



# Polystyrene and Polyethylene Microplastics Decrease Cell Viability and Dysregulate Inflammatory and Oxidative Stress Markers of MDCK and L929 Cells In Vitro

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## Abstract

Microplastics are ubiquitous environmental pollutants that are a growing concern to many ecosystems, as well as human health. Many of the effects of microplastics on mammalian cells and tissues remain unknown. To address this, we treated L929 murine fibroblasts and Madin–Darby canine kidney (MDCK) epithelial cell lines with 1 µg/mL, 10 µg/mL, or 20 µg/mL of polyethylene (PE) or polystyrene (PS) microspheres in vitro for 6 and 24 h and measured the resulting changes in cell viability, metabolism, and transcriptional expression of inflammatory cytokines and antioxidant enzymes. We observed dose-dependent decreases in cell viability corresponding to increases in doses of both PE and PS. We conducted cell metabolism assays and observed dose-dependent increases in metabolism per cell with increasing doses of both PE and PS. Similarly, we also observed increased expression of the superoxide dismutase-3 gene (*SOD3*), indicating oxidative stress caused by the microplastics treatments. We also observed increased expression of *TNFα*, but decreased expression of *IFNβ*, suggesting different mechanisms by which the microplastics regulate inflammatory responses in mammalian cells. Our results contribute new data to the growing understanding of the effects of microplastics on mammalian cells and indicate complex cellular stress responses to microplastics in the environment.

**Keywords** Microplastics · Cell viability · Oxidative stress · Cellular metabolism · Inflammatory cytokines

## Introduction

Microplastics are increasingly recognized as ubiquitous pollutants in the environment, with the same properties that make them durable and desirable synthetic organic polymers also conferring on them the ability to persist and spread. Microplastics 5 mm and smaller decompose slowly, with ultraviolet radiation, hydrolysis, and physical degradation converting them to fragments smaller than 0.0001 mm,

known as nanoplastics, as defined by the European Commission (Alexy et al. 2020).

Microplastics have been found to be able to enter tissues of living organisms, including humans, via ingestion, inhalation, and topical absorption (Deng et al. 2017; Huffer et al. 2018; Pivokonsky et al. 2018; Prata et al. 2020; Zarus et al. 2021; Zhang et al. 2020b). Although the full spectrum of their effects and mechanisms is yet to be determined, microplastic bioaccumulation in multiple biota through higher trophic levels has the potential to amplify their effects by their increasing dosage in top predators (such as humans) and can affect functions as varied as feeding behaviors, metabolism, and fecundity (da Costa Araujo and Malafaia 2021; Foley et al. 2018). Sampling different environments and geographic locations has shown microplastic burdens of varying abundance, types, sizes, shapes, and ages, contributing to a variety of health dangers including bacterial antibiotic resistance (Su et al. 2021; Wang et al. 2020).

As synthetic human-made polymers, microplastic contamination of the environment has numerous diverse sources of origin, including car tires, effluents from clothes washers

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and dryers, and the slow degradation of littered cigarette butts (Belzagui et al. 2021; Karkkainen and Sillanpaa 2020; Koski et al. 2021; Mengistu et al. 2021). Microplastics are not always byproducts, as they can be incorporated specifically for their properties into products such as coatings, cosmetics, cleaning agents, and medical applications (Zarus et al. 2021). Thus, humans have many routes of microplastics exposure such as food (Al-Sid-Cheikh et al. 2018; Razeghi et al. 2021; Ribeiro et al. 2020; Zarus et al. 2021), food packaging (Zarus et al. 2021), medical implants (Hicks et al. 1996; Urban et al. 2000), and baby bottles (Li et al. 2020a), and multiple studies have found microplastics in human stool samples (Schwabl et al. 2019; Zhang et al. 2020a). Estimates put human ingestion of microplastics at 40,000–50,000 particles per person per year (Cox et al. 2019), increasing to 70,000–120,000 particles when including inhalation (Cox et al. 2020; Prata 2018), with possible further exacerbation due to recent increased synthetic mask-wearing due to the COVID-19 pandemic (Li et al. 2020c).

Studies have found that after ingestion microplastics can undergo persorption from the gut to the lymphatics and onto other tissues (Hussain et al. 2001; Volkheimer 1975). Similar translocations of nanoplastics have been found to originate from the lungs via inhalation (Choi et al. 2010). Animal models have found microplastic accumulation in mammalian male (Jin et al. 2021) and female (An et al. 2021; Hou et al. 2021) gonadal tissues, possibly affecting fertility and reproductive capacity. Inside the body, in addition to their physical and mechanical interactions, microplastics can also leach chemical into tissues and circulation, including chemicals applied during manufacturing (e.g., bisphenols, phthalates, perfluorinated compounds, brominated flame retardants, UV stabilizers) (Cox et al. 2019) and for aged plastics, substances absorbed and accumulated from the environment (e.g., organic pesticides, polyaromatic hydrocarbons, heavy metals, and pathogens) (Alimba and Faggio 2019; Cox et al. 2019; Scopetani et al. 2018; Torres et al. 2021).

Until recently, due to frequent pollution of lakes, rivers, and oceans, research articles often focused on wild or model aquatic organisms such as mussels, urchins, and fish to analyze the effects of microplastics (Akhbarizadeh et al. 2018; Browne et al. 2008; Nobre et al. 2015). With increased awareness of the prevalence of microplastics in many of the environments with which humans are in direct and intimate contact, research has shifted from analysis of fish and invertebrates to mammalian animal models and cell lines. Among these, *in vivo* studies have revealed disruptions of gut homeostasis (Choi et al. 2021b; Liang et al. 2021), increased reactive oxygen species generation and inflammation (Ijaz et al. 2021), genotoxicity and lipotoxicity (Deng et al. 2017; Gonzalez-Acedo et al. 2021), and exacerbated bone resorption (Markel et al. 2009). *In vitro* experiments (often using human or rodent cell lines) have similarly demonstrated

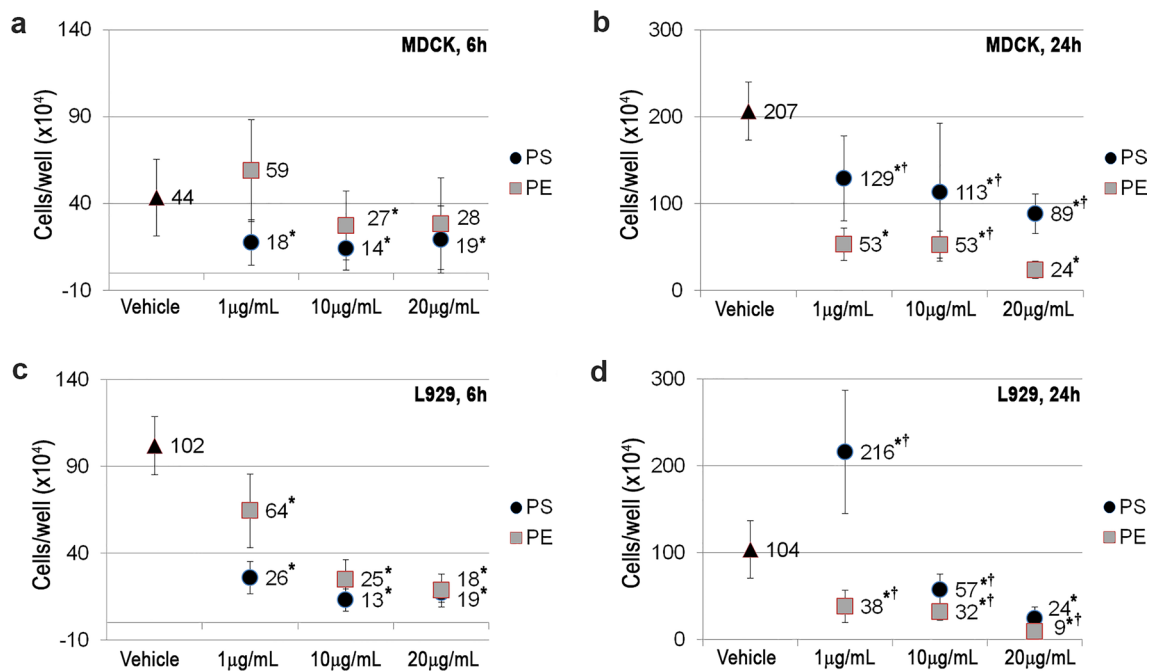
the adverse effects of microplastics, including immune cell activation (Pearl et al. 2011), inflammatory cytokine release (Schirinzi et al. 2017), bone resorption activity (Zhang et al. 2008), decreased survival (Van Tienhoven et al. 2006), cytostructural perturbations and neurotoxicity (Gonzalez-Acedo et al. 2021), and loss of phenotypic cellular behaviors (Lee et al. 2021).

Despite the existing knowledge about microplastics on body burdens and potential impacts on physiology, there are, as of yet, many more molecular mechanisms to be elucidated by which they can impact mammalian health at the cellular level. Furthermore, specific microplastic types, sizes, and shapes can impact various organisms and tissue types differently (Blackburn and Green 2021). Therefore, the goal of this study was to measure the impacts of pristine, virgin microspheres of polystyrene (PS) and polyethylene (PE) on the viability, metabolism, and the transcriptional expression changes in inflammatory and oxidative stress genes of the cell lines MDCK (Madin–Darby canine kidney epithelial cells) and L929 (murine fibroblast cells). The two cell lines were chosen to represent two different mammalian organisms as well as two tissue types (epithelial lining cells and mesenchymal connective tissue cells). The viability, metabolism, and inflammatory and oxidative stress gene expression endpoint assays were selected, as they were oft-observed as targets of dysregulation in previous research studies. Two timepoints were chosen to represent the impacts of the microplastics on the cells, a short-term treatment of 6 h and a longer-term treatment of 24 h. Based on the literature, we hypothesized that there would be differences between the effects of PS and PE on the two cell types on their metabolic activity and inflammatory cytokine gene expression, but that they would be similarly affected in their viability with increased cytotoxicity corresponding to microplastic dosage.

## Results

### Microplastics Decreased Cell Viability in a Dose-Dependent Manner

Relative to vehicle controls, the majority of microplastic treatment conditions, in both the 6 h and 24 h modalities, reduced viability in both cell lines, as measured via hemacytometer (Fig. 1). The two exceptions were both observed in the lowest concentration conditions, at 1 µg/mL PE at 6 h on the MDCK cells (although this was not statistically significant compared to vehicle control), and at 1 µg/mL PS at 24 h on the L929 cells, which was statistically significant (Fig. 1a and d). In almost all of the microplastic treatment conditions, the trend followed a dose-dependent decrease in cell viability, with the highest doses showing the greatest decreases in absolute cell numbers per well. The difference



**Fig. 1** PS and PE microplastics decreased the viability of MDCK and L929 cells in a dose-dependent manner. MDCK (a, b) and L929 (c, d) cells were treated with PS (filled circle) or PE (gray square) microplastics, or vehicle control (filled triangle) for 6 h (a, c) or 24 h (b, d) at 1 µg/mL, 10 µg/mL, or 20 µg/mL, and their viability was measured by total cell count per well. Each experiment was conducted in bio-

logical and technical duplicates. Error bars represent standard deviations. \*Statistical significance of  $P$  values  $< 0.05$ , as determined by student's  $t$  test for each condition relative to vehicle control (vehicle control values = 100%). †Statistical significance of  $P$  values  $< 0.05$  for each condition at 24 h relative to 6 h

in total cells per well between 6 and 24 h for each condition indicates that there was continued proliferation despite the short-term impacts of the microplastics on viability, but that the effects also extended the dose-dependent trend of suppressing viability to suppressing proliferation as well.

### Microplastics Increased Cell Metabolic Rates in a Dose-Dependent Manner

Cell metabolic rates were measured via MTT assay, whose reduction to an insoluble colorimetric precipitate is carried out by mitochondrial and cytoplasmic oxidoreductive enzymes that utilize rate-limiting NADH and NAD(P)H substrates, corresponding to metabolic rate. When normalized to total cells per well, the results of the MTT assay showed mostly dose-dependent increases with microplastics treatments. For both PS and PE, and at 6 h and 24 h, the general trend was increasing metabolic rates as microplastics concentrations were increased, relative to vehicle controls. One of the exceptions to this was observed in the 6 h treatments at 20 µg/mL for both MDCK and L929, which was still much higher than vehicle control conditions, but decreased slightly relative to the 10 µg/mL dose. Two of the experimental conditions (PE at 6 h on the MDCK cells and PS at 24 h on the L929 cells) showed initial decreases in metabolic activity at

1 µg/mL relative to vehicle controls, but were still part of the dose-dependent increases in metabolic rate within their groups. Interestingly, the highest impact on the increase in cell metabolic activity was observed with PE treatment at 20 µg/mL at 24 h for both MDCK and L929 cells, with 8.3-fold and 10.8-fold increases, respectively, relative to vehicle control.

### Microplastics Decreased IL1 $\beta$ and Increased TNF $\alpha$ Inflammatory Cytokine Transcripts

The transcriptional expression of two cytokines, *IL1 $\beta$*  and *TNF $\alpha$* , were measured by qPCR to determine the potential inflammatory effects of microplastics on the two cell lines. As seen with cell viability and metabolism, the expression of the cytokines followed a dose-responsive trend, either directly or inversely correlating with the microplastics doses. However, the dose-dependent trends for the inflammatory cytokine expression changed in a differential manner, varying with cell type, the microplastic type, and occasionally with the 6 h versus 24 h treatment modalities.

Some of the dose-dependent trends that showed increases in cytokine expression, such as with *IL1 $\beta$*  in PS treatment of L929 cells, PE treatment of MDCK cells, and PE treatment of L929 cells at 6 h, had their highest magnitude fold

changes at the 1 µg/mL dose, suppressed below that of vehicle control. In these conditions, the dose-dependent increase actually brought the *IL1β* expression close to baseline by the 20 µg/mL dose. Similarly, the dose-dependent decrease of *TNFα* expression with the PS treatment of MDCK cells at 6 h started with the highest positive magnitude fold change at 1 µg/mL, and the decreasing trendline by 20 µg/mL brought its expression down to baseline.

In the MDCK cells, it was interesting to note that at both 6 h and 24 h PS treatment induced a dose-dependent decrease in both *IL1β* and *TNFα*, and PE treatment induced a dose-dependent increase in both *IL1β* and *TNFα*.

Two generalized observations could also be made about the expression patterns of both *IL1β* and *TNFα*. Although each cytokine displayed dose-dependent changes in expression, *IL1β* expression was almost always below the baseline, indicating that both microplastics suppressed its expression, as compared with vehicle control. Alternatively, the majority of the experimental conditions showed that *TNFα* was increased relative to vehicle control, with the exception of 20 µg/mL PS treatment of MDCKs, and 10 and 20 µg/mL PS treatments of L929s, which were below baseline.

### Microplastics Increased SOD3 Antioxidant Enzyme Transcripts

To evaluate whether the microplastics potentially exerted any oxidative stress on the MDCK and L929 cells, the transcriptional expression of the antioxidant enzyme *SOD3* was measured by qPCR. In most of the experimental conditions, microplastics-induced *SOD3* expression was increased relative to vehicle control cells, albeit its expression exhibited dose-dependent trends similar to those of the inflammatory cytokines. *SOD3* expression was significantly decreased relative to controls in the MDCK cells by PS treatment at 6 h, at 1 µg/mL and 10 µg/mL, and by PE at 6 h, at 1 µg/mL. One of the patterns observed in the dose–response differences between the two cell lines was their 6 h response to both PS and PE, with the MDCK cells' expression trendline showing increased *SOD3* transcripts directly corresponding to dosage, and the L929 cells showing decreasing *SOD3* expression, inversely correlated to dosage. It was also interesting to note that the highest magnitude response in *SOD3* expression, 7.8-fold induction, occurred at 6 h and at the lowest concentration (1 µg/mL) with PE treatment on the L929 cells.

### Discussion

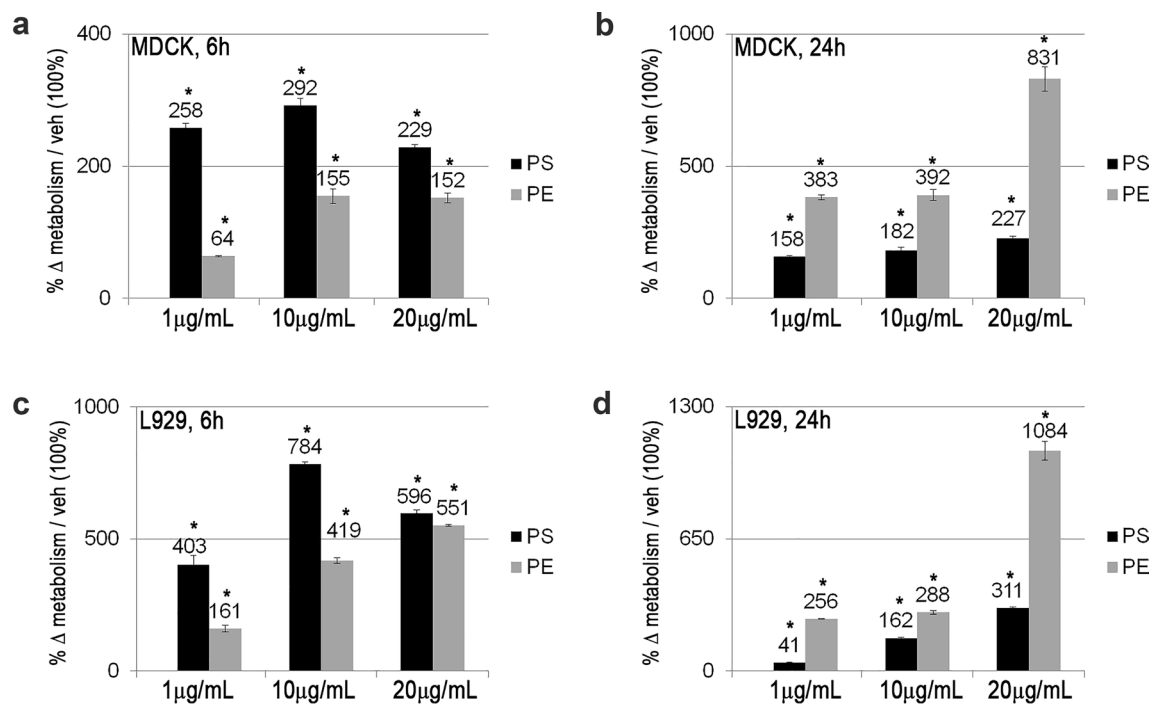
In this study, we report that microplastics treatments of two mammalian cell lines yielded dose-dependent decreases in cell viability, increases in cell metabolism and expression of

the superoxide dismutase-3 gene, and differential expression of inflammatory cytokine genes, with increased *TNFα* and decreased *IL1β*.

Microplastics can impact cells and organisms directly and indirectly, physically and chemically, and via an array of harmful effects, including irritation, oxidative damage, impairment of digestion, genotoxicity, metabolic changes, chemical leaching, and fluctuations of the microbiome (Deng et al. 2017; Ibrahim et al. 2021; Jin et al. 2019; Kumar et al. 2020). As microplastics age and weather in the environment, their properties such as size, shape, surface charges, and surface-bound groups can change, significantly altering the mechanisms with which they interact with cells and tissues, including the ability to be endocytosed (Brachner et al. 2020; Mahadevan and Valiyaveetil 2021; Ramsperger et al. 2020; Zarus et al. 2021). For this study we used virgin, pristine microspheres of PS and PE to decrease the size range and to avoid the variables of different shapes, surface charges and groups, as well as foreign chemicals absorbed from the environment. Although both the PE and the PS were supplied as microspheres of the pure polymers, presumably without plasticizers or copolymers, the leaching of trace amounts of monomers or catalysts used in the manufacture each plastic type cannot be ruled out. The size of the microspheres used in this study was also 1 µm or greater, decreasing the possibility of their endocytosis (Mahadevan and Valiyaveetil 2021), although the measurement of this property was beyond the scope of this study. Thus, the most likely mechanisms by which the microplastics impacted the cells used in this study were physical interactions with the cells and their adjacent in vitro environments.

In the current study, our analysis demonstrated a significant, positively correlated dose-dependent decrease in cell viability at both the 6 h and 24 h timepoints of the assay (Fig. 1). Previous research has shown the toxic effects of microplastics on cells and tissues, with effects such as DNA damage and mitochondrial dysfunction, and these underlying mechanisms, as well as activation of apoptotic pathways, may have contributed to the decreased cell viability observed here (Della Torre et al. 2014; Estrela et al. 2020; Li et al. 2020d; Wei et al. 2021).

The increased metabolic flux measured by our MTT assays (Fig. 2) indicate increased activation of the redox enzymes that utilize NADH and NAD(P)H substrates, albeit the localization of these enzymes within the cytoplasmic or mitochondrial compartments were beyond the scope of this study. However, if the cellular stress responses upregulated the mitochondrial CYP and cytochrome oxidase enzymatic activities, it could explain the corresponding increases in MTT processing per cell observed here (Ding et al. 2020; Wen et al. 2018). The induction of the superoxide dismutase-3 gene (Fig. 4) corroborates this possibility. Alternatively, as observed in some physiologic studies of



**Fig. 2** PS and PE microplastics increased the metabolic enzyme activity of MDCK and L929 cells in a dose-dependent manner. MDCK (**a, b**) and L929 (**c, d**) cells were treated with PS (black bars) or PE (gray bars) microplastics, or vehicle control for 6 h (**a, c**) or 24 h (**b, d**) at 1 µg/mL, 10 µg/mL, or 20 µg/mL, and the activity of their NADH- or NAD(P)H-dependent metabolic enzymes was measured by MTT assay. MTT measurements were normalized to cells per

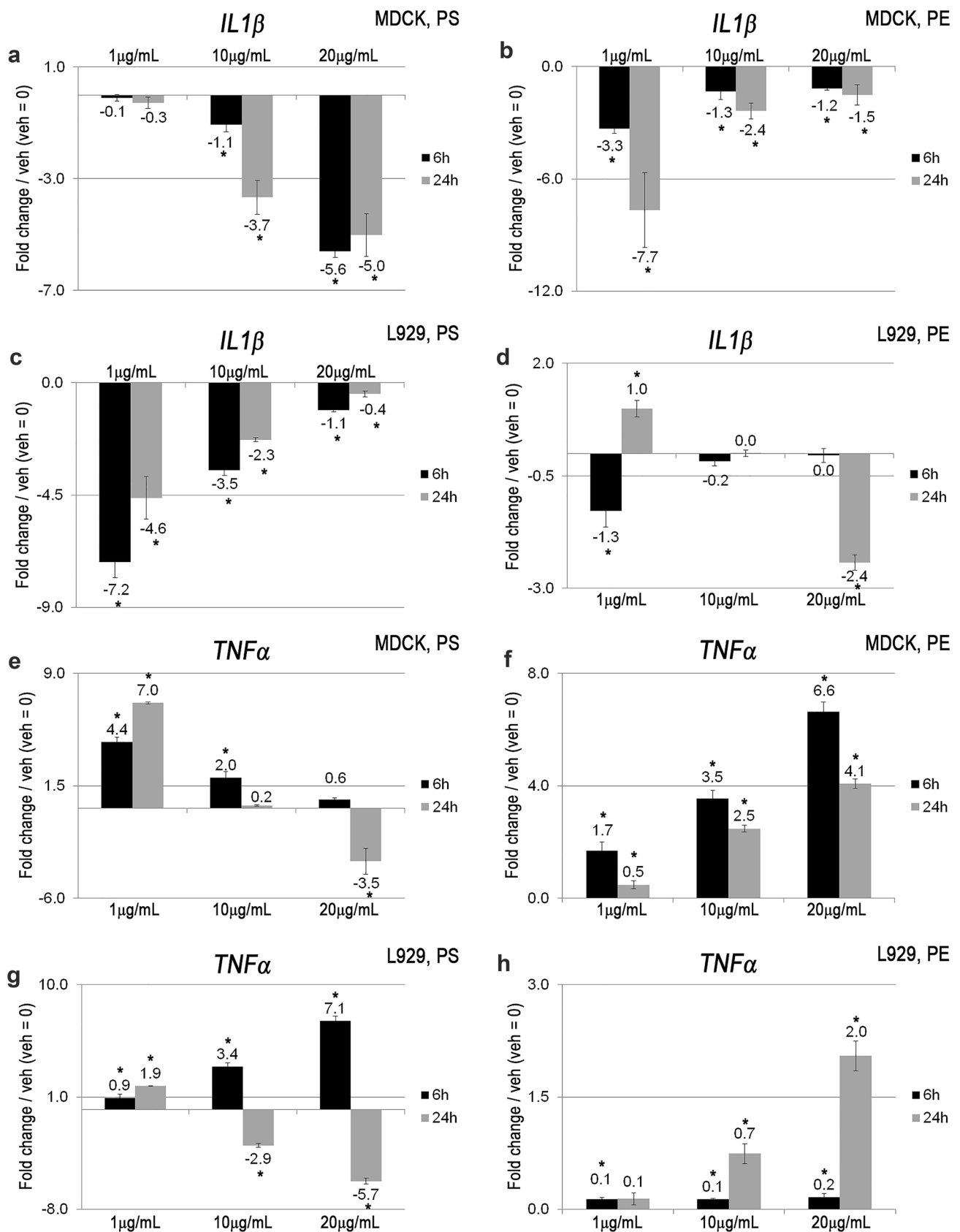
well, and % change was calculated by comparing to the average of vehicle control wells (vehicle control values=100%). Each experiment was conducted in biological and technical duplicates. Error bars represent standard deviations. \*Statistical significance of  $P$  values < 0.05, as determined by student's  $t$  test for each condition relative to vehicle control

microplastics, metabolic dysfunction can shift the availability of ATP or cause a switch in cell fuel sources, causing the cells to react by increasing energetic pathways to compensate (Deng et al. 2017; Jin et al. 2019; Lu et al. 2018).

Although the changes in expression of inflammatory cytokine genes followed dose-dependent trends (Fig. 3), the increase in *TNF $\alpha$*  concomitant with a decrease in *IL1 $\beta$*  may be indicative of the mechanistic differences in how the cells react to the presence of the microplastics in their environment. Although the expression of both *TNF $\alpha$*  and *IL1 $\beta$*  can both be co-regulated by the NF- $\kappa$ B pathway downstream of toll-like receptors (TLRs) and the recognition of damage-associated or pathogen-associated molecular patterns (DAMPs and PAMPs, respectively) (Oeckinghaus and Ghosh 2009), the upregulation of one inflammatory gene and downregulation of the other may be due to changes in expression of co-transcription factors such as HIF1 $\alpha$  or AP-1 (Roy et al. 2016; Walmsley et al. 2014; Wen and Ting 2013), or microRNAs (Li et al. 2020b; Shen et al. 2020; Zhao et al. 2020). However, previous studies have found diverse microplastic-induced inflammatory gene modulations, lending further credence to our findings (Choi et al. 2021a; Wu et al. 2020).

Our study also found that the microplastics overall increased the gene expression of the secreted form of superoxide dismutase (*SOD3*), an indication that the cells were responding to extracellular oxidative stress at both timepoints (Fig. 4), albeit it was beyond the capabilities of our study to confirm the increased secretion or activity of *SOD3* enzyme in the extracellular milieu. This finding is also supported by previous studies that found oxidative stress induced by microplastic-mediated interference in mitochondrial function, suppression of cellular antioxidant systems, and increased generation of reactive oxygen species (Abidli et al. 2021; An et al. 2021; Chen et al. 2021; Cortés et al. 2020; Liu et al. 2020; Wei et al. 2021).

In conclusion, our study provides new data regarding the effects of PE and PS on mammalian cells. With the plethora of effects that can arise from the myriad of variables and interactions between plastics, the environment, and target organisms and tissues, much work remains to be done to gain a broader understanding of the mechanisms of toxicity of microplastics. More thorough studies that include other plastic types and cells representing other mammalian tissues will be needed to shed further light on microplastic toxicity.



**Fig. 3** PS and PE microplastics decreased the transcriptional expression of *IL1 $\beta$*  and increased the expression of *TNF $\alpha$*  inflammatory cytokines. MDCK (**a** and **b**, **e** and **f**) and L929 (**c** and **d**, **g** and **h**) cells were treated with PS or PE microplastics, or vehicle control for 6 h (black bars) or 24 h (gray bars) at 1  $\mu\text{g}/\text{mL}$ , 10  $\mu\text{g}/\text{mL}$ , or 20  $\mu\text{g}/\text{mL}$ , and whole cell lysates from each well were used to obtain the transcriptomes, which were converted to cDNA for qPCR analysis of *IL1 $\beta$*  (**a–d**) and *TNF $\alpha$*  (**e–h**) expression. The comparative  $C_T$  ( $2^{-\Delta\Delta C_T}$ ) method was used to determine fold changes relative to vehicle control (control=0.0). Each experiment was conducted in biological and technical duplicates. Error bars represent standard deviations. \*Statistical significance of  $P$  values < 0.05, as determined by student's  $t$  test for each condition relative to vehicle control

## Materials and Methods

### Cell Culture

Madin–Darby canine kidney (MDCK) epithelial and L929 mouse fibroblast cell lines were grown in complete EMEM (Lonza, Walkersville, MD): supplemented with 10% bovine calf serum (Avantor Seradigm, Radnor, PA) and 1X penicillin–streptomycin–amphotericin-B (Lonza). Cells were grown at 37 °C, 5% CO<sub>2</sub>, and passaged 1:4 at ~70% confluence using 0.25% trypsin (Cytiva Hyclone, Logan, UT). For experiments, cells were seeded in 6-well plates (Greiner Bio-One, Monroe, NC) at  $1 \times 10^4$  cells/well and grown 24 h to reach log growth phase before experimental treatments.

### Microplastics Exposure

1.0–4.0  $\mu\text{m}$  clear polyethylene microspheres and 9.5–11.5  $\mu\text{m}$  polystyrene microspheres (Cospheric, Santa Barbara, CA) were resuspended in complete EMEM + 0.01% tween-20 at 1.0, 10.0, or 20.0  $\mu\text{g}/\text{mL}$  concentrations, and complete EMEM + 0.01% tween-20 without microplastics was used for vehicle control treatments. Concentrations of PS & PE used to dose the cells were based on similar doses described in prior research articles (Hwang et al. 2020; Schirinzi et al. 2017). Cells at their logarithmic growth phase had their media removed, and 2 mL fresh media containing the various microplastic concentrations or vehicle control media was added for 6 h or 24 h. Cell cultures and microplastic treatments were conducted separately and independently for each downstream assay. All treatment conditions and exposure times were conducted in biological duplicates, each of which was subsequently assayed in technical duplicates. For each downstream assay, the statistical significance was determined by comparing the four resulting data points for each experimental condition to the four respective vehicle control data points.

### Cell Viability Assay

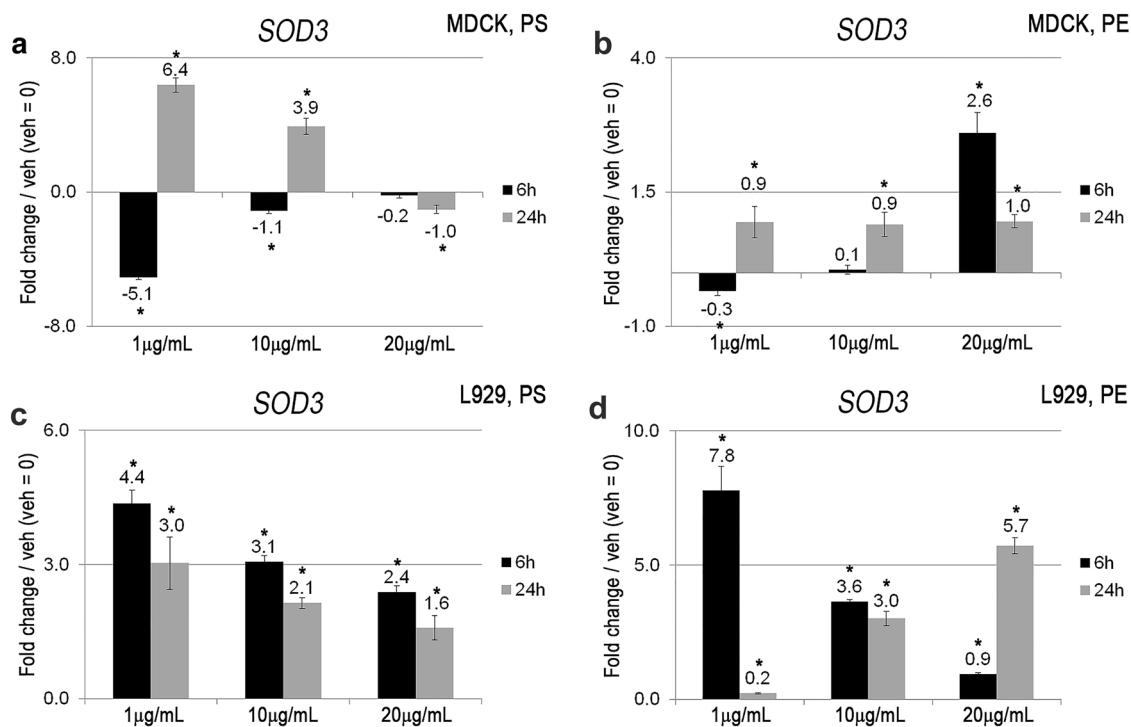
The effects of the microplastics on the viability of cell lines in culture were measured by counting the cells after 6 h or 24 h incubation. Cells treated with microplastics or vehicle controls were washed three times with PBS, detached using trypsin, and resuspended in 2 mL complete EMEM. Cells were counted using a hemacytometer (Sigma-Aldrich, Saint Louis, MO). Changes in cell viability with each microplastic concentration were compared for statistical significance relative to the vehicle control wells for each cell line, at each time point.

### MTT Assay

To quantify changes in cellular metabolic activity, NAD(P)H-dependent enzymatic reduction of the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; MP Biomedicals, Solon, OH) to insoluble formazan precipitate was measured. Stock MTT solution was prepared in PBS at 5 mg/mL. 200  $\mu\text{L}$  of stock MTT was added to the 2 mL complete cell media for a working concentration of ~500  $\mu\text{g}/\text{mL}$ , and incubated during the final 4 h of each treatment condition. Cells were washed three times with PBS and 500  $\mu\text{L}$  dimethylsulfoxide (DMSO) was added to each well to dissolve the formazan, the plates were covered with aluminum foil, and gently agitated on a nutating shaker for 15 min, and optical densities (OD) at 540 nm were measured on a Spectramax i3x spectrophotometer (Molecular Devices, Sunnyvale, CA). Background OD measurements were subtracted out using DMSO from atop cells without MTT incubation, and changes in metabolic activity were calculated relative to respective vehicle control-treated cells never exposed to microplastics. The MTT assay results for each condition were normalized to their respective number of cells per well.

### Quantitative Real-Time PCR Assays (qPCR)

Analysis of relative change in gene transcripts was performed by qPCR assay. Phenol–chloroform RNA extraction was performed with Ribozol (Avantor VWR, Solon, OH) using manufacturer's protocol. Under RNase-free conditions, at the conclusion of each experimental treatment, cells were washed three times with PBS (Lonza), 650  $\mu\text{L}$  of Ribozol was directly added to each well and triturated. Mixtures were collected in 1.5 mL tubes (USA Scientific, Ocala, FL) and 140  $\mu\text{L}$  of chloroform was added to each tube, mixed vigorously, and incubated 3 min at room temperature. Tubes were centrifuged at 12,000 $\times g$  for 15 min at 4 °C, the aqueous phase was transferred to new tubes, and RNA was precipitated by addition of 500  $\mu\text{L}$  isopropanol. Precipitated RNA was pelleted by 12,000 $\times g$  centrifugation for 5 min



**Fig. 4** PS and PE microplastics increased the transcriptional expression of the *SOD3* antioxidant enzyme. MDCK (a, b) and L929 (c, d) cells were treated with PS (a, c) or PE (b, d) microplastics, or vehicle control for 6 h (black bars) or 24 h (gray bars) at 1 µg/mL, 10 µg/mL, or 20 µg/mL, and whole cell lysates from each well were used to obtain the transcriptomes, which were converted to cDNA for qPCR

at 4 °C, the pellet was washed three times with 75% ethanol, and resuspended in 50 µL water. RNA concentrations were obtained using a Nanodrop One-C spectrophotometer (Thermo Scientific, Waltham, MA), and 4 µg equalized volumes of RNA from each sample were used to create cDNA libraries using qScript Supermix (Quantabio, Beverly, MA). For the genes of interest, primer sequences were as follows (5'–3'): *Sod3* Fwd, ATGGTGGCCTTCTTGTTCTGC; *Sod3* Rev, GTGCTGTGGGTGCGGCACACC; *I8S* Fwd, CGGACAGGATTGACAGATTG; *I8S* Rev, CAAATCGCTCCA CCAACTAA; rat *I11B* Fwd, TGAAAGCTCTCCACCTCA ATGGAC; rat *I11B* Rev, TGCAGCCATCTTTAGGAAGAC ACG; rat *TnfA* Fwd, AGCACAGAAAGCATGATCCGAG; rat *TnfA* Rev, CCTGGTATGAAGTGGCAAATCG; canine *I11B* Fwd, TGCAAAACAGATGCGGATAA; canine *I11B* Rev, GTAACCTGCAGTCCACCGATT; canine *TnfA* Fwd, CGTCCATTCTTGCCCAAAC; canine *TnfA* Rev, AGCCCT GAGCCCTTAATTC. qPCR was performed on a StepOne Plus RT-PCR system (Applied Biosystems, Foster City, CA), using Power SYBR-Green Master Mix (Applied Biosystems), and all biological replicates were run in technical duplicates using 10 ng of template, 15 µL reaction volume. The optimized annealing temperature for all primer pairs

analysis of *SOD3* expression. The comparative  $C_T$  ( $2^{-\Delta\Delta C(T)}$ ) method was used to determine fold changes relative to vehicle control (control=0.0). Each experiment was conducted in biological and technical duplicates. Error bars represent standard deviations. \*Statistical significance of  $P$  values < 0.05, as determined by student's  $t$  test for each condition relative to vehicle control

was 48.0 °C, and the thermal cycling conditions were: stage 1, 95.0 °C for 5 min; stage 2 (40 cycles), step 1, 95.0 °C for 30 s; step 2, 48.0 °C for 30 s; step 3, 74.0 °C for 45 s; stage 3, infinite hold at 4.0 °C. The comparative  $C_T$  ( $2^{-\Delta\Delta C(T)}$ ) method was used to calculate transcript fold changes relative to experimental controls, and changes in expression of genes of interest were normalized to *I8S* housekeeping genes. *I8S* expression remained relatively stable in both vehicle control and microplastics-treated experimental conditions, as determined by  $C(t)$  values (data not shown).

### Statistical Analyses

Student's two-tailed  $t$ -tests were used for pair-wise analyses against controls, and comparisons were considered statistically significant at or below  $P < 0.05$ . Standard deviations were derived from the four data points obtained from each set of biological and technical duplicates.

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**Author Contributions** Conceptualization: [SP]; Data curation: [SP, BR]; Formal analysis: [SP, BR]; Investigation: [SP, BR]; Methodology: [BR]; Project administration: [BR, CMS]; Resources: [SP, BR, CMS]; Software: [SP, BR]; Supervision: [BR]; Validation: [BR]; Visualization: [BR]; Writing (original draft): [SP, BR]; Writing (review & editing): [SP, BR, and CMS].

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**Data Availability** The data, codes, and materials generated and analyzed in this published article are available from the corresponding author upon reasonable request.

## Declarations

**Conflict of interest** All authors certify that they have no affiliation with or involvement in any organization or entity with any financial or non-financial interests in the subject matter or materials discussed in this manuscript.

**Ethical Approval** Not applicable.

**Consent to Participate** Not applicable.

**Consent for Publication** Not applicable.

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