NET4mPLASTIC Project

Work Package 4.4

Deliverable 4.4.2

DETERMINATION OF THE EFFECTS OF CONTAMINANTS AT SINGLE CELL LEVELS

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SUMMARY

This document refers to Deliverable 4.4.2, which is related to edit “Determination of the effects of contaminants at single cell levels”. Since the putative toxicity of microplastics (MPs), *in vitro* experiments were performed and literature data was analyzed in order to evaluate the potential toxic effects of these contaminants on human cell lines.

The main aims of the Deliverable 4.4.2 were:

- Evaluation of the effect of MPs on cell viability and proliferation.
- Investigation of the influence of MPs on cell fitness analyzing specific biological processes (i.e. apoptosis and autophagy)

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1. Introduction

Since the significant amount of plastic waste in the environment, a growing concern is the potential toxicity of this material on living organisms, including human. While large plastic items can entangle birds, turtles, mammals (e.g. dolphins), microplastics [MPs] and nanoplastics [NPs] can be swallowed by animals causing physical damage, suffocation and death [Byard R.W. et al.; 2020 – Yong C.Q.Y et al.; 2020]. Moreover, other than a physical hazard, a chemical toxicity related to plastic items could occur as consequence of their composition. The polymer type, the plastic monomers and oligomers resulting from incomplete polymerization reactions, and the additives commonly used for plastic production (i.e. solvent, catalysts antioxidants, plasticizers and elastomers) influence the toxic potential of these materials [Conti I. et al.; 2021 – NET4mPLASTIC, D. 3.4.1]. For example, polyvinyl chloride [PVC] is produced from vinyl chloride which is identified as the most health hazardous monomers since it has been associated with cancer, in particular, a malignant cancer of the endothelial cells of the liver (i.e. liver angiosarcoma) [Brand-Rauf P.W. et al.; 2012 – Rodrigues M.O. et al.; 2019]. Furthermore, plastic particles can adsorb environmental pollutants such as metals, pesticides (e.g. dichlorodiphenyltrichloroethane [DDT]), antibiotics, polycyclic aromatic hydrocarbons [PAHs] and polychlorinated biphenyls [PCBs] acting as chemical carriers [Ashton K. et al., 2010 - Li J. et al., 2018].

While the hazardousness of the plastic additives and environmental pollutants is known and regulated by both national and international laws [Conti I. et al.; 2021], the toxic potential of plastic particles themselves requires more clarification. Several both *in vitro* and *in vivo* studies investigated the impact of plastic particles on biological functions, however conflicting results have been observed [Yong C.Q.Y. et al.; 2020]. 0.2 µm PS-NPs caused lipid accumulation and acute oxidative stress (i.e. increase of reactive oxygen species [ROS]) in RAW 264.7 murine macrophages, conditions that are both related to the development of diseases, such as atherosclerosis [Florance I. et al.; 2021]. Analyses of biochemical markers and metabolomics profile of mice treated with PS-MPs (i.e. 2, 5 and 20 µm) by oral gavage, showed alteration of both lipid metabolism and oxidative stress suggesting that long PS-MPs exposure may be a potential health risk factor [Deng Y. et al.; 2017 – Wang Y.L. et al.; 2021]. Moreover, fatty acid metabolism disorder was observed, in a size-dependent manner, in the offspring of pregnant mice treated with 0.5 and 5 µm PS-MPs [Luo T. et al.; 2019].

Several studies identified the presence of plastic particles within the human body. In particular, 12 MPs fragments (ranging from 5 to 10 µm in size) were found in 4 human placentas [Ragusa A. et al.; 2021] and different plastic types (including PS) of plastic particles and fibres were evaluated within human blood and human stool [Leslie H.A. et al.; 2022 - Schwabl P. et al.; 2019]. However, these studies did not investigate the plastic related toxic effects on human cells.

On the other hand, no relevant toxic effects (including oxidative stress and inflammatory response) were observed by Stock et al. either in *in vitro* systems (i.e. human intestinal epithelial CaCo-2 and

human THP-1 derived macrophages) or in mice both exposed to 1, 4 and 10 µm PS particles [Stock V. et al.; 2019]. Treatment of both human peripheral blood mononuclear cells [PBMCs] and human mast cell line [HMC-1] with 0.46 – 1 – 3 – 10 – 40 – 100 µm PS-particles at a concentration lower than 500 µg/mL did not induce alteration of inflammatory cytokine expression (i.e. TNF-α, IL-6, IL-2 and IL-10) [Hwang J. et al.; 2020].

Since the different toxic effects evaluated among the previously described *in vitro* studies, a better clarification of the negative effects of MPs on cells should be required. In this deliverable, we broadly investigated the putative toxicity of PS-beads with different sizes in three human cell lines. In particular, we analyzed the potential effects of MPs with different diameters on HCT-116, A549 and Mahlavu cell lines (respectively colorectal, lung and hepatocellular carcinoma), in order to explore the effects of plastic particles on processes that are carried out at the cellular level, but are not necessarily restricted to a single cell.

2. Materials and Methods

2.1 Cell lines and treatments

Three different cell lines were exposed to MPs: HCT-116 (human colorectal carcinoma), A549 (human lung carcinoma) and Mahlavu (human hepatocellular carcinoma) (Supplementary Materials - D. 4.4.2, Fig. 1). All the three cell lines were maintained in DMEM medium (Lonza) supplemented with 10% inactivated FBS (Sigma-Aldrich), 1% Penicillin/Streptomycin (Lonza) and 1% L-Glutamine (Lonza) at 37°C and 5% CO_2. Cells were treated with different concentrations of unlabelled PS-MPs (10,000 – 20,000 beads/mm^2 of well plate) for 24 and 48 h and then collected for further analysis.

2.2 Microplastics beads

The PS-MPs were purchased as MPs dispersions in distilled H_2O (10% solid content) from Sigma-Aldrich. In this study, unlabelled PS-MPs with diameters of 0.1 - 1.1 – 3 µm (product number: LB1, LB11 and LB30, respectively) were used. At the beginning of the cellular experiment, PS-MPs samples were sterilized by 70% ethanol treatment for 10 min at +4°C followed by two washes with phosphate buffer saline [PBS] to remove the remaining ethanol.

Characterization of the beads was performed by Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) as described within the D. 4.4.1 (NET4mPLASTIC Project). SEM and TEM pictures of the PS-MPs are shown within the Supplementary Materials – D. 4.4.2 (NET4mPLASTIC Project), Fig. 2 - 3.

2.3 Cell viability assay

Cell viability assay was performed, using the Cell Counting Kit-8 (Dojindo), to evaluate the effect of PS-MPs on cellular viability. Cells were plated in triplicate in 96-wells culture plates at different cell density for 24 and 48 h respectively, to remain sub-confluent at the day of the processing: HCT-116: 5 x 10^3 cells/well and 3 x 10^3 cells/well and A549: 4 x 10^3 cells/well and 2 x 10^3 cells/well, Mahlavu:

1.5 x 10^3 cells/well and 3 x 10^3 cells/well, respectively for the 24 and 48 h of PS-MPs treatment. The following day, cells were treated with 100 µL of fresh medium containing 20,000 PS-MPs/mm^2 of well plate. Cells treated with Doxorubicin 1 µM were used as control for cell cytotoxicity.
After 24 and 48 h, CCK8 reagent was added as described by the manufacture instruction and the resulted optical densities [OD] were detected at both 450 nm (measurement filter) and 620 nm (reference filter) by a microplate reader (Sunrise, Tecan). Following the subtraction of the reference OD to the measurement OD, the values of cell viability for PS-MPs treated cells were calculated as percentage of the control.

2.4 Proliferation assay

HCT-116 cells were plated in 6-wells plates at a cell density of 4 x 10^5 cells/well and 3 x 10^5 cells/well (respectively for the 24 h and 48 h of treatment) and the following day, they were treated with different sizes of PS-MPs at the concentration of 20,000 PS-MPs/mm^2. After 24 and 48 h, cells were collected and counted using Trypan Blue (Sigma-Aldrich) staining to exclude non-viable cells from counts.

2.5 Flow cytometry analysis

To evaluate the effects of PS-MPs on cell cycle, flow cytometry analysis was performed with Propidium Iodide [PI] staining. HCT-116 cells were plated in 6-wells plates at a cell density of 4 x 10^5 cells/well and 3 x 10^5 cells/well (respectively for the 24 h and 48 h of treatment) and the following day, they were treated with different sizes of PS-MPs at the concentration of 20,000 PS-MPs/mm^2. After 24 and 48 h, cells were collected and fixed with 70% ethanol for 30 min at +4°C. Following two washes with cold PBS, cells were stained with 200 µL of PI staining solution (i.e. 20 µg/mL propidium iodide, 10 µg/mL RNase A, 0.1 % Triton X-100 in PBS) at least for 30 min at RT and then, they were analyzed by the flow-cytometer FACSCalibur (BD Bioscences). Data were examined using CellQuest Pro Software (FACSCalibur software, BD Bioscences).

2.6 Protein extraction and Western-blot

To evaluate the effects of PS-MPs on the expression of proteins associated with apoptosis and autophagy, western-blot assays were performed on HCT-116 and A549 treated with the different sizes of PS-beads. Cells were plated in 6-wells plates at a cell density of 3 x 10^5 cells/well (HCT-116) or 4 x 10^5 cells/well (A549) and the following day, they were exposed to 10,000 (HCT-116) or 20,000 (A549) PS-MPs/mm^2 of well plate. Treatment with Doxorubicin 1 µM or BGT-226 0.5 µM was used as control for the apoptosis and autophagy pathway activation. After 48 h, cells were collected and lysed in RIPA Buffer containing protease inhibitors for 30 min at +4°C to extract the cellular proteins. Samples containing equal amount of total proteins (30 – 40 µg) were separated by gel electrophoresis and transferred onto nitrocellulose blotting membranes (Amersham, Cytiva). After blocking with 5% bovine serum albumin [BSA] for 1 h at RT, membranes were incubated with primary antibodies against: LC3A/B (#4108, Cell Signalling Technology), PARP (#9532, Cell Signalling Technology) and β-actin (A5316, Sigma-Aldrich) at appropriate dilutions, based on the manufacturer instructions, overnight at +4°C. The next day, the membranes were incubated with HRP-conjugated secondary antibodies (i.e. Anti-rabbit IgG and Anti-mouse IgG HRP-linked

antibodies, respectively #7074 and #7076, Cell Signalling Technology) for 1 h at RT. Bands detection was performed using Western Lightning ECL Pro Chemiluminescent Substrate (PerkinElmer).

2.7 Statistical analysis

Statistical analyses were performed with GraphPad Prism 8 software (GraphPad Holdings). Comparisons between control and PS-MPs treated group were analyzed using one-way ANOVA followed by Dunnett's test for multiple comparison. $P < 0.05$ was considered as statistically significant.

3. Results

3.1 Effects of PS-beads on cell viability and proliferation

The three human cell lines were exposed to the PS-MPs at concentration of 20,000 beads/mm^2 for 24 and 48 h in order to evaluate the effect of plastic beads on cell viability. No cytotoxic effect on cell viability of the three cell lines was observed for all the tested MPs concentrations and sizes, at the two experimental time points (i.e. 24 and 48 h) (Fig 1).
Cell proliferation was analyzed on HCT-116 cells exposed to 0.1, 1.1 and 3 µm PS-MPs at the concentration of 20,000 beads/mm^2. Results showed a reduction of the cells when treated with 1.1 and 3 µm PS-MPs at both 24 and 48 h compared to control (Fig. 2).
A similar decrease of the proliferation was observed for both the 1.1 µm and 3 µm PS-MPs treated cells at 24 h (60% of the untreated cells), whereas at 48 h were 70 % and 78 %.
Trypan blue staining was used to exclude dead cells from cell counts (Fig. 2).

3.2 Effects of PS-beads on cell cycle

Since we observed the reduction of cell proliferation notwithstanding the unchanged viability of cells, the influence of PS-MPs on cell cycle was therefore investigated. HCT-116 cells treated with 1.1 and 3 µm PS-MPs at the concentration of 20,000 beads/mm^2 for 24 and 48 h showed a block of the cell cycle in S phase at 48 h, especially for the 3 µm treated cells.
In particular, while no changes of the cell cycle were observed for the PS-MPs treated cells at 24 h, an increase of the percentage of the cells in S phase was detected at 48 h for both the 1.1 and 3 µm exposed cells when compared to the control, in a size-dependent manner: the percentage of S phase cells increased from 9.71% of the control to 14.10% and 18.47% of the 1.1 µm and 3 µm treated cells, respectively (Fig. 3).
Noteworthy, this increase was paralleled by the G2/M phase decrease to 14.23 and 7.13% respectively, when compared with a control value of 17,69%.

3.3 PS-beads induced apoptosis and autophagy on human cell lines in a dose and size-dependent manner

Following the Transmission Electron Microscopy [TEM] analyses of Mahlavu cells treated with PS-MPs described within the D. 4.4.1, a cytoplasm enriched in dense organelles and vacuoles,

compatible/suggestive to autophagic ones, was observed in plastic treated cells compared to control (Fig. 4).
To evaluate if the change of the content of the cellular cytoplasm could be related to the activation of autophagy, cells were treated with PS-MPs for 48 h and expression of LC3A/B, as marker for autophagy, was analyzed.
However, no autophagy induction, as detected by LC3A/B isoforms expression, was observable for the three beads size in both A549 exposed to 10,000 beads/mm2 and HCT-116 exposed to 20,000 beads/mm^2 (Fig. 5).
Further, we wanted to explore also the apoptotic cell death process activation using, as a readout of autophagy, the cleavage of PARP. A549 and HCT-116 cells lines were exposed to the same treatment described above for autophagy, and analyzed for the expression of PARP/cleaved-PARP proteins. The 3 µm treated HCT-116 cells when compared to control sample showed some PARP cleavage (Fig. 6).

4. Discussion

Following their uptake by human cell lines as described within the D. 4.4.1 (Net4mPLASTIC Project), MPs could act on cellular pathways influencing biological processes, such as cellular viability and proliferation. Several *in vitro* studies reported reduction of both cell viability and proliferation, up-regulation of the inflammatory genes transcription (e.g. IL-8, TNF-α and NF-kb) and activation of the apoptosis pathway investigating human cell lines (i.e. human lung and human intestinal cell lines) treated with PS-beads [Goodman K.E. et al.; 2021 – Visalli G. et al.; 2021 – Domenech J. et al.; 2021 - Dong C.D. et al.; 2020 – Xu M. et al.; 2019]. For example, alteration of cell viability was observed in A549 cells treated with PS particles. In particular, high concentration of PS-NPs (i.e. 25-30 µg/mL of 25 nm PS-beads and 160-220-300 µg/mL of 70 nm PS-beads) inhibited cell viability in a dose-dependent manner, while PS-size became the main factor, influencing the viability, at lower plastic concentration. Moreover, cell cycle S phase arrest and induction of apoptosis as consequence of PS-NPs treatment was detected [Xu M. et al.; 2019]. Reduction in cell proliferation was evaluated in A549 exposed to both 1 and 10 µm PS-MPs. After 48 h of treatment, PS-MPs treated A549 were consistent at 30% of the unexposed population [Goodman K.E. et al.; 2021]. Induction of autophagy initiation and autophagosome formation was observed in 100 nm PS-NPs human umbilical vein endothelial cells [HUVEC], compared to both the untreated and the 500 nm PS-NPs cells [Lu Y.Y. et al.; 2022].
Here, we investigated the effects of PS-MPs with different sizes on three human cell lines. Mahlavu, HCT-116 and A549 cells (respectively, hepatocellular, colorectal and lung cancer cells) were exposed to unlabelled PS-MPs with 0.1 to 3 µm diameter at different concentrations for 24 and 48 h. PS-MPs did not affect the cellular viability for all the three cell lines. No alteration of viability compared to the control was observed also in the cells treated with the biggest PS-MPs (i.e. 3 µm) and at the highest concentration (i.e. 20,000 beads/mm^2) for the both tested experimental time

points.
However, a reduction of cell proliferation was evaluated. Analysis of HCT-116 cells exposed to 0.1, 1.1 and 3 µm PS-MPs at the concentration of 20,000 beads/mm^2, showed lower number of HCT-116 cells for the 1.1 and 3 µm treated, compared to the 0.1 µm treated cells and the control; but the evaluated reduction of the proliferation in our experiments were higher than those observed by Goodman K.E. et al, probably due to the different analyzed cell line.
Since this inhibition of HCT-116 cells proliferation, effect of Ps-breads treatment on cell cycle was investigated. An arrest of cell cycle in S phase was detected for the 1.1 and 3 µm treated HCT-116 cells after 48 h of plastic beads treatment. In particular, the percentage of cells in S phase of the cell cycle increased in a size-dependent manner: 14.10% for the 1.1 µm treated, 18.47% for the 3 µm treated compared to the control (9.71%).
Finally, investigation of the activation of cell death mechanisms (i.e. autophagy and apoptosis) was performed. Autophagy is a degradative process which is based on the sequestration of parts of cytoplasm and organelles in double membrane autophagic vesicles (autophagosome) where they were hydrolyzed following the fusion to lysosomes, to recycle amino acids and other molecules. While during apoptosis, apoptotic cells show early degradation of the cytoskeleton due to caspase cleavage but the organelles are preserved until late in the process [Thorburn A.; 2008 - Klionsky D.J. et al.; 2021]. Since the evaluation of the accumulation of organelles and vesicles in PS-MPs treated Mahlavu by TEM analysis, we studied if this change in cytoplasm could be caused by cellular death pathways activation. Induction of apoptosis was detected only in 3 µm treated HCT-116 cell (concentration of 20,000 beads/mm^2), suggesting a different effect of plastic particles among the diverse cellular types.

5. Conclusions

Humans are potentially daily exposed to plastic intake although the exposure risk is yet unresolved. Combining inhalation and ingestion data, Cox D.K. et al. estimated an annual human intake of more than 100,000 plastic particles [Cox D.K. et al.; 2019]. Once enter within the human body, plastic fragments could accumulate into tissue and exert possible toxic effects due to the potential toxicity of plastic themselves [Chang X. et al.; 2020].
In this deliverable, we investigated the potential toxic effects of PS-MPs with different sizes and at different concentrations on three human cell lines (colorectal, lung and hepatocellular carcinoma) that could represent the potential human tissue exposed to human plastic intake. Although, plastic particles did not affect cell viability, reduction of cell proliferation and a S phase cell cycle arrest were detected in PS-MPs treated cells in a size-dependent manner. Moreover, induction of cellular death mechanism (i.e. apoptosis) was observed in the 3 µm beads treated HCT-116 cells. Further studies will be required to clarify the toxic effects of plastic beads on human cell lines in order to hypothesize the potential risk of human exposure to plastic.

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