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RESEARCH ARTICLE

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Nobiletin-mediated autophagy mitigates nanoplastic-induced toxicity in human intestinal Caco-2 cells

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Abstract

The presence of nanoplastics (NPs), which cause oxidative stress and damage to the cell structure due to the breakdown of microplastics (MPs), poses considerable ecological and health challenges. This study investigated the protective role of nobiletin (NOB), a flavonoid derived from citrus peel, in modulating autophagy and mitigating NP-induced toxicity in human intestinal Caco-2 cells. The Caco-2 cells were treated with NPs and varying concentrations of NOB to evaluate cell viability, apoptosis, and autophagic activity. We observed that exposure to NPs resulted in a concentration-dependent decrease in cell viability and an increase in the expression of apoptosis markers. Exposure to NPs reduced Caco-2 cell viability and disrupted autophagic processes by decreasing LC3B and increasing p62 levels, indicating impaired autophagy. NOB treatment reversed these effects by enhancing autophagic activity by upregulating LC3B and downregulating p62. Furthermore, NOB improved lysosomal integrity and decreased apoptotic markers such as Bax and cleaved caspase-3 while increasing Bcl-2 expression. NOB also facilitated the nuclear translocation of transcription factor EB through activating AMP-activated protein kinase (AMPK) and inhibiting mechanistic target of rapamycin (mTOR), promoting cellular detoxification and homeostasis. NOB has the potential as a therapeutic agent that leverages the autophagic pathway to mitigate the adverse effects of NPs, suggesting a novel approach for managing NPs toxicity in human intestinal Caco-2 cells.

KEYWORDS

autophagy, Caco-2 cells, nanoplastics (NPs), nobiletin (NOB), oxidative stress

1 | INTRODUCTION

Plastics, with global production exceeding 320 million tons annually, are indispensable in modern life but pose considerable environmental challenges.[1] Plastics infiltrate oceans, degrade water quality, contribute to land pollution, and adversely affect ecosystems and human health.[2] Of particular concern are nanoplastics (NPs), formed from the breakdown of microplastics (MPs) through physical, chemical, or biological processes.[3]

Recent studies have highlighted the toxicological effects of ingesting NPs, including hepatotoxicity, cardiovascular toxicity, and behavioral disorders.[4,5] NPs can induce oxidative stress by generating reactive oxygen species (ROS) via Fenton reactions or pollutant adsorption.[6] This ROS generation can cause cellular damage, as seen in studies with marine organisms and human cells.[7,8] Furthermore, ROS can cause cellular mechanical tears, such as damage to intestinal epithelial cells and loss of the barrier protection ability of the intestinal membrane, and reports suggest that consumption may induce oxidative stress in the intestinal system.[9] Addressing oxidative stress through autophagy as a potential solution has been reported previously.[10]

A promising avenue for mitigating these effects is autophagy, a cellular process that maintains homeostasis by degrading and recycling damaged cellular components.[11] Autophagy is crucial for intestinal health, and the disruption of this process by NPs can lead to their accumulation in lysosomes, thus impairing autophagy and contributing to cellular dysfunction.[12] Emerging studies suggest that interactions between lysosomes and the nucleus are vital for regulating homeostasis in response to stress, involving the nuclear translocation of transcription factor EB (TFEB) and inactivation of mTOR, which rejuvenates cellular metabolism. This interaction is not limited to maintaining intestinal health but also contributes to the overall regulation of biological homeostasis within the body.[13]

Nobiletin (NOB), a citrus peel flavonoid with notable anti-inflammatory and antioxidant properties, has been studied for its role in activating autophagy.[14] NOB promotes autophagy via the AMPK pathway, often suppressed by LPS.[15] Furthermore, studies have indicated that NOB modulates autophagy in cancer cells via p62 and LC3B expression.[14] Despite the potential association between NOB and autophagy activation, further investigation is required to elucidate the exact mechanism underlying NOB-mediated autophagy activation.

Therefore, this study aimed to elucidate the effect of NOB on autophagy regarding exposure to NPs, focusing on its role in nuclear translocation and cytoplasmic distribution of TFEB.

2 | MATERIALS AND METHODS

2.1 | Materials

Nanoplastics (NPs, DiagPoly™ Europium Carboxylate Polystyrene Particles) were purchased from CD Bioparticles (St. Louis, MO, USA). NPs have a diameter of 0.1 μm and are encapsulated with red fluorophores (excitation at 542 nm and emission at 612 nm). The surface contains no additional coatings or specialized functional groups, and the liquid suspension has a density of 1.05 g/mL.

2.2 | Cell cultivation and treatment

Human intestinal epithelial cells (Caco-2, RRID:CVCL_0025) were cultured in an incubator at 5% CO_2 and 37°C in DMEM containing 10% FBS and antibiotic-antimycotic solution. The Caco-2 cells were treated with NOB (12.5, 25, and 50 μM) and NPs (100 μg/mL) for 24 h. Additionally, chloroquine (CQ; 50 μM), the AMPK activator AICAR (10 μM), and the AMPK inhibitor dorsomorphin (Compound C; 5 μM) were co-administered with NPs and NOB-treated Caco-2 cells.

2.3 | Cell viability assay

The Caco-2 cells were cultured in a 96-well plate at a 1×10^4 cells/well density and incubated for 24 h. Cell viability experiments were conducted to determine the non-toxic concentrations of NOB and NPs for the viability of Caco-2 cells. The Caco-2 cells were treated with NPs (50, 100, 200, and 400 μg/mL) and NOB (12.5, 25, 50, 75, and 100 μM), respectively, then incubated at 37°C for 24, 48, and 72 h under a 5% CO_2 atmosphere. The cells were analyzed using a Cell Counting Kit-8 (Dojindo Molecular Technologies, Rockville, MD, USA), and absorbance was measured at 450 nm using a microplate reader (BioTek Inc., Winooski, VT, USA).

2.4 | Immunofluorescence analysis of autophagy

Autophagy was measured using the CYTO-ID® Autophagy Detection Kit. Briefly, samples were washed twice with 1X Assay Buffer and then treated with CYTO-ID and Hoechst 33342. The samples were incubated at 37°C for 30 min in a light-protected environment. Afterward, they were fixed with 4% formaldehyde for 20 min, washed three times with 1X Assay Buffer, and analyzed using a fluorescence microscope (Nikon, Tokyo, Japan).

2.5 | Nucleus/cytoplasm fractionation

The nucleus/cytoplasm fractionation method has been described previously.[16] Briefly, after collecting the cultured

Caco-2 cells, they were swollen using ice-cold hypotonic buffer; subsequently, NP-40 was added, and the nuclear and cytoplasmic layers were separated by centrifugation. The separated nuclear layer was extracted using an isotonic and RIPA buffer. The cytoplasmic layer was extracted by centrifugation.

2.6 | Western blot analysis

The Caco-2 cells treated with NPs and different concentrations of NOB were analyzed by Western blot to investigate the expression of various proteins such as AMPK (1:1000), p-AMPK (1:1000), Bax (1:1000), Beclin 1 (1:2000), Bcl-2 (1:1000), Caspase3 (1:1000), Cleaved caspase-3 (1:250), LAMP1 (1:500), LC3B (1:1000), mTOR (1:1000), p-mTOR (1:1000), SQSTM1 (p62, 1:1000), TFEB (1:2000), LaminB1 (1:5000), and β-actin (1:1000). The Caco-2 cells were extracted using a protein lysis buffer (iNtRON Biotechnology, Seongnam, Korea) containing protease and phosphatase inhibitors (Thermo Fisher, San Jose, CA, USA). Samples containing equal amounts of protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the separated bands were transferred via electrophoresis onto polyvinylidene fluoride membranes. The membrane was blocked for 1 h and incubated with a primary antibody for 2 h. The membrane was probed with a secondary antibody conjugated to horseradish peroxidase for 1 h. Reactive bands of the target proteins were detected using a Quant LAS 500 system (GE Healthcare Bio-Sciences AB, Björkgatan, Uppsala, Sweden) with enhanced chemiluminescence (ECL) reagents (Amersham Pharmacia, Little Chalfont, Buckinghamshire, UK).

2.7 | Statistical analysis

Results are presented as mean ± standard deviation (SD), and all experiments were conducted in triplicate. Statistical analysis was performed using GraphPad Prism 10.2.3 software (RRID:SCR_002798, GraphPad Software Inc., San Diego, CA, USA) with one-way ANOVA and Tukey's post hoc test. Statistical significance was set at a significance level of $p < 0.05$.

3 | RESULTS

3.1 | Cell viability experiments

To determine the effects of NPs and NOB in Caco-2 cells, the cells were treated with nanoparticles (NPs; 0–400 μg/mL) and NOB (0–100 μM) at various concentrations for 24 h. We confirmed that cell viability decreases significantly at 200 μg/mL of NPs. Therefore, we conducted further experiments using a 100 μg/mL concentration of NPs, which did not significantly decrease cell viability (Figure 1A). Furthermore, NOB was observed to reduce cell viability at a concentration of 75 μM. To avoid cell damage, experiments were conducted using final concentrations of NOB at 0, 12.5, 25, and 50 μM, which did not adversely affect cell viability (Figure 1B).

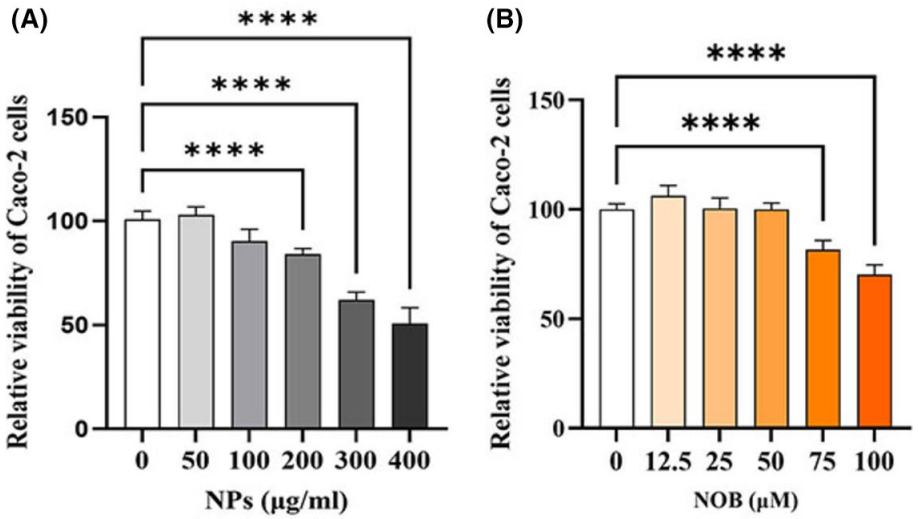

FIGURE 1 Effect of nanoplastics (NPs) and Nobiletin (NOB) on the viability of human intestinal epithelial (Caco-2) cells. (A) Treatment with nanoplastics (50, 100, 200, 300, and 400 μg/mL) for 24 h. (B) Treatment with Nobiletin (NOB; 12.5, 25, 50, 75, and 100 μM) for 24 h. Mean ± Standard deviation (SD) values are presented for five separate experiments. ****$p < .0001$ versus 0 (NPs or NOB).

3.2 | Specific labeling of autophagic compartments using fluorescence analysis

Fluorescence analysis showed red fluorescence intensities in the group treated with NPs, while no red fluorescence was observed in the control group. Additionally, the green fluorescence indicating autophagy revealed that the average intensity decreased in the group treated with NPs but increased in the group treated with NOB. The findings indicate that NPs may suppress the activation of autophagy, while NOB could contribute to its enhancement. Furthermore, after merging the images from the group treated with both NPs and NOB, we observed that autophagy occurred specifically at the locations of the particles (Figure 2).

3.3 | NOB with NPs treated Caco-2 cells exhibited decreased apoptosis

Caco-2 cells treated with NPs showed apparent differences in apoptosis markers compared with the control group. Pro-apoptotic markers were confirmed to have increased protein expression in the NP-treated Caco-2 cells, whereas anti-apoptotic markers were confirmed to have downregulated protein expression (Figure 3A). However, Caco-2 cells co-treated with NOB and NPs showed a significant concentration-dependent decrease in Bax and Cleaved caspase-3/caspase-3 levels (Figure 3B,C). After treatment with NOB in combination with NPs, a significant increase in Bcl-2 levels was observed. These results suggest that NOB inhibits the apoptotic activity in NP-treated Caco-2 cells (Figure 3A).

3.4 | NOB with NP-treated Caco-2 cells triggered autophagy

During autophagy, dysfunctional or unnecessary cellular components are engulfed to form autophagosomes. These autophagosomes then fuse with lysosomes, where hydrolytic enzymes decompose the contents. We examined autophagy-related proteins in Caco-2 cells treated with NPs to determine whether autophagy occurred due to NOB treatment. As shown in Figure 4A, p62 levels increased with NP treatment but significantly decreased when NOB was administered. Similarly, LC3B and Beclin1, proteins associated with autophagy, decreased with NP treatment but significantly increased with NOB treatment (Figure 4B,C). These results suggest that NOB enhances autophagy activity, which NPs inhibit by increasing the expression of autophagy-related proteins.

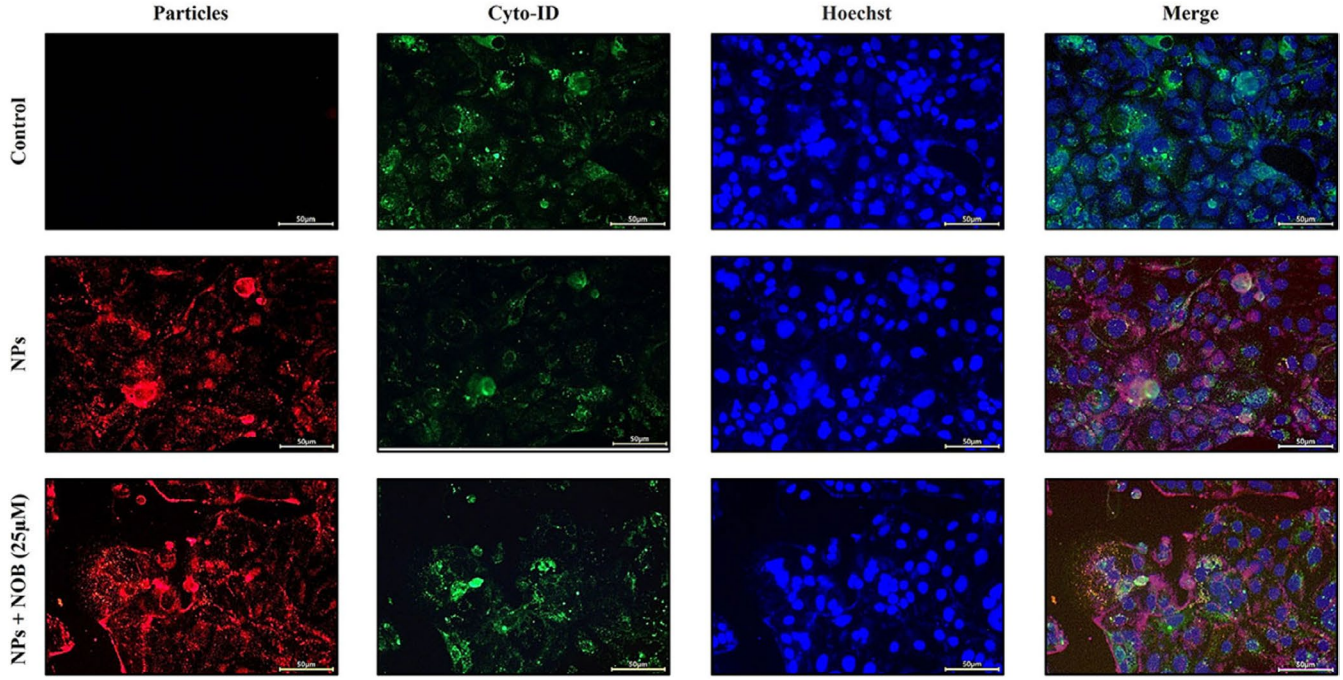

FIGURE 2 Effects of nanoplastics (NPs) on autophagy in human intestinal epithelial (Caco-2) cells. Cyto-ID staining revealed altered autophagy due to NPs, which was ameliorated by NOB treatment. Nanoplastics were shown red (559/635 nm), autophagy was stained green with Cyto-ID (499/548 nm), and the nuclei were stained blue with Hoechst (350/460 nm). The bar size is 50 μm.

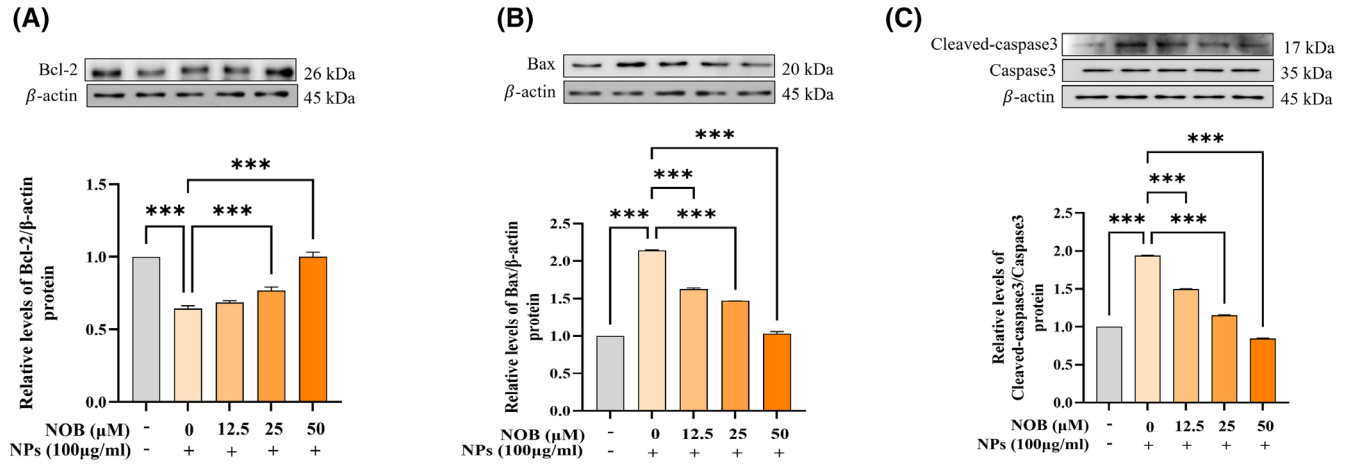

FIGURE 3 Effects of Nobiletin (NOB) on apoptosis in the nanoplastic (NP)-treated human intestinal epithelial (Caco-2) cells. Western blot analysis showed that apoptosis markers were regulated in NP-treated cells after NOB treatment. (A) Relative protein expression levels of Bcl-2, (B) Bax, and (C) Cleaved caspase-3/ caspase. Mean ± Standard deviation (SD) values are presented for three separate experiments. ***$p < .001$ versus NOB (0 μM) + NPs (100 μg/mL).

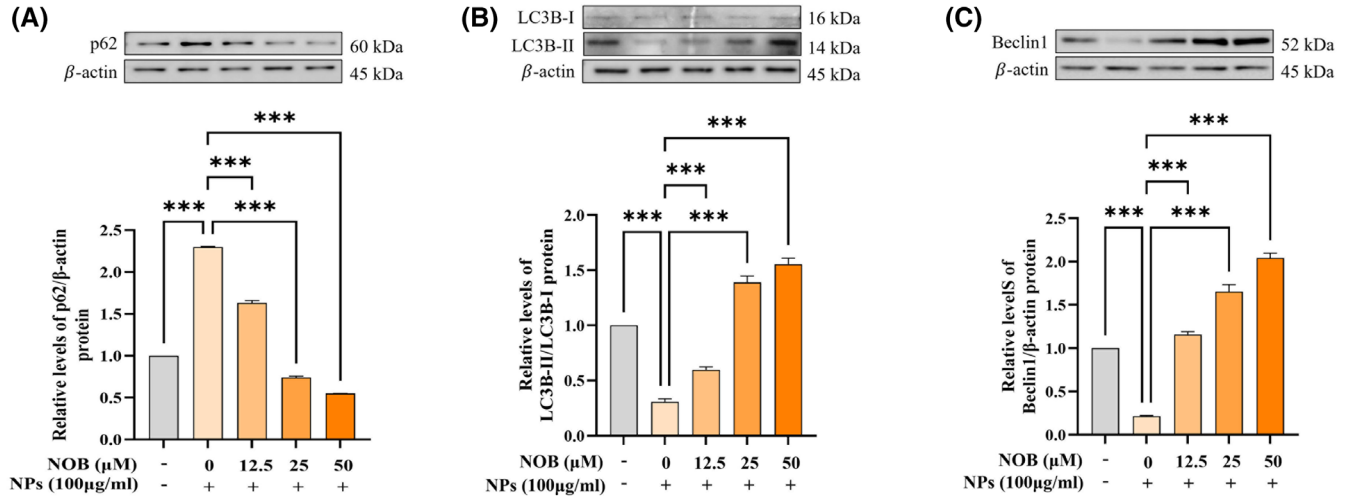

FIGURE 4 Effects of Nobiletin (NOB) on autophagy in the nanoplastic (NPs)-treated human intestinal epithelial (Caco-2) cells. Western blot analysis showed restoration of autophagy-related proteins in NP-treated cells after NOB treatment. (A) Relative protein expression levels of p62, (B) LC3B-II/LC3B-I, and (C) Beclin1. Mean ± Standard deviation (SD) values are presented for three separate experiments. ***$p < .001$ versus NOB (0 μM) + NPs (100 μg/mL).

3.5 | NOB with NP-treated Caco-2 cells improved lysosomal damage

We investigated the damage to lysosomal membrane proteins by assessing the expression of LAMP1, a vital component of the lysosomal membrane. As shown in Figure 5, Caco-2 cells exposed to NPs exhibited a decrease in LAMP1 expression compared to the control group. However, when Caco-2 cells were treated with NOB at varying concentrations, LAMP1 expression significantly increased (Figure 5). These results suggest that NPs negatively impact lysosomal function in Caco-2 cells, whereas NOB treatment improves this function.

3.6 | The relationship between autophagy inhibition and apoptosis in NP-treated Caco-2 cells

To determine whether decreased autophagy negatively affected Caco-2 cells and was associated with cell death, protein expression was compared using CQ, an autophagy inhibitor. CQ is a lysosomal inhibitor that increases the pH of lysosomes and inhibits autophagy by blocking autophagosome–lysosome fusion. Autophagy and apoptosis were evaluated by comparing the expression of p62, LC3B, LAMP1, Bcl-2, Bax, and Caspase-3. Protein degradation of p62 was inhibited, and LC3B-II/LC3B-I

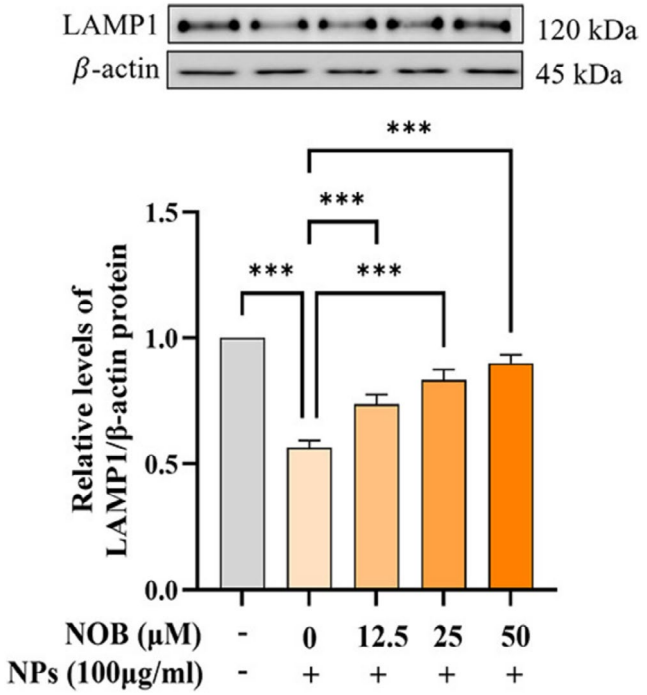

FIGURE 5 Effects of nobiletin (NOB) on restoring lysosomal function damaged by nanoplastics (NPs). Western blot analysis revealed that NOB treatment restored lysosomal function impaired by NPs. Mean ± standard deviation (SD) values are presented for three separate experiments. ***$p < .001$ versus NOB (0 μM) + NPs (100 μg/mL).

expression increased in cells treated with NPs, NOB, and CQ (Figure 6A,B). For LAMP1, no improvement was observed with NOB treatment when CQ was added (Figure 6C). Bcl-2, a protein involved in apoptosis, was downregulated in Caco-2 cells treated with CQ, NPs, and NOB, while Bax and cleaved caspase-3/caspase-3 were upregulated (Figure 6D–F). These findings confirm that autophagy is critical in alleviating apoptosis in Caco-2 cells treated with NPs.

3.7 | NOB-induced nuclear translocation of TFEB in Caco-2 cells via the AMPK pathway

To determine how NOB activates TFEB, which controls the expression of genes involved in the autophagic process following NPs treatment, the study examined the levels of AMPK and mTOR. Caco-2 cells treated with NPs and NOB exhibited an increased nuclear expression of TFEB, indicating a significant translocation of TFEB into the nucleus (Figure 7A). Western blotting showed increased phosphorylated AMPK levels and inhibited phosphorylated mTOR levels (Figure 7B,C). Treatment with AICAR and Compound C (C.C) confirmed that the activation of AMPK promoted the nuclear translocation of TFEB (Figure 7D,E). These findings suggest that NOB can utilize the AMPK pathway to induce the nuclear translocation of TFEB in the Caco-2 cells.

4 | DISCUSSION

This study investigated the effects of NOB on alleviating the side effects of NPs in human intestinal epithelial Caco-2 cells by modulating autophagy. Previous studies have demonstrated that NPs inhibit autophagy in Caco-2 cells, and natural products can reverse this effect. In accordance with these findings, our investigation revealed that NOB, a natural compound, effectively restores autophagy impaired by NPs, thus highlighting its potential as a novel therapeutic agent.[12]

Our findings align with a growing number of studies indicating that NPs derived from the breakdown of microplastics (MPs) debris pose considerable health risks because of their ability to generate ROS and disrupt cellular homeostasis.[15] These findings extend beyond the cellular level and underscore the need for effective therapeutic strategies to combat NP-induced toxicity.

NPs pose considerable toxicity risks via various exposure pathways, including ingestion via the food chain.[17] A significant presence of MPs in the environment through various pathways indicates a high likelihood of human exposure to NPs.[18] Humans encounter these particles through multiple exposure routes, including drinking water, beverages, and food. For instance, in human colorectal tissue samples, Ibrahim et al. (2021) reported that 96% of detected particles were fibroids approximately 1 mm long, with an average concentration of 28 MPs/g.[19] Similarly, in human blood samples, Leslie et al. (2022) observed an average NMP concentration of 1.6 μg/mL.[20]

The impact of NPs on intestinal health is especially important because they interact with other organs.[21] Previous studies have reported that NP exposure can alter the composition of the gut microbiome and cause enteritis through immune and oxidative stress pathways.[22] While their cytotoxicity or bactericidal effects remain debatable, NPs are known to influence mammalian gut microbiota significantly. Some studies have even linked their effects to an increased risk of conditions such as inflammatory bowel disease (IBD).[23]

Our results demonstrated that NP exposure markedly decreased cell viability and increased apoptosis markers, such as Bax and cleaved-caspase-3. These observations are consistent with previous studies that have highlighted the detrimental effects of NPs on various biological systems, including the induction of oxidative stress, mitochondrial dysfunction, and activation of

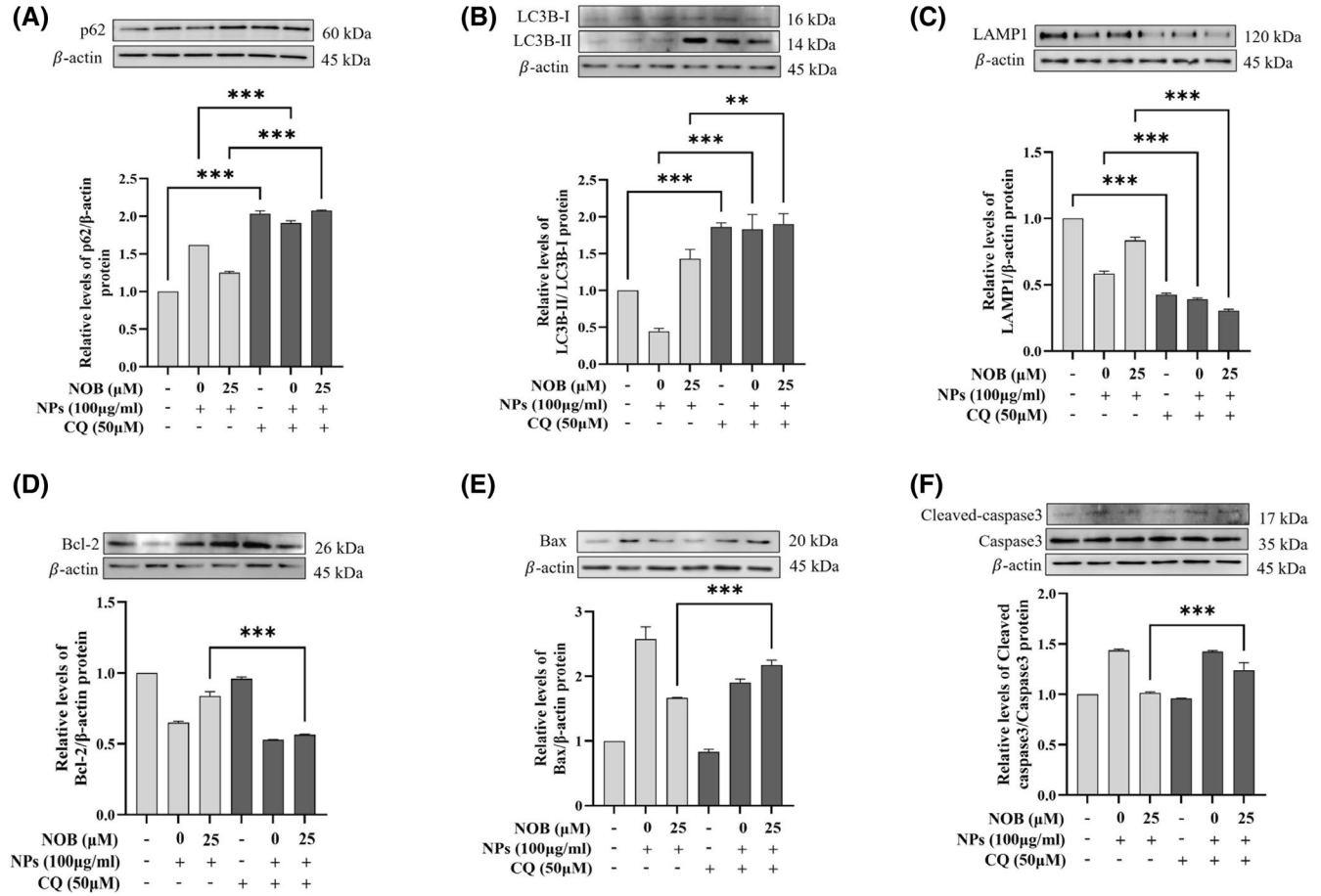

FIGURE 6 Effects of nobiletin (NOB) on autophagy and apoptosis levels in the chloroquine (CQ)-treated human intestinal epithelial (Caco-2) cells. Western blot analysis showed that CQ inhibited autophagy in NP-treated cells, confirming the role of NOB in enhancing autophagy under NP-treated conditions. (A) Relative protein expression levels of p62, (B) LC3B-II/LC3B-I, (C) LAMP1, (D) Bcl-2, (E) Bax, and (F) Cleaved caspase-3/ caspase. Mean ± Standard deviation (SD) values are presented for three experiments. $^{**}p < .01$ and $^{***}p < .001$.

pro-apoptotic pathways.[8,9] ROS generation plays a pivotal role in these processes and leads to cellular damage and death.[15] However, NOB demonstrated the potential to mitigate these effects by enhancing the expression of Bcl-2 protein, stabilizing mitochondrial membranes, and reducing apoptotic markers such as Bax and cleaved caspase-3.[24,25]

Autophagy, a crucial cellular mechanism for maintaining homeostasis, was significantly disrupted following NP exposure.[26] Our study confirmed that NPs impaired autophagy, as evidenced by decreased LC3B and increased p62 levels in treated cells. This impairment likely contributes to accumulating damaged cellular components and initiating apoptosis.[27] Autophagy is vital in degrading and recycling cellular debris for cell survival, particularly under stressful conditions.[11,28]

NOB, a citrus peel flavonoid with known anti-inflammatory and antioxidant properties,[29] has demonstrated a potent ability to counteract NP-induced toxicity. NOB treatment enhances cell viability, reduces apoptotic markers, and restores autophagic activity.[30] Specifically, NOB increased the expression of LC3B and decreased p62 levels, suggesting an upregulation of autophagy. This enhancement of autophagy could be attributed to NOB's role in activating the AMPK pathway and inhibiting mTOR, critical regulators of autophagy and cellular metabolism.[31,32]

In this study, we further confirmed the role of NOB in autophagy regulation by utilizing CQ, an autophagy inhibitor. Interestingly, when comparing the CQ-treated group with those treated with NOB, we observed no significant increase in LC3B-II/I expression, and the degradation of p62 was inhibited. These findings suggest that NOB does not interfere with the formation of autophagosomes. Instead, it appears to promote the fusion of autophagosomes with lysosomes, thereby enhancing the formation of autophagolysosomes.[12,33]

The ability of NOB to promote TFEB nuclear translocation further supports its role in enhancing autophagy. TFEB is a master regulator of lysosomal biogenesis and autophagy, and its nuclear translocation is essential for activating autophagic processes.[13] The NOB-treated cells

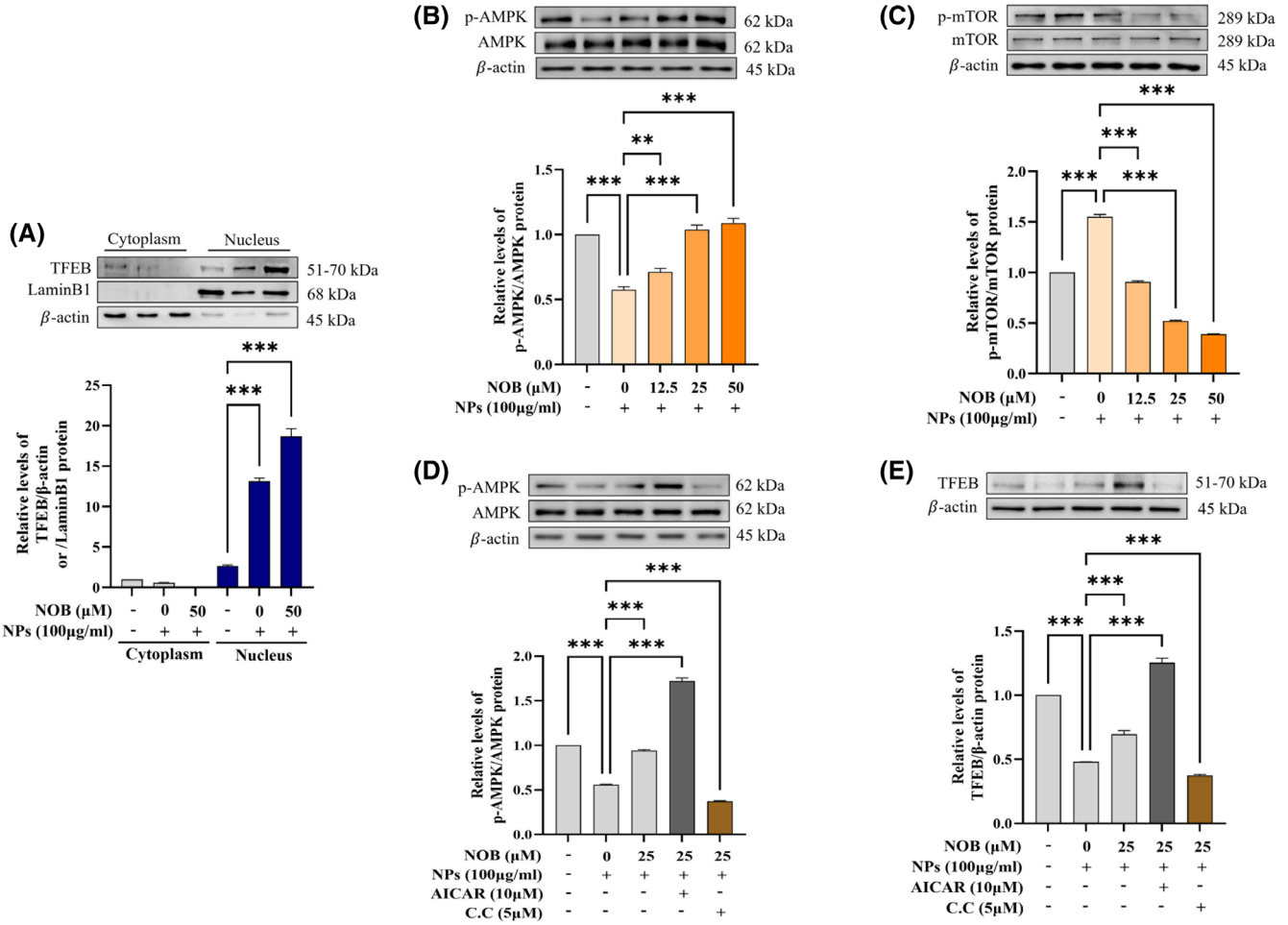

FIGURE 7 Effects of Nobiletin (NOB) in promoting TFEB nuclear translocation via AMPK and mTOR pathways in the Nano-plastics (NPs)-treated human intestinal epithelial (Caco-2) cells. Western blot analysis showed increased TFEB nuclear translocation in NP-treated cells with NOB treatment, confirmed by AICAR and Compound C. (A) Quantification of TFEB protein in the nucleus and cytoplasm. (B) Relative protein expression levels of *p*-AMPK/AMPK and (C) *p*-mTOR/mTOR. (D) Relative protein expression levels of *p*-AMPK/AMPK and (E) TFEB after treatment with AICAR (10 μM) and Compound C (C.C; 5 μM). Mean ± standard deviation (SD) values are presented for three separate experiments. $^{**}p < .01$ and $^{***}p < .001$.

facilitated the nuclear translocation of TFEB, mediated by the activation of AMPK and inhibition of mTOR. NOB promotes the formation of autophagosomes and lysosomes, thereby enhancing the cellular capability to degrade and recycle damaged components induced by NPs.

Although the *in vitro* results are promising, further research is necessary to translate these findings into practical applications. Future studies should focus on *in vivo* models to validate the efficacy of NOB in mitigating NP-induced toxicity within complex biological systems. Additionally, exploring the long-term effects of NOB and its potential interactions with other cellular pathways will be crucial in gaining a more comprehensive understanding of its therapeutic potential.

The findings of this study suggest that NOB holds promise as a therapeutic agent for mitigating the toxic effects of NPs. By enhancing autophagy and reducing oxidative stress-induced damage, NOB could serve as a foundation for developing treatments addressing environmental pollution-related health risks, particularly those resulting from NPs and MPs exposure. Our study provides compelling evidence that NOB mitigates NP-induced toxicity in Caco-2 cells by enhancing autophagy. These findings contribute to a broader understanding of how natural compounds can combat environmental pollutants and protect human health. The potential of NOB as a therapeutic agent warrants further investigation to fully harness its benefits in mitigating the adverse effects of NPs. In future studies, we will conduct *in vivo* experiments utilizing a mouse intestinal inflammation model induced by NP exposure. It is essential to validate the findings of this study and further investigate the clinical potential as a therapeutic approach for intestinal diseases based on these results. These investigations are expected to provide crucial foundational data for elucidating the physiological

effects of NPs and developing efficacious therapeutic agents of NOB.

AUTHOR CONTRIBUTIONS

All the authors have read and agreed to the published version of the manuscript. Junho Yu and Ji-Hwan Yoon performed the laboratory experiments, analyzed the data, prepared the figures, and prepared the original drafts. Miey Park conceived the idea, designed the experiments, and revised the manuscript. Hae-Jeung Lee contributed to the final version of the manuscript and supervised the study.

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The authors have nothing to report.

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DISCLOSURES

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

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