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Exposure to Nanoplastics Cause Caudal Vein Plexus Damage and Hematopoietic Dysfunction by Oxidative Stress Response in Zebrafish *(Danio rerio)*

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Introduction: The proliferation of nanoplastics (NPs) has emerged as a significant environmental concern due to their extensive use, raising concerns about potential adverse effects on human health. However, the exact impacts of NPs on the early development of hematopoietic organs remain poorly understood.

Methods: This investigation utilized fluorescence microscopy to observe the effects of various NP concentrations on the caudal vein plexus (CVP) development in zebrafish embryos. Subsequent RNA sequencing (RNA-seq) identified genes related to CVP deformities and hematopoietic stem/progenitor cells (HSPCs) in zebrafish embryos exposed to NPs. Additionally, single cell RNA sequencing (scRNA-seq) analysis identified genes associated with the development of CVP and HSPCs. RT-qPCR assessed changes in expression of these genes in zebrafish embryos exposed to different NP concentrations.

Results: The impact of NPs on zebrafish embryos was investigated, revealing significant reductions in survival and hatching rates and decreases in body length alongside increased heart rates. Exposure to NPs at 8 mg/L severely impaired zebrafish CVP development. RNA-seq revealed that NPs exposure altered the activity of oxidative enzymes, hydrolases, and the extracellular matrix in zebrafish embryos. Treatment with 10 μM NAC effectively rescued the CVP defects induced by NPs. Additionally, scRNA-seq identified genes associated with EC and HSPC development, and subsequent RT-qPCR validation confirmed significant expression changes in these genes.

Conclusion: The results of this study suggest that NPs induce oxidative stress in vascular ECs and HSPCs, which mediates CVP damage and impairs hematopoiesis in zebrafish embryos.

Keywords: nanoplastics, caudal vein plexus, oxidative stress, endothelial cells, hematopoietic stem/progenitor cells, zebrafish, single cell RNA sequencing

Introduction

Plastic products, especially disposable plastics, are increasingly used in daily life; however, their limited recyclability contributes to rising global production and consumption. The European Plastics Report estimated that global plastic production would reach 390.7 million metric tons by 2021.[1] In natural environments, nearly 80% of plastic waste is discarded through various means.[2,3] Due to their chemical stability and persistence, plastics are challenging to fully decompose, resulting in widespread environmental pollution. Plastic particles can degrade via physical, chemical and biological processes, forming smaller diameters. Microplastics are defined as plastic particles with diameters less than 5 millimeters, whereas NPs measure less than 100 nm and can penetrate the blood-gas, intestinal, and skin barriers, potentially affecting human health.[4–6] NPs can also cross the blood-brain barrier, posing risks of brain damage.[7–9]

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Additionally, NPs have shown detrimental effects on the bone marrow hematopoietic system in mice, reducing the ability of bone marrow cells to self-renew and differentiate.[10,11] However, research on the impact of NPs on hematopoietic tissues and underlying molecular mechanisms remains limited.

Hematopoietic development in vertebrates is typically divided into two stages: primary and secondary hematopoiesis. Primary hematopoiesis generates red blood cells and marrow cells, whereas secondary hematopoiesis produces HSPCs,[12,13] which have the capacity to generate all blood cell types and sustain hematopoiesis throughout life.[14] In mammals, certain ECs in the aorta-gonad-mesonephros region detach from the dorsal aorta and differentiate into HSPCs in response to intracellular and extracellular signaling cues.[15,16] These nascent HSPCs are initially immature and must migrate to the embryonic liver to achieve full hematopoietic functionality.[17] In zebrafish, HSPCs are derived from ECs on the ventral side of the dorsal aorta and initially localize to the CVP, which functions similarly to the embryonic liver in mammals.[18] The CVP, also called caudal hematopoietic tissue, provides a supportive microenvironment for the maturation, proliferation, and differentiation of HSPCs.[19] Furthermore, endothelial cells and macrophages in the CVP interact with HSPCs, facilitating their homing and retention.[20–22] Consequently, the CVP in zebrafish is essential for embryonic blood development.

Zebrafish are increasingly popular for studying bioaccumulation and toxicity effects of environmental pollutants due to their small size, transparent larvae, rapid reproduction and short lifespan. Moreover, the molecular mechanisms of vascular development in zebrafish closely resemble those in humans.[23] As a model organism, zebrafish effectively assess nanoparticle biocompatibility and toxicity through observations of developmental changes, hatchability, and gene expression, making them a reliable model for environmental toxicity research.[24,25] This study aimed to clarify the potential toxicity of 20 nm NPs to the CVP in vivo by leveraging the unique features of the zebrafish model. We hypothesized that NPs may induce CVP damage and hematopoietic disorders by disrupting signaling pathways involved in CVP development in zebrafish embryos. Using the *Tg(kdrl:eGFP)* transgenic zebrafish, commonly used to label vascular ECs, we monitored the effects of NP exposure on CVP development and hematopoiesis. Our results showed that NPs significantly inhibited zebrafish growth and CVP development. Additionally, exposure to H_2O_2 exacerbated the inhibitory effect of NPs on CVP, while the antioxidant NAC rescued CVP development, suggesting that NPs may limit CVP growth via oxidative stress responses. This aligns with previous studies showing that redox balance is crucial in angiogenesis and hematopoiesis.[26–28] Furthermore, based on scRNA-seq and RNA-seq results, we found that NPs induced CVP damage primarily by mediating oxidative stress in vascular ECs and HSPCs in zebrafish. These findings suggest that NPs induce damage to vascular ECs and HSPCs, resulting in developmental abnormalities in CVP and hematopoietic dysfunction through oxidative stress signaling pathways.

Materials and Methods

Ethics Statement

This study adhered strictly to the ethical guidelines set out in Guide for the Care and Use of Laboratory Animals of Sichuan University. All experimental procedures were conducted in accordance with the Guidelines and Regulations issued by the Ministry of Science and Technology of China (Approved No. 2006–398).

Materials

20 nm NPs were purchased from Huge Biotechnology Co., LTD (Shanghai, China). The concentration of the stock solution was 50 mg/mL. 1-phenyl-2-thiourea (PTU) is obtained from Macklin (Shanghai, China). N-acetylcysteine (NAC; CAS No.616–91–1) is obtained from Yeasen Biotechnology (Shanghai, China).

Zebrafish Breeding, Husbandry, and Treatment

Wild-type (WT) and *Tg(kdrl:eGFP)* zebrafish line were obtained from the China Zebrafish Resource Center (Wuhan, China). The adult zebrafish were live in the flow-through tanks and maintained on a 14:10 h light/dark cycle at 28 ± 1°C. Feed these zebrafish with freshly hatched brine shrimp two times in morning and afternoon. Breeding was conducted by separating four

adult zebrafish (two males and two females) with a transparent partition in the breeding tank. The partition was removed the following day at 9 a.m. After spawning was completed within an hour, the embryos were collected and transferred into the E3 medium, which contains 5 mm NaCl, 0.17 mm KCl, 0.33 mm $CaCl_2$ and 0.33 mm $MgSO_4$.

To study the effects of NPs exposure, 2-hours post-fertilization (hpf) embryos of AB or *Tg(kdrl:eGFP)* line were exposed to E3 medium with NPs at concentrations of 2, 5, and 8 mg/L, whereas the control group was exposed to the E3 medium without NPs.[29] The exposure solution was renewed daily at 10 a.m. For *Tg(kdrl:eGFP)* embryos, it is recommended to administer 0.003% PTU to suppress melanin synthesis within each group at 24 hpf.[30]

In the oxidation-reduction study, the *Tg(kdrl:eGFP)* embryos are subjected to treatment with H_2O_2 or NAC.[31] It is essential to prepare H_2O_2 freshly for each exposure. NAC was initially prepared as a 100 μM stock solution in ddH_2O, diluted in E3 medium until a final concentration of 10 μM was reached. A Leica DMi-8 fluorescence microscope was used to observe the images. 90 normal embryos of each concentration of the *Tg(kdrl:eGFP)* transgenic strain were transferred into 6-well plate at 2 hpf, each well have 30 embryos. During the exposure period, 0.003% PTU added into the exposure solution in each group at 24 hpf.

Developmental Toxicity Test

To investigate the developmental toxicity of NPs on zebrafish embryos, the normal embryos of the AB strain of each group were transferred into 100 mm Petri dishes. Survival rates were determined by calculating the percentage of surviving fish embryos at different concentrations 70 hours after NPs treatment (n = 100 for each group). Additionally, the hatching rates were measured by calculating the percentage of embryos successfully hatched at 72 hpf (n = 100 for each group). Heart rates were counted for 15s using a Leica M205FA microsystem (Leica, Germany) at 48 hpf (n = 80 for each group). In addition, body lengths (n = 90 for each group) were determined at 72 hpf embryos.[32] For each concentration, three independent replicates were performed.

Observation of Vasculature in Zebrafish Embryos Treated with NPs

Fluorescence images of the CVP of *Tg(kdrl:eGFP)* transgenic zebrafish embryos were taken using a Leica DMi-8 fluorescence microscope. The images of CVP in different groups were analyzed by using ImageJ software. The morphology of the CVP including loop numbers, area, and maximum ventral extension distance, were measured with ImageJ software.[33]

RNA-Seq Analysis

Following a 46-h exposure to NPs, total RNA was extracted from distinct groups of zebrafish embryos using RNAzol® RT reagent (Molecular Research Center, Inc., Cincinnati, OH). Conduct transcriptome sequencing at BGI Company located in Shenzhen, China. Analyze the resulting counts data through GSVA, utilizing a dataset sourced from the GO dataset within the msigdbr package.[34] Perform differential analysis on the GSVA analysis outcomes using the limma package. For WGCNA, employ the Median Absolute Deviation (MAD) technique to identify the top 5000 genes for subsequent analysis.[35] WGCNA was performed using R (4.4.0) to establish gene modules, with specific parameters such as mergeCutHeight set at 0.3 and minModuleSize at 70, indicating a module merge threshold of 0.3 and a requirement of at least 70 genes per module. These gene modules were then correlated with traits to pinpoint modules associated with zebrafish CVP developmental abnormalities subsequent to NPs exposure, and genes within these modules were chosen for GO enrichment analysis.

Single-Cell RNA-Seq Analysis

The single-cell dataset of GSE146404, which investigates the development of zebrafish tail cells, can be obtained for analysis. Initially, the Seurat package can be utilized for quality control procedures to eliminate low-quality cells and genes. Specifically, cells with more than 300 features and genes expressed in a minimum of 5 cells should be selected to establish a Seurat object. Subsequently, a more stringent quality control process can be implemented by filtering cells based on criteria such as nFeature_RNA > 200, nFeature_RNA < 3000, and percent.mito < 5, resulting in the identification of 25,136 cells suitable for further analysis.

Normalization of the data can be achieved by applying the LogNormalize method within the NormalizeData function with a scaling factor of 10,000. Subsequently, 2000 highly variable genes can be identified using the FindVariableGenes function, followed by data standardization through the ScaleData function. Principal component analysis can then be conducted, and significant principal components can be identified using the RunPCA function. Utilizing the top 15 principal components selected, clustering and dimensionality reduction of the data can be performed using the FindClusters and RunUMAP functions, with a resolution parameter set to 0.5 to define distinct cell clusters. The FindAllMarkers function can be employed to identify characteristic genes associated with each cell cluster. The parameters were configured with verbose set to true, min.pct set to 0.25, and logfc.threshold set to 0.25, stipulating that genes must exhibit high expression in at least 25% of cells and possess a log fold change exceeding 0.25.

Subsequent to the identification of individual cell clusters, the FindAllMarkers function is reapplied to identify genes showing differential expression across distinct cell populations using the Wilcoxon test method, with min.pct and logfc.threshold parameters set at 0.25. Genes exhibiting differential expression between HSPCs and ECs are singled out, with criteria specifying avg_log2FC > 1 and *p_val_adj* < 0.05. Lastly, the enrichGO function within the clusterProfiler package is employed to conduct GO enrichment analysis on these differentially expressed genes.

Total RNA Isolation, cDNA Synthesis, and RT-qPCR Assay

30 embryos were randomly selected from each tested concentration. Total RNA was extracted with RNAzol® RT according to the instructions of manufacturer. Nanodrop 2000 was used to measure RNA quality and quantity. First strand cDNA was reverse transcribed using SMART® MMLV Reverse Transcriptase (Takara Bio Inc., Japan) from approximately 1 μg of total RNA. The CFX Maestro System (Bio-Rad Laboratories, Inc., CA) was used to perform RT-qPCR with EasyTaq® DNA Polymerase (Takara Bio Inc). The RT-qPCR protocol consisted of 35 cycles of 94 °C for 30s, 60 °C for 30s, and 72 °C for 20s after an initial step of 94 °C for 2 min. The mRNA levels of target genes were normalized using the housekeeping gene β-actin, and the RT-qPCR reactions were carried out in triplicate. The primer sequences for the RT-qPCR assay can be found in Table 1.

Statistical Analysis

Experimental data are presented as mean ± SD and were analyzed using GraphPad Prism 10.0 (GraphPad Software, San Diego, CA) for both data and graphical analysis. The data was compared using one-way ANOVA, followed by an examination of the significant differences between the exposure groups and the control groups of different concentrations. A p-value of less than 0.05 was considered statistically significant.

Results

Developmental Toxicity of NPs in Zebrafish

To evaluate the developmental toxicity of NPs on zebrafish embryos, we assessed survival rates, hatching rates, heart rates, and body lengths following NPs exposure. The results indicated that zebrafish embryos exposed to a high NPs concentration (8 mg/L) exhibited distinct tail region abnormalities after 46 hours of exposure (Figure 1A). Additionally, compared to the control group, the survival rates of embryos in the high-concentration treatment group (8 mg/L) decreased to 66.67% at 72 hpf (Figure 1B). Exposure to NPs also significantly affected the hatching rates (Figure 1C). At 72 hpf, hatching rates decreased with increasing NP concentrations, reaching 37.72% in the 8 mg/L treatment group

Table 1 List of RT-qPCR Primer Sequences

Primers	GenBank number	Sense primer (5'-3')	Antisense primer (5'-3')
β-actin	NC_007112.7	ATCCGTAAGGACCTGTATGCC	GAAGCACTTCCTGTGGACGAT
aggf1	NC_007132.7	GAAAGAGCTTCCTGTCCCGA	GCTCCGTCTCCGTGTTTTCT
Ifi30	NC_007113.7	AGGGCGACCAAGGCAATAAA	CCTGACGCGAGTAGTGTTGT
prdx1	NC_007113.7	TCGATTCCCACTTCTGCCAT	CCAAGCGCAGGGTTTCATCA

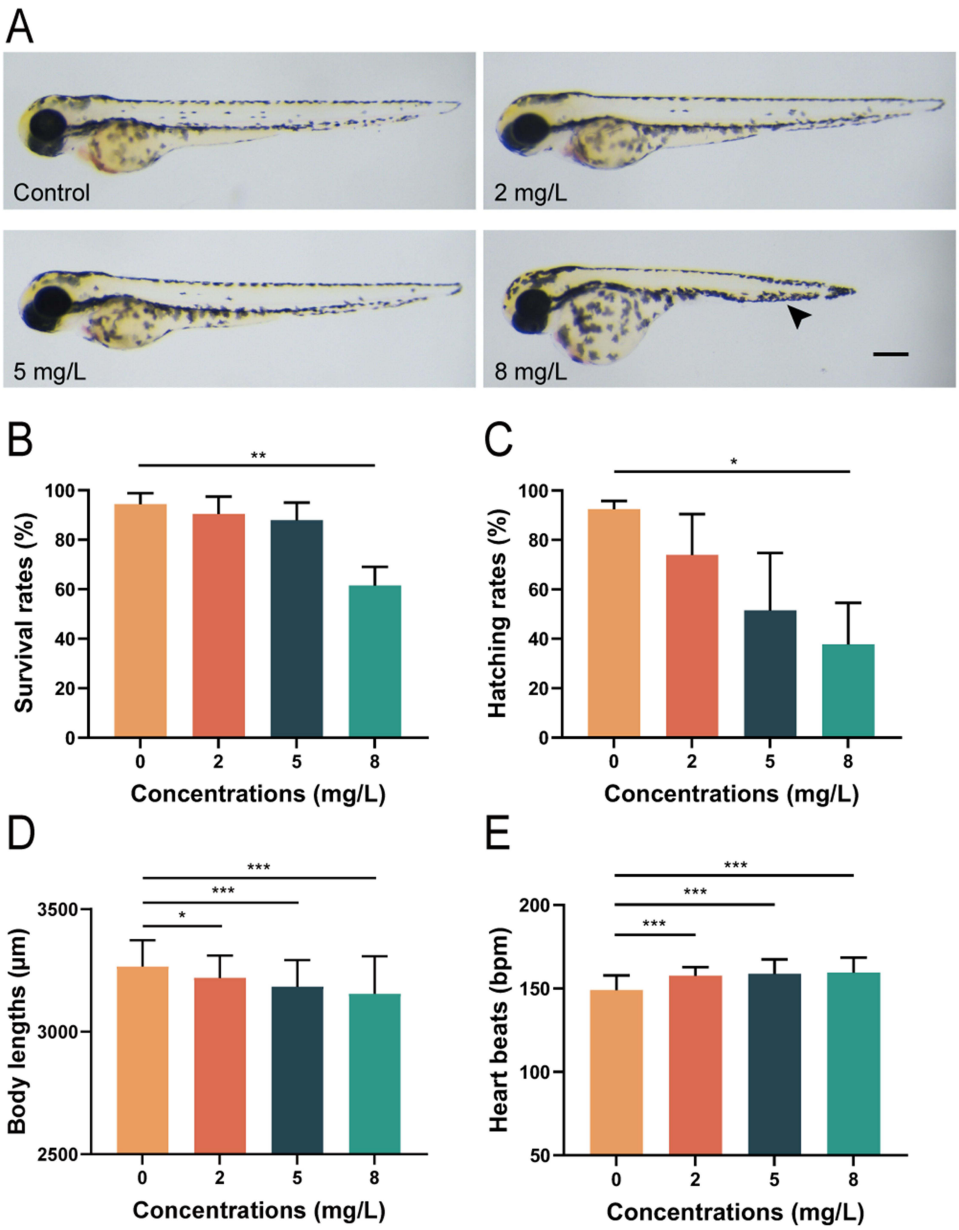

Figure 1 Toxicity of NPs on zebrafish embryonic development. (**A**) Morphological changes in zebrafish embryos exposed to varying NPs concentrations at 72 hpf. Black arrows indicate abnormal tail morphology. (**B**) Survival rates of zebrafish embryos treated with NPs at 72 hpf (n = 100). (**C**) Hatching rates of zebrafish embryos exposed to NPs · at 72 hpf (n = 100). (**D**) Body lengths of zebrafish embryos exposed to NPs at 72 hpf (n = 90). (**E**) Heart rates of zebrafish embryos treated with NPs at 48 hpf (n = 30). Data are presented as mean ± SD. Asterisks indicate statistical significance (**P* < 0.05; ***P* < 0.01; ****P* < 0.001). Scale bar = 100 μm.

(Figure 1C). Moreover, NPs exposure led to dose-dependent reductions in body lengths compared to controls at 72 hpf (Figure 1D). Notably, all treatment groups showed a significant increase in heart rates at 48 hpf (Figure 1E). In summary, NPs exposure decreased survival rates survival rates, delayed hatching, accelerated heart rates, and reduced body lengths in zebrafish embryos.

NPs Exposure Impairs the Development of Zebrafish CVP

The CVP in zebrafish begins developing at approximately 28 hpf and reaches maturity by 48 hpf.[36] At this stage, HSPCs migrate from the dorsal aorta to the CVP, where they begin to proliferate. Normal CVP development establishes a microenvironment that supports the proliferation and differentiation of HSPCs.[37] To investigate the effects of NPs on CVP development in zebrafish embryos, we used the transgenic zebrafish strain *Tg(kdrl:eGFP)*, which expresses green

fluorescent protein in the vascular system and is widely used in *vivo* for vascular development studies. NPs exposure significantly suppressed vascular sprouting in the CVP at 30 hpf compared to the control group (Figure 2A and B; white arrowheads). This exposure also led to a marked decrease in the number of loop structures (Figure 2A and C; red asterisks) and a reduction in maximum ventral extension distance within the CVP (Figure 2A and D; white line). However, the CVP area did not significantly decrease, potentially due to the lack of fusion among filopodia (Figure 2E). After exposing zebrafish embryos to different NPs concentrations (2, 5, and 8 mg/L) for 46 hours, we observed that CVP formation was impaired in embryos

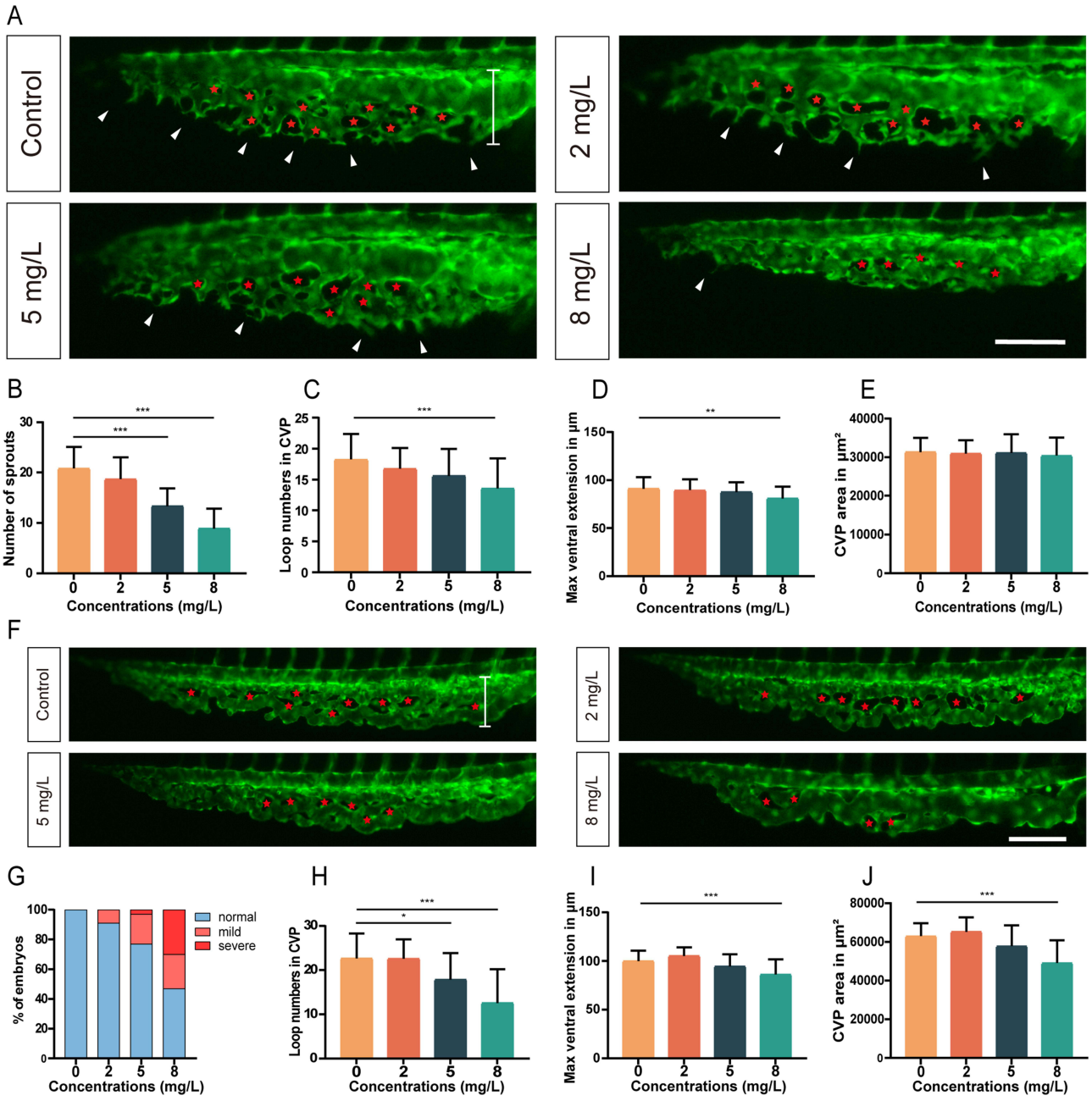

Figure 2 Exposure to NPs leads to developmental defects in the CVP of *Tg(kdrl:eGFP)* embryos. (**A**) Representative images of CVP developmental malformations in *Tg(kdrl:eGFP)* embryos treated with NPs at 30 hpf. (**B-E**) The numbers of filopodia (**B**), numbers of loop (**C**), maximum ventral extension distance (**D**) and area (**E**) of CVP in *Tg(kdrl:eGFP)* embryos after quantified NPs treatment at 30 hpf (n = 30 embryos). (**F**) Representative images of CVP developmental malformations in NPs-treated *Tg(kdrl:eGFP)* embryos at 48 hpf. (**G**) The malformation rate of CVP in *Tg(kdrl:eGFP)* embryos after quantified NPs treatment at 48 hpf (n = 30 embryos). (**H-J**) The numbers of loop (**H**), maximum ventral extension distance (**I**) and area (**J**) of CVP in *Tg(kdrl:eGFP)* embryos after quantified NPs treatment at 48 hpf (n = 30 embryos). White arrowheads, sprouts; red asterisks, loop structure; white vertical line, maximum ventral extension distance. Data are shown as mean ± SD. An asterisk above each bar indicates statistical significance (**P*<0.05; ***P*<0.01; ****P*<0.001). Scale bar, 100 μm.

treated with 8 mg/L NPs (Figure 2F). Embryonic CVP deformities were evaluated and classified by counting loop numbers (Supplementary Figure 1A; red asterisks), revealing a significant increase in severely deformed individuals in the 8 mg/L treatment group (Figure 2G). Furthermore, exposure to 8 mg/L NPs resulted in significant reductions in loop numbers, maximum ventral extension distance, and CVP area in zebrafish embryos after 46 hours compared to controls (Figures 2H–J). These results indicated that NPs exposure led to developmental defects in zebrafish CVP.

NPs Cause Permanent Defects in Zebrafish CVP

To investigate the potential effects of NPs on zebrafish CVP defects, we evaluated whether NPs could induce permanent defects in zebrafish CVP. After treating *Tg(kdrl:eGFP)* embryos with NPs for 46 hours, the exposure medium was replaced with E3 medium to continue treating the embryos until 72 hpf. The findings a significant inhibition of CVP area and maximum ventral extension distance at 72 hpf in the experimental group compared to controls, especially at 5 and 8 mg/L concentrations (Figures 3A–C). This finding suggested that NPs impaired CVP recovery in zebrafish embryos, resulting in persistent CVP defects.

Identification of Signaling Pathways Associated with CVP Defects

To investigate the impact of NPs on the CVP development in zebrafish, we performed RNA-seq analysis to examine transcriptional changes in zebrafish embryos exposed to varying NP concentrations. GSVA enrichment analysis revealed that in the 5 mg/L NPs treated group, biological processes (BP) such as positive regulation of nuclear cell cycle DNA replication, epithelial mesenchymal cell signaling, and positive regulation of neutrophil activation were upregulated, while processes like regulation of polyamine transmembrane transport, negative regulation of neutrophil activation and negative regulation of mitochondrial membrane permeability were downregulated (Figure 4A). In the 8 mg/L treatment group, upregulated BP included positive regulation of centriole elongation, nucleotide excision repair DNA gap filling, and negative regulation of glycogen metabolism, whereas downregulated processes involved regulation of polyamine

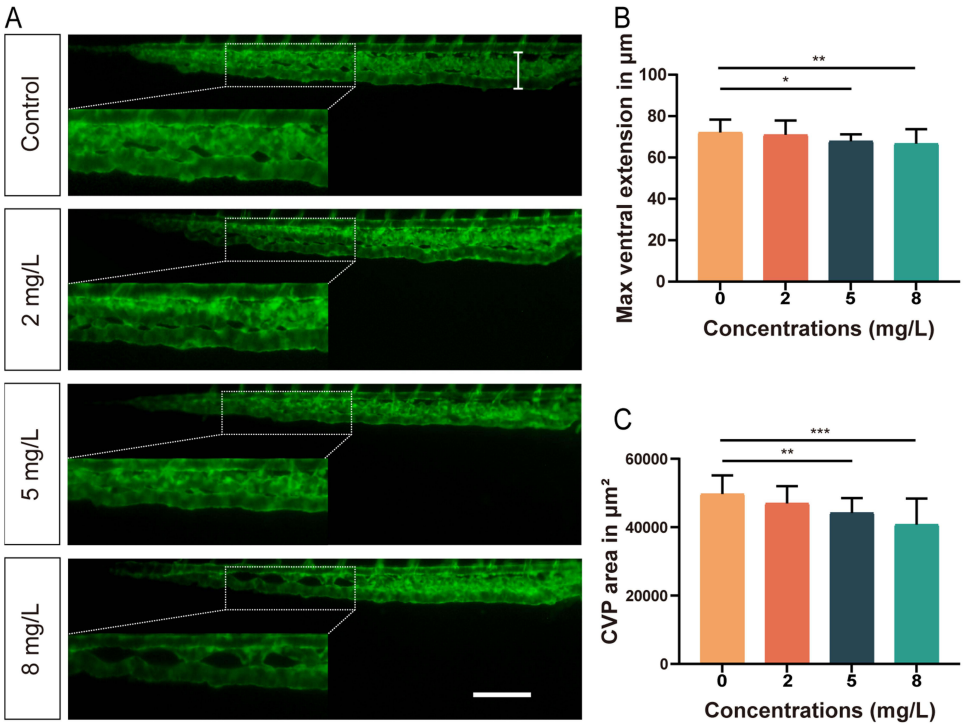

Figure 3 Exposure to NPs causes permanent defects in the CVP of *Tg(kdrl:eGFP)* embryos. (**A**) Representative image of permanent defects in the CVP of *Tg(kdrl:eGFP)* embryos due to exposure to NPs. (**B, C**) The maximum ventral extension distance (**B**) and area (**C**) of larval CVP after quantified NPs treatment (n=30 embryos). White line, maximum ventral extension distance. Data are shown as mean ± SD. An asterisk above each bar indicates statistical significance (*P<0.05; **P<0.01; ***P<0.001). Scale bar, 100 μm.

transmembrane transport, hyaloid vascular plexus regression, and positive regulation of cardiac muscle adaptation (Figure 4B). In terms of molecular function (MF), decreased MFs in the 5 and 8 mg/L treatment groups included oxidoreductase activity acting on paired donors with incorporation, while increased MFs included bubble DNA binding and SNRNP binding (Figure 4A and B).

To further investigate the effect of NPs on CVP damage, WGCNA identified 17 gene modules, with module G strongly correlated with abnormal CVP development and module K associated with normal CVP morphology (Figure 4C). Subsequent GO enrichment analysis showed that module K was enriched in sulfation and biogenic amine metabolic processes, while module C was enriched in negative regulation of hydrolase activity and extracellular matrix organization (Figure 4D). In

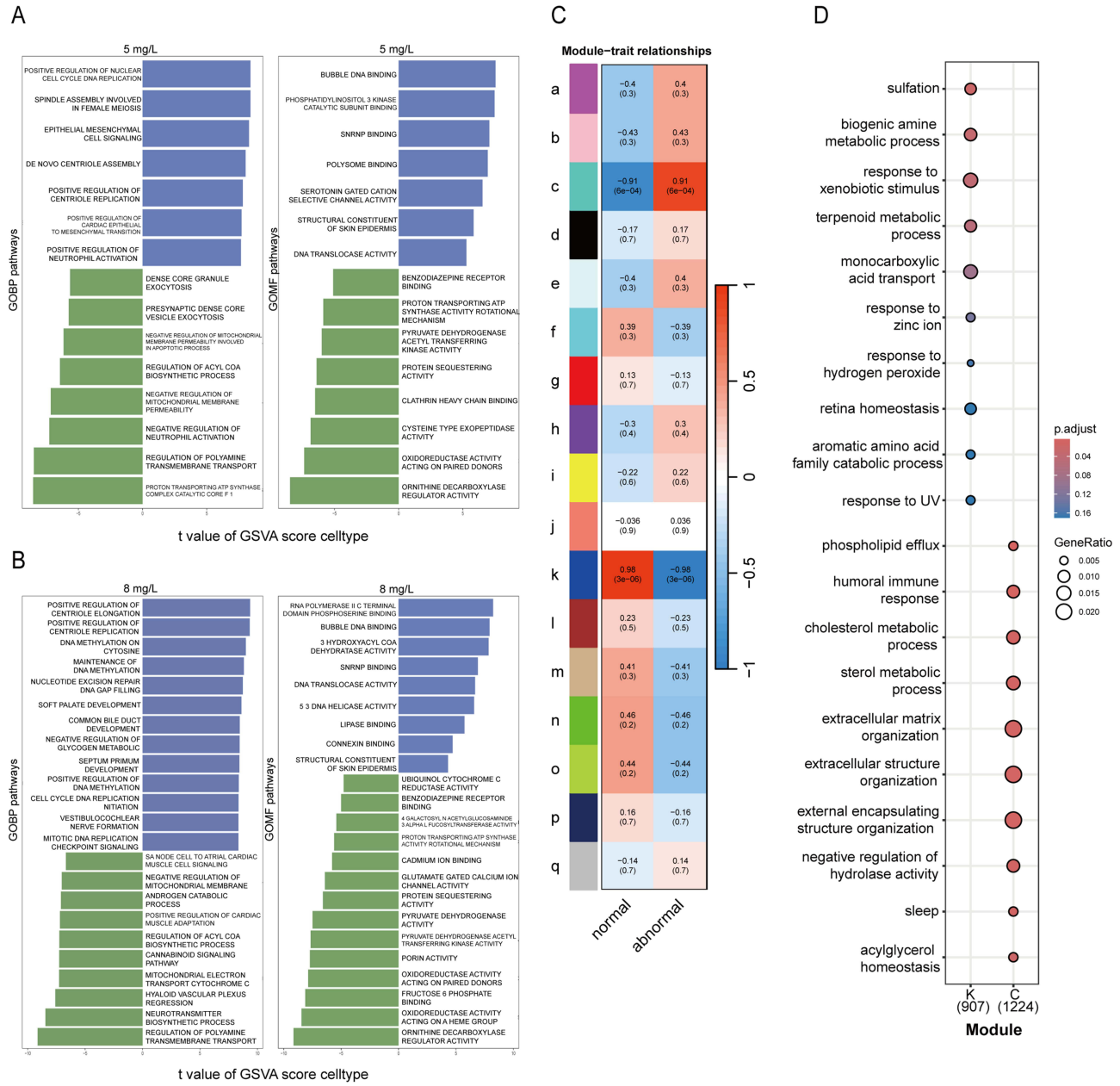

Figure 4 Alterations in the transcriptome of zebrafish embryos following NPs exposure. (**A**) Findings from the GSVA conducted on zebrafish embryos after exposure to a concentration of 5 mg/L of NPs (**B**) Results from the GSVA analysis performed on zebrafish embryos following exposure to 8 mg/L of NPs. (**C**) The WGCNA identified distinct gene modules (labeled a–q) in zebrafish embryos exposed to varying concentrations of NPs. (**D**) The screening conducted using WGCNA revealed GO enrichment analysis findings related to genes associated with CVP defects.

conclusion, these findings suggested that NPs exposure may affect redox levels and extracellular matrix composition in zebrafish embryos, thereby mediating CVP damage and impairing hematopoiesis.

NPs Exposure Induced CVP Damage Through Oxidative Stress Response

Previous research has shown that oxidative stress affects CVP in zebrafish, with 2 mm H_2O_2 treatment causing CVP damage in zebrafish embryos.[31] To further investigate the mechanisms by which NPs exposure impacts CVP development, zebrafish embryos at 2 hpf were simultaneously exposed to 4 mg/L NPs and 1 mm H_2O_2, based on the concentration used by *Song* et al.[38] At 48 hpf, the zebrafish embryos in the control group showed the formation of loop structures in the CVP (Figure 5A and B; red asterisks). Treatment with 4 mg/L of NPs alone did not significantly impact CVP development in zebrafish embryos. However, embryos co-treated with 4 mg/L NPs and 1 mm H_2O_2 displayed more pronounced CVP defects after 46 hours, including reduced loop numbers (Figure 5A and B; red asterisks), shorter maximum ventral extension distance (Figure 5A and C; white line), and decreased CVP area (Figure 5A and D), embryos treated with only 4 mg/L NPs or 1 mm H_2O_2. NAC, an antioxidant known for its ability to penetrate embryos, was used in co-treatment NPs at 2 hpf and alleviated oxidative stress responses in the embryos.

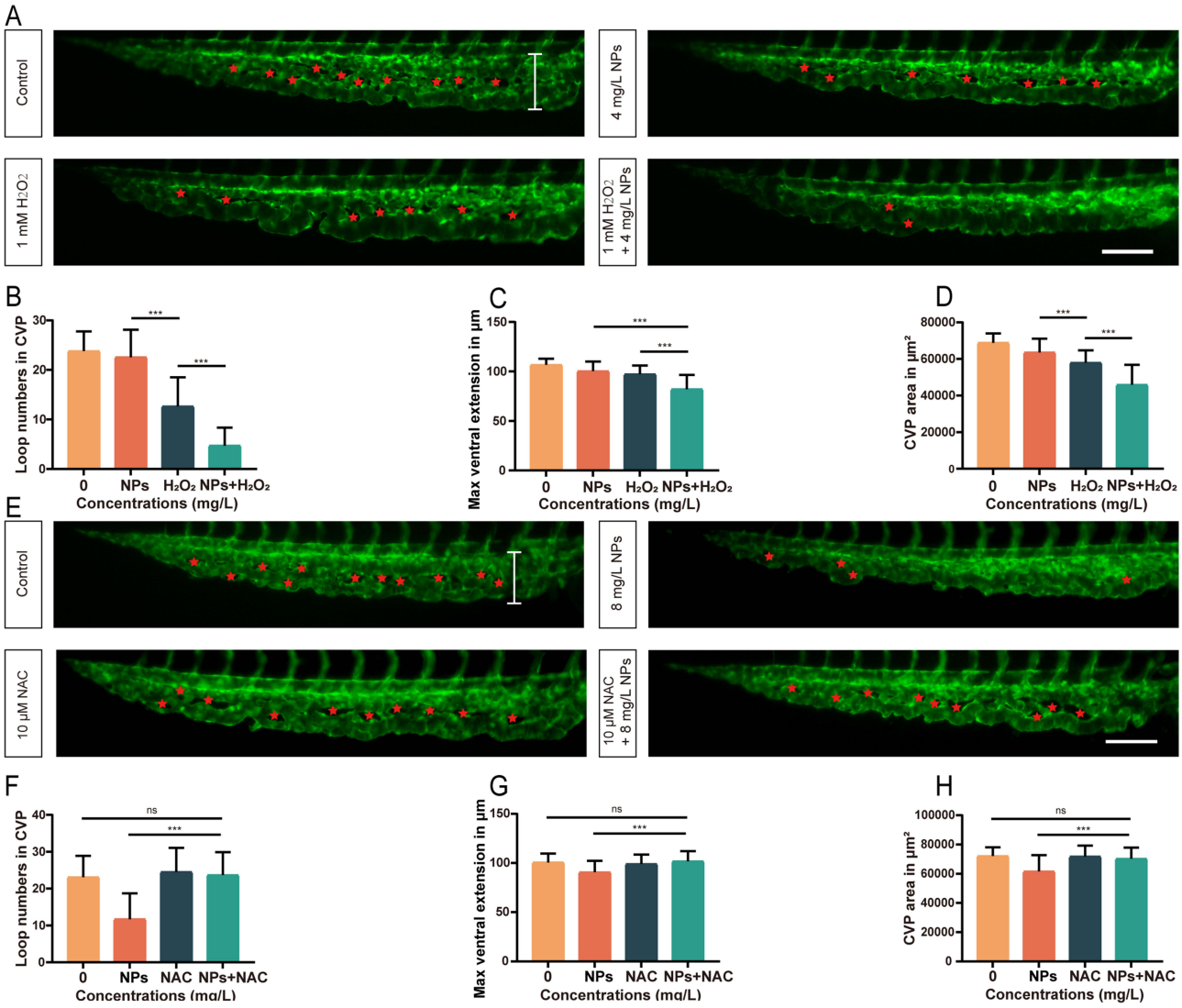

Figure 5 NPs cause oxidative stress damage to embryonic CVP development. (**A**) Representative images of developmental defects in CVP after treatment with low-doses of H_2O_2 and/or NPs for 46 h. (**B-D**) The loop numbers (**B**), maximum ventral extension distance (**C**) and area (**D**) of embryonic CVP after NPs and/or H_2O_2 treatment (n = 30 embryos). (**E**) Representative images of embryonic CVP development after treatment with NAC and/or NPs for 46 h. (**F-H**) The loop numbers (**F**), maximum ventral extension distance (**G**), and area (**H**) of CVP in embryos treated with NPs and/or NAC (n = 30 embryos). Red asterisks, loop structure; white line, maximum ventral extension distance. Data are shown as mean ± SD. An asterisk above each bar indicates statistical significance (***P<0.001; ns: not significant). Scale bar, 100 μm.

Compared to embryos exposed to 8 mg/L NPs alone, those treated with a combination of 10 μM NAC and 8 mg/L NPs for 46 hours showed restored CVP development, including significant increases in loop numbers, maximum ventral extension distance, and CVP area (Figure 5E–H). These findings indicated that NPs impair CVP development in zebrafish by inducing oxidative stress.

NPs Exposure Mediates Vascular ECs and HSPCs Damage in Zebrafish CVP

To investigate the influence of NPs on zebrafish CVP development, we utilized scRNA-seq data from zebrafish embryo tail to identify crucial signaling pathways involved in CVP development. Initially, we created a comprehensive single-cell map of zebrafish tail tissue, identifying 22 distinct cell clusters, which were further categorized into 9 main cell types. Specific marker genes were used for cell categorization: HSPCs were associated with *myb* and *hdr*; ECs were marked by *vegfc* and *fli1a*; neuronal cells were identified by *elavl3* and *snap25a*; erythrocytes were characterized by *hbae3* and *hemgn*; myeloid cells expressed *mmp13a* and *mpx*; epithelial cells were distinguished by *icn2* and *cyt1*; epidermal cells exhibited *cd9b* and areg; fibroblasts were marked by *col2a1a* and *col9a3*; and muscle cells were identified by *mylz3* and *actc1b*. (Supplementary Figure 1B). Of these, HSPCs- and ECs-associated subpopulations accounted for 35.42% and 16.94% of the total cell count, respectively (Figure 6A).

To further explore the regulatory mechanisms of zebrafish CVP development, we analyzed differential gene expression profiles across HSPCs- and ECs-associated subpopulations. GO enrichment analysis for ECs key BP such as the mitotic cell cycle process, organelle fission, and mitotic nuclear division, indicating the active mitotic state of ECs. MF analysis identified tubulin binding, antioxidant activity, and oxidoreductase activity, highlighting the intricate relationship between ECs mitosis and cellular redox status (Figure 6B). In contrast, the GO enrichment analysis of HSPCs identified BP such as ribosome biogenesis, rRNA processing, and hydrogen peroxide metabolism, indicating an active state of protein synthesis in HSPCs. The MF category in HSPCs GO enrichment findings also included antioxidant activity and peroxidase activity, underscoring the importance of redox homeostasis in HSPC development (Figure 6C). We further investigated the expression of genes associated with hematopoiesis and CVP development, including angiogenic factor with G-patch and FHA domains 1 (*aggf1*), interferon gamma-inducible protein 30 (*ifi30*), and peroxiredoxin-1 (*prdx1*). The results indicated high expression levels of these genes in both ECs and HSPCs, with close associations to cell growth, development, and redox processes (Figure 6D).

Subsequent investigations assessed the expression changes of these three genes in zebrafish embryos at 30 and 48 hpf following NPs exposure. Results showed an increase in *ifi30* mRNA at 30 hpf, followed by a decrease at 48 hpf, while *prdx1* mRNA levels remained elevated. In the group exposed to high NPs concentrations, a notable decrease in *aggf1* mRNA expression was observed (Figure 6E). These findings suggested that exposure to 20 nm NPs mediates ECs injury and hematopoietic dysfunction in the CVP of zebrafish by activating oxidative stress responses in both HSPCs and ECs.

Discussion

An increasing number of research indicates that NPs have significant detrimental effects on organisms, disrupting normal growth and development.[39] However, the specific toxic impacts of NPs on hematopoietic tissues remain largely uncertain. This study investigated the developmental toxicity of NPs in zebrafish embryos, revealing that exposure to a concentration of 8 mg/L resulted in decreased hatching rates, survival rates, and body lengths, along with increased heart rates (Figure 1). Additionally, zebrafish embryos exposed to NPs exhibited abnormalities in tail morphology. These findings confirmed that NPs exposure induces severe developmental toxicity in zebrafish embryos, impairing the development of tail tissues.

The CVP in zebrafish, located at the tail end, plays a crucial role in the development of hematopoietic function. The caudal vein primordia emerge at approximately 24 hpf, and by 28 hpf, ECs in the posterior cardinal vein form elongated pseudopods and initiate ventral migration.[36] This process results in the merging of developing blood vessels to create a honeycomb-like vascular network, ultimately forming the CVP structure. By 48 hpf, venous ECs on the ventral side undergo reshaping to establish the caudal vein, and by 72 hpf, the CVP gradually migrates ventrally and detaches from the dorsal aorta.[40,41] The CVP serves as an intermediary niche for HSPCs, significantly influencing their proliferation and

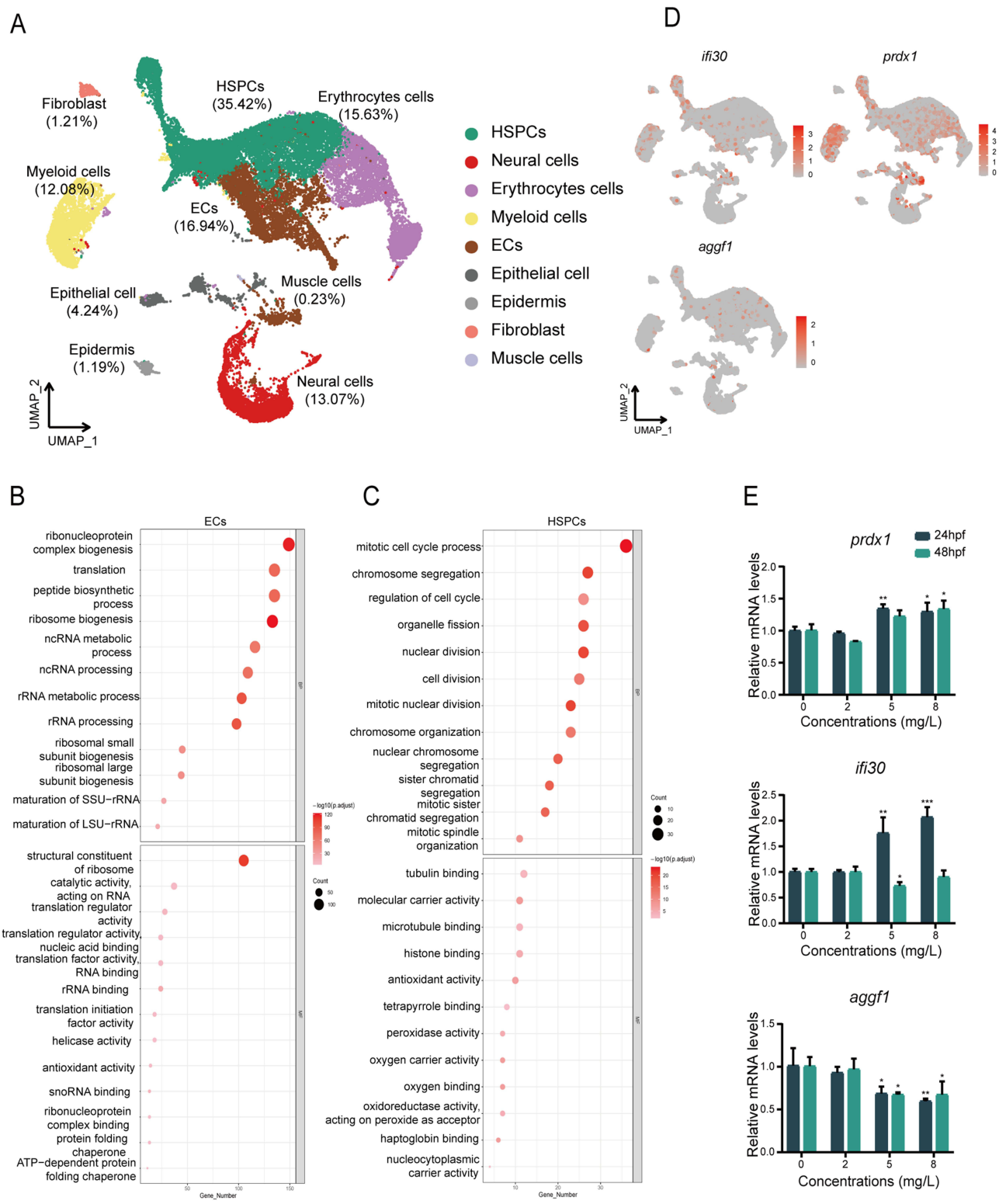

Figure 6 Identification of single cell subpopulation that influence the CVP damage and hematopoiesis after exposure to NPs. (**A**) UMAP visualization of single-cell data derived from zebrafish caudal fin tissue. (**B and C**) GO enrichment analysis findings of differentially expressed genes between HSPCs and ECs. (**D**) Gene expression analysis of angiogenic factors and redox-related genes in zebrafish tail tissue cells. (**E**)The mRNA levels of vascular endothelial growth factor and redox-related genes assessed post-NPs exposure. Data are shown as mean ± SD. An asterisk above each bar indicates statistical significance (*P<0.05; **P<0.01; ***P<0.001).

differentiation.[20] Our results demonstrated that NPs exposure disrupted embryonic CVP development, leading to lasting impairment (Figures 2 and 3), thus indicating a toxic effect of NPs on the zebrafish embryonic CVP.

ROS are generated as byproducts of cellular metabolism and play dual roles as signaling molecules and mediators of inflammation. At low concentrations, ROS serve as secondary messengers modulating processes such as cell growth and differentiation.[42,43] However, excessive ROS production can overwhelm the antioxidant defense mechanism of the body, triggering inflammatory responses and impairing cardiovascular development`.[44,45] NPs exposure frequently induces oxidative stress responses, compromising developmental processes.[26] In this investigation, we employed RNA-seq to assess mRNA alterations in zebrafish embryos exposed to various NPs concentrations. GSVA indicated significant impacts of NPs exposure on oxidative enzyme activity, suggesting a potential induction of oxidative stress responses in zebrafish embryos. Furthermore, WGCNA revealed changes in extracellular matrix and hydrolase activity in zebrafish following NPs exposure (Figure 4). Notably, simultaneous exposure to H_2O_2 and NPs exacerbated CVP damage, while treatment with NAC alleviated the embryonic CVP defects caused by NPs (Figure 5). These results suggested that NPs influence zebrafish CVP development primarily through the induction of oxidative stress.

Recent studies have increasingly demonstrated the ability of NPs to affect vascular development and the hematopoietic system in zebrafish.[46–48] However, the molecular mechanisms by which NPs mediate abnormalities in vascular development and hematopoietic system function remain poorly understood. The zebrafish CVP is a highly vascularized ecosystem characterized by a rapidly expanding population of ECs that form a complex network of dorsal arterioles and foveolar veins.[19] This process also involves various cellular components, including fibroblastic reticulocytes and hematopoietic cells.[49] The proper functioning of the CVP relies on the orderly interaction among all cell types. To elucidate the molecular mechanisms at play, we screened genes highly expressed in the ECs and HSPCs of the CVP using scRNA-seq. These genes are involved in regulating cell development and maintaining redox homeostasis. Our analysis indicated that the expression of these genes changed significantly following exposure to NPs, particularly noting a marked downregulation of aggf1 expression in zebrafish embryos. *Aggf1*, a vascular endothelial growth factor associated with Klippel-Trenaunay syndrome,[50] is primarily expressed in zebrafish CVP during 36 hpf and promotes EC proliferation, migration, and regulation of venous ECs.[51,52] The downregulation of *aggf1* suggests impaired EC function within the CVP. Additionally, we observed significant inhibition of *ifi30*, a lysosomal oxidoreductase critical for immune cell function. Recent studies have shown that *ifi30* is specifically expressed in the ECs of zebrafish embryonic CVP, where its sulfhydryl reductase activity regulates the redox environment and promotes the development of both the CVP and HSPCs.[33,37] Our results demonstrated significant inhibition of *ifi30* expression in zebrafish exposed to 5 mg/L NPs for 48 h, with a notable reduction at 8 mg/L, although the latter decrease was not statistically significant. Suppression of *ifi30* has been linked to abnormal CVP development and reduced HSPC numbers. The observed initial increase in *ifi30* expression after 24 h of exposure may reflect a transient stress response. Furthermore, we noted significant elevation of the peroxidase *prdx1*, suggesting abnormalities in intracellular ROS scavenging and redox homeostasis.[31] Previous studies indicate that *prdx1* deficiency enhances and hinders EC proliferation and migration, leads to CVP injury in zebrafish[31] aligns with our transcriptomic results, supporting the conclusion that NPs exposure mediates CVP injury and hematopoietic abnormalities primarily through oxidative stress responses. Collectively, our findings suggested that NPs exposure adversely affects CVP and hematopoietic function by mediating oxidative stress response in both ECs and HSPCs.

Conclusion

This research aimed to examine the influence of NPs on CVP toxicity and hematopoietic dysfunction in zebrafish embryos. This study revealed NPs have developmental toxicity on zebrafish embryos, resulting in significant abnormalities in tail morphology and permanent CVP permanent defects. Additionally, RNA-seq combined with scRNA-seq analysis indicated that NPs primarily mediated oxidative stress response in ECs and HSPCs, which in turn contribute to CVP damage and hematopoietic dysfunction in zebrafish. Furthermore, co-exposure to H_2O_2 and NPs exacerbated zebrafish CVP damage, while NAC alleviated NPs induced developmental abnormalities, further validating the transcriptome analysis. These findings suggested that NPs impacted the zebrafish CVP damage by triggering oxidative stress responses. However, a limitation of this study is the lack of examination regarding the effects of prolonged exposure to low concentrations of NPs on the CVP and hematopoietic function of zebrafish embryos. Future research should concentrate on investigating the consequences of extended exposure to low concentrations of NPs and other nanomaterials on hematopoietic function, as well as the molecular

mechanisms underlying these effects. Overall, the findings indicated that NPs can impede the development of the CVP and hemopoiesis in zebrafish, providing insights into evaluating the adverse effects of NPs.

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Author Contributions

Juntao Chen designed the experiment and acquired and analyzed the data. Chunjiao Lu, Wenjie Xie, Xiaoqian Cao, Jiannan Zhang analysed the data. Juanjuan Luo and Juan Li supervised the project. All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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