

Polystyrene microplastics induced inflammation with activating the TLR2 signal by excessive accumulation of ROS in hepatopancreas of carp (*Cyprinus carpio*)

Jie Cui^a, Yanhe Zhang^a, Lin Liu^a, Qirui Zhang^a, Shiwen Xu^a, Meng-yao Guo^{a,b,*},¹

^a College of Veterinary Medicine, Northeast Agricultural University, Harbin 150030, People's Republic of China

^b Key Laboratory of the Provincial Education Department of Heilongjiang for Common Animal Disease Prevention and Treatment, People's Republic of China

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ABSTRACT

Polystyrene microplastics (PS-MPs) affect the immune defense function on carp (*Cyprinus carpio*). The PS-MPs model of carp was established by feeding with PS-MPs particle size of 8 μm and concentration of 1000 ng/L water. Hepatopancreas function test revealed the activities of AKP, ALT, AST and LDH abnormal increase. PS-MPs induced tissue damage and lead to abnormal hepatopancreas function. The PS-MPs also induced a oxidative stress with the antioxidant enzymes SOD, CAT, GSH-PX, and T-AOC activities decreasing and reactive oxygen species (ROS) excessive accumulation. PS-MPs activated the Toll like receptor-2 (TLR2) signaling pathway. The mRNA and protein expressions of TLR2, Myeloid differentiation primary response 88 (MyD88), tumor necrosis factor receptor-associated factor 6 (TRAF6), NF- κB p65, Tumor necrosis factor (TNF- α), Interleukin-1 β (IL-1 β), Inducible Nitric Oxide Synthase (iNOS), and cyclooxygenase 2(COX2) was revealed increased in both hepatopancreas and hepatocytes with the qPCR and Western blotting analysis mode. ELISA showed the expressions of TNF- α , IL-1 β , iNOS, and COX2 inflammatory molecule were increased in both hepatopancreas and hepatocytes. The results showed that PS-MPs caused a serious injury in the hepatopancreas and brought serious effects on the inflammatory response of carp. The present study displayed the harm caused by PS-MPs in freshwater fish, and provided some suggestions and references for toxicological studies of microplastics in freshwater environment.

1. Introduction

Plastic products have very many advantages, it is lightweight, have excellent chemical stability and excellent electrical insulation properties. Therefore, with the discovery of the 20th century to today, plastic products are used in a large number of daily lives. But while it brings great convenience to life, it also brings harm (Wang, 2022b; Cao et al., 2022c). Every year, about 8 million tons of plastic flow into the ocean, causing a serious impact to the Marine ecological environment and bringing great harm to aquatic animals (Chen et al., 2022; Yang et al., 2020). A variety of environmental polluting wastes from human activities can seriously harm the health of carp and restrict their growth (Kim et al., 2021; Grabicova et al., 2020). According to the United Nations Environment Programme, global plastic production is growing rapidly, and by 2030, the world could produce 619 million tons of plastic each

year, with a plastic recycling rate of only 9%. These plastic products are physically and chemically broken down into smaller particle sizes, and plastic particles smaller than 5 mm are called microplastics (MPs), which are more common in aquatic environmental pollutants (Estrela et al., 2021; Zhang et al., 2019).

MPs are causing increasing harm to aquatic organisms (Xia et al., 2020). Some studies have shown that the concentration of MPs in freshwater ecosystems are more than that in marine environment (Nam et al., 2022). MPs of different particle sizes have different levels of impact on aquatic organisms (Hoyo-Alvarez et al., 2022). Micron-sized and nano-sized MPs can penetrate cell membrane and affect the intracellular physiological environment (Banaei et al., 2022; Jeyavani et al., 2022). It had reported that the MPs showed a serious damage effect on liver, intestines and gills of freshwater fish, such as zebrafish (*Danio rerio*), freshwater perch (*Perca fluviatilis*), grass carp, and silver carp.

* Correspondence to: College of Veterinary Medicine, Northeastern Agricultural University, Harbin 150000, People's Republic of China.

E-mail address: gmy@neau.edu.cn (M.-y. Guo).

¹ ORCID: <https://orcid.org/0000-0002-7596-3632>.

(Bobori et al., 2022; Hamed et al., 2022; Cao et al., 2022a,b). Studies have shown that MPs can accumulate in different degrees in body tissues and organs after being ingested by aquatic organisms. After consuming the same particle size and same content of MPs, the deposition in the liver was the most compared to the intestine and gills (Espinosa et al., 2019; Solomando et al., 2022; Santos et al., 2022). At the same time, with the enrichment effect of the food chain, the harm to higher-level organisms is more obvious (Wang et al., 2022c; Gasca-Perez et al., 2019). *Cyprinus carpio* is a common freshwater fish (Zhang et al., 2022b), there are few studies on micron-sized Polystyrene microplastics (PS-MPs) in the hepatopancreas tissue of carp. (Wang et al., 2022a; Pei et al., 2022). In present study, we also chose the *Cyprinus carpio* to define model with feeding micron-sized PS-MPs and took the hepatopancreas tissue to carry out the study.

PS-MPs induced hepatopancreas inflammation in freshwater carp, but the toxicological mechanisms involved have not been fully demonstrated. Toll-like receptors (TLRs) are representative immune receptors in the innate immune response, recognizing many types of ligands and playing a critical role in the immune response of the organism (Cao et al., 2022a). TLR2 and TLR4 are the relatively common TLRs types in freshwater fish. It has been shown that TLR2 gene is more expressed in carp hepatopancreas tissue (Jia et al., 2021). In present study, the TLR2 was used as the main receptor study molecule, and MyD88 signaling molecule activated after TLR2 were detected, combined with NF- κ B and later TNF- α , IL-1 β , iNOS, and COX2 inflammatory molecules expression to analyze the inflammatory damage caused by PS-MPs on the hepatopancreas of carp. It was to analyze the effects of PS-MPs on freshwater organisms in a comprehensive manner in vivo and in vitro. This study provides some research basis for the harm of PS-MPs to freshwater fish.

2. Materials and methods

2.1. Animal treatment

A total of 60 *Cyprinus carpio* (carp) were used in the experiment. the fish were purchased from the Harbin Water Body Market. The weight of each carp be controlled in the range of 140–150 g. PS-MPs was purchased the SuZhou NanoMicro Technology Co Ltd. PS-MPs size 8 μ m, Solid content: 1.0% w/v, Refractive Index: 1.59 (589 nm). Store in water at room temperature. The carp were randomly divided into two groups on average. C Group: control group, there were 30 in this group, the carp were kept in water without PS-MPs and food also contains no PS-MPs; M Group: treatment group, there were 30 in this group, the carp were kept in water with PS-MPs at concentration of 1000 ng/L, and the food was mix PS-MPs together. The carp were kept in a tank containing 50 L of water, with an oxygen pump inserted to maintain sufficient oxygen and the water temperature maintained at 25 °C, and the water in the tank was changed every 48 h. The tap water was exposed to sunlight for 3 days to remove chlorine, and then the PH was adjusted to 7.2–7.4 using sodium chloride and buffer salt. The concentration of sodium chloride is three parts per thousand, which is used to remove some bacteria. All experiments were performed in a humanitarian manner, following the Institutional Animal. The standards of the Care and Use Committee of the Northeast Agricultural University were implemented. The carp were euthanized after 21 days of feeding and the hepatopancreas tissue were removed for this study, and the remaining carp tissues were stored at –80 °C environment.

2.2. Carp hepatocyte culture

10 extra carp was harvest. hepatopancreas tissue was obtained under sterile conditions and cut into small pieces within 0.2 cm². Clear using PBS buffers containing antibiotics. Pancreatic enzymes are digested into cell suspensions, cultured in M199 medium. 10% proportion of fetal bovine serum and 0.5% mixture of penicillin and streptomycin were added inside the medium. The cells were incubated in an incubator at

28 °C with 5% CO₂ concentration. The cells were divided into three groups to study: CC group (no PS-MPs and NAC added), MC group (PS-MPs concentration of 100 ng/L in the medium), and NMC (PS-MPs concentration of 100 ng/L in the medium and N-acetylcysteine concentration of 1 mM) (1 mM dose of NAC showed no toxicological response to carp hepatocytes).

2.3. Histological analysis

The obtained carp hepatopancreas tissue was cut into small pieces of 5 cubic millimeters and placed in formalin at 4 °C for 12 h to fix. Subsequently, dehydration was performed in 80%, 85%, 90%, and 95% concentrations of alcohol. Transparent treatment was done in a solution of one to one ratio of ethanol to xylene. The immersion and embedding operation was carried out after 30 min. The obtained wax blocks were trimmed, sectioned, patched, and baked. Then HE staining. The first step is to dewax with xylene, then rehydrating with different concentrations of alcohol, staining with hematoxylin and eosin, then dehydrating with different concentrations of alcohol and clearing in one-to-one ratio solution of ethanol to xylene. Finally, it is sealed with neutral gum and dried in natural environment.

2.4. Hepatopancrea function test

Accurately weigh 0.1 g of carp hepatopancrea tissue, add 900 μ L of pre-chilled saline, and centrifuge at 2500 g for 15 min to obtain carp hepatopancrea tissue homogenate. The operation was performed according to the instructions and the levels of AKP, ALT, AST and LDH were determined. Carp hepatopancrea cells were washed 1–2 times with saline, centrifuged at 1000 g for 10 min, the supernatant was discarded, pre-cooled saline was added, homogenizer was ground, and OD values were measured without centrifugation. Hepatopancrea function test assay kits were purchased from Nanjing Jiancheng Biotechnology Co.

2.5. ROS analysis

0.1 g of carp hepatopancrea tissue was weighed, and centrifuged at 1000 g for 5 min, the supernatant was removed and added to a 96-well plate at a dose of 200 μ L per well, and then 2 μ L of DHE fluorescent probe dye was added to each well, and the absorbance was measured at 510/610 nm. The carp hepatocytes were washed twice with PBS. DHE dye and M199 was added to a six-well plate at a ratio of 1:1000, incubated for 30 min protected from light and observed under fluorescence microscope. The ROS kit was purchased from Nanjing Jiancheng Biotechnology Co.

2.6. Antioxidant enzyme assay

The hepatopancrea tissue homogenization was performed in 1:9 w/v. After centrifugation, supernatant was selected for analysis. The hepatocytes were cracked and then centrifuged for analysis. The MDA level was measurement according to the specification for reflecting the antioxidant capacity. The SOD, CAT, GSH-PX, and T-AOC enzyme activity value was measurement according to the specification for reflecting the antioxidant capacity. Corresponding detection and analysis were performed with enzyme label instrument at the wavelength 496 nm or 520 nm. All the antioxidant enzyme assay kit was purchased from Nanjing Jiancheng Biotechnology Co.

2.7. Real-time PCR

The total mRNA was extracted with the Trizol both hepatopancrea tissue and hepatocyte. And then The concentration was detected by microanalyzer. The RNA was adding Enzyme mix and Hybrid mix, and reverse transcription was carried out. The final cDNA was obtained. Frozen reserve. The primers were designed synthesis with Primer 5.0,

referring to the gene sequence from genebank, showing in Table 1. The PCR reaction system was a-20 μ l. The 35 cycles amplification were taken. The expressions of TLR2, MyD88, TRAF6, NF κ B P65, TNF- α , IL-1 β , iNOS, COX2 gene were detected by β -actin as the reference gene. The expressions of hepatopancreas genes in carp were calculated using the $2^{-\Delta\Delta t}$ method, Consistent with statistical analysis.

2.8. Western blotting

The hepatopancreas tissue and hepatocytes were fully cleaved, total protein was extracted. Protein concentration was measured using the BCA kit according to the specification. After protein samples were homogenized and denaturation. The SDS-PAGE gel electrophoresis was performed.

The protein glue concentration is configured according to the size of the protein in each analysis. According to the size of the target protein, the glue was cut and the membrane was transferred. The skim milk was used to blocking, and washed with TBST. The primary antibody of TLR2, MyD88, TRAF6, p-p65 et al. were diluted at a ratio of 1:1000, incubation at 4 °C overnight. And then incubated second antibody. All the antibodies were purchased from CST(shanghai) Biotechnology Co. A chemiluminescence imaging system was used to detect the protein signal (A chemiluminescence imaging instrument was purchased from Hangzhou Deepening Technology Co).

2.9. ELISA

The hepatopancreas tissue was homogenate and centrifuge supernatant. The supernatant was also collected from cell culture hepatocytes. The level of TNF- α , IL-1 β , iNOS and COX2 was analysed according to the specification. All the ELISA assay kit was purchased from Jiangsu Jingmei Biotechnology Co. The results measure with OD value by a microplate reader at 450 nm. According to the standard curve of the pure product established, The content of each molecule is calculated separately.

2.10. Statistical analysis

GraphPad Prism 8.2.3 software was used to analyze and process the experimental results, which were expressed as mean $\pm$ standard deviation (SD). The tests used to verify the normality distribution of the variance. One-way comparisons were used to analyze the different groups ($P < 0.05$).

Table 1

The primers used in the present study.

Gene (Full name)	Primer	Reverse primer (5'-3')
TLR2 (Toll-like receptor 2)	Forward Reverse	CCATCGGACTCTCTTTGTTC TCATCGACTATGCGGAAATG
MyD88 (Myeloid differentiation primary response 88)	Forward Reverse	TCCAGAGGCAACAGTAGAA CTTCCTGCAGTCATCATCTATC
TRAF6 (TNF Receptor Associated Factor 6)	Forward Reverse	ATCCACAATGAGACACAGAAA CATACGTAGACGCTTGATAC
NF- κ B p65 (nuclear factor kappa-B p65)	Forward Reverse	AACCAAGAACCAGCCGTACAAGC ACTGTGTATCCTCCGCTCTGTAG
TNF- α (Tumor necrosis factor- α)	Forward Reverse	TCAGGCGGCTTGAAATTAG GTCCTCAGTCATGTTAGCTTG
IL-1 β (Interleukin-1 β)	Forward Reverse	GTTGAGAGAGACCAGTATAAAG CTTCCTCTGACGTTTGTTTATG
iNOS (Inducible Nitric Oxide Synthase)	Forward Reverse	CAACGTACCTGGTGCTTATC CAGTAGGTGAAGCGCATATC
COX2 (Cycooxygenase 2)	Forward Reverse	GGAAACAGACCACCGAATAG GGATGGAACAGCTCAAGAAT
β -actin (beta-actin)	Forward Reverse	CTAAGCGACCTCTACTACGA CTGTGGGACATTGTCTTG

3. Result

3.1. In Vivo

3.1.1. Hepatopancreas histological analysis and hepatopancreas function test results

The results of HE staining of carp hepatopancreas are shown in Fig. 1A. From the figure, it can be seen that the hepatic cords and the central hepatic blood sinusoid in the carp hepatopancreas were well arranged in group C at 10 $\times$ low magnification. At 40 $\times$ high magnification, normal hepatocytes with the complete cell membrane and no rupture could be observed more clearly. At 40 $\times$ high magnification in group M, the hepatic cords were disordered, the hepatocytes were ruptured and the hepatic blood sinusoid was ruptured and infiltrated with inflammatory cells. It can be seen that the hepatopancreas of carp fed with PS-MPs had severe damage.

To evaluate the effect of PS-MPs on the hepatopancreas of carp, we measured AKP, ALT, AST, and LDH levels in carp hepatopancreas. From Fig. 1B-E, it was shown that AKP, ALT, AST, and LDH activities were enhanced in group M compared to group C. This leads to the conclusion that when carp inhaled PS-MPs caused activities changes in hepatopancreas function indicators AKP, ALT, AST, LDH.

3.1.2. Oxidative damage to hepatopancreas caused by PS-MPs

ROS is a marker molecule of oxidative stress. Firstly, we examined the ROS content in the hepatopancreas tissue of carp. It is concluded from Fig. 2A that when carp inhaled PS-MPs, ROS content increased compared to group C. Fig. 2B concluded that MDA content increased compared to group C. SOD, CAT, GSH-PX, and T-AOC activities were reduced due to the inhalation of PS-MPs in carp (Fig. 2C-F). A large amount of ROS production caused the increase in hepatopancreas MDA content and the decrease in antioxidant enzyme activity, resulting in oxidative stress in the organism.

3.1.3. Effect of PS-MPs on inflammatory factors expressions in hepatopancreas of carp

TLRs are important immune signaling molecules that recognize and bind a variety of biological signals, triggering the MyD88 signaling pathway to induce the production of various cytokines (Cao et al., 2022a). TLR2 is an important immune signaling molecule for TLRs in carp. In RT-PCR experiments, we found that TLR2, MyD88, TRAF6, and NF- κ B p65 gene expressions were increased in group M compared with group C (Fig. 3A-D). The expressions of TNF- α , IL-1 β , iNOS, and COX2 inflammatory molecule gene were also increased compared with group C (Fig. 3E-H). RT-PCR experiments could reveal that PS-MPs activated TLR2-MyD88-NF- κ B p65 signaling pathway and caused the expressions of inflammatory molecules genes, resulting in inflammatory response in the hepatopancreas. Further combined with Western blotting experiments, it was found that TLR2, MyD88, TRAF6, NF κ B P-P65 protein expressions were increased in group M compared with group C (Fig. 3I-J). ELISA assay revealed that TNF- α , IL-1 β , iNOS, COX2 inflammatory molecule expressions were increased compared with group C (Fig. 3K-N). It was further concluded that PS-MPs caused a large amount of ROS production in the organism, and induced a large amount of inflammatory molecules production in the hepatopancreas of carp through TLR2-MyD88-NF κ B P65 signaling pathway, resulting in the inflammatory reaction in the carp hepatopancreas.

3.2. In Vitro

3.2.1. Effect of PS-MPs on hepatopancreas function of carp hepatocytes

From the vivo experiments of carp, it was concluded that when carp inhaled PS-MPs, the hepatopancreas function indexes AKP, ALT, AST, and LDH activities increased. These liver function index was also detected in hepatocytes. The results were shown in Fig. 4. Compared to the CC group, the AKP, ALT, AST, and LDH activities were increased

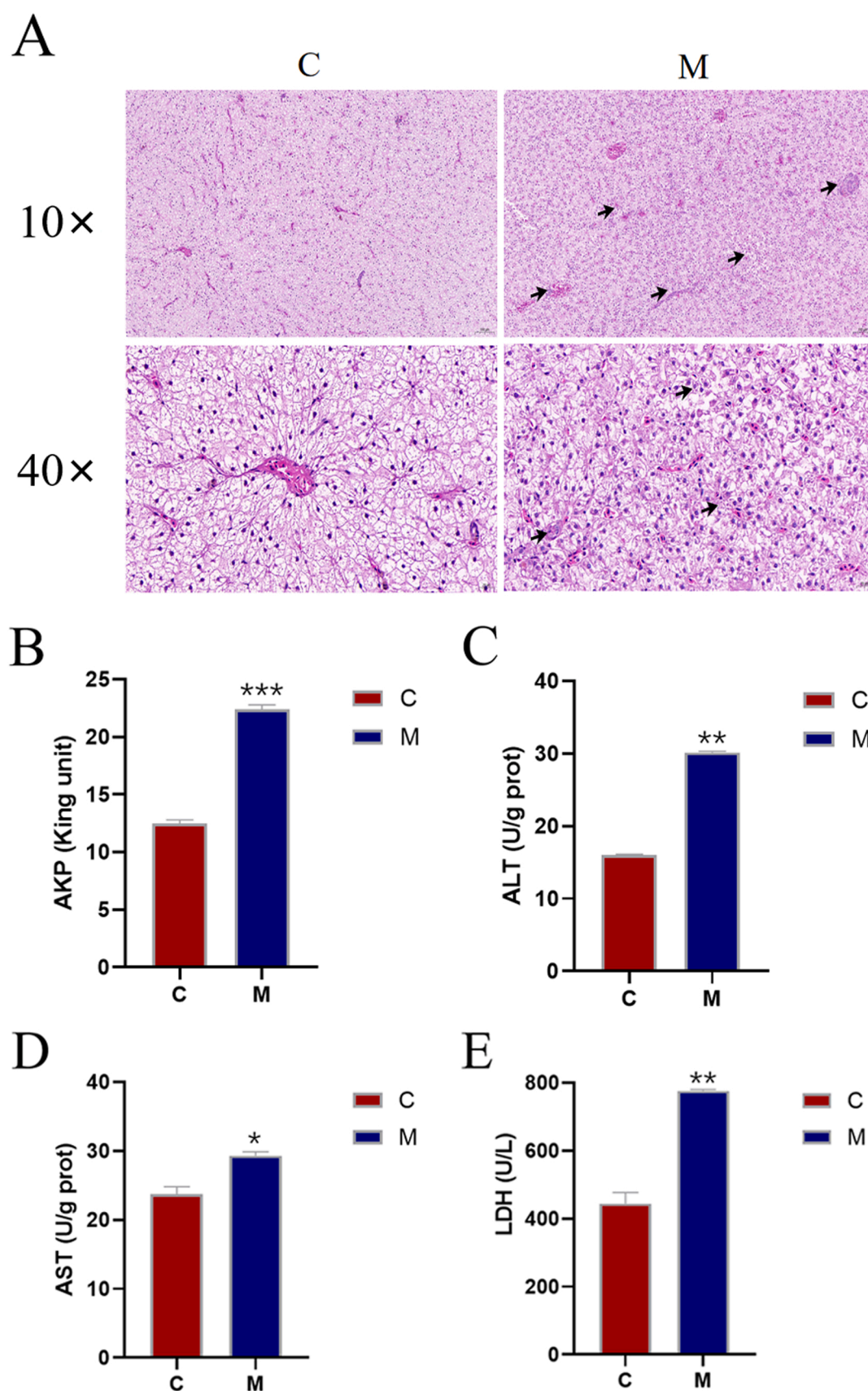


Fig. 1. Histopathological and Serum parameters analysis of hepatopancreas. (A) HE staining of C group and M group at 10× and 40× magnification, respectively. (B-E) Serum parameters results of hepatopancreas tissue. (B) AKP level in the hepatopancreas of C and M. (C) ALT level in the hepatopancreas of C and M. (D) AST level in the hepatopancreas of C and M. (E) LDH level in the hepatopancreas of C and M. C, control group; M, reared in water with PS-MPs particle size of 8 μm and concentration of 1000 ng/L. Values are expressed as the mean ± SD. **p < 0.01 as compared to C, ****p < 0.05 as compared to C.

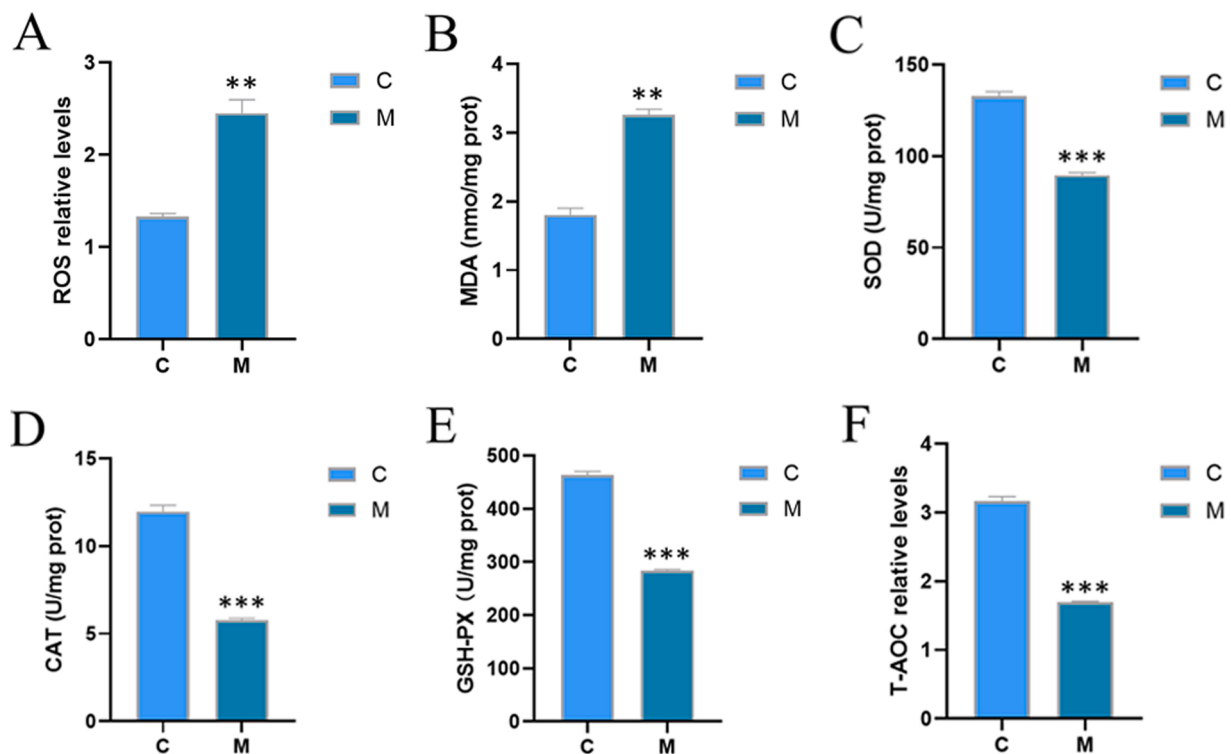


Fig. 2. Results of oxidative damage to hepatopancreas tissue. (A) ROS level in the hepatopancreas. (B) MDA level in the hepatopancreas. (C) SOD level in the hepatopancreas. (D) CAT level in the hepatopancreas. (E) GSH-PX level in the hepatopancreas. (F) T-AOC level in the hepatopancreas. C, control group; M, reared in water with PS-MPs particle size of 8 μ m and concentration of 1000 ng/L. Values are expressed as the mean $\pm$ SD. ** p < 0.01 as compared to C, *** p < 0.05 as compared to C.

both in the MC and NMC group. But the increased trend in NMC group was significantly ($P < 0.05$) weaker than that in MC group. It was concluded from the cellular level that the activities of AKP, ALT, AST, and LDH were increased after PS-MPs stimulated, and this increased enzyme activity caused by hepatocyte damage can be mitigated by the oxidative stress inhibitor NAC.

3.2.2. Effect of PS-MPs on oxidative stress in carp hepatocytes

Carp hepatocytes were observed under a fluorescence microscope, and the fluorescence intensity of carp hepatocytes in MC group was enhanced compared with CC group, and the fluorescence intensity of NMC group was weakened after the addition of NAC (Fig. 5A-C). The MDA activity was similar to ROS, and the MDA activity in MC group was enhanced due to the addition of PS-MPs, and the activity was weakened after the addition of NAC inhibitor. Compared with the CC group, SOD, CAT, GSH-PX and T-AOC activities were weakened in the MC group, and the antioxidant capacity of carp hepatocytes was reduced. After adding NAC inhibitor, SOD, CAT, GSH-PX, and T-AOC activities were enhanced, but did not return to normal levels (Fig. 5D-I).

3.2.3. Effect of PS-MPs on the inflammatory factors in carp hepatocytes

The expressions of inflammatory molecules were similar to hepatopancreas tissue in carp hepatocytes. The expressions of TLR2, MyD88, TRAF6, and NF- κ B p65 gene were increased in the MC group compared to the CC group, and decreased in the NMC group due to the addition of NAC (Fig. 6A-D). The expressions of TNF- α , IL-1 β , iNOS, COX2 inflammatory molecule were also increased in the MC group compared to the CC group (Fig. 6E-H), and the expressions of inflammatory molecule were decreased due to the addition of NAC. Western blotting experiment shown that the expressions of TLR2, MyD88, TRAF6, NF κ B P-P65 protein were increased in the MC group compared to the CC group. In ELISA assay, the expressions of TNF- α , IL-1 β , iNOS, COX2 inflammatory molecule were increased in MC group compared with CC group, and the

expressions of inflammatory molecule were decreased in NMC group due to the addition of NAC. It was further confirmed from the cellular level that PS-MPs could induce a large amount of ROS production and cause a serious inflammatory response in the organism, and the addition of NAC oxidative stress inhibitors could alleviate some PS-MPs-induced inflammatory damage and play a protective role for the cells.

4. Discussion

With the massive accumulation of plastics and their decomposition products, it brings serious harm to the organisms of the water environment, especially freshwater fish (Wang et al., 2022a). In this study, we investigated the damage of PS-MPs on the hepatopancreas of carp by rearing them in microplastic-contaminated water environment. The hepatopancreas of the carp was stained with H&E. The results showed that microplastics can cause liver tissue damage in carp, with a large number of hepatocytes ruptured and red blood cells infiltrated in the hepatic sinusoids, which caused serious damage to the hepatopancreas of the carp. It was same as the report on the toxic effects of PS-MPs on the Zebrafish and *Carassius auratus* (Liu et al., 2016; Jabeen et al., 2018).

The breakdown of organizational structure can lead to impairment or loss of function, while AKP, ALT, AST, and LDH have crucial roles in the metabolism of aquatic organisms (Xie et al., 2020; Sun et al., 2021). And there were a few studies on hepatopancreas function with PS-MPs stimulated. In present study, the hepatopancreas function assay was with AKP, ALT, AST, and LDH. With PS-MPs treated, the liver function indexes abnormal growth both in tissues and cells. The previous study had reported the hepatopancreas function indicators increase and cause inflammatory damage in carp hepatopancreas (Liu et al., 2021). Damage to liver cells can lead to the release of various enzymes, corresponding indicators of rapid increase, which indicates that the liver has been seriously damaged. our study yielded similar results to previous studies, indicating that PS-MPs caused inflammatory damage to the carp

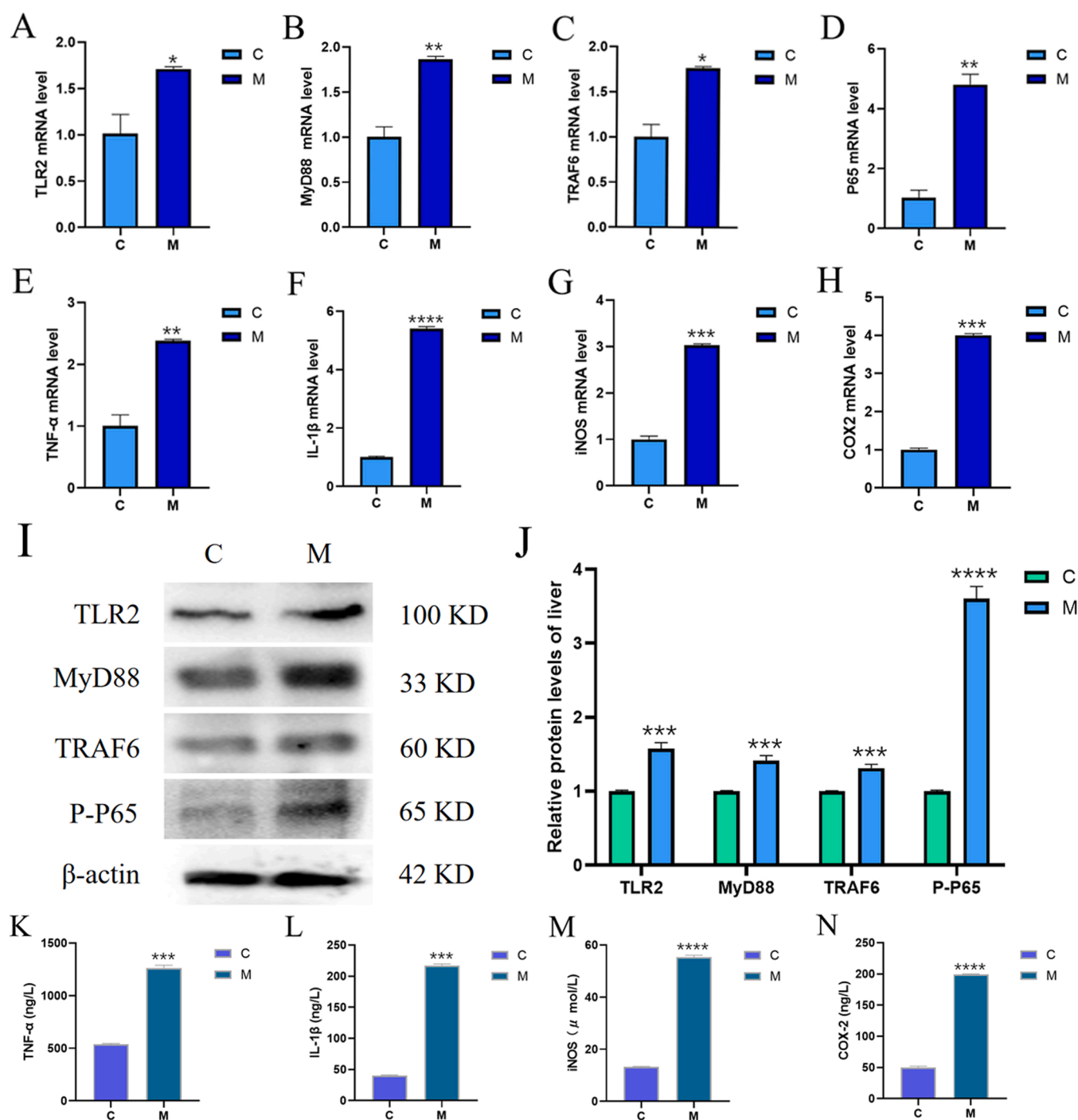


Fig. 3. Expression of inflammatory molecules in the hepatopancreas of carp. (A-H) RT-qPCR analysis of mRNA levels of inflammatory molecules (TLR2, MyD88, TRAF6, P65, TNF-α, IL-1β, iNOS, COX2). β-Actin was used as a control. (I and J) Analysis of protein levels of hepatopancreas tissue inflammatory molecules (TLR2, MyD88, TRAF6, P65). (K-N) ELISA results of hepatopancreas tissue. (K) AKP level in the hepatopancreas of C and M. (L) ALT level in the hepatopancreas of C and M. (M) AST level in the hepatopancreas of C and M. (N) LDH level in the hepatopancreas of C and M. C, control group; M, reared in water with PS-MPs particle size of 8 μm and concentration of 1000 ng/L. Values are expressed as the mean ± SD. ***p* < 0.01 as compared to C, *****p* < 0.05 as compared to C.

hepatopancreas (Montero et al., 2022; Qiao et al., 2019). The results showed that adding NAC can effectively reduce the excessive increase of enzyme activity caused by PS-MPs. These results suggest that liver tissue damage and liver function abnormalities caused by microplastics are closely related to oxidative stress.

Numerous studies have shown that some toxicants induce excessive production of ROS by reducing antioxidant enzyme activity, causing tissue and organ damage (Zhang et al., 2022a; Cao et al., 2022b). Studies in carp have shown that Bisphenol A can promote excessive aggregation

of ROS and induce hepatopancreas damage in carp (Afzal et al., 2022). In present study, the results displayed that PS-MPs significantly inhibit antioxidant enzyme activity and promote ROS accumulation. This showed that PS-MPs induce oxidative stress in hepatopancreatic tissue. The addition of NAC at the cellular level showed an increase in antioxidant enzyme activity and a significant decrease in ROS levels. Excessive ROS accumulation can cause tissue damage (Hodkovicova et al., 2019; Xu et al., 2022a,b; Zhang et al., 2022b). These results are similar to the findings of the damage caused by microplastics in other

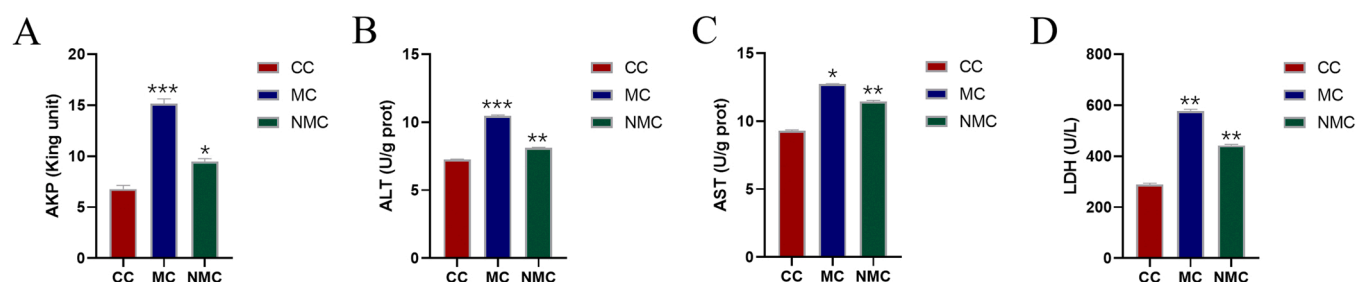


Fig. 4. Results of Serum parameters analysis of Hepatocytes. (A) AKP level in the Hepatocytes of CC, MC and NMC. (B) ALT level in the Hepatocytes of CC, MC and NMC. (C) AST level in the Hepatocytes of C and M. (D) LDH level in the Hepatocytes of CC, MC and NMC. CC, control group; MC, microplastic concentration of 100 ng/L in the medium; NMC, microplastic concentration of 100 ng/L in the medium and N-acetylcysteine concentration of 1 mM. ** $p < 0.01$ as compared to CC, *** $p < 0.05$ as compared to CC.

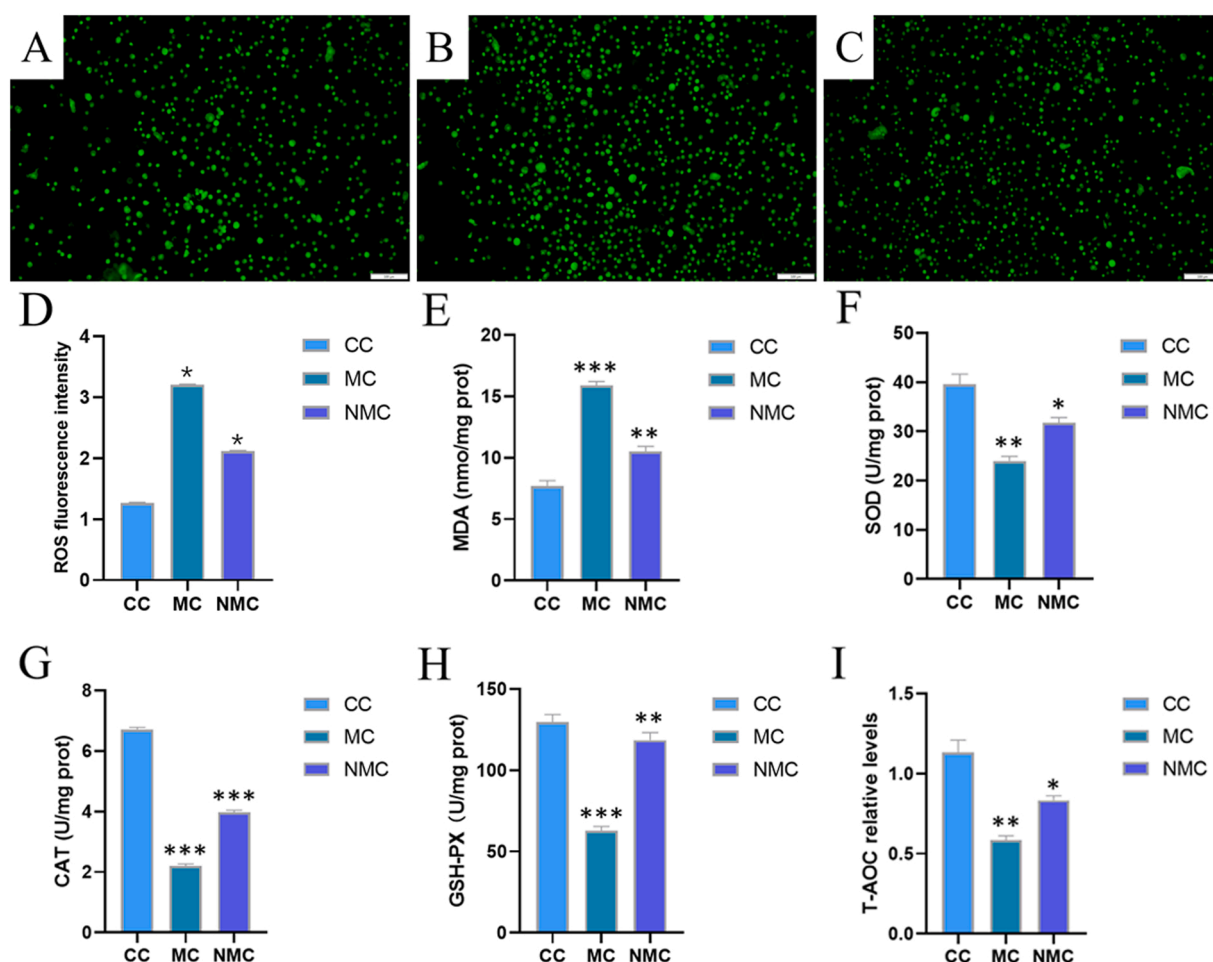


Fig. 5. Results of oxidative injury of hepatocytes. (A-I) Determination of hepatocytes oxidative damage. (A-C) Hepatocytes ROS level in the fluorescence microscope of CC, MC, NMC group. (D) ROS level in the hepatocytes of CC, MC, NMC group. (E) MDA level in the hepatocytes of CC, MC, NMC group. (F) SOD level in the hepatocytes of different types of CC, MC, NMC group. (G) CAT level in the hepatocytes of CC, MC, NMC group. (H) GSH-PX level in the hepatocytes of CC, MC, NMC group. (I) T-AOC level in the hepatocytes of CC, MC, NMC group. CC, control group; MC, microplastic concentration of 100 ng/L in the medium; NMC, microplastic concentration of 100 ng/L in the medium and N-acetylcysteine concentration of 1 mM. ** $p < 0.01$ as compared to CC, *** $p < 0.05$ as compared to CC.

tissues, indicating that the inflammatory damage caused by microplastics is closely related to the excessive accumulation of ROS, thus activating inflammatory signal transduction and forming inflammatory damage to a certain extent (Li et al., 2022).

ROS-induced tissue injury is closely related to the activation of inflammatory signaling (Miao et al., 2021). TLRs are very important immune receptor in the innate immune response (Wu et al., 2022). TLRs signaling can be activated by ROS, which promotes the release of pro-inflammatory molecules and induces tissue inflammatory damage

(Jing et al., 2020). In this study, it was found TLR2 expression was significantly increased by PS-MPs. with NAC adding in hepatocytes the increased expression of TLR2 was inhibited. This suggested that PS-MPs induced an increase of ROS, which promoted TLR2 expression. This is similar to the study that Lipophosphoglycan lead to increased ROS and oxidative stress, which further activate TLR2 receptor (Zhang et al., 2021).

TLRs are transmembrane receptors, which can activate downstream signals with incitant, such as bacteria, ROS and other. the activation of

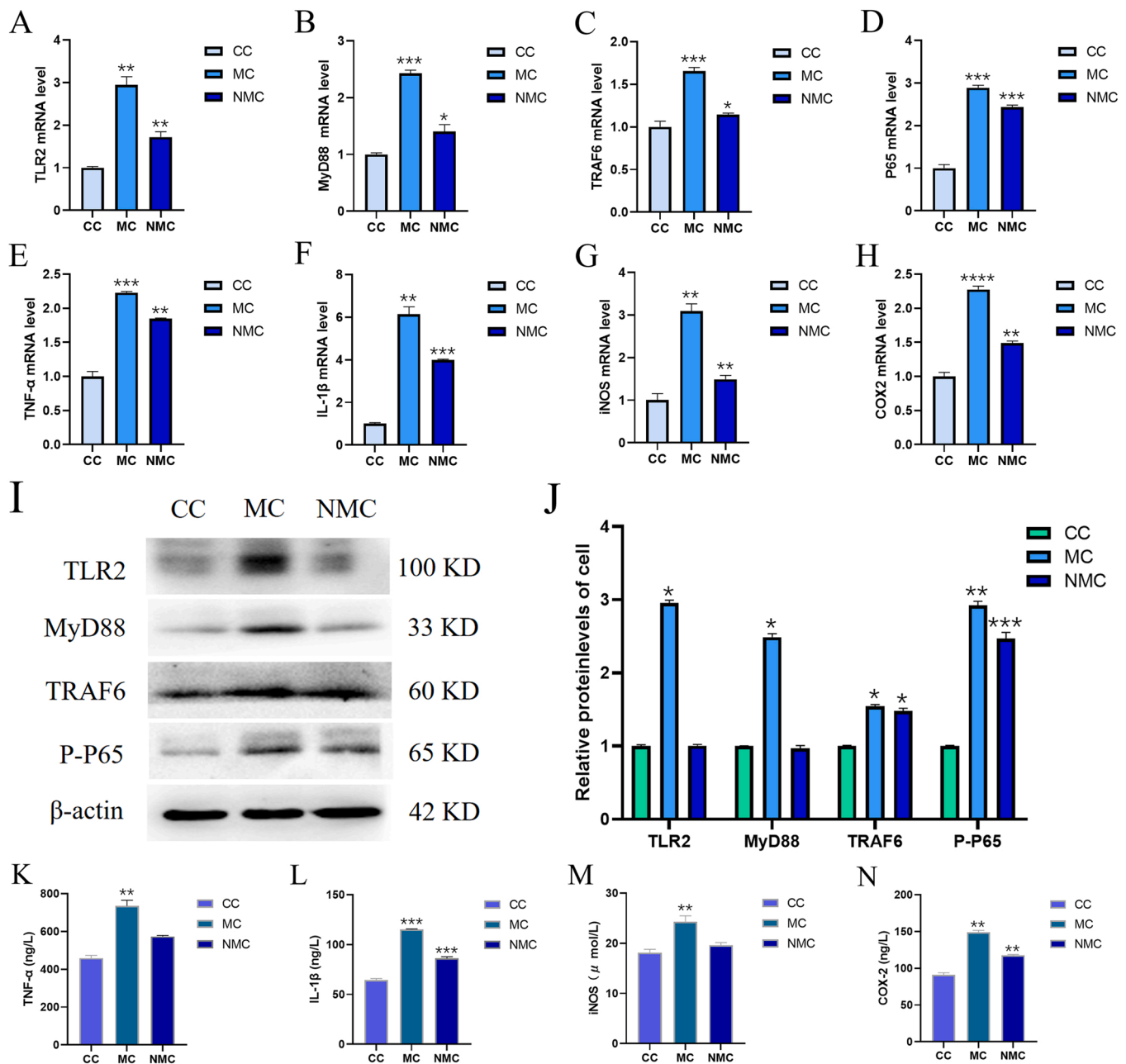


Fig. 6. Expression of inflammatory molecules in the hepatocytes. (A-H) RT-qPCR analysis of mRNA levels of inflammatory molecules (TLR2, MyD88, TRAF6, P65, TNF-α, IL-1β, iNOS, COX2). (I and J) Analysis of protein levels of hepatocytes inflammatory molecules (TLR2, MyD88, TRAF6, P65). (K-N) ELISA results of hepatocytes. (K) AKP level in the hepatocytes of CC, MC, NMC group. (L) ALT level in the hepatocytes of CC, MC, NMC group. (M) AST level in the hepatocytes of CC, MC, NMC group. (N) LDH level in the hepatocytes of CC, MC, NMC group. CC, control group; MC, microplastic concentration of 1000 ng/L in the medium; NMC, microplastic concentration of 100 ng/L in the medium and N-acetylcysteine concentration of 1 mM. ** $p < 0.01$ as compared to CC, **** $p < 0.05$ as compared to CC.

downstream molecules could promote the release of inflammatory molecules (Xu et al., 2022a,b). The key role of the TLR2/MyD88/NF-κB pathway were analysed in this study. The MyD88 and TRAF6 were increased with the PS-MPs. However, the expressions of MyD88 and TRAF6 in hepatocyte were decreased after adding NAC. This suggests that reducing ROS production can reduce the activation of signaling pathways. This further suggests that the inflammatory response caused by microplastics is related to the induced oxidative stress. The finding is similar to other reports on PS-MPs in Aquatic and other animals (Wang et al., 2020; Zhao et al., 2021). The NF-κB is a key nucleus transcription molecule (Tian et al., 2021). the p65 subunit is the one most closely associated with inflammatory responses. When phosphorylated, it can enter the nucleus and initiate transcription of inflammatory factors,

inducing inflammatory responses (Shanaka et al., 2020). The only downstream splicing molecule of TLR2 is MyD88. Therefore, the expression of MyD88 molecule is increased after TLR2 was activated. After TRAF6 activation by MyD88, it continues to phosphorylate NF-κB p65. The studies on NF-κB showed protein phosphorylation level was significant increase with the stimulation of PS-MPs. NF-κB is inhibited similarly by NAC in hepatocyte. The findings suggest that the TLR2/MyD88/NF-κB p65 signaling pathway was activated by PS-MPs with ROS over-accumulation, it was supported the reports of immunological and oxidative stress parameters in common carp (Hodkovicova et al., 2019). To further confirm our findings on inflammatory signaling pathways, we analyzed inflammatory cytokines. PS-MPs stimulation promoted the release of pro-inflammatory molecules TNF-α, IL-1β,

iNOS, and COX2 in large amounts. It was confirmed that the secretion of inflammatory factors promoted the occurrence of inflammatory injury (Shanaka et al., 2020). but when ROS (NAC effect) decreased, the release of these inflammatory molecules decreased significantly. The results further indicate that the PS-MPs could induce a large release of inflammatory factors with the increase of ROS, leading to inflammatory damage in carp hepatopancreas.

5. Conclusion

In conclusion, the high presence of PS-MPs in the aqueous environment could generated hepatopancreatic tissue damage and consequently hepatopancreas dysfunction with a inflammatory response, which induced by a burst increase of ROS. The ROS promoted the expression of TLR2 and activated TLR2 signaling downstream molecule MyD88 and TRAF6. And then the occurrence of NF- κ B p65 phosphorylation, leading to the inflammatory factors of TNF- α , IL-1 β , iNOS, and COX2 release. The present study had shown a investigate on the new toxicological mechanisms of microplastics on carp hepatopancreas from the perspective of Oxidative stress, innate immune recognition receptors and inflammatory response, providing a new strategy for the study of microplastics in freshwater fishes. It also gives some warning to the environmental problems caused by microplastics.

CRedit authorship contribution statement

Jie Cui: Methodology, Conceptualization, Writing – original draft. **Yanhe Zhang:** Formal analysis, Data collection. **Lin Liu:** Data curation. **Qirui Zhang:** Data analysis. **Shiwen Xu:** Writing, Supervision. **Meng-yao Guo:** Experimental design, Writing and modification.

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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