

Human osteoblast-like cells exhibit anabolic attenuation and catabolic phenotypic signals after exposure to polystyrene microplastics

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Abstract

Live organisms exposed to microplastics (MPs) develop several organ dysfunctions. Recently, MPs were detected in human bones. However, their role in the pathophysiology of bone diseases requires a better understanding at the cellular level. We investigated the effects of polystyrene (PS-MPs) in MG-63 osteoblast-like cells. Internalization of PS-MPs was observed by confocal microscopy, and cell proliferation by MTT assay. Mineralization was evaluated by alkaline phosphatase (ALP) activity and calcium uptake. Oxidative metabolism was assessed by mitochondrial oxygen consumption rate, glucose consumption, and lactate release. Intracellular superoxide production was evaluated using dihydroethidium assay. Protein expression was analyzed by Milliplex™ bone panel and Western blot, and gene expression by RT-qPCR. PS-MPs were internalized by MG-63 cells. Exposure to up to 25 µg/mL PS-MPs did not affect proliferation, whereas 50 µg/mL significantly increased superoxide production and cell death. Mitochondrial bioenergetics and calcium uptake were preserved. The protein levels of osteocalcin, osteoprotegerin, and DKK1 were reduced, while β -catenin, p-SMAD4, and NF- κ B were increased. Downregulation of *ALPL* and *BMP4* genes was also observed, while other osteogenic and inflammatory signaling gene expressions (*RUNX2*, *CTNNB1*, *OPG*, *OC*, *DKK1*, *TGF β* , *NF- κ B-p65* and *IL-1 β*) remained unchanged. In conclusion, MG-63 osteoblast-like cells internalize PS-MPs; at high concentrations reduce cell proliferation and increase oxidative stress levels. PS-MPs exposure seems to induce a distinct uncoupling between gene transcripts and key bone proteins, independently of changes in mitochondrial bioenergetics or impairment of matrix mineralization. These data may suggest that human osteoblast-like cells exhibit anabolic attenuation and catabolic phenotype signals after PS-MPs exposure.

INTRODUCTION

Microplastics (MP) have emerged as pervasive environmental contaminants, resulting in unavoidable human exposure through inhalation, ingestion, and dermal contact (1–3). Evidence indicates that MP can cross mucosal and endothelial barriers (4, 5), entering systemic circulation (6, 7) and accumulating within distant physiological compartments (8), including the bone microenvironment (9, 10). Among the various polymer constituents, polystyrene microplastics (PS-MP) represent a major fraction of detected microparticles in biological systems (11, 12). However, despite growing concerns regarding environmental toxicants and bone health, the direct cellular and molecular mechanisms by which PS-MP interact with bone cells remain insufficiently defined.

Osteoblasts play a critical role in maintaining skeletal integrity by driving matrix mineralization, secreting regulatory cytokines, and coordinating bone remodeling pathways (13, 14). Disruptions in osteoblastic activity caused by exogenous toxicants can impair matrix deposition and alter intracellular signaling networks, predisposing bone tissue to metabolic imbalance (15, 16). While the physical presence of microparticles is known to induce localized tissue responses, the internalization capacity and the physiological consequences of PS-MP exposure on osteoblast survival, oxidative homeostasis, energy

metabolism, and functional differentiation capacity require comprehensive investigation to elucidate the mechanisms of PS-MP-induced bone cell toxicity.

Recent studies have begun to explore MP toxicity in skeletal tissue, demonstrating that PS-MPs can induce oxidative stress, promote pro-inflammatory responses, and disrupt osteogenic differentiation in progenitor stem cell models and animal tissues (17–19). However, significant mechanistic knowledge gaps remain. Most existing investigations rely on undifferentiated stem cells or static cytotoxicity assays, leaving the direct bioenergetics and functional kinetics in committed human osteoblasts poorly understood. Specifically, there is no precise information about PS-MPs internalization, if any, by osteoblastic cells, and what their consequences are for mitochondrial bioenergetics and downstream mineral deposition, alongside dual gene and protein expression of core osteogenic pathways.

To address this knowledge gap, the present study investigated the toxicological profile of characterized PS-MPs in human MG-63 osteoblast-like cells, a widely validated model in bone biology due to their stable phenotype, reproducibility, and responsiveness to osteogenic stimuli. (20, 21).

We evaluated interactions by verifying particle internalization and examining their subsequent impact on cell proliferation, oxidative stress parameters and mitochondrial bioenergetics. Furthermore, we assessed whether PS-MPs exposure alters functional osteogenic outcomes, such as alkaline phosphatase activity and calcium mineral deposition, alongside targeted profiling of key osteogenic and inflammatory signaling pathways at both the gene and protein expression levels. We aimed to explore potential toxicological mechanisms of PS-MPs in human osteoblast-like cells.

MATERIAL AND METHODS

This study protocol was approved by the local ERB under registration number CEP/PRP no. 031/2024.

Polystyrene microplastics (PS-MP) type and characterization

Fluorescent 0.5 µm PS-MP particles (excitation 360 nm/emission 407 nm) were purchased from Polysciences, Inc. (Fluoresbrite TM, Warrington, PA, USA). The confirmation of the PS-MPs morphological characteristics and chemical composition was made by Fourier-transform infrared (FT-IR), scanning electron microscopy (SEM-FEG Mira 3 XMU Electron Microscope – Tescan), and by RAMAN spectroscopy (Witec Alpha 300-R), through the identification of characteristic PS vibrational bands.

Cell type and conditions of treatment

The effects of PS-MP on OBL MG-63 cells (CRL 1427, ATCC, USA) were evaluated in comparison with non-exposed controls. Cells were routinely cultured in T75 cm² bottles at 37°C in a humidified incubator with 5% CO₂. Cells were maintained in Dulbecco's modified Eagle's standard medium (DMEM)

supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 44 mM sodium bicarbonate (3.7 g/L of DMEM). The culture medium was always changed every two days.

For alkaline phosphatase (ALP) and calcium uptake assays, cells were treated for 21 days with an osteoblast differentiation cocktail to induce mineralization: ascorbic acid (Sigma A4544, 125 μ M), β -glycerophosphate (Sigma G9422, 10 mM), and dexamethasone (D4902, 0.1 μ M), which were supplemented to the standard culture medium, every two days. Proliferation test was performed at 24 h of culture, and glucose/lactate measurements at 24 and 48 h. Other experiments were performed with cells cultured for 7 days.

Cell density was adjusted for each experiment: for cells internalization of PS-MP test, the density was 1×10^4 cells/ petri dish; for cell viability assay, 2×10^4 /well in 96-well plates; for ALP test, 5×10^3 cells/well, for calcium uptake measurement, 1×10^4 cells/well, both in 48-well plates; for oxygen consumption rate (OCR) assessment, 7.5×10^5 cells were used; for glucose and lactate determinations, 1×10^6 cells/well in 48-wells plates; for superoxide production 62 cells/well in 96-well plates, and for proteins and qPCR analysis, 2×10^5 cells/well in 6-wells plates.

PS-MPs internalization by MG-63 cells

Following exposure for 7 days, cells were fixed with 4% formaldehyde and incubated with bromodeoxyuridine-neuronal nuclei-TOPRO-3 (1:500 diluted, Thermo Fisher, R37113) for 15 minutes. The cellular incorporation of PS-MPs was observed by confocal microscopy (Leica, TCS SP5 II), with a thickness of 3.09 μ m in each image, strictly set to the excitation and emission parameters for the PS-MPs, as stated by the manufacturer.

Cell proliferation assay

Proliferation of MG-63 cells was assessed by the MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazoline bromide] method. Cells were incubated at 37°C and 5% CO₂ for 24 hours. In sequence, the culture medium was replenished with medium containing the range of tested PS-MPs concentrations. After 24 h of incubation and washing the wells with PBS 1X, 100 μ L of DMEM with MTT at a concentration of 0.5 mg/mL was added to each well. The plates were kept at 37°C for 3 hours to allow formazan crystals to develop. Then, 100 μ L of dimethyl sulfoxide (DMSO, D0231, BioBasic, Canada) was added. Plate reading used the absorbance in the spectrophotometer (PowerWave X2, Biotek, USA) at 450 nm with 630 nm as reference. Data are presented as mean $\pm$ standard deviation (SD) from twelve biological samples (n = 12).

Functional and metabolic tests

The ALP activity was determined by hydrolysis of p-nitrophenylphosphate to p-nitrophenol as previously reported (22). Calcium uptake as a marker of cell mineralization influenced by PS-MP treatment in MG-63 cells was measured by quantification of o-cresolphthalein complexone according to a previous study (23). Both assays were performed after 21 days of culture. Data are presented as mean $\pm$ standard deviation (SD) from twelve biological samples (n = 12).

The OCR was determined using a high-resolution oxygraph (Oroboros Oxygraph-2k, Innsbruck, Austria). For this, the cells were trypsinized and suspended in DMEM containing 20 mM HEPES- Na^+ (pH 7.3–7.4). Cells were added to the 2 mL oxygraph chamber. Firstly, OCR was measured under basal conditions. Then, 1 μL oligomycin was added to inhibit complex V and assess leak respiration. Then, CCCP was titrated in sequential 0.5 μM doses to achieve maximum uncoupled respiration. Data are presented as mean $\pm$ standard deviation (SD) from seven independent biological samples (n = 7).

Glucose and lactate concentrations at baseline, 24, and 48 h were measured in the cell culture supernatant using commercial enzymatic-colorimetric kits (K082-2 and K084-2, Quibasa Química Básica Ltda –Bioclin, Belo Horizonte, Brazil). For these assays, cells were incubated for up to 48 h with DMEM without phenol red (D5523, Sigma-Aldrich) and PS-MPs. Absorbances were measured on a microplate spectrophotometer (Biotek, K082) in baseline, 24, and 48 h (505 nm for glucose and 545 nm for lactate). Quantification was performed by interpolating the sample absorbance values against standard glucose and lactate calibration curves, and the results were expressed in mg/dL. Data are presented as mean $\pm$ standard deviation (SD) from twelve independent biological samples (n = 12).

Assessment of superoxide production

The medium from cell culture was removed, and 100 μL of dihydroethidium (DHE, Invitrogen D11347, 5 μM) diluted in PBS was added to each well for 30 min at 37°C under 5% CO_2 . Samples were washed with DMEM without phenol red (D5523, Sigma-Aldrich), and fluorescence images were captured using an inverted microscope (Ex/Em 488/574–595 nm, red channel, Leica). Cells were then fixed with 4% formaldehyde and stained overnight with 0.05% crystal violet (Sigma-Aldrich C0775). After staining, 10% acetic acid was added to each well, and absorbance was measured at 590 nm using a microplate reader (Biotek K082). Superoxide production was quantified as the optical density of DHE normalized to the optical density of crystal violet. Data are presented as mean $\pm$ standard deviation (SD) from twelve independent biological samples (n = 12).

Bone-related proteins expression

Protein lysates from the cells were obtained using RIPA lysis buffer (Tris-HCl pH 7.4, 50 mM; NaCl, 150 mM; Triton X-100, 1%; Sodium deoxycholate, 0.5%; SDS, 0.1%; H_2O). This buffer was supplemented with 25x protease inhibitor cocktail (Merck Sigma REF 11873580001, USA), 0.1 M phenylmethylsulfonyl fluoride (PMSF, Sigma P7626, USA), and 0.1 M sodium orthovanadate (Sigma S6508, USA). Total protein was extracted from the cells following 7 days of exposure to PS-MPs and their concentration was

determined using the Bio-Rad DC Protein Assay kit, following the manufacturer's instructions, and expressed in $\mu\text{g}/\mu\text{L}$.

Quantitative immunoassays using the MILLIPLEX® Multiplex Assays with Luminex technology (Bone Panel HBNMAG-51K, USA. Assays range: DKK1 5–20,000 pg/mL; OPG 7–30,000 pg/mL; OC 146–600,000 pg/mL; OPN 98–400,000 pg/mL; SCL 24–100,000 pg/mL; FGF-23 37–150,000 pg/mL) were performed according to manufacturer's protocols. The levels of dickkopf-1 (Dkk1), osteoprotegerin (OPG), osteocalcin (OC), osteopontin (OPN), sclerostin (SCL), and fibroblast growth factor 23 (FGF-23) were analyzed in both the cellular protein lysate and the corresponding supernatant.

Western blot analysis was performed according to the protocol previously published (20). Briefly, 50 μg of total protein from each sample was diluted in ultrapure water to a final volume of 15 μL , in which 5 μL of 4× Laemmli sample buffer (1.610.747, Bio-Rad, USA) was added. The samples were then denatured at 95°C for 5 minutes and separated by gel electrophoresis. Then, a transfer was carried out using a wet transfer system with a buffer composed of 25 mM Tris, 192 mM glycine, and 20% ethanol, at a constant voltage of 60 V for 120 minutes. The membranes were blocked in a solution containing 2% bovine serum albumin (BSA, Goldbio, 9048–46 – 8, USA) in Tris-buffered saline containing 0.1% Tween 20 (TBST) (20.605, ICI America Inc., USA) for 30 minutes. Washing steps were performed four times, for 5 minutes each, using TBST.

For protein detection, the membranes were incubated overnight at 4°C with the primary antibodies of interest. Next, incubation was performed with a peroxidase (HRP)-conjugated anti-rabbit secondary antibody (Cell Signaling, 5174), diluted 1:10,000 in 5% BSA, for 1 h at room temperature. Protein bands were visualized using the ECL Prime Western Blotting Detection Reagent (Cytiva, Amersham, USA), with signal acquisition performed on an ImageQuant LAS 500 imaging system (GE Healthcare, Japan) in “incremental” mode. Additionally, the anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase; Cell Signaling, 5174) antibody, also diluted 1:1,000 in TBST with 5% BSA, was incubated for 1 h at room temperature and was used as a housekeeping control. Information on primary antibodies is listed in supplementary Table S1.

The optical density of these protein bands was quantified using ImageJ software (using “gel” tool), with quantifications based on the area under the curve and normalization relative to the intra-experimental control (GAPDH). Data are presented as fold-change related to the control, representing six independent biological samples ($n = 6$). Statistical significance was determined using one-way ANOVA followed by Tukey's multiple comparisons test.

Gene expression by quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from the cells following 7 days of exposure to PS-MPs using TRIzol Reagent (15596018, Thermo Fisher Scientific Invitrogen), according to the manufacturer's instructions. The quantification (in $\text{ng}/\mu\text{L}$) was performed using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher

Scientific, Waltham, MA, USA). Transcription to cDNA was performed using the GoScript™ reverse transcription system (Cat A5001, Promega, USA) according to the manufacturer's instructions, consistently using 0.6 µg of total RNA input. The qPCR reactions were performed using the QuantStudio 3 system (Thermo Fisher Scientific), with 2X PowerUp SYBR™ Green Master Mix, containing 10 µL of Master Mix, 0.25 µL of forward primer, 0.25 µL of reverse primer, 7.5 µL of nuclease-free water, and 2 µL of cDNA (1–10 ng), in addition to NTC (no-template control) controls. Serial standard curves were used to determine primer amplification efficiency, with values between 90–110%, a correlation coefficient (R^2) > 0.99, and a slope of -3.32 considered ideal.

The primers were designed using the Primer-BLAST tool, followed by structural validation via the IDT OligoAnalyzer platform (<https://www.idtdna.com/pages/tools/oligoanalyzer>). Parameters of formation of secondary structures (hairpins) and homologous and heterologous dimers were evaluated, using ΔG values as a criterion for stability. Only primer pairs that met the specific IDT criteria quality control were selected for synthesis and experimental use.

Amplification specificity was also confirmed by melt curve analysis, ensuring the presence of a single amplified product. The absence of genomic DNA contamination was verified by reverse transcriptase (RT-) controls, using primers designed in intragenic regions. No-template controls (NTCs) were also included globally as the ultimate non-specific amplification control. Data normalization was performed using the *YWHAZ* gene as reference (24). Information on target primers is listed in supplementary Table S2.

Gene expression of the selected primers was quantified by quantitative qPCR (QuantStudio 3), with the $2^{-\Delta\Delta CT}$ method for relative quantification. Resulting values were normalized relative to experimental control (without exposure to PS-MPs) and normalized against the housekeeping gene, representing six independent biological samples ($n = 6$).

Statistical analysis

Data were expressed as mean $\pm$ standard deviation, or percentages. Data normality was assessed using the Shapiro-Wilk test. Depending on the distribution, comparisons between two conditions were performed using either the Student's *t*-test or the Mann-Whitney test. For multiple comparisons, either one-way ANOVA followed by Dunnett's post hoc test or the Kruskal-Wallis test followed by Dunn's post hoc test was used. For Luminex multiplex secretome assays, sample sizes ($n = 4$ biological samples per group) were selected based on standard exploratory immunoassay protocols, and findings near the significance threshold ($p \approx 0.03$ – 0.05) were interpreted conservatively. Statistical analyses were performed using GraphPad Prism 10.1.1 (v. 323), and a significance level of $p < 0.05$ was considered statistically significant.

RESULTS

The morphological and physicochemical characteristics of the PS-MPs suspension were confirmed by Fourier-transform infrared (FT-IR), Scanning Electron Microscopy (SEM), and Raman spectroscopy (Fig. 1), as stated by the manufacturers.

PS-MPs internalization

Fluorescent signals corresponding to the PS-MPs particles (blue) were consistently detected within the cytoplasmic region, surrounding the nuclei stained by TO-PRO-3 (red). The distribution pattern indicated that PS-MPs were taken up by the cells rather than remaining exclusively in the extracellular space. No fluorescence was detected in the control condition, confirming the specificity of the signal. These observations demonstrate that MG-63 cells internalize PS-MPs under the tested conditions (Fig. 2a-b).

Cell proliferation with PS-MPs

Differences in optical density between the different PS-MPs concentrations were observed; indeed, proliferation slightly but significantly decreased at the highest PS-MPs concentration when compared to the control (50 µg/mL: 0.958 ± 0.007 ; control: 1.009 ± 0.008 ; $p = 0.004$) (Fig. 3).

Functional tests

Exposure to PS-MPs did not significantly affect ALP activity or calcium uptake by MG-63 cells. Enzymatic ALP activity, measured through pNPP conversion to pNP, remained comparable between control and treated groups, and no detectable differences were observed in mineralized formation as assessed by calcium quantification. These findings indicate that PS-MPs, in tested concentrations, did not impair the osteogenic differentiation capacity of MG-63 cells (Fig. 4a-b).

The mitochondrial bioenergetics of MG-63 cells were assessed by high-resolution respirometry, glucose consumption, and lactate release. The mitochondrial oxygen consumption rate was similar between the control group and cells treated with 5 or 50 µg/mL PS-MPs. Neither basal oxygen consumption (control: 40.6 ± 4.8 vs. 50 µg/mL: 41.7 ± 14.5 pmol/s/ 10^6 cells) nor maximum oxygen consumption (control: 114.8 ± 20.5 vs. 50 µg/mL: 125.9 ± 20.6 pmol/s/ 10^6 cells) was altered in the presence of PS-MPs (Fig. 4c-d). Glucose consumption from the culture medium and lactate release were evaluated at baseline, 24 h and 48 h. No differences were observed among the control, 5 µg/mL and 50 µg/mL PS-MP exposure groups (Fig. 4e-f).

Superoxide production

Exposure to PS-MPs modulated superoxide generation, with a marked and significant increase in all concentrations tested (control: 5.8 ± 2.5 AU vs. 5 µg/mL: 32.3 ± 4.0 AU vs. 10 µg/mL: 32.3 ± 4.2 AU vs. 25 µg/mL: 33.5 ± 4.1 AU vs. 50 µg/mL: 44.0 ± 3.9 AU, $p < 0.0001$). Quantitative analysis of DHE fluorescence, normalized to crystal violet staining, revealed an increasing but overall non-significant effect among groups, except at the highest PS-MP concentration (Fig. 5a). Consistent with the quantitative data,

fluorescence microscopy showed low basal ROS levels in control cells, whereas cells exposed to 50 µg/mL PS-MPs exhibited a pronounced increase in DHE-derived fluorescence (Fig. 5b).

Bone-related proteins expression

Protein expression assessed by Milliplex assay revealed that the concentration of OC levels was significantly decreased in the lysate at both 10 and 25 µg/mL PS-MPs [(137 vs. 109 pg/mL, $p = 0.04$) and (137 vs. 100 pg/mL, $p = 0.03$)], but no differences were detected in the supernatants. OPG in the supernatant was significantly reduced at 10 and 25 µg/mL PS-MPs compared to the control [(7402 vs. 8719 pg/mL, $p = 0.046$) and (6950 vs. 8719 pg/mL, $p = 0.011$)], whereas no differences were observed in the cell lysate (Fig. 6a). Dkk-1 concentrations were also reduced in the supernatants at 25 µg/mL PS-MPs compared to control (7789 vs. 10,431 pg/mL, $p = 0.04$) (Fig. 6a). OPN, SCL, and FGF-23 concentrations remained unchanged across treatments in both cell lysates and supernatants (supplementary material, Figure S1).

In the protein expression analysis assessed by Western blot (WB) assay, β -Catenin, p-SMAD4, and NF- κ B expressions were significantly increased at PS-MPs 25 µg/mL (Fig. 6b). The representative Western blot pictures corresponding to these quantified proteins are shown in Fig. 6c. Other bone-related proteins, such as SMAD3 and SMAD1/5/9, were not altered by PS-MPs (supplementary material, Figure S2).

Quantitative reverse transcriptase polymerase chain reaction (RT qPCR)

The expressions of *ALPL* and *BMP4* were lower in the presence of the PS-MPs (Fig. 7). Conversely, no significant changes were observed in the expression of *RUNX2*, *BGLAP*, *TNFRSF11B*, *CTNNB1*, *IL-1 β* , *NF κ B-p65*, and *DKK1* genes at the tested PS-MP concentrations (supplementary material, Figure S4).

DISCUSSION

In the present study, the effects of PS-MPs on MG-63 cells were associated with signals of cytotoxicity and increased oxidative stress at the highest concentration (50 µg/mL), while at the concentrations of 10 and 25 µg/mL, we observed changes related to inflammation and modulation of osteogenic and catabolic pathways. Mineralization capacity and mitochondrial function were preserved, suggesting the activation of adaptive or compensatory mechanisms to maintain cellular mitochondrial bioenergetic functionality in the face of the induced stress.

Interestingly, cell proliferation displayed a non-linear dose-response profile across the tested concentration range. Exposure to 2.5 µg/mL PS-MPs resulted in a slight, significant increase in cellular proliferation compared to control, whereas intermediate concentrations (5–25 µg/mL) showed no significant alteration and a significant decrease in proliferation was observed only at 50 µg/mL. This pattern resembles a hormetic response, a well-documented phenomenon in toxicology where low-dose stress stimulates metabolic activity or transient proliferation as an adaptive survival mechanism prior to cytotoxicity at higher concentrations (25). Similar non-linear dose responses have been observed in

osteogenic and soft tissue models exposed to micro- and nanoplastics, where lower particle burdens trigger metabolic hyper-activation or cellular defense mechanisms without compromising viability (26).

The observed alterations in secreted OC and OPG protein levels indicate significant changes in the MG-63 osteoblast-like phenotype. The decrease in OC and OPG may be interpreted as signals of decreased capacity for bone formation and a lower ability to inhibit osteoclastogenesis, since OC is crucial for calcium deposition in the bone matrix and OPG is essential for blocking RANKL action (27). The unaltered expression of their corresponding regulatory genes (*RUNX2* and *CTNNB1*) suggests that sub-cytotoxic concentrations of PS-MPs exposure may primarily impact post-translational processing. Such uncoupling of transcriptional and translational profiles is a well-documented cellular response to sub-lethal particulate stress, where PS-MPs-induced oxidative stress selectively compromises protein maturation, vesicular trafficking, or extracellular export mechanisms without necessarily dismantling the underlying intracellular transcription factors (18, 28, 29). Intriguingly, this particulate stress may not affect all pathways exclusively at the post-translational level. The present data also show a transcriptional downregulation of the *ALPL* gene. Alkaline phosphatase (encoded by *ALPL*) is a foundational enzymatic driver of early osteoblast maturation and mineralization (30, 31). This selective downregulation of the *ALPL* gene suggests that chronic PS-MPs exposure could repress specific early-stage differentiation transcripts without compromising the functional enzymatic levels of ALP (10, 32).

This complex mechanistic crosstalk is further highlighted by the concurrent modulation of NF- κ B, BMP4/p-SMAD4 and canonical Wnt/ β -catenin components. In our experimental study, we observed a significant upregulation of NF- κ B, β -catenin, and p-SMAD4 protein levels alongside reduced levels of DKK1 and OPG, as well as a transcriptional downregulation of *ALPL* and *BMP4* genes. Based on prior literature, inflammatory and osteogenic cascades in skeletal physiology are intimately intertwined, where early activation of NF- κ B acts as a recognized inhibitor of bone anabolic signaling (33). Specifically, elevated NF- κ B signaling can suppress BMP4 expression and alter downstream SMAD dynamics, which aligns with the observed downregulation of *BMP4* gene expression and shifts in p-SMAD4 protein (34, 35).

Taken together, one plausible hypothesis is that while the upstream signaling crosstalk is primed, the structural export of key regulatory proteins, such as OC, DKK1, and OPG, could be selectively stalled (36–38). Consequently, the intracellular β -catenin could remain uncoupled from its usual extracellular feedback loops, leaving its core signaling intact despite the drop in secreted proteins (39, 40). While these parallel alterations suggest a potential signaling crosstalk between inflammatory and osteogenic axes, we emphasize that this cascade remains a proposed conceptual framework.

Of note, the observed elevation in NF- κ B protein levels, without changes in the expression of pro-inflammatory genes (i.e., *NF- κ B-p65* and *IL1B*), likely reflects an early cellular warning system or a primed state rather than a chronic inflammatory response (41). These molecular discrepancies may highlight a state of early cellular transition, where the OBL is managing PS-MPs-induced stress (9, 42).

The duration of exposure appears to play a critical role in determining the biological consequences of PS-MPs exposure. In *in vitro* models, short-term exposures (24 h – 72 h) are primarily associated with initial responses, such as oxidative stress, whereas prolonged exposures (≥ 7 days) result in more pronounced functional changes (43). In mice, significant chronic effects were observed following exposures lasting several weeks (≥ 8 weeks), including cellular senescence, chronic inflammation and impaired bone homeostasis (10, 15).

This study has important limitations, and the results must be interpreted as a hypothesis-generating study rather than conclusive. First, evaluating a single 7-day time point captured uncoupled gene-protein expression, necessitating future time-course kinetics to map transient transcriptional dynamics. Second, as an exploratory study, targeted pathway inhibition or rescue experiments (e.g., NF- κ B, Wnt) are required to establish causality. Third, extended longitudinal studies are also needed to assess whether these early signaling alterations impair long-term bone mineralization and tissue structure. Additionally, despite MG-63 osteoblast cells being commonly used as a model for studies in bone biology, the observed results may not be translated to primary cells or complex systems.

Conversely, a major strength of this work is the highly integrated evaluation of cellular, molecular and functional parameters within human-derived osteoblast-like cells. By combining analyses of PS-MPs internalization, oxidative stress assays, mitochondrial activity, mineralization capacity, inflammatory signaling and osteogenic markers, this study provides mechanistic insights into the maladaptive responses induced by MPs. Furthermore, utilizing an extended 7-day exposure model intended to reproduce the chronic, low-grade accumulation conditions to which humans are continuously subjected through environmental contact, ingestion, and inhalation (44, 45).

CONCLUSIONS

MG-63 osteoblast-like cells internalize PS-MPs, which at high concentrations reduce cell proliferation and increase oxidative stress levels. PS-MPs exposure seems to induce a distinct uncoupling between gene transcripts and key bone proteins, independently of changes in mitochondrial bioenergetics or impairment of matrix mineralization. These data may suggest that human osteoblast-like cells exhibit anabolic attenuation and catabolic phenotype signals after PS-MPs exposure. Future studies using primary human bone-derived cells or animal models, focusing on targeted pathway inhibition or rescue experiments, are required to establish the broader systemic relevance and long-term consequences of microplastic exposure on skeletal health.

Declarations

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Declaration of generative AI and AI-assisted technologies in the manuscript preparation process

During the preparation of this work, the authors used AI to review grammar and language. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

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Figures

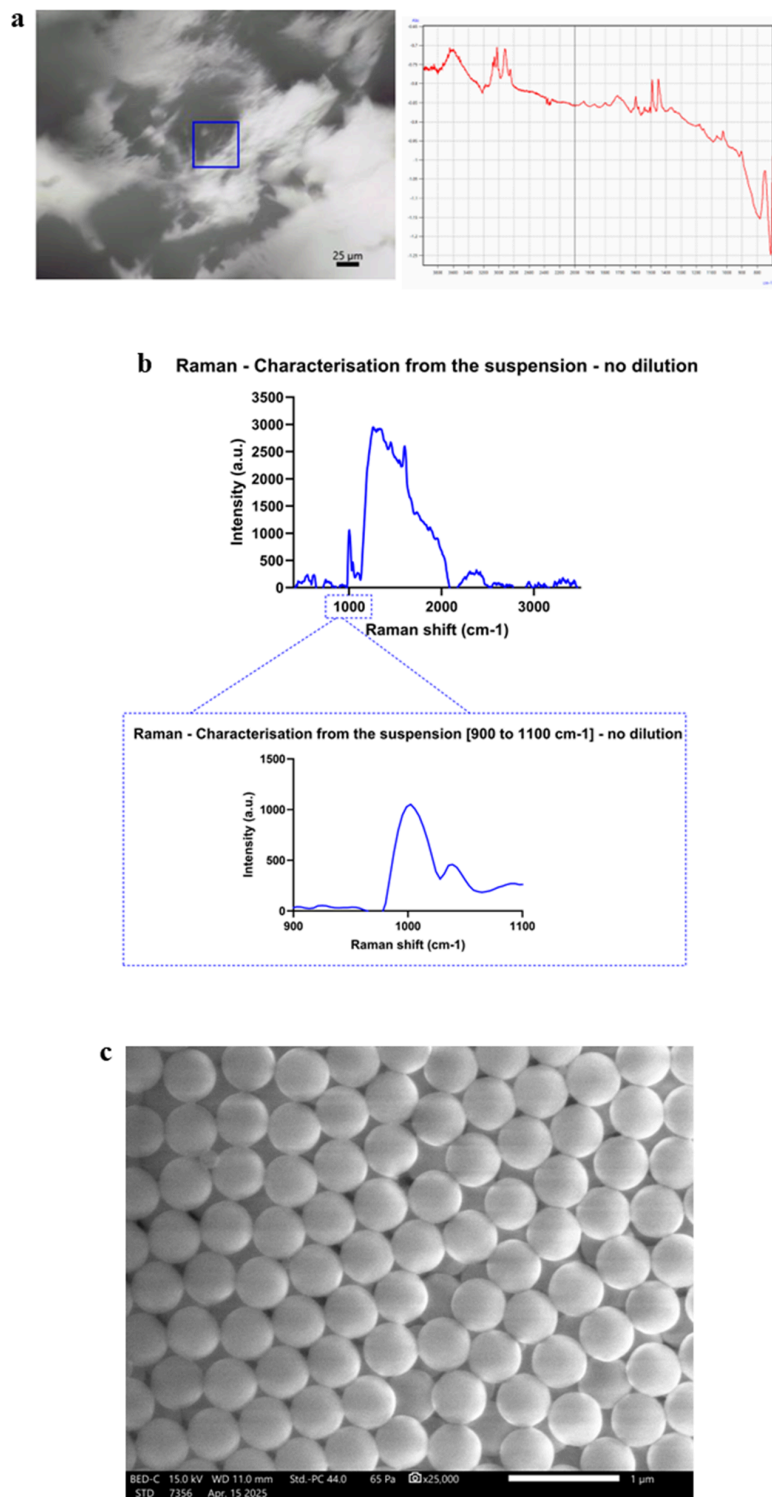


Figure 1

Physicochemical characterization of polystyrene microplastics (PS-MPs). **(a)** Fourier-transform infrared (FT-IR) spectrum of the PS-MP suspension showing characteristic functional groups, alongside an optical micrograph (25 µm) revealing the presence of particulate agglomerates. **(b)** Scanning electron microscopy (SEM) analysis at 25,000× magnification, showing the smooth, regular, and spherical

morphology characteristic of synthetic PS-MPs. **(c)**Raman spectroscopy spectrum confirming the identity of the PS-MPs, highlighted by an intense peak at a Raman shift in 900–1100 cm^{-1} region

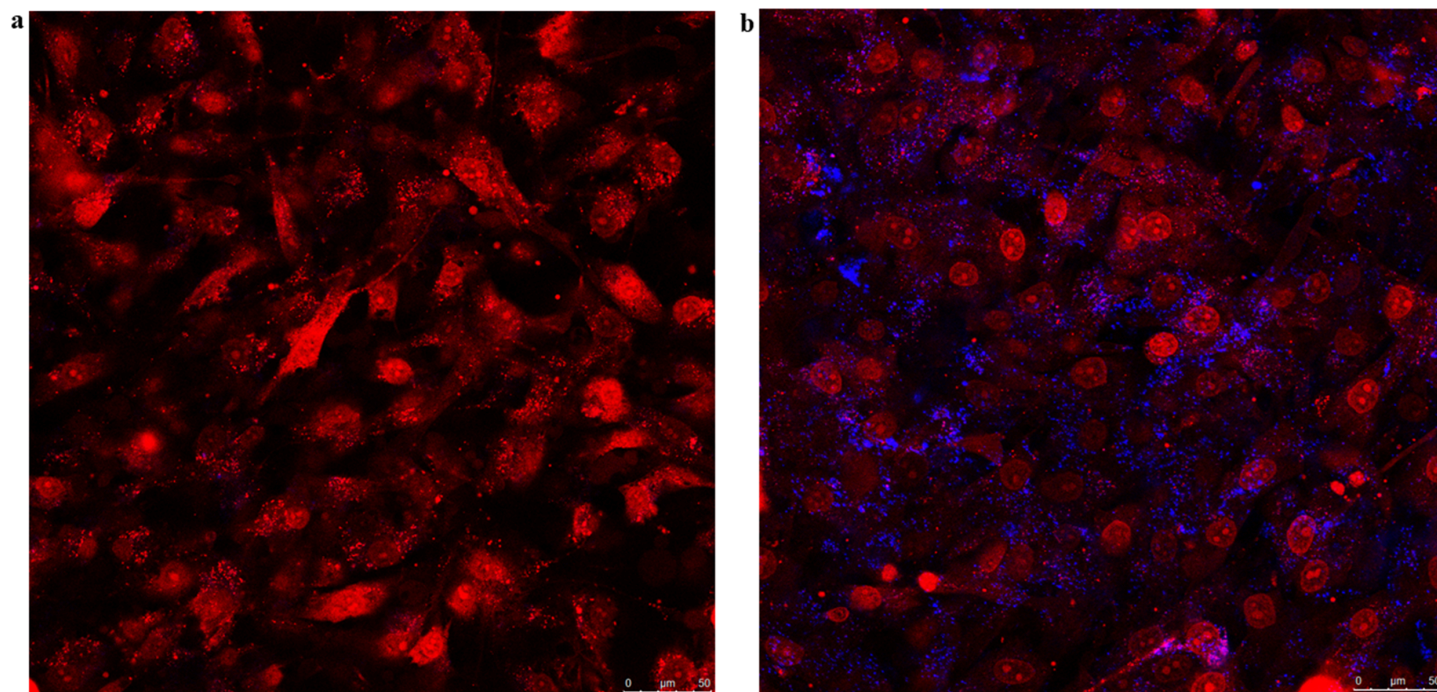


Figure 2

Uptake of polystyrene microplastics (PS-MPs) by MG-63 cells. Confocal microscopy single optical sections (thickness of 3.09 μm per image) showing: **(a)**Control cells show no detectable blue fluorescent particles (360 nm Excitation; 407 nm Emission). **(b)** PS-MPs (25 $\mu\text{g/mL}$) are shown in blue fluorescence, and cell nuclei are stained with TO-PRO-3 (red). Scale bar = 50 μm . Images were acquired at 200 $\times$ magnification with a confocal microscope (Leica, TCS SP5 II)

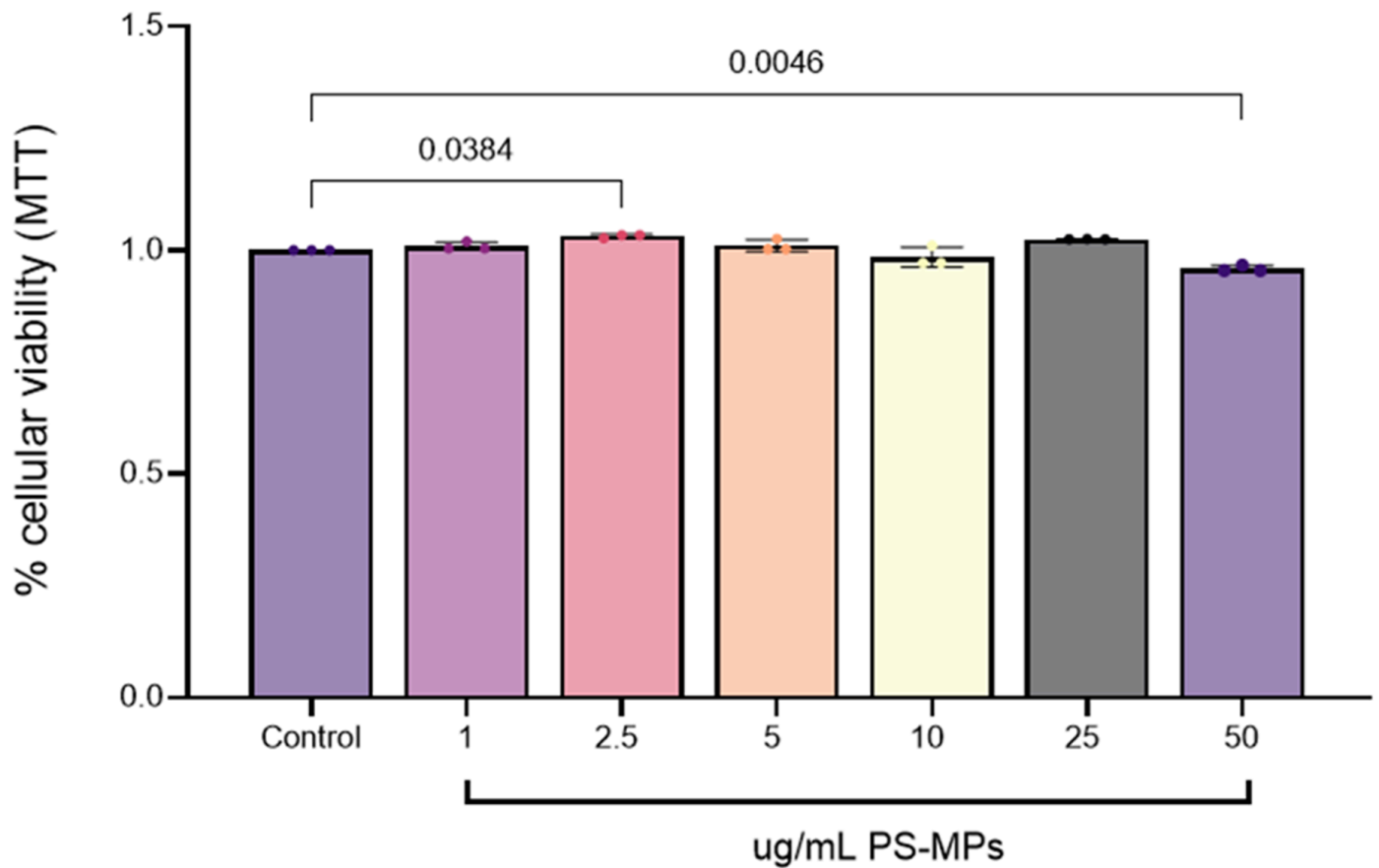


Figure 3

Cell viability assessed by MTT assay after exposure to increasing concentrations of PS-MPs (2.5–50 $\mu\text{g/mL}$). Cells cultured in DMEM 10% FBS and non-exposed to PS-MPs were used as a negative control ($n = 12$). A significant reduction in viability was observed at 50 $\mu\text{g/mL}$. Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparisons test. Data are presented as mean $\pm$ standard deviation (SD) and were analyzed using GraphPad Prism 10.1.1 (v. 323). The significance level of $p < 0.05$ was considered.

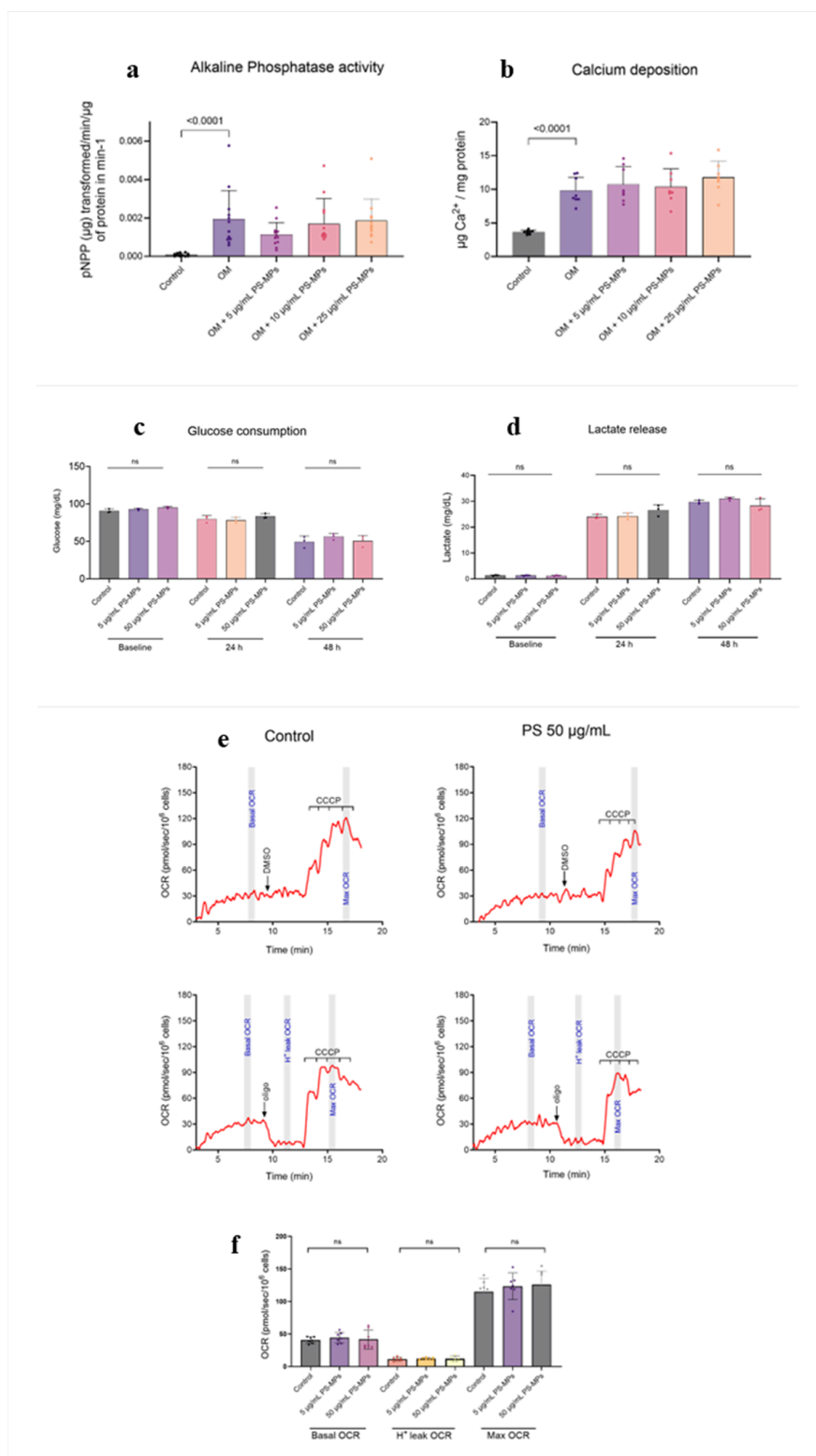


Figure 4

Functional tests of MG-63 cells exposed to microplastics of polystyrene (PS-MPs). **(a)** Alkaline phosphatase activity (n = 12), expressed as pNPP transformed per minute per μg of protein, and **(b)** calcium concentration (μg Ca²⁺/mg protein) (n = 12), were not significantly affected by PS-MP exposure after mineralization induction for 21 days (ascorbic acid, dexamethasone, and β-glycerophosphate). Glycolytic activity assessed by enzymatic-colorimetric kits (n = 3) **(c)** glucose consumption and **(d)**

lactate release at baseline, 24 h, and 48 h after exposure to PS-MPs (5 and 50 µg/mL). No significant changes were observed compared to the control condition. Representative traces of the experimental protocol used to assess the oxygen consumption rate (OCR) (n = 7) of MG-63 non-treated (control) and treated with 50 µg/mL of PS-MPs. Where indicated, 0.5 µM DMSO, 1 µg oligomycin, and 0.5 µM CCCP were added. **(e)** Control cells and cells treated with 50 µg/mL of PS showed no difference in OCR. **(f)** Basal OCR, proton leak, and maximal respiration were determined in control cells and after treatment with PS at 5 and 50 µg/mL. Statistical analysis was performed using one-way ANOVA or Kruskal-Wallis followed by Dunnett's multiple comparisons test. Data are presented as mean ± standard deviation (SD) and were analyzed using GraphPad Prism 10.1.1 (v. 323). The significance level of $p < 0.05$ was considered.

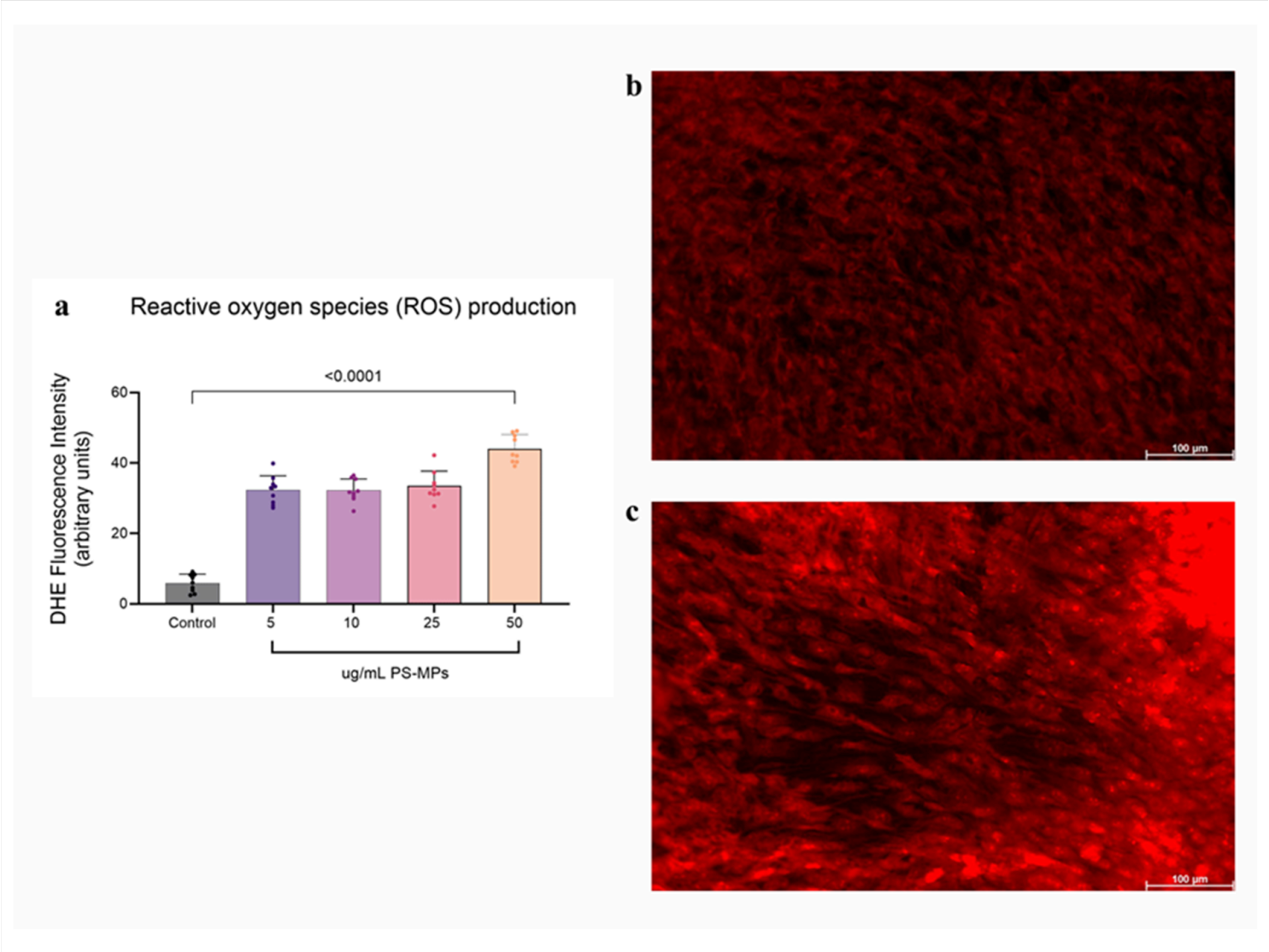


Figure 5

Intracellular superoxide production levels measured by DHE fluorescence in MG-63 cells under osteogenic conditions (ascorbic acid, dexamethasone, and β -glycerophosphate) and exposure to PS-MPs (5–50 µg/mL). **(a)** Superoxide production increased in the higher PS-MPs concentration tested, 50

µg/mL PS-MPs (ANOVA, $p < 0.0001$). **(b)** Representative fluorescence microscopy images of DHE staining showing basal superoxide levels in control cells (top) and increased superoxide signal after exposure to 50 µg/mL PS-MPs (bottom). Scale bar = 100 µm. Fluorescence intensity was normalized to crystal violet staining and expressed as arbitrary units ($n = 9$). Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparisons test. Data are presented as mean $\pm$ standard deviation (SD) and were analyzed using GraphPad Prism 10.1.1 (v. 323). The significance level of $p < 0.05$ was considered.

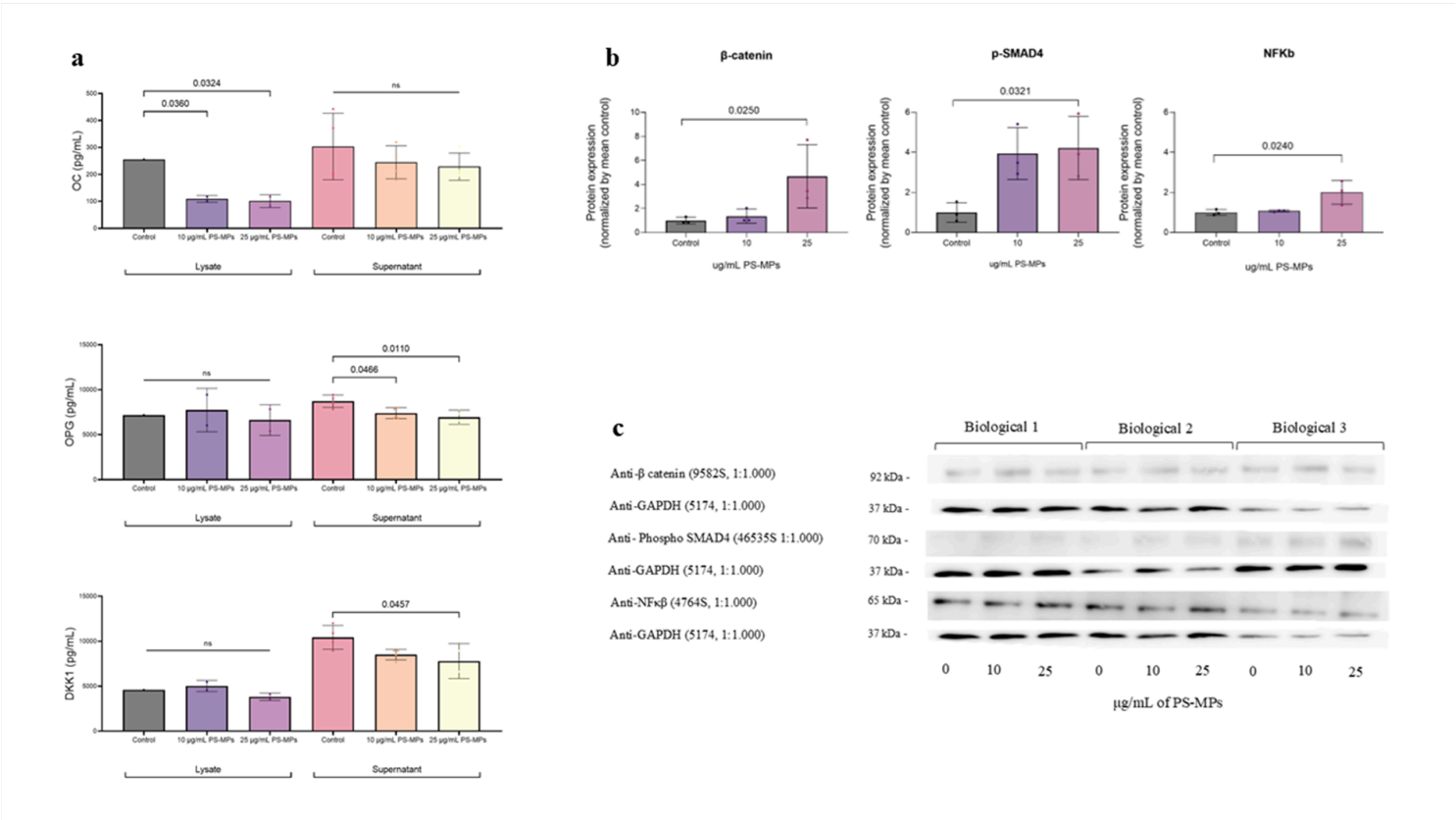


Figure 6

Effects of polystyrene microplastics (PS-MPs) on bone-related protein secretion and intracellular signaling pathways in MG-63 cells. Quantification of bone-related proteins in cell lysates and supernatants using the Milliplex™ Bone Panel after exposure to PS-MPs (10 and 25 µg/mL) ($n = 4$). **(a)** Osteocalcin (OC) levels in cell lysates were significantly decreased at both concentrations compared to control; Osteoprotegerin (OPG) levels in the supernatant were significantly decreased at 10 and 25 µg/mL; Dickkopf-related protein 1 (Dkk-1) levels in the supernatant were decreased at 25 µg/mL. **(b)** Representation of the Western blot data: β-catenin expression was increased at 25 µg/mL; p-SMAD4 expression was significantly increased at 10 and 25 µg/mL, and NF-κB expression was increased at 25 µg/mL. **(c)** Representative Western blot images corresponding to the quantified proteins. Protein expression levels were normalized to GAPDH and expressed relative to control cells. Band quantification was performed using the ImageJ gel analysis tool, based on the area under the curve, and normalized to the internal loading control (GAPDH). Molecular weights (kDa) are indicated for each protein according

to the molecular weight marker. Statistical analysis was performed using one-way ANOVA or Kruskal-Wallis followed by Dunnett's multiple comparisons test. Data are presented as fold-change related to the control, representing three independent biological and two technical replicates (n = 6), and were analyzed using GraphPad Prism 10.1.1 (v. 323). The significance level of $p < 0.05$ was considered.

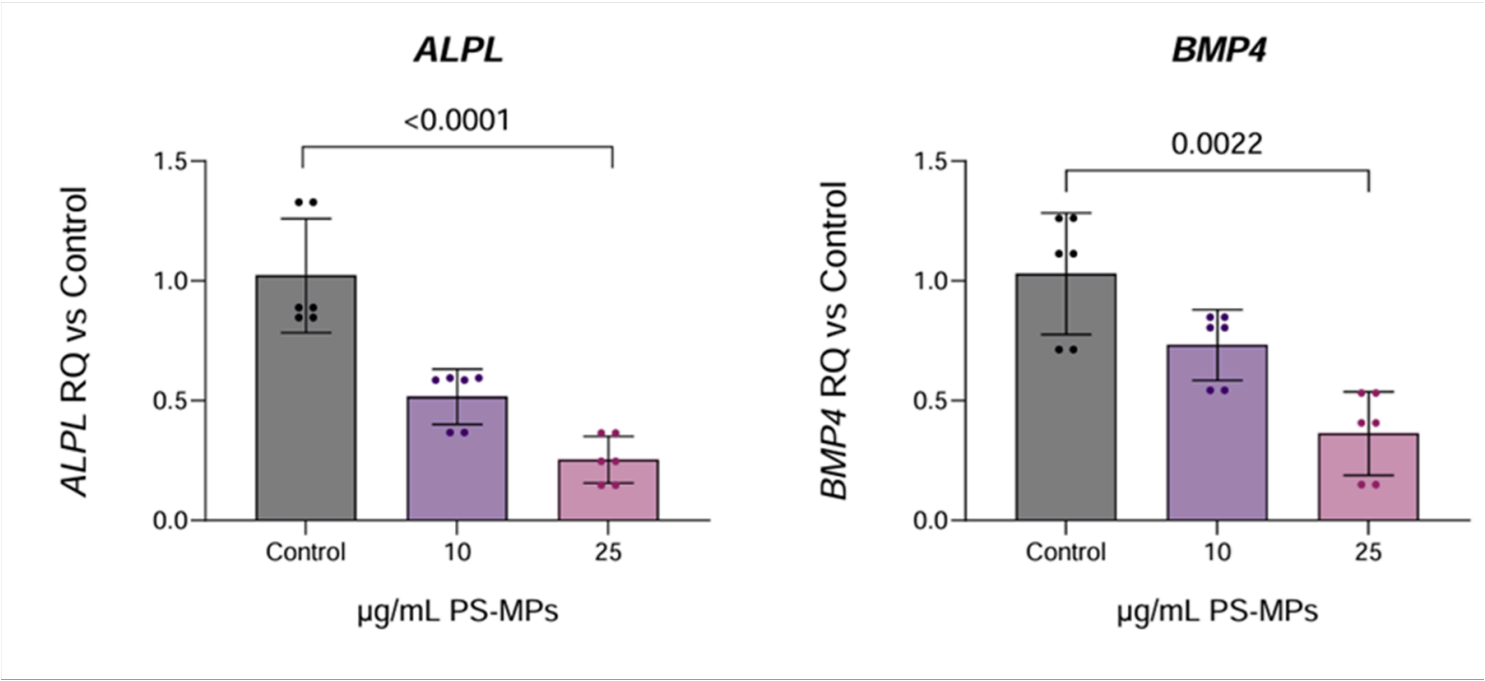


Figure 7

Relative gene expression in MG-63 cells exposed to polystyrene microplastics (PS-MPs) for 7 days. Gene expression was quantified by quantitative qPCR (QuantStudio 3), with the 2-CT method for relative quantification and normalized using the *YWHAZ* reference gene (n = 6). Lower expression of *ALPL* and *BMP4* genes was observed in the presence of PS-MPs. Other osteogenic and inflammatory signaling genes were not altered. Statistical analysis was performed using one-way ANOVA or Kruskal-Wallis followed by Dunnett's multiple comparisons test. Data are presented as fold-change related to the control, representing three independent biological and two technical replicates (n = 6), and were analyzed using GraphPad Prism 10.1.1 (v. 323). The significance level of $p < 0.05$ was considered.

Supplementary Files

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- [SupplementarymaterialPSMG63Calcified.docx](#)