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Preliminary Study on the Toxic Effects of Polystyrene Microplastics in Human Colorectal Cells

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Abstract: According to this rapid urbanization background, the widespread presence of microplastics in the environment, caused by plastic pollution and poor waste management, poses a huge threat to public health. This research aims to evaluate the colorectal health risk associated with microplastics and provides data for managing urban water and food security. We have evaluated the toxic impact of different sizes (80 nm and 500 nm) of polystyrene microplastics (PS-MPs) on human colorectal cells (NCM460). Analyses were conducted to assess changes in cell viability, cell cycle, and apoptotic response after exposure to PS-MPs of varying particle sizes and concentrations. Our results revealed that 80 nm and 500 nm PS-MPs significantly reduced cell viability, induced cell apoptosis, and prolonged the time that cells stay in the S-phase. These results demonstrated a size-and concentration-dependent toxic effect. Therefore, this investigation provides experimental evidence for evaluating the intestinal health risks of microplastics, and calls for the optimization of the water treatment process in the field of environmental engineering to achieve targeted removal of these high-risk microplastics.

1 Introduction

Polystyrene microplastics (PS-MPs) have emerged as pervasive environmental pollutants; they are already found to persist in water[5] and soil[9]. These particles can enter human body by oral intake and cause a potential threat to overall health[2][10]. Since plastics are widely used in food packaging and daily plastics, humans may intake polystyrene microplastics (PS-MPs) ranging from hundreds of micrograms to several milligrams every week[1], and the gastrointestinal tract is the main exposure route. Recent studies have demonstrated that microplastics can accumulate in the gastrointestinal tract through oral ingestion and affect intestinal epithelial cells directly by inducing intestinal inflammation[6].

Although the biological toxicity of microplastics is uncertain, some investigations indicate that long-term exposure to microplastics can harm human health by damaging the physical barrier, leading to an imbalance of intestinal bacteria, and causing an abnormal inflammatory response[12]. Some researchers concentrate on the toxicity of PS-MPs on the nervous system: they pass through the blood-brain barrier and launch direct toxic effects on neurons[8]. Others discussed sample pretreatment methods and detection techniques for microplastics[13]. However, current research on the toxic effects of polystyrene microplastics is still concentrated on examining aquatic organisms[3][11] and the gastric tissue cells of mice[4][7]. There still lacks a systematic analysis of the direct impact caused by PS-MPs on normal human colorectal epithelial cells.

My study is aimed on investigating the toxic effects of polystyrene microplastics with concentrations of 1, 10, and 100 μg/mL and diameters of 80 nm and 500 nm on human colorectal cells (NCM460). Combining with the control group, the data of cell viability, cell apoptosis, and cell cycle, the interactions between microplastics and human colorectal cells, this investigation aims to determine if microplastics exhibit size-dependent toxicity towards human colorectal cells.

2 Materials and methods

2.1 Materials

Polystyrene microspheres with diameters of 80 nm (Catalog #: PS-080-NH_2) and 500 nm (Catalog #: PS-500-NH_2) were from Baseline Chrom Tech Research Center (Tianjin, China). Human colorectal cells (NCM460) were from ACCA. This study selected the normal human colon epithelial cell line NCM460 as the in vitro model. Compared to cancer cell lines, NCM460 cells have a normal genetic background and physiological functions, which can more accurately simulate the healthy human intestinal environment, providing more physiologically relevant experimental evidence for evaluating the intestinal toxicity of microplastics. MTT kit and formazan were from Yeasen Biotechnology (China). Cell Cycle Detection Kit was from KeyGen BioTECH (Jiangsu,

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China). Hoechst 33258 / PI apoptosis assay kit was from Leagene Biotechnology (Beijing, China).

2.2 Cell Culture

Human colorectal normal cells (NCM460) were pre-cultured in RPMI-1640 from Gibco (USA) with 10% FBS and 1% triple antibiotics at 37℃ under 5% CO2. When adding to the culture plate, the cell concentration was about 4.025×103 cells/mL.

2.3 Cytotoxicity Assay

Seed normal human colorectal epithelial cells (NCM460) at a density of 4×103cells/well into a 96-well plate, add 200 μL of complete culture medium, which is composed of RPMI-1640, 10% FBS, and triple antibiotics to each well, seed the cells into the 96-well plate, and pre-culture them in a 37°C, 5% CO2 incubator for 24 hours. Sterilize the microplastic reagents in the autoclave for 20 minutes under 120℃ and 120 kPa conditions to remove bacteria from the microplastic reagents. Add the reagents to 10 mL of RPMI-1640 to prepare solutions with different concentrations of 0, 1, 10, and 100 μg/mL.

Discard the original culture medium and add fresh medium containing varying concentrations (0, 1, 10, and 100 μg/mL) of polystyrene microplastics (PS-MPs), using 6 replicate wells per concentration group. Then, continue to culture for one group for 24 hours and another for 48 hours, with RPMI-1640 culture medium as a negative control. Add 20 μL of MTT solution with concentration of 5 mg/mL to each well, then incubate them in the dark at 37℃, 5% CO2 condition for 4 h. Carefully discard the previous culture medium and add 100 μL of formazan solution into each well, then incubate them in the dark at 37℃, 5% CO2 condition for another 4 h. After incubating, put the 96-well plate into a microplate reader, then choose 450 nm wavelength to evaluate each well's light absorbance and record the results. For the cytotoxicity assay, let the OD value of cells that haven't been treated be 100%, and calculate the percentage of cells that have been treated with medicines, and draw histograms.

2.4 Cell Cycle

Seed normal human colorectal epithelial cells (NCM460) into 6-well plates, add 2 mL of complete culture medium, which was configured of RPMI-1640, 10% FBS, and triple antibiotics to each well, and pre-culture the 6-well plate in a 37°C, 5% CO2 incubator for 24 hours.

Incubate with 500 μL working solution per sample. Before use, prepare RNase A: PI working solution into 10 mL working solution with a volume ratio of 1:9.

Cells were washed with PBS and centrifuged at 2000 rpm for 5 minutes. Collect and adjust the cell concentration to 1×106 cells/mL, and take 1ml of single-cell suspension. Then, remove the supernatant and fix the cells with 500 μL of 70% cold ethanol (one group for 2 hours, and the other overnight), and store them at 4℃. Before staining, wash the fixative with PBS and centrifuge it at 1000 rpm for 3 minutes. Then, 500 μL of PI/RNase A staining solution was added to each sample, followed by incubation in the dark at room temperature for 30 minutes. Finally, samples were analyzed by a flow cytometer, and red fluorescence was recorded at a wavelength of 488 nm.

2.5 Cell Apoptosis

Seed normal human colorectal epithelial cells (NCM460) into 7 small culture plates, add 2 mL of complete culture medium, which was composed of RPMI-1640, 10% FBS, and triple antibiotics to each dish, and pre-culture the cells in a 37℃, 5% CO2 incubator for 24 hours.

Use trypsin to remove all adherent cells, and gently disperse the cells. Then, collect the cell suspension into a centrifuge tube, centrifuge at 4℃ 1000 g for 3-5 minutes, in order to enable the cells to settle to the bottom of the centrifuge tube, and carefully discard the supernatant. Add 1mL PBS, resuspend the cells, and transfer them to a 1.5 mL sterile centrifuge tube. Centrifuge at 1000 g at 4℃ for 3 minutes until the cells settle to the bottom of the tube. Carefully discard the supernatant.

Mix the Cell Stain Buffer with distilled water in a 2:1 ratio to obtain the working solution of Cell Stain Buffer and store it at 4℃. Take the collected 1×106 cells and add 0.9ml of Cell Stain Buffer working solution to resuspend the cell sediment.

Add 5 μL Hoechst 33258 Stain and 5 μL PI Stain, mix them gently, and incubate cells in an ice bath for 20-30 minutes. Centrifuge the cells at 1000g at 4℃ for 3-5 minutes to precipitate cells, wash once with PBS, and then observe red and blue fluorescence using a laser confocal microscope.

Based on laser confocal microscopy image, normal cells exhibited weak blue and red fluorescence, whereas apoptotic cells were characterized by intense blue and weak red fluorescence.

3 Results and discussions

3.1 Results and Discussion of Cytotoxicity Experiments

3.1.1Cytotoxic Effects of 80 nm PS-MPs after 24h Treatment

The experimental group with low concentration microplastics (1 μg/mL) showed significantly reduced cell viability (Fig. 1), which indicates that 80 nm PS-MPs had significant cytotoxicity towards human intestinal epithelial cells at low concentrations.

When treating with middle and high concentrations of microplastics (10 μg/mL and 100 μg/mL), the cell viability showed a slight increase, but it was still lower than that of the control group(Fig. 1). It suggested that the cytotoxicity of microplastics did not continuously increase with concentration within a certain concentration range.

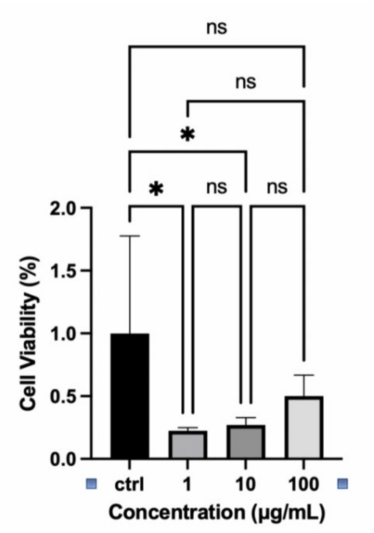

Figure 1. Cytotoxicity of NCM460 cells after 24h exposure to 80 nm PS-MPs.

3.1.2 Cytotoxic Effects of 500 nm PS-MPs after 24h Treatment.

Low and middle concentration microplastics (1 μg/mL and 10 μg/mL) did not affect cell viability significantly(Fig. 2), indicating that 500 nm PS-MPs had no significant cytotoxicity on human intestinal epithelial cells at low and medium concentrations.

High concentration of microplastics (100 μg/mL) enhanced cell viability(Fig. 2), which was contradictory to conventional understanding. This suggested a complex dose-response relationship or a potential stimulating effect of microplastics on cells at high concentrations. Since the diameter of the PS-MPs in this group was 500 nm, their size could be too large for the cell to absorb.

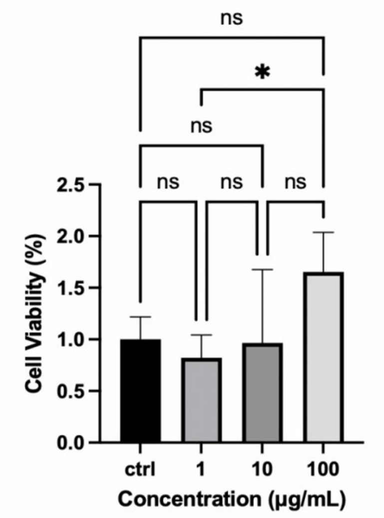

Figure 2. Cytotoxicity of NCM460 cells after 24h exposure to 500 nm PS-MPs.

3.1.3Cytotoxic Effects of 80 nm PS-MPs after 48h Treatment

The 1 μg/mL group significantly reduced cell viability, while the 10 μg/mL group showed no significant effect(Fig. 3). These results demonstrated a concentration-dependent toxic effect of 80 nm PS-MPs on human intestinal epithelial cells at low concentrations.

The high concentration microplastics (100 μg/mL) treatment significantly increased cell viability(Fig. 3), which was consistent with the results of the 24-hour treatment. This suggested that high-concentration microplastics may have a stimulating effect on cells.

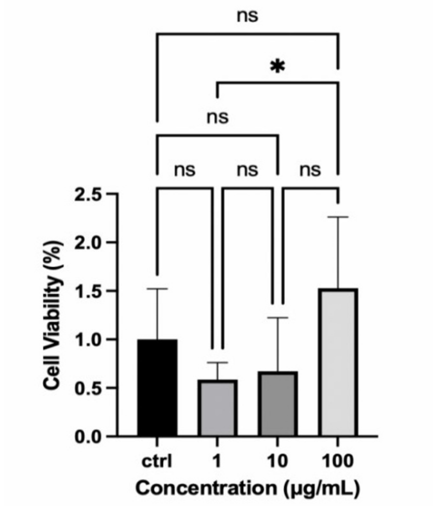

Figure 3. Cytotoxicity of NCM460 cells after 48h exposure to 80 nm PS-MPs.

3.1.4 Cytotoxic Effects of 500 nm PS-MPs after 48h Treatment

The 1 μg/mL group reduced cell viability significantly. However, the 10 μg/mL group had no significant effect(Fig. 4), indicating that 500 nm PS-MPs exhibited a concentration-dependent toxic effect on human intestinal epithelial cells at low concentrations, the group with low concentration has high toxicity.

The high concentration microplastics (100 μg/mL) group significantly increased cell viability(Fig. 4). The result was consistent with the results of 24-hour treatment, suggesting that high-concentration microplastics might have had a stimulating effect on cells.

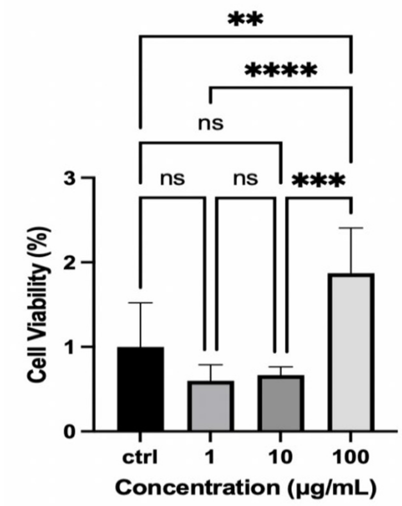

Figure 4. Cytotoxicity of NCM460 cells after 48h exposure to 500 nm PS-MPs.

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3.1.5 Discussion of Cytotoxicity

According to the experimental results, after 48 hours, the cells' ability to recover outweighed the harm caused by microplastics, allowing them to recover from the damage. Moreover, when the diameter of the polystyrene microsphere was 500 nm, it would surpass the scale of endocytosis and exocytosis, making it difficult for the cells to absorb. As the concentration of polystyrene microspheres increased, the cell capacity reached its upper limit; it would not take in Polystyrene microplastics anymore.

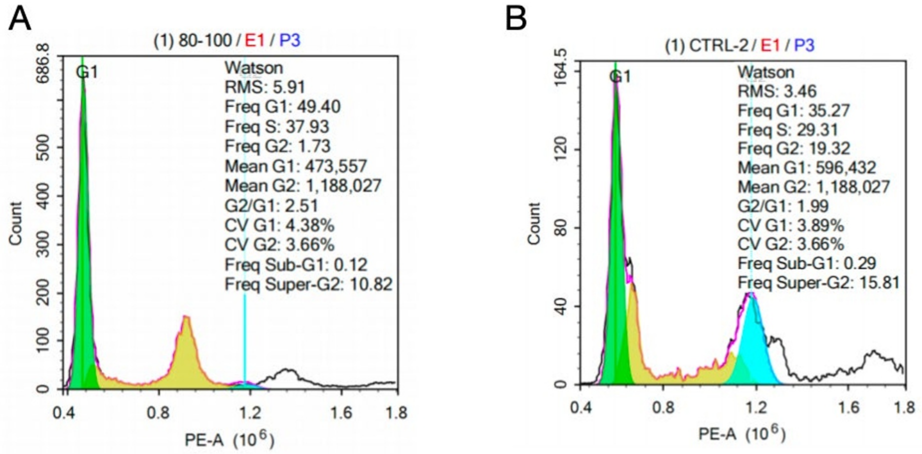
3.2 Results and Discussion of Cell Cycle

3.2.1 Colorectal Cell Cycle Affected by PS-MPs

The exposure to PS-MPs caused notable alterations in the cell cycle of human colorectal cells, and the most prominent effect would be a disturb on the S and G2 phases.

For the 24-hour groups, the flow cytometry analysis indicated that cells treated with 80 nm PS-MPs (100 μg/mL) showed a notable increase in the proportion of cells in the S phase (37.93% vs 29.31% in the control) and a following drop in the G2/M phase population (1.73% vs 19.32% in the control) (Fig. 5). Moreover, a similar but more obvious S phase arrest was observed in cells that were treated with 500 nm PS-MPs at 1 μg/mL, where the S phase population increased to 51.62% (Fig. 6).

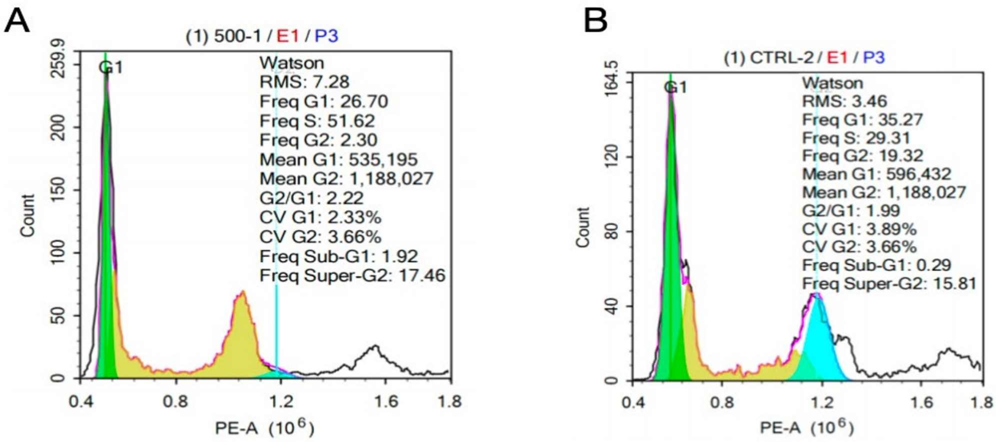
In the 2-hour groups, comparing to the control, the group with 500 nm PS-MPs at 1 μg/mL resulted in a decrease of cells in both S phase (29.59% vs 39.07%) and G2 phase (14.17% vs 26.01%). The proportion of sub-cells was increased in several treatment groups, especially in the 500 nm, 1 μg/mL, 2-hour group (1.47% vs 0.66% in control) (Fig. 7).



RMS	Freq G1	Freq S	Freq G2	G2/G1	CV G1
5.91	49.40	37.93	1.73	2.51	4.38%

RMS	Freq G1	Freq S	Freq G2	G2/G1	CV G1
3.46	35.27	29.31	19.32	1.99	3.89%

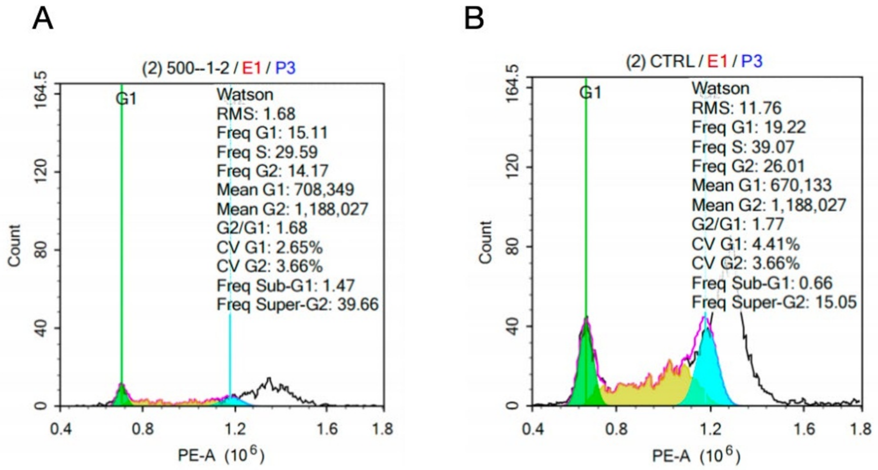
Figure 5. Cell cycle of NCM460 cells after 24h exposure to 80 nm 100 μg/mL PS-MPs.



RMS	Freq G1	Freq S	Freq G2	G2/G1	CV G1
7.28	26.70	51.62	2.30	2.22	2.33%

RMS	Freq G1	Freq S	Freq G2	G2/G1	CV G1
3.46	35.27	29.31	19.32	1.99	3.89%

Figure 6. Cell cycle of NCM460 cells after 24h exposure to 500 nm 1 μg/mL PS-MPs.



RMS	Freq G1	Freq S	Freq G2	G2/G1	CV G1
1.68	15.11	29.59	14.17	1.68	2.65%

RMS	Freq G1	Freq S	Freq G2	G2/G1	CV G1
11.76	19.22	39.07	26.01	1.77	4.41%

Figure 7. Cell cycle of NCM460 cells after 2h exposure to 500 nm 1 μg/mL PS-MPs.

3.2.2 Discussion of Cell Cycle

The most significant finding of the cell cycle study is that the exposure to PS-MPs, particularly 80 nm in diameter, induces S phase arrest in human colorectal cells, because the cell number increased in the S phase and there's a reduction in population in the G2/M phase. The S phase is the period that DNA replication occurs, and an arrest in this phase suggests that PS-MPs hindered the DNA synthesis process (Hu, 2023).

Intriguingly, high concentration (100 μg/mL) PS-MPs sometimes exhibited a less obvious effect than lower concentrations. At high concentrations, cells might induce emergency response mechanisms that alter their typical cycle arrest profile, potentially leading to apoptosis, as supported by our apoptosis assay results.

3.3 Colorectal Cell Apoptosis Affected by PS-MPs

We used the laser confocal microscope to observe the cells. The image of the staining patterns between the experimental group and the control group showed a distinct difference (Fig. 8). In the control group, cells exhibited only weak blue fluorescence and negligible red fluorescence, indicating a healthy cell with intact membranes and minimal apoptosis.

In contrast, in the experimental groups treated with both 80 nm and 500 nm PS-MPs across all concentrations of 1, 10, and 100 μg/mL for 24 hours, a marked increase in bright blue fluorescence (Hoechst 33258) was observed. This bright blue staining is an indicative feature of apoptotic cells. At the same time, the red fluorescence (PI) in these groups remained relatively weak. These observations suggested that most cells' membrane remained intact and the cells experienced apoptosis rather than necrosis.

These observations indicate that exposure to PS-MPs for 24 hours induced apoptosis in NCM460 cells, regardless of particle size or reagent concentration.

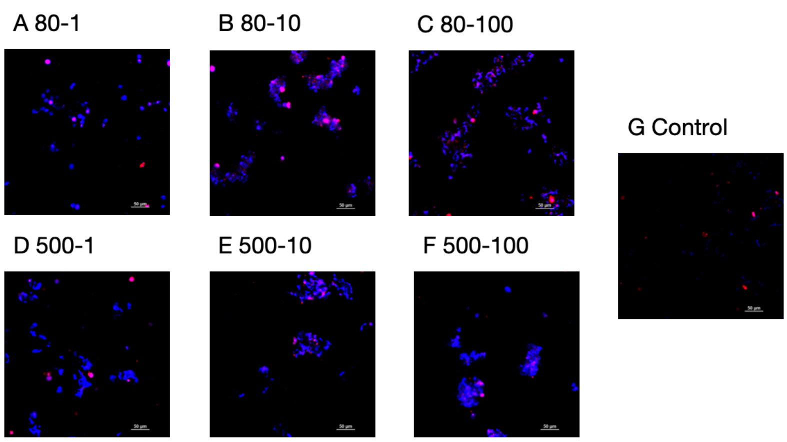


Figure 8. Cell apoptosis of NCM460 cells after 24h exposure to PS-MPs.

4 Conclusion

My study demonstrated that PS-MPs exert toxic effects on human normal colorectal epithelial cells (NCM460), and these effects depend on the size of the PS-MPs, the concentration of the reagents, and the exposure time. These findings provide crucial parameters for assessing environmental health risk, and directly inform the development of targeted strategies.

Firstly, in terms of cytotoxicity, smaller diameter PS-MPs (80 nm) exhibited more significant toxicity than larger ones (500 nm). After exposure to low concentrations (1 μg/mL and 10 μg/mL) of PS-MPs for both 24 and 48 hours, we found that cell viability was reduced. Interestingly, we discovered a non-monotonic dose-response relationship, where high concentrations (100 μg/mL) of both sizes of PS-MPs unexpectedly increased cell viability. This result suggests a potential complex response of cells to high-dose microplastic stress. This precise identification of high-risk, smaller-sized pollutants provides a clear priority for environmental engineering, underscoring the urgent need to enhance water treatment processes for the efficient removal of these specific microplastics.

Secondly, regarding the impact on the cell cycle, PS-MPs exposure caused significant disruptions, primarily presenting as S-phase arrest. This indicates a delay in DNA replication. Such fundamental disruption of cellular function serves as a crucial scientific basis for re-evaluating the public health risks posed by microplastic exposure, reinforcing the need for preventative public health policies.

Thirdly, the results from the laser confocal microscope indicated that exposure to both 80 nm and 500 nm PS-MPs for 24 hours significantly promoted apoptosis. This observed cell death mechanism provides direct biological evidence of the potential harm caused by the microplastics to the intestinal barrier.

In conclusion, our findings provide direct in vitro experimental evidence that exposure to PS-MPs can damage intestinal epithelial cells by inhibiting cell viability, disrupting the cell cycle, and inducing apoptosis. These results underscore a clear pathway from environmental pollution to potential health risk. Therefore, this study advocates for integrated action on both Environmental Engineering and Public Health field: To advance filting technologies for targeted microplastic removal, and to inform policies that reduce human exposure and contribute to the development of sustainable, healthy cities resilient against such emerging contaminants.

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When selecting a research topic, Ms. Yang provided essential advice that helped me narrow down my focus to the toxic effects of polystyrene on colorectal cells, building on my interest in digestive system physiology and gut function.

During the experimental design stage, she offered critical input on key methodological aspects, including cell culture procedures, MTT assay protocols, and approaches for analyzing cell cycle and apoptosis, thereby ensuring the scientific validity and feasibility of the study.

Throughout the experimental process, Ms. Yang provided timely assistance when technical challenges arose. During confocal microscopy for apoptosis observation and cell cycle analysis, she assisted with equipment calibration and image acquisition. She also introduced me to data analysis tools such as Prism, enabling me to independently perform statistical analysis and interpret the results.

During manuscript preparation, Ms. Yang provided multiple rounds of feedback on the logical structure, analytical methods, figure presentation, and language clarity, significantly improving the quality of this paper.

I hereby declare that all guidance received from Ms. Yang was strictly academic. Any errors or shortcomings in this work are my own.

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