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Acute *In Vitro* Effects of Polystyrene Microplastics on Cell Viability, Oxidative Status, and DNA Integrity in Zebrafish Erythrocytes

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Simple Summary

This study examined the detrimental impact of polystyrene microplastics on zebrafish erythrocytes. The use of nucleated erythrocytes as a cellular model to investigate cellular mechanisms triggered by potentially harmful substances is supported by numerous studies demonstrating their high sensitivity to oxidative stress and their ability to respond in a measurable way to toxic stimuli. The results revealed a time-dependent decrease in genomic stability, accompanied by increased DNA fragmentation and reactive oxygen species production in treated erythrocytes. These molecular alterations were observed in parallel with reduced cell viability, suggesting a potential ability of these particles to interfere with and damage genetic material.

Abstract

In recent decades, intensified human activities, including industrialization and urban expansion, have given rise to significant environmental challenges, with plastic pollution emerging as a critical concern. Microplastics, particularly those derived from polystyrene (PS-MPs), are widespread in soils and oceans, posing risks to biodiversity and human health through ingestion, inhalation, and contact. This study examined the detrimental impact of PS-MPs on Zebrafish-*Danio rerio* (Hamilton, 1822) erythrocytes. The zebrafish (*Danio rerio*) is a well-established model organism in biomedical research, whereas its erythrocytes served as the cellular model investigated in this study. *In vitro* experiments involved exposing zebrafish erythrocytes, isolated from 25 adult zebrafish, to 105 µg/mL of PS-MPs for 30, 60 and 90 min to assess cytotoxicity, genotoxicity and oxidative stress. The results revealed a time-dependent and statistically significant reduction in erythrocyte viability (−30% to −50%), genomic stability (%GTS −20% to −40%), and a concomitant increase in DNA damage (DFI ~30–38%) and ROS production (~27–35%) compared to controls (all $p \leq 0.05$). These molecular disruptions related to reduced cell viability, indicating that PS-MPs are cytotoxic and genotoxic, causing genetic damage and oxidative imbalance. The results obtained confirmed the toxicity of PS-MPs. These data suggest that PS-MPs can interfere with genetic material by inducing apoptosis through oxidative imbalance.

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1. Introduction

Environmental pollution is defined as the introduction of chemical, physical, and biological substances into the environment, primarily resulting from human activities such as industrialization (greenhouse gas emissions, chemical discharges, industrial waste), transportation (exhaust emissions and smog), agriculture (use of pesticides, fertilizers, intensive farming), and waste management (improper disposal of urban and special waste) [1]. A significant yet often overlooked aspect of global pollution is the contamination of aquatic environments, which has multiple and varied direct and indirect impacts on human health. Around 90% of pollutants produced are transported by rivers to the sea, adding to the direct releases associated with the fact that 70–80% of the world's population live in coastal areas [2].

Of the many contaminants causing great concern, plastics certainly play a prominent role. In fact, microplastics from 1 μm to 5 mm are listed as one of the four main global environmental threats alongside ocean acidification, ozone depletion, and climate change [3]. Microplastics infiltrate the food chain, accumulate in organs, and cause cellular and molecular damage. These effects pose significant risks to biodiversity, aquatic life and human health. Therefore, addressing the impact of microplastics is just as important as tackling climate change to protect the environment and public health [4].

The degradation of plastics occurs mainly through physico-chemical processes, such as exposure to ultraviolet light, physical abrasion and oxidation. These processes are extremely slow and inefficient, and do not lead to the complete mineralisation of the material. As a result, plastics fragment into small pieces, which can persist in the environment indefinitely [5]. Plastic fragments are classified based on their size resulting from degradation into nanoplastics (<1 μm), microplastics (1 μm–5 mm), mesoplastics (0.5–2.5 cm), and macroplastics (>2.5 cm) [6].

MPs are attracting scholarly attention due to their widespread abundance and distribution and their impact on natural ecosystems, and the health risks they pose to humans [7,8].

Polystyrene (PS) is a plastic polymer known for releasing MPs into the environment. PS is soluble in organic solvents such as ketones, esters, and aromatic hydrocarbons. Its resistance to acids, alkalis, salts, oils, and alcohols makes it ideal for durable applications such as transparent food packaging and laboratory equipment [9]. Expanded PS is an excellent thermal insulator used in construction and appliances, and its strength, chemical stability, large surface area, and low cost explain its widespread use [10]. While PS is not a major plastic pollutant in marine ecosystems as most environmental debris originates from other types of plastic, it remains one of the most extensively studied polymers due to its associated ecological risks [11]. Environmental weathering transforms PS into microplastics (PS-MPs), increasing their surface area, porosity and amorphous structure, thereby enhancing their capacity to absorb other contaminants [12]. Due to their low density, PS-MPs float and are easily transported by surface seawater, spreading throughout marine environments and accumulating in organisms such as mussels, other invertebrates, and fish, thereby causing a broad spectrum of detrimental effects [13–15]. Once internalized, MPs can be transported to different organs and exert multiple lethal and sublethal effects on a wide range of aquatic and terrestrial organisms, from invertebrates to vertebrates, due to their sensitivity to MPs [4–8].

Although the Zebrafish-*Danio rerio* (Hamilton, 1822) model has already been used to evaluate the environmental toxicity of microplastics (MPs), our understanding of the underlying molecular mechanisms remains incomplete [16,17]. In particular, the specific effects of polystyrene microplastics on cellular parameters such as genomic stability, reactive oxygen species (ROS) production, and apoptosis induction have not yet been fully elucidated.

In this study, we analyzed the impact of PS-MPs on zebrafish erythrocytes *in vitro*. The use of nucleated erythrocytes as an experimental model to investigate cellular mechanisms triggered by potentially harmful substances is supported by numerous studies demonstrating their high sensitivity to oxidative stress and their ability to respond in a measurable way to toxic stimuli. Nagasaka et al. [18] analyzed the response of nucleated erythrocytes from common carp exposed to the free radical initiator AAPH, observing lipid peroxidation and membrane alterations, thus confirming their usefulness in studying oxidative damage. Similarly, Kramer et al. [19] showed that nucleated erythroid cells from zebrafish (gata1:DsRed+) produce high levels of ROS in response to pro-oxidants such as 1-naphthol, highlighting their high sensitivity to oxidative stress. Comet assays on *Cyprinus carpio* erythrocytes, which are used to measure DNA damage induced by pharmaceuticals and chemicals, demonstrate that these red blood cells are excellent indicators for monitoring the genotoxic impact of environmental contaminants [20]. These findings support the use of nucleated erythrocytes, including those from zebrafish, as an effective biological system for evaluating the toxicity of environmental contaminants, such as MPs, at the cellular and molecular levels. Zebrafish is also a particularly advantageous animal model: it is a small-sized vertebrate, easy to maintain in the laboratory, with well-characterized physiological and molecular features [21].

By integrating cellular and molecular endpoints, our study enables a more detailed investigation of the mechanisms of MP-induced toxicity and provides new data that contribute to expanding our understanding of the cellular and molecular effects induced by these particles in a biologically relevant genetic model. Specifically, we investigated whether exposure to PS-MPs induces DNA damage and reduces cell viability by comparing the recorded effects to those caused by hydrogen peroxide (H_2O_2), a well-established genotoxic agent.

Together, these techniques provide a more comprehensive view of genotoxicity, capturing both specific apoptotic effects and general genome instability.

A single PS-MP concentration of 105 μg/mL was selected to obtain preliminary insights into the biological and genotoxic effects of PS-MPs and to establish a baseline response before designing more extensive experiments. This concentration was selected based on previous *in vitro* studies conducted on different experimental models, which employed comparable or even higher exposure levels to investigate microplastic-induced cellular responses [22–24]. Elevated exposure concentrations represent a useful experimental approach to induce measurable biological effects and characterize specific cellular endpoints, including genomic stability, DNA fragmentation, ROS production, and apoptosis.

Although the selected concentration exceeds commonly reported environmental levels (<0.05 μg/mL), its use is justified by the characteristics of *in vitro* systems, which do not fully reproduce the physiological mechanisms that may modulate or compensate for microplastic effects *in vivo*. Therefore, higher concentrations are frequently applied under controlled laboratory conditions to facilitate the detection of cellular responses and to investigate the underlying toxicological mechanisms of microplastic exposure. This approach provides valuable information for understanding potential biological effects and supporting risk assessment evaluations.

2. Materials and Methods

2.1. Chemistry

The study employed polystyrene microplastics with a nominal diameter of 1.0 µm, a specific gravity of 1.05 g/cm^3 and a solid content of 2% (*w*/*w*). The particles were supplied by Sigma-Aldrich (St. Louis, MO, USA; product code 72.938; batch BCCH6977) as a concentrated aqueous suspension of 21,000 mg/L, corresponding to approximately 3.43×10^{10} particles per mL, and certified as an analytical-grade standard. The particle suspension was prepared immediately before exposure by dilution in ultrapure water and briefly sonicated to reduce aggregation.

2.2. Specimens and PS-MPs Exposure

Adult wild-type zebrafish (*Danio rerio*) were obtained from a commercial supplier and used for blood collection. No transgenic or mutant lines were used in this study. Adult wild-type *Danio rerio* (zebrafish) specimens sourced from a fish farm were housed at the Department of Biology, University of Naples Federico II. A total of 25 adult zebrafish (approximately 4 months old), with a mean body weight of 0.4556 ± 0.007 g and a mean total length of 3.49 ± 0.017 cm, were used in this study. The fish were kept in an 80 L tank with optimal conditions mimicking natural growth and were acclimated to laboratory conditions for 1 month. Water quality parameters were monitored daily and maintained at the following values: a water temperature ≥ 25 °C, dissolved oxygen = 7.78, pH 7.2, a 14 h light/10 h dark photoperiod, and commercial feed. For the *in vitro* experiments, the fish were gently positioned on a moist support without distinguishing sex, and the lateral branchial vessel was exposed. Blood was collected by puncturing the superficial vasculature in the anterior region of the fish using a fine 29 G needle and promptly transferred into microtubes containing anticoagulant and gently mixed to prevent clotting. The volume of blood collected did not exceed ~2% of body weight per fish, corresponding to ~6–10 µL of blood per individual. No mortality was observed following the procedure. This procedure was adapted from previously described minimally invasive blood sampling methods in small teleosts, allowing collection of small blood volumes while minimizing tissue damage and ensuring the survival of the fish [25,26].

Cells from each individual fish were processed separately and considered as independent experimental units. Erythrocytes were suspended in an anticoagulant solution (sodium citrate), and the final cell concentration was adjusted to approximately 1×10^6 cells/mL, using a Neubauer counting chamber (BRAND GmbH + Co. KG, Wertheim, Germany), for *in vitro* assays. To minimize stress, we employed gradual cooling (12–17 °C), an approved and widely used anesthetic method in zebrafish research that ensures a non-invasive and ethically acceptable procedure. The fish were then transferred to a container, and the temperature was gradually reduced to ~12 °C over several minutes. Anesthesia was confirmed by cessation of movement, reduced opercular activity, and lack of response to handling. Immediately after the procedure, the fish were transferred to a system containing water at 28.5 °C for recovery. Each sample was handled gently and individually to further reduce handling stress [27].

Blood samples collected from 25 zebrafish were randomly assigned to five independent biological pools, each consisting of blood from five individual fish, to obtain a sufficient volume for the *in vitro* experiments. Each pool was considered an independent biological replicate. The pooled blood samples were centrifuged at 3200 rpm for 5 min, and the erythrocyte fraction was isolated, resuspended in PBS 1×, and adjusted to a final concentration of approximately 1×10^6 cells/mL. The erythrocyte suspensions obtained from each biological pool were then divided into equal aliquots and assigned to the different experimental conditions. Aliquots derived from the same pool were considered technical

replicates and were not treated as independent biological samples for statistical analysis. Cells were exposed to PS-MPs (105 μg/mL) for 30, 60, and 90 min. Negative controls consisted of erythrocytes exposed to the same volume of PBS 1× used as vehicle, while positive controls were treated with H_2O_2 (1 μL/mL) for the corresponding exposure times. Hydrogen peroxide was selected due to its well-established genotoxic potential, documented in multiple *in vitro* and *in vivo* models. It has indeed been shown to induce DNA damage and chromosomal aberrations in bacterial and mammalian cells *in vitro*, as well as genotoxic and cytotoxic effects in zebrafish embryos [28,29]. The exposure solution was prepared by diluting the hydrogen peroxide stock solution (3% *w*/*v*, provided by the manufacturer) in ultrapure water to obtain the final working concentration.

For the experimental exposure, a concentration of 105 μg/mL of PS-MPs was used because this value was selected based on previous *in vitro* studies conducted on different experimental models [22–24]. In particular, this concentration was chosen to ensure the detection of specific endpoints, including genomic stability, DNA fragmentation, reactive oxygen species (ROS) production, and apoptosis.

Exposure times of 30, 60, and 90 min were selected to capture early and dynamic cellular responses to PS-MPs, in line with standard acute *in vitro* toxicity assays [30,31]. Each treatment time included negative and positive controls. Short-term exposures ranging from a few minutes to several hours are commonly employed in erythrocyte studies investigating oxidative and genotoxic stress [32,33]. Furthermore, a recent literature review reported that *in vitro* studies on the acute toxic effects of microplastics typically employ short exposure times to evaluate early cellular responses [34]. These precedents validate our choice of an exposure window of 30–90 min to evaluate acute oxidative and genotoxic effects *in vitro.*

Each experimental group contained a total of 10^6 cells per tube, and each condition was performed in triplicate. This setup aimed to assess the effects of PS-MPs exposure over time, ensuring animal welfare and reliable results when evaluating potential cytotoxicity and genotoxicity [35]. After exposure, the samples were centrifuged at 2000 rpm for 5 min. The pellet was then recovered and suspended in PBS 1X. The methods for assessing the cytotoxic and genotoxic activity of PS-MPs (viability test, RAPD-PCR technique, TUNEL test, NBT test) were then performed.

The handling of animals and the experimental procedures were in accordance with local regulations and international guidelines for the use and care of laboratory animals. They were approved by the Italian Ministry of Health (Legislative Decree No. 26 of 4 March 2014—"Implementation of Directive No. 2010/63/EU on the protection of animals used for scientific purposes") and received approval from the Italian Ministry of Health (Permit No. 529/2019-PR, 19 July 2019). Efforts were made to minimize both animal suffering and the number of samples used.

2.3. Viability Test

Cell viability was assessed using Trypan Blue staining, as previously described [36]. The slides were pre-treated with methanol, after which 10 μL of cell solution (containing approximately 1×10^4 cells) was mixed with 10 μL of a 0.4% Trypan Blue solution and applied to the slides. The slides were then observed immediately under an optical microscope (ZEISS Axioskop microscope (Carl Zeiss AG, Oberkochen, Germany)) at 60× magnification in order to perform a viable versus non-viable cell count. For this viability test, slides were prepared in triplicate for each PS-MP exposure time, as well as for the negative and positive controls. A total of 200 cells were counted per slide, and three independent slides were analyzed for each experimental condition, resulting in 600 cells per condition.

2.4. RAPD-PCR Technique

The RAPD-PCR method was adapted from that described by Rong and Yin [37], who successfully applied it to the zebrafish genome. To correctly execute the RAPD-PCR technique, DNA was extracted from zebrafish erythrocytes using a High Pure PCR Template Preparation Kit (Roche Diagnostics® GmbH, Mannheim, Germany), following the manufacturer's protocol. Genomic DNA was amplified in a total reaction volume of 25 µL using FastStart™ PCR Master (Roche Diagnostics® GmbH, Mannheim, Germany), 40 ng of genomic DNA and the P6 primer (5′-CCCGTCAGCA-3′; 10 µM stock solution) [35]. RAPD-PCR amplification was performed using 45 cycles, preceded by an initial denaturation step at 95 °C for 5 min. Each cycle consisted of denaturation at 95 °C for 1 min, annealing at 36 °C for 1 min and extension at 72 °C for 2 min, followed by a final extension at 72 °C for 1 min. The DNA fragments amplified by RAPD-PCR were separated by electrophoretic running on a 1% agarose gel containing ethidium bromide (Thermo Fisher Scientific, Waltham, MA, USA) at a final concentration of 0.5 µg/mL. Electrophoresis was performed at 120 V for 1 h and 10 min, thus creating a polymorphic DNA profile. By comparing the profile of the treated sample with that of an untreated control sample, it is possible to identify bands that have disappeared or new bands that have appeared due to DNA instability [38]. The number of bands that disappeared or appeared is an indicator of the level of DNA damage. RAPD-PCR products were visualized using a UV transilluminator (ECX-F26.M UV transilluminator (Vilber Lourmat, Collégien, France)) and an imaging system. Only clear and reproducible bands were considered, while faint or ambiguous bands were excluded. The reproducibility of the profiles was assessed through independent PCR amplifications, including only bands consistently present across triplicates. A percentage estimate of GTS (genomic template stability) of the sample can be obtained by a simple calculation: %GTS = $(1 - a/n) \times 100$, where a is the number of polymorphic bands present in the profile of each treated sample (i.e., the number of bands that appeared and/or disappeared compared to the negative control) and n is the total number of bands present in the negative control profile [35,39].

2.5. TUNEL Test

The TUNEL test is a method of measuring and quantifying DNA fragmentation. After the PS-MPs exposure time and cell centrifugation, the slides were prepared according to the protocol of Mottola et al. [36]. Briefly, the slides were fixed in 4% paraformaldehyde for 1 h at room temperature, left to air dry, and incubated in the permeabilising solution (0.1% sodium citrate and 0.1% Triton X-100) for 2 min. After rinsing in 1× PBS, the slides were incubated in the ready-made reaction mix of the In Situ Cell Death Detection Kit (Roche Diagnostics® GmbH, Mannheim, Germany), following the manufacturer's instructions. The slides were then immersed in a DAPI solution for counterstaining the cells in blue and for allowing the quantification of fragmented nuclei in relation to intact nuclei. The resulting slides were carefully observed under a Nikon ECLIPSE E600 epifluorescence microscope (Nikon Corporation, Tokyo, Japan) equipped with a mercury vapour lamp that emits energy over a broad spectrum with several peaks, a DAPI filter (400–450 nm) and a FITC filter (520–570 nm), at 100× magnification. Images were acquired with a CCD camera and analyzed using Genikon Imaging System software (version 3.7; Nikon Corporation, Tokyo, Japan).

The TUNEL technique is a valid tool for determining the extent of DNA damage, which is measured by the DNA fragmentation index (DFI): %DFI = FITC-stained nuclei/total nuclei × 100. Slides were prepared in triplicate for each PS-MP experimental group across various exposure times, as well as for the negative and positive controls. As a positive control for the TUNEL assay, samples treated with hydrogen peroxide (1 µL/mL H_2O_2) were included to induce DNA damage.

For each slide, approximately 250 nuclei were manually counted; image analysis software was used only for image acquisition and visualization and not for automated cell counting.

2.6. NBT Test-ROS Production Determination

The determination of ROS was performed using the NBT test. In brief, 20 µL of an erythrocyte suspension from each experimental group was diluted in 1 mL of a 0.2% Nitroblue tetrazolium (NBT) solution (Sigma-Aldrich, St. Louis, MO, USA) and incubated at 37 °C for 15 min. After centrifugation at 2000 rpm for 1 min, the pellet was suspended in 200 µL of PBS 1× and 100 µL of the cell solution was deposited onto slides. After air drying, the slides were fixed in 100% methanol for one minute and then rinsed with highly purified water. Safranin (Sigma-Aldrich, St. Louis, MO, USA) was then used as a counterstain to highlight the cells. The slides were air-dried and observed under a light microscope (ZEISS Axioskop microscope (Carl Zeiss AG, Oberkochen, Germany)) at 40× magnification. The results of this method are expressed as a percentage of reactive oxygen species (%ROS), i.e., the ratio of cells exhibiting the formazan reaction to the total number of cells, multiplied by 100. Therefore, %ROS is determined as the ratio of cells showing the blue formazan crystal reaction to the total number of cells (%ROS = n° cells with ROS/n° total cells × 100) [40]. As with the TUNEL assay, 250 cells from each experimental group were analyzed.

2.7. Physicochemical Characterization of Polystyrene Microplastics

The physicochemical properties of the commercially available 1 µm polystyrene microplastics were characterized following a 1:2 dilution in phosphate-buffered saline (PBS). The hydrodynamic diameter and zeta potential were measured using a NanoLink SZ902 Nanoparticle Size and Zeta Potential Analyzer (Linkoptik Instruments Co., Ltd., Shanghai, China), based on dynamic light scattering (DLS) and electrophoretic light scattering (ELS), respectively. Measurements were performed at room temperature according to the manufacturer's instructions. The mean hydrodynamic diameter was 1098.4 ± 40.1 nm, and the zeta potential was −35.3 ± 0.57 mV. Measurements were performed at 25.0 °C using a U-shaped capillary cell, with a measurement duration of 160 s, a measurement position of 6.00 mm, an attenuator value of 206, and a detection angle of 173°. The obtained measurements provided information on the hydrodynamic particle size and zeta potential of the suspension under the tested conditions. These measurements were used to assess particle size in suspension, aggregation state, and colloidal stability under the tested conditions.

2.8. Statistical Analysis

Data are expressed as mean ± SD. Data distribution was evaluated using the Shapiro–Wilk normality test. Based on data distribution, appropriate parametric or non-parametric tests were applied (unpaired Student's *t*-test and Mann–Whitney U test, respectively). Comparisons were performed between each experimental condition and its corresponding control group. Multiple comparisons were accounted for by applying the appropriate correction in the multiple t-test analysis performed using GraphPad Prism 10 (GraphPad Software Inc., San Diego, CA, USA). A p-value ≤ 0.05 was considered statistically significant.

3. Results

3.1. Erythrocytes Viability

Exposure to PS-MPs at a concentration of 105 µg/mL induced a progressive and statistically significant decrease in the viability of zebrafish erythrocytes as the exposure time increased, in the same way as in the control group (erythrocytes treated with 1 µL/mL H_2O_2). After 30, 60 and 90 min, the percentage of viable cells decreased progressively by approxi-

mately 30%, 40% and 50%, respectively, compared to the negative control, which presented values of 94.2% at 30 min, 93.8% at 60 min, and 92.1% at 90 min (all treatments were statistically significant compared to the negative control), as shown in Figure 1. Results were considered statistically significant when $p \leq 0.05$.

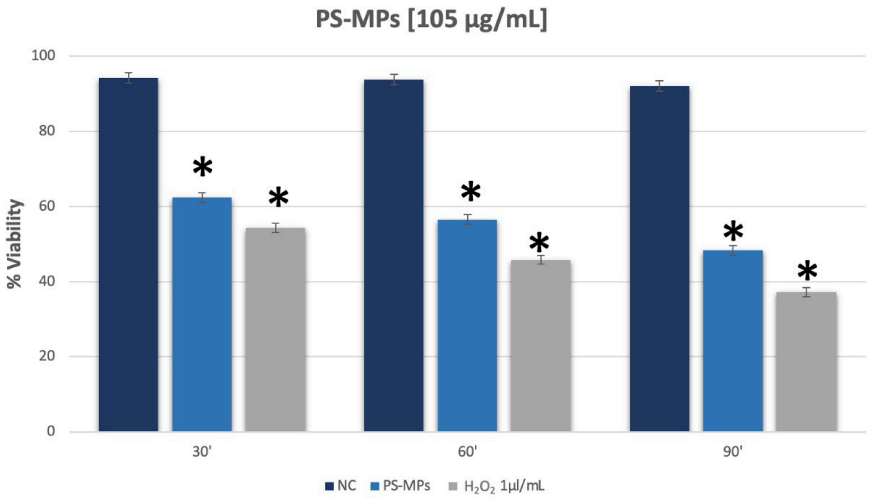

Figure 1. Percentage of viability in zebrafish erythrocytes following exposure to 105 µg/mL PS-MPs and 1 µL/mL H_2O_2 at different time points. Data are presented as mean ± SD of five independent experiments. Error bars represent the standard deviation. * $p \leq 0.05$ compared with the negative control group.

Figure 2 shows a representative image of zebrafish blood cells after 90 min of exposure to 105 µg/mL PS-MPs.

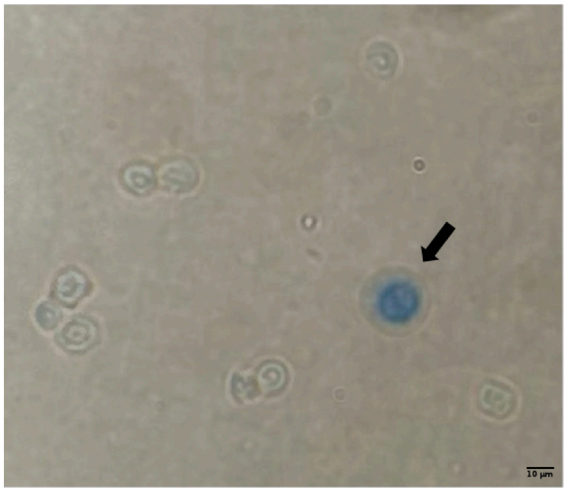

Figure 2. Representative image of zebrafish blood cell viability after 90 min of exposure to 105 µg/mL PS-MPs, obtained by Nikon Eclipse E600 microscope at 60× magnification. Viable cells appear translucent, while non-viable cells are stained (arrow).

3.2. Random Amplified Polymorphic DNA Profiles

The control pattern showed the presence of bands at 200 bp, 300 bp, 400 bp, 500 bp, 550 bp, 650 bp, 700 bp, 800 bp, 980 bp and 3000 bp. Variations in bands were observed at all three exposure times compared to the negative control. After 30 min, two new bands appeared at 220 and 320 bp, respectively. Even at the intermediate exposure time of 60 min, a band appeared at 220 bp and a band disappeared at 200 bp. At the maximum exposure time, two bands disappeared at 800 bp and 980 bp, while two new bands appeared at 220 bp and 320 bp (Table 1, Figure 3).

Table 1. RAPD-PCR profiles of DNA isolated from zebrafish erythrocytes treated with PS-MPs 105 µg/mL for different exposure times.

Exposure Time	Disappearance of Bands (bp)	Appearance of Bands (bp)
PS-MPs 30′	-	220–320
PS-MPs 60′	200	220
PS-MPs 90′	800–980	220–320
H_2O_2 90′	300–550–700–800–980–3000	-

Control bands: 200, 300, 400, 500, 550, 650, 700, 800, 980, 3000 base pairs (bp).

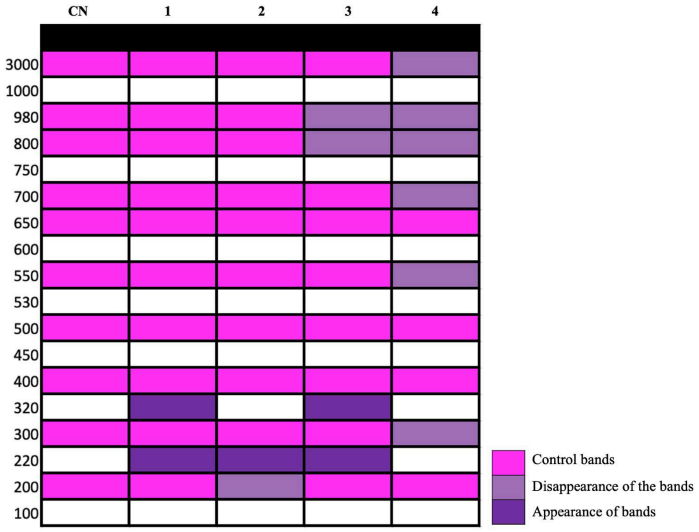

Figure 3. Molecular sizes (bp) of zebrafish DNA samples analyzed with Genesis software 1.8.1 and bands that appeared and disappeared after amplification with the P6 primer following *in vitro* exposure to PS-MP 105 µg/mL and H_2O_2. CN = Negative Control; 1 = PS-MP exposure 30′; 2 = PS-MP exposure 60′; 3 = PS-MP exposure 90′; 4 = H_2O_2 exposure 90′. Dark purple indicates newly formed bands, light purple indicates disappeared bands, and fuchsia indicates control bands. Control bands are at 200, 300, 400, 500, 550, 650, 700, 800, 980, and 3000 bp.

3.3. Genomic Template Stability

The polymorphic profiles obtained using the RAPD-PCR technique were used to calculate the percentage of genomic template stability (%GTS). After 30 min of PS-MPs exposure, a statistically significant reduction in the genomic stability of 20% compared to the negative control (100%) was detected; the same value was also found at the intermediate treatment time of 60 min. After 90 min, the GTS percentage had decreased by 40% compared to the negative control, which was statistically significant (Figure 4).

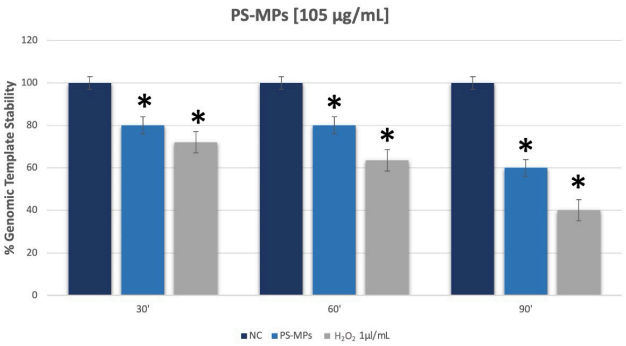

Figure 4. The percentage of genomic template stability (%GTS) in zebrafish erythrocytes determined following exposure to 105 µg/mL PS-MPs and 1 µL/mL H_2O_2 at different time points. Data are presented as the mean ± standard deviation (SD) of five independent experiments. Error bars represent the standard deviation. * $p \leq 0.05$ compared with the negative control group.

3.4. DNA Fragmentation

TUNEL analysis revealed a statistically significant rise in DNA fragmentation index (DFI) in cells exposed to PS-MPs (Figure 5).

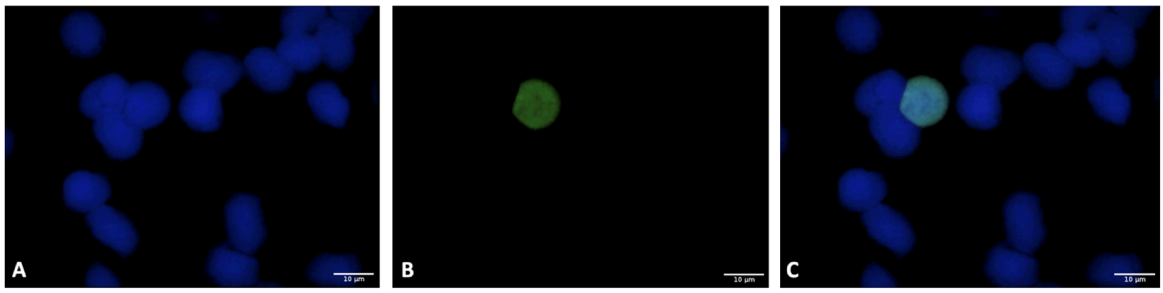

Figure 5. DNA fragmentation in zebrafish blood cells exposed for 30 min to PS-MPs 105 µg/mL, analyzed under a fluorescence microscope (Nikon Eclipse E600 (Nikon Corporation, Tokyo, Japan) at 100× magnification). (**A**) DAPI, no DNA fragmentation (blue nuclei). (**B**) FITC, fragmented DNA (green nuclei). (**C**) Merge. Bar: 10 µm.

Specifically, a DFI of around 30% was observed after 30 min, increasing to approximately 32% and 38% after 60 and 90 min, respectively, thus indicating that MPs induce DNA damage in a time-dependent manner (Figure 6). These values were significantly higher than those of the negative control (approximately 17%) and in line with the increase observed in the positive control (erythrocytes treated with 1 µL/mL H_2O_2) (Figure 6).

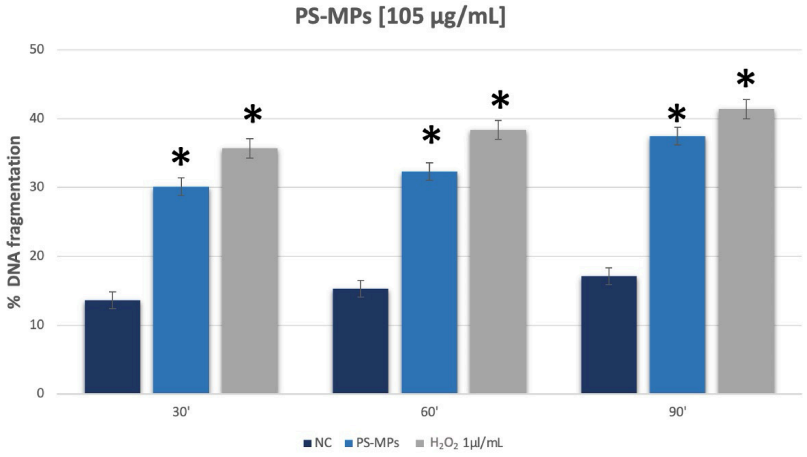

Figure 6. Percentage of DNA fragmentation (%DFI) in zebrafish erythrocytes following exposure to 105 µg/mL PS-MPs and 1 µL/mL H_2O_2 at different time points. Data are presented as mean ± SD of five independent experiments. Error bars represent the standard deviation. * $p \leq 0.05$ compared with the negative control group.

3.5. Percentage of NBT-Positive Cells

Exposure to PS-MPs was found to significantly increase intracellular ROS in zebrafish erythrocytes, as determined by the NBT test (Figure 7). The test revealed a progressive increase in the proportion of formazan-positive (NBT-positive) erythrocytes, indicating enhanced superoxide anion production over time (Figure 8). The analysis showed that the percentage of NBT-positive cells was approximately 27% after 30 min, compared to ~ 15% in the negative control. This increased to around 30% and 35% after 60 and 90 min, respectively. This is consistent with the increase observed in the positive control (erythrocytes treated with 1 µL/mL H_2O_2) (Figure 8).

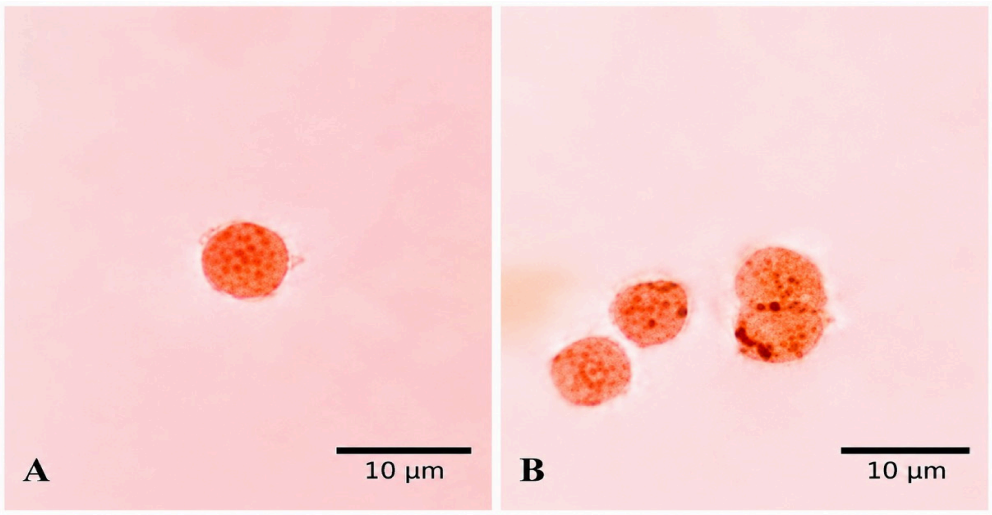

Figure 7. Representative image of zebrafish erythrocytes showing ROS production, visualized by the NBT reaction (**A**). Images were acquired using a ZEISS Axioskop microscope (Carl Zeiss AG, Oberkochen, Germany) at 100× magnification. (**B**) After 90 min of exposure to 105 µg/mL PS-MPs; and zebrafish erythrocytes showing no detectable ROS production.

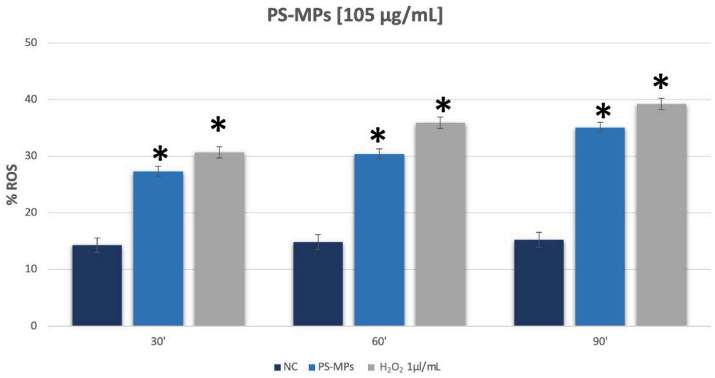

Figure 8. Proportion of formazan-positive erythrocytes in zebrafish following exposure to 105 µg/mL PS-MPs and 1 µL/mL H_2O_2 at different time points. Data are presented as mean ± SD of five independent experiments. Error bars represent the standard deviation. * $p \leq 0.05$ compared with the negative control group.

3.6. Physicochemical Characterization

The physicochemical properties of the particles used in this study were characterized by commercially available, non-functionalized polystyrene microplastics and were used as supplied by the manufacturer, without additional surface modification. Dynamic light scattering analysis showed a mean hydrodynamic diameter of 1098.4 ± 40.1 nm. These indicate that, under the tested conditions, the particle suspension was mainly composed of micrometre-sized particles without evidence of extensive aggregation. The zeta potential of the suspension was −35.3 ± 0.57 mV, indicating a negatively charged particle surface and suggesting a moderate-to-good electrostatic stability in PBS. These measurements provide a baseline characterization of the particles used for subsequent erythrocyte exposure experiments.

4. Discussion

To evaluate the genotoxic effects of MPs, we employed a well-established model commonly used in ecotoxicological research. The zebrafish is an optimal model for investigating fundamental biological processes such as DNA damage, oxidative stress responses, and cellular toxicity due to its high degree of genetic conservation with other vertebrates [41].

The methods we chose address different aspects of the same overarching research question: how does exposure to MPs affect the cellular and genomic stability of zebrafish erythrocytes? Specifically, the TUNEL assay detects DNA fragmentation, which is consistent with, but not specific for, apoptosis, providing insights into cellular damage at the level of the individual cell [42]. In contrast, RAPD-PCR detects broader changes in genomic structure by revealing variations in DNA amplification patterns [43,44]. The Genomic Template Stability (GTS) index is derived from these data, enabling a quantitative comparison of the overall extent of genomic alterations across experimental groups. The NBT assay was used to measure intracellular superoxide anion production, serving as a marker of oxidative stress. An increase in NBT reduction following PS-MP exposure indicates elevated ROS levels and supports the idea that oxidative imbalance plays a role in the observed DNA damage and reduced cell viability. Furthermore, evaluating cell viability alongside genotoxicity assays is crucial for differentiating between primary DNA damage and secondary effects resulting from cytotoxicity. This enables a more precise and thorough evaluation of the biological impact of the tested compound.

The results obtained are consistent and converge towards a single result: PS-MPs exert clear cytotoxicity and genotoxicity in a time-dependent manner. The cytotoxic effect is manifested by a marked reduction in cell viability in zebrafish erythrocytes. Specifically, prolonged exposure to PS-MPs at a concentration of 105 µg/mL resulted in a statistically significant decrease in erythrocyte viability in zebrafish, with progressive impairment over time. Compared to the negative control, cell viability was significantly reduced after 90 min of exposure to MPs, with an approximate 50% decrease.

Genotoxicity tests also confirmed time-dependent damage to genetic material, with oxidative stress being the main underlying mechanism of the observed cytotoxicity and genotoxicity. RAPD-PCR analysis revealed a significant decrease in the stability of the genomic template of erythrocyte DNA treated with 105 µg/mL of PS-MPs, compared to the control. This decrease was consistent for the first 60 min of exposure, increasing further after 90 min, when a 40% reduction in GTS was observed. Similarly, TUNEL analysis showed time-dependent DNA damage in *D. rerio* erythrocytes after treatment with PS-MPs, compared to the negative control. %DFI increased to approximately 38% after 90 min of exposure. Furthermore, exposure to MPs induced a progressive and statistically significant increase in ROS in zebrafish erythrocytes, with the highest %ROS detected after 90 min (35%). To better contextualize the magnitude of the observed toxicity, the effects induced by PS-MPs were compared with those observed in the H_2O_2 positive control. Overall, H_2O_2 induced the most pronounced cytotoxic, oxidative, and genotoxic alterations, as expected for a strong oxidative stress inducer. However, PS-MPs exposure showed a consistent pattern of toxicity, with effects increasing over time and approaching those observed after H_2O_2 treatment, particularly at the longest exposure time.

Our *in vitro* findings, which reveal increased DNA fragmentation and elevated oxidative stress in zebrafish erythrocytes subsequent to PS-MP exposure, impair cellular metabolism and reduce cell viability, as has been observed in other *in vitro* experiments [45]. These results are in line with the previous literature using the same experimental model. The mechanism by which MPs induce the observed damage appears to be indirect, creating an inflammatory and oxidative environment conducive to the formation of genotoxic reactive species that may contribute to DNA damage and cell death [46]. Inflammation and oxidative stress are closely related and often influence each other [47]. According to these authors, the toxic mechanism of PS-MPs could indeed be linked to increased ROS, leading to the upregulation of pro-inflammatory factors such as TNF-alpha, which are involved in key processes such as apoptosis, differentiation, and cell growth. Several studies have shown that MPs can generate ROS and cause genotoxic effects without significant cellular

internalization, suggesting that particle–cell surface interactions alone may be sufficient to trigger these effects [48]. However, the mechanisms underlying these effects have not yet been fully clarified. Furthermore, elevated ROS levels could affect the activity of antioxidant enzymes such as SOD and CAT, which are responsible for detoxifying oxidant species. In zebrafish, exposure to PS-MPs has already been associated with increased SOD and CAT expression, resulting in increased ROS production [49]. Although the concomitant increase in ROS production and DNA damage suggests a possible role of oxidative stress in PS-MP-induced toxicity, further mechanistic studies, including antioxidant rescue experiments, are required to confirm whether ROS generation represents a primary driver of the observed effects.

Furthermore, in our study, we observed that exposure to PS-MPs induces increased DNA fragmentation, as detected by the TUNEL assay. This finding is consistent with a previous study reporting a possible mechanism underlying PS-MP-induced DNA damage; in fact, Qiang and Cheng [50] demonstrated that ROS upregulate the transcription of the p53 gene, which in turn activates caspase 3b. Activated caspase 3b then promotes GADD45B transcription, causing DNA damage and apoptosis. Furthermore, Gupta et al. reported that exposure to high concentrations of polystyrene microplastics significantly increased the percentage of TUNEL-positive cells by 2.1- and 3.9-fold, respectively [51]. Although these findings suggest a possible mechanism underlying PS-MP-induced toxicity, the present study did not evaluate p53 expression or caspase activation; therefore, the involvement of this pathway cannot be confirmed from our data.

The observed biological effects are in line with previous studies reporting oxidative stress, DNA damage and probably activation of apoptotic pathways induced by polystyrene micro- and nanoplastics under similar experimental conditions [47]. However, further gene expression studies are necessary to confirm the molecular cascade involving p53 activation in our experimental model and to improve our understanding of it. Additional endpoints, such as antioxidant enzyme activities or lipid peroxidation, could also help to clarify the oxidative stress response. Future investigations should also consider potential biological variables, including donor sex, to better characterize inter-individual variability in response to PS-MP exposure.

The toxicity of MPs and their ability to generate ROS also depends on their size. The physicochemical characterization performed in PBS showed a hydrodynamic diameter (1098.4 $\pm$ 40.1 nm) close to the nominal particle size and a negative zeta potential (-35.3 ± 0.57 mV), indicating that the particle suspension was predominantly composed of micrometre-sized particles with moderate-to-good electrostatic stability under the tested conditions. However, physicochemical characterization was not performed in the erythrocyte suspension, and therefore possible medium-dependent changes in particle size, aggregation state, or surface properties cannot be excluded. Despite this limitation, our findings indicate that 1 µm PS-MPs induce oxidative stress, DNA damage, and reduced cell viability in zebrafish erythrocytes, in agreement with previous studies reporting similar biological effects for polystyrene microplastics [52]. Moreover, our results demonstrate a clear time-dependent effect: oxidative stress, DNA damage and reduced cell viability become progressively more pronounced with longer exposure durations. This is consistent with studies suggesting that prolonged exposure to MPs exacerbates their toxic effects through both extracellular and intracellular processes [52]. However, the presented results should not be directly extrapolated to environmentally relevant exposure scenarios. This study was designed to evaluate the time-dependent effects of PS-MPs at a single exposure concentration. Future investigations involving multiple concentrations will help define dose–response relationships and further enhance the environmental relevance of PS-MPs.

5. Conclusions

Taken together, these findings demonstrate that PS-MPs induce early cellular stress responses in zebrafish erythrocytes, characterized by increased ROS production and DNA damage. This study provides further insights into the mechanisms underlying microplastic-induced toxicity and highlights the potential of erythrocyte-based *in vitro* models as useful tools for investigating cellular responses to environmental contaminants. Although *in vitro* approaches cannot fully reproduce the complexity of an organism, including metabolism, tissue interactions, immune responses, and long-term exposure dynamics, they represent valuable models for identifying early molecular and cellular effects of emerging pollutants. Therefore, the present findings contribute to the understanding of PS-MP toxicity and may support future approaches for environmental risk assessment.

Further studies integrating environmentally relevant exposure conditions, *in vivo* models, complementary genotoxicity assays, and detailed physicochemical characterization of microplastics (including size distribution, aggregation behaviour, surface properties, and cellular uptake) will be essential to better define how particle characteristics influence bioavailability, toxicity, and the induction of oxidative and genotoxic responses.

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