested that mtROS mediated BPA-induced LMP and subsequent CTSS leakage and iron efflux.

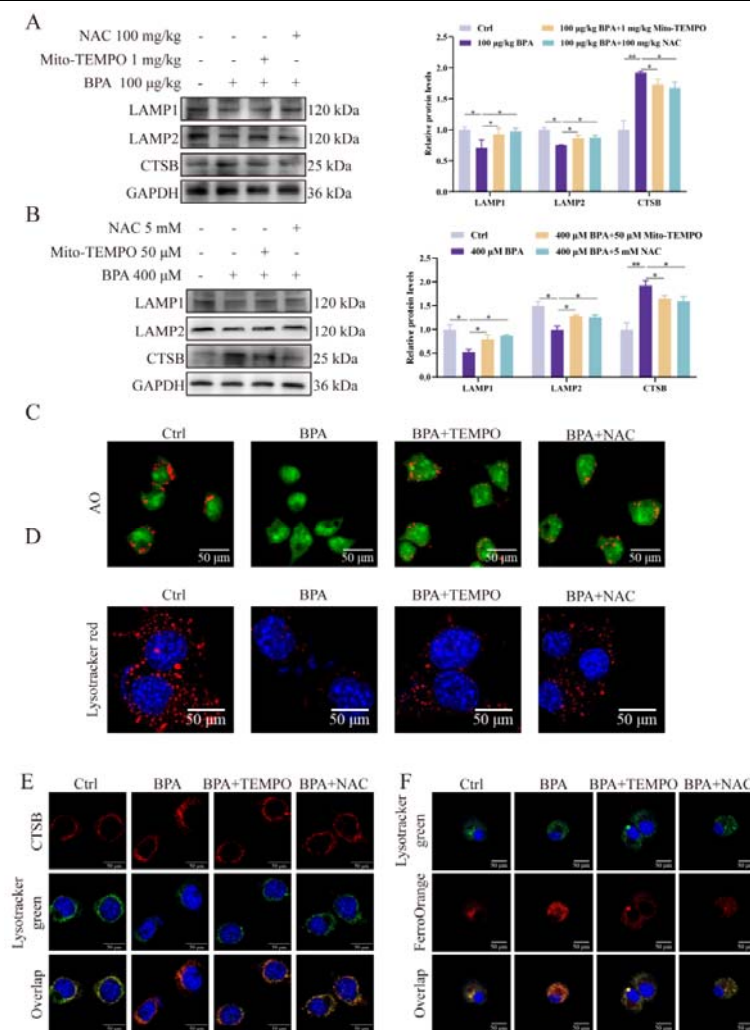


Fig. 11. mtROS mediated BPA-induced LMP and subsequent CTSB leakage and iron efflux. (A, B) Quantitative study and protein expression profile for LAMP1, LAMP2 and CTSB. (C) AO was used to dye AML12 cells (scale bar, 50 µm). (D) LysoTracker red was utilized to stain AML12 cells (scale bar, 100 µm). (E) CTSB and LysoTracker green colocalization staining (scale bar, 50 µm). (F) LysoTracker green and FerroOrange colocalization staining (scale bar, 50 µm). * $P < 0.05$, ** $P < 0.01$.

3.9. mtROS was involved in BPA-induced hepatocytes pyroptosis and ferroptosis

To investigate the effects of mtROS on BPA-triggered hepatocytes pyroptosis and ferroptosis, we examined pyroptosis and ferroptosis after administration of Mito-TEMPO and NAC. As shown in Fig. 12A and B, both Mito-TEMPO and NAC efficiently mitigated the elevated levels of NLRP3, GSDMD-N, caspase-1, IL-18 and IL-1 β caused by BPA *in vivo* and *in vitro*. Meanwhile, Mito-TEMPO and NAC alleviated the BPA-induced reduction in GPX4 protein level and increase in ACSL4 protein level in mice liver (Fig. 13A). In comparison to the liver of BPA group, Mito-TEMPO and NAC markedly recovered the aberrant levels of GSH, MDA, and iron ion content (Fig. 13B-D). The *in vitro* results also validated these effects (Fig. 13E-H). Furthermore, Mito-TEMPO and NAC significantly inhibited BPA-induced lipid ROS overproduction in AML12 cells (Fig. 13I). These findings collectively confirmed that mtROS was implicated in BPA-induced hepatocytes pyroptosis and ferroptosis.

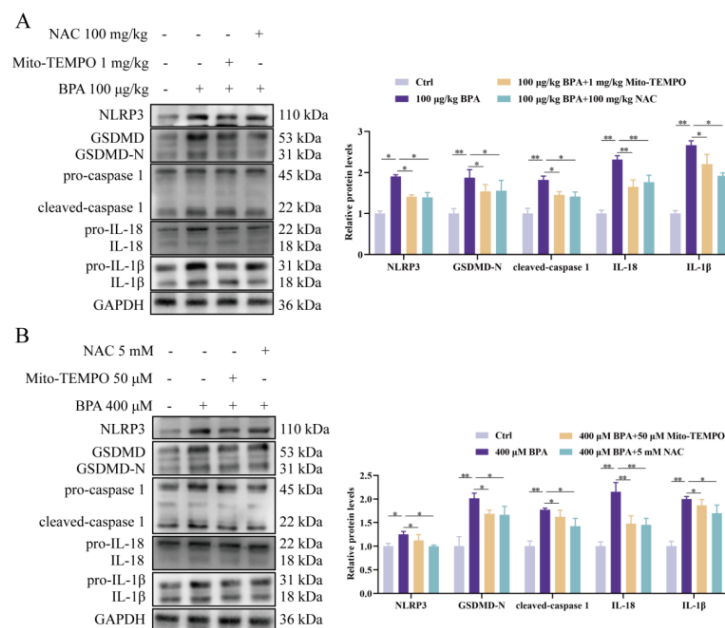


Fig. 12. mtROS was involved in BPA-induced hepatocytes pyroptosis. (A, B) Western blot analysis of NLRP3, GSDMD-N, caspase 1, IL-1 β , and IL-18 protein expression. * $P < 0.05$, ** $P < 0.01$.

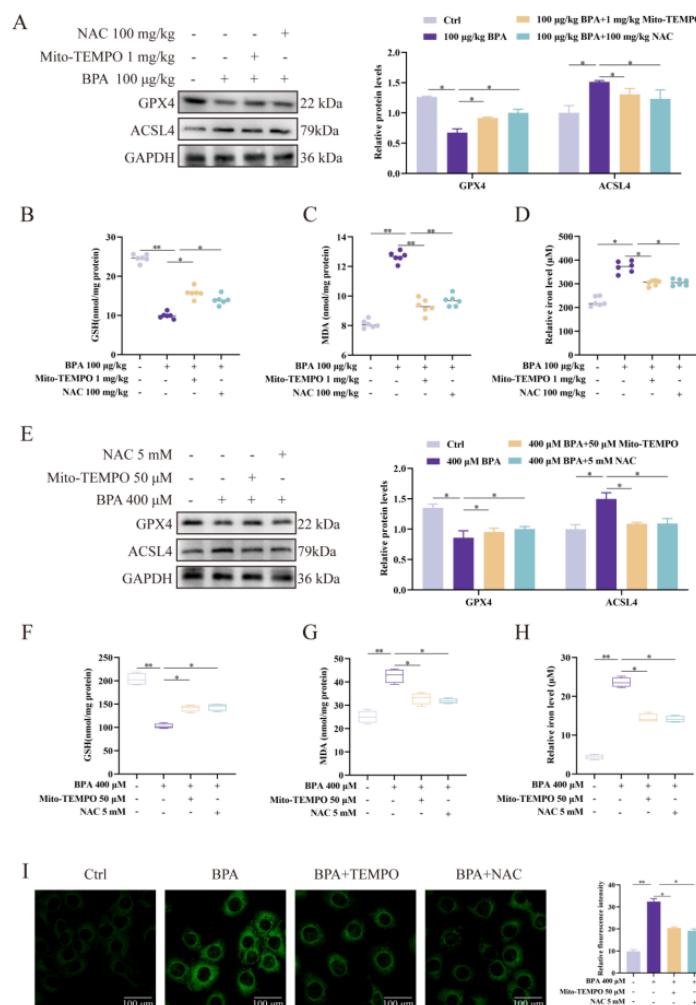


Fig. 13. mtROS was involved in BPA-induced hepatocytes ferroptosis. (A) Quantitative study and protein expression profiles for GPX4 and ACSL4. (B-D) GSH, MDA and liver iron ion amount contained were calculated using kits. (E) Quantitative study and protein expression profiles for GPX4 and ACSL4. (F-H) GSH, MDA and AML12 cells iron ion amount contained were calculated using kits. (I) Lipid ROS was detected with BODIPY TM 581/591 C11 (scale bar, 100 µm). * $P < 0.05$, ** $P < 0.01$.

4. Discussion

Although previous studies have investigated BPA-induced liver injury, the doses employed were excessively high (exceeding 50 mg/kg)^[32]. BPA can migrate from food packaging materials into food, which represents a major route of human exposure^[33,34]. Quantitative migration data indicated BPA levels ranging from 0.25 nmol/L to 1000 nmol/L across food types, with canned foods reaching mean values of 134.57 µg/kg (59.17 µg/kg -223.25 µg/kg) and consumer practices such as in-package heating further elevating exposure^[35]. This range overlaps substantially with concentrations known to induce cellular effects, as even 1 nmol/L -100 nmol/L BPA disrupts mitochondrial function *in vitro*^[36]. In the present study, we demonstrated that mice exposed to environmentally relevant doses of BPA (50 µg/kg bw/day) for 30 days developed significant liver injury. Further investigations revealed that BPA induced lysosome-dependent pyroptosis and ferroptosis in hepatocytes. Mechanistically, BPA induced mtROS overproduction through destroying mitochondria. Subsequently, mtROS-mediated LMP facilitated the CTSB leakage and iron efflux, which led to NLRP3 inflammasome activation and iron ion accumulation, ultimately triggering pyroptosis and ferroptosis.

The role of lysosomal dysfunction in liver injury has garnered significant attention. However, previous research has primarily focused on the accumulation of harmful metabolites resulting from autophagic impairment^[37], with limited investigation into the driving role of LMP. During LMP, the lysosomal membrane ruptures, followed by the shedding of LAMP1 and LAMP2 from the membrane into the cytosol and their subsequent degradation by cytosolic proteases, leading to decreased protein expression levels. Consequently, the expression levels of LAMP1 and LAMP2 serve as core indicators for assessing LMP. We found that BPA significantly reduced the expression of LAMP1 and LAMP2 both *in vivo* and *in vitro*, indicating BPA induced LMP in hepatocytes. In this study, administration of mannose rescued BPA-induced LMP and significantly alleviated BPA-induced liver injury, demonstrating that LMP was a pivotal event in BPA-driven liver injury.

LMP-driven liver injury depends on LDCD, triggered by the release of lysosomal contents into the cytosol^[38]. Indeed, the molecular mechanisms underlying LDCD are complex, while directly initiated by LMP, its downstream cascades frequently intersect with other programmed cell death pathways^[17]. Unlike other lysosomal hydrolases whose activity is strictly dependent on an acidic environment, CTSB retains catalytic activity in the neutral cytosol^[39]. Cytosolic CTSB can directly interact with the leucine-rich repeat (LRR) domain of NLRP3, activating the NLRP3 inflammasome and subsequent pyroptosis^[40]. Our results demonstrated that BPA promoted CTSB expression in hepatocytes and activated NLRP3-dependent pyroptosis. Both mannose and the CTSB inhibitor CA074-ME alleviated BPA-induced pyroptosis, indicating that BPA induced hepatocytes pyroptosis through LMP-mediated CTSB leakage. In addition to cathepsins, lysosomes serve as significant reservoirs of iron ions. Iron ions accumulate within lysosomes during the degradation of iron-containing proteins (e.g., ferritin) and damaged organelles (e.g., dysfunctional mitochondria)^[41]. In this study, BPA induced lysosomal iron efflux and triggered ferroptosis. Both mannose and the iron chelator DFO mitigated BPA-induced ferroptosis, demonstrating that BPA induced hepatocytes ferroptosis via LMP-mediated iron efflux. Persson et al. reported that pulmonary allografts induce LMP and

cell death in pulmonary macrophages concomitant with increased iron content, although the precise mechanisms were not explored^[42]. Our findings may provide a reliable mechanistic explanation for this phenomenon. LMP-mediated lysosomal iron efflux caused intracellular iron overload, leading to strong Fenton reactions and subsequent lipid peroxidation, ultimately inducing ferroptosis. Substantial evidence has confirmed the existence of synergistic interactions among multiple cell death modalities within a single pathological insult^[43]. Our study revealed that LMP-mediated CTSB leakage and lysosomal iron efflux triggered pyroptosis and ferroptosis, respectively, synergistically driving BPA-induced hepatic injury.

The catalase deficiency and iron-rich environment render the lysosomal membrane susceptible to ROS damage^[30]. Our results showed that BPA led to an excess of mtROS and ROS in AML12 cells. Moreover, mtROS inhibitor Mito-TEMPO and ROS scavenger NAC exhibited similar mitigating effects on BPA-induced LMP, suggesting that mtROS mediated BPA-induced LMP. Mitochondrial quality control is a cellular endogenous protective program to maintain mitochondrial homeostasis and function, in which mitochondrial fission/fusion dynamics and mitophagy are critical^[30]. Impaired mitochondrial quality control caused dysfunction within the mitochondria and mtROS excessive production^[44]. Mfn1 and Fis1 are central components in the regulation of mitochondrial fusion and fission, respectively. Our study showed that BPA decreased the level of Mfn1 protein and increased the expression of Fis1 protein, suggesting that BPA disrupted mitochondrial dynamics in hepatocytes. Consistent with our research, Yao et al. have reported that BPA induce colonic mitochondrial dynamics disorder. Mitophagy is a metabolic process that removes damaged mitochondria, and the PINK1/Parkin pathway is the predominant mechanism^[45]. VDAC1, an outer mitochondrial membrane protein, is a mitochondrial substrate for Parkin. Impaired mitophagy results in the inability to remove damaged mitochondria, leading to the accumulation of VDAC1^[46]. We found that BPA inhibited mitophagy as evidenced by the decreased levels of PINK1 and Parkin proteins and increased abundance of VDAC1. Our results showed that BPA led to mitochondrial dysfunction and mtROS overproduction by disrupting mitochondrial dynamics and inhibiting mitophagy. The triggering role of mtROS in pyroptosis and ferroptosis has been previously documented. In this study, both Mito-TEMPO and NAC similarly suppressed BPA-induced pyroptosis and ferroptosis. Distinct from prior research, our findings demonstrated that mtROS-mediated LMP served as a shared upstream pathway for pyroptosis and ferroptosis induction by BPA. Intriguingly, the pyroptosis executioner protein GSDMD-N has been reported to form pores in the mitochondrial outer membrane, exacerbating mitochondrial impairment and mtROS generation. This implied a potential feedback circuit between mitochondrial damage and pyroptosis, which will be the focus for our future investigations.

In this study, AML12 hepatocytes were used for *in vitro* experiments, which are genetically consistent with the C57BL/6 mouse strain. This ensured mechanistic findings on BPA-induced mitochondrial and lysosomal effects were aligned between *in vitro* and *in vivo* systems. However, as a murine cell line, AML12 may not fully capture human-specific differences in BPA metabolism and cellular pathways. Therefore, extrapolation to human hepatocytes should be made with caution, and future studies using human-derived

hepatocyte models would be valuable for translational validation. Furthermore, while the once-daily oral gavage regimen employed in this study represents a standard approach in toxicological research, it differs from the continuous, multi-route exposure patterns experienced by humans. Human exposure typically occurs through diet, dermal contact, and inhalation, resulting in sustained low-level presence rather than the peak concentrations following a single bolus dose. Therefore, future studies should focus on simulating real-world exposure scenarios, such as through dietary admixture or administration in drinking water, to further validate our findings at environmentally relevant concentrations.

In conclusion, our results showed that BPA induced lysosome-dependent pyroptosis and ferroptosis via signal crosstalk between mitochondria and lysosomes in hepatocytes. mtROS overproduction caused by mitochondrial dysfunction subsequently led to LMP, which mediated the CTSS leakage and iron efflux, eventually inducing pyroptosis and ferroptosis. Our study indicated that lysosomes acted as the hub linking pyroptosis and ferroptosis. The mtROS/LMP axis may represent a potential shared pathway for pyroptosis and ferroptosis, providing a mechanistic framework for BPA-induced hepatotoxicity and novel insights into the intricate crosstalk between cell death modalities.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Data availability

Data will be made available on request.

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Supporting Information

Supporting data, including the effects of BPA on mitochondrial autophagy and mitochondrial fusion-fission proteins in the livers of mice, as well as quantitative immunofluorescence analysis, etc.

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