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All that Glitters may be Toxic: Understanding the Impact of Exposure to Glitter Microplastics upon Animal Health

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ABSTRACT

Glitter, a type of primary microplastic, has multiple applications including in cosmetics, embellishments in arts and crafts, decoration items and jewelry. The current study was designed to determine the potential effects on ingestion of microplastics upon hematology, histology and DNA integrity in relation to varying doses in experimental groups of Mus musculus along with their retention within the body. Commercial glitter (Polyethylene terephthalate), (0.03 µm in size) was selected and fed to experimental groups mixed with basal diet in escalating doses (0.0 µg/kg, 100 µg/kg, 200 µg/kg, 400 µg/kg and 800 µg/kg). Fecal matter was collected on daily basis and analyzed for amounts of MPs present in them. After a trial duration of 21 days, blood samples were collected following standard protocol and hematological parameters analyzed. Statistical analysis indicated no significant difference of doses upon blood profile at $\alpha = 0.05$ except for Lymphocytes ($p = 0.01$) which showed a significant impact at higher doses i.e. 400 µg/kg and 800 µg/kg. Histological examination of Gastrointestinal Tract (GI) indicated degeneration and sloughing of mucosal cells, and necrosis in intestines of treatment group 3 and treatment group 4. Degeneration and disruption of villi was also visible in these groups. Likewise, DNA damage was noted to increase with increase in doses. It was also observed that the number of MPs present in excreta reduced as the dose increased.

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INTRODUCTION

Microplastics (MPs) are small plastic particles that are <5mm in diameter which are used in consumer products (e.g. as exfoliants in cosmetics) and in other activities such as air blasting (Cole et al., 2011). In aquatic ecosystems, plastics degrade into MPs and have a direct influence on aquatic organisms (Frias & Nash, 2019). UV radiation, biological and light degradation can break down the plastic into small pieces in the environment (Cozar et al., 2014). Primary and secondary types of MPs are found all over the world that cause pollution in the environment. Minute or tiny size of MPs called as primary MPs are commonly used in makeup, or as air-blasting media, although their use in medicine might also be investigated (Patel et al., 2020). MPs are formed by everyday actions such as opening of water bottles with the help of scissors, tearing with hands, cutting with a knife, or even manual twisting, and they may be found in

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aquatic and terrestrial environments (Liu et al., 2017). Because the entire human population lives on land and most human activities are land-based, the bulk of plastic litter, including macroplastics, MPs, and nanoparticles, is discharged directly to lands. Plastic waste has the potential to directly affect marine ecology through activities involving the sea such as fishing and aquaculture (Kershaw et al., 2011).

MPs are not only found in oceans, seas, and rivers but also found in sediments and soil (Sekudewicz et al., 2021). Animals are affected by MPs in a variety of ways, including disrupted feeding in *Calanus helgolandicus*, reproductive performance in oysters, physical ingestion in fish, disturbances in energy metabolism in freshwater detritivores, and hepatotoxicity in tadpoles, according to scientific evidence (Coppock et al., 2019; Kratina et al., 2019; Araujo et al., 2020). It was observed that about 80% of plastic originates from its original sources and is recorded as the second more significant problem of the world which caused a negative impact on living organisms (Wright & Kelly, 2017). Plastic particles can destroy the food chain including food stuffs and human consumption and contaminate the marine environment with the passage of time. MPs are found in food items such as mussels, commercial fish, table salt, sugar and bottled water (Neves et al., 2015; Karami, 2017; Obmann et al., 2018). Microplastic ingestion can harm the digestive pathway and cause inflammation and other physical blockages in living organisms. These inflammations sometimes cause death in aquatic organisms. MP consumption has an influence on the aquatic ecosystem, but it also poses a concern to food safety, as fish are a vital source of protein for people (Law, 2017). According to recent research less data is available regarding effects of MPs on damage in mammals and it is known that mammal's genes are more similar to human genes (Ding et al., 2019).

With multiple types of plastics being used worldwide, and hence the MPs being produced by their wear and tear, it is necessary to understand the impact of these variants upon animal and ecosystem health. While various doses of different MPs have reported multiple harmful effects in different experimental species, the fact that their toxicity threshold levels are yet to be determined calls for further research. Moreover, with ingestion being the major route of exposure of these MPs, the possibility of removal of maximum or some proportion of the ingested amount cannot be ruled out. Glitter (Polyethylene terephthalate-PET) is a commonly used product in various fields such as cosmetics, jewelry, embellishments, arts and craft projects, and decoration items and so on.

Mice (*Mus musculus*) due to their well-known physical features, quick metabolic activities, and quick response to chemicals present in the environment and well-designed genome has contributed in understanding mammalian hematology. The present study investigated the impact of MPs upon growth and hematology of living tissues using *Mus musculus* as model animals.

MATERIAL AND METHODS

Healthy adult male albino mice *Mus musculus* (n=25) were purchased from an animal breeding house from University of Veterinary and Animal Sciences, Lahore, Pakistan. The trial animals were screened and checked up thoroughly for any kind of disease or physical injury and only healthy mice were selected for experimental work. The mice were kept in labeled plastic cages in the control temperature room (29±3°C) with 50-70% relative humidity and regulated light and dark schedule and acclimatized for 1 week (Jin et al., 2019). The experiment was conducted after getting the approval certificate from the ethical committee for animal use at the University of Veterinary and Animal Sciences Lahore, Pakistan.

Feed preparation

The basal feed to be given to the mice was grinded into fine powder. A primary source of MPs widely used in beauty products (glitter; PET-polyethylene terephthalate) with a mean size

0.33 mm was added to the standard feed given to mice in doses as described here: Group 1 was the control group (T_0) with no MP provided to them and received only commercial laboratory pelleted food and water ad libitum. T_1 was fed upon 0.1 mg MPs/gm of BW, T_2 was fed 0.2 mg MPs/gm of BW, T_3 was fed 0.4 mg MPs/gm of BW, and T_4 was fed 0.8 mg MPs/gm of BW. Body weight and mortality rates were noted regularly, and mice were maintained for 21 days. (Jin et al., 2019).

Fecal sample analysis

Fecal waste was manually collected on daily basis from bedding material into pre-labelled sterile glass vials. The waste for each day was then dried and weighed before manually grinding them coarsely. The grinded samples were then added to 10% KOH solution and kept overnight at room temperature to digest the organic content of the samples. The samples were then filtered and subjected to density separation overnight to isolate and enumerate the MPs excreted by the mice. For this purpose, 145 g of NaCl was dissolved in 400 ml of distilled water. 20 ml of this salt solution was then used for density separation for each sample. The bottom residue was drained off and the remaining contents were vacuum filtered onto a cellulose acetate membrane filter. The density separation funnel was rinsed thrice with deionized water to ensure that no MPs remain attached to the glass apparatus. The filters were dried and observed under stereomicroscope to enumerate the number of MP items recovered from fecal waste.

Hematology

After completion of the trial, the animals were anesthetized with chloroform and 1 mL blood was taken for hematological analysis. The blood obtained from the samples were kept in EDTA tubes and sent to the lab for further analysis.

Histopathology

After sampling for blood, the experimental animals were dissected and GIT organs were isolated. The tissues obtained (esophagus, stomach, small and large intestine) were dipped into neutral buffered formalin, then dried, embedded into the paraffin. The 4 mm sections of GIT were made through microtome and were then stained with the hematoxylin and eosin (H&E) method of staining. Inflammation, necrosis, and degeneration were observed following (Li et al., 2020).

DNA damage

Comet assay was performed in the Department of Biochemistry and Biotechnology, UVAS Lahore according to the method proposed by (Collins et al., 1996) and improved by (Kienzler et al., 2012) on blood cells. 0.3ml of blood and Histopaque was mixed (equal ratio of blood and histopaque) to remove lymphocytes from small volumes of whole blood. Blood was transferred to Eppendorf tubes from EDTA tubes via blue tips (pipette). Centrifugation of this mixture was done at 10000 rpm for 12 minutes. The supernatant of centrifugation was transferred to another Eppendorf tube to remove all the blood suspensions except lymphocytes. This supernatant was then washed with 10X PBS solution (1ml PBS in 9ml dist. water).

PBS added was equal to the amount of supernatant. It was centrifuged again at 12000 rpm for 5 to 7 minutes. Washing with PBS solution and centrifugation was continued until white transparent settlement in the eppendorf tube was achieved. Finally, Lymphocytes settled down in the bottom. Then LMA (low melting agarose) solution was prepared (0.16g LMA in 2ml PBS) and kept in the water bath to avoid the hardening of gel. Lymphocytes were added to the LMA solution. This solution of LMA+ lymphocytes was then transferred to pre-coated agarose gel slides (normally used agarose gel 1g of agarose in 100ml 1X TAE). Hardening of gel was avoided strictly. Slides were covered with long coverslips and kept on ice cubes for 10 min to

support fixing. Slides were then labeled with a permanent marker and dipped in lysis buffer in a tray. Coverslips were removed and there was enough buffer in a tray that all slides were fully covered. A tray was placed in the fridge at 7 to 8 °C for 50 minutes. Then 1X tris buffer was used for dipping slides for 10 minutes. In the last step, electrophoresis was done at 110V for 20 minutes until bubbles appeared in the tank. Slides were taken out from the tank and labeled with ethidium bromide dye (50µl per slide). Slides were again covered with long glass slides and observed under the fluorescent microscope. DNA damage was analyzed by DNA tail formation. Total number of cells visualized along with cells where tail formation was observed were enumerated and fragmentation percentage determined using the following formula:

*Fragmentation percentage = Number of damaged cells*100/Total number of cells observed*

RESULTS AND DISCUSSION:

Recent global attention has been focused on MPs' environmental contamination. MPs adversely affect human health because plastics are highly resistant to degradation and can endure in the environment for a long time. In living organisms, MPs accumulate in their cells and tissues, potentially causing chronic biological effects, such as gastrointestinal disorders, immunity, respiratory problems, cancer, infertility, and chromosome modifications. The health effects of MPs and mechanisms of toxicity must be extensively studied because of the threat they pose to human health (Haindongo et al., 2023; Mamun et al., 2023). More recently, mice models have been used to investigate the potential impacts of MPs on host cellular and metabolic damages of mammalian tissues (Osman et al., 2023). MPs having a size of 1–10 µm were found in the pet animal tissues (Prata et al., 2022). It is assumed that MPs having a size less than 20 µm have the capacity to cross the vascular wall and mixed with blood circulation (FAO, 2017).

Respiratory and the digestive system are the major route of the MPs entry into the body of an organism. First of all, these MPs change the kinetics and in result cause inflammation. Few MPs are found in the tissues of wild animals, while these plastics when exposed to domestic animals, are commonly found in their feces (Zhao et al., 2021). These MPs are also found in the digestive system of invertebrates e.g mollusks (Reguera et al., 2019) as well as in vertebrates e.g. fishes (Neves et al., 2015), birds (Carlin et al., 2020) and some mammals of marine ecosystem (Nelms et al., 2019). MPs found in dissolved mice gut tissues (Abdel-Zaher et al., 2023). These MPs, when get entry into the body of animals, overcome the natural barrier of tissues and make their way into the internal environment. The presence of these MPs in the fish liver (Su et al., 2019) muscles, liver, stomach and intestine of wild animals located in the coasts of Norway and liver, intestine, kidneys, lungs and blood of domestic animals (Prata et al., 2022) is the evidence of their entry into internal environment (Su et al., 2019). However, little is known regarding how MPs affect vascular biology or humans/mammals. In this study, mice were administered varying doses of a widely used primary microplastic (glitter) to determine any potential toxic impacts upon organ integrity.

Body Weight

No significant reduction in body weight of the experimental animals was noted during the trial while weight gain was noted for each experimental group hereby indicating that MP ingestion did not affect body growth (Fig. 1, Table 1).

However, it must be noted that since almost 42 to 67 % of the ingested glitter particles were being retained within the body while the rest was excreted (Fig. 2), they might not pose an immediate health risk for the animal as their quantities within the body were further reduced.

Hematotoxicity

The hematological analysis showed that red blood cells (RBCs) of the control group

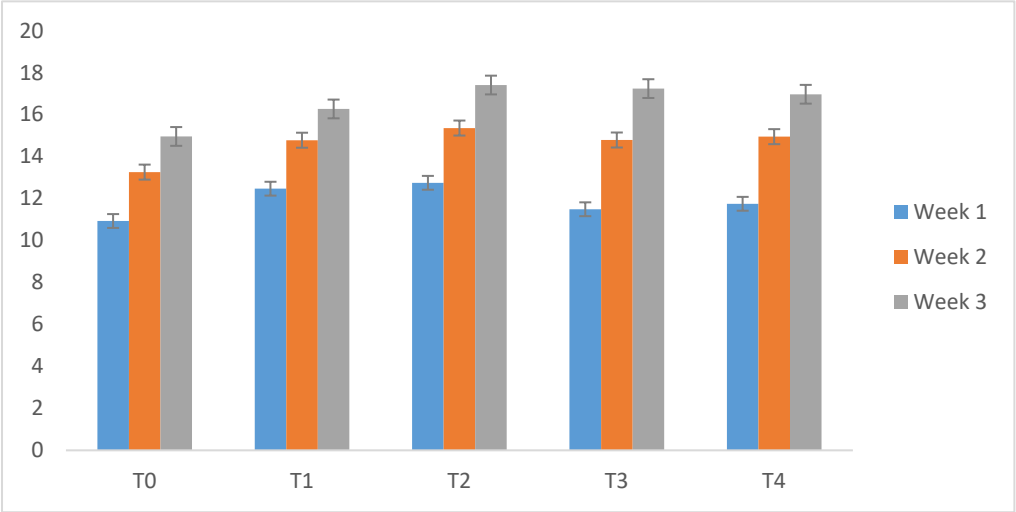

Fig. 1. Changes in body weight during the experimental trial (21 days). One-way Anova indicated no significant difference in mean values across all groups at $\alpha = 0.05$.

Table 1. Body weight changes in control and treated groups.

Treatment Groups	Week 1	Week 2	Week 3
Control group	10.93 ± 1.12	13.26 ± 0.91	14.96 ± 0.89
T1	12.47 ± 2.44	14.78 ± 1.66	16.27 ± 1.32
T2	12.75 ± 2.54	15.36 ± 2.85	17.41 ± 2.51
T3	11.49 ± 2.59	14.79 ± 2.25	17.24 ± 2.60
T4	11.75 ± 1.90	14.95 ± 1.53	16.97 ± 1.37

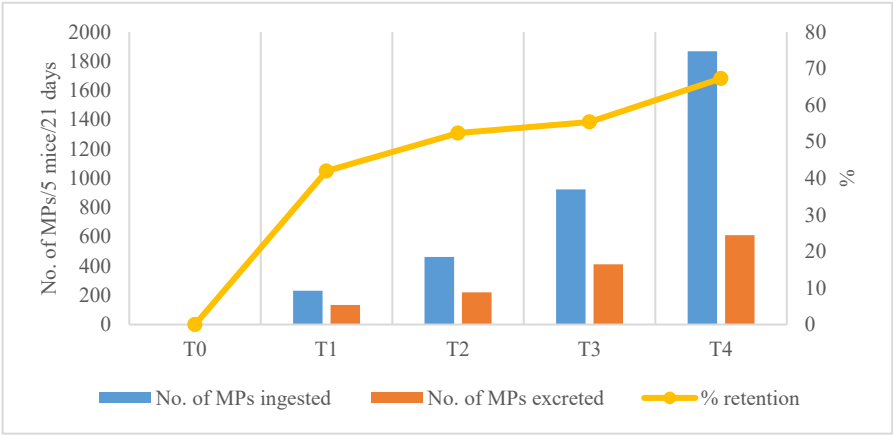

Fig. 2. Retention of MPs within the GIT of mice

maintained their quantity whereas a minor decrease was observed in group 2 which were fed with 100 µg/kg MPs. The decrease in quantity of RBCs in group 3 and 5 remained almost same. Group 4 treated with 400 µg/kg MPs was observed with a great decrease in RBCs quantity with reference values (Table 2). In the present research, it was found that hematology depends upon the dose given to mice. Plastics in small particles are really damaging to organisms directly or indirectly (Lu et al., 2018; Jin et al., 2019). Formation of reactive oxygen species and in result, development of oxidative stress causes harmful effects for blood parameters. (Jeong & Choi, 2019).

Table 2. Hematological parameters

	Ref values	T_0	T_1	T_2	T_3	T_4
RBCs	8.09	8.27+0.62	8.83+1.82	7.03+1.56	5.76+0.33	7.30+0.05
Plt	--	403.50+170.41	496.50+502.75	328.00+29.70	265.50+48.79	768+147.08
WBCs	6.04	3.75+0.49	5.60+5.23	2.90+1.13	2.20+0.00	3.25+0.07
Hb	13.9	13.15+1.06	13.85+1.63	11.55+1.91	8.95+0.21	11.55+0.07
Hct	41.43	44.35+5.73	45.65+4.31	37.40+7.78	27.70+2.40	35.65+1.20
MCH	17.32	15.95+0.07	15.85+1.34	16.60+0.99	15.60+0.57	15.80+0.14
MCHC	33.61	29.85+1.48	30.30+0.71	31.10+1.41	32.60+2.12	32.35+1.20
Lym	4.03	71+7.07	73.50+0.71	79+1.41	57+1.41	78.50+6.36
Neutro	1.9	14+11.31	10.50+2.12	11.50+0.71	18.0+4.24	5.50+0.71
NLR	0.47	0.21+0.18	0.14+0.03	0.15+0.01	0.31++0.07	0.07+0.01

Comparison with reference values for white blood cells (WBCs) showed a great deviation except for T1. Hemoglobin of experimental groups 1 and 2 showed no deviation from the reference group. Group 3 and 5 also showed a negligible decrease whereas group 4 showed a significant decrease in hemoglobin quantity. The hematocrit (HCT) values of group 1 and 2 showed a minor increase in quantity as compared to group 3, 4 and 5 which showed a great decrease in quantity of Hct from reference values. The values for mean corpuscular hemoglobin (MCH) in all groups almost decreased whereas mean corpuscular hemoglobin concentration (MCHC) showed a gradual decrease. Lymphocytes (Lym) of MPs treated groups were compared with reference values of Lym. It was observed that values of Lym showed a greatest increase in quantity from reference values when quantity of ingested MPs was increased from group 1 to 5 except for group 4 which was treated with 400µg/kg MPs. Neutrophils (Neutro) also showed an increased deviation from reference values which is too less than the Lym values. So, its deviated values could be neglected.

In our research, when MPs are added in the feed, they cause a significant increase in the number of monocytes beyond the physiological norm due to the formation of macrophages for MPs excretion which in turn cause an increased number of monocytes in the blood of all experimental mice treated with different doses of MPs in feed (Lieshchova et al., 2019). Most MPs, when added in the feed of mice, prolonged the life of monocytes in the connective tissues by affecting the macrophages which originate from these monocytes (Anon, 2007).

Histopathology

The previous study by Xiong et al., (2023) reported the effects of MPs on organ injury and fibrosis under long-term accumulation in mice. Furthermore, it has been reported that MPs induced kidney injury through enhancing oxidative stress, autophagy, apoptosis, and fibrosis (Zou et al., 2022).

After MPs enter the body's circulatory system, most of them are transferred to the liver and kidneys and metabolized (Goodman et al., 2022). These MPs cause the decrease in the formation of gastric juices because MPs added to food have no nutritional value and pass rapidly through the gastrointestinal tract resulting in the decrease in the relative mass of the stomach. In another related study, exposure to 0.5 mg/kg of PVC MPs for sixty days resulted in oxidative stress, marked hepatic damage and changes in gut microbiota of mice (Chen et al., 2022). Few studies have shown that PS-MPs exposure has little effect on mouse body weight; the effect of the exposure may be dependent on the size of the microplastic and the duration of exposure (Hou et al., 2021; Zhang et al., 2021).

Histological examination of esophagus tissues revealed distinct histopathological alterations

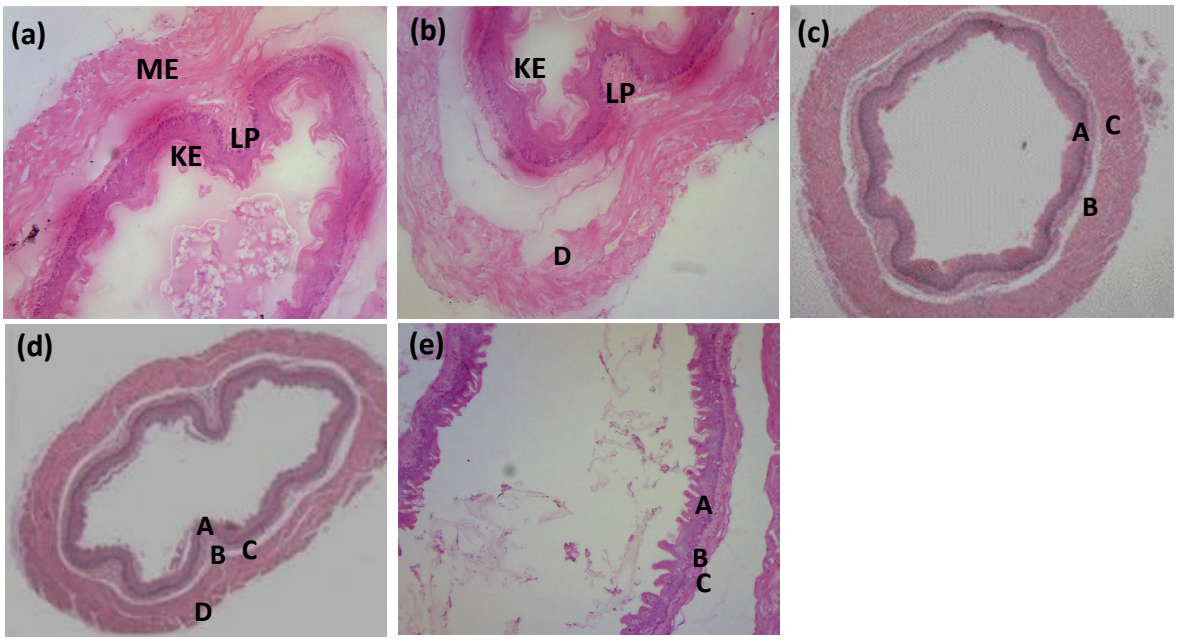

Fig. 3. Micrograph of sections esophagus tissues (a) Control Group: (A) Stratified squamous keratinized epithelium (KE), (B) Lamina propria (LE), (C) Muscularis externa (ME); (b) Treatment group 1: (A) Stratified squamous keratinized epithelium, (B) Lamina propria, (C) Submucosa, (D) Disorganized Muscularis externa; (c) Treatment group 2: (A) Stratified squamous keratinized epithelium, (B) Submucosa, (C) Muscularis externa; (d) Treatment group 3: (A) Stratified squamous keratinized epithelium, (B) Lamina propria, (C) Submucosa, (D) Disorganized Muscularis externa; (e) Treatment group T4: (A) Stratified squamous keratinized epithelium, (B) Damaged Submucosa, (C) Inflammation in Muscularis externa

between control group and treatment groups. In the control group, the esophageal structure appears well preserved and normal with an intact stratified squamous keratinized epithelium, a clearly defined lamina propria, and an organized muscularis externa (Fig. 3a). In contrast, treatment group 1 receiving a low dose of (0.1 mg MPs/gm) MPs exhibited mild histological changes. While the epithelial layer remained intact, the lamina propria and submucosa became more prominent, and the muscularis externa showed slight disorganization (Fig. 3b). In treatment group 2 given 0.2 mg MPs/gm of MPs, lamina propria was not distinctly visible, and only the stratified squamous keratinized epithelium, submucosa, and muscularis externa were identifiable (Fig. 3c). Whereas, the treatment group 3 receiving 0.4 mg MPs/gm of MPs show disorganized muscularis externa with stratified squamous keratinized epithelium (Fig. 3d). Treatment group 4 given a high dose of MPs (0.8 mg MPs/gm) showed squamous stratified epithelium, damaged submucosa and inflammation in muscularis externa (fig. 3e).

Histopathological alterations in stomach tissue were observed in the groups administered with MPs in a dose-dependent manner. Mice in the control group revealed normal histological features including a well-defined columnar epithelium, intact gastric cells and submucosa and compact muscularis externa (Fig. 4a). In contrast, treatment group 1 given low dose of MPs (0.1 mg MPs/gm) showed no significant deviations from control group exhibiting simple columnar epithelium, intact gastric gland, submucosa and muscularis externa (Fig. 4b). Meanwhile, the treatment group 2 receiving 0.2 mg MPs/gm of MPs presented damaged muscularis externa with disorganized epithelium (Fig. 4c). Treatment group 3 treated with medium dose of MPs (0.4 mg MPs/gm) showed damaged muscularis externa with disorganized epithelium (Fig. 4d). On the other hand, extensive degeneration, sloughing of epithelial lining and necrosis of tunic mucosa was observed in treatment group 4 receiving high dose of MPs (0.8 mg MPs/gm) (Fig. 4e).In the small intestine, treatment of mice with MPs exposed significant histopathological

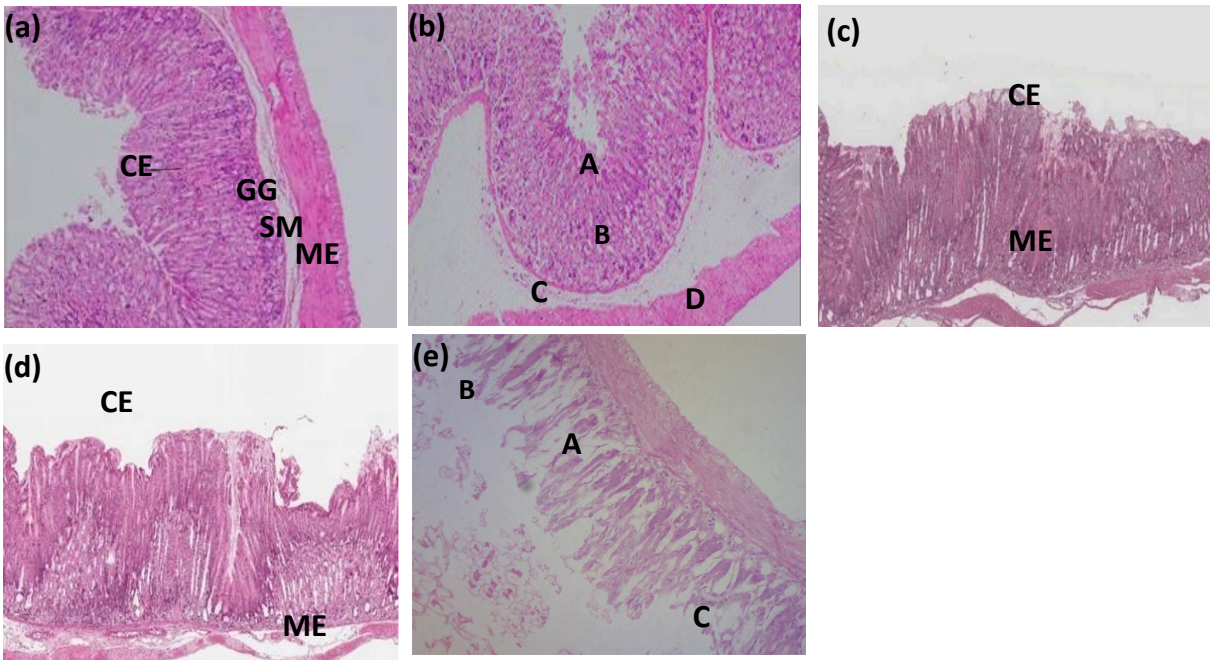

Fig. 4. Micrograph (4X) of sections of stomach tissues (a) Control Group: (A) Simple columnar epithelium (CE), (B) Gastric glands (GG), (C) Submucosa (SM), (D) Muscularis externa (ME); (b) Treatment group 1 (A) Simple columnar epithelium (B) Gastric glands (C) Submucosa (D) Muscularis externa; (c) Treatment group 2: damaged muscularis externa (ME), disorganized cellular epithelium (CE); (d) Treatment group 3: damaged muscularis externa (ME), disorganized cellular epithelium (CaE) (e) Treatment group 4: Degenerated (A), sloughed off (B), necrosed tunica musoca (C)

changes in a dose-dependent manner. In the control group, the intestinal tissue exhibited normal architecture with intact columnar epithelium, well-organized lamina propria and muscularis externa (Fig. 5a). The treatment group 1 treated with low dose of MPs (0.1 mg MPs/gm), significant histopathological alterations were observed including disorganized epithelium, damaged villi and muscularis externa (Fig. 5b). Meanwhile, treatment group 2 treated with (0.2 mg MPs/gm) of MPs showed moderate alterations with intact columnar epithelium, blunted villi and swelling in muscularis externa (Fig. 5c). However, treatment group 3 given medium dose of MPs (0.4 mg MPs/gm) displayed simple columnar epithelium with damaged villi and lamina propria (Fig. 5d). In contrast, the group receiving high doses of MPs (0.8 mg MPs/gm) exhibited severe tissue damage such as villi showing extensive degeneration and disruption (Fig. 5e). A previous study investigated the uptake, bioaccumulation, and the toxic effects of MPs in the intestine of mice (Meng et al., 2022). Additionally, polystyrene MPs induced nephrotoxicity associated with oxidative stress, inflammation, and endoplasmic reticulum stress in juvenile rats (Wang et al., 2023).

Histological analysis of the large intestine revealed significant structural changes in treatment groups in a dose-dependent manner. Control group showed intact columnar epithelium, preserved goblet cells and muscularis externa (Fig. 6a). Treatment group 1 treated with low MPs dose (0.1 mg MPs/gm), displayed mild inflammation in epithelium lining causing mucosal damage (Fig. 6b). In treatment group 2, intact columnar epithelium, cellular swelling and damaged mucosa was observed (Fig. 6c). In contrast, treatment group 3 receiving medium dose of MPs (0.4 mg MPs/gm), columnar epithelium was disorganized with swollen goblet cells and damaged muscularis externa (Fig. 6d). On the other hand, treatment group 4 treated with high dose of MPs (0.8 mg MPs/gm) demonstrated necrosis and sloughed off epithelial layer (Fig. 6e).

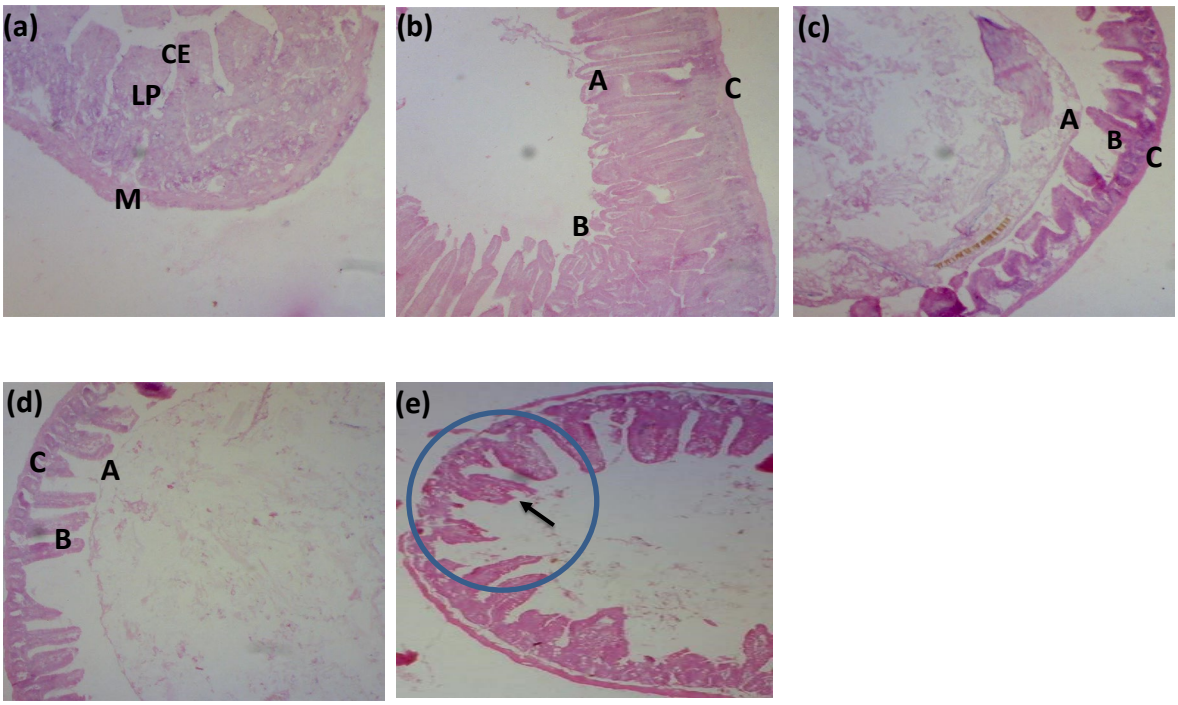

Fig. 5. Micrograph of tissue sections of small intestine (a) Control Group: (A) Simple columnar epithelium (CE), (B) Lamina propria (LP), (C) Muscularis externa, (ME); (b) Treatment group 1: (A) Disorganized columnar epithelium, (B) Damaged villi, (C) Muscularis externa; (c) Treatment group 2: (A) Intact columnar epithelium, (B) Blunted villi, (C) Swelling in Muscularis externa; (d) Treatment group 3: (A) Simple columnar epithelium, (B) damaged villi, (C) Muscularis externa; (e) Treatment group 4: degeneration and disruption of villi (black arrow)

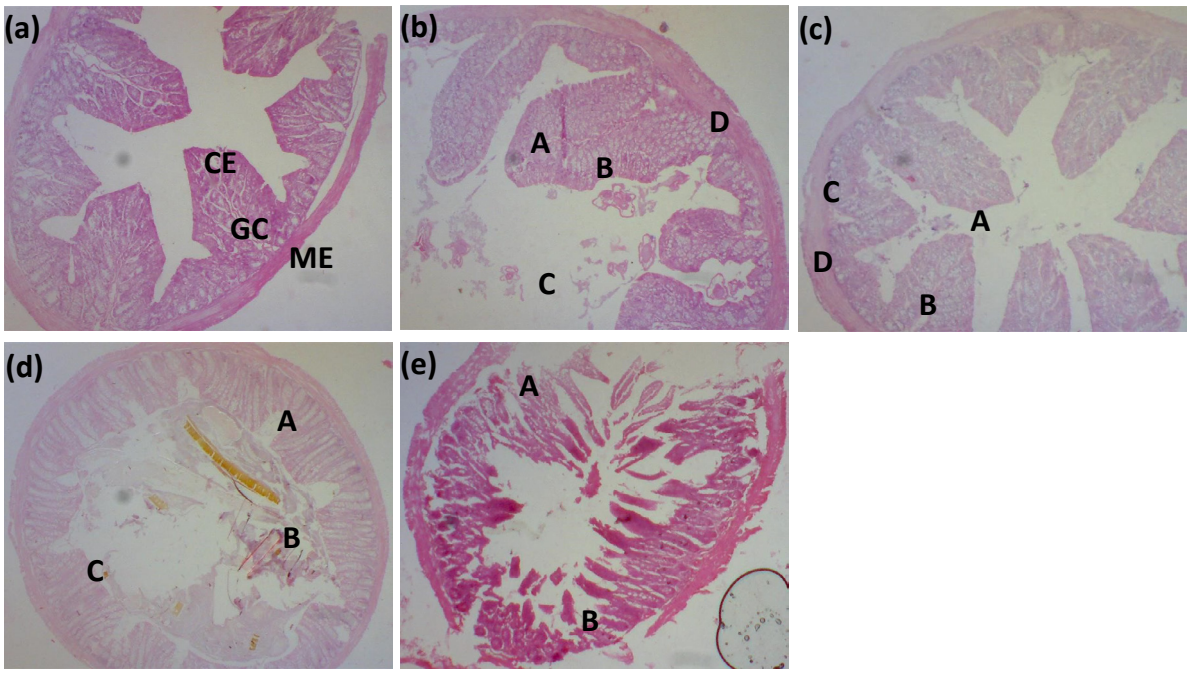

Fig. 6. Micrograph of tissue sections of large intestine (a) Control Group: (A) Simple columnar epithelium, (B) Goblet cells, (C) Muscularis externa; (b) Treatment group 1: (A) Intact epithelium, (B) Inflammation, (C) Damaged submucosa (D) Muscularis externa; (c) Treatment group 2: (A) Intact columnar epithelium, (B) Cellular Swelling, (C) Damaged submucosa (D) Muscularis externa; (d) Treatment group 3: (A) Disorganized columnar epithelium, (B) Swelling in Goblet cells, (C) Damaged Muscularis externa; (e) Treatment group 4: necrosis (A) and sloughed off epithelium (B)

Table 3. DNA Damage Index and fragmentation % observed in mice of each treatment group receiving different doses of MPs

Experimental group	Total number of cells observed Mean + SD	Total number of damaged cells Mean + SD	Fragmentation %
Control group	275.8 ± 14.77	Nil	Nil
T1	281.6 ± 10.69	18.2 ± 1.30	6.46
T2	288.0 ± 8.46	45.2 ± 2.86	15.70
T3	280.2 ± 7.98	61.6 ± 5.55	22.00
T4	282.8 ± 12.32	78.6 ± 2.97	27.84

DNA damage

Results of comet assay showed that damage index increased with the increase in number of MPs. Control group showed no cell damage while dose dependent increase in damage was observed in treatment groups receiving different concentrations of MPs as shown in Table 3. The comet assay, as recommended by ICH S2 (R1), showed that PE-MP exposure caused significant DNA damage, likely due to increased ROS levels, which induce oxidative DNA damage (Roursgaard et al., 2022). Exposure to PET and PP MPs can cause DNA damage, possibly through direct interaction with DNA rather than oxidative stress. While these findings suggest potential genotoxic effects, they did not reveal direct toxicity (Ballesteros et al., 2020).

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CONFLICT OF INTEREST

The authors report no conflict of interest.

LIFE SCIENCE REPORTING

No life threat was practiced in this research.

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