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ORIGINAL ARTICLE

Development of a histological grading system for assessing microplastic-induced structural damage in rat aorta, trachea, and bladder tissues following acute exposure

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Abstract

Introduction: Microplastic (MP) exposure has increasingly been associated with tissue-level impact in toxicology studies, including inflammatory changes and histopathological injury in aquatic and mammalian models. However, there is currently no consistently developed histological scoring approach for assessing the structural damage across different tissue types induced by MPs following acute exposure. This study aimed to develop and apply a semi-quantitative histological scoring framework to investigate the histological alterations of polyethylene terephthalate (PET) MPs in Sprague-Dawley rat aorta, trachea, and bladder *ex vivo*. *Materials and Methods*: Rat tissues were excised and incubated for 22 to 24 hours at 4°C with different concentrations of PET particles (0.3 – 5.0 mg/mL). Trachea and bladder tissues were processed for haematoxylin and eosin staining, whereas aorta sections were stained using CD31 immunohistochemistry to assess the endothelial integrity. Images were randomised and scored independently by three blinded observers using tissue-specific grading criteria for aorta endothelium, tracheal epithelium, and bladder urothelium. *Results*: Across the three tissues, the scoring system suggests organ-specific differences in terms of sensitivity to acute exposure, with bladder urothelium appearing as the most structurally vulnerable under the tested conditions. *Conclusion*: This method provides a structured framework for screening MPs-induced tissue damage and may improve reproducibility in histological evaluation of tissue structural integrity.

Keywords: microplastics, polyethylene terephthalate, histology, severity grading, aorta, trachea, bladder

INTRODUCTION

Microplastics (MPs) are widely investigated as emerging contaminants because they may interact with biological tissues through physical, chemical, and inflammatory mechanisms.[1,2] Experimental studies have reported histopathological changes in different organisms and tissues associated with MPs exposure, including gut tissue injury in marine medaka larvae, tissue damage in oysters, and gill, intestinal, hepatic or other organ-level lesions in aquatic exposure models.[3–5] These findings, hence, emphasise the value of histology as a direct structural endpoint in MP toxicology. This is important when functional changes need to be linked with visible disruption of tissue architecture.

There is not yet a consistent and widely developed method for grading MP-induced tissue damage across different types of tissues, despite the frequent reporting of histopathology in MP studies. Many studies describe lesions qualitatively or focus on organ-specific abnormalities[6,7], which can make comparison

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across experiments difficult when the tissue of interest is not covered by an established scoring scheme. Not only that, methodological reviews of MPs also emphasised the need for uniform assessment approaches and better standardisation to improve cross-study comparability. [8,9]

The absence of a standardised scoring system is particularly relevant for tissues such as the aorta, trachea and bladder, as different interface levels bring different structure and function. The aorta is lined by endothelium, the trachea by epithelium, and the bladder by urothelium. Damage to these lining layers may indicate early compromise of tissue barrier integrity, but the morphology and severity features that define damage need to be adapted to each tissue type.[10] A structured grading method is therefore needed to translate histological observations into reproducible semi-quantitative scores.

This paper presents the development of a histological grading system for assessing structural damage in rat aorta, trachea, and bladder tissue following exposure to polyethylene terephthalate (PET) MPs. The grading system was designed to provide a transparent and reproducible method by randomising the image slides and having three independent blinded observers to score tissue-specific damage.

PET was chosen as the polymer of interest due to their widespread use in beverage bottles, food packaging, textiles, and other consumer products. PET chemical composition contributes to its excellent mechanical strength, making it highly resistant to degradation.[11] It is also one of the most frequently detected polymers in the environment and biological samples.[12,13] Hence, PET is not only ubiquitous in the environment but also is directly linked to daily human consumption via PET disposable plastic bottles.[14,15] This study is PET-focused rather than using a cocktail of MPs.

The aim of this methodological paper is to describe the development of a semi-quantitative histological grading system for assessing MP-induced structural tissue damage in rat aorta, trachea, and bladder tissues. The grading system focuses specifically on damage to the endothelial lining of the aorta, the epithelial lining of the trachea, and the urothelial lining of the bladder.

MATERIALS AND METHODS

Ethical approval and animals

Ethical approval was obtained from the University of Nottingham's Animal Welfare and Ethics Review Body (Reference:310). Male Sprague-Dawley rats weighing 245–462 g and aged approximately 2–3 months were used in the experiment. Animals were purchased from University Kebangsaan Malaysia and euthanised by carbon dioxide (CO_2) asphyxiation on the day of the experiment. Following euthanasia, the animals were placed in an ice box and transported to the laboratory for dissection and tissue excision.

Tissue preparation and incubation

The aorta, trachea, and bladder were excised and carefully cleaned to remove surrounding connective tissues. The aorta was cut into 4 mm rings. The trachea was cut into 2 mm rings and placed in Krebs solution. The bladder was cut longitudinally into strips measuring 8 mm × 2 mm (length x width) and kept in Krebs solution until use. The tissue storage and incubation approach was adopted and modified from.[16]

A standard Krebs-Henseleit bicarbonate solution (referred to as Krebs solution) at pH 7.4 was freshly prepared on the day of the experiment with the following composition (mM): NaCl 120, KCl 5.4, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, D-glucose 11.7, and $CaCl_2$ 1.26. All salts used for the preparation of Krebs solution were purchased from Chemiz (Malaysia). After dissolving all salts in Type II purified water of Milli-Q quality (> 15 MΩ), the solution was aerated with 95% O_2 and 5% CO_2 for at least 15 minutes.

PET and MPs were generated by grinding pieces of disposable PET plastic drinking water bottles. The average length and width of the MP particles were 85.95 ± 13.66 µm and 21.30 ± 3.78 µm, respectively. PET MPs were weighed and added to separate glass scintillation vials to achieve final test concentrations of 0.3, 1, 3, and 5 mg/mL. The glass scintillation vials were agitated and vortexed to disperse the PET MPs as thoroughly as possible. Tissue segments or strips were then added into the respective vials containing the MP suspensions. One vial containing Krebs solution only served as the negative control. Vials were kept static (non-agitated) at 4°C, and tissues were incubated for 22–24 hours. The low temperature was used to help preserve tissue viability during the incubation period.

Tissue fixation and histological processing

Following incubation, tissues were transferred into neutral buffered formalin for preservation. Tissue processing and haematoxylin and eosin (H&E) staining were performed by the Faculty

of Veterinary Science, University Putra Malaysia following the protocol described by by Pauzi *et al.*[17] Trachea and bladder were stained with H&E to assess general tissue morphology, epithelial integrity, and urothelial structure.

For the aorta, H&E staining alone did not provide sufficient contrast for reliable scoring of endothelial damage. Therefore, immunohistochemistry (IHC) was performed by Qube Scientific Sdn. Bhd. Aortic sections were stained using an anti-CD31 antibody (Anti-CD31 antibody, monoclonal clone EPR17259, Abcam; primary antibody dilution 1:1000) and visualised with a ready-to-use biotinylated goat anti-polyvalent secondary antibody (Abcam), followed by 3,3'-Diaminobenzidine (DAB) chromogen. Primary and secondary antibodies were each incubated for 30 minutes, DAB chromogen was applied for three minutes, and sections were counterstained with haematoxylin. CD31 staining was selected because the grading system focused specifically on endothelial integrity along the luminal surface.

In brief, the aorta was initially stained with H&E. As the endothelial layer was not visually distinct enough for confident scoring, subsequent CD31 IHC was employed to enable more accurate assessment of endothelial integrity.

Image acquisition, randomisation, and blinding

Digital images were captured from stained sections using light microscopy (Leica DM500). Trachea and bladder images were obtained from H&E-stained sections, while aorta images were obtained from CD31 IHC-stained sections. A total of three rats (n = 3) were used. Not all three tissue types were obtained from the same individual rat, but for each tissue type, one representative section per rat was selected for imaging. For each concentration group (control, 0.3, 1, 3, and 5 mg/mL), three biological replicates (three rats) were assessed. From each stained section, three sites were randomly selected for grading. Each concentration group, therefore, yielded nine scored images per tissue type (3 rats × 3 sites). Across all five concentration groups, a total of 45 images were assessed for each tissue type.

All image files were randomised and compiled into a single PowerPoint file. Three blinded observers were provided with the randomised image set and the corresponding scoring criteria. Observers were blinded to treatment groups, exposure conditions, and sample identity. Each observer scored the same image set independently according to the severity grading criteria.

Blinding and randomisation were incorporated to reduce expectation bias during histological interpretation. Similar blinded scoring approaches are commonly used in histological method evaluation studies to support reliability assessment and reduce observer-dependent interpretation bias.[18]

Development of scoring criteria

The scoring criteria were developed to assess the features most relevant to each tissue type. As PET MPs were incubated directly with excised tissues in an acute exposure setting, structural damage was expected to be limited primarily to the innermost lining rather than the underlying muscle layers. The grading system therefore targeted the aortic endothelium, tracheal epithelium, and bladder urothelium.

For the aorta, the grading system focused on endothelial integrity, assessed by the continuity of the brown CD31-positive endothelial lining along the luminal surface. For the trachea, three parameters were assessed: cilia loss, erosion, and thinning of the epithelial layer. For the bladder, three parameters were assessed: urothelial surface integrity, urothelial thickness/layering, and superficial umbrella cell integrity. The method is inspired by and modified from Howard *et al*[19] and Carter *et al*.[20]

Each parameter was scored from Grade 0 to Grade 4 (TABLE 1). Grade 0 indicated no observable damage, while Grade 4 indicated severe or near-complete disruption of the assessed feature.

TABLE 1: General severity scale. The general 0 to 4 grading structure

Grade	Severity	General definition
0	No damage observed	Normal tissue architecture with intact target structure
1	Minimal damage	Slight focal disruption of the investigated characteristic
2	Mild damage	Clear but limited focal disruption of the investigated characteristic
3	Moderate damage	Significant multifocal disruption with extensive loss of the investigated characteristic
4	Severe damage	Extensive or widespread disruption, potentially approaching total loss of the investigated characteristic

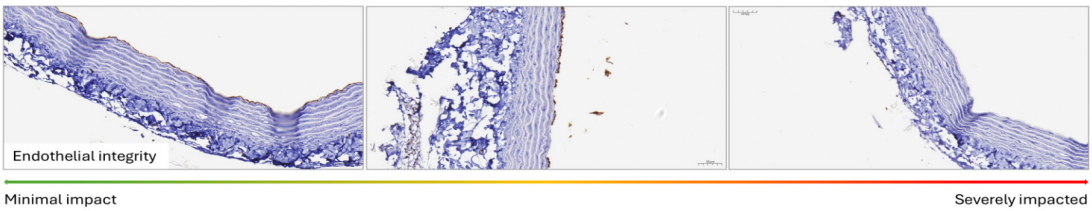

FIG. 1. Grading scheme used to grade endothelial damage of rat aorta tissue following polyethylene terephthalate (PET) microplastic (MP) exposure. From left to right, examples of images from least to most impacted, respectively. Image taken at 20× magnification. Scale bar = 50 µm.

Aorta grading criteria

Aorta damage was graded according to endothelial integrity. Brown CD31/DAB-positive staining along the luminal surface was used to identify preserved endothelial lining (FIG 1). The parameter definition for aortic endothelial integrity was listed in TABLE 2.

To illustrate the parameter, FIG 2 compares an aortic section graded as 0 with one graded as 4. Note the continuous brown endothelial lining in FIG 2A compared with the absence of endothelial staining in FIG 2B.

Trachea grading criteria

Tracheal damage was graded using three parameters, including cilia loss, erosion, and thinning of the epithelial layer. Each parameter was scored from Grade 0 to Grade 4 (TABLE 3). Cilia loss was defined as the degree of ciliated epithelial cell loss from the luminal surface. Erosion was defined as the focal damage and discontinuity of the tracheal mucosal epithelium. Thinning of the epithelial layer is defined as a reduction in mucosal thickness and loss of normal epithelial layering architecture (FIG 3).

A comparison between Grade 0 and Grade 3 tracheal tissue is shown in FIG 4. The comparison illustrates the distinction between intact ciliated cells, with normal mucosal thickness and the heavily eroded, thinned mucosa of a Grade 3 tissue.

Bladder grading criteria

Bladder damage was graded using three parameters, including urothelial surface erosion, urothelial thickness/layering, and superficial umbrella cell integrity (FIG 5). Each parameter was scored from Grade 0 to Grade 4 (TABLE 4). Urothelial surface integrity is assessed by the degree of epithelial continuity and erosive damage. Urothelial thickness and layering are assessed by the deviation from normal urothelial layer organization (typically ~2 to 3 cell layers) and thickness. Superficial umbrella cell integrity was assessed by the preservation and integrity of the specialised umbrella cell layer.

To facilitate comparison, FIG 6 shows a Grade 0 bladder tissue alongside a Grade 4 bladder tissue.

Data handling and analysis

For each image, individual observer scores were entered into a master spreadsheet. For trachea and bladder, scores for each parameter (e.g. cilia loss, erosion, epithelial thinning) were analysed separately, and one graph was generated per parameter for each tissue type. In addition, a composite "total damage score" was calculated

TABLE 2: Parameter definition for aortic endothelial integrity scoring

Grade	Parameter definition
0	A continuous, uninterrupted brown endothelial lining is present around essentially the full luminal circumference, with strong and even staining intensity.
1	The endothelial lining is mostly continuous, with only very small focal gaps or mild attenuation of brown staining in a negligible area.
2	Definite focal areas of endothelial loss or weak staining are present, but more than half of the luminal circumference still shows preserved endothelial staining.
3	The endothelial lining is incompletely preserved, with long interrupted segments and only scattered portions remaining DAB-positive.
4	Brown endothelial staining is absent from most or nearly all of the luminal surface.

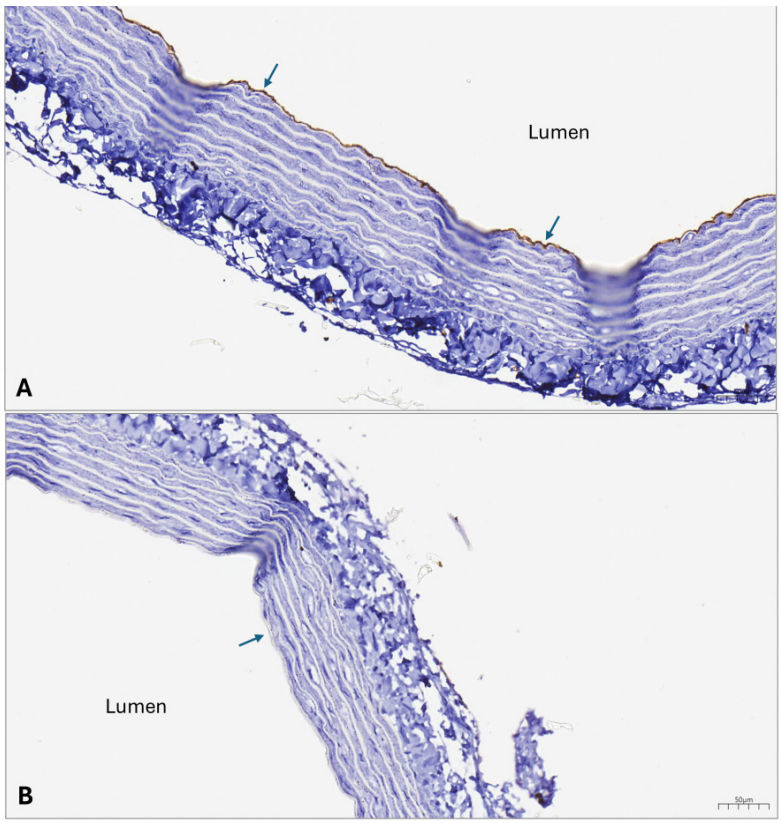

FIG. 2. Grade 0 anti-CD31-stained aortic tissue (A), compared to Grade 4 aortic tissue (B). Blue arrows in (A) indicate the intact endothelial lining, whereas the blue arrow in (B) indicates the absence of endothelial lining.

for each image by summing the mean scores of the three parameters within a tissue and was presented in a separate summary graph.

For the biological effect analyses, scores from the three reviewers were first averaged for each image to obtain a single mean score per image. These mean scores (n = 9 images from 3 rats per concentration) were then analysed by one-way ANOVA with PET concentrations as the factor, followed by Dunnett's multiple comparison test to compare each concentration group with the control. Effects were considered statistically significant when $p < 0.05$. GraphPad Prism v10 was used for all statistical analysis and graphical visualisation. Data are presented as mean ± standard error of mean (SEM).

TABLE 3: Parameter definition for trachea epithelium integrity scoring

Grade	Parameter definition
0	Normal tracheal mucosal structure with intact ciliated epithelium.
1	Slight disruption of the investigated characteristic, such as focal cilia loss or minimal erosion.
2	More extensive disruption at focal areas, such as patchy cilia loss or focal epithelial thinning.
3	Significant disruption with extensive loss of the investigated characteristic, such as widespread cilia loss or mucosal attenuation.
4	Extensive disruption and widespread loss of the characteristic, potentially including complete cilia loss or severely attenuated epithelium.

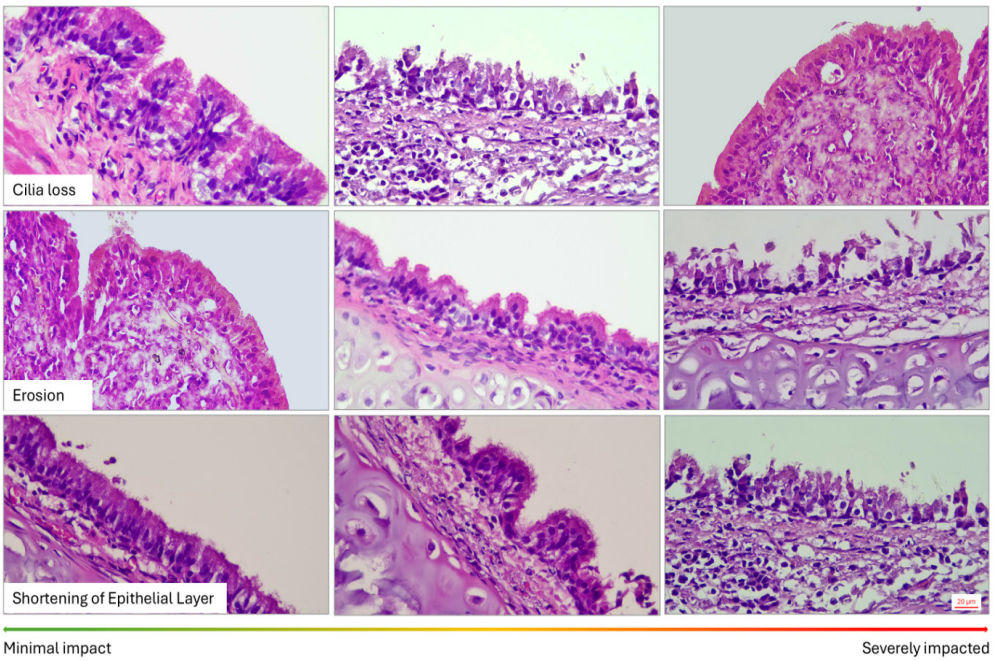

FIG. 3. Grading scheme used to assess epithelial damage in rat trachea tissue following polyethylene terephthalate (PET) microplastic (MP) exposure. From left to right, examples of images from least to most impacted, respectively. Images were taken at 40× magnification. Scale bar = 20 µm is applicable to all images.

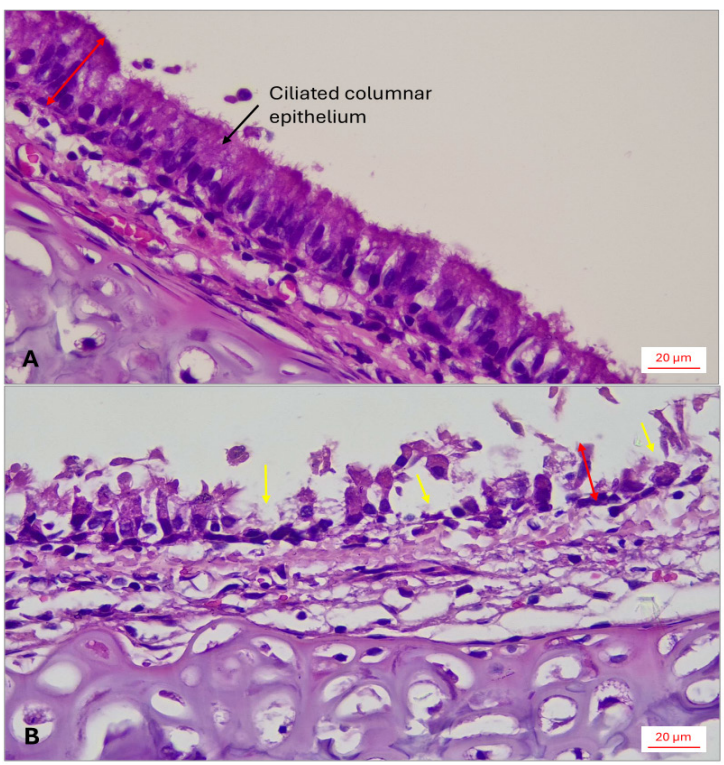

FIG. 4. Grade 0 trachea tissue (A) compared with Grade 3 trachea tissue (B). Red arrows indicate the epithelium thickness. Note the reduction in epithelium thickness in (B) compared with (A). Yellow arrows in (B) indicate eroded sites.

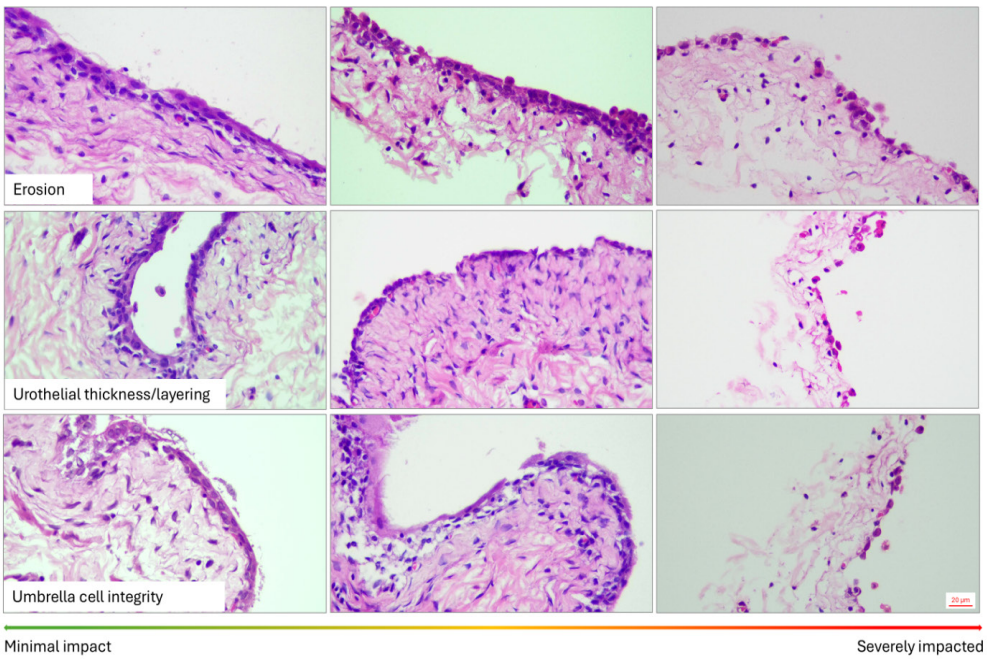

FIG. 5. Grading scheme used to grade urothelium damage of rat bladder tissue following polyethylene terephthalate (PET) microplastic (MP) exposure. From left to right, examples of images from least to most impacted, respectively. Image taken at 40x magnification. Scale bar = 20μm is applicable to all images.

Inter-observer reliability was assessed in a separate analysis to determine the consistency of the scoring method. For each tissue and scoring parameter, the raw scores from the three reviewers were analysed using a two-way ANOVA. A non-significant reviewer × concentration interaction was taken to indicate that any differences between the reviewers were consistent across concentrations. Tukey's multiple-comparison test was used to assess pairwise differences among reviewers.

RESULTS

Histopathological changes of aortic endothelium induced by polyethylene terephthalate (PET) microplastic (MP)

FIG 7 shows the comparison of damage severity between control and PET MPs of different concentrations using one-way ANOVA. Incubation of rat aortic rings with PET MPs significantly affected the endothelial integrity score ($p < 0.0005$). However, post hoc

TABLE 4: Parameter definition for bladder urothelium integrity scoring

Grade	Parameter definition
0	Intact urothelium with normal morphology and architecture, such as 2 – 3 well-organised cell layers, and the presence of large dome-shaped umbrella cells.
1	Slight focal disruption of the investigated characteristic, such as tiny erosive sites, minimal focal thinning or thickening cell layer, and focal loss or flattening of umbrella cells.
2	More extensive disruption at focal areas, such as small erosions, clear thickening or thinning in defined regions, and mild focal loss of umbrella cells.
3	Significant disruption with extensive loss of the investigated characteristic, such as multifocal erosions and loss of umbrella cell, and large segments of clearly thinned or thickened layering.
4	Extensive disruption and widespread loss of the characteristic, potentially including extensive denudation or severe degeneration.

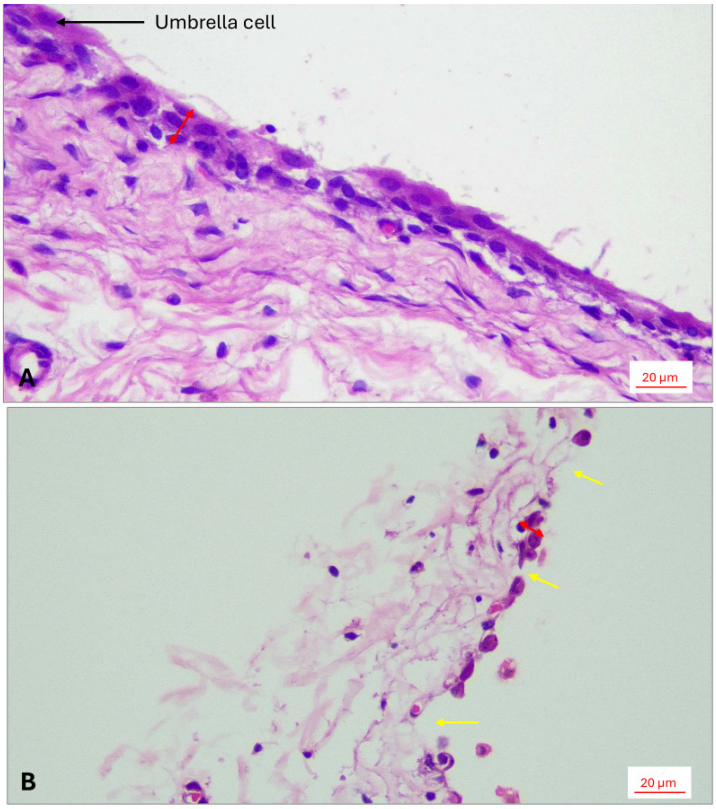

FIG. 6. Grade 0 bladder tissue (A) compared with Grade 4 bladder tissue (B). Red arrows indicate the thickness of the urothelium. Note the reduction in urothelium thickness and cell layer in (B) compared to (A). Yellow arrows in (B) indicate eroded sites. Superficial umbrella cells are evident in (A) but are not identifiable in (B) due to severe urothelial erosion.

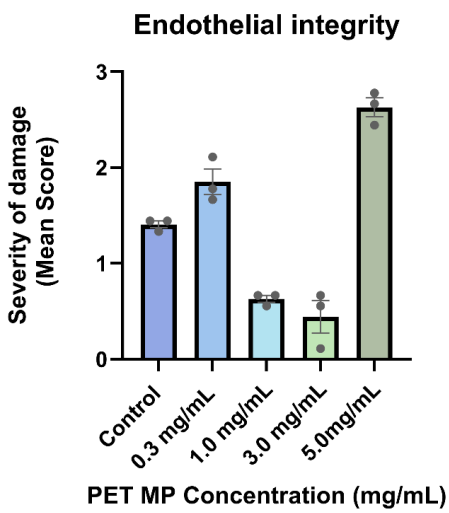

FIG. 7. Endothelial damage in rat aorta, characterised by endothelial scores after incubation with different polyethylene terephthalate (PET) microplastics (MPs) concentrations (0.3, 1.0, 3.0, and 5.0 mg/mL). Data are expressed as mean ± SEM of n = 9 tissue sections per group. Individual points represent the mean score of each reviewer at each concentration. Statistical comparisons were performed on the mean scores of each image averaged across the three reviewers.

comparisons did not identify any individual concentration that differed significantly from the control group (Tukey's test, all $p > 0.12$). Overall, these data indicate a trend towards altered endothelial integrity across PET MP concentrations, but no single PET concentration produced a statistically distinct difference in endothelial damage relative to control.

Histopathological changes of tracheal epithelium induced by polyethylene terephthalate (PET) microplastics (MPs)

In tracheal tissue, PET exposure had a parameter-specific effect on epithelial damage (FIG 8). For cilia loss, there was a significant overall effect of concentration analysed using one-way ANOVA ($p = 0.0008$). Dunnett's post hoc test showed that 3.0 mg/mL PET MP significantly increased cilia loss compared with control ($p = 0.0014$), whereas there were no significant differences in other PET MP concentrations. In contrast, no significant concentration-dependent effects were detected for epithelial erosion ($p = 0.077$), and epithelial thinning ($p = 0.29$). A composite total damage score was also included by summing the three parameters to provide an integrated measure of endothelial injury. Dunnett's test showing a statistically significant increase of endothelial damage at 0.3 mg/mL compared to control ($p = 0.0493$).

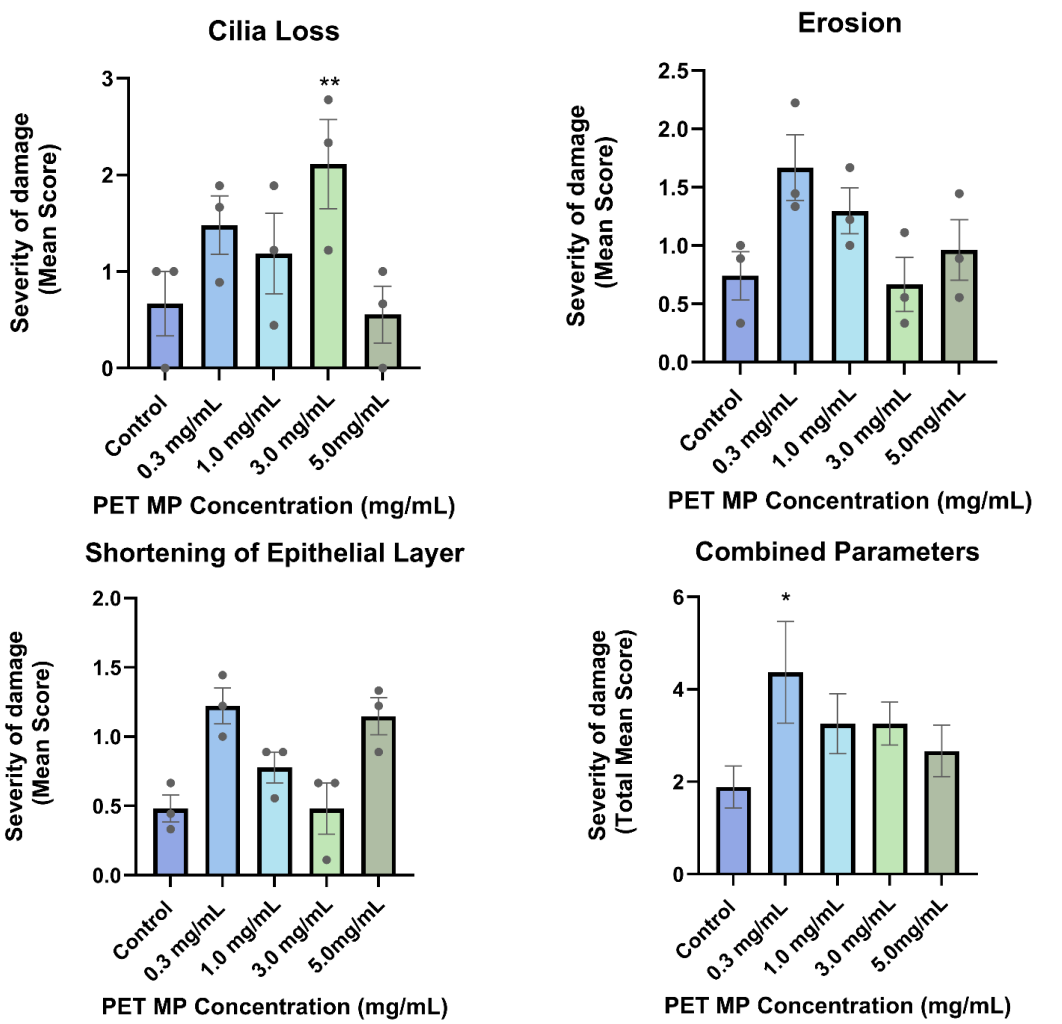

FIG. 8. Epithelial damage in rat trachea, characterised by cilia loss, erosion, and shortening of epithelial layer after incubation with different polyethylene terephthalate (PET) microplastics (MPs) concentrations (0.3, 1.0, 3.0, and 5.0 mg/mL). A composite total damage score is shown at bottom right panel. Data are expressed as mean ± SEM of n = 9 tissue sections per group. Individual points represent the mean score of each reviewer at each concentration. Statistical comparisons were performed on the mean scores of each image averaged across the three reviewers (one-way ANOVA with Dunnett's post hoc test; *p < 0.05, **p < 0.01 versus control).

Histopathology changes of on bladder urothelium induced by PET MPs

FIG 9 shows the comparison of damage severity of bladder urothelium between control and PET MPs of different concentrations using one-way ANOVA. PET MP exposure causes a clear, concentration-dependent increase in bladder urothelial damage across all three parameters. For urothelium erosion, all PET MPs concentrations showed significantly higher scores than control (Dunnett's test: 0.3 mg/mL, $p < 0.0001$; 1.0 mg/mL, $p < 0.0001$; 3.0 mg/mL, $p = 0.0006$; 5.0 mg/mL, $p = 0.0121$). Urothelial thickness/layering was similarly affected, with all PET MPs concentration significantly differed than control (0.3 mg/mL, $p < 0.0001$; 1.0 mg/mL, $p < 0.0001$; 3.0 mg/mL, $p = 0.0034$; 5.0 mg/mL, $p = 0.0105$). Superficial umbrella cell integrity also showed a significant difference compared to control (0.3 mg/mL, $p < 0.0001$; 1.0 mg/mL, $p < 0.0001$; 3.0 mg/mL, $p = 0.0009$; 5.0 mg/mL, $p = 0.0012$). The composite total damage score that combined all three parameters showed an increased urothelial damage compared to control (0.3 mg/mL, $p < 0.0001$; 1.0 mg/mL, $p < 0.0001$; 3.0 mg/mL, $p = 0.0004$; 5.0 mg/mL, $p = 0.0021$), as shown by the asterisk in FIG 9.

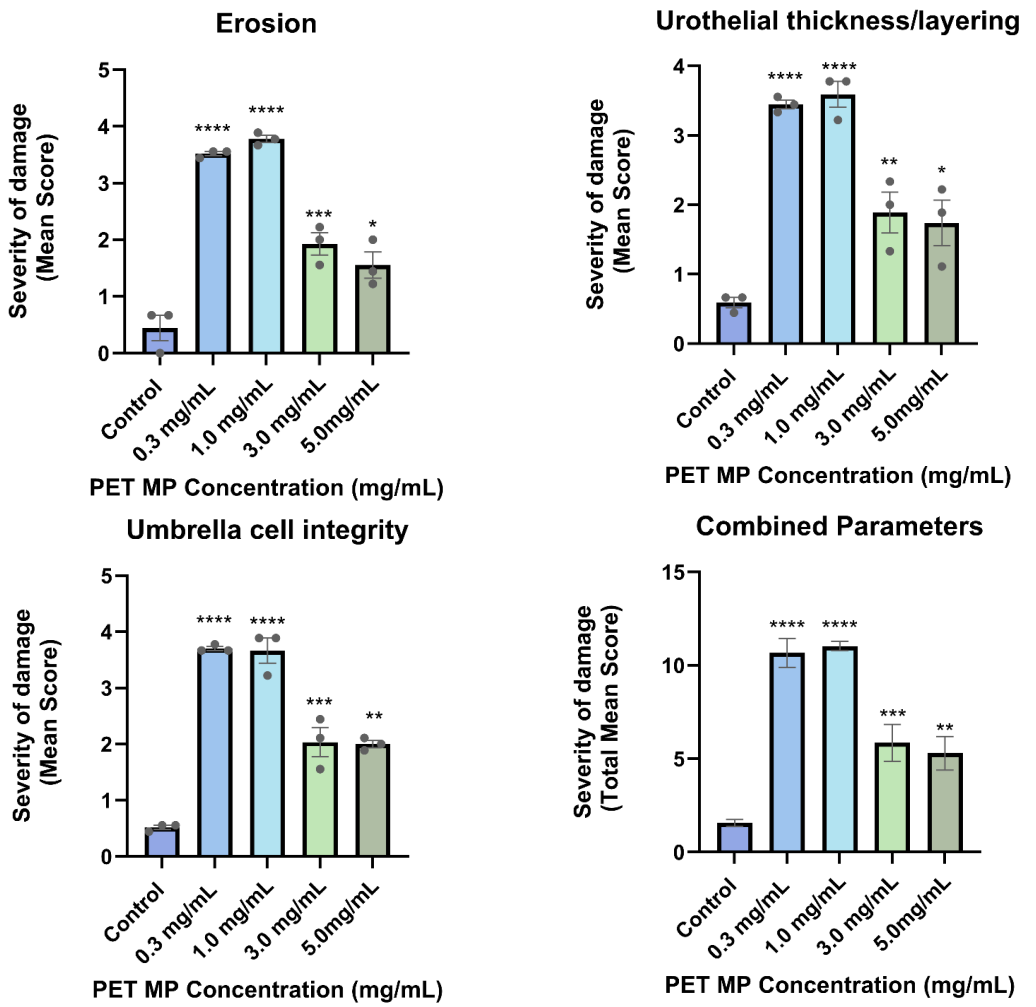

FIG. 9. Urothelial damage in rat bladder, characterised by erosion, urothelial thickness/layering, and umbrella cell integrity after incubation with different polyethylene terephthalate (PET) microplastics (MPs) concentrations (0.3, 1.0, 3.0, and 5.0 mg/mL). A composite total damage score is shown in the bottom-right panel. Data are expressed as mean ± SEM of n = 9 tissue sections per group. Individual points represent the mean score of each reviewer at each concentration. Statistical comparisons were performed on the mean scores of each image averaged across the three reviewers (one-way ANOVA with Dunnett's post hoc test; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 versus control).

Inter-observer reliability

Inter-observer reliability of the scoring system was evaluated using a two-way ANOVA for each tissue and parameter (TABLE 5). The reviewer main effect tests whether the reviewers differ in their average scoring when scores are averaged across all PET MP concentrations. In contrast, the reviewer × concentration interaction tests whether the magnitude of these differences between the reviewers varies with PET MPs concentration.

For aortic endothelial integrity, neither the reviewer main effect nor the reviewer × concentration interaction was significant. This indicates a high level of agreement between the three observers for this parameter.

For tracheal tissue, the degree of agreement varied between parameters. For cilia loss, there was a significant main effect of the reviewer but no reviewer × concentration interaction. This indicates that one reviewer tends to score consistently higher or lower than the others. However, all reviewers showed a similar scoring pattern across different MP concentrations. A similar pattern was observed for epithelial erosion. In contrast, shortening of the epithelial layer showed neither a significant reviewer main effect nor interaction, suggesting good agreement among observers for this parameter.

In bladder tissue, inter-observer agreement was generally high for all three urothelial parameters. For erosion, urothelial thickness/layering, and umbrella cell integrity, there were no significant reviewer main effects and reviewer × concentration interactions. This indicates that observers provide similar scores across all concentrations for these parameters.

DISCUSSION

Development of a histological grading system

This study shows that a semi-quantitative histological scoring system can be applied to multiple rat tissues to characterise the structural damage induced by PET MP *ex vivo*. The grading framework is built around 0 – 4 severity score, with definitions tailored to the specific luminal interface of each tissue, specifically the endothelial integrity for aorta, epithelial integrity for trachea, and urothelial integrity for bladder. In addition, the method separated the scoring into distinct parameters for each tissue type, and the criteria were applied in a blinded fashion. This approach aligns with the key principle for valid histopathologic scoring, including clear, operational definitions, semi-quantitative ordinal scales, and minimisation of observer bias.[21]

An important feature of the approach is that it supports both analysis at the parameter-level and the analysis at the composite level via the "total damage" scores. The parameter-level scores allow mechanistic interpretation while composite scores provide a summary of overall tissue injury. This structure can also be observed in other scoring systems developed for joint cartilage and intervertebral disc degeneration, where multiple morphological domains are scored separately and combined into global

TABLE 5: Two-way ANOVA results for inter-observer reliability

Tissue	Parameter	Reviewer main effect (*p*-value)	Reviewer × concentration interaction (*p*-value)	Interpretation
Aorta	Endothelial integrity	0.9268	0.9663	Good agreement
Trachea	Cilia loss	<0.0001****	0.7377	Systematic reviewer offset, no pattern change
	Erosion	0.0005***	0.9526	Small reviewer offset, no pattern change
	Shortening of epithelial layer	0.4556	0.9149	Good agreement
Bladder	Erosion	0.4046	0.3794	Good agreement
	Urothelial thickness/layering	0.0615	0.2358	Good agreement
	Umbrella cell integrity	0.6498	0.5312	Good agreement

severity index.[22,23] Therefore, this study not only reports tissue-specific effects of PET MPs, but it also offers a reusable scoring framework for other *ex vivo* or *in vivo* MPs and toxicology studies.

Tissue-specific patterns of PET MPs-induced structural damage

The scoring results show clear differences in sensitivity and injury patterns among aorta, trachea, and bladder under the same acute *ex vivo* exposure conditions.

In aortic tissue, endothelial integrity scores showed a statistically significant overall effect of PET MP concentration, but no individual concentration differed significantly from control in post hoc comparisons. This combination suggests that although there is variability across the concentration groups, PET exposure at the experimental conditions does not consistently produce concentration-dependent damage of the endothelial lining detectable by the present method. CD31 is a widely used endothelial marker, and immunohistochemical staining for CD31 or related endothelial antigens is commonly employed to assess microvascular integrity and endothelial preservation.[24] Several reasons could account for this insignificant effect. The aortic endothelium in this acute *ex vivo* model may be relatively resilient. Besides that, any subtle changes may be obscured by variability in staining intensity, section quality, or limited particle-surface interaction during incubation.

This pattern contrasts with several *in vivo* and *in vitro* studies in which prolonged exposure to micro- or nanoplastics, often under physiological shear stress, caused clear endothelial injury. For example, chronic oral PET-MP exposure in rats led to endothelial glycocalyx degradation and structural impairment of aortic elastic fibres.[25] Polystyrene MPs have been shown to induce marked endothelial damage via NLRP3-mediated pyroptosis *in vivo* and in cultured endothelial cells.[26] Multi-omics analyses further indicate that MPs can disturb endothelial transcriptomic and metabolic profiles and promote vascular dysfunction.[27] From a conservative perspective, the data support the conclusion that, under the conditions tested, PET MPs do not cause extensive CD31-defined endothelial loss in rat aorta.

In trachea, the response to PET exposure differed. Cilia loss exhibited a clear concentration effect, with significantly higher scores at 3.0 mg/mL compared to control, while epithelial erosion and thinning did not show significant changes across concentrations. The composite tracheal total damage score was elevated at 0.3 mg/mL and did not differ significantly from control at higher concentrations. These findings suggest that ciliated cells are the most sensitive component of the tracheal mucosa to acute PET MP exposure. With this observation, the early injury may be localised predominantly to the apical cilia, while the deeper epithelial architecture remains relatively preserved over the short incubation period.

Similar patterns, in which ciliary damage was observed in an earlier stage compared to deeper epithelial disruption, have been described in inhalation toxicology and airway irritation models, where loss or dysfunction ciliated cells is a recognised early hallmark of epithelial damage and mucociliary impairment.[28–30] Although MP-specific airway studies to date have focused mainly on inflammatory, barrier, and molecular endpoints in nasal or bronchial epithelial models, they also report cilia-related alterations and epithelial stress at exposure levels that do not immediately cause full epithelial loss.[31–33] The findings are compatible with an interpretation in which the ciliated surface acts as a sensitive early target for PET MPs in this *ex vivo* model, while more extensive damage to the epithelium may require longer, repeated, or *in vivo* exposure under physiological temperature, airflow, and immune conditions.

Bladder urothelium showed the most pronounced and consistent damage. All PET concentrations significantly increased the scores for urothelial surface erosion, abnormal thickening/layering, and loss of umbrella cell integrity. The composite bladder total damage score was also significantly elevated at every concentration. A stronger effect was observed at 0.3 and 1.0 mg/mL, whereas higher concentrations (3.0 and 5.0 mg/mL) showed smaller differences. These findings indicate that even the lowest tested concentrations are sufficient to disrupt the urothelial surface and barrier structure in this experimental model.

There is growing evidence that the urinary tract is a sensitive target for micro- and nanoplastic toxicity. For example, a scoping review reported the detection of MPs in a high proportion of human urine, kidney, and bladder cancer samples. The study also summarized *in vitro* data showing that urinary tract cell lines exposed

to MPs exhibit cytotoxicity, reduced viability, increased inflammatory signalling, and disrupted key pathways such as mitogen-activated protein kinases (MAPK) pathway.[34] Experimental study in kidney models also reports dose-dependent histological injury, including glomerular atrophy, tubular degeneration, and interstitial inflammation after polyethylene MPs exposure.[35] Although these studies focus on functional endpoints such as viability, cytokines, and oxidative stress rather than systemic urothelial histology, they converge with the present results to indicate that urothelial and renal epithelia and vulnerable to MPs-induced injury.

These results contrast with the more subtle tracheal changes and the absence of robust, concentration-specific endothelial damage in aorta under the same conditions. Therefore, the bladder mucosa may act as a particularly sensitive histological endpoint for MPs-related epithelial injury in this experimental setup.

Inter-observer reliability for the scoring system

Inter-observer reliability is a critical component of any semi-quantitative scoring system, and inadequate reliability has been highlighted as a common weakness in histological scoring studies.[21] In this study, reliability was assessed using two-way ANOVA with two factors (reviewer and concentration) for each tissue and parameter. This allows the separation of reviewer differences (main effect) from differences that depend on PET MP concentration (interaction).

For aortic endothelial integrity and all bladder parameters, neither the reviewer main effect nor the reviewer × concentration interaction was significant. This indicates good agreement between the three observers in both their overall scoring level (Grade 0 to Grade 4), and the relative differences between PET concentrations. To explain in simpler terms, different reviewers gave similar scores across all groups, and their ranking of concentrations in relation to structural damage was consistent.

In the tracheal tissue, the pattern was more complicated. For cilia loss and erosion, the reviewer main effect was significant, whereas the reviewer × concentration interaction was not. This combination indicates that one reviewer tended to assign higher or lower scores than the others across all concentrations. However, all three reviewers captured the same underlying concentration-response pattern. For shortening of the epithelial layer, both the reviewer main effect and interaction remain insignificant, indicating a strong agreement for this parameter. Modest baseline offset between observers are common in semi-quantitative histology, and similar issues have been reported in evaluations of cartilage and intestinal scoring systems, where overall reliability can be acceptable despite some systematic differences between the raters.[23,36]

The decision to average scores across the three reviewers at the image level before performing one-way ANOVA is supported by these reliability results and is in line with general recommendations for handling multiple raters in semi-quantitative scales.[21] By averaging the scores between three reviewers, the random scoring noise can be reduced, and the impact of systematic baseline differences can be mitigated. This is particularly important for the tracheal parameter where the reviewer main effect was detected. The lack of significant reviewer × concentration interactions across parameters is important as it indicates that the main conclusions about concentration effects are not driven by any single reviewer. Hence, presenting the individual reviewer means as individual points in the figures enhances transparency about inter-observer variability.

Limitations and future directions

There are several limitations in the present work. First, the experimental design reflects an acute *ex vivo* exposure of MPs at 4°C. These conditions are appropriate for preserving tissue structure but do not mimic *in vivo* physiology. In addition, the MP concentrations used in this study may not be reflective of the actual concentrations that would be present within tissue *in vivo*. At present, it is still challenging to define a specific tissue-level MP burden that reliably produces a given toxic effect *in vivo*. This uncertainty arises from several limitations as seen in current literature, including variability in analytical techniques, incomplete documentation of tissue-level MP concentrations, uncertainty in internal dose estimation, and poor comparability between experimental exposure conditions and realistic *in vivo* exposure scenarios.[37] The observed damage therefore should be interpreted as a surrogate observation of potential tissue structural response under controlled *ex vivo* conditions, rather than as a direct representation of physiological exposure levels or an exact *in vivo* toxicity threshold. Chronic *in vivo* studies and long-term organ culture models would be valuable to determine whether the same parameters remain sensitive

under more physiological conditions.

Second, although inter-observer reliability was generally good, the significant main reviewer effects for some tracheal parameters indicate that additional scorer training could further improve agreement. The inclusion of representative images for each grade is an important component for standardization, and future implementations could add structured training sessions or consensus scoring on a subset of images before formal data collection to further improve the inter-observer reliability. Another limitation is that this study used clean, single-polymer PET MPs, whereas environmental MPs are commonly present as complex mixtures of different polymer types, particle sizes, morphologies, and weathering states. Environmental MPs may also contain additives or absorb contaminants, which may alter biological responses and contribute to toxicity.[2] Therefore, future studies should consider testing mixed polymer types, weathered MPs, and MPs carrying environmentally relevant adsorbed contaminants to improve ecological and toxicological relevance.

CONCLUSION

The semi-quantitative scoring system was successfully applied across aorta, trachea, and bladder. In this model, the bladder showed the clearest and most consistent structural injury. The trachea showed more selective surface damage via cilia loss, and the aorta showed no concentration-specific endothelial denudation. These findings support the use of the framework for detecting tissue-specific patterns of injury while also reporting that the sensitivity to PET MPs is organ-dependent. Although the method is inherently observer-dependent and less precise than fully quantitative image analysis, it provides a practical and structured approach for screening histopathological effects in *ex vivo* exposure studies. The scoring framework should be readily adaptable to other polymer types, exposure durations, and experimental models. It can also serve as a useful basis for future integration with digital pathology or computational image analysis.

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Not applicable (this study used only nonidentifiable rat tissues from animals euthanised in separate, ethics-approved studies and involved no human participants or identifiable human data).

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Conceptualization: Kang Nee Ting, Man Yee Chin, Geok Chin Tan; Formal analysis: Man Yee Chin, Geok Chin Tan, Ungku Maryam Jamilah Ungku Mohsin; Writing: Man Yee Chin; review and editing: Kang Nee Ting, Man Yee Chin, Geok Chin Tan; Supervision: Kang Nee Ting, Kim Yeow Tshai, Sivathass Bannir Selvam; Final approval of the manuscript: Kang Nee Ting and Geok Chin Tan

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