Tesi di Dottorato

Università degli Studi di Genova

XXXIV ciclo

Emato oncologia e medicina interna clinico-traslazionale

The Burden of Plastic in Human Health: presence of microplastics in human kidney and urine and their prospective nephrotoxicity

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Background

Plastic debris is defined on the basis of their dimensions in microplastics (between 5 mm and 1 μm) and nanoplastics (< 1μm). Micro and nano-plastic (NPs) pollution is growing exponentially in the last decades, and there are increasing evidences of the ubiquitous distribution of plastic contaminants in the environment. They can be derived from larger plastic pieces by degradation or industrially made as microspheres (healthcare, personal care products, detergents etc…) [1]. In the last century, the global production of plastics has reached 370 million tons. Annual production volumes are expected to continue rising in the following decades, up to double by 2050. Europe produced roughly 55 million metric tons of plastic in 2020 [2]. Microplastics (MPs) were reported in soil [3], food [4], in marine ecosystem [5], drinking water [6], and air [7-8]. Some studies evaluated that MPs with a range of 4.5 to 0.3 mm account for the 90% of the plastic debris present on the ocean's surface [9]. Uptake of MPs has been confirmed by detection in the gastrointestinal tract of many marine animals [10-11] and the human intestine [12]. Indeed, MPs and NPs pass through a trophic level to another, and finally reach the human food chain.

Deleterious effects of MPs and NPs on the fauna have been widely described [13-14]. Most frequent biological effects of MPs ingestion described are oxidative stress [15], inflammation [16], alteration of metabolic pathways, and immune response [17].

Furthermore, MPs can lead to cellular and tissues damage by the release of contaminants adsorbed in the environment or of chemicals additives used in the plastic production [18]. MPs

have physical and chemical properties that enable the absorption of a wide range of environmental pollutants such as heavy metals and persistent organic pollutant (POPs) that includes polycyclic aromatic hydrocarbons, bisphenol A (BPA), and others. This capacity of MPs and NPs can lead to the accumulation of multiple pollutants, causing the enhancement of their toxicity, the so called "Trojan Horse" effect [19-20]. Anyway, the real impact of the "trojan horse" effect is still debated, especially in consideration of the lack of standardization of the methods for in vitro studies. Moreover, some researchers criticized the higher concentrations of MPs used in vitro to investigate this phenomenon compared to concentrations of MPs in the environment [9].

In consideration of the above-mentioned evidences, MPs and NPs deserve attention for their potential negative role on human health.

Only recently, few studies investigated the presence of MPs in human beings. Evidences of the presence of MPs in human placenta [21], lungs [22] and blood [23] have been reported.

The first evidence of the presence of MPs in humans is the study by Ragusa et al 2020. They found a total of 12 MP fragments in 4 out of 6 placentas. Samples were digested through alkaline hydrolysis (KOH 10%), filtered and analyzed at the optical microscope. MPs debris had a size within a range from 5 to 10 μm and they were characterized by Micro-Raman spectroscopy.

More recently, MPs have been detected in human blood. Researchers developed a sensitive method with double shot pyrolysis-gas chromatography/mass spectrometry analysis to investigate the presence of plastic debris (> 700 nm dimension) on human blood of healthy volunteers. They

found a mean concentration of plastic particles (mainly polyethylene and polymers of styrene) of 1.6 µg/mL. Analysis was generated for polymethyl methacrylate (PMMA), polypropylene (PP), materials containing polymerized styrene (PS), polyethylene (PE) and polyethylene terephthalate (PET) demonstrating that 17 out of 22 blood donors carry plastic debris in their blood.

Finally, the manuscript by Jenner et al. demonstrated MPs in human lung tissues. Thirty-nine MPs have been detected in 11 out of 13 samples analyzed when µFTIR spectroscopy has been used to characterize MPs with a size > 3 µm. A total of 12 polymers were identified for a mean concentration of 0.69 ± 0.84 MP/g. Most abundant were PP, PET, resin and pPE.

Plastic debris are entered in the human food chain, and have been described in human lungs, placenta and blood but the consequences on human health is still unknown and poorly investigated.

Furthermore, very limited in vitro and in vivo data are available on the possible consequences of MPs and NPs on kidney tissue. A pioneering study by Deng et al. investigated different tissues accumulation and the effect of polystyrene MP diet in a mouse model [24]. They used fluorescent microspheres of two sizes 5 and 20 µm. They found a markedly high deposition of MPs in kidney, especially for 5 µm diameter MPs. They further performed a metabolomic analysis that revealed differences based on the size and concentration of the MP used. Mice treated with mild and high concentrations of 5 µm MPs can be differentiated from control mice, while the groups treated

with 20 µm MPs can be clearly separated from mice with 5 µm MPs and controls. In conclusion they found that MPs induce various effects in biomarkers and metebolomic profile of mice.

In a recent in vitro and in vivo study, MPs have been shown to increase in HK2 kidney tubular cells the levels of mitochondrial ROS, Bad (apoptosis regulator), ER (endoplasmatic reticulum) stress-related proteins, inflammation-related proteins, and autophagy-related proteins [25]. They further found that protein expression of the MAPK signaling pathways (p38, JNK, and ERK1/2 signaling pathways) and AKT/mTOR signaling pathways were differently phosphorylated between cells. Finally, they demonstrated in male C57BL/6 mice that MPs increase ER stress, inflammatory markers, autophagy-related proteins, and increase levels of creatinine and proteinuria. Also Meng et al investigated the effect of polystyrene microparticles in the kidney of mice [26]. They showed that NPs and MPs accumulate in the kidneys and induce inflammation and oxidative stress. Moreover, they demonstrated that the agglomeration of microparticles increase adverse effects and affect kidney tissue leading to nephrotoxicity.

Due to the wide literature of the negative effects on different organisms, also in mammalians, and in vitro on kidney tissues, and finally in consideration of the first evidences of MPs accumulation in human tissues, it is mandatory to push for new and prompt investigations of the presence and the potential deleterious effects of MPs on human beings.

This is the first study aimed to investigate the presence of MPs in human kidney. Moreover, we analyzed the effect of microparticles of PE on HK2 kidney tubular cells and the potential additive

effects of BPA, a common compound of MPs in the environment and a well-known toxic agent linked to several health disorders [27].

Materials and Methods

Design of the study

Our study consists of two parts: in vivo and in vitro study.

In vivo we investigated the presence of MPs in urine and kidney tissues and we characterized the microparticles found through Micro-Raman Spectroscopy.

In vitro we analyzed the effect of PE-MPs, BPA, and co-treatment BPA-MPs on HK2 kidney tubular cells. We performed different cells culture conditions and we analyzed various biomarkers of inflammation, oxidative stress, and mitochondrial activity.

Cell cultures

HK-2 cells, an immortalized proximal tubular epithelial cell line from normal adult human male kidney, were obtained from ATCC. Cells were grown in DMEM/F12 medium supplemented with 5% [v/v] FBS, 2 mmol L-glutamine, 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. Cells were grown at 37°C in a humidified 5% CO_2 condition.

Cell treatments

HK-2 were exposed for 5-24 hours to Bisphenol A (BPA) (10-100 nmol) (Merck Group, Milan, Italy), Polyethylene Microspheres (PE-MP) (1-4um diameter; 0.2 mg/mL) (Cospheric LLC, Aurogene, Rome, Italy) and MP (0.2 mg/mL) pre-treated with BPA (100 nmol) stored at 37 °C overnight into the cell culture medium.

Cellular Uptake of Microplastics

HK-2 cells (4 × 104) were seeded on chamber slides for 5-24 hours and treated with Green Fluorescent Microspheres (1-5um) (Cospheric LLC, Aurogene), at concentration of 0.2 mg/ml for 5-24 hours. Then, the slides were washed with phosphate-buffered saline (PBS) for 5 min, fixed with cold methanol for 5 minutes, and stained with 4,6-dimidyl-2-phenylindole (DAPI) at room temperature for 5 min. Finally, the slides were examined with a fluorescence microscope (Leica)

Cell Viability Assay

This assay for cell viability 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. HK-2 were seeded in a 96-well culture plate and incubated overnight at 37°C. Then, HK-2 cells were treated with BPA (10-100 nm), PE-MPs and PE-MPs+BPA for 24 hours. After the incubation period, MTT (final concentration 0.5 mg/ml) was added to each well and cells were incubated for 4 hours at 37° C. After that, MTT was solubilized by DMSO and the absorbance of the samples was measured using a microplate (ELISA) reader. The wavelength to measure absorbance of the formazan product was 570 nm and the number of viable cells was expressed as percentage of no treated cells (CTR)

Immunocitochemistry

HK-2 grown on chamber slides to sub-confluence were incubated for 24 hrs with or without BPA, PE-MPs, PE-MPs+BPA. After a five-minute incubation in cold methanol, cells were incubated with anti PGC-1α (Santa Cruz Biotechnology, DBA Italia, Segrate, Italy) or HSP-90 (Santa Cruz Biotechnology) monoclonal antibodies. After a-1 hour incubation, cells were washed extensively with PBS and exposed to Ultrapolymer Goat anti-mouse IgG, HRP conjugated for 45 minutes (ImmunoReagents Inc, Microtech s.r.l., Napoli, Italy). Peroxidase was developed with diaminobenzidine detection kit (Roche, Microtech s.r.l.). Slides were counterstained with

haematoxylin and examined by light microscopy. The signal intensity was evaluated by the MetaMorph®NX (Molecular Devices) and expressed as integrated intensity.

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, 2.5 mM DTT, 10µg/L aprotinin, leupeptin, pepstatin A, 1 mM PMSF, and Na3VO4). Protein concentration was determined by using the Bicinchonic Protein assay kit (Euroclone S.p.A. Pero, Italy.) and 10-100 µg were resolved on SDS-polyacrylamide gels and electro-transferred to a PVDF membrane (Serva, Euroclone S.p.A.). Blots were incubated in anti NRF2 (Life Technology, Monza, Italy), anti PGC1α and anti NRF-2 (Santa Cruz Biotechnology) monoclonal antibodies, anti Nox-4 polyclonal antibody (Proteintech, DBA Italia) (o.n. at 4°C) and re-probed with β-actin (Santa Cruz Biotechnology) and, then, in horseradish peroxidase secondary antibodies (Cell Signaling Technology, Euroclone) for 1 hour. Immunoblots were developed with Immobilon Western chemiluminescent HRP substrate (Merck Group, Milan, Italy). Band intensities were determined using Alliance imaging system (Uvitec, Cambridge, UK).

mRNA Analysis

Total RNA was isolated using the Trizol Lysis reagent (Life Technology). The RNA concentration and integrity were evaluated on a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). 1 µg RNA was used for cDNA synthesis with Wonder RT synthesis 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 kit (Meridian Bioscience, Aurogene) (0,5 µL each primer (Tib Molbiol S.r.l., Genova. Italy), 3 µL of nuclease-free water. β-actin was quantified, and used for the normalization of expression values of the other genes. Fluorescence signals measured during the amplification were considered positive if the fluorescence intensity was more than 20-fold greater than the standard deviation of the baseline fluorescence. The ΔΔ*C*T method of relative quantification was used to determine the fold change in expression (50). Here, the threshold cycle (*C*T) values of the target mRNAs were first normalized to the *C*T values of the internal control, β-actin, in the same samples (Δ*C*T = *C*Ttarget − *C*Tcon), and then further normalized with the internal control (T0). Assays were run in triplicate with Universal PCR Master Mix on MasterCycler realplex (Eppendorf, Hamburg, Germany) PCR system.

Primers	**Forward**	**Reverse**
Human MCP-1	accgagaggctgagactaac	aatgaaggtggctgctatgag
Human IL-1β	ttcgacacatgggataacgagg	tttttgctgtgagtcccggag

Human AHR	taacccagaccagattcctc	gcaaacaaagccaactgag
Human NRF2	ttcccggtcacatcgagag	tccctgttgcataccgtctaaatc
Human PGC1α	tcctctgaccccagagtcac	cttggttggctttatgaggagg
Human NOX4	cacagacttggctttggatttc	ggatgacttatgaccgaaatgatg
Human β-Actin	catcccccaaagttcacaat	agtggggtggcttttagga

Sample collection and hydrolysis

We performed the following procedures to digest tissues and cells and to denature proteins in kidney and urine samples.

We added KOH 10% to urine samples and we stored them at 60 °C for 24 hours.

Differently, we placed kidney tissues in glass petri dishes. We crumbled samples and we added 300 mL deionized water plus KOH 10%. Samples were incubated in glass flask for 5 days at 60 °C and shacked every day for at least 2 hours in an incubator shaker at 60 rpm, until the fluid became clean and no fragments were visible.

Further, we filtered urine and kidney sample through paper micropore filters (diameter 0.2 μm). using a vacuum pump.

Filter papers were then stored and dried at room temperature. Since not all the procedures were made under laminar-flow we didn't considered fibers in the samples. To prevent contamination, we used metal and glass and we avoid any kind of plastics. Procedural blanks were made using the procedures above mentioned.

Identification and analysis of microplastics in vivo

When electromagnetic energy interacts with a material, it can either be reflected, absorbed, transmitted, or scattered. One type of scattering is Raman scattering and is the basis for Raman spectroscopy. Raman spectroscopy is useful as the Raman scattered light yields data of the vibrational modes of the sample molecules. A Raman spectrum thus enables the identification of molecules and their functional groups, similar to IR spectroscopy, but with visible range light.

Raman spectroscopy is the study of the interaction between light and matter where light is inelastically scattered: a process upon which Raman spectroscopy is based. Raman microspectroscopy is where a Raman microspectrometer is used in place of a standard Raman spectrometer. A Raman microspectrometer consists of a specially designed Raman spectrometer integrated with an optical microscope. This allows the experimenter to acquire Raman spectra of microscopic samples or microscopic areas of larger samples.

Micro-Raman analysis of MPs was performed at the Department of Chimica and Chimica Industriale, University of Genoa (Genoa, Italy). Filter membranes were first inspected by visible light using a ×20 objective (Leica). The detected MPs were morphologically characterized by a ×50 and then directly analyzed on the filter by Raman Microspectroscopy (633 nm laser diode, 16 mW/100%, spectral range 100-4000 cm^{-1},1200 lines per mm grating). A Raman Nano Microspectrometer (System2000, Renishaw) was used. The collected Raman spectra were compared with those reported in the SLOPP Library of Microplastics ("SLOPP Library of Microplastics," n.d.) and in the spectral library of the Galactic software (Renishaw plc Spectroscopy Product Division.)

Statistical analysis

Statistical analysis was performed with GraphPad Prism®, version 5.02 [Graph Pad, San Diego, CA, USA]. The one-way analysis of variance [ANOVA] and the Tukey-Kramer multiple comparison test were used to test the significance of differences. Results are expressed as mean±SEM and are the expression of at least three experiments, with two wells for each experiment. Differences were considered statistically significant if $p < 0.05$.

Results

Cellular up-take of PE-MO

The effect of Fluorescent Microspheres was evaluated at concentration of 0.2 mg/ml for 5-24 hours. Cellular uptake showed cytoplasmatic and perinuclear deposition of MPs into HK2 cells (Figure 1). Cellular uptake was already visible after 5 hours. Fluorescent analysis revealed a time dependent aggregation increase of MPs after 24 hours with the evidence of intracellular and perinuclear clusters of fluorescent MPs.

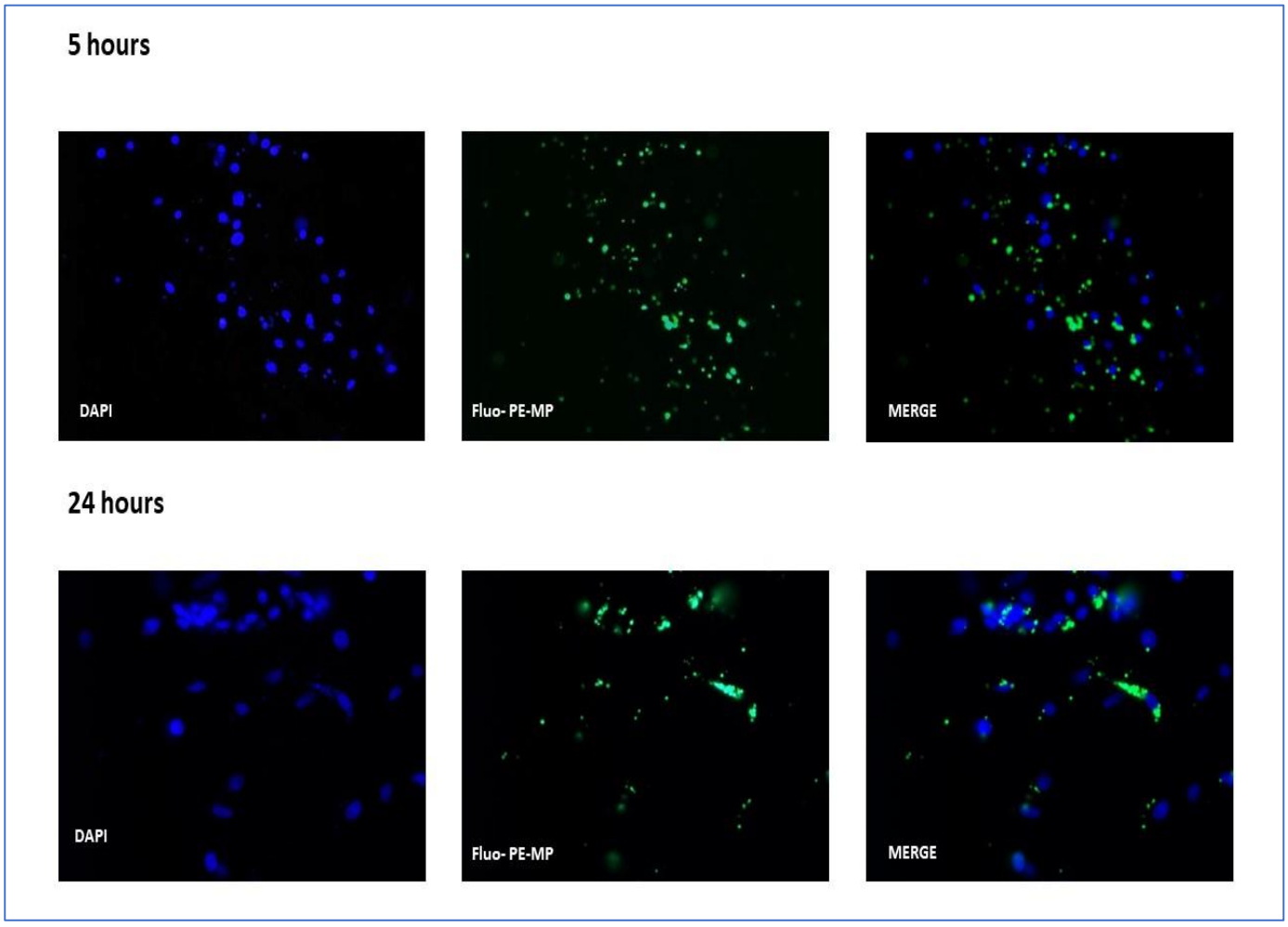

FIGURE 1. *HK2 kidney tubular cells uptake of green fluorescent microspheres at 5 and 24 hours. Nuclei were stained with 4,6-dimidyl-2-phenylindole (DAPI)*

HK2 cells viability after treatment with BPA, MPs and co-treatment

As shown in Figure 2 viability of HK2 after treatment with BPA ($p < 0.5$), MPs ($p < 0.001$), and MPs pre-treated with BPA ($p < 0.01$) was decreased very significantly compared to controls. After 24 hours all the groups were significantly different compared to control. Interestingly, cell viability decreased in a greater manner after treatment with MP alone, and MP pre-treated with BPA compared to HK2 treated with BPA alone.

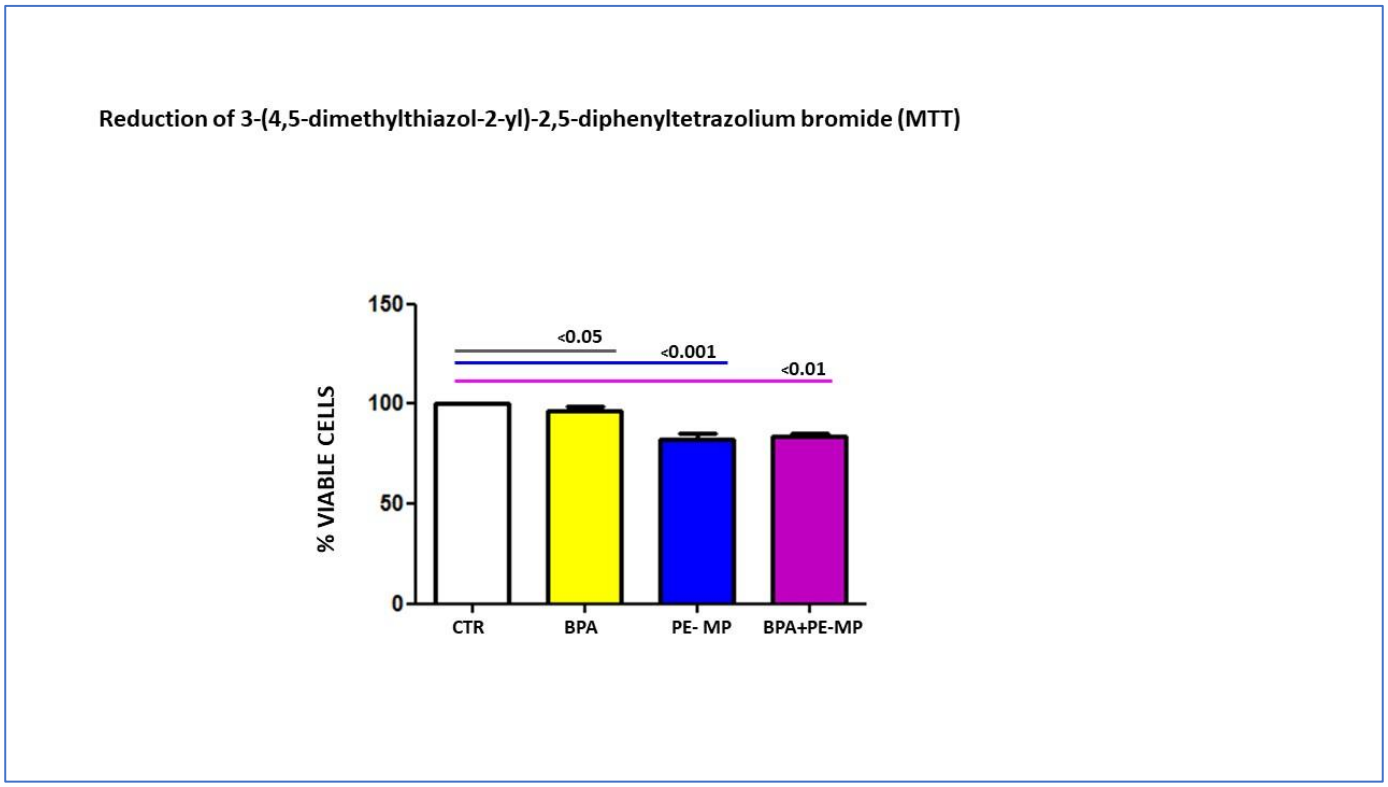

FIGURE 2. *MTT assay for evaluation of cells viability after 24 hours treatment with BPA, MPs and co-treatment BPA-MPs*

BPA, MPs and co-treatment induce MCP-1 and IL-1β expression in HK2 cells

We investigated the treatment and co-treatment of BPA and MPs on kidney proximal tubules cells by evaluating the mRNA expression of the pro-inflammatory cytokine Monocyte chemotactic protein-1 (MCP1) and mediator IL-1β. As shown in Figure 3, exposure to BPA and MPs induced 1.8-3.8 fold increase in MCP-1 expression. MPs alone ($p < 0.01$) and co-treatment with BPA ($p < 0.05$) induced a significantly higher increase as compared to that with BPA alone (respectively 3.8 vs 2.5 vs 1.8). Also IL-1 β showed an increase by the treatment with MPs and BPA and by co-treatment even if not statistically significant.

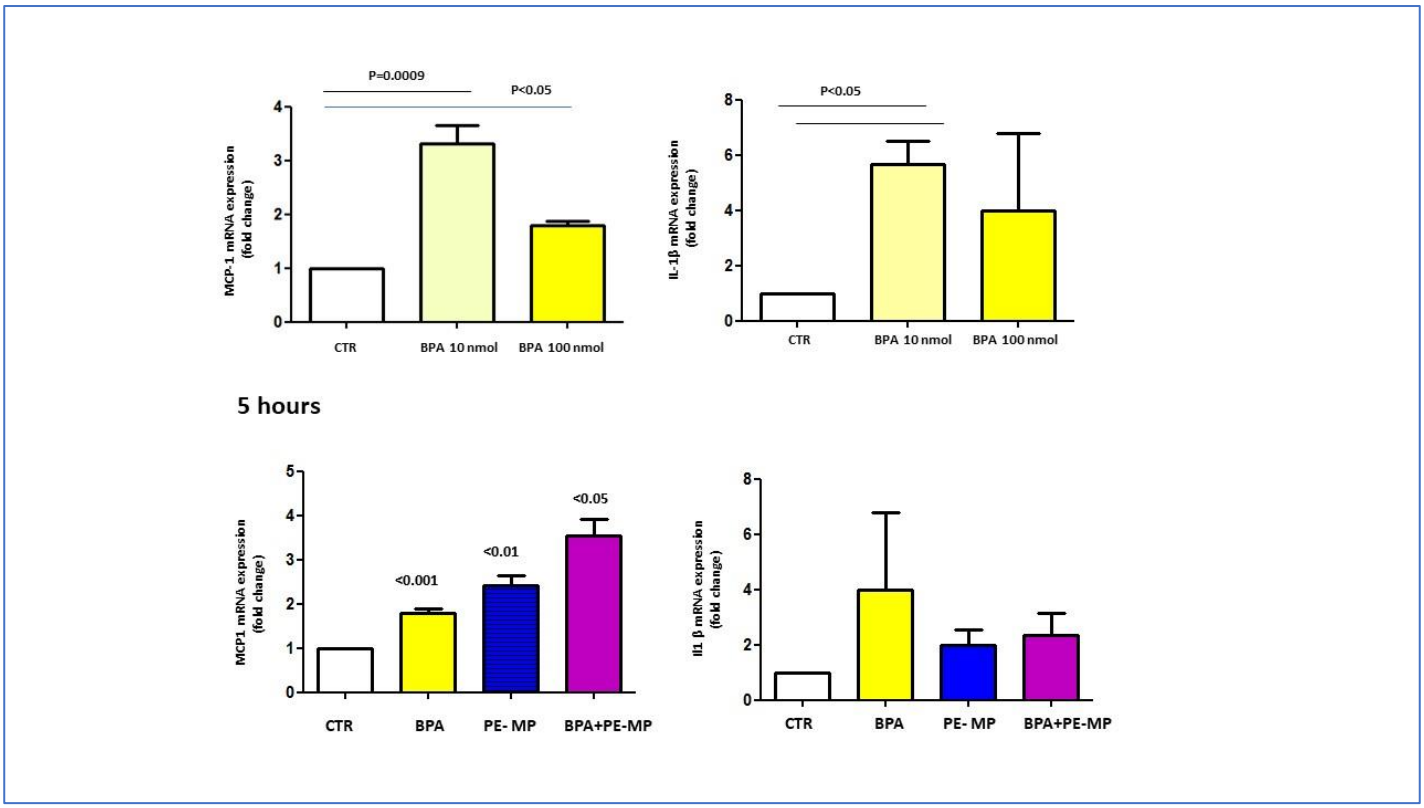

FIGURE 3. *mRNA expression of MCP-1 and IL-1β evaluated with PCR after 5 hours treatment with BPA, MPs and cotreatment with BPA-MPs*

BPA and MPs promote inflammation and fibrosis through the expression of AhR and HSP90

The pro-inflammatory and pro-fibrotic complex composed by heat shock protein 90 (HSP90) and Aryl hydrocarbon receptor (AhR) was significantly induced by BPA and MPs treatment. AhR gene expression was significantly increased both with BPA ($p < 0.5$) and co-treatment BPA-MPs ($p < 0.5$) after 24 hours compared to controls (Figure 4). Interestingly, co-treatment with BPA-MPs induces

a double fold increase compared to BPA alone (fold increase BPA-MPS vs BPA respectively 2.9 vs 1.7; $p < 0.001$). Furthermore, we evaluated HSP90 protein expression through western-blot and immunohistochemistry (Figure 5). In contrast to AhR, HSP90 protein expression was significantly reduced by treatment and co-treatment with BPA and MPs. In particular, co-treatment BPA-MPs induced the strongest reduction in HSP90 protein.

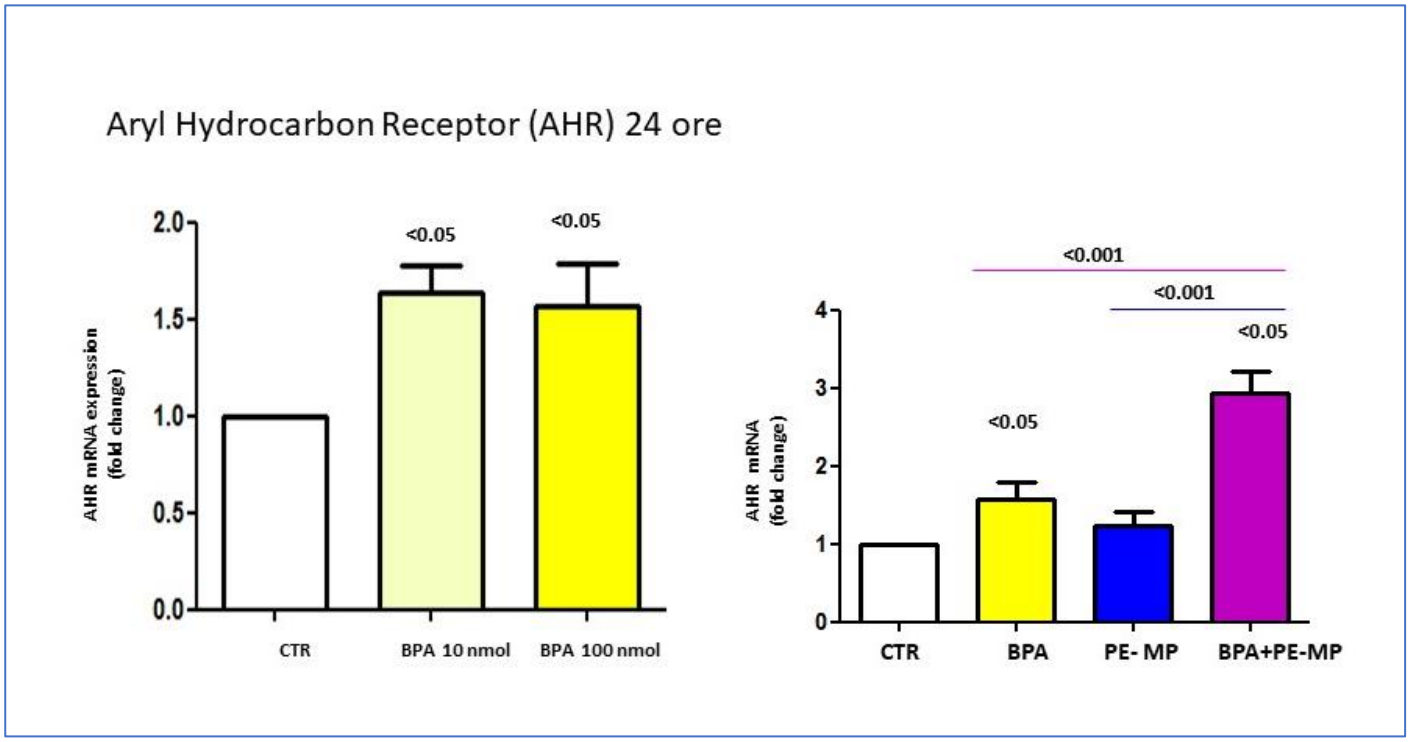

FIGURE 4. *mRNA expression of AhR evaluated with PCR after 24 hours treatment of BPA, MPs and cotreatment with BPA-MPs*

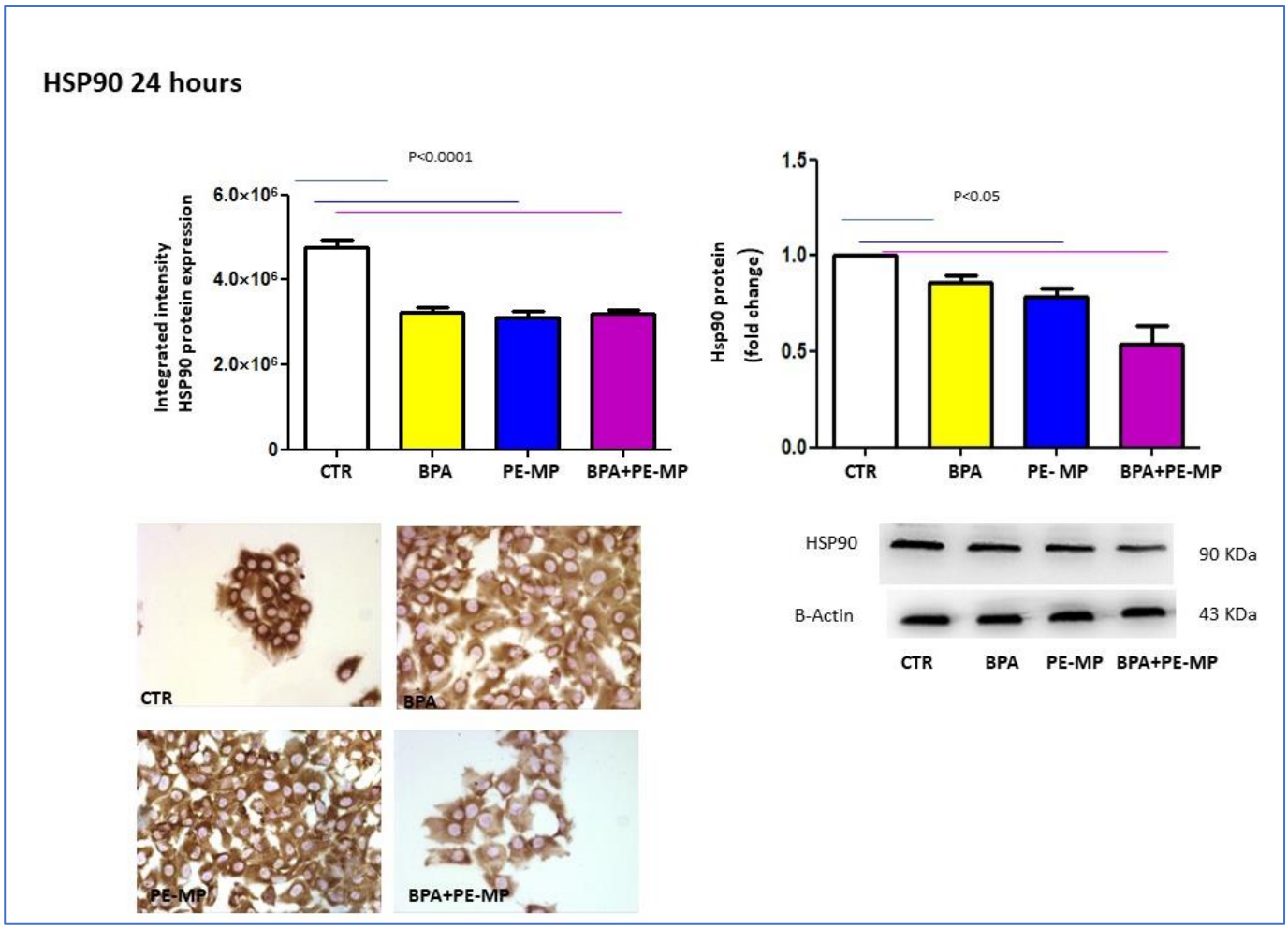

FIGURE 5. *HSP90 protein expression evaluated through western blot and IHC after 24 hours treatment with BPA, MPs and cotreatment with BPA-MPs*

PGC-1α is down-regulated by BPA and MPs treatment

PGC-1α, a mitocondrial biogenesis regulator that acts through the downstream of transcription factors, was down-regulated in HK2 cells after 24 hours of treatment with BPA ($p < 0.001$), MPs and co-treatment with BPA and MPs ($p < 0.0001$). We showed protein expression reduction

through IHC and western blot (Figure 6). Also in this case, the co-treatment BPA-MPs seems to enhance the effect of BPA and MPs alone on kidney tubular cells.

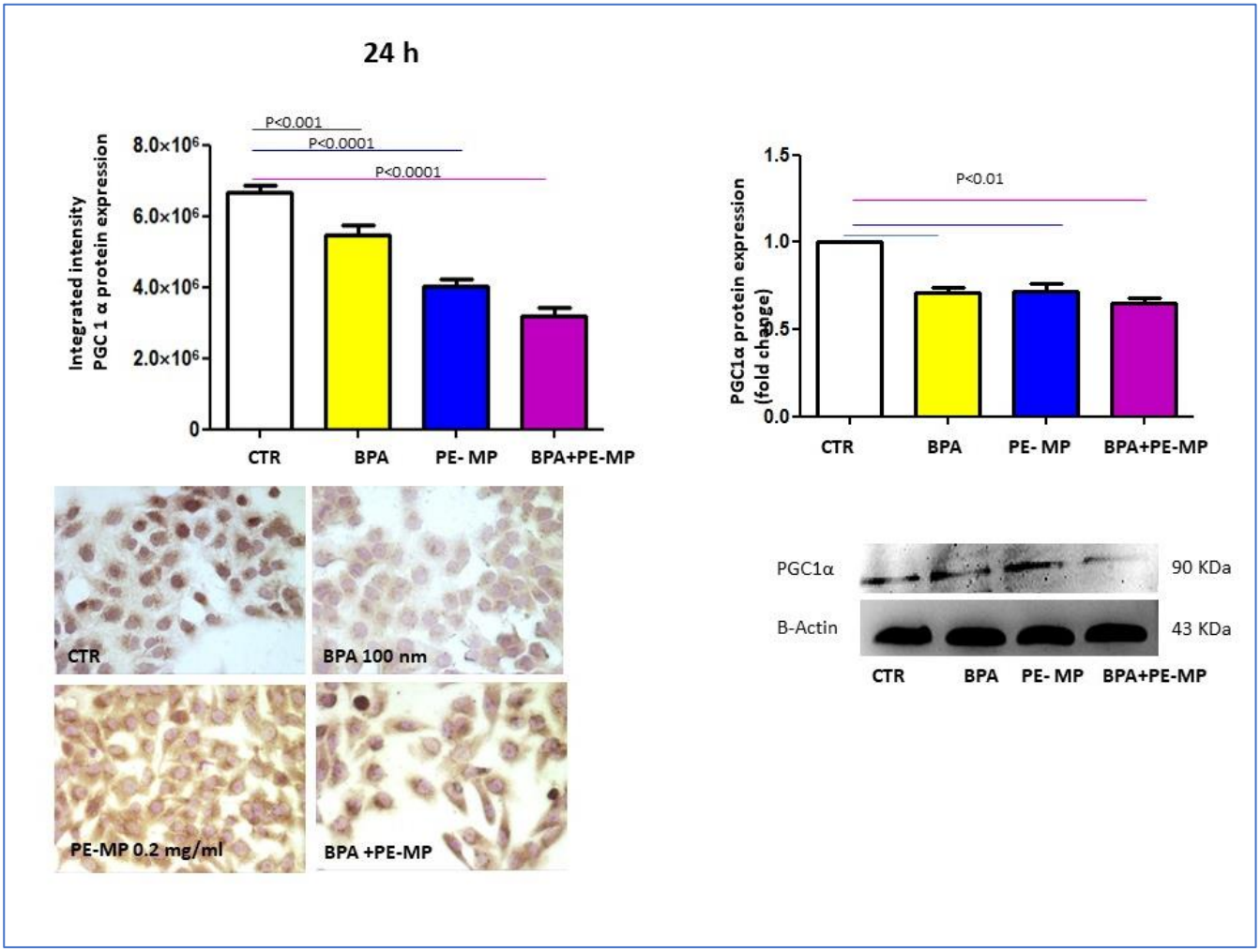

FIGURE 6. *PGC1α protein expression evaluated through western blot and IHC after 24 hours treatment with BPA, MPs and cotreatment with BPA-MPs*

Effect of BPA and MPs on the axis NRF-2 and NOX-4

HK2 protein expression of NRF-2, an antioxidant mediator, revealed a different response to BPA, MPs and co-treatment after 24 hours (Figure 7-8). While BPA induce a significant downregulation of NRF2 ($p < 0.05$), MP and co-treatment with BPA and MPs caused a slight but significant increase of protein expression ($p < 0.05$).

Differently, treatment with MPs and BPA caused a significant increase ($p < 0.05$) in protein expression at 24 hours of NOX-4, an oxidative stress effector (Figure 7-8).

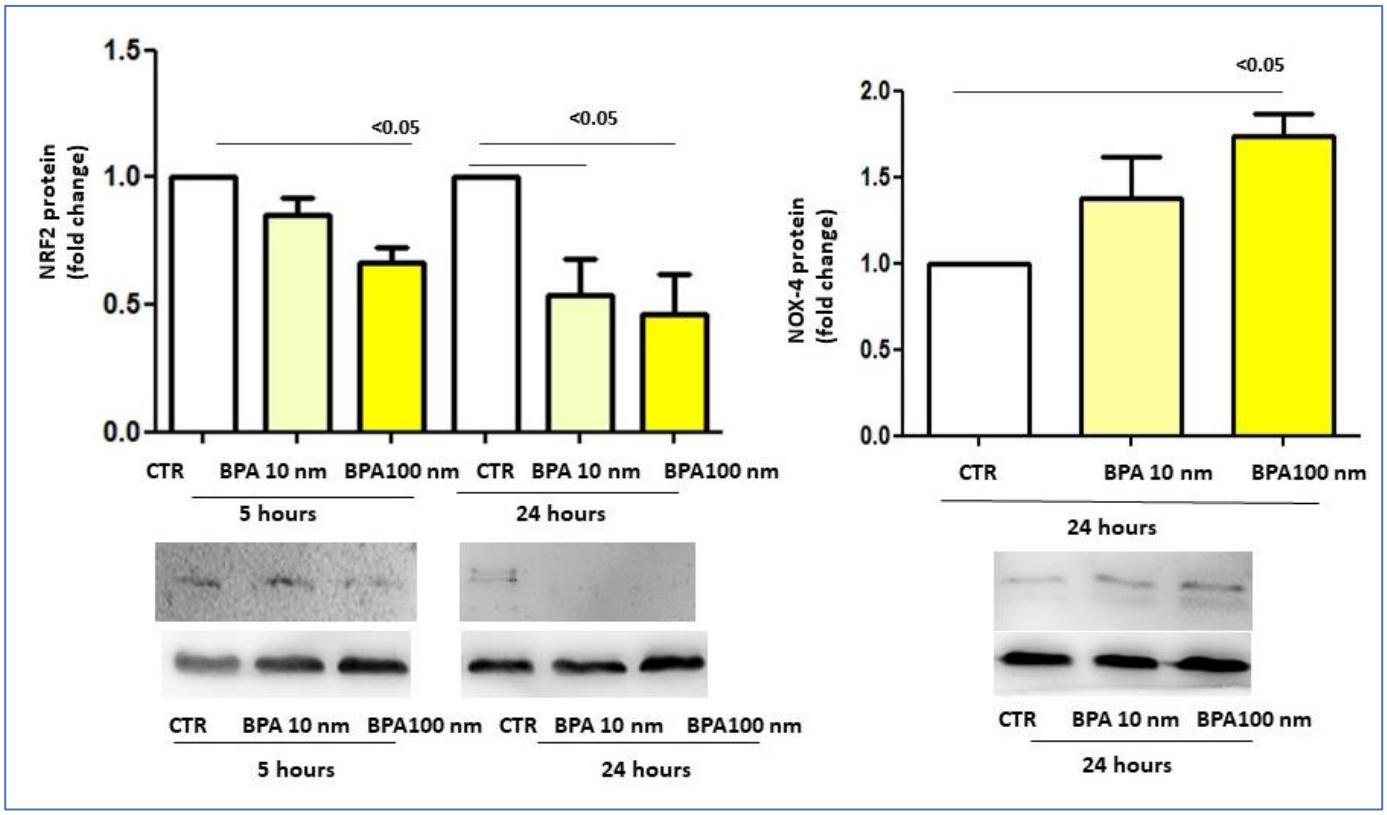

FIGURE 7. *Protein expression of NRF2 after 5 and 24 hours treatment with BPA (10 and 100 nm) and NOX-4 protein expression after 24 hours treatment with BPA (10 and 100 nm) evaluated through western blot analysis.*

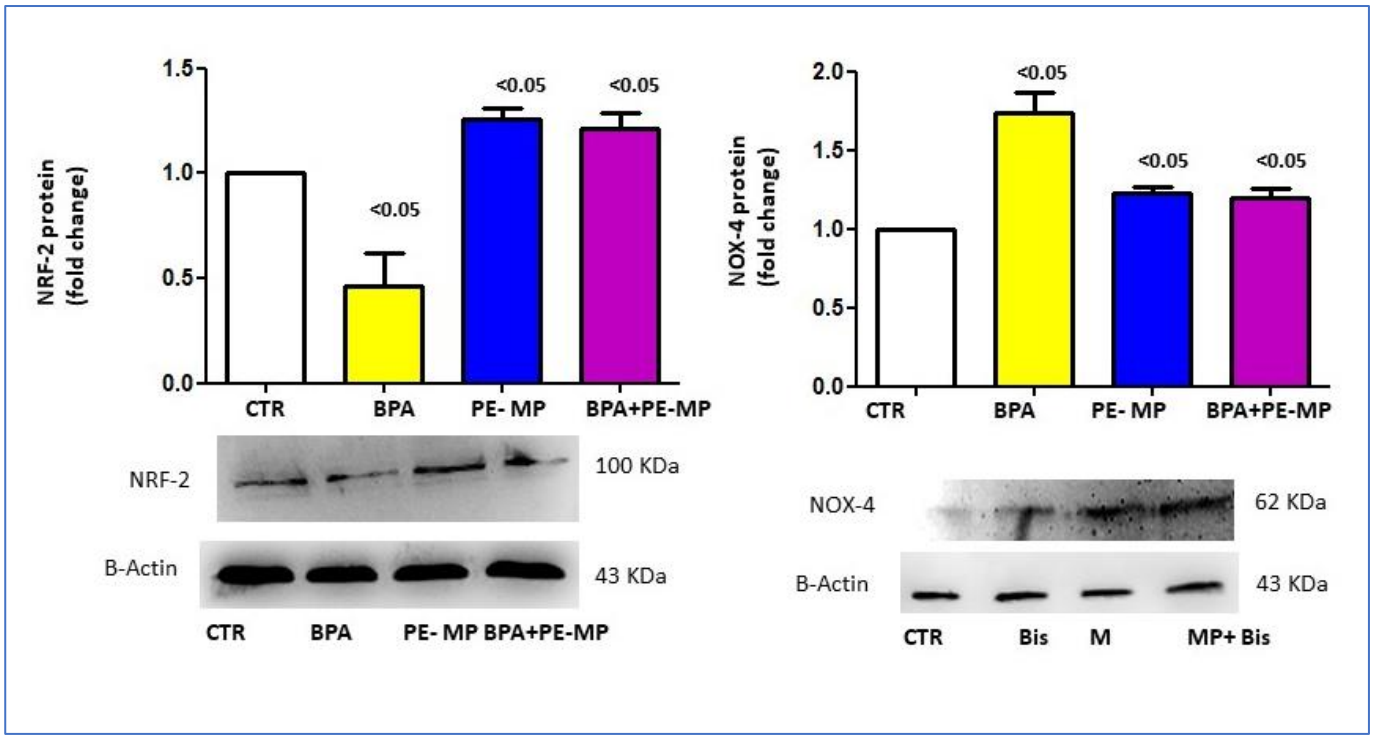

FIGURE 8. *Protein expression of NRF2 and NOX-4 after 24 hours treatment with BPA, MPs and cotreatment BPA-MPs evaluated through western blot analysis.*

Analysis of Kidney Tissues and Urine Samples by Raman Microspectroscopy

At the time I am writing, we already collected 10 samples of urine (300 cc) by healthy donors and 10 sample of normal kidney tissues obtained by nephrectomies in patients affected by renal carcinoma.

We already processed with alkaline hydrolysis and filtered, as above-mentioned in the methods section, five urine samples (300 cc each) and 10 healthy tissue's collected by nephrectomies for

carcinoma with a mean weight of 2.47 ± 1.27 g, and we already analyzed six samples of kidney and one of urine by Raman Microspectrometry.

Retrospective analysis of spectra is still ongoing and partially performed on tree samples of kidney and 1 of urine.

A total of 45 particles were found in kidney samples and analyzed (K_1, 6 particles; K_2, 12 particles; K_3, 27 particles). Particles were named K_x_y (K=kidney; x= sample number; y= particle analyzed).

In the first urine sample analyzed a total of 26 particles were found. Particles were named U_x_y.

Firstly, we analyzed a paper filtered with a solution of PE microspheres used for cell treatment as benchmark for further analysis of spectra (Figure 9). Furthermore, we analyzed kidney and urine samples. In all the kidney samples we found black particles (Figure 10) with a spectra potentially consistent with the following materials: gilsonite, thucholite, retinet, cannel coal, jet coal or chalcocite (Figura 11). Moreover, we identified various particles which spectra were consistent with hematite, a common iron oxide compound (Figure 12). Finally, in urine sample we found again the black particles above mentioned and a particle of polyethylene (Figure 13)

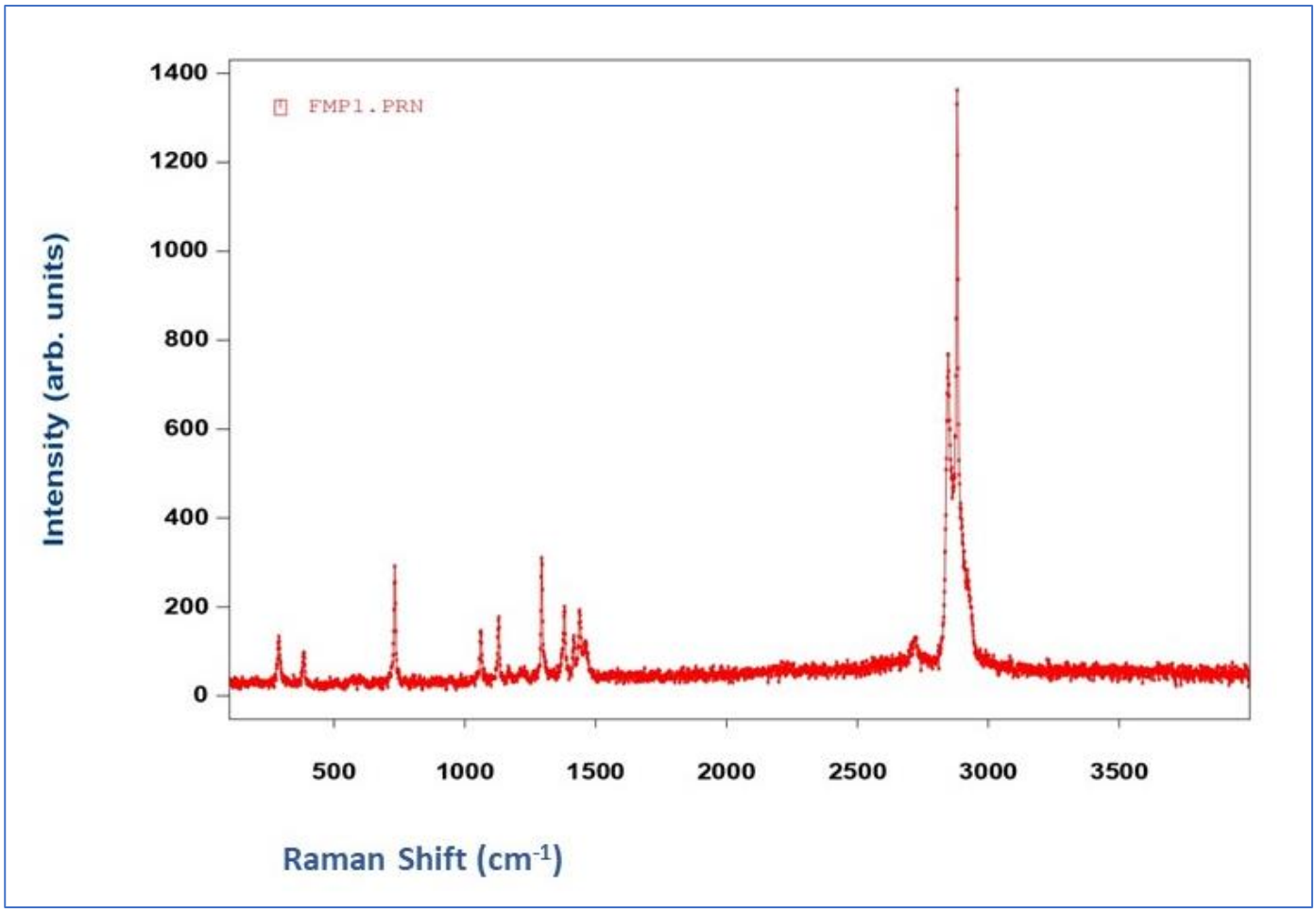

FIGURE 9. *Micro-Raman spectra of Polyethylene Microspheres (1-4um diameter)*

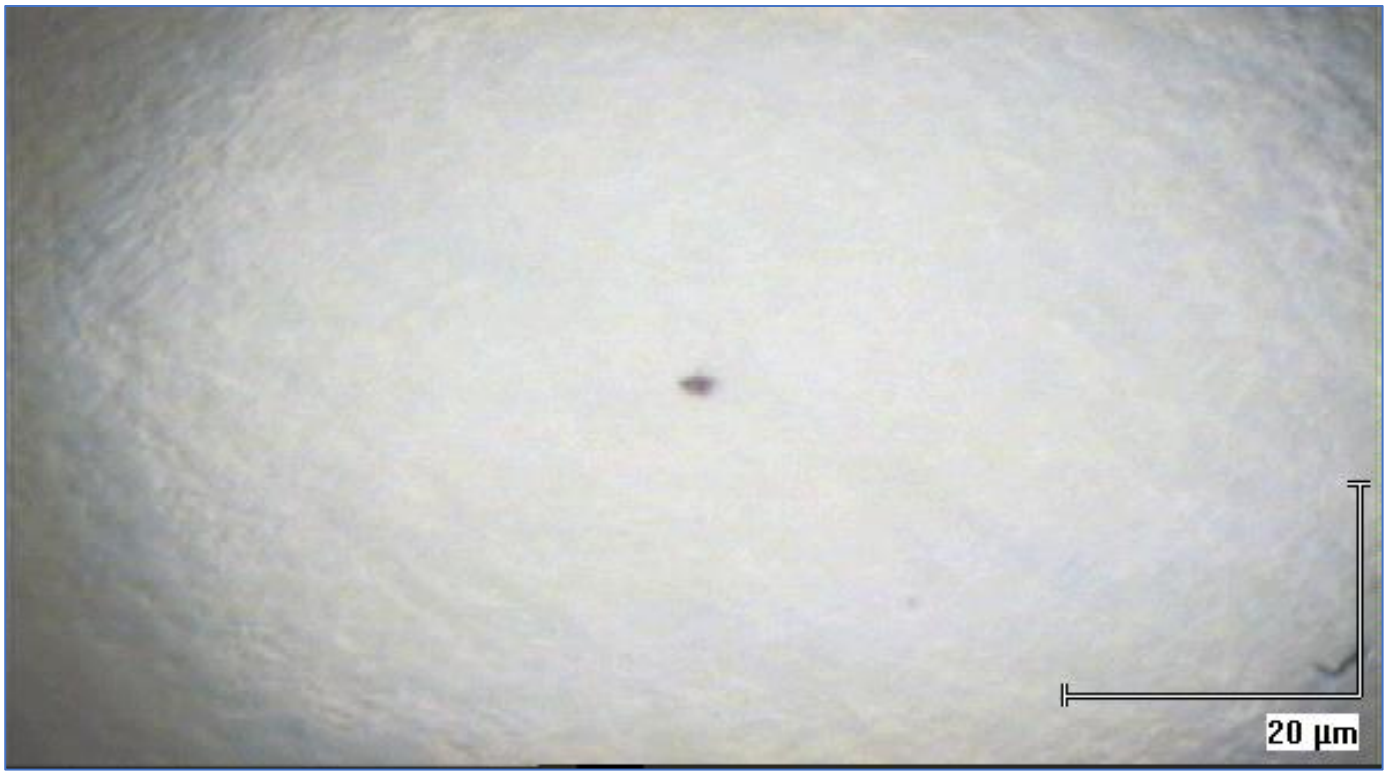

FIGURE 10. *One of the black particles found in our samples*

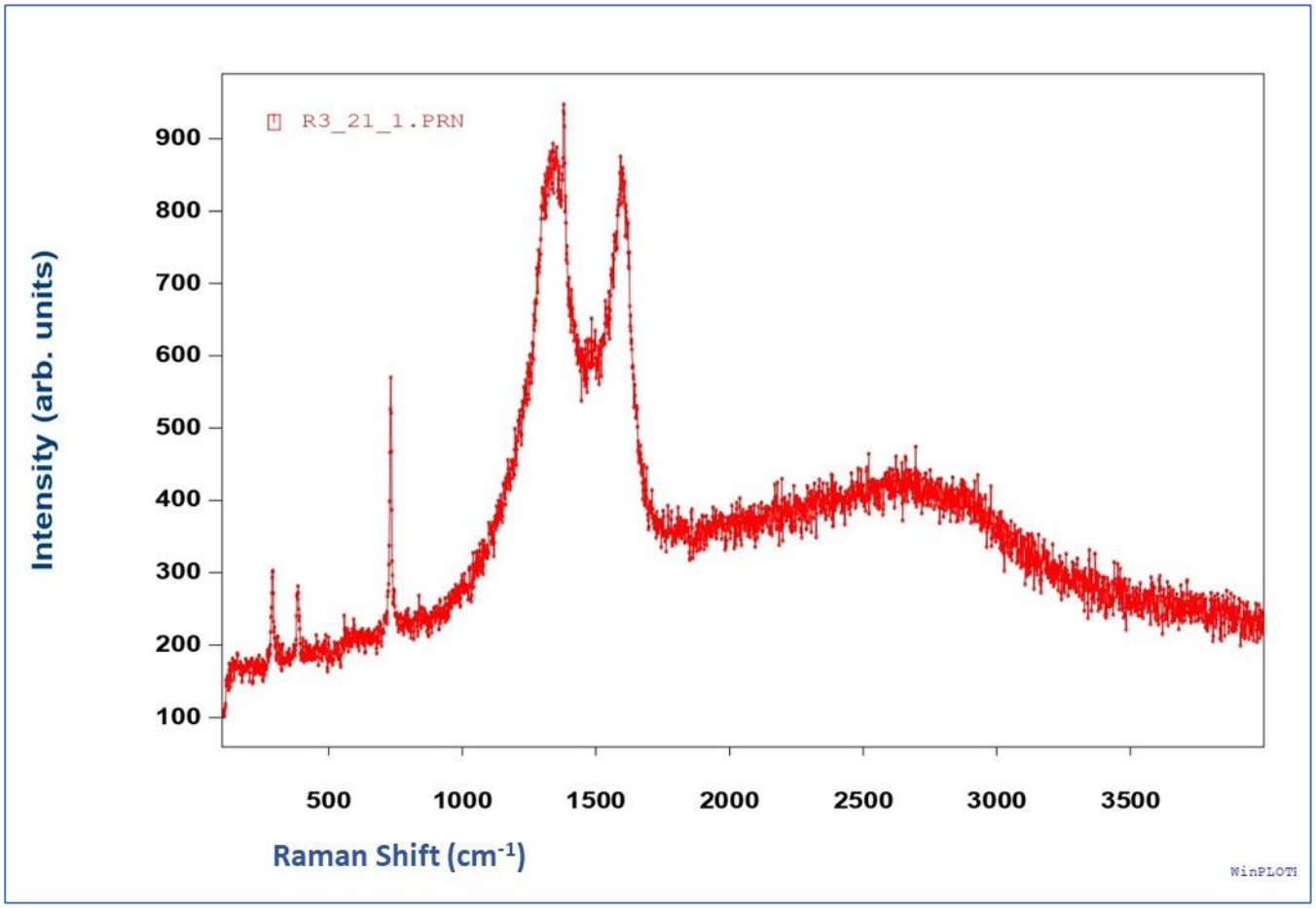

FIGURE 11. *Micro-Raman spectra of the black particles. The spectra is potentially consistent with the following materials: gilsonite, thucholite, retinet, cannel coal, jet coal or chalcocite*

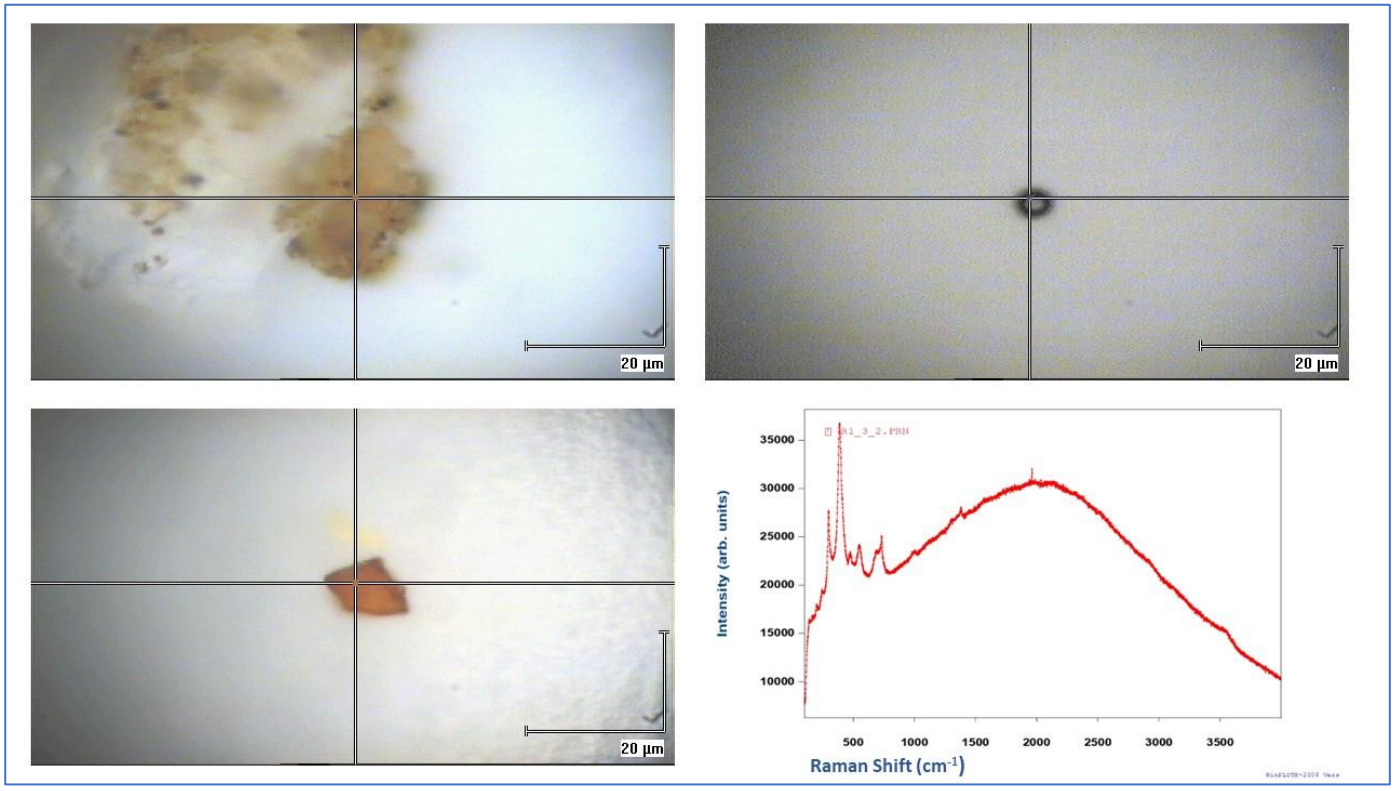

FIGURE 12. *Various particles found in our kidney samples, and spectra were consistent with hematite*

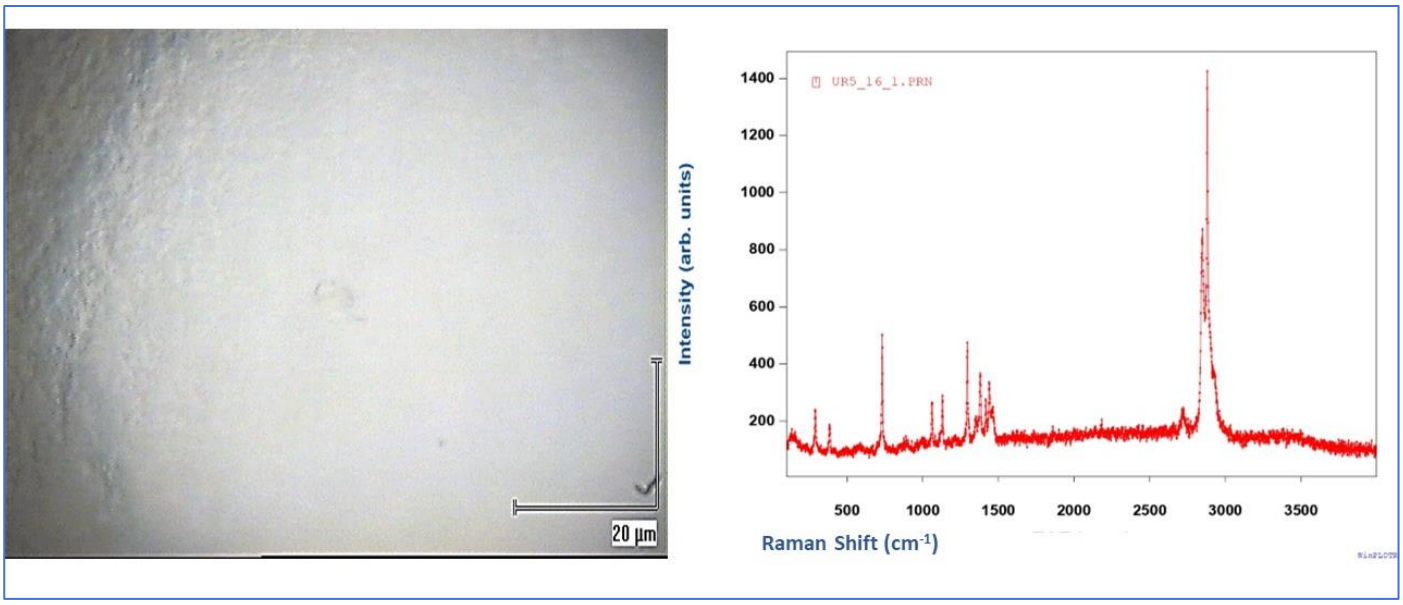

FIGURE 13. *A particle found in the urine and related spectra consistent with polyethylene*

Discussion

To the best of our knowledge, this is the first study that demonstrated the presence of MPs and their compounds in human urine and kidney tissue. In particular, this is the first study that advance evidence of MPs clearance in humans. Moreover, we added new evidences of their potential nephrotoxic effect in vitro on HK2 tubular cells using PE-microspheres and BPA. In particular, we performed the first study aimed to evaluate the toxicity of PE-MPs on kidney tubular effect and their “trojan horse” effect performing a wide biomolecular analysis.

In human kidney samples, collected by the “healthy” part of nephrectomies performed for renal carcinoma, we found various human-made particles. Some particles spectra were consistent with hematite, while others showed a spectra consistent with chalcocite or gilsonite or coals.

Hematite is a common heavy metal pollutant present in different environments [28] and recent studies analyzed its interaction with plastic particles [29]. Hematite is also used as polymer filler to reduce costs and enhance tensile properties [30-31].

We also found, both in kidney and urine, black particles in all the samples analyzed with a spectra potentially consistent with different elements: thucholite, retinet, chalcocite, gilsonite, cannel coal or jet coal (Figure 10). Thucolite is a mixture of hydrocarbons, uraninite and some sulphides [32]. It is a radioactive element, thus it could be probably excluded. Retinet is a natural resin, brown colored and could be probably be excluded too. Chalcocite, as hematite, is used for shielding purpose in Polyvinyl chloride (PVC) production, one of the most widely produced synthetic plastic polymer [33]. Gilsonite is a natural bitumen with various industrial uses such as additive in steel industry and in drilling fluids, production of aluminium and it is widely employed in black inks production [34].

Cannel coal, jet coal are polycyclic aromatic hydrocarbons naturally present in the environment [35].

Moreover, we analyzed the first sample of urine collected and we found various black particles consistent with the above-mentioned found on kidney samples. We also identified a particles of

PE. These findings are consistent with previous studies that evidences the presence in human organs of such MPs fragments and their compounds in particular, PVC and PE [21-22] and corroborate the hypothesis of a diffuse deposition of MPs in human organs and tissues.

In the study by Ragusa et al the identification of human-made particles was made mostly through the analysis of the particles pigments spectra. These pigments are also ubiquitous in the environment not only as MPs compounds but also linked to paints and coatings [36].

Raman analysis of sample and retrospective analysis of the spectra collected through Raman dedicated software and SLOPP Library of Microplastics is still ongoing.

Moreover, we performed one of the first in vitro analysis aimed to analyze the effect of MPs, its compounds and their combined exposure on kidney, by using HK2 cells treated with PE-MPs and BPA. We performed different conditions to investigate the potential toxicity of PE-MPs and the combined effect of BPA and MPs. BPA is a well-known pollutant, and various previous studies confirmed its deleterious effect on human cells [27]. Our analysis seems to confirm the few previous evidences of the potential toxic effects of MPs and compounds on kidney tubular cells and tissues.

Firstly, we demonstrated the reduced cell viability in HK2 caused by BPA and MPs through MTT reduction assay. This effect was much more obvious in MPs and BPA+MPs compared with BPA. Hence in our study MPs alone and paired with BPA were found to be toxic after 24 hours. This data is in contrast with previous studies in vitro. D.M. Monti et al analyzed the effect of engineered

polystyrene (PS) NPs on renal human cortical cells. They found that 44 nm PS-NPs didn't reduce cells viability and proliferation [37]. Our results on cells viability are also in contrast with another study performed by Wang et al. that analyzed the effect of PS-MPs [25]. None of the treated groups had significant differences compared with the control group except after 3 days with a MPs concentration of 0.8 mg/dL. In our study we used lower concentrations of PE microspheres and BPA as compared to previous studies.

These previous studies also evaluated the cellular uptake of PS-MPs and NPs. As in our study, they found a great and quick intracellular accumulation of MPs that overtime became preferably located in the perinuclear region and they didn't evidence a cellular clearance of MPs.

Furthermore, we analyzed different pathways of cell damage: inflammation, fibrosis, oxidative stress and mitochondria biogenesis. We found the PE-MPs, BPA and co-treatment to cause an activation of the inflammation and inflammation-fibrosis signaling with an increased expression of MCP-1 and IL-1β and a significant alteration of the axis AhR/HSP90.

AhR inactive form is present in the cytoplasm as complex with chaperons such as HSP90 [38]. Various exogenous ligands, such as heavy metals, polycyclic aromatic hydrocarbons and biphenyls can induce AhR upregulation and conformational alteration of AhR its ligands [39]. Its activation triggers the transcription of different mediators such as cytochrome P450, family 1, member 1A (*CYP1A1*), cytochrome P450, family 1, sub family B (*CYP1B1*) and cyclooxygenase-2 (*COX-2*).

The study of Wang et al investigated different signaling related to inflammation, autophagy, apoptosis and they conclude that PS-MPs injured kidney tissues. They found an increased expression of inflammation related proteins cPLA2 and COX-1 by MPs concentrations above 0.4 mg/mL. This study is interesting because they evaluated different concentrations of PS-MPs from 0.05, to 0:8 mg/mL for 24 or 48 h.

We also investigated the inflammation response and the oxidative stress induced by MPs and BPA evaluating various biomarkers such as MCP-1 that is a crucial regulator of the mitochondrial biogenesis [40], NOX4 and NRF-2 well known biomarkers of inflammation, vascular inflammation and mediators of the oxidative stress in kidney disease [41-42], IL-1β, a ubiquitous mediator that involves different pathways of inflammation mainly activated by the molecular mechanism of inflammosome [43] and finally NRF-2 a nuclear respiratory factor serving as a target of coactivators by MCP-1. Our results strongly indicate that BPA and PE-MPs induce a pro-inflammatory and pro-oxidative response in HK2 tubular cells.

Moreover, our initial results seem to indicate that the combined effects of MPs and BPA seem to induce a worst effect on HK2 than BPA and MPs alone corroborating the theory of the “trojan horse” effect mediated by MPs and NPs. Some authors refused the theory of the “trojan horse” effect in consideration of the fact that the concentration of MP in the environment are much lower than that used in vitro study [19].

The growing body of evidence of great accumulation of MPs and NPs in the environment, their presence in human body, their peculiar features (trespassing tissues and membrane, protein corona, trojan horse effect etc…), their potential tissues toxicity through different cell signaling (inflammation, fibrosis, oxidative stress, mitochondrial damage etc…) and the lack of information on human exposure indicate that more information about their potential risk for human health and suitable analytic methods are needed [44].

Recent evidences on the consequences of plastic pollution on human being shifted under the spotlight a topic that was previously considered trivial by medical scientific community.

It's undeniable that the presence of MPs in human body is an issue that potentially concern the whole human population and deserve a rapid and widespread call to action by the medical scientific community for its possible global implications.

Nowadays, the medical research has the responsibility to deeply investigate this field with the aim of eventually contrast a potential huge public health concern.

Firstly, researchers should try to quantify and monitor the presence of MPs and NPs in different organs and tissues. Thereafter, it's of utmost importance to investigate the potential detrimental effects of micro and nano plastic and their compounds on human health.

It’s possible that MPs will affect human health, thus we need to understand their toxicity and epidemiology, planning systematic researches to follow the fate of MPs in the body and reveal the disorders they cause.

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