# Oxidative stress induced by combined glyphosate and TBBPA exposure promotes gill autophagy and inflammation via the PI3K/AKT/mTOR pathway

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**Abstract**

Glyphosate and tetrabromobisphenol A (TBBPA) are pollutants that pose a serious threat to the ecological safety of aquatic environments. However, there has been no report on the effects of combined exposure on the toxicity of carp fish gills in water. Therefore, we constructed a model of carp gill tissue and EPC cells exposed to glyphosate and/or TBBPA, established a control group, a glyphosate group, a TBBPA group, and a glyphosate +TBBPA group, and added PI3K/AKT pathway activator *in vitro* to establish a glyphosate +TBBPA+Musk ketone group. qRT-PCR and western blotting methods were used to detect the expression of oxidative stress-related indicators (CAT, GSH-PX, T-AOC, $H_2O_2$) and related genes. *In vitro* and *in vivo* results showed that glyphosate and/or TBBPA exposure resulted in overproduction of ROS, decreased activity of CAT, GSH-PX, T-AOC, and increased $H_2O_2$ content. Glyphosate and/or TBBPA exposure inhibited the PI3K/AKT/mTOR signaling pathway, further resulting in increased autophagy related genes LC3, ATG-5, Beclin-1, and decreased P62 expression. Inflammation related genes TNF-α, IL-1β, IL-6, IL-18 increased. And it was more significant when exposed in combination than when exposed alone. The addition of PI3K/AKT signaling pathway activator musk ketone *in vitro* can significantly alleviate the changes of autophagy and inflammation-related indicators. In summary, glyphosate and/or TBBPA induce oxidative stress by promoting gill autophagy and inflammation via the PI3K/AKT/mTOR pathway.

Keywords : Glyphosate, TBBPA, ROS, PI3K/AKT/mTOR, Autophagy, Inflammation.

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## 1. Introduction

Glyphosate is one of the most widely used broad-spectrum herbicides (Baylis 2000). Glyphosate is popular worldwide for its effective control of weeds in water, agriculture and forests (Yang, et al. 2019). In 2022, the global glyphosate industry production is about 900,000 tons, of which the domestic glyphosate industry will produce up to 810,000 tons. Glyphosate continues to be used, resulting in its eventual deposition in water and soil, causing damage to the ecological environment. The water toxicity of glyphosate is extensive, and microorganisms, plants, and fish can be affected by it. (Tarazona, et al. 2017). Glyphosate can cause liver, kidney and nervous system damage in mammals and fish. (Jia, et al. 2022b; Madani and Carpenter 2022). Glyphosate was found to cause liver damage in common carp (Nešković, et al. 1996) and Nile tilapia (Jiraungkoorskul, et al. 2003). The report also found pesticide residues in fish gill tissue caused gill damage (Menéndez-Helman, et al. 2020). Studies have shown that glyphosate can cause inflammation in human glioblastoma (Bianco, et al. 2023), and that glyphosate exposure can increase ROS production in amphoteric neutrophils with increased oxidative stress damage. (Leblanc, et al. 2024). Glyphosate and polyethylene microplastics alone or in combination interfered with carp behavior, gut microbiome homeostasis, and metabolites (Chen, et al. 2022). In addition, glyphosate regulates the IRS-1/PI3K/Akt pathway by promoting the production of a large number of reactive oxygen species (ROS) in skeletal muscle, promoting insulin resistance and ultimately leading to Diabetes mellitus type 2 (T2DM). (Jayaraman, et al. 2023).

PI3K/AKT/mTOR pathway is critical in physiological and pathological conditions such as cell survival (Porta, et al. 2014). Experiments have shown that the coronavirus-2 (SARS-CoV-2) spike protein increases ROS, thereby inhibiting this pathway and promoting autophagy leading to inflammation and apoptosis (Li, et al. 2021). At the same time, it has been proved that oxidative stress can induce this pathway to lead to macrophage autophagy to protect atherosclerosis (Fang, et al. 2021). Experiments have shown that the addition of PI3K pathway inhibitors can inhibit the pathway and activate autophagy to inhibit peritoneal fibrosis (Jia, et al. 2022a). Selenoprotein U knockdown is closely related to this pathway, and autophagy occurs by inhibiting this pathway in chicken support cells (Sattar, et al. 2018). $H_2S$ induced oxidative stress inhibits this pathway and promotes LPS-mediated hepatocyte autophagy (Guo, et al. 2021). TRIM22 induces psoriasis by activating this pathway to increase the number of cells and promote inflammation (Ren, et al. 2022). Tanshinone IIA effectively suppresses inflammation, ferroptosis, and apoptosis mediated by ischemia-reperfusion injury through activation this pathway. (Zhang, et al. 2023b). Brg1 inhibits increased inflammation of the respiratory tract through this pathway (Zou, et al. 2018). Zhenwu Decoction protects chronic glomerulonephritis by inducing mitochondrial autophagy through this pathway (Liu, et al. 2021). By activating this pathway, scopolamine reduced lipopolysaccharide-induced (LPS) autophagosome and autophagy substrate accumulation, reactive oxygen species production and inflammatory response (Liu, et al. 2020).

Tetrabromodiphenol A (TBBPA) is an effective brominated flame retardant and is in great demand in the current market (Zhou, et al. 2020). Large-scale production and not timely treatment, resulting in more and more serious environmental pollution. In China, demand increased from 5.21 million tons to 6.43 million tons from 2014 to 2017. (Han, et al. 2019). TBBPA is a serious threat to human and animal life, because they can be found everywhere. TBBPA may induce neurological, endocrine, liver and kidney function damage. (Cope, et al. 2015; Jia, et al. 2022c; Szychowski and Wójtowicz 2016; Wang, et al. 2021). TBBPA can cause irreversible damage to aquatic animals

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(Khan, et al. 2023). Studies have shown that liver antioxidant enzyme in crucian carp can cause damage if 0.50-0.71 mg/L TBBPA is exceeded during a 32-day exposure period (Yang SuWen, et al. 2013). It has also been found that TBBPA can induce gill tissue damage in fish. In addition, TBBPA induces inflammation and uterine injury in mice by inhibiting PI3K signaling and activating NF-κB signaling (Zhang, et al. 2021). TBBPA induces liver oxidative stress and autophagy by triggering oxidative damage in Rana nigromaculata (Han, et al. 2023). The heavy use of herbicides and brominated flame retardants has greatly increased the risk of glyphosate and TBBPA being present in water at the same time. In recent years, with the aggravation of water pollution, fish survival has been seriously threatened, and the research on glyphosate and TBBPA has begun, however, current studies on single pollutants are limited. Studies have shown that both glyphosate and TBBPA are present in water bodies. The toxicity of glyphosate and TBBPA exposure to fish gills still needs to be explored. ROS is crucial in toxicological studies. PI3K/AKT/mTOR pathway can mediate autophagy and inflammation. However, the relationship between oxidative stress, the PI3K/AKT/mTOR pathway, autophagy, and inflammation after exposure to glyphosate and TBBPA remains unclear. Therefore, we established glyphosate and/or TBBPA exposure models of fish gill tissue. The gill morphology was observed by hematoxylin and eosin (H&E) staining. Oxidative stress index ($H_2O_2$, GSH-PX, T-AOC, CAT) in fish gill tissue was detected by oxidative stress kit. Real-time quantitative PCR (qRT-PCR), western blot and immunofluorescence were used to detect mRNA and protein expression levels of components of PI3K/AKT/mTOR pathway, autophagy and inflammatory genes. In this study, the carp epithelioma cells (EPC) cells were cultured *in vitro* to further verify the relationship between the two poisons and oxidative stress, PI3K/AKT/mTOR signaling pathway, autophagy and inflammation. The purpose of this study is to clarify the mechanism of glyphosate and TBBPA induced gill injury, and provide a new research direction for the toxicology research of glyphosate and TBBPA.

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## 2. Material method

### 2.1 Establishment and grouping of animal models

Sixty healthy carp of similar weight were raised in 15 litre buckets of water in Changling Lake Fish Farm in Harbin, with 5 carp per barrel. Carp were divided into four groups: Control, glyphosate, TBBPA and glyphosate+TBBPA. Glyphosate (MacLean, Shanghai, China) and TBBPA (98% purity, Aladdin) in water were 50 μg/L and 500 μg/L, respectively. 1/3 of the dechlorinated water is replaced daily with glyphosate and TBBPA. Carp were euthanized on the 31st day of feeding, and jejunum was immediately extracted for washing and then fixed at 4% paraformaldehyde or placed at -80 °C. The Experimental Animal Protection and Use Committee of Northeast Agricultural University (SM-11) approved all procedures for the use of this experiment.

### 2.2 Cell treatment

The EPC cells were removed from the cell storage tank, rapidly vibrated in a 37 ℃ water bath, and quickly transferred to a centrifuge tube with 3mL complete medium when the cells were not completely melted, and centrifuged at 1000 rpm for 5 min. The complete medium consisted of 90% L15 (11415064, Gibco), 10% fetal bovine serum (1705124, VivaCell, Shanghai, China), and 1% penicillin-streptomycin - amphotericin B solution (Beyotime, China). Add 3 mL medium into the cell culture bottle. After centrifugation, discard the supernatant, add 2 mL complete medium into the centrifuge tube to form suspension, transfer to the cell bottle and shake well. It was cultured in an incubator without $CO_2$ at 28.5 ℃. 24 h to change the liquid once, 48 h to pass a generation, and all operations were carried out under aseptic operation.

### 2.3 Cell viability determination

EPC cells were distributed in 96-well plates at a density of $1\times10^5$ cells per well, and different concentrations of glyphosate or/and TBBPA were added to the cells and placed in the incubator for 24 h The dilution method of glyphosate and TBBPA is as follows: Glyphosate was dissolved in PBS (phosphate buffered saline) to obtain the initial concentration of 10 mg/mL, and diluted to 1, 10, 100, 200, 250, 300, 400 μM with L15 complete medium, respectively. First, TBBPA was dissolved with DMSO (Solarbio, China) to obtain the initial concentration of 100 mg/mL, and diluted to 0.1, 1, 2, 4, 8, 10, 16, 20, 32 μM in L15 complete medium, respectively. Musk ketone (81-14-1, MCE) was placed in DMSO to obtain 1mM and then diluted to an experimental concentration of 1.8 μM. CCK-8 (40203ES80, Yeasen) was mixed with L15 medium at a ratio of 1:9, and then added into 96-well plates with 10 μL per well. EPC cells were then cultured at 28.5 ℃ for 1.5 h, and the absorbance of 450 nm was determined by Multiskan SkyHigh (Thermo).

### 2.4 Hematoxylin and eosin (H&E) staining

To visually observe the damage caused by glyphosate and/or TBBPA to fish gill tissue. The fixed carp gill tissue was dehydrated with alcohol. After paraffin embedding, sections were made with a thickness of 5 μm, three pieces per group. The slices were dewaxed and hydrated, then H&E stained, and finally photographed with the CaseViewer software.

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## 2.5 Oxidative stress index detection

The gill tissues of the four groups were treated with 0.9% physiological salt homogenate, and about $1\times10^6$ EPC cells in each group were cleaved with 500 μL normal saline. The supernatant was used to detect oxidative stress kit.The protein concentration was calculated according to the instructions of Coomasil Bright Blue (TP, A045-2-2, Nanjing). The activity or content of $H_2O_2$ (A001-1-2, Nanjing), GSH-PX (A005-1-2, Nanjing), T-AOC (A015-2-1, Nanjing) and CAT (A007-1-1, Nanjing) were calculated according to the protein concentration.

## 2.6 ROS detection

EPC cells were cultured according to the above treatment. Follow the instructions of reactive oxygen species assay kit (E004-1-1, Nanjing). After staining for about two minutes, check under the microscope for staining. After dyeing, the dye is removed and replaced with PBS. We used fluorescence microscopy (Thermo Fisher Scientific, USA) to measure the fluorescence intensity of cells to assess intracellular ROS levels in EPC cells. The fluorescence comprehensive density was analyzed by ImageJ software.

## 2.7 Autophagy was detected by MDC

EPC cells were cultured according to the above treatment. according to the instructions of the autophagy staining assay kit (Solarbio, Beijing). Images were collected by Olympus inverted fluorescence microscope, and ImageJ was used to analyze the fluorescence intensity of autophagy.

## 2.8 Immunofluorescence staining of fish gill tissue and EPC cells

EPC cells were cultured according to the above treatment. Wash gently with 4% paraformaldehyde histiocytic fixative and add 1 mL fixative. After 8h, the fixing solution was extracted and washed with the washing solution. After washing, it was sealed for 1.5 h, and the first antibody was added at 4 ℃ overnight. The primary antibody was recovered, the cells were washed and the fluorescent secondary antibody was added for 1.5 h. The whole operation process was shielded from light. After the second antibody was recovered, the cells were washed, and the second primary antibody was added and incubated at 37 ℃ for 2 h. The second primary antibody was recovered and washed and the second fluorescent antibody was added for 1 h. After the second antibody was recovered, the cells were washed and DAPI nuclear dye was added. The cells were observed under a fluorescence microscope for 5 minutes. After dyeing, DAPI is sucked out and anti-fluorescence quencher is added. The above washing conditions are 3 times for 5 minutes. The required antibodies were formulated as follows: LC3 Rabbit mAb (1:200, wanleibio), p62 Rabbit mAb (1:200, Abclonal), TNF-α Rabbit mAb (1:300, wanleibio), IL-1β (1: 200, wanleibio). Dlight 594 goat anti-rabbit (1:500, Biodragon), Dlight 488 goat anti-rabbit (1:500, Biodragon), DAPI staining (1:1000, Beyotime). Images were collected by Olympus inverted fluorescence microscope, and Image J was used to analyze fluorescence intensity.

## 2.9 RNA extraction and qRT-PCR

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Refer to the article for specific experimental methods of RNA extraction(Li, et al. 2022). Then the total RNA was reverse-transcribed into cDNA (BSB40M1, BioFlux) according to the instructions, and SYBR green fluorescent dye (BSB25L1 B, BioFlux) was used in LightCycler®480 II (Roche, Switzerland) system for quantitative detection. Primers in this experiment are listed as follows **Table 1**. The β-actin sequence plays a normalized role in endogenous control and $2^{-\Delta\Delta CT}$ was used to calculate the target gene.

**Table 1**

The primers used in the present study.

| Gene | Forward primer (5′-3′) | Reverse primer (3′-5′) |
|---|---|---|
| β-Actin | ACCTGAGCGTAAATACTCTGTCTGG | TCGTCATACTCCTGCTTGCTAATCC |
| IL1β | GAACAACGGCAACAGTCAGAGTG | AGATGGTGTGCTCGTCATGGC |
| IL-6 | GTCTGCTACACTGGCTACACTCTTC | GTCCACATCCTGAACTTCGTCTCC |
| IL-18 | GAACAACGGCAACAGTCAGAGTG | AGATGGTGTGCTCGTCATGGC |
| TNF-α | GCTGTCTGCTTCACGCTCAA | CTTGGAAGTGACATTTGCTTTT |
| Beclin-1 | CAGTTGGACACAGAGGAGCTACAG | CTGAACCTGGCAATAACGCATCTG |
| Atg5 | AAGGCAGAGGATGTGGAGGAAATG | CCAGGGCAGAGCGGAGTTTG |
| LC3 | ATGCCTTCGGAAAAGACATTTAAAC | TTACTGAGGACACGCAGTTCC |
| P62 | GGTGGCTGGAACTCGCTTCAAG | AGGGTGAAAGATGGGCAGGAGAG |
| mTOR | TGCGGAGTATGTGGAGTT | CCGTGGAGTCTTCTGCCTTC |
| PI3K | AGTCAGTGCCTGTGGCTGAG | CGTGTCCATGACCTCAGAGC |
| AKT | CCTGGTGATGAAGGAGCTGA | CTGTCAGAGAGCCTCCAGCA |

### 2.10 Protein extraction and Western blot

Refer to the article for specific experimental methods of protein extraction(Miao, et al. 2021). The primary antibodies used in this experiment were LC3 (1:1000, wanleibio), p62 (1:1500, Abclonal), Beclin-1 (1:1500, wanleibio), ATG5 (1:1500, wanleibio), TNF-α (1:1000, wanleibio), IL-1β (1:1500, wanleibio), IL-6 (1:1000, wanleibio), IL-18 (1:1500, wanleibio), β-actin (1:50,000, Abmart). The second antibody is the anti-rabbit IgG (1:100,000) that binds to the first antibody. Finally, the X-ray film was used to detect the proteinsignal.

### 2.11 Statistical analysis

In this paper, all the results were obtained using GraphPad Prism8.0.1 software. One-way ANOVA and Tukey's post-hoc multiple comparison were used to analyze the significance of differences between groups. N represents the sample size (biological replication), and the above experiments were conducted at least three times ($n \geq 3$). The resulting data are expressed as the standard error (SEM) of the mean ± mean. The same letter indicated no significant difference ($P > 0.05$); Different letters indicated the difference was statistically significant ($P < 0.05$).

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## 3. Result

### 3.1 H&E staining of carp gill tissue

The gill tissue was stained by H&E to see directly how glyphosate and TBBPA affect gill tissue damage (Fig. 1). In the control group, the gill lamella arranged regular epithelial cells were normal. In the glyphosate and TBBPA monotoxic groups, the gill lamellas were arranged in an incomplete or disintegrated tilt (represented by red arrows), a small number of epithelial cells were necrotic (represented by green arrows), a small amount of eosinophilic infiltration (represented by yellow arrows). In the glyphosate+TBBPA group, epithelial cells damage were aggravated and eosinophils increased. The above results showed that glyphosate and TBBPA both caused gill tissue damage, and glyphosate+TBBPA group caused more gill tissue damage. We speculate that it may be caused by autophagy and inflammation in epithelial cells.

### 3.2 Effects of glyphosate or/and TBBPA on cells viability

To investigate the toxicity of glyphosate and TBBPA alone or in combination on EPC cells, the CCK-8 method was used to detect the viabilities of EPC cells treated with glyphosate, TBBPA, and glyphosate+TBBPA. As shown in (Fig. 2). EPC cell activity was measured at 1, 10, 100, 200, 250, 300, 400 μM glyphosate concentration and 0.1, 1, 2, 4, 8, 10, 16, 20, 32 μM TBBPA concentration. The results showed that the cell viability decreased with the increase of glyphosate and TBBPA concentrations (n = 3). The IC50 for glyphosate was 378.4 μM (95% confidence interval 362.4 μM to 397.3 μM). The IC50 of TBBPA was 42.37 μM (95% confidence interval 35.96 μM to 54.09 μM). In subsequent experiments, the concentration selected for glyphosate group was 200 μM, the concentration selected for TBBPA group was 10 μM, and the concentration selected for glyphosate +TBBPA group was (200 μM glyphosate +10 μM TBBPA).

### 3.3 Glyphosate or/and TBBPA exposure caused oxidative stress in carp gill tissue and EPC cells

To explore whether glyphosate or/and TBBPA cause oxidative stress in fish gill tissue and EPC cells, we detected oxidative stress relevant indicators (n = 5). As shown in (Fig. 3A and B), glyphosate and TBBPA inhibited the activities of antioxidant enzyme CAT ($P<0.05$) and free radical reaction related enzyme GSH-PX ($P<0.05$), the total antioxidant capacity T-AOC decreased, and the $H_2O_2$ content increased ($P<0.05$), and the effect was more obvious in glyphosate+TBBPA group ($P<0.05$), indicating that oxidative stress was more severe in the double exposure group

To further evaluate the effects of glyphosate or/and TBBPA on oxidative stress of EPC cells, the DCFH-DA probe was used to determine the intracellular ROS in EPC cells under different treatment (Fig. 3C and D). According to quantization result (Fig. 3D), the ROS production in glyphosate and TBBPA groups was higher than that in control group ($P<0.05$) , and glyphosate+TBBPA group had the highest ROS level ($P<0.05$). These results suggest that glyphosate and TBBPA induce overproduction of ROS, and dual exposure to both reaches higher levels.

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### 3.4 Effects of glyphosate or/and TBBPA exposure on the PI3K/AKT/mTOR pathway

We analyzed gene and protein expression levels of PI3K/AKT/mTOR pathway, which often plays a key role in autophagy (n = 5). Compared with the control group (Fig. 4), glyphosate and TBBPA single exposure groups were significantly decreased, which was manifested in PI3K, AKT and mTOR ($P<0.05$) levels, and these changes were aggravated in glyphosate+TBBPA group ($P<0.05$). However, in the glyphosate+TBBPA+Musk ketone group, the decreased expression levels of the above genes and proteins were moderated ($P<0.05$). The results showed that glyphosate and TBBPA could inhibit this pathway, and the effect of glyphosate and TBBPA dual exposure was more obvious. The addition of musk ketone mitigated the effects of combined glyphosate and TBBPA exposure.

### 3.5 Effects of glyphosate or/and TBBPA exposure on autophagy of carp gill tissue and EPC cells

To investigate whether glyphosate or/and TBBPA can cause autophagy, the expression of LC3 and p62 in fish gill tissue was studied by immunofluorescence technique (Fig. 5A and B), LC3 fluorescence intensity was higher in glyphosate and TBBPA signal exposure group than in control group ($P<0.05$), and the highest in glyphosate + TBBPA groups ($P<0.05$). p62 fluorescence intensity was opposite to LC3.

The autophagy related genes and proteins expression were detected. As shown in (Fig. 5C-E), after exposure to glyphosate and TBBPA, LC3II, Beclin-1 and ATG5 expression were higher than control group ($P<0.05$), p62 bucked this trend ($P<0.05$). The expression levels of LC3II, Beclin-1 and ATG5 were the highest and the expression levels of p62 were the lowest in the group exposed to glyphosate and TBBPA. This suggests that glyphosate and/or TBBPA can induce autophagy in fish gill tissue.

To determine whether autophagy is also induced in cells, we tested autophagy with the MDC kit. As shown in (Fig. 5F-G), glyphosate and TBBPA could induce autophagy in EPC cells ($P<0.05$), and autophagy occurred more stronglye in glyphosate+TBBPA group ($P<0.05$). However, after glyphosate, TBBPA and musk ketone treatment, the fluorescence intensity of autophagosomes decreased in EPC cells. Then, immunofluorescence was used to detect LC3 and p62 in EPC cells (Fig. 5H-I). The result was the same as above, LC3 levels in EPC cells treated by musk ketone were reduced ($P<0.05$), and p62 has the opposite trend ($P<0.05$)

Next, genes and proteins in EPC cells were detected (Fig. 5J-L), after exposure to glyphosate and TBBPA, LC3II, Beclin-1 and ATG5 expression were higher than control group ($P<0.05$), p62 bucked this trend ($P<0.05$). The expression levels of LC3II, Beclin-1 and ATG5 were the highest and the expression levels of p62 were the lowest in the group exposed to glyphosate and TBBPA. However, after glyphosate, TBBPA and musk ketone treatment, autophagy expression levels were reduced. The above results indicate that glyphosate and TBBPA can cause autophagy, and autophagy is aggravated by their combined exposure, and autophagy is alleviated after the addition of musk ketone.

### 3.6 Effects of glyphosate or/and TBBPA exposure on inflammation of carp gill tissue and EPC cells

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In order to investigate whether glyphosate or/and TBBPA exposure could induce inflammation, we used immunofluorescence to detect TNF-α and IL-1β expression levels in fish gill tissues (Fig. 6A and B). TNF-α and IL-1β fluorescence intensity was higher in glyphosate and TBBPA signal exposure group than in control group ($P<0.05$), and the expression of glyphosate+TBBPA group was the highest ($P<0.05$).

The inflammation-related genes and proteins expression were detected (Fig. 6C-E). After exposure to glyphosate and TBBPA, the genes and protein levels of TNF-α, IL-1β, IL-6 and IL-18, were increased compared with control group ($P<0.05$), and the highest levels were found in glyphosate+TBBPA group ($P<0.05$).

To further validate, genes and proteins associated with inflammation were detected in EPC cells (Fig. 6F-H), the results were the same as above. After EPC cells were treated with musk ketone, the inflammatory response could be slowed down. These results indicate that compared with glyphosate and TBBPA alone, combined exposure to glyphosate and TBBPA could aggravate inflammation in gill tissues and EPC cells, and inflammation is alleviated after the addition of musk ketone.

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## 4. Discussion

In recent years, the co-occurrence of glyphosate and TBBPA on biological health has caused widespread concern (Lu, et al. 2021; Zhang, et al. 2023a). Many experiments prove that glyphosate and TBBPA cause oxidative stress while causing tissue and organ damage (Feng, et al. 2022; Xu, et al. 2020). The PI3K/AKT/mTOR pathway is associated with both autophagy and inflammation (Han, et al. 2022; Sul and Ra 2021). Studies have shown that ammonia inhibits PI3K/AKT/mTOR pathway by inducing ROS, causing inflammation and autophagy (Sun, et al. 2023). This experiment elucidated the mechanism of glyphosate and TBBPA co-occurrence leading to gill injury. The results show that the simultaneous occurrence of glyphosate or TBBPA can increase ROS, inhibit PI3K/AKT/mTOR pathway, resulting in autophagy and inflammation.

Description of previous experiments that glyphosate promotes ROS generation causing oxidative damage and carp hepatitis, and alters the physical barrier of the intestine. Meanwhile, ROS has also been found to be associated with oxidative DNA damage and mitochondrial dysfunction (Cao, et al. 2022). TBBPA generates ROS in the gastric mucosa, which cause inflammation and cell death (Xu, et al. 2023). The experiment shows that the simultaneous occurrence of DEHP and PS-MPs can inhibit the increase of antioxidant enzymes, at the same time, increase oxidase levels and the level of ROS in mice, thus leading to oxidative stress. (Wu, et al. 2023). Consistent with previous studies, CAT, GSH-PX and T-AOC activities decreased and $H_2O_2$ content increased in gill tissues and cells after glyphosate or TBBPA exposure. Indicates oxidative stress. Compared with single exposure, glyphosate and TBBPA synergistic exposure increased oxidative stress and excess ROS production in EPC cells in fish gill tissue. The experiment shows that ROS upregulation inhibits PI3K/Akt/mTOR pathway, thereby preventing myocardial damage caused by LPS (Chen, et al. 2017). Enhancing ROS levels inhibits PI3K/AKT/mTOR pathway to trigger apoptosis (Jiang, et al. 2024). In this experiment, we detected pathway mRNA and protein levels. After glyphosate or TBBPA exposure, the levels of passage-related genes in fish gill tissues and cells showed a downward trend, and the levels of pathway indicators were lowest when glyphosate and TBBPA were synergistically exposed compared with single exposure. After adding PI3K pathway activator, the indexes were increased. We demonstrated that glyphosate or/and TBBPA exposure induced ROS production and inhibited PI3K/Akt/mTOR pathway. Experiments have shown that autophagy and inflammation can be induced by inhibition of the PI3K/Akt/mTOR pathway (Idriss and Naismith 2000; Sun, et al. 2024). P62 is an important autophagy receptor that accumulates when inhibition occurs, so P62 can be used as a marker to study autophagy flux. Beclin-1 is an important molecule in regulating autophagy maturation (Lopez-Castejon and Brough 2011). ATG5 plays an important role in initiation, nucleation, elongation and closure of autophagosomes, while LC3-II is the only protein that remains on the autophagosome bilayer membrane (Tanaka, et al. 2016), which is the most direct evidence to confirm autophagy and can directly reflect the activity of autophagy (Ihim, et al. 2022). It has been proved that BPA can aggravate ROS and increase autophagy genes ATG5, LC3-II/I and Beclin-1, while decrease autophagy flux marker P62, resulting in increased autophagy injury (Saitoh and Akira 2010). Meanwhile, in our study, when glyphosate and TBBPA were exposed at the same time, the increase and decrease of each index were consistent with the study results. In addition, immunofluorescence results showed that LC3 and P62 were consistent with the above trend after glyphosate and TBBPA exposure. Subsequently, for further verification, musk ketone, an activator of PI3K pathway, was added and autophagosomes were observed with MDC staining. These results proved that glyphosate

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and TBBPA exposure induced oxidative stress and gill autophagy inhibited PI3K/AKT/mTOR pathway. What is more noteworthy is that autophagy plays different roles in different inflammatory responses. The transport of IL-1β to vesicular intermediates during autophagy mediated secretion has been demonstrated experimentally (Zhang, et al. 2015). The unconventional secretion pathway of autophagy delivers IL-1β outside the cell (Dupont, et al. 2011). Autophagy causes the body to produce IL-6 to support the maintenance of breast cancer stem cells (Maycotte, et al. 2015). In the gill study, we further analyzed whether glyphosate and TBBPA exposure can trigger PI3K/AKT/mTOR pathway to induce inflammation. The results showed that IL-1β, IL-6, IL-18 and TNF-α increased when glyphosate and TBBPA were present. Meanwhile, the immunofluorescence results of TNF-α and IL-1β were the same as above. Musk ketone, a PI3K pathway activator, was added to detect the mRNA and protein of inflammation-related genes. The results demonstrated that glyphosate and TBBPA exposure induced gill inflammation through the PI3K/AKT/mTOR pathway.

In conclusion, we demonstrated that glyphosate and TBBPA toxicity have a synergistic effect, and that simultaneous exposure to both causes more damage to fish gills than exposure alone. In the mechanism, glyphosate and TBBPA induce ROS overproduction, inhibit PI3K/AKT/mTOR pathway, and lead to gill autophagy. At the same time, autophagy promotes the release of inflammatory factors, leading to inflammation. In short, oxidative stress induced by glyphosate and TBBPA combined exposure promotes gill autophagy and inflammation through the PI3K/AKT/mTor pathway. This study enriched the theoretical mechanism of toxicity of glyphosate and TBBPA to fish gill injury, and provided a reference for the risk of glyphosate and TBBPA to human health.

**Abbreviations**

| | |
|---|---|
| Autophagy-related 5 | ATG-5 |
| Catalase | CAT |
| Diabetes mellitus type 2 | T2DM |
| Glutathione peroxidase | GSH-Px |
| Hydrogen peroxide | $H_2O_2$ |
| Immunofluorescence staining | IF |
| Microtuble-associated protein light chain 3 | LC3 |
| Interleukin-1β, 6,18 | IL-1β, 6,18 |
| reactive oxygen species | ROS |
| sequestosome 1 | P62 |
| Tetrabromobisphenol A | TBBPA |
| The carp epithelioma cells | EPC |
| Total antioxidant capacity | T-AOC |
| tumor necrosis factor-α | TNF-α |

**Compliance with ethics requirements**

All procedures used in this research were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University (SRM-11).

**Credit author statement**

**Mingyue Li:** Investigation, Formal analysis, Writing-original draft. **Shuang Lou and Kai**

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**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.

**Data availability**

Data will be made available on request.

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