The mechanism of polystyrene nanoplastics hepatotoxicity in zebrafish (Danio rerio)

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Abstract: The mass production of plastics and improper disposal of plastic waste has led to their widespread presence in the ecosystem. Plastics entering the natural environment degrade into irregularly shaped and differently sized nanoplastics (NPs), exacerbating global pollution. The research object to study toxic mechanisms of the liver polystyrene nanoplastics (PS NPs) of zebrafish, providing key scientific insights for the environmental risk assessment of NPs. The zebrafish were exposed to different concentrations with a particle size of 100 nm PS NPs containing Eu, and the accumulation of nanoplastics in each tissue/organ was quantified. The mechanism of liver toxicity of nanoplastics to zebrafish was explored. Where the mass of PS NPs was linearly related to the mass of Eu with $R^2 = 0.999$. PS NPs were accumulated in gills, ovaries, liver, spleen, intestine, kidney, skin, muscle and brain. The top three accumulations were: intestine > gills > liver. The histopathological analysis of liver indicated that 0.1 mg/L and 1 mg/L PS NPs caused increased liver inflammation. 0.1 mg/L PS NPs significantly increased the activity of SOD and the content of ROS and MDA in liver. 1 mg/L PS NPs significantly increased the activity of SOD, CAT and GSH and the content of MDA in liver, while significantly decreased the content of ROS. These results will improve the appreciation of the cumulative impact of PS NPs and the mechanism of toxicity on target organs in organisms.

1. Introduction

Plastic waste degrades into microplastics (MP, < 5 mm) or even smaller nanoplastics (Nanoplastics, NPs, < 1 μm)[1], which can enter the food chain and affect the environment and living organisms (plants, animals and humans). Both different size PS NPs were found in the gills and digestive tract of the sea mussel (*Mytilus galloprovincialis*); the digestive glands were the tissues in which PS NPs accumulated most, and PS NPs accumulated in tissues in correlation with their sizes, with 50 nm PS NPs translocating to the hemolymph to a greater extent over a 24-hr period; nanoplastics uptake pathway may be critical for hemocyte motility, and internalization within hemocytes triggers changes in immune responses such as motility, apoptosis, ROS, and phagocytosis[2]. The 96-h LC_{50} of 75 nm PS NPs in juvenile Japanese marsh shrimp (*Macrobrachium nipponense*) was 396.391 mg/L, and at higher concentrations (10, 20, and 40 mg/L), the antioxidant and immune defenses of juvenile shrimp were adversely affected; after 28 days of exposure, the survival of shrimp larvae was improved by 5 mg/L PS NPs[3].The organisms exposure to PS NP will lead to reproductive abnormalities, gastrointestinal dysfunction and oxidative stress, increased mortality, neurological damage, and growth inhibition and disruption[4].

Zebrafish, as a model organism[5], has biological characteristics and advantages such as ease of culture, rapid development, high spawning capacity (spawning in all seasons), and high homology with the human genome[6]. In this study, we developed a method for the quantitative analysis of PS NPs in various tissues of zebrafish using PS NPs commonly found in the environment, and also explored the mechanism of toxicity of nanoplastics to the liver, a target organ for specific accumulation. This study provides an important scientific basis for the environmental and health risk evaluation of nanoplastics.

2. Materials and Methods

2.1. Chemicals and reagents

PS NPs labeled as 100 nm (encapsulated with the rare earth element europium, Europium) were purchased from Shanghai HuiQi Biotechnology Co. The concentration of the 100 nm PS NPs stock solution was 10 mg/mL, and it was stored at 4 ℃ protected from light. HNO3 and H2O2 (ICPMS PURE) were purchased from Shanghai Aoban Technology Co.

2.2. Subjects

Adult female zebrafish (Danio rerio), 3-4 months old, averaging 24 ± 1 mm and weighing 229 ± 10 mg, were purchased from Shanghai FeiXi Biotechnology Co. and were domesticated in dechlorinated tap water for two weeks. During the period of domestication, the natural

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mortality rate was less than 5 %, and they were accepted for the experiments.

2.3. Experimental setup

The 0.1 mg/L and 1 mg/L PS NPs exposure groups were set up, as well as a control group, and three parallels were set up for each experimental group. The PS NPs containing Eu were diluted with ultrapure water, ready to use, and ultrasonicated for 5 min before use.

There was no feeding for 2 days prior to the experiment, and 90 zebrafish were randomly selected in 40 L glass tanks loaded with 0.60 ± 0.2 g fish (wet weight) per liter. During the experimental period, the tanks were illuminated for 12 h per day, the water temperature was maintained at 23.2 ± 0.5 ℃ (20-25 ℃ is the optimal temperature for zebrafish), an aeration device was passed through to ensure sufficient oxygen content, the dissolved oxygen content was 6.70 ± 0.18 mg/L, and the pH was monitored to be 7.88 ± 0.07 in each tank.The fish were fed with brine shrimp once a day, at a rate of 1% of their body weight.

The experiment was set up for a 30-day exposure cycle. Zebrafish were collected before feeding, and 8 fish were randomly taken from each tank, placed in pre-prepared ice water to make them unconscious, and then the water was removed with absorbent paper, and then dissected quickly to obtain the liver biosamples, which were weighed, placed in liquid nitrogen, and stored at -80 ℃ to be processed. In addition, one liver from each experimental group was immersed in 4% paraformaldehyde for section observation.

2.4. Experimental Methods

2.4.1. Characterization and quantification of PS NPs

The 100 nm PS NPs were placed on the conductive adhesive, and the morphology of the NPs was observed by Scanning Electron Microscope (SEM). And the PS NPs solution was diluted 2000 times, the particle size was counted by NPs Tracking Analysis (NTA).

A standard curve was configured with ultrapure water, and the concentrations of PS NPs nanoplastics were 0, 1, 3, 6, 10, 50, 100, 500, and 1000 μg/L. The digestion was carried out using a high-pressure microwave ablator with nitric acid, hydrogen peroxide and ultrapure water in the ratio of 3:1:4 in the ablating tube.The Eu content determined by ICP-MS was used to make PS (μg) and Eu (ng) standard curves for subsequent experiments.

2.4.2. Determination of PS NPs content in zebrafish tissues

Four zebrafish were taken as one parallel and three parallels for each treatment group and dissected to obtain gills, gonads, liver, spleen, intestine, kidney, skin, muscle and brain. The mass of Eu measured by Inductively coupled plasma-Mass Spectrometry (ICP-MS) after digested using the digestion method of 2.4.1.

2.4.3. Fertility and Hepatosomatic Index

The morphological indicators Fatness Condition Factor (FCF) and Hepatic Steatosis Index (HSI) can be visualized as indicators of the toxic effects of PS NPs on zebrafish[7], and the FCF is usually used as a general health status indicator. indicators are used. Liver body index, a measure of liver stress in fish, is also used as an indicator of aquatic pollution. These two indicators can be used as a preliminary predictor of the toxic effects of PS NPs on zebrafish and as a reference for further studies on the mechanisms of toxicity at a deeper level[8].

2.4.4. Histological sections

Four zebrafish were randomly dissected out of the liver and fixed in 4 % paraformaldehyde for 24 h. The liver was stained using i.e. hematoxylin-eosin staining (H&E), which is one of the commonly used staining methods in paraffin sectioning technique. Observation of zebrafish liver microstructure after exposure to PS NPs.

2.4.5. Determination of ROS content in zebrafish liver

Livers of three zebrafish were taken as one parallel and three parallels for each treatment group to determine the ROS content.

2.4.6. Antioxidant enzyme activity assay

Ten zebrafish were taken as one parallel and three parallels for each treatment group. Catalase (CAT), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and malondialdehyde (MDA) content were measured using kits from Nanjing Jianjian Bioengineering Institute.

2.4.7. Transcriptome sequencing

Twelve zebrafish livers were taken as one parallel and three parallels per treatment group. Put in liquid nitrogen and placed at -80 ℃ for storage. The cryopreserved zebrafish liver samples were taken and used for transcriptome sequencing.

3. Results

3.1. Characterization and quantification of PS NPs

The particle size of PS NPs was counted by NTA (Fig. 1a). The hydrodynamic diameters of PS NPs with nominal 100 nm were 109 ± 1.5 nm, respectively.

Standard curves were configured with ultrapure water at concentrations of 0, 1, 3, 6, 10, 50, 100, 500, and 1000 μg/L. After digestion, the mass of Eu determined by ICP-MS was used to make a standard curve with the mass of PS NPs, and the PS NPs were found to have a linear relationship with the mass of Eu (Fig. 2b):

TEM of nominal 100 nm PS NPs (Fig. 1c). SEM was used to observe the surface morphology and size of the PS NPs used in this experiment. SEM showed the PS NPs with the shape of regular spherical round balls.

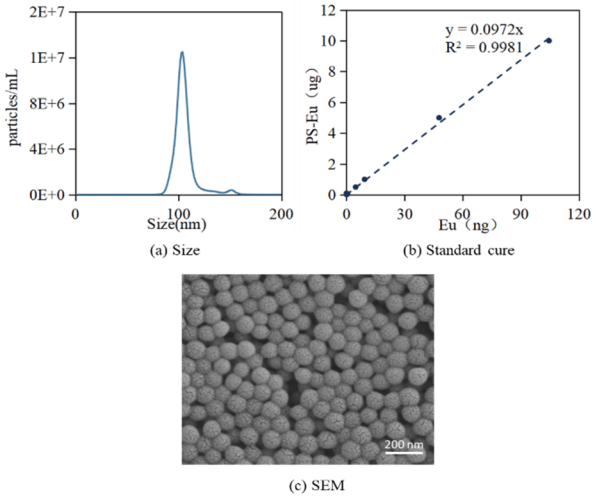


Fig. 1 The characterization and quantification of 100 nm PS NPs

3.2. Accumulation of PS NPs in tissues

After 30 days of exposure, the content of PS NPs were determined in gills, gonads, liver, spleen, intestines, kidneys, skin muscles and brain, where the highest accumulation was in the intestines, gills and liver, respectively. Among them, the presence of PS NPs was also determined in the brain. As the liver is the most important metabolic organ and accumulates high in all tissues, we next investigated the liver physiological indices.

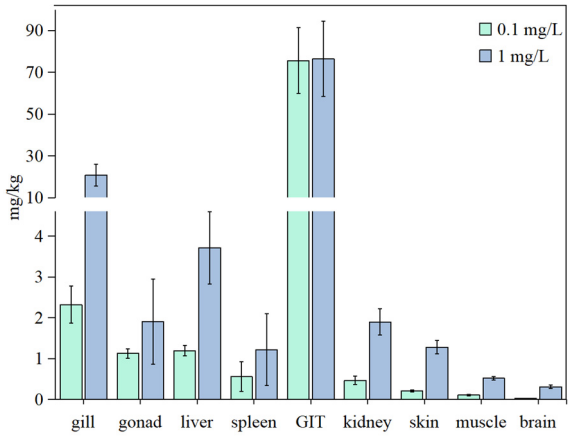


Fig. 2 The total PS NPs content in tissue of zebrafish after a 30-day exposure

3.3. Changes in physiologic indices

3.3.1. Hepatic body index and fattening degree

In the exposure experiment with a period of 30 days, the liver weight, body length, and body weight indexes of zebrafish in different treatment groups were measured, and the hepatosomatic index (HSI) and fattening capacity (FCF) were calculated. The results showed that there was no significant difference between the exposure treatment groups and the control group (Fig. 3a, b).

3.3.2. Changes in liver physiological indexes

0.1 mg/L PS NPs led to a significant increase in SOD activity, and MDA content, and a significant decrease in ROS content in the liver; 1 mg/L PS NPs led to a significant increase in hepatic SOD, CAT and GSH enzyme activities, and MDA content, and a significant decrease in ROS content. (Figure 3c-g)

3.3.3. Histological sections

Tissue sections can directly reflect the degree of damage caused by pollutants to the tissues of organisms. The liver of zebrafish was selected to observe the histological changes after exposure to PS NPs (Fig. 3h). While the liver underwent extravasation of erythrocytes into the liver and infiltration of inflammatory cells from the central vein, again the high.

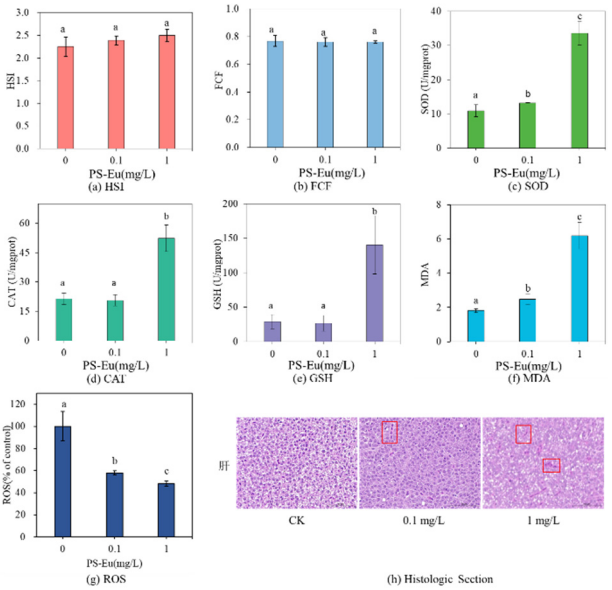


Fig. 3 Changes in the activity/content of biomarkers in the liver of zebrafish after a 30-day exposure

3.3.4. Transcriptome analysis

(A) The results of differential expression analysis were screened, as shown in the volcano plot in Fig. 4, and differentially expressed genes were screened under the conditions that the significant level was less than 0.05 and the multiplicity of expression difference was greater than 1. In the volcano plot, there were 96 differentially expressed genes, which can be found that differentially expressed genes only accounted for a small proportion, indicating the small effect of 0.1 mg/L nanoplastics treatment group on zebrafish, while the gray color indicates non-significant genes, which represents that non-significant genes accounted for most of the genes.

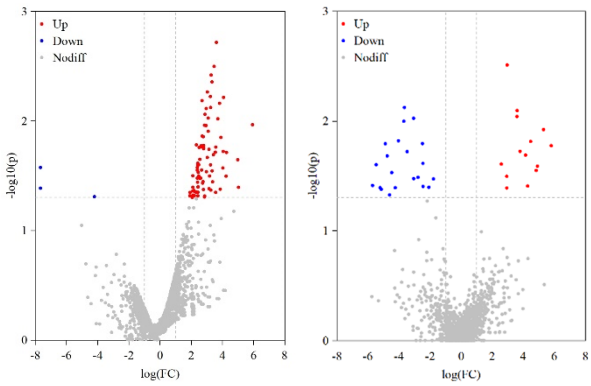
(B) GO enrichment analysis

GO enrichment analysis was performed on the differential gene transcriptomics data obtained from the above analysis (Fig. 5a, b). To investigate the response of zebrafish liver to PS NPs at the transcriptomic level, oxidative stress-related transcriptomics data after 30 days of exposure were subjected to GO enrichment analysis. GO covers three aspects, which characterize the Molecular Function (MF), Cellular Component role (CC), and Biological Process (BP) involved. There were both up- and down-regulated genes in the entries of the 0.1 mg/L nanoplastics treatment group, interestingly, whereas there were only up-regulated genes in the 1 mg/L nanoplastics treatment group.

(C) KEGG enrichment analysis

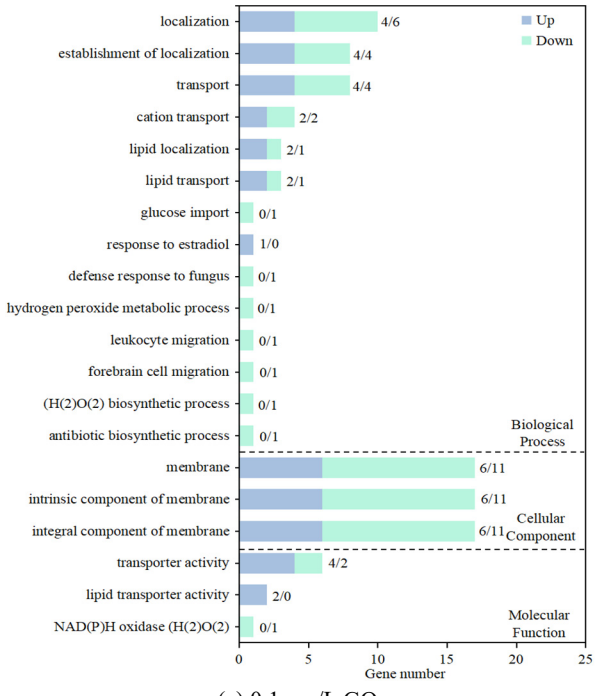
KEGG enrichment analysis of DEGs was further done (Figure 5c, d).

In 0.1 mg/L nanoplastics treatment group, there were three categories of related DEGs, including organismal systems, environmental information processing and cellular processes, while in the 1 mg/L nanoplastics treatment group, the category of metabolism was added, and the number of metabolic pathways accounted for the largest proportion.

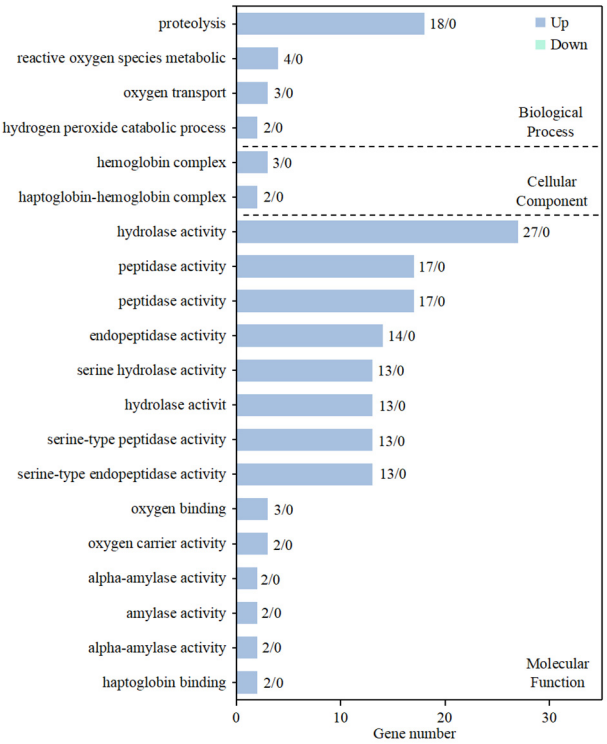


(a) 0.1 mg/L (b) 1 mg/L

Fig. 4 Volcanos of differential genes under treatment



(a) 0.1 mg/L GO



(b) 1 mg/L GO

Fig. 5 GO annotation and KEGG enrichment maps of differential expression genes of treatment group compared with control group after a 30-day of exposure

4. Discussion

In this study, we found that the accumulation and translocation of PS NPs in zebrafish is tissue-specific and can cross the physiological barriers of gill and gastrointestinal tract, thus accumulating in various tissues and organs of zebrafish, and intestines, gills, and liver are the target organs with the highest accumulation of nanoplastics, and PS NPs were found to accumulate in the brain, which suggests that the PS NPs can cross the blood-brain barrier. The above results are also consistent with several studies in aquatic organisms, where PS NPs are small hydrophobic particles that can be transferred from the intestines to the liver and kidneys via blood circulation[9]. Overall, 100 nm PS NPs could accumulate in various zebrafish tissues and organs.

In this study, we investigated the mechanism of toxicity of 0.1 mg/L and 1 mg/L of 100 nm PS NPs on zebrafish liver, which is the main accumulation organ of nanomaterials. Histopathological sections can visualize the changes in the zebrafish liver, and we found that PS NPs were able to cause the liver to undergo erythrocyte extravasation to the liver and inflammatory cell infiltration from the central vein, which was most pronounced in the 1 mg/L nanoplastics treatment group. In a study, nanoplastics (< 100 nm) caused structural deformation of the liver, loss of hepatocyte necrotic plaque boundaries, and hepatocyte degeneration in carp (*Cyprinus carpio*)[7]. Exposure to 100 nm PS NPs at a concentration of 0.1 mg/L for 19 days resulted in infiltration of hemocytes in the liver, capillary dilatation, aneurysms, and fibrous swelling in the gills of largemouth

bass (*Micropterus salmoides*). This is more consistent with our findings[10].

NPs induces oxidative stress in organisms when there is an imbalance between the production and elimination of reactive oxygen species[11]. In this study, 0.1 mg/L PS NPs could lead to a significant increase in SOD activity and a significant decrease in ROS content in the liver; 1 mg/L PS NPs led to a significant increase in hepatic SOD, CAT, and GSH enzyme activities, and MDA content, and a significant decrease in ROS content; this suggests that the antioxidant system of the zebrafish liver, in the early stage of oxidative stress, will scavenge oxidized free radicals by accelerating the oxidative radical scavenging, thus leading to a decrease in ROS content. It has been shown that after 19 days of exposure, 0.1 mg/L PS NPs significantly elevated SOD and CAT activities; 0.01 mg/L PS NPs resulted in significantly higher GSH content than that of the control group[10]. This is similar to the results of our study. In another study, 100 nm PS NPs resulted in significantly higher SOD, GSH and CAT activities, as well as significantly higher MDA content in the livers of juvenile larvae of larvae of Larimichthys crocea[11]. In addition, in a 100 nm PS NPs exposure study in medaka fish (*Oryzias latipes*), an increase in SOD and CAT enzyme activities was observed in the liver at low concentrations compared to the control, indicating activation of the antioxidant defense system, and chronic exposure to NPs inhibited SOD in the liver of medaka fish at higher treatments.

In summary, the results indicate that PS NPs are able to accumulate in zebrafish, cause an inflammatory response in the zebrafish liver, and activate oxidative stress in the zebrafish liver. Therefore, nanoplastics will negatively affect the health status of fish and impact the ecosystem, which may be passed on to humans through the food chain, posing a risk to human health.

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