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Assessment of the Toxicity of Polystyrene Microplastic in the Colon and Liver of Adult NMRI Mice

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ABSTRACT

Background: Microplastics (MPs), which are emerging environmental contaminants measuring approximately less than 5mm in diameter, have garnered significant attention in recent years.

Objectives: This study was conducted to evaluate the impact of microplastics on the colon samples, which are directly exposed to microplastics that enter the digestive tract through food, and the liver, which is responsible for processing chemicals from the digestive tract in mice.

Methods: During this experiment, 36 adult male mice were randomly divided into four groups of nine animals each. Three groups received PS-MPs at doses of 0.001, 0.01and 1 (gavage) for 42 days; a control group was also considered. 24 hours after the last treatment, tissue samples were collected for histomorphological, histomorphometrical, inflammatory factors, and gene expression analyses.

Result: The findings showed that receiving PS-MPs led to negative effects on the histomorphology and histomorphometry of the colon and liver. Also, receiving PS-MPs caused a significant increase ($P<0.05$) in the inflammatory factors, such as TNF-α and PGE2, compared to the control group. In addition, a significant increase ($P<0.05$) in β-catenin and HIF-1α mRNA expression was observed in the groups treated with PS-MPs compared to the control group.

Conclusions: It seems that PS-MPs can cause negative effects on histomorphology, and histomorphometry and increase the concentration of TNF-α, and PGE2 factors and the expression of the HIF-1α, and β-catenin genes in the colon.

Keywords: Colon, Liver, Microplastic, Inflammation, Polystyrene

Introduction

Microplastics (MPs) are of global concern with their widespread distribution and complex effects on living organisms. Recently, terrestrial ecosystems and biological health hazards, including human health hazards, have become the focus of parliamentarians' attention (Ghosh *et al*., 2023). MPs, a novel category of environmental contaminant composed of plastic particulates with a diameter of less than 5 microns, emerged due to the extensive utilization and mass production of plastic on a global scale (Barnes *et al*., 2009). In contrast, scientists have begun to devote considerable attention to MP pollutants in the twenty-first century, particularly in the last ten years (Chia *et al*., 2020). In 2015, MP pollution was ranked as the second most significant scientific challenge in the realm of environmental and ecological science, following global threats such as ozone depletion, climate change, and ocean acidification, at the second United Nations Conference on the Environment (Jin *et al*., 2019).

Animals' intestinal mucosa serves as the first line of defense against intestinal infections (Kinnebrew and Pamer, 2012). Several prior investigations have demonstrated that gut (Guvenc *et al*., 2024) microbiota can stimulate the growth of intestinal epithelial cells, fortify the intercellular adhesion of the intestinal mucosal epithelium, impede pathogenic bacterial damage to the intestinal mucosa, and preserve the integrity of the intestinal barrier (Hemarajata and

Versalovic, 2013). Therefore, the intestinal microbiota plays a critical role in regulating the metabolism of the host and contributing to the pathogenesis of certain metabolic disorders. A growing body of evidence has substantiated the notion (Guinane and Cotter, 2013).

It has been demonstrated that various types of environmental pollutants exhibit toxicological effects on the gastrointestinal microbiota (Singh *et al*., 2022). Furthermore, several investigations have demonstrated that MPs are capable of interacting with microorganisms and have even proposed that they may function as a unique habitat for microbes (Parsaeimehr *et al*., 2023). Prior studies have demonstrated that polystyrene microplastic (PS- MPs) could induce gut microbiota dysbiosis in rodents and zebrafish (Jin *et al*., 2018). Tumor necrosis factor α (TNF-α), a cytokine, impacts different cell types differently. TNF-α typically signals biological processes including inflammation and cell death via binding to TNFR1 and TNFR2 receptors (Jang *et al*., 2021,Zigam et al., 2023). Prostaglandin E2 (PGE2), which regulates numerous physiological and pathological processes, can be synthesized by an extensive variety of cell types within the body, including epithelia, fibroblasts, and infiltrating inflammatory cells in particular (Cheng *et al*., 2021). Significantly increased PGE2 production is observed in damaged tissue. PGE2 is produced from arachidonic acid (AA), which is released from membrane phospholipids via phospholipase A2 catalyzed by stressors including inflammation (St-Onge *et al*., 2007; Tithof *et al*., 2007). β-catenin serves as a significant diagnostic and prognostic indicator for colon cancer (Bhattacharya *et al*., 2019). This protein is involved in internal signaling and may play a significant role in colon cancer (Mesgari *et al.*, 2023) and tumorigenesis (Zhao *et al*., 2022). Besides β-catenin, hypoxia-inducible factor 1-alpha (HIF-1α) is also crucial for tumor growth in mammals (Burslem *et al*., 2017). This protein product greatly improve oxygen availability.

Increasing erythropoiesis and angiogenesis increases oxygen access. Events activate genes involved in glucose transport and metabolism (Watts *et al*., 2020).

In this investigation, we subjected mature male NMRI mice to 2 µm PS- MPs. Previous studies showed that PS-MPs disrupt the structure of different body tissues. Therefore, the present study aims to investigate the effects on histomorphology, histomorphometry, inflammation factors, and expression of β-catenin and HIF-1α genes on colon and liver tissue in mice treated with PS-MPs for 42 days.

Materials and Methods

Chemicals and materials

The 2 µm Microplastic composed of PS- MPs solutions (78452-5ML-F) were acquired from Sigma Chemical (Germany). Before utilization, PS-MPs were evenly distributed in deionized water and subjected to agitation by supersonic waves for 30 minutes (Jin *et al*., 2019).

Animals and experimental scheme

Five-week-old NMRI mice were purchased from the Razi Vaccine and Serum Research Institute (Tehran, Iran). All mice were housed in independent cages (standard size) in an animal room with a cycle of 12 hours of light and dark. After a week of accommodation, they were weighed and randomly divided into four groups. The first group (nine in each group) was the control group exposed to distilled water (0.1 ml/kg body weight), and three other groups were taken

different doses of 2 µm PS-MPs (0.001, 0.01 and 1 mg/kg body weight) for the toxicological experiment (Wen *et al*., 2023). The PS-MPs were diluted in Distilled water, the animals were continuously exposed for six weeks by gavage. During the whole experiment, the basic diet and water were always available. At the end of the experiment, all the mice were fasted for 12 h, anesthetized with ketamine/xylazine (0.10 mL xylazine and 1 mL ketamine and 8.90 mL distilled water with the dose of 0.1 mL/10 g body weight), and sacrificed. Tissues such as liver, and colon were collected quickly and flash-frozen in liquid nitrogen; the samples were stored at −70 °C until the following study. All experiments were performed following the Guiding Principles for the Use of Animals of the University of Tehran, and Every endeavor was put forth to reduce animal suffering (Anbara *et al*., 2021).

Histopathological analysis

Small sections of the colon and liver were promptly preserved in a 10% (v/v) formaldehyde solution after being removed. After dehydration with ethanol, hyalinization with xylene, and subsequent embedding in paraffin wax at 56°C, the fixed tissues were prepared. Five micrometer-thick sections were subsequently cut from three colon samples and three liver samples from each group. Hematoxylin-eosin solution (H&E) was subsequently used to stain liver and colon tissue. An imaging (DinoCapture 2.0) was employed.In the histological study using hematoxylin-eosin staining, the stained sections were graded based on the degree of tissue abnormality (Anbara *et al*., 2022). This abnormality was assessed using five parameters, which are features and characteristics of colonic dysplasia and neoplasia (Sarikoç *et al*., 2013; Ilić *et al*., 2019. Vajed *et al*., 2024):

1. Nuclear pleomorphism (0 for absence of pleomorphism, 1 for mild to moderate pleomorphism, and 2 for severe pleomorphism).

2. Mucosal stratification (0 for monolayer covering and lack of stratification, 1 for moderate stratification, and 2 for severe stratification).

3. Nuclear polarity (0 for proper nuclear polarity, 1 for mild lack of nuclear polarity, and 2 for severe lack of nuclear polarity).

4. Goblet cell content (0 for normal goblet content, 1 for moderate absence of goblet cells, and 2 for severe absence of goblet cells).

5. Crypt abnormality (0 for normal crypts, 1 for moderate disorganization of the crypt, and 2 for severe disorganization of the crypt).

Normal colon crypts score 0, while colon cancers can have a maximum score of 10.

Measurement of inflammatory factors

The pro-inflammatory factor TNF-α and the anti-inflammatory factor PGE were quantified using the manufacturer's method and an ELISA kit (eBioscience).

DNA extraction, qPCR amplification

In a sterile porcelain mortar, liquid nitrogen pulverized colon sections. Total RNA was extracted from 50 mg of powdered colon tissue. In summary, total RNA extraction involved these stages. Start by adding 50 mg of tissue sample to one milliliter of Trizol (Invitrogen, USA). Add 250

microliters of chloroform and centrifuge for 15 minutes at 4°C and 12000 rpm, then transfer the supernatant containing Total RNA to a microtube, add isopropanol, and precipitate RNA at 4°C and 1400 rpm. Following a 70% alcohol wash and drying, add 50 µl of DEPC-treated water to the microtube and store at -80°C (Table 1).

Table 1. Primer sequence.

Primers	Forward	Reverse
HIF-1	CACAGGACAGTACAGGATGCTT	CGTGCTGAATAATACCACTTACAACAT
B-catenin	CTAAGCAGGAAGGGATGGAAGG	GATGGCAGGCTCAGTGATGTC
β-actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT

Statistical analysis

Data were analyzed with SPSS 22.0 software. Values of the measured parameters are shown as mean values ± SD. The differences between groups were evaluated by one-way analysis of variance (ANOVA) followed by Tukey post hoc test.

Results

Microplastic polystyrene causes histomorphometric changes in colon and liver tissue

The mucosa and muscula layers of the colon tissue structure of mice in the control group are devoid of any abnormalities. Furthermore, it was observed that the colons of mice in the medium and high-dose treatment groups with PS-MPs contained substantial lymphocyte masses. A multitude of inflammatory cells were identified within the colon of PS-MP-treated mice. Masses containing immune cells were measured in experimental groups. On the other hand in mice treated with PS-MPs, we observed portal vein hyperemia (PVc), and hepatocyte lysis (HL) through histological analysis (Figure 1).

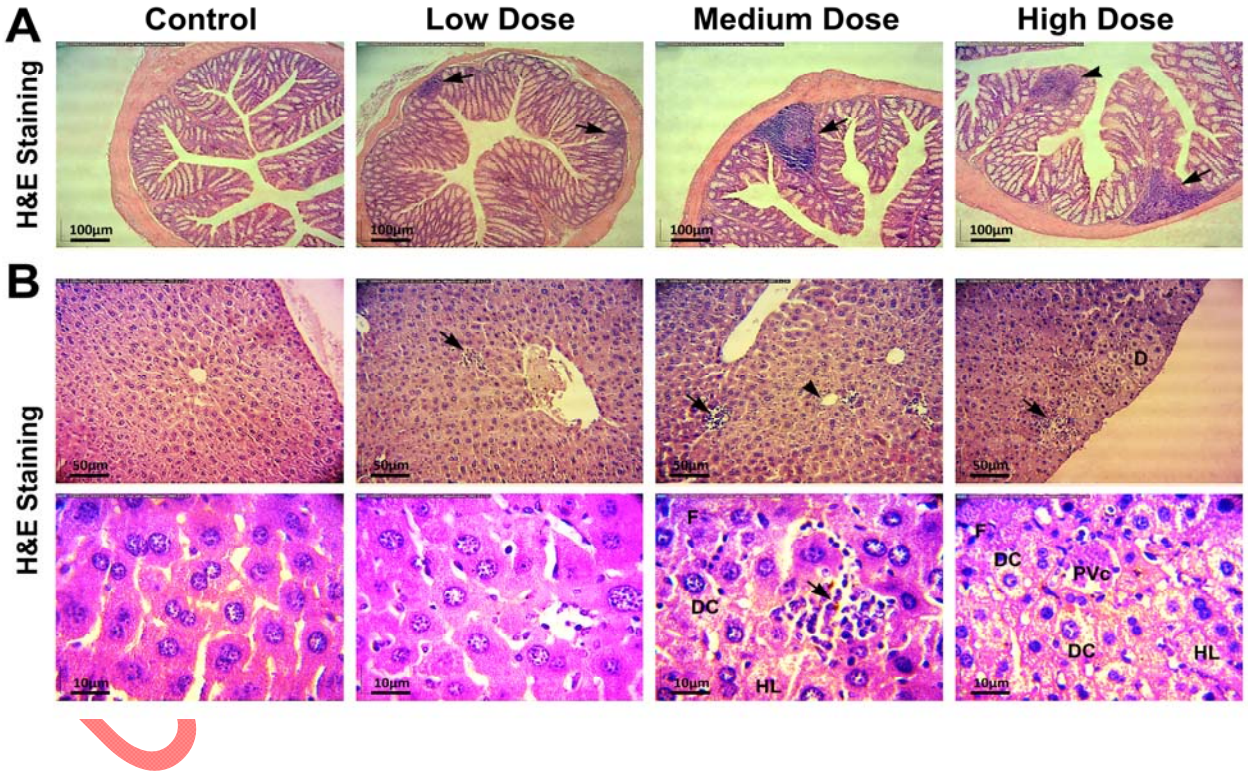

Figure 1. (A) Cross section of the colon; scattered lymphoid accumulations (Arrow), scattered ectopic lymphoid accumulation (Head arrow), and decrease of muscle layer thickness in groups with PS-MPs. (B) Hepatocytes with clear nucleus, cytoplasmic area ,and central venule of healthy liver in the control group; Hyperemia (PVc), Lysed, fragmented cell nuclei (F), disruption of the cytoplasmic area of cells (DC), infiltration of lymphoid cells in the portal space (Head arrow), and the presence of inflammatory mononuclear cells (Arrow), and deformed cells (D) in groups with PS-MPs.

Effect of PS-MPs on the deforma cells

An assessment was conducted to determine the mean quantity of deformed cells (characterized by a pyknose nucleus and furrowed cytoplasm) in both the control and PS-MPs -treated groups. A notable increase in a dose-dependent manner was noted among the groups that were exposed to PS-MPs (Table 2).

Table 2. Changes in histopathology index in colon tissue with hematoxylin-eosin staining after PS-MPs consumption.

	Control	Low dose	Medium dose	High dose
Nuclear pleomorphism	0.4±0.27	0.97±0.28	1.66±0.31*	1.78±0.39*
Mucous stratification	1.3±0.35	1.62±0.31	1.71±0.31*	1.85±0.08*
Lack of nuclear polarity	0.38±0.08	0.68±0.21*	1.39±0.33**	1.52±0.27**
Reduction of goblet cells	1.44±0.19	1.27±0.37	0.82±0.27	0.64±0.13***

Crypt anomaly	0.26±0.21	0.86±0.38	1.63±0.16**	1.73±0.19**

Degrees of tissue abnormalities: semi-qualitative. Scoring using the following five indicators: The asterisks indicate significant differences compared to control group, * (P<0.05), ** (P<0.01), *** (P<0.001) and **** (P<0.0001). Values presented are mean ± SD (N=9).

Effect of PS-MPs on the Kupffer cells

The quantification of Kupffer cells in the high dose group was found to be substantially greater than that of the control group, as determined by comparing the PS-MPs treated dermatological groups to the control group (P<0.05. Figure 2).

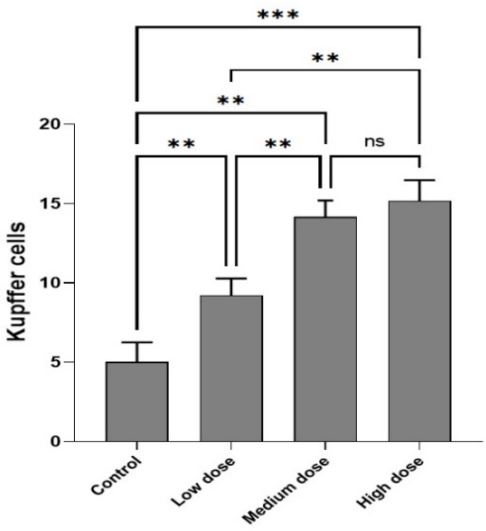

Figure 2. Average number of Kupffer cells. The asterisks indicate significant differences between groups, * ($P<0.05$), ** ($P<0.01$), *** ($P<0.001$) and **** ($P<0.0001$). Values presented are mean ± SD (N=9).

The mRNA expression level of HIF-1α and β-catenin

HIF-1 and β-catenin are significant genes implicated in colon cancer. Consequently, their expression in colon tissue serves as an indicator of its cancerous nature. The study's findings revealed elevated expression of HIF-1 and β-catenin genes in groups exposed to polystyrene compared to the control group ($P<0.05$). HIF-1 and β-catenin is a known cancer gene whose expression is increased by microplastic treatment (Figure 3).

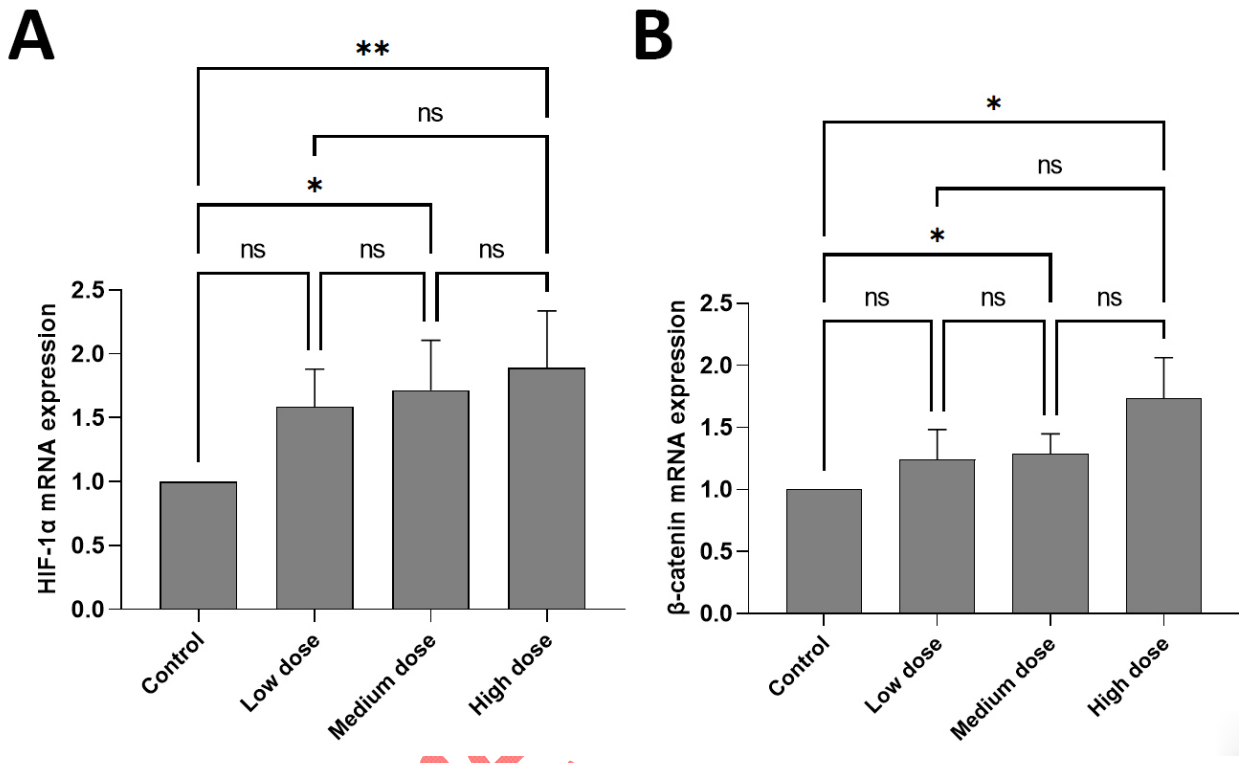

Figure 3. The relative mRNA levels of (A) HIF-1α, (B) β-catenin in mouse colon were detected with q-PCR by normalizing to β-actin. The asterisks indicate significant differences between groups, * (P<0.05), ** (P<0.01), *** (P<0.001) and **** (P<0.0001). Values presented are mean ± SD (N=9).

Effect of PS-MPs on TNF-α and PGE2 factors

Variations in the concentration of TNF-α and PGE2 indicated that the medium and high dose groups exhibited a significant increase (P<0.05) in this factor relative to the control group (Figure 4).

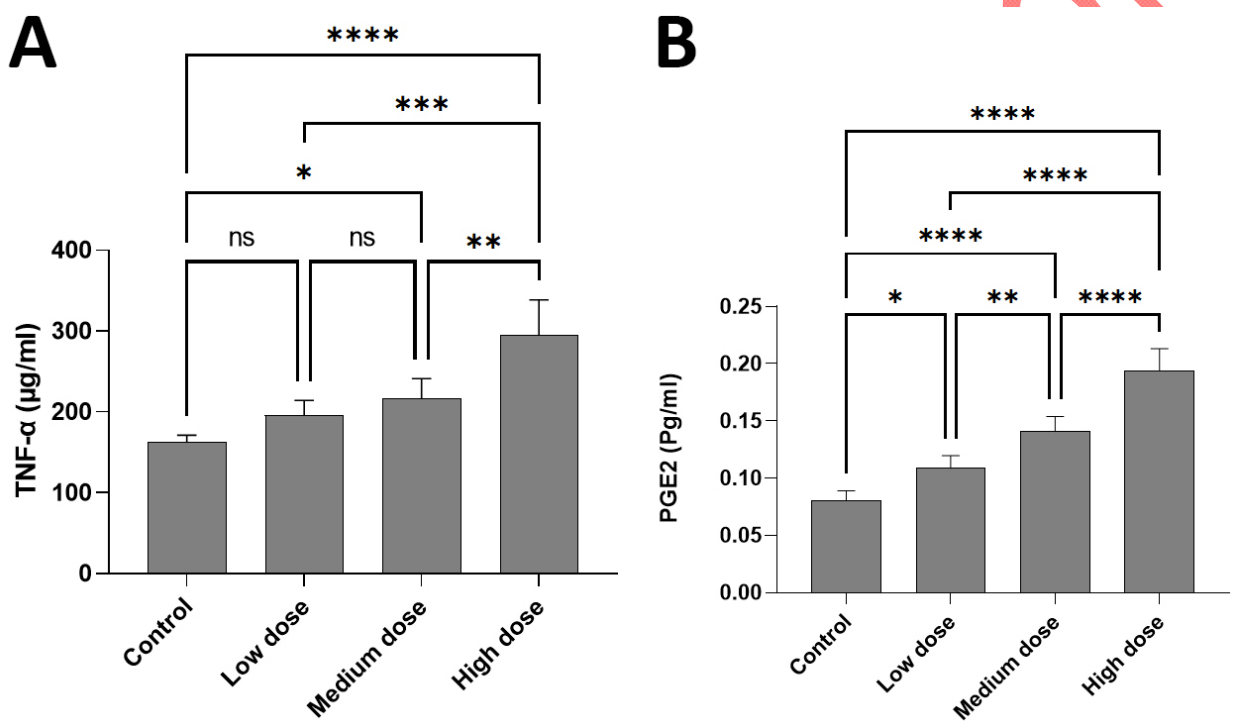

Figure 4. The relative levels of (A) TNF-α, (B) PGE2 in mouse colon. The asterisks indicate significant differences between groups, * (P<0.05), ** (P<0.01), *** (P<0.001) and **** (P<0.0001). Values presented are mean ± SD (N=9).

Discussion

There is a satisfactory body of research concerning the impacts of MP on the physiological processes of aquatic organisms within the worldwide ecological system (Jalaudin Basha *et al*., 2023). However, there is a paucity of knowledge regarding the effects of MPs on sophisticated terrestrial animals, particularly mammals (Liu *et al*., 2023). Indeed, MPs have become pervasive in terrestrial ecosystems due to human activities. For instance, plastic particulates generated during the manufacturing process of widely utilized products like toothpaste, facial cleansers, detergent, and scrub, constitute a significant environmental contributor to MP (Kannan and Vimalkumar, 2021). Sajjad et al. (2022) identify the substantial quantity of plastic film utilized in agriculture as a significant source of MPs in the land environment (Sajjad *et al.,* 2022). Furthermore, the application of sludge has resulted in the presence of MPs in certain agricultural soils across Europe, with concentrations ranging from 1000 to 4000 particles per kilogram of soil. And MPs in these terrestrial ecosystems are not only detrimental to terrestrial fauna but also have the potential to impact human health through contact (Ren *et al*., 2020. Hussain *et al*., 2024).

MPs have the potential to ascend the food chain and reach higher levels of living organisms, or they may penetrate the human food chain via salt (Saeedi, 2023). Furthermore, MPs have the potential to accumulate in the intestines of animals and could enter the bloodstream via the intestinal barrier (Huang *et al*., 2021). To substantiate this notion, a prior investigation documented that MPs could traverse the intestinal barrier and accumulate within the hepatic tissue of commercial European anchovies. In patients afflicted with intestinal diseases,

alterations in tissue permeability brought about by inflammatory conditions will substantially enhance the transport efficacy of MPs (Vagner *et al*., 2022). MPs affect the intestinal barrier and intestinal microbiota and disrupt the intestinal barrier (Takiishi *et al*., 2017).

A course of MPs treatment induced a wide range of physiological responses, including increased inflammatory cytokines in the gut and reduced mucus secretion and composition of the gastrointestinal microbiota (Choi *et al*., 2021a). In particular, chronic constipation has been reported in rats with oral administration of PS-MPs. This condition is characterized by impaired mucin secretion, chloride ion and water transport across the colon, and impaired gastrointestinal (GI) motility (Choi *et al*., 2021b). This study investigated the inflammatory response in the colon induced by MPs. The findings showed that the colonic expression of TNF-α inflammatory cytokines has increased significantly in the colon and liver.

β-catenin and HIF-1 are two crucial genes implicated in the progression and metastasis of colon cancer; therefore, they are regarded as viable therapeutic targets for the diagnosis of colon cancer (Y. Chen *et al*., 2023). Although MPs have been observed to influence the expression of these genes, no investigation into the impact of MPs on gene expression has been undertaken to date. Colonic cancer examination is not feasible; therefore, the current research is not accessible. This pertains to the expression level of these genes and the impact of MPs on that expression level.

An additional discovery made in our research was that the Cirrhosis in the liver was substantially elevated by polystyrene MP. Liver tissue cirrhosis can have a direct or indirect impact on the generation of bile acids. The liver has a significant role in the synthesis of bile acid (Sauerbruch *et al*., 2021. Poudineh *et al*., 2022). Any impairment or abnormality in liver function, such as

cirrhosis of the hepatic tissue, can result in a reduction or elevation in bile acid production (Johnson and Sherding, 2006). The hepatic diseases can lead to obstruction of the bile ducts as a result of liver inflammation, resulting in this consequence. Bile acid circulation from the intestines to the liver can be utilized to facilitate the digestion and absorption of lipids and recycle scarce bile acids (Chiang, 2013). The digestion and assimilation of lipids would be disrupted if this circulation were to cease, rendering the bile acids unusable (Ticho et al., 2020). All of these findings demonstrated conclusively that polystyrene MP poses a risk to host health due to its ability to induce metabolic disorders. In contrast to prior research, the in-vivo consequences of MPs ingestion in aquatic organisms are considerably more pronounced. Illustratively, MPs have the capacity to perturb the organism's dietary behavior, growth rates, and reproduction (Amran *et al*., 2022). Additionally, they can stimulate inflammatory reactions including vacuolation, infiltration, and necrosis (S. Chen *et al*., 2023).

Conclusions

In our investigation, the histological composition of the mice's colon and liver was modified after a 6-week exposure to PS-MPs. The results of our study showed that mice exposed to PS-MPs displayed increased levels of nuclear pleomorphism, mucosal stratification, and loss of nuclear polarity, decreased goblet cells, and aberrant crypts. These findings indicate that PS-MPs may play a role in the progression of colon cancer in mice. The liver tissue shows signs of cirrhosis, hyperemia, lysed and fragmented cell nuclei, wrinkling, disruption of the cytoplasmic area of cells, infiltration of lymphoid cells in the portal space, and the presence of inflammatory

mononuclear cells and malformed cells. It has a direct impact on liver function and can lead to issues in the synthesis of bile acids.

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ارزیابی سمیت میکروپلاستیک پلی استایرن در کولون و کبد موش های بالغ NMRI

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چکیده

زمینه: میکروپلاستیک‌ها (MPs)، آلاینده‌های محیطی نوظهور با قطری تقریباً کمتر از 5 میلی متر هستند، که در سال‌های اخیر توجه قابل توجهی را به خود جلب کرده اند.

هدف: این مطالعه به منظور بررسی تأثیر میکروپلاستیک‌ها بر روی نمونه‌های بافتی کولون که مستقیماً در معرض میکروپلاستیک‌هایی هستند که از طریق غذا وارد دستگاه گوارش می‌شوند و کبد که مسئول پردازش مواد شیمیایی از دستگاه گوارش در موش است، انجام شد.

روش کار: در این آزمایش 36 موش نر بالغ به طور تصادفی به چهار گروه 9 تایی تقسیم شدند. سه گروه در دوزهای 0.001، 0.01 و 1 میلی گرم بر کیلوگرم وزن بدن PS-MPs را (از طریق گاواژ) به مدت 42 روز دریافت کردند. یک گروه کنترل نیز در نظر گرفته شد. 24 ساعت پس از آخرین درمان، نمونه‌های بافتی برای بررسی هیستومورفولوژیکی، هیستومورفومتری، عوامل التهابی و بیان ژن جمع‌آوری شد.

یافته‌ها: یافته‌ها نشان داد که دریافت PS-MPs اثرات منفی بر هیستومورفولوژی و هیستومورفومتری کولون و کبد دارد. همچنین دریافت PS-MPs باعث افزایش معنی دار ($P<0/05$) در عوامل التهابی مانند TNF-α و PGE2 نسبت به گروه کنترل شد. علاوه بر این، افزایش معنی‌داری ($P<0/05$) در بیان mRNA ژن β-کاتنین و HIF-1α در گروه‌های تحت درمان با PS-MPs نسبت به گروه کنترل مشاهده شد.

نتیجه‌گیری: به نظر می‌رسد PS-MPs می‌تواند اثرات منفی بر هیستومورفولوژی و هیستومورفومتری داشته باشد و غلظت فاکتورهای TNF-α و PGE2 و بیان ژن‌های HIF-1α و β-کاتنین را در روده بزرگ افزایش دهد.

کلمات کلیدی: کولون، کبد، میکروپلاستیک، التهاب، پلی استایرن