Molecular effects of polystyrene nanoplastics toxicity in zebrafish embryos (*Danio rerio*)

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Abstract

Plastics pose a health hazard to living beings and the environment. Plastic degradation produces nano-sized plastic particles (NPs) that end up in terrestrial and aquatic ecosystems, including oceans, rivers, and lakes. Their presence in air, drinking water, sediments, food, and personal care products leads to a variety of exposure routes for living beings, including humans. The toxicity mechanisms of these nanomaterials (NMs) in living organisms and ecosystems are currently unknown, making it a priority to understand their effects at the molecular and cellular levels. The zebrafish (Zf) (*Danio rerio*) is a model organism which has a high homology with humans and has been widely used to assess the hazard of different xenobiotics. In this study, the expression changes of different genes in 120 hpf Zf embryos (Zfe) after exposure to polystyrene (PS) NPs (30 nm) at concentrations of 0.1, 0.5 and 3 ppm were investigated. The results showed that the gene encoding heat shock protein (*hsp70*) was down-regulated in a dose-dependent manner. The genes encoding superoxide dismutase (*SOD 1* and *SOD 2*), apoptotic genes (*cas 1* and *cas 8*) and interleukin 1-β (*il1β*) were activated at the concentration of 3 ppm PS NP, while the anti-apoptotic gene *Bcl2α* was inhibited at 0.5 and 3 ppm. In addition, the neurotransmitter-related gene Acetyl-Cholinesterase (*ache*) was significantly inhibited and the DNA repair genes

(*gadd45α* and *rad51*) were also down-regulated. In contrast, the mitochondrial metabolism-related gene *cox1* did not alter its expression in any of the treatments. Most of the changes in gene expression occurred at the highest concentration of NPs. Overall, the results indicated that NPs generated cellular stress that caused certain alterations in normal gene expression (oxidative stress, apoptotic and inflammatory processes, neurotoxicity and anti-apoptotic proteins), but did not cause any mortality after 120 hpf exposure at the three concentrations assayed. These results highlight the need for further studies investigating the effects, at the molecular level, of these materials in humans and other living organisms.

1. Introduction

Plastics are widely used in our daily lives and consequently a large amount of plastic waste is discharged into the environment. Plastic waste degradation generates particles of micro or nano-size. Microplastics (MPs) have a diameter of less than 5 mm, while nanoplastics (NPs) have a diameter of 1 to 100 nm, although some authors consider it to be <1000 nm (Gigault et al., 2018). Plastics are synthetic products that have many commercial applications due to their ease of production, low cost, and hydrophobicity, and their production is increasing every year. In 2019, the global production reached 370 million tons, being almost 58 million tons produced in Europe (Plastics Europe, 2020). However, only 10% of the waste was properly recycled (Rhodes, 2018; Zhang et al., 2020). Plastic that is not recycled is incinerated or ends up in the environment (Rhodes et al., 2018). This leads to plastic contamination of water, air, and soil. Once plastics enter the environment, physical, chemical, and biological interactions produce large plastic fragments to degrade and form smaller plastic fragments. In addition, industry also manufactures tiny plastic particles to add to personal care products, generating an additional source of population exposure.

Currently MP and NPs have been detected in both aquatic and terrestrial ecosystems, and their harm to biota for both humans and other living organisms cannot be ignored. However, the molecular mechanisms of toxicity involving these nanomaterials and their impact on human health remain an unknown. The study of the toxicology of NPs is a priority in public health and environmental health.

Zfe have been used as a model in environmental toxicology, human disease, and drug development. (Lleras-Forero et al., 2020; Deveau et al., 2017; Pereira et al., 2019; Garcia et al., 2016; Dai et al., 2014). Zebrafish is a prototypical organism with great advantages due to its small size and short development time. Furthermore, they do not present bioethical problems if larvae up to 120 hpf are used (Directive 2010/63/EU). Moreover, the Organization for Economic Co-operation and Development (OECD) and the Environmental Protection Agency (EPA) have standard guidelines for toxicity studies with the Zfe (OECD 236, 2013). Therefore, the Zfe is a suitable model for assessing the effects of MPs and NPs at the biological, molecular, and cellular levels (Torres-Ruiz et al., 2021; de Marco et al., 2022). This organism has a high genetic homology with humans (>70%), which allows predicting the possible health effects (Howe et al., 2013). The study of the toxicity of NPs in the Zfe model has only recently begun (Bhagat et al., 2020; Torres-Ruiz et al. 2021), but there is insufficient information on the effects of these nanomaterials.

Research on the molecular effects of PS NPs is very scarce and it is a priority to understand the mechanisms of action of these nanomaterials present in the environment and their potential hazards to living beings, including humans.

The aim of the present study was to determine the specific molecular response of genes involved in different metabolic pathways (cellular stress response, oxidative stress, DNA damage, apoptosis, inflammation, mitochondrial and neurotransmission), using Zfe exposed to PS NPs of about 30 nm at three concentrations of 0.1, 0.5 and 3 ppm for 120 h.

2. Material and Methods

2.1. Nanoparticle preparation

Pristine commercial polystyrene round particles with a mean diameter of 30 nm were purchased from Thermo Scientific™ (Madrid, Spain), catalogue number 5003A. NP of 1.05g/cm³ density were provided as a 10% solution in water with < 2% surfactant (SDS) to prevent agglomeration. This stock solution was diluted to working concentrations in embryo medium and the surfactant was diluted enough not to

cause effects in embryos (Pedersen et al. 2020). As per manufacturer instructions, the stock solution was shaken and sonicated for 15 sec prior to use. In addition, every time ZFe medium was changed, solutions were shaken and sonicated for 60 sec prior to use. NP working concentrations in the range of those predicted to be encountered in nature (Torres-Ruiz et al., 2021) were tried in a pilot study to select the ones that best allowed appreciating effects and avoiding mortality. As a result, solutions of 0.1, 0.5 and 3 ppm were used in Zfe exposure.

2.2. Nanoparticle characterization

NP solutions in embryo water at the concentrations used for exposure were characterized by observation under a transmission electron microscope (FEC Tecnai 12) operated at 120 kV. Briefly, samples were sonicated for 1 min, then incubated on copper grids for 10 min, washed with deionized water from a MilliQ system (Merck RiOs^{TM} Essential 24) and then dyed with 2% uranyl acetate before observation.

2.3. Zebrafish maintenance

Adult wild type zebrafish (*Danio rerio*) were maintained in glass recirculating aquariums at 25 - 26°C, pH 7-7.5, oxygen 6-7 mg/L, conductivity 500-600 µS/cm, NO_3 < 5 mg/L, NH_4 and NO_2 < 0.05 mg/L. Water for fish was prepared using deionized water from a MilliQ system (Merck RiOs^{TM} Essential 24) and a mixture of salts: $Cl_2Ca.2H_2O$ (294 mg/L), $MgSO_4.7H_2O$ (123 mg/L), $NaHCO_3$ (64.7 mg/L), and KCl (5.8 mg/L). Fish were maintained in a light/dark cycle of 14:10h and they were fed twice a day with frozen brine shrimp (*Artemia salina*, Ocean Nutrition^{TM}, Belgium), shell free brine shrimp eggs (Ocean Nutrition^{TM}, Belgium) or Tropica Basic fish flakes (Dajana®, Czech Republic). The fish laboratory adheres to all animal welfare according to Directive 2010/63/EU.

2.4. Embryo culture and NP exposure

Embryos were obtained by placing a breeding tank inside the aquarium. Eggs were laid at the onset of light (8:30 am) and were collected within 1h of oviposition. Embryos were washed with embryo water (same as fish water, see above) and placed in large Petri dishes where they were immediately selected under a stereoscope and placed in experiment plates. Embryos were treated at NPs concentrations of 0.1, 0.5 and 3 ppm. Three plates (24 wells) per NP concentration and three plates (24 wells) for negative

control (embryo water) were used resulting a total of 72 embryos per treatment/control groups. Plates were kept in an incubator (Nuaire) at 28 °C until the end of the study (120 hpf) and medium was renewed every 24 h. Mortality was assessed every 24 h in control and exposed larvae that were used for molecular analysis.

2.5. RNA extraction and cDNA synthesis

Total RNA was extracted from exposed and control Zfe using a guanidine isothiocyanate-based method, performed with a commercial kit (Trizol, Invitrogen) according to the manufacturer's protocol. The embryos frozen at -80 °C were homogenised in 500 µl Trizol and left for 5 minutes at room temperature. Next, 0.2 volume of chloroform was added to each sample, mixed, and left for 5 minutes at room temperature. The samples were then centrifuged for 15 minutes at 4 °C and 15,000 g. After transfer of the aqueous phase, the RNA was precipitated with isopropyl alcohol (0.5 v/v), washed with 70% ethanol, and resuspended in DEPC water. The quality and quantity of total RNA was determined by agarose electrophoresis and absorbance (Biophotomer Eppendorf), and the purified RNA was finally stored at -80 °C.

cDNA synthesis was performed with 500 ng total RNA, 500 ng Oligo dT20 (Invitrogen) and 100 u/µl of MMLV enzyme (Invitrogen, Germany). The synthesised cDNA was stored at -20 °C until used for PCR reactions.

2.6. Real-time PCR

The synthesised cDNA was used as a template in real-time PCR to analyse the messenger RNA (mRNA) expression profile of genes related to cell stress (*hsp90*, *hsp70* and *hsp27*), oxidative stress (*SOD 1*, *SOD 2*, *cat*), DNA repair (*gadd45α* and *rad51*), apoptosis (*cas 1*, *cas 3a*, *cas 8* and *Bcl2a*), and inflammatory, mitochondrial, and neurotransmission (*il1β*, *cox 1* and *ache*) responses. Treated embryos RNAs were compared with RNAs extracted from control embryos. The reaction was performed under the following conditions: initial denaturation at 95 ◦C for 3 min and 40 cycles of denaturation at 95 ◦C for 5 s; annealing at 58 ◦C for 15 s; and elongation at 65 ◦C for 10 s. The sequences of the oligonucleotides designed in this study for each of the used genes are shown in Table 1. All samples were analysed in duplicate, and two replicates of each plate were performed. Actin, beta 2 (*actb2*) and Glyceraldehyde 3-phosphate

dehydrogenase (*GAPDH*) genes, with a coefficient of variation < 0.25 and an M-value < 0.5, were used as endogenous reference controls to normalise the expression data of the selected study genes.

Table 1. Oligonucleotides sequences

Gene	Function	Accession nº	Primer (5'–3')	bp
actb2	Reference gene	NM_181601.5	F: TGACCGAGAGAGGCTACAG R: CAATGGTGATGACCTGTCCG	174
GAPDH	Reference gene	NM_001115114	F: CACCAGGTTGTGTCCACTGA R: GATGGGAGAATGGTCGCGTA	225
cox1	Mitochondrial metabolism	NC_002333	F: GGAATACCACGACGGTACTCT R: AGGGCAGCCGTGTAAT	195
SOD 1 (*Cu/Zn- -*	Oxidative stress	BC055516	F: GTGTGAGACACGTCGGAG R: TGCCGATCACTCCACAGG	222
SOD 2 (*Mn-SOD)*	Oxidative stress	NM_199976	F: CCCACTGCTTGGGATAGATG R: ATAGACACTCGGTTGCTCTC	188
hsp90	Stress	NM_131328	F: GAGCTTGACAGATCCGAGCA R: CCTGCAGAGCCTCCATGAAA	185
hsp70	Stress/Antiapoptotic	AB062116	F: CCAGCTATGTGGCCTTCAC R: GGCTTTCCTCCATCTCTGAC	188
hsp27	Stress	EU000061	F: ATGCGAGGGCCGTCCCTG R: AGAAGGCTTTCTGGAGG	111
cat	Stress	NM_130912	F: AGGGCAACTGGGATCTTACA R: TTTATGGGACCAGACCTTGG	499
gadd45α	DNA repair BER/NER	BC152637.1	F: CTCAGTCATCCACATGGAAAG R: GATGACTGGCACCCACTGG	92
rad51	DNA repair	BC062849	F: CGTCTCGCTGATGAGTTTGG R: CTTACATATCCTCGTCTCACC	180
ache	Neurotransmission	NM_131846	F: AATGAGCAAAAGCATGTGGGC R: ACTCCACTTTCCACTGTCGC	154
cas 1	Apoptosis/inflammation	NM_131505	F: TTCTCTGATGTCGTGCACCC R: AGTCCGGGGAACAGGTAGAA	237
cas 3a	Apoptosis-related cysteine peptidase a	NM_131877	F: GGACATGCGGATACGGAGAC R: TGCAGATGCCCCATCCTTAC	204
cas 8	Apoptosis-related cysteine peptidase/ inflammation	NM_131510	F: GTTTTGGGCACAGATGGTAA R: TACTGTGGCCATTCCGATCA	258
Bcl2a	Antiapoptotic	NM_001030253	F: AACCGACTCTTTCCTGCTCG R: GACATTTCCACACATATCCTCGC	138
il1β	Inflammatory response	AY340959.1	F: GTCACACTGAGAGCCGGAAG R: GGAGATTCCCAAACACACAGG	111

Abbreviations: bactin2- bactin2;*GAPDH*- Glyceraldehyde-3-phosphate dehydrogenase; *coxI* – cytochrome C oxidase subunit I; *sod1*- superoxide dismutase type 1; *sod 2*– mitochondrial superoxide dismutase; *hsp90*- heat shock protein; *hsp70*- heat shock protein; *hsp27*- heat shock protein; *cat*- catalase ; *gadd45α* – growth arrest DNA damage; *ache*- Acetyl-Cholinesterase; *cas 1*- caspase 1; *cas 3a*- caspase 3a ; *cas 8*- caspase 8; *Bcl2α*- B-cell lymphoma 2; *il1β*- Inflammatory response. In grey are the reference genes used.

2.7. Statistical analysis

The statistical analysis of the gene expression was performed using IBM SPSS Statistics 25. Normality and homogeneity were tested using Levene's tests. Analyses were performed using values obtained from a pool of Zfe in triplicate. Comparisons were made between control and treated larvae using analysis of

variance (ANOVA) with Dunnett's multiple comparison tests. A difference was considered significant at a $p < 0.05$.

3. Results and discussion

3.1. Nanoparticle characterization

Transmission electron microscopy (TEM) characterization of 30 nm polystyrene nanoparticles in embryo medium showed that these particles were round with a mean size of 25.1 ± 4 nm, according to commercial specifications. However, we observed agglomeration of these particles at all concentrations assayed, and especially at 3 ppm (Figure 1) even after mixing and sonicating according to manufacturer instructions. Agglomeration of polystyrene particles has been observed before in *in vitro* experiments (Eitzen et al., 2020) and is expected to be the more probable presentation in nature (Summers et al., 2018), which may alter their toxicological profiles. There are some studies that have shown lower toxicity for particles that are more aggregated (Manfra et al., 2017). On the other hand, a reduction of acute toxicity in *Daphnia magna* following sonication of PS NPs compared to non-sonicated NPs has been described by Vaz et al., 2021. Sonication may reduce or eliminate agglomerates during the time of exposures, but they are likely to re-form over time (Zhao et al., 2021). NPs are found in nature both, in aggregated and non-aggregated states, and their level of aggregation will depend on characteristics of aquatic medium (i.e. pH, salt type, ionic strength) in which they are found (Wang et al., 2021).

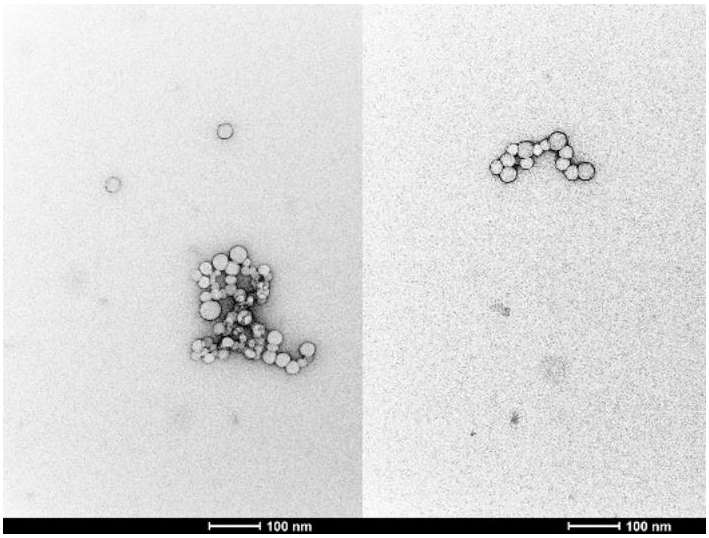

Fig. 1. Transmission electron microscopy (TEM) characterization of sonicated 30 nm polystyrene nanoparticles used in the study at 0.5 ppm.

3.2. Mortality

The percentages of mortality observed were very low in all the treatments, no mortality was found in the control and 3 ppm exposed groups, 1.4% in the 0.1 ppm group, and 0.7% in the 0.5 ppm group. This indicates a minimal mortality, independent of treatment, which we commonly observe in our laboratory, under the conditions tested. These results agree with previous studies in Zfe, with most showing that NPs do not affect embryo mortality in the ranges of NPs concentrations assayed in the present research (Torres-Ruiz et al., 2021; Lee et al., 2019). However, at higher concentrations (around 50-200 μg/ml of NPs), increased mortality has been observed in Zfe (Brun et al., 2018; Duan et al., 2020; Zhang and Goss, 2020) but it is very possible that these concentrations are higher than those present in the environment. The concentrations chosen for this study are based on the limited previous literature (Gallego-Urrea et al., 2010; Torres-Ruiz et al., 2021) which concludes that concentrations of approximately 0.55 to 8.6 ppm would be those found in the environment and therefore the most realistic for use in toxicity testing. Future studies will be needed to confirm real NP concentrations in different ecosystems, as currently this is not known with precision (Torres-Ruiz et al., 2021).

3.3. Gene expression

Recently published studies indicated that exposure to MPs and NPs can induce oxidative stress and inflammatory process at the molecular, cellular, and tissue level (Moyan et al., 2020; Yee et al., 2021), but more evidence is needed to understand the mechanism of action of these nanomaterials. In the present study, we investigated the changes in the expression of different genes involved in different metabolic pathways (cellular and oxidative stress responses, DNA damage, apoptosis, inflammatory, mitochondrial, and neurotransmission) in Zfe exposed to PS NPs of about 30 nm. The Zfe is a robust model in which whole-organism molecular and cellular effects can be assessed. In addition, this aquatic organism shows a high homology with humans, making it an excellent candidate for evaluating the molecular and cellular effects of these nanomaterials in humans.

3.4. Stress response: HSPs proteins

Heat shock proteins (HSPs) are a family of proteins that are expressed in response to stress factors (Lindquist and Craig, 1988). In addition, these proteins are anti-apoptotic, associating with apoptotic factors to block apoptotic cell death (Joly et al., 2010; Wang et al., 2014). The Zfe stress response to NPs showed a dose-dependent down-regulation at all concentrations studied in *hsp70* gene expression, although only at the highest concentration tested was this significant. On the other hand, *hsp90* and *hsp27* mRNA levels did not change (Figure 2). A similar result was obtained in the inhibition of *hsp70* gene expression in human stem cells (Im et al., 2022) and an aquaculture species *Apostichopus japonicus* (Liu et al., 2022) exposed to NPs. This result indicates an altered stress response and possible anti-apoptosis regulation. This decrease in *hsps* genes has been previously described after exposures to other nanomaterials. In human lung fibroblasts and keratinocytes, inhibition of HSP90 protein after exposure with Single-walled carbon nanotubes (SWCNTs) has been described (Ong et al., 2017), and in *C. riparius* after exposure to Multi-walled carbon nanotubes (MWCNTs) (Martinez-Paz et al., 2019). These studies link this heat shock genes down-regulation to an activation of apoptosis. Possibly, and based on the results of these investigations, nanomaterials would inhibit the expression of some of these anti-apoptotic proteins.

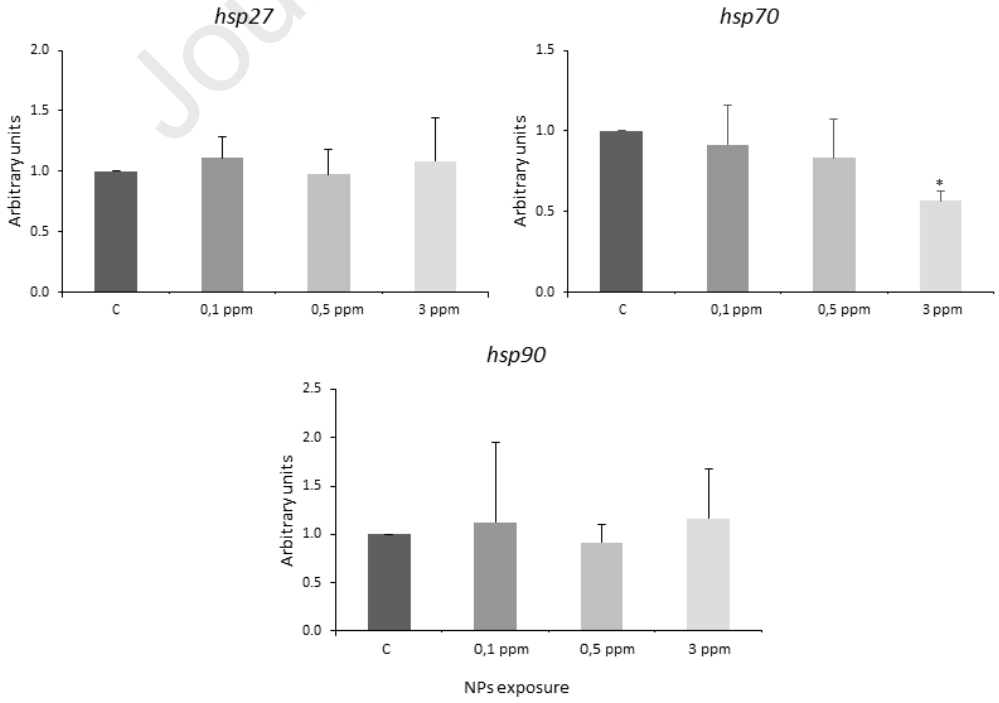

Fig. 2. Expression of the *hsp27, hsp70 and hsp90* genes. Asterisks indicate statistical differences compared to controls ($p < 0.05$).

3.5. Oxidative stress response: SOD and CAT

Recent toxicity studies of PS NPs in model organisms (*D. pulex, D.rerio, etc.)* and in human cells have shown that they can stimulate ROS production (Liu et al., 2020; Lu et al., 2016; Yu et al., 2018; Poma et al., 2019; Im et al., 2022). ROS production can generate oxidative stress, mitochondrial damage, increased inflammatory cytokines and pro-apoptotic factors that could induce cell death in mammals (Banerjee et al., 2021), and also can stimulate apoptosis in cells (Zucker et al., 1997). Antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) have a role in protecting against ROS (Livingstone, 2001) and help maintain cellular homeostasis. Superoxide dismutase (SOD) is an antioxidant detoxification enzyme involved in the superoxide anions metabolisation (Ighodaro and Akinloye, 2018). Specifically, SOD 1 (Cu/Zn SOD) binds Cu an Zn and localizes to the cytoplasm and mitochondrial intermembrane space and SOD 2 (Mn SOD) binds manganese at the catalytic core and is located within the mitochondria (Wang et al., 2018). Catalase (CAT) is a key enzyme in the biological defence system (Winston, 1991). In the present study, changes in mRNA expression of the mentioned genes involved in antioxidant responses, were evaluated in Zfe exposed to NPs. We have found that the molecular expression of CAT did not change and this result is consistent with previously published findings assessing its enzymatic activity (Parenti et al., 2019; Torres-Ruiz et al., 2021). Instead, a significant increase in the expression of SOD 1 (Cu/Zn SOD) and SOD 2 (Mn-SOD) could be observed at the highest concentration studied (Figure 3). Therefore, it is logical that the PS NPs used in the present study (at 3ppm) produced oxidative stress, stimulating the up-regulation of SOD 1 and SOD 2. In support of this assertion, the SOD results are in agreement with data obtained in Zfe exposed to PS NPs (Liu et al., 2021), as well as in other aquatic organisms (Liu et al., 2020). On the other hand, there are studies showing that NPs can induce a decrease in SOD levels in Zfe (Hu et al., 2020b; Zhang et al., 2020; Lu et al., 2018). The decreased expression of these genes could be due to a depletion of the antioxidant response, as a consequence of toxicity (Zhang et al., 2020). The fact that at the concentrations studied,

the expression of CAT was not altered but SOD 1 and SOD 2 (3 ppm) were altered may be due to catalase not being able to compensate for the excessive production of ROS. Previous studies with microplastics inhibit the activity of CAT enzymes. This inhibition is associated with the energetic cost of the oxidative stress response induced by microplastic exposure (Yu et al., 2018). Another possible explanation is that SOD 1 and SOD 2 respond in a first phase to ameliorate the oxidative stress produced by PS NPs.

The speed of the response reaction will depend on the concentration of the PS NPs and therefore activation is seen at the highest concentration. To test these hypotheses, it would be necessary to analyse the mRNA expression of SOD 1 and SOD 2 in Zfe at times before and after 120 hpf. Some reports suggest that oxidative stress is the main cause of MP and NP damage (Prata et al., 2020). ROS production causes oxidative stress enzymes to be activated, but at some point, they may not be able to activate due to the energetic cost of oxidative stress (Yu et al., 2018).

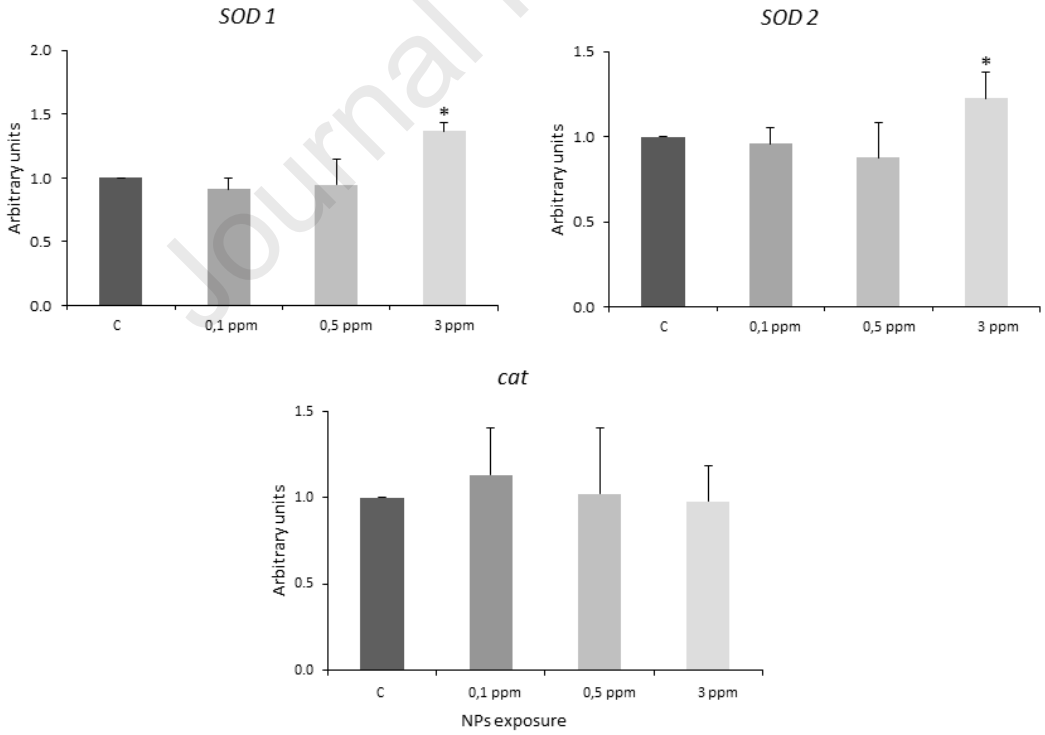

Fig. 3. Expression of *SOD 1, SOD 2* and catalase genes. Asterisks indicate statistical differences compared to controls ($p < 0.05$).

3.6. DNA Damage response: *gadd45α* and *rad51*

DNA damage activates DNA repair mechanisms, and in this study we analysed two genes related to this DNA repair process, *gadd45α* and *rad51*, in Zfe treated with different concentrations of PS NPs. *Rad51* is a gene involved in DNA repair of double-strand breaks, and its malfunction is linked to diseases such as cancer (Bonilla et al., 2020). *Gadd45α* is activated following DNA damage (Salvador et al., 2013). The results of this study showed that *gadd45α* mRNA levels were increased at the lowest NP concentration and inhibited at the two highest NP concentrations, although in neither case significantly. In the case of *rad51*, transcriptional activity decreased non-significantly at 0.1 and 0.5 ppm NP (Figure 4). The down-regulation of *gadd45α* and *rad51* seems to indicate that DNA repair mechanisms are being altered by the presence of NPs, affecting the general DNA repair capacity. At this stage, it cannot be established that NPs damage DNA, although it has been reported that they are capable of producing ROS (Liu et al., 2020; Lu et al., 2016; Yu et al., 2018; Poma et al., 2019; Im et al., 2022) and this may induce DNA damage (Romdhani et al., 2022). However, further research is needed to fully understand the mechanisms involved.

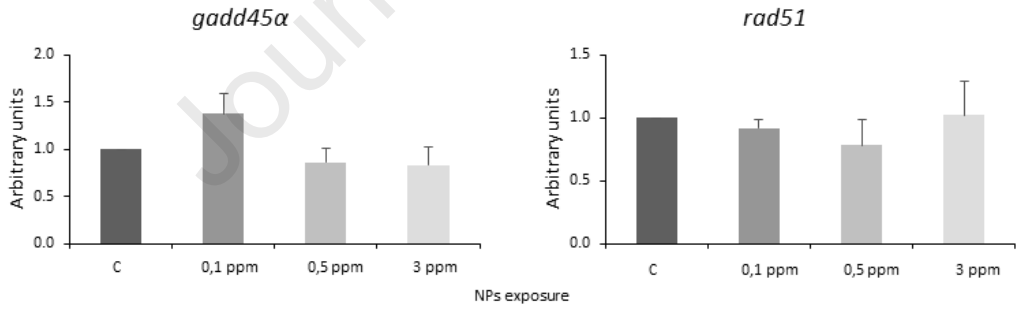

Fig. 4. Expression of *gadd45α and rad51* genes. Asterisks indicate statistical differences compared to controls ($p < 0.05$).

3.7. Apoptotic response: caspases and anti-apoptotic genes

Caspases are a family of highly conserved, cysteine-dependent intracellular proteases, well known for their role in inflammatory responses and apoptosis (McIlwain et al., 2013). Caspases are classified as inflammatory (Caspases-1, -4, -5 and -12), initiators (Caspases-2, -8, -9 and -10) or apoptosis

executioners (Caspases-3, -6 and -7) (Spead et al., 2018). However, caspases (cas) have also other functions in cell proliferation, tissue regeneration and repair, differentiation, motility, and migration (Spead et al., 2018). In this study, we have analysed the expression changes of three caspases (*cas 1*, *cas 3a* and *cas 8*) involved in inflammatory response and apoptosis mediation. The results show a significant activation of *cas 1* and *8* at the highest NP concentration of 3 ppm (Figure 5). In the case of *cas 3a*, we observed an increase in expression at the lowest concentration and inhibition at the highest concentration, although these changes were not significant (Figure 5). Caspase-1 has an inflammatory function that is activated by inflammasome complexes in response to pathogens or endogenous factors (Sun et al., 2016). Inflammation is a mechanism responsible for defence against pathogens and noxious agents, including xenobiotic or toxic compounds. Caspase-8 induces apoptosis as a consequence of cell membrane rupture (extrinsic pathway). Caspase-8 initiates the apoptotic cell signalling pathway in vertebrates and is involved in the control of cell death. However, it has also been found to be involved in the optimal transcription of certain inflammatory cytokines (Spead et al., 2018; Orning and Lien, 2021). Recently, an increase in *cas 8* expression has been described after NPs exposure from *P. lividus* hedgehog embryos (Della Torre et al., 2014), according to the results obtained in our study. Caspases are specific initiators of cellular apoptosis and inflammatory responses (Riedl et al., 2004). Different types of caspases are activated depending on the organelle in which they trigger apoptosis. The up-regulation of caspase-8 induced by NPs in Zfe may be due to cell membrane rupture and/or inflammasome activation. Previous studies suggest that caspase-8 may mediate IL-1b and IL-18 processing, independently of the conventional inflammasome and cas 1 pathway (Maelfait et al., 2008; Orning and Lien, 2021). Our results show, for first time, that PS NPs induce the activation of *cas 1* and *cas 8* genes involved in inflammatory processes related to the inflammasome and the initiation of apoptosis in Zfe. A possible hypothesis for the response of *cas 3a* in this study could be that this executioner protein is activated at longer exposure times. In view of the response of caspases to the presence of NPs, the role of these proteins in inflammatory processes and cell apoptosis needs to be further investigated.

297 On the other hand, *Bcl2a* belongs to the family of anti-apoptotic genes whose function is to inhibit pro-
298 apoptotic genes (Youle et al., 2008). In this study, we evaluated the response of *Bcl2a* mRNA expression
299 after exposure of Zfe to NPs. The results show a down-regulation of its expression at 0.5 and 3 ppm,
300 only significant at the highest concentration used (Figure 5). These results suggest that NPs inhibit the
301 expression of this anti-apoptotic gene. The down-regulation of this gene supports the result obtained in
302 our study with the antiapoptotic gene *hsp70*. In both genes (*hsp70* and *Bcl2a*) an inhibition of mRNA
303 expression is observed. The down-regulation of these antiapoptotic genes may be related to an activation
304 of apoptosis. It would suggest that NP inhibit the expression of these antiapoptotic proteins. It is likely
305 that the down-regulation of both anti-apoptotic genes (*hsp70* and *Bcl2a*) is due to altered apoptosis. The
306 activation of apoptosis may need inhibition of antiapoptotic proteins. The increased mRNA levels
307 observed for *cas 1* and *cas 8* support this idea because they are key proteins in apoptotic signal
308 transduction and execution (Spead et al., 2018). A previous study in Zfe has shown that NPs induce the
309 expression of other apoptosis-related genes (Bhagat et al., 2021). It can be suggested that activation of
310 caspases and down-regulation of *hsp70* and *Bcl2a* genes show the progression of apoptosis. The failure
311 of DNA repair genes to be activated could explain, in part, the activation of apoptosis as a consequence
312 of the cell's failure to repair DNA double-strand breaks. All these events could be the consequence of
313 elevated ROS production that can stimulate apoptosis and inflammation (Lu et al., 2018; Redza-
314 Dutordoir and Averill-Bates, 2016). Further research is needed to gain insight into each of the above
315 proposed mechanisms.

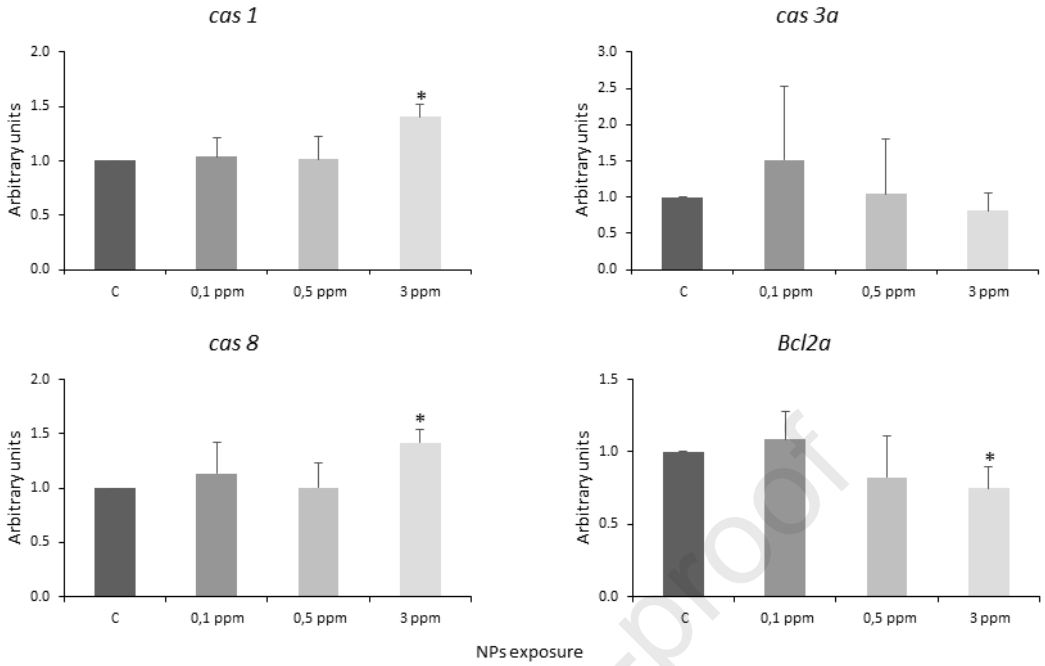

Fig. 5. Expression of *caspases (1, 3a* and *8)* and *Bcl2a* genes. Asterisks indicate statistical differences compared to controls ($p < 0.05$).

3.8. Inflammatory, neurotransmission and mitochondrial response: *il1β, ache* and *cox1*

In addition, other genes, *il1β*, *ache* and *cox1*, related to inflammation, neurotransmission, and mitochondrial metabolism, were analysed (Figure 6). Our results show that *Il1β* was up-regulated, in concentration-dependent manner, in Zfe exposed to NPs. Moreover, at the highest concentration of NPs the expression of *il1β* is significantly increased. Pathogens entering an organism can trigger a nonspecific inflammatory response that is mediated by the proinflammatory cytokine interleukin-1β (Il1β). The response of cells to the presence of NPs could generate an inflammatory response like that of an infection. Activation of the inflammatory response has been previously described in Zf exposed to MPs (Qiang and Cheng, 2019) and NPs (Bhagat et al., 2021, Brun et al., 2018). Our results are in agreement with these findings, suggesting that NPs could activate an inflammatory response in the cell. Taken together, our results show that NPs induce the expression of three proteins involved in inflammatory response: CAS 1, CAS 8 and IL1β. Caspase-1 has an inflammatory function that is activated by inflammasome complexes in response to pathogens or endogenous factors. The

inflammasome promotes the maturation of proinflammatory cytokines such as interleukin 1-β and interleukin-18 (Sun et al., 2016). Recent studies show that caspase-8 has several key roles in inflammation and immunity (Orning and Lien, 2021).

Acetylcholinesterase (ache) is an enzyme belonging to the cholinesterase family and its primary function is to degrade Acetylcholine (ache) and terminate neurotransmission. This enzyme is essential for the proper functioning of the nervous system of both vertebrates and invertebrates (Massoulié et al., 1993, Deng et al., 2017; Lei et al., 2020; Chen et al., 2017; Sarasamma et al., 2020). The inhibition of its activity leads to the accumulation of acetylcholine and to very severe muscle deterioration (Massoulié et al., 1993). Our results at the molecular level showed that the expression of *ache* mRNA was inhibited. Especially significant down-regulation occurs at the two highest concentrations of NPs used. These dysregulations raise the possibility that exposure to NPs may induce adverse effects on neurotransmission. Previous studies in several marine organisms such as *Hediste diversicolor* (Missawi et al., 2021), *Scrobicularia plana* (Ribeiro et al., 2017), *Mytilus galloprovincialis* (Avio et al., 2015) and *Eriocheir sinensis* (Yu et al., 2018) showed neurotoxicity induced by environmental MPs due to inhibition of AChE activity. Recent reports suggest that MPs and NPs can inhibit ache enzymatic activity and generate changes in neurotransmitter levels (Prüst et al., 2020). Our data are in agreement with previous results obtained with NPs in the down-regulation of ache mRNA levels in Zfe (Bhagat et al., 2021). Future research should be conducted to further investigate the neurotoxic effects of these materials.

Cytochrome c oxidase (COX) has a regulatory role in electron transport (Little et al., 2010). However, little is known about the role of COX1 in the response to pollutants. Some studies have shown that many drugs and xenobiotics inhibit COX activity and produce mitochondrial stress, but their mechanisms of action are unknown (Chandran et al., 2009; Srinivasan et al., 2012). There are no previous data in the literature on *cox1* expression in response to NPs in Zfe. However, Parenti et al (2019) found decreased COX activity in Zfe exposed to 500 nm NP, and other mitochondria alterations have been observed in response to NP exposure (Trevisan 2019, 2020). Our results showed that Zfe exposed to the

concentrations and times of the study did not modify *cox1* expression. These results contradict previous data and possible differences in NP size and/or concentrations could be responsible for these differences.

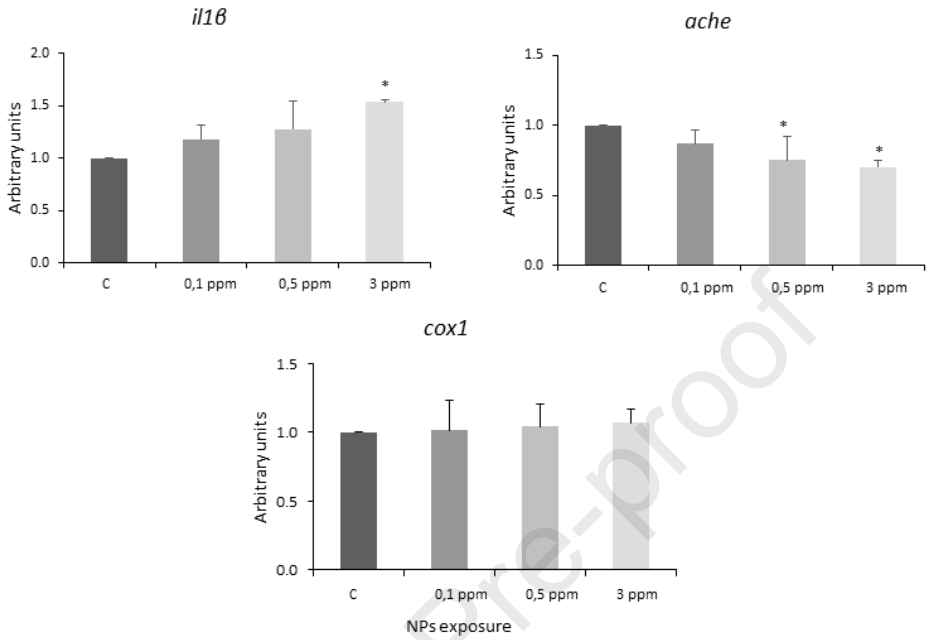

Fig. 6. Expression of *Il1β*, *ache* and *cox1* genes. Asterisks indicate statistical differences compared to controls ($p < 0.05$).

4. Conclusions

In this study, the effects of PS NPs on oxidative stress response genes, cellular stress, DNA damage, apoptosis, anti-apoptosis, inflammatory, mitochondrial and neurotoxic responses were evaluated.

The PS NPs concentrations used in this study did not affect Zfe mortality but were able to alter the expression of genes involved in oxidative stress, anti-apoptosis, inflammatory response, apoptosis, and neurotoxicity. The greatest changes in gene expression were observed after exposures to the highest concentration of NPs, although this concentration is very possible in nature. Significant changes were observed in *hsp70*, *SOD 1*, *SOD 2*, *cas 1*, *cas 8*, *Bcl2a*, *il1β* and *ache* genes. The expression of *hsp70*, *il1β* and *ache* was modified in a dose-dependent manner at the concentrations studied. The NPs did not affect the expression of *hsp27*, *hsp90*, *cat* and *cox1* genes.

Taken together, our results of this work suggest that NPs may induce oxidative stress that could lead to activation inflammatory response and apoptosis, together with inhibition of anti-apoptotic genes. In

addition, these nanomaterials affect neurotoxicity. However, the specific mechanisms of action of these emerging contaminants need to be studied in more detail. Further research is needed to unravel the relevance of each of the mechanisms postulated above. Our results show the advantages of molecular techniques to assess the effect of NPs and show the risk that these emerging pollutants pose to living organisms.

Credit author statement

All authors contributed to the conception and design of the study. Raquel Martín Folgar: Conceptualization, Methodology, Analysis, Research, Writing - preparation of the original draft, Writing - review and editing Resources. Mónica Torres-Ruiz: Methodology, Analysis, Research, Writing - review and Characterization of carbon nanomaterials. Ana I. Cañas-Portilla: Methodology, Writing - review and Supervision. Mercedes de Alba: Methodology, Analysis, Research, Writing – review. Mª Carmen González: Methodology, Analysis, Research. Monica Morales: Conceptualization, Methodology, Writing of original draft, Preparation, Supervision.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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- PS NPs exposures did not significantly affect Zfe mortality
- NPs increased oxidative stress genes *(SOD 1*, *SOD 2)*
- NPs down-regulated antiapoptotic genes (*hsp70*, *Bcl2a)*
- DNA repair (*gadd45α, rad51*) and mitochondrial (*cox1*) genes were not altered
- NPs activated the inflammatory and apoptosis response through *cas1*, *cas8* and *IL1β*

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: