Mesenchymal Stromal Cells showed an alteration of differentiation potential under Environmental Micro and Nanoplastics Exposure

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Abstract

Background: Humans are exposed to environmental microplastic (MPs) that can be frequent in water and food. The mesenchymal stromal cells are a heterogeneous population, which contain fibroblasts and stromal cells, progenitor cells and stem cells. They are part of the stromal component of most tissue and organs in our organisms. Any injury to their functions may impair tissue renewal and homeostasis.

Methods: We evaluated the effects of different size MPs that could be present in water bottles on human bone marrow mesenchymal stromal cells (BMMSCs) and adipose mesenchymal stromal cells (AMSCs). MPs of polyethylene terephthalate (PET) (<1 μm and <2.6 μm) were tested in this study.

Results: PET treatments induced a reduction in proliferating cells associated either with the onset of senescence or increase in apoptosis. The AMSCs and BMMSCs exposed to PET showed an alteration of differentiation potential. AMSCs remained in an early stage of adipocyte differentiation as shown by high levels of mRNA for *PPARG* and reduction in *LPL* mRNA levels. A loss of differentiation capacity was also observed for the osteocyte phenotype in BMMSCs.

Conclusions: This pioneering mesenchymal cell response survey demonstrated that environmental plastic particles are bioavailable for uptake into cells and thus may lead to irreversible diseases.

1. Introduction

Each year, over 8 million tons of plastic gets dumped into the environment, both on land and in the sea. Indeed, the world plastic production has skyrocketed nearly 200 times in the last years [1]. Nevertheless, consumption is still growing exponentially.

The name microplastics (MPs), was first reported by Thompson in 2004 [2] and designates very small plastic particulates and plastic fibers having a diameter less than 5 mm. MPs also include nanoplastics (NPs), whose diameters are below 0.1μm (100 nm). Due to their small size, MPs can be potentially dangerous, since they can be easily absorbed by the organism's tissues and organs. Nevertheless, MPs are used in certain industries.

MPs are classified as primary and secondary. Primary MPs have industrial use, and they are among the raw materials for certain consumer products too, while secondary MPs are those that are derived from the decomposition of macroplastics due to the different processes of environmental mechanical degradation, biodegradation and chemical degradation [3].

For these reasons, in recent years, research has been carried out with a focus on the risk MPs pose to human health. Human could be MP exposed through several routes: inhalation, diet, dermic.

As MPs persistently remain in the environment, they accumulate in organisms [4], especially in marine ecosystems [5]. In this context, the marine living species area major contamination source.

A recent study demonstrates the presence of plastic particles in human bloodstream [6]thus providing robust proofs of the occurrence of plastic contamination in human body. Moreover, studies involving humans have shown the transfer of MPs through the intestine to the lymphatic system [7].This transfer is even more highlighted in the colon of patients with inflammatory bowel disease, which correlates with an increase in intestinal permeability [7, 8] The presence of MPs in the intestine can be toxic due to its intrinsic ability to induce intestinal blockages or tissue abrasions. Furthermore, the passage of MPs to the lymphatic system suggests that they can reach any part of an organism. This is due also to another worrying aspect, i.e., their microscopic size; in fact, MPs and NPs could find different paths of absorption either by phagocytosis or endocytosis[9].

Mesenchymal Stromal Cells (MSCs), residing in the bone marrow (BM), adipose tissue (AT), and in the stromal component of other tissues, are made of subpopulation of stem cells that can differentiate in chondrocytes, osteocytes, adipocytes, and smooth muscle cells. In addition, MSCs sustain hematopoiesis process, support the organismal's homeostasis, and regulate the inflammatory response. MSCs also have a crucial therapeutic value [10]. The valuable effect of MSCs in homeostasis and tissue repair occurs through the secretion of many factors. The presence of MSCs in the BM of skeletal bones and in the AT, which is, widely distributed in our body (beneath the skin, between muscles, around the kidneys, heart, and abdominal membranes) increases the possibility that they may suffer from genotoxic damage, including exposure to MPs [5]. As a result of their long lifespan, stem cells can undergo multiple rounds of damage, MPs that individually do not have a major impact on cell physiology, but collectively can severely impair cell function.

In view of the afore said factors, we hypothesized that neither the microenvironment of the BM nor the adipose tissue (AT) that is a dense vascular network are safe from being penetrated by MPs [11].

The present study aims to analyze the effects of MPs of different sizes (1 and 2.6 μm) on human bone marrow mesenchymal stromal cells (BMMSCs) and adipose mesenchymal stromal cells (AMSCs). We evaluated the potential of MPs to affect the biology of MSCs by inducing cytotoxic or damaging effects.

2. Materials And Methods

2.1. Human bone marrow and adipose MSCs

The human BMMSCs and AMSCs were obtained by Lonza (CH) and cultivated according manufacturer's instruction.

2.2. Preparation of MPs

The MP particles derived from uncolored PET water bottles. The polymer type of the bottles was verified by FTIR spectroscopy (FTIR, Thermo-Scientific, MS, USA). In brief, a bottle sample (10 grams) was flash-frozen with liquid nitrogen and then grounded in a swing mill to obtain irregular particles having heterogeneous form and size. The obtained samples were filtered with a 30 μm sieve and then diluted in

100 ml of MQ water. The obtained plastic dust was successively filtered through small pores of cellulose nitrate filters (Ø = 47 mm, 2.76 µm, and 1 µm) (Healthcare Life Science, UK) to obtain two solutions with two size ranges of MPs (2.67–1 µm and under 1µm).We performed a Scanning Electron Microscopy qualitative analysis of the particle surface structure (Cambridge Instruments, UK - Mod. Stereoscan 360). The microscopy analysis was associated with SEM-EDX (X Energy Dispersion Detector) using the Inca software. The SEM-EDX was also used to count the number of particles per ml for each size range and further transformed to µg/L based on PET density [12].

2.3. MSC treatment with PET

For an evaluation of PET effects on *in-vitro* MSC functions, the cells were incubated for 48 hours in aMEM containing FBS (10%), FGF2(3ng/ml) and PETs (10µg/ml). We used two different sizes—<1µm (PET1) and <2.6µm (PET2.6). After 48 hours, the cultures were used for the programmed experiments.

2.4. Proliferating assay

Cell proliferation was determined by CCK-8(Colorimetric Cell Counting)assay by Dojindo (MD, USA) according manufacturer's instruction.

2.5. Immunocytochemistry and Senescence-Associated Beta-Galactosidase

After PETs exposure, the cells grown in 24 multi-wells were fixed in a 2% formaldehyde solution for 10 minutes. Cells were then stained for detecting Senescence-Associated Beta-Galactosidase as already reported [13].

Following senescence staining, cells were permeabilized with 0.3% Triton-X100 (Roche, CH) and further incubated at room temperature in a blocking solution maid of 0.1% Triton-X100and5% FBS. The samples were then incubated with the antibodies against pRPS6 (Cell Signaling, MA, USA, code 4858) and Ki67 (Santa Cruz Biotechnology, CA, USA, code sc7846) at 4 °C overnight. We used a goat anti-rabbit antibody (FITC-conjugated) secondary antibody(Gtx-Rb-003D488) and a goat anti-mouse antibody(TRITC-conjugated) secondary antibody (Gtx-Mu-003D594) that were from Immuno Reagents (NC, USA). DAPI staining was employed to recognize nuclei and images were obtained using a DM2000Leica fluorescence microscope., For each analyzed marker, the percentage of positive cells was calculated as reported by Alessio and co-workers(2021) [14].

2.6. Immunocytochemistry (ICC) for detection of Ataxia-telangiectasia mutated kinase (ATM) and gamma-H2AX

The ICC procedure was performed according our previous published [13].

2.7. DCF-DA assay

Reactive oxygen species (ROS)were evaluated by intracellular conversion of fluorescent DCFH-DA (2,7-dichlorodihydrofluorescein). Cells were incubated with 0.1% Pluronic F-127 and DCFH-DA (2 µM) for 30

minutes at 37°C, then were PBS washed analyzed with easyCyte™ flow cytometer (Millipore, MS,USA) with easyCyte™ software.

2.8. Annexin-V assay

Apoptotic cells were identified with a fluorescein-conjugated Annexin V kit (Millipore, MS, USA) on a Guava easyCyte cytometer following the manufacturer's instructions. We grouped together early and late apoptotic cells.

2.9. Colony Forming Units assay (CFU)

Following treatments with MPs, 1,000 cells were seeded in 100 mm plate and incubated in a growth medium for 14 days according our previous published procedure [13].

2.10. Spontaneous differentiation

MSC cultures were trypsinized after being treated with MPs and $1.5x10^4$ cells were seeded in six-well plates pre-coated with gelatin solution (Sigma Aldrich, MO, USA). Cells were incubated in aMEM medium supplemented with 10% FBS for 21 days.

2.11. *In-vitro* differentiation and staining

After being treatment with MPs, $1.5 \times 10^4/cm^2$ cells were seeded and cultivated either in osteogenic or chondrogenic or adipogenic medium at 37°C for 21 days as already reported [15]. Cell staining for detecting osteocytes, adipocytes and chondrocytes was performed as previously described [15].

2.12 Western blotting

Cells were lysed in buffer containing 0.1% Triton-X100 (Roche, CH) and analyzed for western blot as already reported [16]. The following primary antibodies were used: RB2/P130 from BD Biosciences (CA, USA); P27KIP1 and RB1 from Cell Signaling Technology (MS, USA), P107 (code sc-318), P53 (code DOI-1), and P21CIP1 (code C-19) from Santa Cruz Biotechnology (CA, USA); P16INK4A from ABCAM (UK).

2.13 Soft agar

After being treated with MPs, 2,500 cells were incubated in 0.5 mL DMEM containing agarose plus FBS in 35 mm Petri dishes and incubated for 21 days as already reported [13].

2.14 Statistical analysis

Statistical significance was processed using the one-way test ANOVA followed by post hoc test (Tukey test). All data were analyzed using the statistical software package GraphPad Prism version 5.01 (GraphPad, CA, USA).

3. Results

3.1 Effects of (polyethylene terephthalate) PET on the MSC biology

MPs used for this study were obtained from uncolored PET water bottles. We exposed the AMSC and BMMSC cultures to MPs for 72 hrs. Then, we assessed the effect of MPs on the biological properties of MSCs such as: proliferation rate, cell cycle, apoptosis, and senescence. The culture exposure to different sizes of MPs showed modification in the MSC proliferation rate only for PET 2.6 μm (Figure 1a). We also carried out ICC to identify the percentage of cycling cells (SA-β-gal-, Ki67 +, pRPS6 +). We evidenced a reduction in the cycling cells in all of the analyzed conditions (Figure 1b). These results are in line with the cell cycle analysis (Figure 1c), where the level of cells in S phase decreased in both AMSCs and BMMSCs after being treated with PET 1 μm and 2.6 μm (hereinafter indicated as PET1 and PET2.6, respectively).

Reduction of cycling cells could either indicate that cells entered quiescence or, alternatively, the PET may produce genotoxic damages, which trigger senescence or apoptosis. Thus we assessed the percentage of senescent and apoptotic cells using the Senescence-Associated Beta-Galactosidase (SA -β-gal) and Annexin V assay, respectively. Some findings evidenced that SA -β-gal is highly active even after persistent stress, such as forced culture conditions, changes in temperature and pH values [17, 18].This event may limit the use of SA -β-gal as a senescence marker. We then performed cytochemistry analysis for SA -β-gal together with Ki67 and pRPS6 immunodetection to specifically identify senescent cells, which are SA -β-gal(+), Ki67(-), and pRPS6(+) [14].

The percentage of senescent cells (Figure 1d) showed an increase in BMMSC and AMSC cultures incubated with PET1, while PET2.6 treatment showed an increase in senescence only for BMMSCs.

The analysis of apoptosis, instead, showed an increase in apoptosis in AMSCs treated with PET 2.6 (Figure 1e). The PET2.6 genotoxic damages may be more severe in AMSCs, since in these samples we detected cell death while in BMMSCs we observed senescence phenomena.

We then studied the molecular signaling circuits associated with PET-induced senescence and/or apoptosis. We analyzed the levels of key proteins (P53- RB-pathways) involved in these phenomena. In most of the analyzed conditions, we detected an upregulation of P27KIP1, P21CIP1, and P16INK4A, which are cyclin kinase inhibitors (CKIs) (Figure 1f).In detail, in samples with increased senescence we observed the upregulation of all three reported CKIs, while in AMSCs treated with PET2.6 the apoptosis onset appeared related mainly to P21CIP1 increase.

3.2 PET induce modification in DNA damage repair (DDR)

In recent study Chang-Bum et al. (2020) showed that the MP cellular toxicity mainly depends on oxidative stress via reactive oxygen species(ROS). This event may contribute to the senescence and apoptosis we observed following MSC treatment with PETs. ROS may induce macromolecule damages, including DNA.

For these reasons, we determined the intracellular ROS levels after MP exposure. PET1 treatment induced an increase of ROS level in BMMSCs (Figure 2a) and AMSCs (Figure 2a), while PET 2.6induced ROS increase only in BMMSCs. We also evaluated the percentage of stressed cells by immunocytochemistry against Ki67, RPS6, and beta-Gal positive cells. The results highlight that only PET1 is able to induce a stress phenotype in BMMSCs (Figure 2b).

We then performed a detailed analysis of potential MP-induced unrepaired/misrepaired DNA damages by evaluating the persistence of active H2AX (γ-H2AX) and ATM nuclear foci. Histone H2AX is an important regulator of cellular responses to DNA damage and is considered a hallmark of damaged DNA nuclear foci. ATM is a kinase that regulates DNA repair. Activation of ATM by auto-phosphorylation at Ser1981 (pATM) signals the presence of DNA injury [19]. A correct DNA repair is achieved within a few hours from alteration, the permanence of H2AX and ATM foci for hours or days following genotoxic events may indicates the presence of unrepaired/misrepaired DNA damages, which can harm cell's physiological function by promoting either senescence or, alternatively, neoplastic transformation.

PET treatments induced accumulation of damaged DNA foci in all the experimental conditions (Figure 2c,2d). This result is in line with senescence phenomena we above reported. In order to have a clear insight on PET effects on MSCs we also evaluated the occurrence of neoplastic transformation, since recent studies suggest a link between microplastics and cancer risk [20]. Transformed cells can grow independently of a solid surface. This feature is a typical carcinogenesis hallmark and was examined by soft agar colony formation assay. Cells cultured with MPs for 72 hours and matched controls showed no anchorage-independent growth until 21 days after exposure (Figure 2e).

3.3 Effects of PET on MSC stemness.

Any stress may alter stem cell functions. In this context, we studied the MPs effects on the properties of stem cells present in MSCs. In detail, we analyzed self-renewal and multipotentiality, which are fundamental for preserving the performance of a stem cell, i.e. contribution to tissue regeneration and organismal homeostasis. We carried out a CFU assay to check clonogenicity, which is a basic characteristic for stem cell self-renewal.

PET1 treatment induced a decrease in the number of CFUs in both AMSCs and BMMSCs, whereas PET2.6 showed such a decrease only in AMSCs (Figure 3a).

The differentiation potential of MSCs based on their spontaneous ability to be committed into adipocytes, osteocytes, and chondrocytes was assessed (Figure 3b). We observed modifications in this capacity: the AMSCs showed a bias toward the adipocyte lineage specification in presence of PET1 and to lesser extent with PET2.6 (Figure 3b), while BMMSCs showed a propensity to osteocyte lineage commitment (Figure 3b), associated with a decline in chondrocyte lineage specification (Figure 3b).

We then determined the capacity of these cells to external cue-induced differentiation in adipocytes, osteocytes and chondrocytes. The results showed that, in presence of PET1 and PET2.6, AMSCs

remained in an early stage of adipocyte differentiation as shown by the high levels of *PPARG* mRNA and the reduction in *LPL* mRNA, which are early and late differentiation markers, respectively (Figure 3c). The PET1 and PET2.6 treatments induced a loss in osteocyte differentiation while the PET 2.6 impaired the chondrocyte differentiation. Similar results were observed in PET-treated BMMSC cultures with alteration of differentiation processes. In detail, PET1 and PET2.6 arrested osteocyte differentiation in a early stage, as evidenced by an increase in *BGLA* mRNA, associated with reduction of *SSP1* (early and late differentiation markers, respectively). Of note, the PET treatments significantly impaired the BMMSCs ability to differentiate into adipocytes and chondrocytes (Figure 3c).

Discussion

Over the last years, MPs have increased in a variety of products, from water bottles to synthetic clothing, cosmetics and plastic bags. The toxicity of MPs also depends on the increased bioaccumulation potential that has rendered toxicological effects on the environment and also on human health through the food chain [5, 21]. Not only, but also the shape and size of these MPs fall among the harmful aspects of these substances[22]. For these reasons, detailed research is required to characterize these compounds and understand their effect on human health.

The simple ingestion of MPs by fish and their transfer to humans through the food chain where they move from the intestines to the lymphatic system suggests that these microparticles can reach any part of the organism and present a dangerous problem [22]. For these reasons, we hypothesized that the highly vascularized tissues such as bone marrow and AT are not immune to this phenomenon. The bone marrow microenvironment contains a heterogeneous cell population of stromal cells called MSCs [16].

To better understand the effects of MPs on the fate of stem cells, we carried out several experiments with two different sizes of PET—1 and 2.6 μm on MSCs present in the bone marrow. The presence of MSCs within all the organs and tissues that contain stroma prompted us also to study the MSCs isolated from AT, where these cells may be more prone to MP contamination than those present in the bone marrow. The MSC stem cells component can differentiate into adipocytes, osteocytes, and chondrocytes. MSCs locate in various tissues and they receive strong attention for their therapeutic capacities for these reasons represent a good study model.

Besides supporting hematopoiesis, the MSCs contribute to tissue renewal. They also sustain through paracrine effects, site-specific epithelial and endothelial responses, secrete growth factors and ECM proteins, and also display a local anti-inflammatory capability [23].

The treatment of MSCs with MPs significantly changed the fate of these cells. Indeed, after 3days of treatment, we observed a significant decrease in the proliferation rate of MSCs isolated from both the bone marrow and AT. The reduction of this proliferation was associated with the onset of senescence, as demonstrated by the acid beta-galactosidase assay. The increase in senescence is in line with the reduction of the proliferation and signified that a single dose of MPs could cause a dangerous effect in

cells. This is a general problem caused by MPs, in fact, in several papers, different authors showed that the treatment with MPs reduced the proliferation rate and the cell growth of different cell lines [24].

The effects observed on the fate of these cells led us to focus on two important aspects that characterize stem cells: self-renewal and differentiation capacity. We carried out a CFU assay to test their clonogenicity, which is an important feature of self-renewing stem cells, as well as their differentiation into adipocytes, osteocytes and chondrocytes. We observed significant differences between control and MP-exposed cells, suggesting that MPs may be dangerous to MSCs.

Several experiments have showed that the exposure of living cells to MPs may cause the overproduction of free radicals, in particular ROS [25, 26]. An excessive generation of ROS and their accumulation in cells cause oxidative stress, this increase induces higher probability of cancer risk and/or senescence. Our study evidenced that MP exposure by promoting ROS accumulation in MSCs may in turn induce unrepaired DNA damaged leading to senescence. Increase in ROS was not detected in AMSCs treated with PET2.6. This observation may be at odds with the other results, it must be underlined that the PET2.6 treatment induced apoptosis rather than senescence in AMSC, dead cells with high levels of intracellular ROS may detach from cell plates and hence ROS increase may be overlooked.

Senescence occurring in stem cell compartments, such as those hosting MSCs, are particular dangerous since any damage to stem cell pools may have profound consequences for human health. Indeed, PET-induced senescence of MSCs altered the self-renewal capacity of stem cells and undermine their differentiation potential.

Several studies claim that there are risks induced by MPs, specifically, an increase in the possibility of tumors. To ascertain whether MPs had caused genetic changes to MSCs that would induce neoplastic changes, we carried out a soft agar assay, which demonstrated that the exposure of MSCs to PET1 and PET2.6 could not genetically modify MSCs, even when maintained in culture for an additional 21 days. These results do not exclude that MP long exposure time-periods and or cultivation for periods longer than 21 days may promote some neoplastic events.

Conclusions

Our study has found that MP exposure modified the fate of MSCs *in-vitro*, inducing senescence with the loss of several properties of stem cells. Our *in-vitro* study is innovative in demonstrating that there are significant changes in the biology of MSCs after their exposure to MPs and NPs. This could pave the way to *in vivo* studies to evaluate effect of MPs on stem cell niches to evaluate which kinds of damages may affect stem cells following exposure to such potentially genotoxic agents.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the

corresponding author on reasonable request.

Competing interests

The Authors declare that there is no conflict of interest.

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Authors' contributions

NA, HN, and TS provided substantial contributions to conception and design, data acquisition and data analysis and interpretation;

NA, HN, GO, MF, MB: Writing - original draft, Writing - review & editing, and critically revising it for important intellectual content;

GC, MF, GB, UG, IM, SM, MB, they provided final approval of the version to be published;

NH, NA, TS, GC, MF, GB, UG, IM, SM, MB are agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy and integrity of the work are appropriately investigated and resolved.

All the authors revised to approved the final version to be submitted.

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Figures

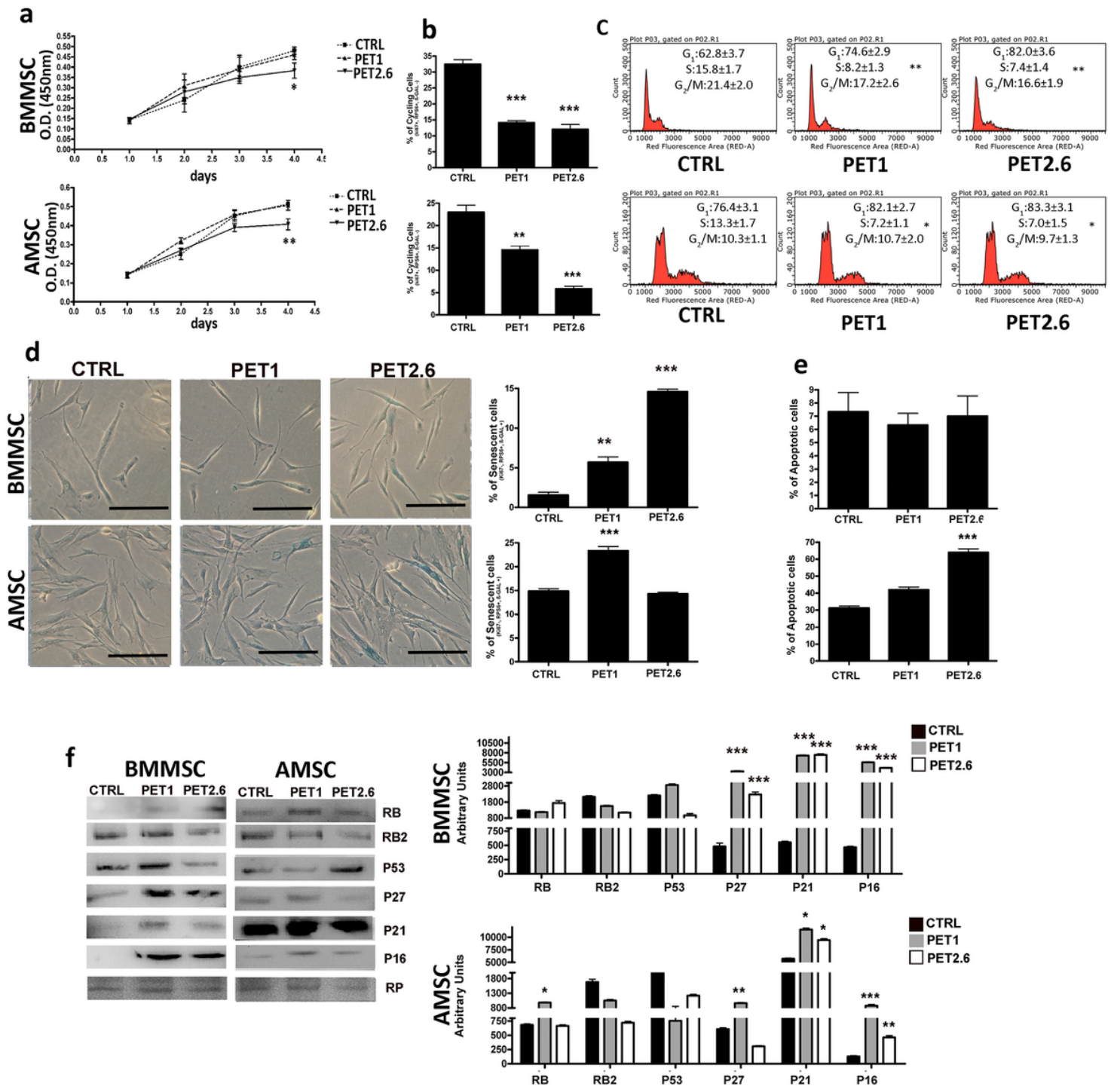

Figure 1

Biological properties of AMSCs and BMMSCs after treatments with PET1 and PET2.6.

a – Cell proliferation measured by Cell Counting Kit-8,one, two and three days after treatments. (n = 3± SD; * p<0.05, **p<0.01).

b –Histograms show the percentage of cycling MSCs (Ki67+, pRPS6+, β-Gal-), after PET treatments (n = 3± SD;** p<0.01, ***p<0.001).

c – The graphsshow representative FACS analysis. Percentages of different cell populations (G1, S, and G2/M) are indicated(n = 3± SD; * p<0.05, **p<0.01).

d –Representative microscopic fields of senescence-associated beta-galactosidase–positive cells. On the right, histograms show the percentage of senescent MSCs (Ki67 -, pRPS6 +, β-Gal +).(n = 3 ± SD; ** p<0.01, ***p<0.001). The black bar corresponds to 100 mm.

e – Histograms show the percentage of apoptotic cells (n = 3, ± SD; ***p<0.001).

f – Expression levels of proteins involved in senescence and/or apoptosis phenomena. Ponceau Red (RP) was used as a loading control. On the right, the histograms show the quantitative western blot analysis (n = 3± SD; *p<0.05, ** p<0.01, ***p<0.001).

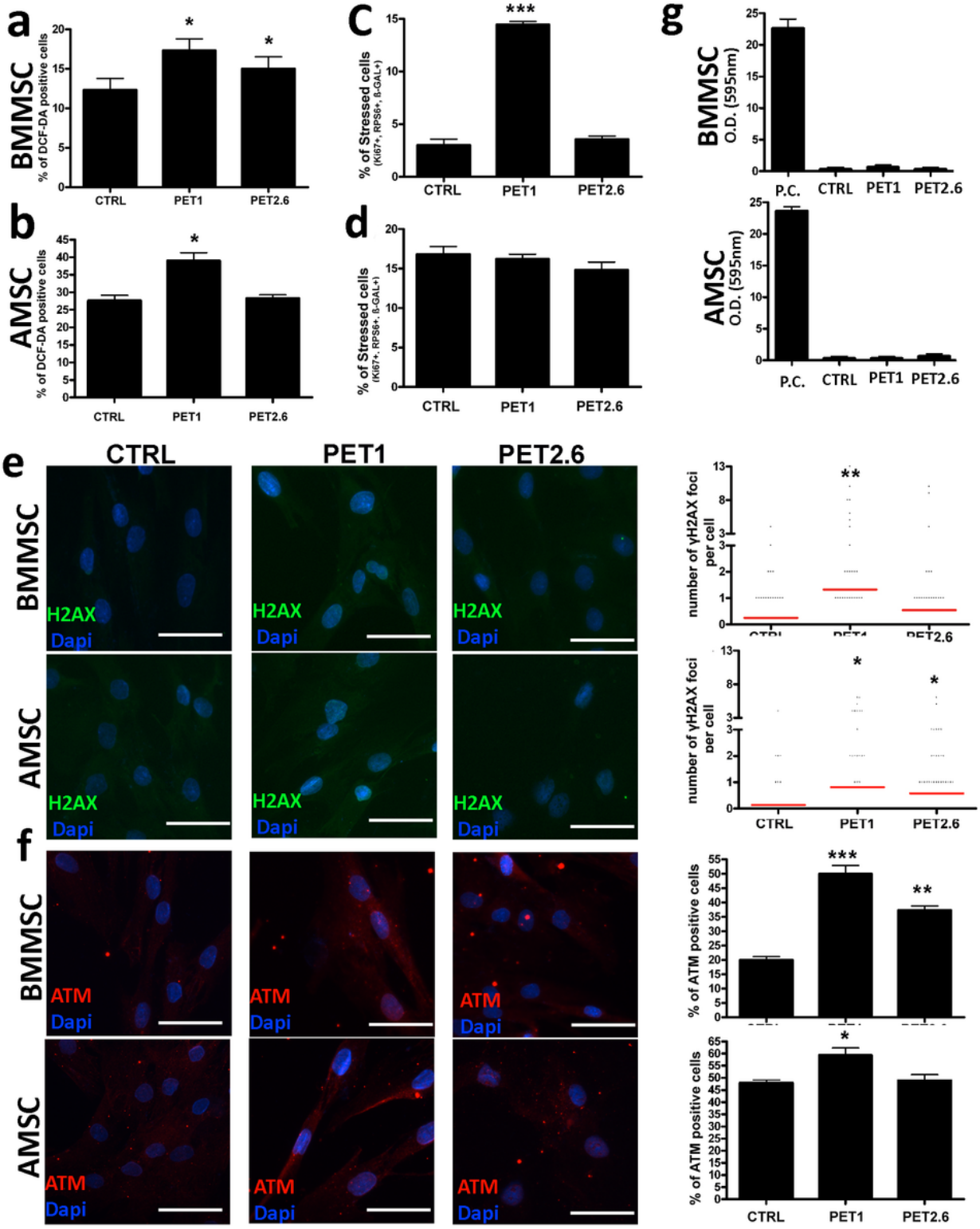

Figure 2

Evaluation of DNA damage in AMSCs and BMMSCs after treatments with PET1 and PET2.6.

a- The histograms show the percentage of DCF-DA positive cells (n = 3± SD; *p<0.05).

b – The histograms show the percentage of stressed MSCs (Ki67 +, pRPS6 +, β-Gal +) .(n = 3 ± SD; ***p<0.001).

c -.Fluorescence images show the merge of cells stained with anti-γH2AX and nuclei stained with DAPI. On the right, graphs show the degree of γH2AX foci per cell (n = 3 ± SD; *p<0.05 and **p<0.01).

d - Fluorescence images show typical cells stained with anti-ATM and nuclei stained with DAPI. On the right the histograms indicate the mean percentage of ATM-positive cells for each condition (n=3± SD; *p<0.05, **p<0.01 and ***p<0.001).

e – Histograms show optical density readings at 595 nm obtained from methanol elution of the crystal violet stain for each condition (n = 3, ± SD).

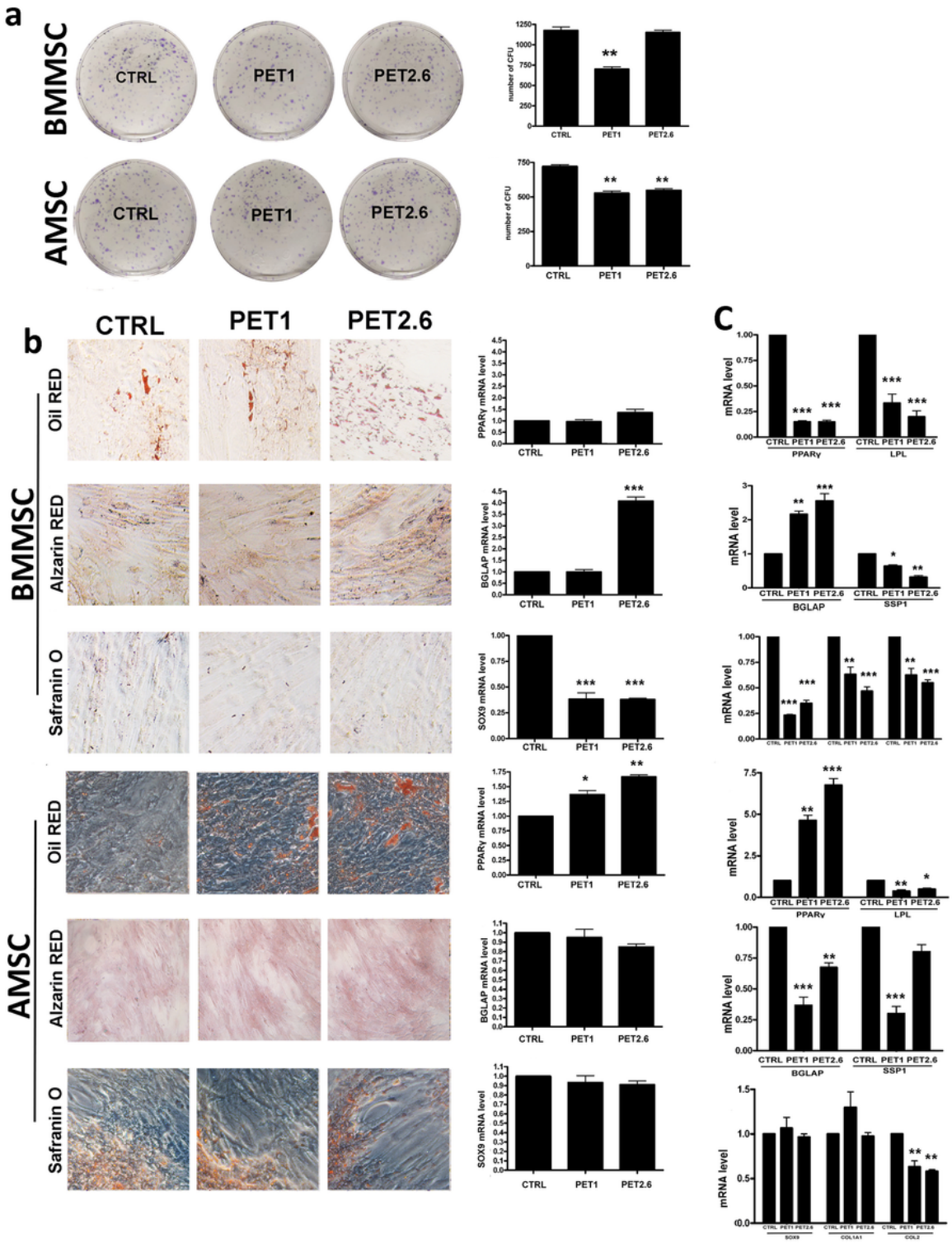

Figure 3

Stemness properties of AMSCs and BMMSCs after treatments with PET1 and PET2.6.

a – The pictures show representative crystal violet staining of clones. On the right, the histograms show the number of clones obtained (n = 3, ± SD; ** p<0.01).

b – Microscope images of differentiated cells: adipocyte (Oil RED), osteocyte (Alizarin RED) and chondrocyte (Safranin O). On the right, the histograms show mRNA levels of differentiation markers in spontaneous condition. GAPDH was selected as internal control (n= 3± SD;*** p<0.001).

c - The histograms show mRNA levels of differentiation markers in cue-differentation. GAPDH was selected as internal control (n = 3± SD;*p<0.05, **p<0.01 and *** p<0.001).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupFile1.pdf