



Polyethylene Microplastics Induced Hepatic Oxidative Stress and Early Non-alcoholic Fatty Liver Disease in Wistar Rats

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ABSTRACT

Background: Polyethylene microplastics (PE-MPs) are persistent environmental pollutants found extensively in various ecosystems. However, their specific impact on liver function remains inadequately investigated.

Methods: Adult male wistar rats were orally exposed to PE-MPs (1-10 μ m) at doses of 0.1, 1 and 5 mg/kg/day over 28 days. Attenuated total reflectance-fourier transform infrared spectroscopy (ATR-FTIR) and fluorescence microscopy identified accumulations of PE-MPs. Liver tissues were examined histologically using Haematoxylin and Eosin staining. Oxidative stress was measured by measuring malondialdehyde (MDA), superoxide dismutase (SOD) and reduced glutathione (GSH) levels. Transcriptomic analysis was performed to detect differentially expressed genes (DEGs). Functional enrichment analyses were conducted using gene ontology (GO) and kyoto encyclopaedia of genes and genomes (KEGG) pathways. Key genes were validated by quantitative reverse transcription polymerase chain reaction (qRT-PCR).

Result: Accumulation of PE-MPs was detected in liver tissue. Histological evaluation revealed dose-dependent liver injury, including inflammatory infiltration and periportal fibrosis. Elevated MDA levels indicated increased oxidative stress via lipid peroxidation (LPO). qRT-PCR investigation confirmed the upregulation of mitochondrial dysfunction-related genes (*NDUFC*, *UQCRC*, *MT-CO₂*), pro-inflammatory genes (*TNF- α* , *CXCL1*, *IL-1 β*) and fibrosis markers (*IL-6*, *Col1A1*, *α -SMA*). Transcriptomic analysis identified 189 DEGs linked with oxidative stress, inflammation and lipid metabolism pathways. Altogether, PE-MPs exposure induces oxidative stress, mitochondrial dysfunction, inflammation and fibrosis, contributing to non-alcoholic fatty liver disease (NAFLD) like hepatic alterations. This highlights the potential risk of PE-MPs as emerging environmental threats to liver health.

Key words: FTIR, Lipid peroxidation, MPs, NAFLD, Polyethylene microplastics, Transcriptome-seq.

INTRODUCTION

Globally, the issue of microplastic (MP) contamination is becoming more and more problematic. By 2050, global plastic production is predicted to reach 33 billion tons, a sharp rise from 1.5 million tons in 1950 and 400 million tons in 2020 (Lei *et al.*, 2024). MPs are composed of multiple polymer categories, specifically polyethylene terephthalate (PET), polypropylene (PP), polyethylene (PE), polyvinyl chloride (PVC) and polystyrene (PS). MPs are accumulated from birth and continue to accumulate throughout life (Braun *et al.*, 2021). Faecal samples from adults and newborns contained MPs (Wen *et al.*, 2022). Furthermore, both humans and animals have demonstrated that microplastics disrupt the intestinal barrier, enter the bloodstream and accumulate in organs, including the gastrointestinal tract, kidneys, liver and brain (Deng *et al.*, 2017a).

The liver is an essential organ accountable for detoxification and metabolism, making it a primary target for microplastic (MP) accumulation. MPs can cause liver damage, disrupt digestive functions, impair bile acid secretion and interfere with metabolic processes, leading to serious health concerns (Hou *et al.*, 2024). Recent studies have documented hepatotoxicity and disruptions in lipid metabolism associated with MPs, induced oxidative stress and inflammation (Chiang *et al.* 2024). Humans consume

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MPs every day through food, drink and the air. We previously reported that MPs were present in bottled drinking water, with adults consuming around 883 and children consuming 553 particles per capita per day, respectively (Patil *et al.*, 2024). The incidence of MPs in the airborne was also reported by our group earlier (Narmadha *et al.*, 2020). The assessment of MP-induced hepatotoxicity has been

investigated, with particular emphasis on PS-MPs (Hamza *et al.*, 2023). However, the specific impact of PE-MPs on liver toxicity is highly elusive. In this study, we evaluate the impact of oral doses of PE-MPs administered to wistar rats (0.1, 1 and 5 mg/kg/day) serving as the independent variable on dependent variables such as oxidative stress biomarkers, histopathological changes (inflammation, fibrosis) and differential gene expression profiles in exposed liver tissue. Therefore, the main objective of present study to identify accumulation of PE-MPs in liver tissue, potential dose-dependent biological responses, mechanistic pathways contributing to liver dysfunction and NAFLD-like alterations.

MATERIALS AND METHODS

Microplastic (MP) particles

MPs Nanochemazone supplied the High-Density Polyethylene (HDPE)-MPs that had diameters between 1 to 10 μm (CAS No 9002-88-4, Canada).

Rats and experimental protocols

The study was conducted at Priyadarshini J.L. College of Pharmacy, Nagpur, under ethical approval protocol number PJLCP/2021-2022IAEC/36, dated November 29, 2021, granted by the Institutional Animal Ethics Committee of Priyadarshini J.L. College of Pharmacy. 24 male Wistar rats were chosen to avoid variability caused by estrous cycle. Rats were housed in a 12-hour light/dark cycle, at a temperature of $22\pm3^\circ\text{C}$ and with humidity levels ranging from 55% to 65%. Animals were randomly divided into four groups (6 animals in each group, as per IAEC and CPCSEA guidelines to minimize animal use): Group 1 (control) received corn oil along with food and water free from microplastics, groups 2 received 0.1 mg/kg/day, group 3 received 1 mg/kg/day and group 4 received 5 mg/kg/day dosage of PE-MPs per day for 28 days. The dose range (0.1, 1 and 5 mg/kg/day) and 28-day exposure period were selected based on prior microplastic toxicology studies and OECD guidelines to represent environmentally relevant, moderate and high exposures and to allow for detection of early hepatic alterations (Lu *et al.*, 2018b; Patil *et al.*, 2024; Djouina *et al.*, 2023). On day 29, the rats were anesthetized and subsequently euthanized, organ removed and kept at -80°C for further analysis.

MP accumulation in liver tissue digest

Preserved organs (0.1 g) were digested following the given protocol (Deng *et al.*, 2017b). Nile Red (lipophilic dye that binds to plastic polymers) staining was used to evaluate PE-MPs in liver tissue digest. Using glass microfiber filter paper (Whatman, Cat. No. 1822-047), the liver tissue digest was vacuum-filtered. Each filter paper was stained with 2-3 drops of Nile Red dye (HI-MEDIA, TC707) and incubated for 10 minutes at 60°C to dry. Fluorescence imaging of the filter paper was performed using an Olympus BX51 microscope to determine the presence of the accumulated PE-MPs. Subsequently, Liver tissue digests were filtered and analyzed using ATR-FTIR analysis. PE-MPs showed

characteristic peaks: CH_2 stretching at 2913 and 2847 cm^{-1} , in-plane bending at 1462 and 1377 cm^{-1} and CH_2 rocking at 717 cm^{-1} (Narmadha *et al.*, 2020).

Direct visualization of MP in the liver

The PHAD method was employed to directly visualize PE-MPs in the liver sections (Marinho and Hanscheid, 2023).

Biochemical analyses of liver sample

A 10% homogenate was made from rat liver samples in phosphate buffer saline (PBS) and the supernatant was extracted by centrifuging the samples at $2665g$ for five minutes at 4°C (Lohiya *et al.*, 2017). Commercially available kits were employed to detect oxidative stress biomarkers following the manufacturer's guidelines such as reactive oxygen species (ROS), superoxide dismutase (SOD; HIMEDIA), malondialdehyde (MDA, HIMEDIA), catalase (CAT), glutathione S-transferase (GST, HIMEDIA) and total antioxidant capacity (TAC; HIMEDIA). All assays were carried out in triplicate.

Histopathology in liver tissue

Liver tissues from control and treated groups were fixed in formalin and sectioned using a microtome. Hematoxylin and eosin (H and E) staining was performed on $5\text{ }\mu\text{m}$ -thick sections for histopathological characterisation (Deng *et al.*, 2017b). Brightfield images were taken with Olympus BX51 microscope (Ravikumar *et al.*, 2022). Leukocyte infiltration and fibrotic score were analysed with Image J software.

qRT-qPCR analysis

Total RNA was extracted using TRIzol reagents (Ambion, Cat. No. 15596018). Reverse transcription was performed using a cDNA synthesis kit (Applied Biosystems, Cat. No. 4368814). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Wang *et al.*, 2023) served as the housekeeping gene and the primer sequences are accessible in Table 1.

Transcriptome investigation and differential expression genes expression

Liver samples were from control and higher doses (5 mg/kg/day) group was analysed for multiplex transcriptome sequencing. The total RNA was isolated, purified using TRIzol method and Nucleospin RNA kit. KAPA HyperPrep kit was used for synthesis, amplification and purification of cDNA. The cDNA library was measured using a bioanalyzer and transcriptome sequencing was achieved on the Illumina NovaSeq 6000 platform. All differentially expressed genes were plotted using Enhanced Volcano and a Venn diagram showed the distinct and frequently expressed transcripts. Using ClusterProfiler, detailed GO tree diagrams and quality visuals were created and GO and pathway enrichment analyses revealed key biological mechanisms (Love, Huber and Anders 2014).

Statistical analysis

The data were expressed as mean $\pm$ standard deviation (SD). Student t-test (a non-parametric) was performed

using Prism 8 (Graph pad software). P-value <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

PE-MPs accumulation in liver tissues

PE-MPs were detected in the livers of the experimental groups, whereas no such accumulation was observed in

the control group. Nile red staining revealed the presence of PE-MPs in liver tissue sections (Fig 1A). A clear progressive accumulation of PE-MPs with a concentration-dependent pattern was observed across the experimental groups (Fig 1B), consistent with previous findings on MP biodistribution in mammalian organs (Deng *et al.* 2017a). Additionally, ATR-FTIR analysis of the liver tissue extracts further confirmed the occurrence of PE-MPs (Fig 1C). The liver sections were examined using the PHAD method showed the occurrence of PE-MPs in the liver tissues of exposed rats. These observations confirmed an increase in hepatic deposition of PE-MPs in a dose-dependent manner (Fig 1D-E), suggesting a proportional relationship between exposure level and bioaccumulation. These results not only confirm hepatic uptake of ingested PE-MPs but also align with earlier studies on tissue retention and distribution of MPs, reinforcing the liver's role as a primary accumulation and detoxification site.

Histopathological analysis of liver tissues

PE-MPs accumulation in hepatic tissue induced a gradual increase in multifocal centrilobular necrosis and inflammation with increase in dosage of PE-MPs. Compared to the controls, livers from PE-MPs-treated rats also showed notable Leukocyte infiltration and periportal fibrosis (Fig 2A). These histological changes were supported by quantitative morphometric data, which demonstrated significant increases in neutrophil infiltration and fibrotic scores with escalating PE-MP dosages (Fig 2B). This aligns with earlier observations reported by (Deng *et al.*, 2017a) where fluorescent polystyrene microplastics (PS-MPs) were shown to accumulate in mouse liver and induce oxidative damage and inflammation, laying a foundational understanding of microplastic-induced tissue toxicity. The dose-dependent progression of fibrosis and inflammation observed in our study is consistent with earlier work by (Lu *et al.*, 2018b) who demonstrated that PS-MP exposure in mice disrupted hepatic lipid metabolism and promoted hepatic steatosis and inflammation. Similarly, (Zhao *et al.*, 2021) found that chronic PS-MP ingestion triggered immune cell activation and natural killer cell infiltration in the liver, contributing to fibrosis *via* immunopathological pathways.

Evaluation of oxidative stress by biochemical assay

Liver tissue extracts from rats were analyzed for ROS levels following PE-MP exposure. No significant changes in ROS levels were detected across any of the exposed doses after 28 days ($p>0.05$) (Fig 3A), possibly due to the inherently unstable nature of ROS (Andrés Juan *et al.*, 2021). The PE-MP-exposed groups exhibited significantly higher MDA levels, reflecting enhanced LPO compared to the control group ($p<0.05$) (Fig 3B). Dose-dependent increase GST activity was showed on PE-MP exposure ($p<0.05$) (Fig 3C). Furthermore, significant increases in SOD, catalase CAT and total antioxidant capacity were observed in high exposure group (5 mg/kg/day) (Fig 3D-F). At low doses of experiments groups (0.1 mg/kg/day and 1 mg/kg/day), no

Table 1: RT-qPCR primer sequence.

Gene special primers used in the real-time quantitative reverse transcription PCR

Gene name	Primer sequence (5'3')
Gapdh	F- CCAGCCCAGCAAGGATACTG R- CCCCTCCTGTTGTTATGGGG
Rpl12	F- GAAAGCTGGCCTTTTGTGTC R- CCCCTTCTCGTTGCTTCTTA
Lcn2	F- CTGATGGTTCAGGTCCCACC R- TGCTTGGTGAATCATGGCT
Aunip	F- GAGCCGTCTAATACAGCCCC R- ATCTGCCACACAGAATGCCA
Ccnb1	F- CCCTCCCCTTGCATTCTCAA R- AGGTAACAGGGGTCAAGTCT
Ccna2	F- GCTCCTCGCAGCTCTGAAAA R- ATGGTCAGCTCTGGTAGCAA
Xbp1	F- AGGGGCTGTCTTCCTTAT R- ATCATTCAACGGGGATGCTG
Uqcrh	F- GCACTGTGAGCAGTTGGAGA R- GAAGACACGCGCCTATCACA
Uqcrb	F- CAAATGGCTGGATGGTTTCCG R- CATGCATTGTGTCATCTCGCA
Mt-co2	F- CTCATGAGCCGTCCCTTCAC R- AAGCTGTGATTGAGCCGCA
Ndufc	F- CACTCATGAGCCGTCCCTTC R- AGACCTGGTCGGTTTGATGTC
COL1A1	F- TACTTCCCTACCCAGCACCT R- ATGTGTGGCCGATGTTTCCA
α -SMA	F- GTGTGAAGAGGAAGACAGCAC R- GTGATGATGCCGTGTTCTATCG
CXCL1	F- CTAGAAGGTGTTGAGCGGGA R- GTCCAAGGGAAGCTTCAACAC
IL-6	F- GCAAGGTGTTTGCTGAAGGG R- ACGGCTTCGTAGCTTTGTA
IL-8	F- GTG CAG TTT TGC CAA GGA GT R- TTATGAATT CTC AGC CCT CTT CAAAAA CTT CTC
IL-1 β	F- TCCTCTGCCAAGTCAGGTCT R- CAGGGAGGGAACACACGTT
IL-10	F- ACGAATGCCAGTAGACCACC R- GGGAAGCTGGCTGACACAAA
ICAM-1	F- TGAAGGGGCACAACAGAGTC R- TTGAGGAAAGCACCCCTGACC
TNF- α	F- ACAGCAACTCCAGAACACCC R- GGCCAGTGTATGAGAGGGAC

notable changes were detected in CAT, SOD, or total antioxidant levels (Fig 3D-F). The increase in MDA levels confirms ROS involvement. Djouina *et al.* have shown elevated levels of MDA in mice exposed to PE-MPs, which is further responsible for aggravating liver dysfunction (Djouina *et al.*, 2023). These data corroborate earlier reports by Lu *et al.* (2018c) and Zhao *et al.* (2021) who documented similar upregulation of antioxidant defenses in response to polystyrene microplastic (PS-MP) exposure, suggesting a conserved cellular adaptive mechanism across microplastic types.

Transcriptomic analysis, GO and KEGG pathway analysis of DEGs

Fan *et al.* 2022 reported 293 upregulated and 351 downregulated genes in mice livers after 20 weeks of PS-

MPs ingestion. Wang *et al.* (2022) observed 69 DEGs gene (low-exposure dose) and 178 (high-exposure dose), with a mix of upregulated and downregulated genes. To explore transcriptional alterations, transcriptome sequencing was performed on liver tissues. A higher dose 5 mg/kg/day PE-MPs were chosen to investigate the molecular mechanisms of liver toxicity following 4 weeks of exposure. The analysis identified 162 differentially expressed genes (DEGs) compared to the control group, comprising 59 down regulated and 103 upregulated genes ($|\log_2FC| > 0$), as depicted in the volcano plot (Fig 4A). A heat map of the top 50 DEGs further illustrates the gene expression changes in 5 mg/kg/day group (Fig 4B). Transcriptomic data, validated by five randomly chosen DEGs, selected for quantitative PCR (qPCR) which includes three down regulated genes (CCNB1, CCNA2 and AUNIP) and two

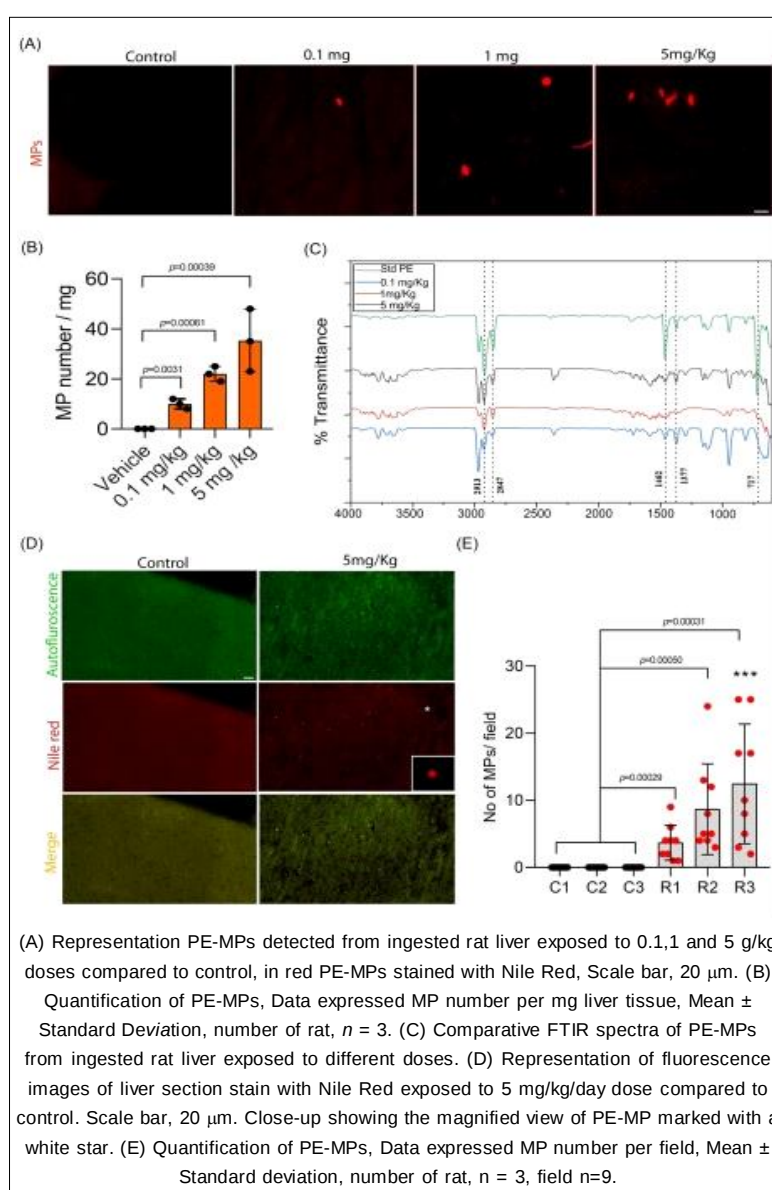


Fig 1: Accumulation of PE-MPs in rat liver tissue.

upregulated genes (LCN2 and RPL12). The qPCR results corroborated the sequencing findings, supporting the accuracy and reliability of the transcriptomic analysis (Fig 4C-D). KEGG pathway analysis of DEGs showed alterations in lipid metabolism pathways, including prolactin signaling, alcoholic liver disease, PPAR signaling, NAFLD, retinol metabolism and drug metabolism. Gene ontology (GO) annotation further highlighted enrichment in pathways related to cell cycle suppression and negative regulation of apoptosis. Additionally, GO analysis pointed to mitochondrial involvement, with enrichment in oxidative stress-related processes, including mitochondrial

transport chains and electron transport functions. These findings suggest that PE-MP exposure disrupts mitochondrial function and lipid metabolism as part of the liver's adaptive response. Among the top 50 DEGs, several key genes, such as RGD1565355, Hsd17b13, Lpin1, (CD36-like), Car3, Spc25, Xbp1, Pdk4 and Sgms2, were associated with lipid metabolism processing pathways implicated in NAFLD. Analysis of transcriptomic profiles alongside KEGG pathway mapping indicated NAFLD pathway activation in PE-MP-treated liver samples. Given that LPO emerged as a major contributor to liver injury, we further validated its upstream and downstream effects.

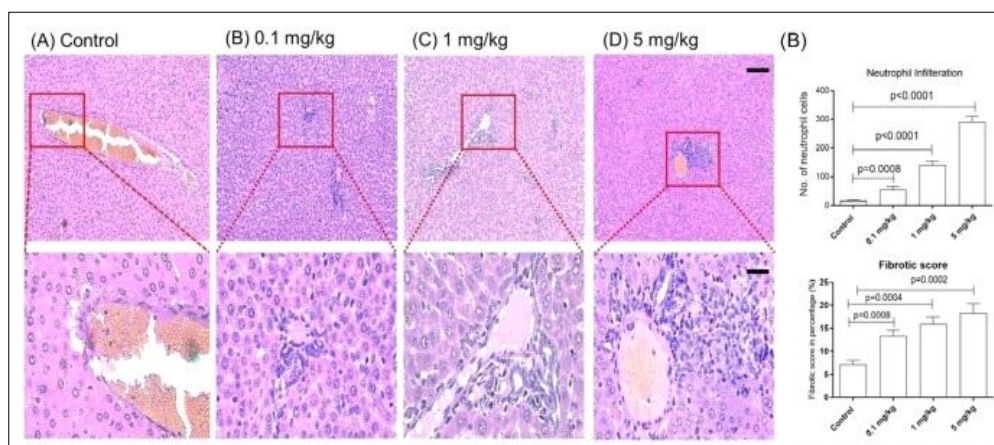


Fig 2: (A) Representative images of H and E-stained liver sections from rats exposed for 28 days. Control group shows normal histopathology of liver but experimental group shows notable gradual increase in neutrophil infiltration and periportal fibrosis with increasing dosages. Upper panel, 10X Scale bar, 50 µm and lower panel with inset 40X Scale bar, 10 µm. n = 6 rat/group; 2 section/slide. (B) Quantification of neutrophil infiltration in liver sections of rats treated with increasing doses of PE-MPs (0.1, 1 and 5 mg/kg/day) for 28 days. (B) Fibrotic score expressed as percentage area affected in liver tissue, demonstrating a progressive increase in fibrosis severity with higher PE-MP exposure. Data are expressed as mean ± SD. rat n = 6 per group.

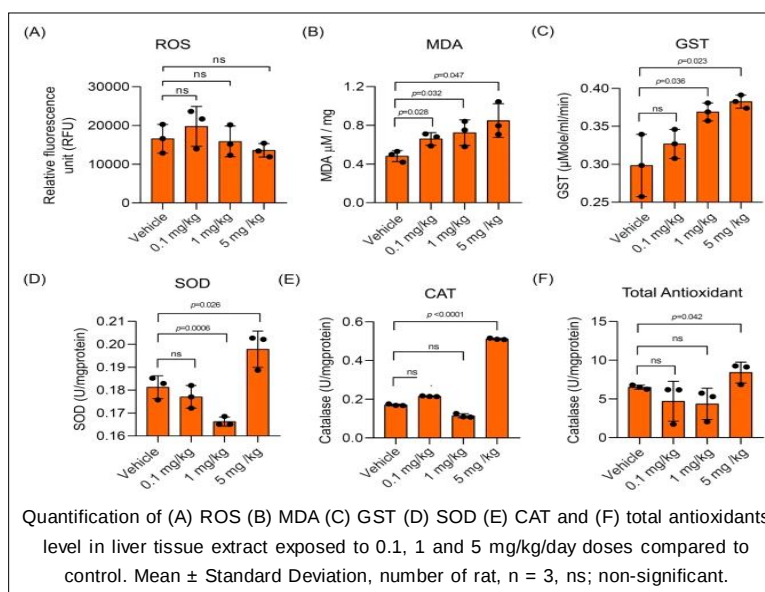


Fig 3: Evaluation of oxidative stress and anti-oxidant markers.

Activation of the NAFLD pathway

Exposure to 5 mg/kg/day PE-MPs led to transcriptomic changes affecting lipid metabolic processes and NAFLD-associated pathways, with evident mitochondrial participation. We suggest that mitochondrial impairment drives LPO, leading to inflammatory responses, neutrophil infiltration and fibrosis characteristic of NAFLD. To verify this observation, qPCR analysis showed a significant upregulation of mitochondrial dysfunction-related genes *UQCRRH*, *NDUFC* and *MT-CO₂* in 5 mg/kg/day group compared to controls (Fig 5A). qPCR validation showed a surge in mRNA levels of *IL-1 β* , *CXCL1* and *TNF- α* key markers of neutrophil infiltration and inflammation involved in NAFLD in the high exposure 5 mg/kg/day PE-MPs group (Fig 5B). Additionally, fibrosis-related genes (Liu *et al.*, 2021) *IL-6*, *α -SMA* and *Col1A1*, were also significantly

upregulated (Fig 5C). Elevated expression of *CXCL1* facilitates the transition from hepatic steatosis to steatohepatitis by increasing oxidative stress and promoting neutrophil infiltration. *IL-1 β* expression is crucial in the transition from steatosis to NASH and fibrosis, mediated by NLRP3 inflammasome pathway. Experimental data suggest that MPs, including PE-MPs, stimulate pro-inflammatory cytokines expression such as *IL-1 β* , *TNF- α* and *CXCL1*, thereby aggravating hepatic inflammation and promoting NAFLD (Musso *et al.*, 2018). These transcriptomic and qPCR findings are consistent with prior studies using other microplastic types such as PS-MPs, which have similarly demonstrated disruption of mitochondrial bioenergetics, inflammation and fibrogenesis in murine models (Fan *et al.*, 2022; Wang *et al.*, 2022).

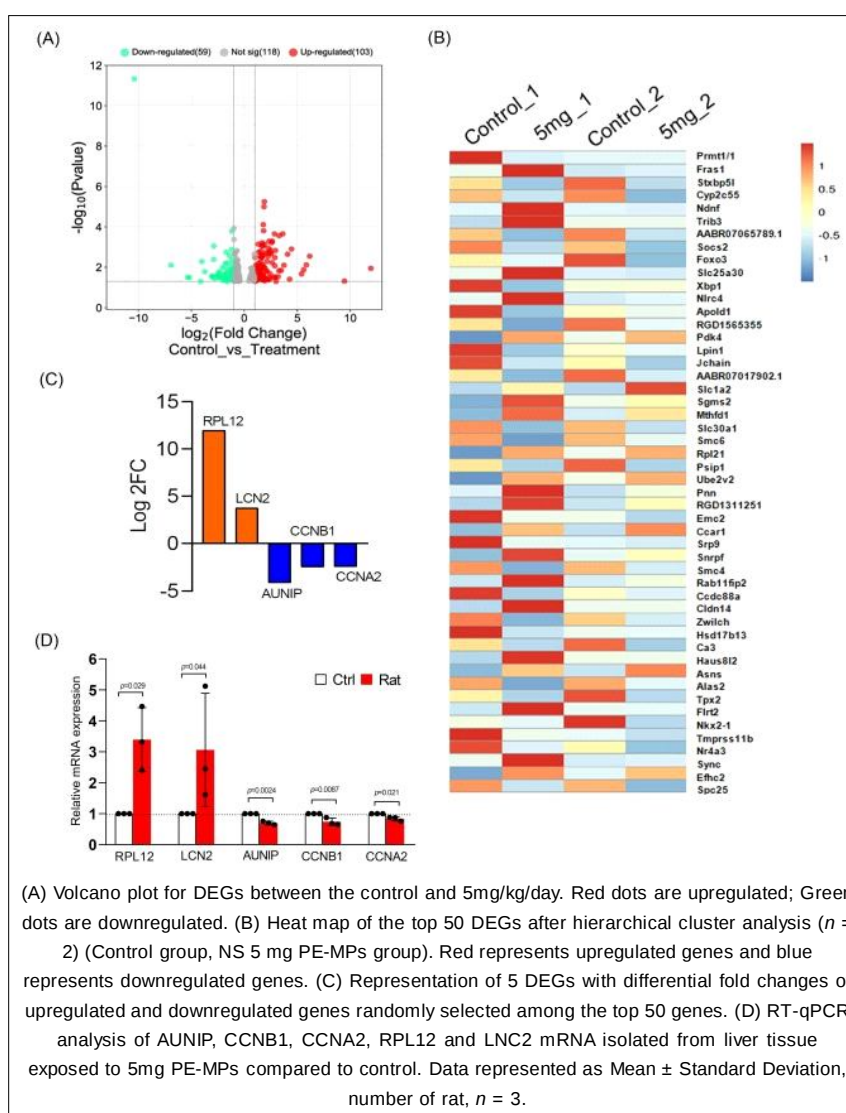


Fig 4: Transcriptomic analysis.

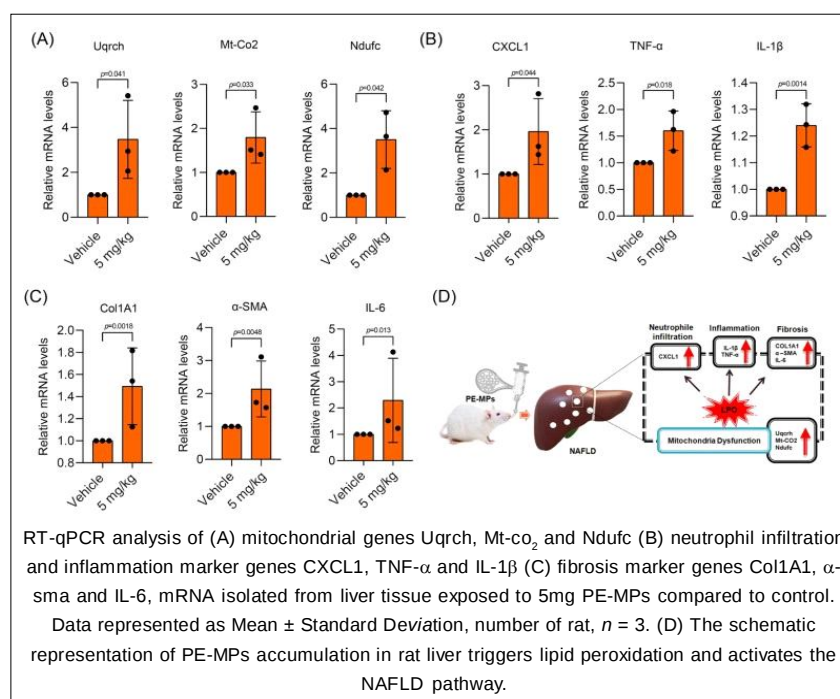


Fig 5: NAFLD pathway activation leads to neutrophil infiltration, inflammation and liver fibrosis.

CONCLUSION

In conclusion, this study demonstrates that PE-MPs have hepatotoxic effects in the Wistar rat models. The comprehensive analysis includes PE-MPs accumulation, biochemical profiling, histopathological examination and NAFLD pathway evaluation. We sense a clear association between PE-MPs exposure and liver toxicity. These findings suggest that PE-MP accumulation induces LPO, leading to mitochondrial dysfunction, inflammation and fibrosis, thereby activating the NAFLD pathway. Although this research primarily focuses on the hepatotoxicity and NAFLD pathway, future studies should investigate PE-MPs toxicity in other organs.

Conflict of interest

All authors declared that there is no conflict of interest.

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