Polystyrene Nanoplastic Exposure Induces Developmental Toxicity by Activating the Oxidative Stress Response and Base Excision Repair Pathway in Zebrafish (*Danio rerio*)

Meilan Feng, Juanjuan Luo, Yiping Wan, Jiannan Zhang, Chunjiao Lu, Maya Wang, Lu Dai, Xiaoqian Cao, Xiaojun Yang,* and Yajun Wang*

ABSTRACT: The widespread accumulation of nanoplastics is a growing concern for the environmental and human health. However, studies on the mechanisms of nanoplastic-induced developmental toxicity are still limited. Here, we systematically investigated the potential biological roles of nanoplastic exposure in zebrafish during the early developmental stage. The zebrafish embryos were subjected to exposure to 100 nm polystyrene nanoplastics with different concentrations (0, 100, 200, and 400 mg/L). The results indicated that nanoplastic exposure could decrease the hatching and survival rates of zebrafish embryos. In addition, the developmental toxicity test indicated that nanoplastic exposure exhibits developmental toxicity via the inhibition of the heart rate and body length in zebrafish embryos. Besides, behavioral activity was also significantly suppressed after 96 h of nanoplastic exposure in zebrafish larvae. Further biochemical assays revealed that nanoplastic-induced activation of the oxidative stress responses, including reactive oxygen species accumulation and enhanced superoxide dismutase and catalase activities, might affect developmental toxicity in zebrafish embryos. Furthermore, a quantitative polymerase chain reaction assay demonstrated that the mRNA levels of the base excision repair (BER) pathway-related genes, including *lig1*, *lig3*, *polb*, *parp1*, *pold*, *fen1*, *nthl1*, *apex*, *xrcc1*, and *ogg1*, were altered in zebrafish embryos for 24 h after nanoplastic exposure, indicating that the activation of the BER pathway would be stimulated after nanoplastic exposure in zebrafish embryos. Therefore, our findings illustrated that nanoplastics could induce developmental toxicity through activation of the oxidative stress response and BER pathways in zebrafish.

1. INTRODUCTION

Microplastics and nanoplastics refer to all plastic particles less than 5 mm in diameter.1 In recent decades, the plastic pollution has been listed as the second largest environmental science problem in the world and is as famous as global threats such as ocean acidification, climate change, and ozone depletion.2,3 The main sources of plastic pollution are poor waste management practices, garbage dumping, improper disposal, or runoff in industrial and agricultural activities.4,5 Different from the environmental contamination by larger plastic pieces, microplastics and nanoplastics can be ingested due to their smaller sizes and may thus accumulate along the food chain6−9 and subsequently be introduced to animals and humans.10−16 After analyzing microplastics in food and water, studies revealed that up to 250 plastic microparticles per liter were present in mineral water for human consumption,17 and microplastics were also detected in sugar, salt, alcohol, and honey.18−20 Therefore, it is speculated that humans consume 80 g of microplastics per day by eating plants.21 Accumulated evidence illustrates that microplastics are harmful to the human body. Ingestion of microplastics often causes oxidative stress, inflammation, and DNA damage.22 It was found that microplastics containing heavy metals could cause lipid peroxidation and other oxidative damage in the hippocampus, leading to increased mortality.23 Mate and Schuelke’s study showed that microplastics exposure can increase the level of reactive oxygen species (ROS) in crabs, thus turning on the antioxidant defense mechanism to cope with oxidative stress.24 ROS are intracellular chemical species that contain oxygen (O₂) and are reactive toward lipids,
proteins, and DNA. Superoxide dismutase (SOD) and catalase (CAT) are important components of antioxidant enzymes in the biological system. The accumulation of polystyrene microplastics leads to lipid accumulation and liver inflammation in fish. In addition, antioxidant enzymes, including CAT and SOD, were significantly activated, indicating that microplastics are responsible for the recovery of oxidative stress.

Notably, a recent study revealed that microplastics can be divided into smaller nanoplastics (less than 1 μm in diameter), which might more easily infiltrate or accumulate in animal or human organs because of their smaller size. Based on the literature review, the European Food Safety Agency (EFSA) concluded that particles less than 150 μm in diameter might cross the intestinal mucosal barrier, whereas particles less than 1.5 μm in diameter could be transported to deeper tissues. In addition, nanoplastics are generally difficult to isolate from the environment or organism, which would greatly affect human health. Thus, plastic waste in water might affect human health, the biological toxicity effect and the underlying mechanism the effect of nanoplastic exposure on the development in organisms have still not been fully characterized. As an aquatic vertebrate animal, the zebrafish model is extensively utilized to study the toxicity of environmental pollutants, benefitting from their small size, easy reproduction, short life cycle, and lower maintenance cost. This encouraged us to illustrate the potential developmental toxicity of nanoplastic exposure in vivo. Herein, our study explored the effects and underlying mechanisms of nanoplastic exposure on developmental toxicity in zebrafish embryos. The analysis of developmental parameters showed that the exposure of zebrafish to nanoplastics can affect embryonic development. Further analyses showed that nanoplastics can boost ROS accumulation, increase CAT activity, affect SOD activity, induce apoptosis, and alter the base excision repair (BER) pathway-related gene expression at the mRNA level. Thus, our findings revealed the mechanism of nanoplastic exposure-induced developmental toxicity via the activation of the oxidative stress response and BER pathway in zebrafish embryos.

2. RESULTS

2.1. Characterization of Polystyrene Nanoplastics. The polystyrene nanoplastic particles of average 106 nm
diameter were detected by using scanning electron microscopy (SEM) analysis (Figure 1A) and dynamic light scattering (DLS) (Figure 1B), respectively. The particles were non-fluorescent and white in color. The aggregation effect of microplastics resulted in the larger hydration diameter. The average zeta potential was $-22.3333 \text{ mV}$. 

2.2. Polystyrene Nanoplastics Affect the Survival and Hatching Rates of Zebrafish. To gain insights into the effects of nanoplastics on zebrafish embryonic development, we first identified the median lethal concentration (LC$_{50}$) of 100 nm nanoplastics in zebrafish embryos. The results indicated that the LC$_{50}$ of nanoplastics was 431.1 mg/L after the treatment of 24 hpf zebrafish embryos for 96 h ($n = 100$ for each group) (Figure 2A). Therefore, the zebrafish embryos were treated to a range of nanoplastics from 100 to 400 mg/L in the following experiments.

To further study the effects of nanoplastics on zebrafish embryonic development, we identified the median lethal concentration (LC$_{50}$) of nanoplastics in zebrafish embryos. The results indicated that the LC$_{50}$ of nanoplastics was 431.1 mg/L after the treatment of 24 hpf zebrafish embryos for 96 h ($n = 100$ for each group) (Figure 2A). Therefore, the zebrafish embryos were treated to a range of nanoplastics from 100 to 400 mg/L in the following experiments.

To further study the effects of nanoplastics on zebrafish embryonic development, we determined the survival rates of 24 hpf zebrafish embryos treated with nanoplastics for 96 h (Figure 2B). In the higher concentration group (400 mg/L), we noticed that the survival rate of zebrafish embryos was significantly decreased to 68.33% ($P < 0.05$) after nanoplastic treatment for 96 h. In addition, we found that 100 mg/L nanoplastic treatment was almost insufficient to affect the hatching rates, whereas 200 and 400 mg/L nanoplastic exposure resulted in obviously delayed hatching rates in zebrafish embryos ($P < 0.0001$) (Figure 2C). These results suggested that nanoplastic exposure, especially at higher concentrations, significantly decreased the survival and hatching rates of zebrafish embryos.

2.3. Nanoplastic Exposure Impairs the Heart Rate, Body Length, and Behavioral Activity of Zebrafish. To determine the developmental toxicity of nanoplastic exposure in zebrafish, we next determined the effects of exposing 24 hpf zebrafish embryos to nanoplastics for 48 and 72 h on the heart rate and body length. The results indicated that the heart rate was significantly decreased after 72 h of nanoplastic exposure ($P < 0.0001$) (Figure 3A), suggesting that nanoplastic exposure may impair the development of zebrafish embryos. In addition, we noticed that a decreased zebrafish larvae length was detected in 200 mg/L ($P < 0.05$) and 400 mg/L groups ($P < 0.0001$) after 48 h of nanoplastic exposure, while for only the 400 mg/L group compared with the control group, the body length decreased significantly ($P < 0.05$) after 72 h of nanoplastic exposure (Figure 3B,C).

Further investigations revealed that the 96 h nanoplastic treatment significantly ($P < 0.05$) affects the locomotor activity of zebrafish larvae at 5 days post-fertilization (dpf). Notably, the behavioral analysis of zebrafish larvae treated with different concentrations demonstrated significant differences in the distance travelled and trajectories. The weaker the swimming ability of larvae in each hole, the more the vacancy left in the well. In this context, exposure to a lower concentration (less than 100 mg/L) of nanoplastics seems to be insufficient to
inhibit the behavioral activity, whereas the distances swam by zebrafish larvae in the higher concentration (more than 200 mg/L) groups were markedly decreased (P < 0.05) after nanoplastic exposure (Figure 4A–C), suggesting that nanoplastic exposure concentrations might be positively correlated with developmental disruption in zebrafish embryos.

Figure 4. Nanoplastic exposure impairs the behavioral ability of zebrafish larvae. (A) Average distance traveled by larvae treated with nanoplastics in 1 min under 5 min light and dark (black bars on the x-axis) conditions for 30 min (n = 24 for each group) (B) Total distances of zebrafish swimming. (C) Trajectory graph of zebrafish larvae with or without nanoplastic treatment at different concentrations after 96 h exposure. Data are shown as the mean ± SD. *P < 0.05 and ****P < 0.0001 compared with the control.

Figure 5. Nanoplastic exposure induces the oxidative stress response and apoptosis in zebrafish embryos. (A-C) Determinations of ROS accumulation (A) and SOD (B) and CAT (C) activities in 24 hpf zebrafish embryos after 24 h nanoplastic treatment (n = 30 for each group). (D) PCA plot of biomarkers (ROS, SOD, and CAT) in nanoplastic-treated and control groups of zebrafish embryos. (E,F) mRNA levels of bcl2 (D) and bax (E) in 24 h nanoplastic-treated zebrafish embryos (n = 30 for each group). Data are shown as the mean ± SD. *P < 0.05, ***P < 0.001, and ****P < 0.0001 compared with the control.
2.4. Nanoplastic Exposure Induces an Oxidative Stress Response and Apoptosis in Zebrafish Embryos. Since developmental toxicity is usually accompanied by an oxidative stress response and apoptosis,

we then evaluated the effects of nanoplastic exposure on ROS accumulation and the regulation of CAT and SOD activities after treatment with nanoplastics at different concentrations in zebrafish embryos. As expected, the nanoplastic-treated zebrafish embryos displayed significantly enhanced ROS accumulation (Figure 5A). After 24 h of 100, 200, and 400 mg/L nanoplastic treatment, the levels of ROS were increased by 1.27- (P < 0.001), 1.47- (P < 0.0001), and 2.23-fold (P < 0.0001), respectively, compared with that of the nontreated control group (Figure 5A). In addition, the results indicated that CAT activity was increased after nanoplastic exposure at different concentrations in all three groups of zebrafish embryos (Figure 5B), suggesting that the CAT synthesis pathway might be activated by nanoplastic treatment in zebrafish. Intriguingly, we found that the SOD activity was increased in the lower concentration group (less than 100 mg/L) but decreased in the higher concentration groups (more than 200 mg/L) after the exposure of 24 hpf zebrafish embryos to nanoplastics for 24 h (Figure 5C). Considering that multiple biomarkers were evaluated in this process, a principal component analysis (PCA) was performed for determining the differences between experimental groups after nanoplastic exposure in zebrafish embryos. Notably, we found that the expression of antiapoptotic genes was decreased in zebrafish after nanoplastic exposure for 24 h (Figure 5F). Therefore, these results demonstrated that apoptosis is associated with the developmental toxicity of microplastic exposure in zebrafish embryos.

2.5. Activation of the BER Pathway Is Involved in Nanoplastic-Induced Developmental Toxicity. Previous studies indicated that the BER pathway performs an important part in oxidative stress-related DNA damage and subsequently prevents developmental abnormalities.

In this context, we therefore attempted to measure the regulation of potential key genes of the BER pathway in zebrafish embryos after nanoplastic exposure. BER is the primary DNA repair pathway that corrects base lesions induced by oxidation, alkylation, and deamination. In this process, BER facilitates the repair of damaged DNA via two general pathways, including short- and long-patch. Importantly, several key regulators, such as endonuclease III-like (NTHL1), 8-oxoguanine DNA glycosylase (OGG1), apurimic endonuclease (APEX), flap endonuclease 1 (FEN1), DNA polymerases (POLB and POLD), X-ray repair cross complementing 1 (XRCC1), DNA ligases 1 and 3 (LIG1 and LIG3), and poly (ADP-ribose) polymerase 1 (PARP1), were found to be essential for the BER pathway (Figure 6A).

In nanoplastic-treated zebrafish embryos, the expression levels of several genes, including lgl1, pold, nthl1, parp1, apex, and xrc1, were statistically increased (P < 0.05), whereas only the fen1 expression was decreased (P < 0.05) after microplastic exposure in zebrafish embryos. Notably, we found that the expressions of lig3, polb, and ogg1 were increased in the lower concentration groups (less than 200 mg/L) and slightly decreased in the 400 mg/L microplastic exposure group in zebrafish (Figure 6B). Thus, our results demonstrated that the activation of the BER pathway may be a stress response for the oxidative DNA damage induced in nanoplastic-treated zebrafish embryos.

3. DISCUSSION

In a realistic aquatic ecosystem, the nanoplastics usually persist over long-term periods (months to years) with lower
concentrations. Previous reports indicated that the typical nanoplastic concentrations were 150–2400 particles/m³, whereas in a harbor adjacent to a plastic production facility, the concentration was 102,000/m³.44 We have tested the toxicological effects of nanoplastics using a median lethal concentration (LC₅₀) assay,45 followed by for a series of concentrations lower than LC₅₀ for subsequent experiments. However, for investigating the toxic effects and mechanisms of nanoplastics on aquatic organisms, the complicated environmental factors might disturb the effects of nanoplastics in organisms. Therefore, the laboratory condition usually focuses on one or two organisms and lasts for shorter periods (hours to days), and the outcomes from simplified and individual studies in laboratorial conditions with a higher concentration might accurately evaluate the risk for human health and partially reflect the toxicity effects of nanoplastics in realistic environmental ecosystems.

The chorion of zebrafish embryos can effectively block polystyrene nanoparticles with a diameter of 100 nm.46 The adsorption of nanoplastics on the outer surface of the chorion changes the mechanical properties of the chorion, which may lead to an anoxic microenvironment that subsequently extends the incubation period of zebrafish embryos.46 A previous study indicated that hypoxia caused by microplastics is likely to result in the death of zebrafish embryos and therefore reduce the survival rate.47 Microplastics or nanoplastics can also combine with other chemicals, such as heavy metals, influencing their bioavailability and toxicity in the organisms.48 In this study, we used nanoplastics alone, and Lee’s work has indicated that nanoplastics synergistically accelerated the inhibition of hatching.49 In view of the large number of marine pollutants, the adsorption of microplastics means that they can combine with a variety of toxic compounds to deepen the toxicity.

It is noted that the survival of vertebrates under hypoxia includes reducing various processes, such as heart function and cell cycle processes, to match the energy supply with the energy demand. Nanoplastic exposure also creates a hypoxic environment for zebrafish, which slows the heart rate. We found that the body length was decreased after the exposure of zebrafish embryos to nanoplastics, which might be initially caused by delayed hatching and then limited by poor nutrient absorption in later development, for the nanoparticles initially accumulate in the yolk sac and the head and later in other regions, such as the liver, pancreas, gall bladder, pericardium, and GI tract.50 Zebrafish embryos obtain nutrients from their yolk sac until 5 dpf, which can eliminate the differences caused by nutrients.51 However, the results indicated that the growth rates of zebrafish embryos in the 400 mg/L nanoplastic treatment group were inhibited within the first 72 h developmental stage, implying that nanoplastic exposure might permanently impair the development of zebrafish embryos.

A previous study indicated that hypoxia leads to heart damage and reduced ATP synthesis, resulting in decreased behavioral ability,52 which is consistent with our results that the behavioral ability of zebrafish larvae is inhibited after microplastic exposure (Figure 4A–C). In addition, Chen et al. concluded that oxidative damage is one of the main reasons for the behavior inhibition in zebrafish larvae.53 It may also be that nanoplastics affect the neural development of zebrafish larvae, resulting in a reduction in their behavioral ability. In addition, a recent report demonstrated that the accumulation of polyethylene microplastics could trigger a behavioral disorder and subsequently cause an impact on the anxiety behavior and defensive anti-predatory response in mice through the food chain,15 indicating that microplastics might induce neurotoxicity in mice. In a realistic environment, microplastics and nanoplastics often work in synergy with other chemicals in nature. Together with other natural compounds, they reduce the secretion of acetylcholine and induce hypoaactivity and a disorganized swimming pattern in zebrafish larvae.54

It is known that excessive oxygen radicals are the main culprit of oxidative stress in vivo.55 SOD and CAT are regarded as important components of antioxidant enzymes in the oxidative stress response. The dysfunction of ROS and oxidative stress in the cell would lead to severe disorders and diseases.56 Our results indicated that SOD first increased and then decreased with the increasing microplastic concentration (Figure 5B), which may be because lower nanoplastic concentrations cause oxidative damage to the body, resulting in an increase in the SOD concentration. When the nanoplastic concentration exceeds the self-regulated concentration of the zebrafish body, the synthesis of SOD will be affected, resulting in a decrease in the SOD concentration.57 As discovered, SOD can catalytically convert the superoxide radical or singlet oxygen radical generated in tissues through the metabolism or reactions in cells to hydrogen peroxide and molecular oxygen.58 CAT decomposes hydrogen peroxide into water and oxygen. We therefore speculated that the oxidative damage to cells caused by nanoplastics is mostly decomposed by CAT.

Several previous studies indicated that microplastics and nanoplastics could induce the ROS metabolism and oxidative stress through regulating antioxidants, including SOD and CAT, in zebrafish. After microplastic or nanoplastic exposure, significant induction was determined in the activities of SOD and CAT.26,29 In contrast, other reports showed no significant change in CAT activity after microplastic exposure.50,61 Our results indicated that SOD activity were increased in the lower concentration group but decreased in higher concentration groups in zebrafish embryos. The upregulation of CAT activity was also observed in zebrafish embryos exposed to nanoplastics. We expected that these differences might be caused by the sizes of microplastics or nanoplastics, exposure times, exposure concentrations, and different developmental stages of zebrafish embryos. Furthermore, regulation of bcl2 and bax expressions demonstrated the activation of oxidative stress-induced apoptosis in nanoplastic-treated zebrafish embryos (Figure 5D,E), suggesting that nanoplastic exposure probably causes apoptosis by boosting ROS accumulation and/or affecting SOD and CAT activity.52,63

As an important pathway for DNA damage repair, the regulation of BER pathway-related gene expression levels suggested that the BER pathway was related to nanoplastic-induced DNA oxidative damage in zebrafish embryos (Figure 6B). In this context, OGG1 and NTHL1, as complex glycosylases, are able to recognize and detach damaged bases and create an AP site in DNA.64 Then, APE acts on this site to continue the BER pathway repair.65 The expression levels of ogg1, nthl1, and apex generally display an upward trend after nanoplastic exposure in zebrafish embryos. Additionally, DNA polymerases, POLB and POLD, were mainly associated with the gap-fill work. FEN1 participates in the long patch BER pathway to complete the repair process.66 Moreover, as a central scaffolding protein in the BER pathway, XRCC1 can interact with LIG3 and PARP and undertake important tasks in sealing the DNA ends.67 During the early embryonic
developmental stage, the embryos develop rapidly, and the mechanism by which the embryos face pressure in the early stage is imperfect, which may make the embryos more sensitive to harmful compounds.\textsuperscript{67} Our results indicated that the mRNA levels of most of the BER pathway-related genes were upregulated after nanoplastic exposure, except fen1, suggesting that the BER pathway might be a protective mechanism triggered at higher concentrations in nanoplastic-treated zebrafish embryos.

4. CONCLUSIONS

The microplastic and nanoplastic pollution has been an emerging threat to human health.\textsuperscript{68} They have entered the human food chain either by inhalation or by ingestion, particularly of shellfish and crustaceans.\textsuperscript{69} In addition, nanoparticles are potentially more hazardous than microplastics because they might easily permeate biological membranes.\textsuperscript{29} Zebrafish have been increasingly used to investigate the toxicity of microplastics and nano-durability due to their low cost, optical clarity, high fecundity, and short life cycle.\textsuperscript{70} Herein, we discovered that polystyrene nanoplastic exposure could lead to developmental toxicity by promoting ROS accumulation in zebrafish embryos. Interestingly, further investigations revealed that apoptosis was also involved in nanoplastic-induced development toxicity in zebrafish, suggesting that nanoplastic exposure might trigger oxidative stress-mediated apoptosis in this process.\textsuperscript{71} In addition, the results indicated that the expression levels of several BER pathway-related genes, including lgg1, pold, nthl1, apex, srrc1, lgg3, polb, parp1, and ogg1, in zebrafish embryos were significantly changed after microplastic treatment, suggesting that DNA damage was probably caused by the activation of oxidative stress and inflammation induced by nanoplastic exposure in zebrafish embryos.\textsuperscript{72} Therefore, these findings highlight that nanoplastic exposure could induce an oxidative stress response and activate the BER pathway to defend against oxidative DNA damage, which distributes the potential risks that come along with nanoplastic exposure.

5. MATERIALS AND METHODS

5.1. Ethics Statement. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Sichuan University. All experiments were performed according to the regulations and guidelines established by the Ministry of Science and Technology of the People’s Republic of China (Approval number: 2006-398).

5.2. Characterization of Nanoplastics. Nanoplastics were obtained from Huge Biotechnology Co., Ltd. (DS100, density of 1.05 g/cm\(^3\), CV % = 3, Shanghai, China). The microplastic morphology was photographed by SEM (SU8100, Hitachi, Japan). The DLS spectra and zeta potential were determined using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). The sample was dispersed in deionized water.

5.3. Zebrafish Breeding, Husbandry, and Exposure Test. Wild-type strain (AB) zebrafish were purchased from the China Zebrafish Resource Center (Wuhan, China). Zebrafish were raised at 28 °C with a 14:10 h light/dark cycle and fed freshly hatched brine shrimp (Artemia nauplii) at 9 am and 6 pm per day. For breeding, three male and three female adult zebrafish were matched to produce embryos in each breeding tank. The zebrafish embryos were collected by siphoning the bottom of the tank the next day and maintained in an E\(_3\), embryonic medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl\(_2\), and 0.33 mM MgSO\(_4\) in 1 L of distilled water). At 24 hpf, zebrafish embryos were assigned to 100 mm Petri dishes (100 embryos per dish) and treated with nanoplastics with different concentrations. The potential toxicity of chemical substances was determined using an LC\(_{50}\) assay that exposed 24 hpf zebrafish embryos to nanoplastics for 96 h. Briefly, taking the common logarithm of concentration as the abscissa and the probability unit of mortality as the ordinate, the LC\(_{50}\) values of 96 h and 95% confidence limit were obtained through a regression equation. In this study, the concentrations of 100 nm diameter nanoplastics used for experiments were 100, 200, and 400 mg/L. Untreated zebrafish embryos were defined as the negative control. All experiments were performed independently in three replicates.

5.4. Developmental Toxicity Test. The zebrafish embryos with or without the nanoplastic treatment were characterized for developmental toxicity at different exposure time points. The survival rates were measured as the percentages of surviving zebrafish embryos at different concentrations for 72 h (\(n = 100\) for each group). The hatch rates were recorded every 24 h after microplastic exposure in 24 hpf zebrafish embryos (\(n = 100\) for each group). In addition, the heart rates (\(n = 20\) for each group) and body lengths (\(n = 50\) for each group) of zebrafish embryos were measured under a Stereo microscope (Leica M205FA, Leica Microsystems, Germany) at 48 and/or 72 h after nanoplastic exposure.

5.5. Behavioral Test. At the 96 h nanoplastic exposure time point, the motor capabilities of the nanoplastic-treated zebrafish larvae were assessed by determining swimming activities,\textsuperscript{73} which was slightly modified from the previously reported protocol.\textsuperscript{74,75} Briefly, the 5 dpf zebrafish larvae of the different groups (\(n = 24\) for each group) were assigned to a 24-well plate (one larva in each well). The swimming trajectory and total distance travelled by each larva were measured using a ZebraLab Video-Track system (ViewPoint Life Science, France) through the 5 min cycle light-to-dark photoperiod and the following 30 s cycle sound and vibration stimulus. All experiments were performed independently in three replicates.

5.6. Biochemical Assay. To determine the activation of the oxidative stress response in nanoplastic-treated zebrafish embryos, 24 hpf embryos (\(n = 30\) for each group) treated with or without nanoplastics at different concentrations (0, 100, 200, and 400 mg/L) for 24 h were collected for ROS, SOD, or CAT assays. All experiments were performed independently in three replicates.

5.6.1. ROS Assay. ROS levels in nanoplastic-exposed zebrafish embryos were detected using a reactive oxygen species assay kit (S0033S, Beyotime, Shanghai, China) according to the standard procedure. In brief, the zebrafish embryos (\(n = 30\) for each group) in different groups were collected and homogenized in ice-cold lysis buffer. All the samples were centrifuged at 15,000g at 4 °C for 20 min. The 24 μL supernatants were then transferred to a 96-well plate and incubated at room temperature for 5 min. According to the experimental protocol, 1 × PBS (PH 7.4) and a 10 μM DCF-DA solution were added, and the plates were incubated at 37 °C for 30 min. The fluorescence intensities of each sample were determined using a microplate reader (BioTek Synergy H1, USA) with excitation at 485 nm and emission at 530 nm.
The protein concentrations were detected using a BCA protein quantification kit (E112-02, Vazyme, China). The ROS generation was in relation to protein quantity. All experiments were performed independently at least three times.

5.6.2. Measurement of SOD and CAT Activities. The homogenized samples from nanoplastic-treated zebrafish embryos (n = 30 for each group) were centrifuged and harvested before evaluating SOD and CAT activities. Thereafter, the SOD and CAT activities of the samples from different groups were measured by using an SOD activity examination kit (D799598, Sangon Biotech, Shanghai, China), respectively, according to the manufacturer’s instructions. The CAT and SOD contents were in relation of protein quantity. All samples were analyzed in three independent replicates.

5.7. Total RNA Extraction and qPCR Assay. After exposing 24 hpf zebrafish embryos to nanoplastics for 24 h, the total RNA from the different groups (n = 30 for each group) was extracted by using TRIzol (Invitrogen, Thermo Fisher Scientific, Waltham, MA) according to the manufacturer’s instructions. Thereafter, the purity and quality of the RNA were detected using a spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The PCR assay using a reverse transcription PCR system on the CFX Maestro System (Bio-Rad Laboratories, Inc., CA) was performed independently at least three times. The PCR protocol was started with a denaturation step at 95 °C for 5 min, followed by 36 cycles at 95 °C for 10 s, 60–62 °C for 30 s, and 72 °C for 30 s. Three replicate samples were performed for different groups. The sequences of the primers for the qPCR assay are provided in Table 1.

5.8. Quantification and Statistical Analysis. Statistical significance was accepted at P < 0.05, and values were presented as means ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to determine the significant differences between mean values, and the Dunnett’s test was used to determine the significant difference (P < 0.05) between microplastic-treated and control groups. The ANOVA results and the figures were obtained and plotted using Graphpad Prism 8 (GraphPad Software, San Diego, CA). A PCA was performed by Bioinformatics (http://www.bioinformatics.com.cn) for visualization to see the group differences after nanoplastic exposure.

### Table 1. Primer Sequences Used to Target BER Pathway Genes in Zebrafish Embryos

| Primer | Forward (5′−3′) | Reverse (5′−3′) | Length (bp) | Annealing Temperature (°C) |
|--------|----------------|-----------------|-------------|---------------------------|
| β-actin | CTACAATGAGCTGCGGTGTTG; R: CCTCATAGATGGGCACTGGT | 239 | 62 |
| bcl-2 | TTCTAACCCTGGCAGGGAAG; R: GGCAGCTTGAAGTGCATGC | 162 | 62 |
| bax | TACCTGCGGTGTTGCTGTTG; R: CAGGAGGGAAGACCTCGAC | 193 | 62 |
| pold | TTCTCCCTCCTGCTGTTGC; R: CATATACCCCGAACACTGCT | 466 | 62 |
| fen1 | TCAGGACGTTGAAAGCTCA; R: TCCCTGTCGAACCGCAATCAA | 138 | 60 |
| lig3 | AGCAAAGGCTGCGAAAACAG; R: CCTAGCGTGTGTGTGGCTAA | 300 | 62 |
| polb | TCCCTGACGAGGAAATCAC; R: ATCTTTGCACCGACTCCAC | 179 | 60 |
| xrc1 | ACCCTCTTTTTGCGGGGTATC; R: ACCCTTATCTGCCTTTTGCT | 489 | 60 |
| nthl1 | TCAAGGCTTCCTGTGCACTCA; R: TCGCAACTATAACGGCTCCC | 100 | 60 |
| apex1 | AATATAAGGTGTTGGGTATACGG; R: CAGGGAGGGAATCTTCATATGG | 250 | 60 |
| lg1 | ATGAGACGCGCCCAAGGGAAG; R: CTAGATTGCCTGACTGCT | 117 | 60 |
| parp1 | CTTCAACAGGCGAGGCCGCTT; R: TCAACAGATCCCTCTACCATG | 246 | 60 |
| ogg1 | CAAGATCTTACAGCACCTTGTG; R: CAAACTTGTCAGTAGATCAG | 232 | 60 |

### Corresponding Authors

Xiaojun Yang — Guangdong Provincial Key Laboratory of Infectious Disease and Molecular Immunopathology, Shantou University Medical College, Shantou S15041, China; orcid.org/0000-0002-0007-3715; Email: yangx@stu.edu.cn

Yajun Wang — Key Laboratory of Bio-resources and Eco-environment of Ministry of Education, College of Life Science, Sichuan University, Chengdu 610065, China; Email: cdwyjhk@gmail.com

### Authors

Melin Feng — Key Laboratory of Bio-resources and Eco-environment of Ministry of Education, College of Life Science, Sichuan University, Chengdu 610065, China

Jianjuan Luo — Key Laboratory of Bio-resources and Eco-environment of Ministry of Education, College of Life Science, Sichuan University, Chengdu 610065, China

Yiping Wan — Key Laboratory of Bio-resources and Eco-environment of Ministry of Education, College of Life Science, Sichuan University, Chengdu 610065, China

Jiannan Zhang — Key Laboratory of Bio-resources and Eco-environment of Ministry of Education, College of Life Science, Sichuan University, Chengdu 610065, China

Chunjiao Lu — Guangdong Provincial Key Laboratory of Infectious Disease and Molecular Immunopathology, Shantou University Medical College, Shantou S15041, China

Maya Wang — Key Laboratory of Bio-resources and Eco-environment of Ministry of Education, College of Life Science, Sichuan University, Chengdu 610065, China

Lu Dai — Key Laboratory of Bio-resources and Eco-environment of Ministry of Education, College of Life Science, Sichuan University, Chengdu 610065, China

Xiaoqian Cao — Key Laboratory of Bio-resources and Eco-environment of Ministry of Education, College of Life Science, Sichuan University, Chengdu 610065, China

Complete contact information is available at: https://pubs.acs.org/doi/10.1021/acsomega.2c03378

### Author Contributions

M.F. and J.L. contributed equally to this work. J.L., C.L., X.Y., and Y.W. established the research ideas and designed methods; M.F., J.L., Y.W., C.L., J.Z., M.W., L.D., and X.C. conducted the experimental operations; M.F. and J.L. analyzed the data; M.F., J.L., X.Y., and Y.W. wrote and revised the manuscript. J.L. and...
Y.W. supervised the research. All authors read and approved the manuscript for publication.

Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS
This work was financially supported by the National Natural Science Foundation of China (32072706 and U1901206) and the Science and Technique Foundation of Guangdong Province (210728156901639).

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