Heliyon 10 (2024) e39426

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Coexposure to microplastic and Bisphenol A exhacerbates damage to human kidney proximal tubular cells

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ARTICLE INFO

Keywords:
Polyethylene-microplastics
Bisphenol A
Kidney tubular cells
Cell damage

ABSTRACT

Microplastics (MPs) accumulate in tissues, including kidney tissue, while Bisphenol A (BPA) is a plasticizer of particular concern. At present, the combined effects of MPs and BPA are unexplored in human renal cells. Therefore, we exposed a proximal tubular cell line (PTECs) to polyethylene (PE)-MPs and BPA, both separately and in combination. When co-exposed, cells showed a significantly reduced cell viability (MTT test) and a pronounced pro-oxidant (MDA levels, NRF2 and NOX4 expression by Western blot) and pro-inflammatory response (IL1β, CCL/CCR2 and CCL/CCR5 mRNAs by RT-PCR), compared to those treated with a single compound. In addition, heat shock protein (HSP90), a chaperone involved in multiple cellular functions, was reduced (by Western Blot and immunocytochemistry), while aryl hydrocarbon receptor (AHR) expression, a transcription factor which binds environmental ligands, was increased (RT-PCR and immunofluorescence). Our research can contribute to the study of the nephrotoxic effects of pollutants and MPs and shed new light on the combined effects of BPA and PE-MPs.

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https://doi.org/10.1016/j.heliyon.2024.e39426
Received 28 August 2024; Received in revised form 8 October 2024; Accepted 14 October 2024
Available online 15 October 2024

1. Introduction

Plastic has become an important material for everyday use due to its versatility, resistance, and relatively low-cost production. These features have contributed to the enormous production of plastic materials, which has increased by 218 times between 1950 and 2022 [1]. However, the durability and growing usage of plastics create a significant waste management problem. Plastic debris accumulates in the environment, and sunlight, wind, and waves continuously break down plastic into smaller particles [2]. Microplastics (MPs) are defined as synthetic solid particles or polymeric matrices with regular or irregular shapes and $\leq$ 5 mm size with functional additives as well as other intentionally and unintentionally added chemicals [3]. MPs are not only secondary byproducts, but can be incorporated into different products, such as personal care and cosmetic products (scrubbing agents, shower gels, creams) [4,5] and medical implants [6]. MPs are ubiquitous in the air [7], water [7], soil [7] and wastewater treatment plant [8], and their presence in the food chain is a cause for serious concern [7]. MPs encompass many substances, including polystyrene, polyethylene terephthalate, polypropylene, polyvinyl chloride and polyethylene (PE).

PE is one of the most widely used plastics in household products, and its fragments have been found in human blood [9], urine [10,11], and several human tissues, including the liver [12], lungs [13], placenta [14], heart [15], and kidney [11]. Moreover, Marfella et al. [16] have recently demonstrated that patients with evidence of PE-MPs in carotid artery plaques are at an increased risk of death and cardiovascular events compared with those without MPs.

Despite the aforementioned evidence, knowledge regarding the impact of MPs (and especially PE-MPs exposure) on human health is still scarce. Further clinical and epidemiological studies are needed to investigate the toxic effects that MPs exert on humans. Nevertheless, several experimental studies have shown that MPs may have harmful effects in different *in vitro* and *in vivo* models, provoking pro-inflammatory, pro-oxidant, and cytotoxic actions, as reviewed by Khan and Jia [17]. Conversely, fewer data have been reported regarding renal toxicity due to MPs [18]. In this regard, it has been found that exposure to PE may affect viability and stimulate oxidative stress, and inflammation of the Madin–Darby canine kidney (MDCK) epithelial cell line [19].

Interestingly, beyond the direct toxic effects of MPs, there is growing evidence for the ability of MPs to act as carriers, adsorbing multiple pollutants on their surface [20], such as heavy metals [21], polychlorinated biphenyls, polycyclic aromatic hydrocarbons [22], pharmaceutically active compounds [23] and organic pesticides [24], consequently promoting their bioaccumulation and biomagnification and increasing their toxicity, leading to the phenomenon called the "Trojan horse effect" [20]. In addition to the adsorption properties of plastics, toxic molecules, such as plasticizers, may also be released during plastic fragmentation [25].

Among these, Bisphenol A (BPA), a commonly used plasticizer, is a pollutant of particular concern. Its presence in the environment is widely documented, and human exposure to BPA occurs via several routes, such as contact and food intake [26]. BPA is an endocrine disruptor and its exposure has been correlated to several human chronic diseases, including diabetes, obesity, reproductive disorders, congenital disabilities, and breast cancer, as well as cardiovascular, chronic respiratory, and kidney diseases [27].

Once ingested, BPA is eliminated through the kidney, and in patients with chronic kidney disease (CKD), it accumulates as the glomerular filtration rate decreases [28]. Several *in vitro* studies have described how BPA can induce apoptosis [29,30], oxidative stress [31,32], mitochondrial dysfunction [29,31], and inflammation [32].

Considering this complex picture, it seems highly probable that multiple mechanisms act synergically to mediate and enhance MP-related toxicity.

Interestingly, while the effects of the combination of MPs and pollutants are well documented in acquatic organisms [33–39], they have been scarcely studied in mammal system cells. Regarding renal cells, only recently has the co-exposure of polystyrene MPs and di (2-ethylhexyl) phthalate been evaluated in a human embryonic renal cell line [40,41].

To address this point, we evaluated the individual and mixed impact of PE-MPs and BPA on a human renal proximal tubular cell line (PTECs) (HK-2). Different assays were performed to investigate cell viability and expression of inflammatory and oxidative stress mediators. Moreover, we assessed the impact of BPA/PE-MP exposure on the aryl hydrocarbon receptor (AHR) and the heat shock protein 90 (HSP90) expression.

2. Materials and methods

2.1. PE-MP characterization

Hydrodynamic radius, polydispersity index (PDI), and ζ-potential (surface charge) measurements on PE-MP samples were performed by means of dynamic light scattering (DLS) analyses, using a Zetasizer Instrument, Nano ZS90 Series (Malvern Panalytical, Malvern, UK), working with He-Ne laser (emission $\lambda = 633$ nm). A 40 kHz sonication was performed on each sample prior to analysis in order to keep MPs dispersed within the solution. Measurements were performed at a fixed temperature (T = 25 °C) by means of a Peltier thermostatic system, with an equilibrating time set to 120 s before each analysis. Laser radiation was collected at 90°, and outcomes were calculated using triplicate analyses. Clear PE-MPs (PE-Microspheres, 1–4 μm, PE >70 % + proprietary additive <30 % (Trade Secret) and reported density of 0.96 g/cc; CPMS-0.96 1–4 μm; Cospheric, Aurogene, Rome, Italy) suspensions (0.05 mg/mL) were prepared in different media: mQ water, NH_3 15 % (v/v) aqueous solution and HNO_3 (0,026 M) aqueous solution. Samples were analyzed in triplicate, and each measurement consisted of 20 runs.

2.2. Cell culture

HK-2 cells were obtained from American Type Culture Collection (ATCC) (LGC Standards S.r.l. Sesto S. Giovanni, Italy).

Cells were grown in DMEM/F12 medium supplemented with 5 % [v/v] Foetal Calf Serum (FCS) (Euroclone S.p.A., Milan, Italy), 5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL sodium selenite, 5 pg/mL T3, 5 ng/mL hydrocortisone, 5 pg/mL PGE1 and 10 ng/mL epidermal growth factor (Merck Group, Milan, Italy). Cells were grown at 37 °C in a humidified 5 % CO_2 condition.

2.3. Uptake of PE-MPs

PTECs were seeded and treated with 1–5 μm green fluorescent microspheres (Fl-MPs) (FMG-1.3 1-5μm - 500 mg FMG Cospheric) at 0.2 mg/mL concentrations for 5–24 h.

- *Analysis by flow cytometry*

Cells from a 6-well plate were detached, centrifuged, resuspended in 500 μL of PBS in flow cytometry tubes, and run without further fixation. Fl-MP uptake was evaluated using side scatter (SSC) median values, as measured by flow cytometry Cytoflex-S flow cytometer, and data were analyzed with Flow Jo 10.8. For all the analyses, debris, dead cells, and isolated Fl-MPs were excluded by gating over physical parameters (FSC/SSC). A minimum of 15,000 cells per sample were analyzed.

Fl-MP uptake was evaluated using Staining Index (SI), a normalized metric which quantifies the fluorescent signal in relation to negative CTR and is calculated according to the formula $SI = \frac{\text{Mediane positive cells} - \text{mediane negative control}}{2 \text{ Standard Deviation (SD) mediane negative control}}$. Moreover, using dot plot analysis it was possible to evaluate the percentage of cells which incorporated Fl- MPs.

- *Analysis by confocal microscope*

HK-2 cells were seeded onto EZ chamber slides (Merck Group) and treated with Fl-MPs. Slides were then washed with phosphate-buffered saline (PBS) for 5 min, fixed with 2 % paraformaldehyde for 5 min at room temperature, and stained with 5 U/mL Alexa-Fluor 594-conjugated phalloidin (ThermoFisher Scientific, Monza, MB, Italy). Nuclei were counterstained with DAPI and examined under a Nikon AX R confocal microscope.

2.4. Cell treatments

Cells were grown to sub-confluence and exposed for 5–24 h to complete medium (CTR), 100 nmol/l BPA (Merck Group), 1.0–4.0 μm clear PE-MPs (0.2 mg/mL) (Cospheric) and BPA + PE-MPs. Previously, all treatments were pre-incubated at 37 °C overnight (o.n.) and only used for cell exposure afterwards. All treatment conditions and exposure times were conducted in duplicate. We chose 100 nmol/L BPA since it is the average environmental concentration to which humans are exposed (10–100 nmol/day) [42].MP concentration was similar to those used in Refs. [41,43,44].

2.5. MTT assay

This assay, intended for cell viability measurement, is based on the reduction of 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) (Merck Group) by mitochondrial dehydrogenase in viable cells to produce a purple formazan product. Each experiment was performed according to the protocol previously described [45].

2.6. mRNA analysis

Total RNA was isolated using the Tri-Reagent (Zymo Research, Aurogene). A total of 1 μg RNA was used for cDNA synthesis with Wonder RT kit (Euroclone s.p.a.). PCR amplification was carried out in a total volume of 10 μL, containing 1 μL cDNA solution, 5 μL SensiFAST SYBR no ROX mix (Meridian Bioscience, Aurogene, Rome, Italy), 1 μL of each primer (Tib Molbiol, Genoa, Italy) and 3 μL of nuclease-free water. β-actin was quantified and used for the normalization of expression values of the other genes. Assays were run in triplicate on MasterCycler realplex PCR system (Eppendorf, Hamburg, Germany). The primer sequences are reported in Table S1. Gene expression was evaluated as fold change in comparison with No treated cells (CTR).

2.7. Immunofluorescence and immunocytochemistry

HK-2 cells grown on chamber slides were incubated for 5–24 h as described above. After a 5-min fixation in cold methanol, cells were exposed to anti-HSP90 (Santa Cruz Biotechnology Inc., DBA Italia s.r.l., Seregno, Italy), anti-AHR (Invitrogen, Life Technologies Italia, Monza, Italy) and anti-CCR2 antibodies (Novus Biologicals, DBA Italia s.r.l.). For immunocytochemistry, cells were probed with an ultra-polymer goat anti-mouse IgG conjugated to horseradish peroxidase (HRP) (ImmunoReagents Inc, Microtech, Pozzuoli, Italy) and developed with DAB detection kit (Roche, Microtech). Slides were counterstained with hematoxylin and examined using light microscopy. For immunofluorescence, secondary antibodies conjugated to Alexa Fluor 594 were used (anti-rabbit antibody, Invitrogen, Life Technologies; anti-mouse antibody, Cell Signaling Technology, Euroclone s.p.a.). Antibody details are reported In Table S2.Nuclei were stained with DAPI. For immunocytochemistry, images were analyzed using MetaMorph® NX software (Molecular Devices) and the levels of positivity were expressed as arbitrary units. The levels of cell fluorescence were determined by

ImageJ as described by Russo et al. [46]. For both methods, almost 200 cells were analyzed.

2.8. Western blot analysis

The cell layers were lysed in cold buffer (20 mM HEPES, 150 mM NaCl, 10 % (v/v) glycerol, 0.5 % (v/v) NP-40, 1 mM EDTA) supplemented with protease inhibitor cocktail (Merck Group). The protein concentration was determined by using the Bicinchonic Protein assay kit (Euroclone S.p.A.), and 30–50 μg was resolved on SDS-polyacrylamide gels and electro-transferred to Amersham™ Hybond™ PVDF membrane (Euroclone S.P.A.). Blots were incubated with anti-NOX4 (Proteintech, DBA Italia s.r.l.), anti-NRF2 (Thermofisher Scientific, Life Technologies), anti-HSP90 (Santa Cruz Biotechnology Inc, anti-AHR (Invitrogen), anti CCR5 antibodies (Novus Biologicals), and re-probed with anti-β-actin antibody (Santa Cruz Biotechnology Inc.). Then, they were incubated in (HRP) secondary antibodies (Cell Signaling Technology). Antibody details are reported In Table S2. Immunoblots were developed with Immobilon Western chemiluminescent HRP substrate (Merck Group) and band intensities were determined using the Alliance imaging system (Uvitec, Cambridge, UK).

2.9. Measurement of malondialdehyde (MDA)

Thiobarbituric acid reactive substance levels, such as MDA in HK-2 cells, were measured to track lipid peroxidation, following the method of Ohkawa et al. [47], with minor changes. Briefly, after treatment, cellular pellets were homogenated and mixed with a solution containing 8.1 % SDS, 20 % acetic acid (pH 3.5), 0.8 % thiobarbituric acid, to be heated at 95 °C for 1 h. After cooling in ice water, the organic phase was extracted by adding 4 mL of n-butanol and pyridine (15:1, vol/vol), centrifuged at 950×*g* for 10 min. The absorbance of each sample was evaluated at a wavelength of 532 nm. MDA concentrations were calculated from a standard curve and expressed as nanomole MDA equivalent per milligram protein.

2.10. Statistical analysis

T-test or one-way analysis of variance (ANOVA) was used to compare differences between groups and $p < 0.05$ was considered statistically significant. Results are expressed as mean ± SEM and are the expression of at least three experiments, with two duplicates for each experiment. All statistical analyses were performed using Graph Pad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA.)

3. Results

3.1. Characterization of PE-MPS

The hydrodynamic radius of PE-MPs was investigated at two different concentrations and the results are reported in Table 1A.

According to the analysis, it was possible to confirm the size range reported by the manufacturer in both concentrations. The associated PDI values (PDI<0.2) suggest a narrow particle size distribution. The Z-potential of the same particles was investigated employing solutions with different pH conditions; the concentration of PE MPs was set at 0,05 mg/mL and temperature at 25 °C. Results are reported in Table 1B.

As elsewhere reported [48,49], PE MPs show a negative surface charge proportionally to the pH of the environment. The negative Z-potential is predominant in a very broad range of pH, whereas Z-potential turns positive below the isoelectric point of PE. The average conductivity of the solutions is very different within the investigated conditions; a more in-depth analysis would be helpful to shed light on the influence exerted by this parameter.

3.2. Uptake of PE-MPs BY HK-2 cells

The uptake kinetics were evaluated at 5 and 24 h as depicted in Fig. 1A and B. SI increased significantly after 5 and 24 h ($p < 0.001$-0.0001 vs. CTR, respectively) as well as the percentage of cells that took Fl-MPs. After 5 h, 4.4 ± 1 % of HK-2 incorporated Fl-MPs, and after the longest exposure time this percentage rose to 24.8 ± 3.3 % (both $p < 0.05$ vs. CTR). Furthermore, confocal microscope analysis confirmed the internalization of Fl-MPs in cells. MPs were mainly diffused in the cytoplasm and localized in small discrete clusters in the perinuclear region, but absent in the nucleus (Fig. 1C).

Table 1A
Hydrodynamic radius and polydispersity index (PDI) of PE-MPs.

Dispersion Medium	Concentration (mg/mL)	Average size (nm)	PDI
DW	0,1	2437	0,15
DW	0,05	2253	0,17

Table 1B
Z-potential measurements performed at different pH conditions of PE-MPs.

Dispersion Medium	pH	Average Z-Pot (mV)	Average Conductivity (mS/cm)
DW	6,5	−50,1	0,01
HNO_3	1,8	12	10,9
NH_3	12,5	−54,3	1,5

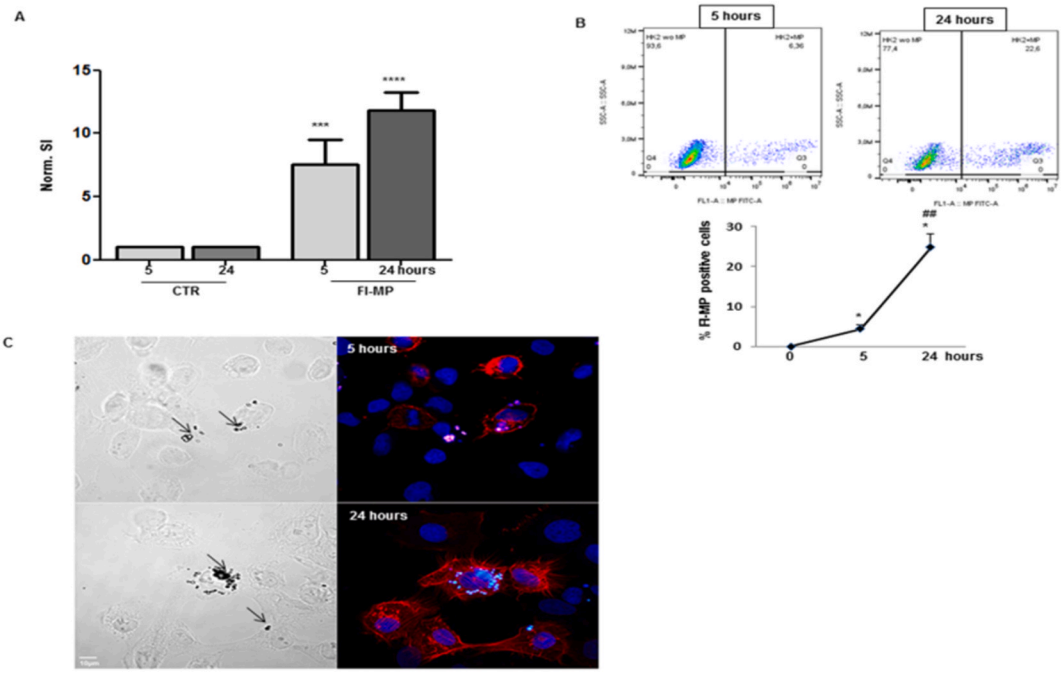

Fig. 1. Uptake of Fl-MPs by HK-2 at 5–24 h. A time-dependent increase in SI was observed (A) as well as in the percentage of Fl-MP positive cells (B). All uptake measurements were done by SSC-A mediane in flow cytometry and normalized over CTR. By confocal microscope (C), Fl-MPs were observed in the cytoplasm (red), stained with Alexa-Fluor 594-conjugated phalloidin. Nuclei were counterstained with DAPI (blue). Black arrows point MPs observed by bright field scale. Bar = 10 μm. Results represent means ± SEM obtained from three independent experiments. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$ vs. CTR; $^{\#\#}p < 0.001$ vs. 5 h MP exposure. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
Abbreviations: Fl-MPs = fluorescent microplastics; CTR=No treated cells; Norm. = normalized; SI = stain index.

3.3. CELL viability

Fig. 2 shows the cell viability of HK-2 after exposure to 100 nmol/L BPA, 0.2 mg/mL PE-MPs and BPA + PE-MPs. After 24 h, no pronounced loss of cell viability was observed in the presence of BPA, whereas PE-MP and BPA + PE-MP treatments reduced it (both

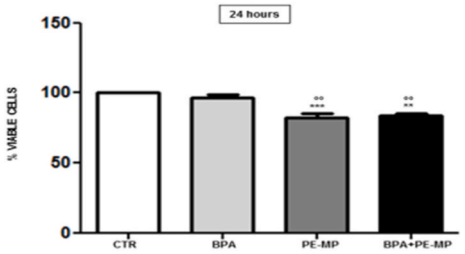

Fig. 2. Effects of treatments on HK-2 viability at 24 h (MTT test). A decrease in cell viability was observed with PE-MPs and BPA + PE-MPs. For each treatment group the number of CTR cells served as baseline value 100 % and was used to express the percentage of living cells. **$p < 0.01$, ***$p < 0.001$ vs. CTR.°$p < 0.01$ vs. BPA.
Abbreviations: MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CTR=No treated cells; BPA = Bisphenol A; PE-MP = polyethylene microplastics.

−20 %, $p < 0.001–0.01$ vs. CTR, respectively). In addition, the decreased percentage of viable cells exposed to PE-MPs or BPA + PE-MPs was statistically significant with respect to BPA treatment ($p < 0.01$).

3.4. Effects of treatments on oxidative stress

To determine whether the treatments exerted any oxidative stress on HK-2, we evaluated the expression of NOX4, the primary source of ROS in the kidney [50], and NRF2, a master regulator of the antioxidant response and xenobiotic metabolism [51]. As shown in Fig. 3A and B, a 5-h exposure to BPA, PE-MPs and BPA + PE-MPs saw a rise in NOX4 (1.3–1.8 fold vs. CTR, $p < 0.05$) and NRF2 (1.7–2.4 fold vs. CTR, $p < 0.05$-0.01) protein levels compared with CTR. To confirm the induction of oxidative stress, we then measured MDA content, which was significantly increased by all treatments (1.7–2.6 fold vs. CTR, $p < 0.05$-0.01) (Fig. 3C).

3.5. Effects of treatments on inflammation

To determine whether the treatments acted as inflammatory triggers on renal tubular cells, we examined the genic expression of IL-1β, CCL-2, CCL-5 and their receptors CCR-2 and -5 (Fig. 4A–C). The transcriptional analysis showed that the treatments increased their expression, with a more significant effect of BPA + PE-MPs. A 5-h co-exposure induced a 3.8, 3.5 and 2.7 fold increase of IL-1β, CCL-2 and -5 ($p < 0.01–0.05$ vs. CTR) (and a more powerful effect was observed on CCL-2 and CCL-5 receptors (6.7–8.4 fold vs. CTR, CCR-2 and -5 respectively, $p < 0.01–0.05$)). Finally, as shown in Supplementary Fig. S1, all treatments significantly induced CCR-2 and CCR-5 protein expression.

3.6. HSP90

HSP90, a highly conserved and abundant chaperone, is involved in multiple cellular functions. It drives conformational changes to facilitate client protein binding, stabilization, and activation [52]. Furthermore, HSP90 can preserve renal tubule viability after injury and maintain cell homeostasis [53]. As reported in Fig. 5A, we found that after 24 h HSP90 protein levels were significantly decreased

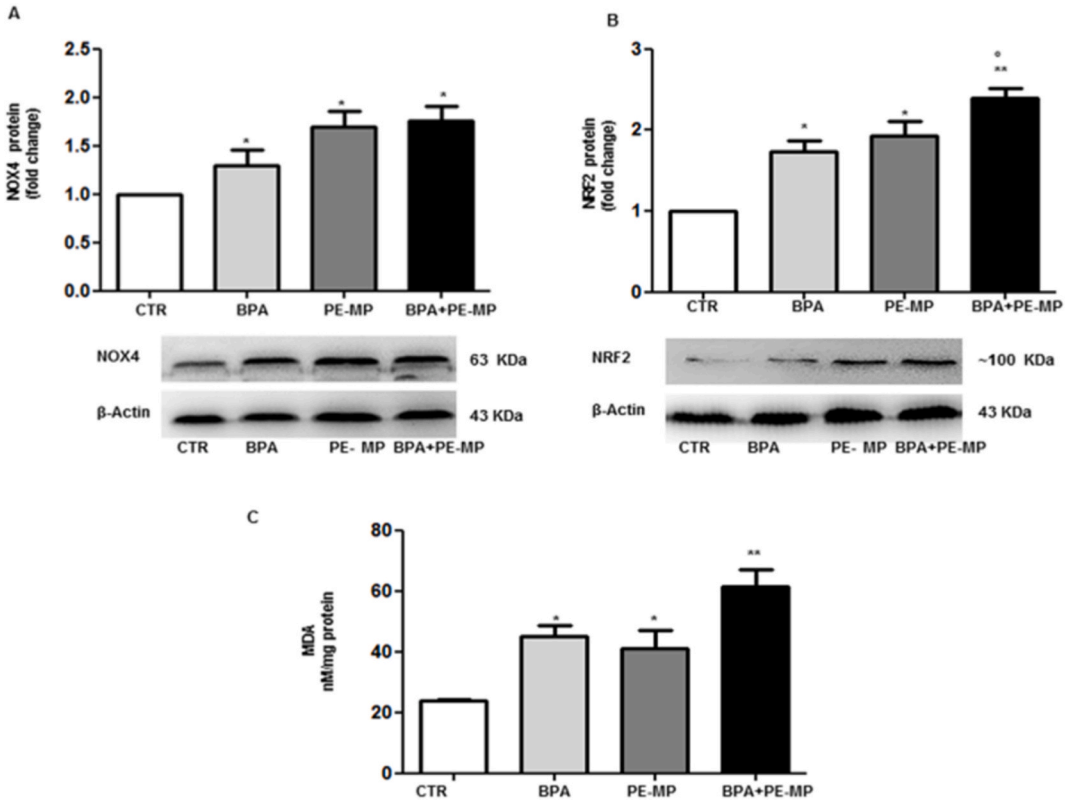

Fig. 3. Effects of treatments on NOX4 (A), NRF2 (B) protein expression and MDA levels (C). A 5-h exposure saw a rise in NOX 4, NRF2 and MDA. NOX4 and NRF2 were evaluated by Western blot. Blots were stripped and reprobed with antibody to anti β-actin. All results represent means ± SEM obtained from three independent experiments and are expressed as fold change to CTR. *$p < 0.05$; **$p < 0.01$ vs. CTR; °$p < 0.05$ vs. BPA. Abbreviations: CTR=No treated cells; BPA = Bisphenol A; PE-MP = polyethylene microplastics; NOX4 = NADPH Oxidase 4; NRF2 = nuclear factor erythroid 2-related factor 2; MDA = malondialdehyde.

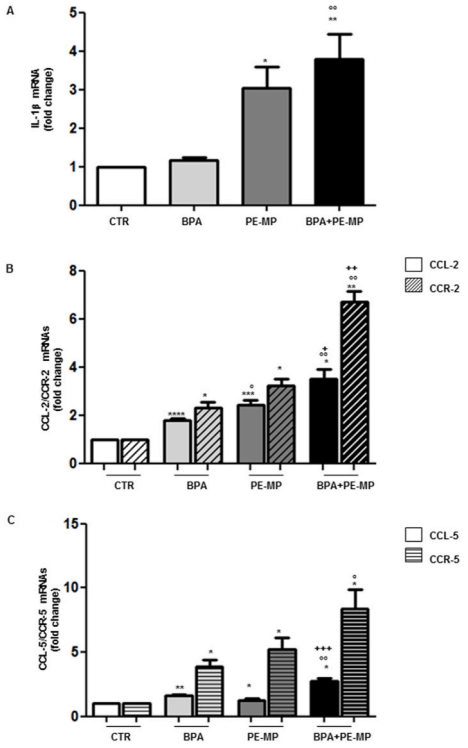

Fig. 4. Effects of treatments on inflammatory molecules. 5-hour exposure increased IL-1β (A), CCL-2 (B), CCL-5 (C) and their receptors CCR-2 (B) and CCR-5 (C). mRNAs were evaluated by RT-PCR. The results represent means ± SEM obtained from three independent experiments and are expressed as fold change to CTR. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 vs. CTR; °p < 0.05,°p < 0.01 vs. BPA; $^{+}$p < 0.05, $^{++}$p < 0.01, $^{+++}$p < 0.001 vs. PE-MPs.
Abbreviations: IL-1β = interleukin-1β; CCL-2 = chemokine (C-C motif) ligand 2; CCL-5 = chemokine (C-C motif) ligand 5; CCR-2 = C-C chemokine receptor type 2; CCR-5 = C-C chemokine receptor type 5; RT-PCR = real-time polymerase chain reaction; CTR=No treated cells; BPA = Bisphenol A; PE-MP = polyethylene microplastics.

by all treatments (p < 0.05 vs. CTR), but BPA + PE-MPs had a more substantial effect (−40 % vs. ctr p < 0.05). This result was confirmed by immunocytochemistry (Fig. 5B).

3.7. *Effects of BPA and PE-MP MIXTURE on AHR expression*

BPA can activate AHR [54]; we asked whether PE-MPs exposed to BPA could have a similar effect. As graphed in Fig. 6A, a 5-h exposure to BPA and BPA + MP raised mRNA levels ((~1.5–2.9 fold vs CTR respectively, p < 0.05), which then decreased after 24 h. In addition, the treatments enhanced protein expression both at 5 (p < 0.05-0.001 vs. CTR) and 24 h (p < 0.01-0.001) (Fig. 6B, Supplementary Fig. 2). Using immunofluorescence analysis we observed that although the amount of AHR did not vary over time, its localization changed, being nuclear at 5 h and perinuclear after 24 h (Fig. 6C). Moreover, a significant AHR increase was observed during co-exposure, compared with treatments using BPA or PE-MPs singly (p < 0.05-0.001 vs. BPA or PE-MPs) (Fig. 6 and Supplementary Fig. 2).

4. Discussion

Extensive research indicates that humans are exposed to MPs, which may accumulate in various organs and tissues [11,13–15]. Once inside the human body, MPs can potentially exert harmful effects through various processes [20,55].

However, the extent of this toxicity and its underlying mechanisms remain largely undetermined. Based on these observations, we designed the present study to assess the possible detrimental effects of PE-MPs on PTECs and to investigate the interaction between MPs and BPA, a widespread environmental pollutant.

Using DLS, we characterized MPs' physicochemical properties, such as size, aggregation kinetics and zeta potentials, which are the main determinants of uptake mechanisms and intracellular fate of MPs. We found that PE-MPs had an average size of 2437 nm and a zeta potential above (±) 30 mV, with a PDI of 0.16, suggesting a homogenous and stable dispersion in solution [56,57].

We subsequently observed that MPs accumulated in the HK-2 cytoplasm with time-dependent kinetics. Both plastic particles and associated BPA could cause an array of detrimental effects, once inside cells. Unlike polystyrene-MPs [43], PE-MPs and BPA-MP

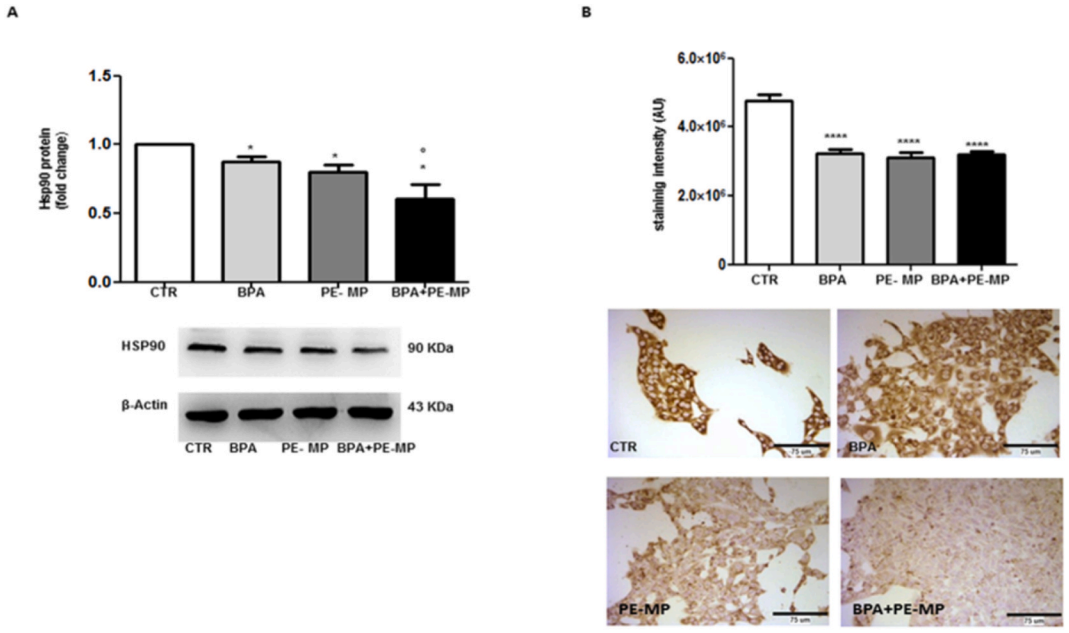

Fig. 5. Treatments changed HSP90 expression. A 24-h exposition decreased HSP90 levels, as evaluated by Western blot (A) and confirmed by immunocytochemistry and image analysis (B). Blots were stripped and reprobed with antibody to β-actin. Results represent means ± SEM obtained from four independent experiments and expressed as fold change to CTR. For immunocytochemistry, staining intensity was expressed as AU and at least 200 cells for condition from three independent experiments were evaluated. *p < 0.05, ****p < 0.0001 vs. CTR; °p < 0.05 vs. BPA. Magnification = ×200, scale bar = 75 μm.
Abbreviations: CTR=No treated cells; BPA = Bisphenol A; PE-MP = polyethylene microplastics, HSP90 = heat shock protein 90; AU = arbitrary units.

co-exposure decreased cell viability. BPA single exposure was not toxic for tubular cells, probably due to the presence of albumin in the cell culture media [58], as only extreme BPA concentrations >50 μM are able to reduce cell viability [29]. While the effects of PE-MPs on cell viability have been scarcely studied, different concentrations of PE-MPs have been tested in 6 different human cell lines and it was lowered only in intestinal epithelial Caco-2 and lung epithelial A549 [59]. Similarly, PE-MPs induced a dose-dependent cell death in an MDCK epithelial cell line [19]. To date, our study demonstrates, for the first time, that PE-MPs act negatively on human PTEC cell viability. Cell death, as well as inflammation and fibrosis, can serve as triggers for oxidative stress, which has emerged as a major cause of kidney damage [50], and is caused by the excessive production of reactive oxygen species (ROS) mainly produced by NOX4, a member of the NADPH oxidase family in PTECs [50], frequently implicated in various kidney diseases [60]. Furthermore, ROS can activate NRF2, a master regulator of cellular responses against oxidative stress [61,62].

Overproduction of ROS can lead to lipid peroxidation, which is monitored by MDA content. In our experimental setting, all treatments acted as pro-oxidant triggers, as supported by NOX4, NRF2 and MDA overexpression. *In vitro* and *in vivo* studies indicate that in renal tubular cells exposed to BPA [29,63], oxidative stress is increased, as well as in mice exposed to 5 μm MP [64]. Moreover, we observed that the pro-oxidant effect was boosted when BPA was synergized with PE-MPs. Furthermore, NRF2 can also participate in the clearance of oxidized proteins and organelles, activating both chaperone-mediated autophagy and macroautophagy [65]. In the kidney, it has been demonstrated that both BPA [63] and MPs [43] can modulate autophagy; we can thus suppose that increased levels of NRF2 enable the maintenance of a stable internal environment. Evidence has shown that BPA and MPs can exert pro-inflammatory actions in the kidney. In rats, oral administration of BPA for 30 days led to an increase of the release of proinflammatory cytokines and to a decline of renal function [66]. In contrast, in mice the systemic administration of BPA induced the renal gene expression of IL-6, CCL-2/MCP-1, CCL-5/Rantes, and the inflammatory response was boosted by the presence of CKD [63]. In addition, in mice, exposition to MPs promoted macrophage infiltration and increased mRNA levels of CCL-2/MCP-1 and IL-1β [64], indicating the activation of inflammation. These data support our findings, demonstrating that BPA and PE-MPs (especially when considered in combination) exhibit pro-inflammatory properties and significantly contribute to renal damage. HSP90 is highly expressed in PTECs, and after injury caused by heat [67], anoxia [67] or several toxic agents such as cisplatin [68] or gentamicin [69], its levels can rise further, reducing renal damage during stress insult. Moreover, it has been demonstrated that intrarenal transfection of HSP90 protects the kidney against ischemia/reperfusion injury, reducing tubular damage and improving renal function [70]. Therefore, HSP90 is crucial in cell homeostasis because it inhibits apoptosis [71], facilitates cell proliferation [71], preserves protein folding and regulates cellular processes such as signal transduction, protein trafficking and DNA damage [72,73]. The effects of BPA and MPs on HSP90 expression in PTECs are unknown, with limited and inconsistent studies on other cell types. After a 6-h treatment, BPA increased HSP90 in the murine uterus [74]. In marine organisms, BPA's effect on HSP90 transcription was variable: it remained unchanged in the dinoflagellate *Prorocentrum minimum* [75], increased after 5 h, and then decreased at 24 h in the freshwater snail *Physa acuta* [76]. Similarly,

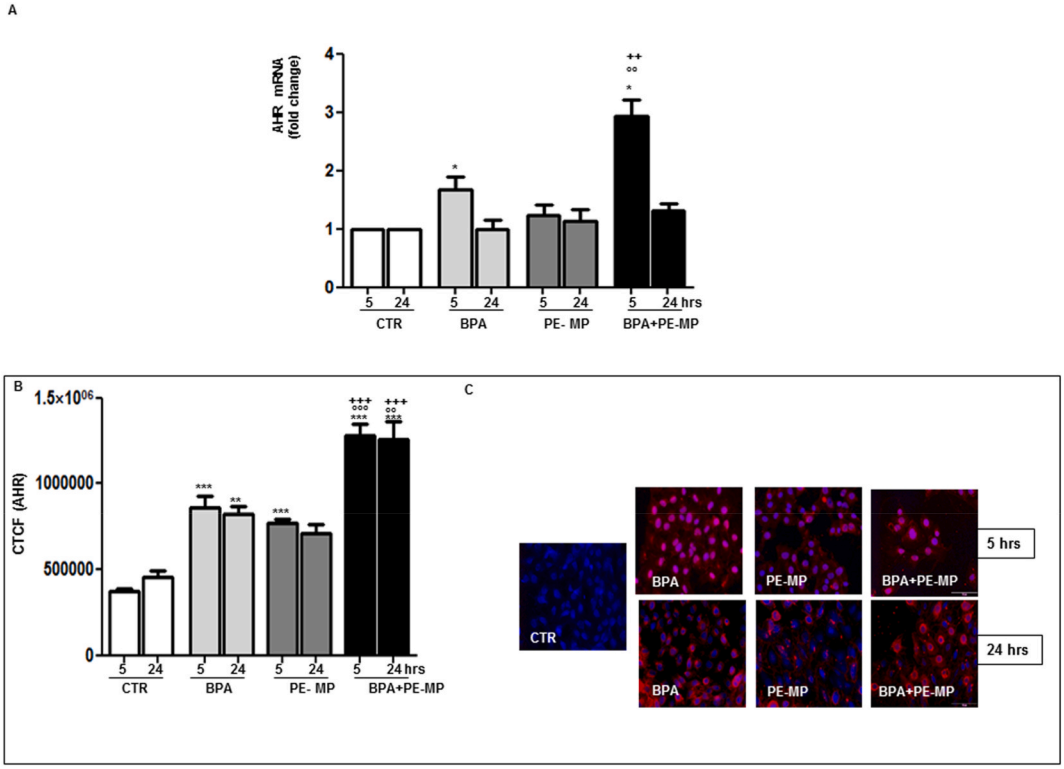

Fig. 6. Effects of treatments on AHR mRNA (A) and protein expression (B). A 5-h exposition increased AHR levels, but after 24 h only protein was enhanced, compared to CTR. Immunofluorescence analysis (C) at 5 h revealed AHR in nuclei, whereas after 24 h AHR was perinuclear. AHR signal was red and nuclei counterstained with DAPI (blue). mRNA was evaluated by RT-PCR. The results represent means ± SEM obtained from four independent experiments and are expressed as fold change to CTR. *$p < 0.05$ vs. CTR; **$p < 0.01$ vs. CTR; ***$p < 0.001$ vs. CTR; °$p < 0.01$ vs. BPA; °$p < 0.001$ vs. BPA; $^{++}p < 0.01$, $^{+++}p < 0.001$ vs. PE-MPs. Magnification = ×400, scale bar = 75 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
Abbreviations: AHR = aryl hydrocarbon receptor; HRS = hours; RT-PCR = real-time polymerase chain reaction; CTR=No treated cells; BPA = Bisphenol A; PE-MP = polyethylene microplastics; DAPI = 4′,6-diamidino-2-phenylindole; CTCF = corrected total cell fluorescence.

HSP90 mRNA levels increased in gilthead seabreams (*Sparus aurata*) [77] (Del Piano et al., 2023) but decreased in juvenile sea cucumber *Apostichopus japonicas* [78] when exposed to MPs. We found that all treatments decreased HSP90 levels, which is notable, given the many proteins interacting with HSP90. Reduced HSP90 expression can significantly affect its client proteins, modifying cell homeostasis and influencing human health and disease [73].

AHR, a transcription factor activated by several environmental, dietary, microbial and metabolic ligands, combines with HSP90 and the co-chaperone p23 to constitute a stable protein complex in the cytoplasm [79]. Upon ligand binding, AHR translocates into the nucleus, to regulate target genes' transcription, including phase I metabolism enzymes, phase II conjugation enzymes [79] and other transcriptional factors, such as activator protein 1, NRF2 and nuclear factor kappa B (NF-κB), Smads, β-catenin, mitogen-activated protein kinase (MAPK) family p38, NADPH oxidase and extracellular signal-regulated kinase affecting multiple cell pathways [80]. Emerging evidence from murine models of kidney diseases shows that AHR is involved in the ischemia/reperfusion injury [81]. Benzopyrene, an AHR ligand, causes oxidative stress and alters renal function in mice [82], in a similar manner to tryptophan metabolites, which induce glomerular lesions and inflammation [83], apoptosis and mitochondria depolarization in HK-2 [84]. AHR is engaged by BPA as observed in endocrine [85] and immune cells [86], and by MPs as demonstrated *in vitro* using the H4IIE-luc assay [87]. For the first time, our results show that in HK-2 exposed to BPA + PE-MPs, the AHR gene and protein expression increased and that AHR had a nuclear localization, supporting the hypothesis that it could be activated by MPs and pollutants. Our research shows that all the treatments had detrimental effects on human PTECs. The reasons could be several. Due to their high surface-to-volume ratio and high surface hydrophobicity, MPs have strong adsorption affinities for many environmental pollutants, and thus, they work as carriers of contaminants in living organisms [88]. Therefore, MPs may boost the accumulation of chemicals, by introducing a larger amount. On this matter, in whiteleg shrimps exposed to BPA and BPA + MPs, it has been demonstrated that, the co-exposure group had significantly less BPA in the seawater than the BPA exposure group [89], confirming that MPs can load seawater and act as vectors. In addition, since the toxicity of BPA is dose-dependent [90], MPs may boost the accumulation of BPA in the organisms. Furthermore, the interaction between MPs and BPA may have a synergistic enhancement, that is pollutants and MPs can individually induce toxic reactions, but the combined effect on the same targets is stronger than that of acting individually [91]. This has been demonstrated in marine invertebrates, in which the co-presence of BPA and MPs aggravated the immunotoxicity and neurotoxicity [92], disrupted

humoral immune responses [93] or hampered the gonadal development [89] in respect to the single treatment. Regarding mammal cells, coexposure to BPA and MPs strengthened the cell death in Caco2 [94] and had the detrimental effects on metabolism in liver cells [95]. In conclusion, our research shows that the single treatments or the coexposure BPA MPs were toxic for PTECs inducing over-expression of oxidative stress and inflammatory molecules potentially leading to kidney damage.

Finally, although this study reports experimental results, the growing evidence of the potential harmful effects of MPs and related contaminants on human health could significantly influence future environmental policy. Our results suggest that the health risks posed by MPs are not only linked to the particles themselves, but also to the bioavailability and toxicity of other environmental pollutants they may carry. Further research is essential to deepen our understanding of the physiological mechanisms through which MP contamination impacts both experimental models and humans, as well as their potential toxicity. Moreover, epidemiological and longitudinal studies are crucial to assess variations in exposure across different human populations and geographic regions.

5. Limitations of this study

Several limitations of this study should be highlighted. While we exposed cells to PE-MPs pre-treated with BPA, we did not quantify the concentration of BPA adsorbed onto the microplastics. Additionally, we used PE-MPs with varying diameters (1–4 μm), but we did not assess whether particles with different diameters were incorporated with different efficiency or had different biological effects. Furthermore, the use of a single concentration of BPA and microplastics—chosen based on average environmental levels and prior experimental data—limits our understanding of potential dose-response relationships. It's also important to note that while we used spherical MPs, environmental studies, such as those by Kamani et al. [5], report that PE in the environment commonly appears as foam, fragments, fibers, or films.

Finally, we described some molecules involved in BPA-PE-MPs damage, but the underlying pathways were unexplored. Future studies should address these points.

CRediT authorship contribution statement

Daniela Verzola: Writing – original draft, Investigation, Conceptualization. **Noemi Rumeo:** Investigation. **Stefano Alberti:** Methodology. **Fabrizio Loiacono:** Investigation. **Sebastiano La Maestra:** Methodology, Investigation. **Mario Passalacqua:** Methodology. **Cristina Artini:** Writing – review & editing, Funding acquisition. **Elisa Russo:** Supervision, Investigation. **Enrico Verrina:** Investigation, Data curation. **Andrea Angeletti:** Formal analysis. **Simona Matarese:** Investigation, Data curation. **Nicoletta Mancianti:** Data curation. **Paolo Cravedi:** Resources, Investigation. **Micaela Gentile:** Investigation. **Francesca Viazzi:** Writing – review & editing. **Pasquale Esposito:** Writing – review & editing, Supervision. **Edoardo La Porta:** Writing – original draft, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Edoardo La Porta reports article publishing charges was provided by IRCCS Istituto Giannina Gaslini. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This research was supported by Ricerca Corrente of Italian Ministry of Health, Fondazione Malattie Renali del Bambino ETS, and IRCCS Istituto Giannina Gaslini which will cover the pubblications costs.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e39426.

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