Histopathological Assessment of Kidney Toxicity Induced by Polystyrene

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Abstract

The kidneys are essential organs that maintain homeostasis through complex physiological processes, including blood filtration, electrolyte balance, and blood pressure regulation. This study investigates the nephrotoxic effects of polystyrene microplastics (PS-MPs) by examining structural and functional changes in renal tissues after exposure in a controlled mouse model. Over a 28-day period, BALB/c mice were exposed to varying doses of PS-MPs, and several histopathological analyses, including Haematoxylin and Eosin (H&E), Periodic Acid-Schiff (PAS), and Picrosirius Red (PSR) staining, were conducted to assess kidney damage. The results indicated significant changes in renal architecture, including glomerular hypertrophy, tubular degeneration, and interstitial fibrosis, particularly at higher exposure levels. Additionally, there was a notable impact on organ weights and body compositions, suggesting a link between PS-MP exposure, oxidative stress, and metabolic alterations. The findings underscore the potential health risks posed by plastic pollution, especially regarding chronic exposure to microplastics, ultimately contributing to a growing concern about their effects on renal health and function. Further studies are warranted to elucidate the mechanisms underlying these nephrotoxic effects and to evaluate their implications for human health in the context of environmental exposure to microplastics.

Introduction

The Kidney is vital organ responsible for maintaining homeostasis within the human body through their complex physiological functions. Located bilaterally in the retroperitoneal space, these bean-shaped organs play crucial roles in filtering metabolic waste products from the blood, regulating electrolyte balance, controlling blood pressure, and contributing to red blood cell production via erythropoietin synthesis [1,2]. Structurally, each kidney consists of an outer renal cortex and inner renal medulla, housing approximately one million nephrons— the functional units responsible for filtration and urine production [3]. Each nephron comprises a renal corpuscle, which includes the glomerulus and Bowman's capsule, and a renal tubule extending through proximal convoluted tubules,

loops of Henle, distal convoluted tubules, and collecting ducts [4]. The glomerulus acts as a filtration unit where blood plasma is initially filtered, and subsequent re-absorption and secretion occur along the renal tubule to regulate the composition of urine [3,4]. This intricate structural arrangement allows the Kidneys to perform essential functions crucial for maintaining physiological balance and overall health. Given their critical roles, any disruption or damage to Kidney structure or function can lead to significant health implications, including renal disorders and diseases [5]. Understanding the fundamental anatomy and physiology of the Kidneys is essential for comprehending their susceptibility to various pathological conditions and the impact of nephrotoxic agents.

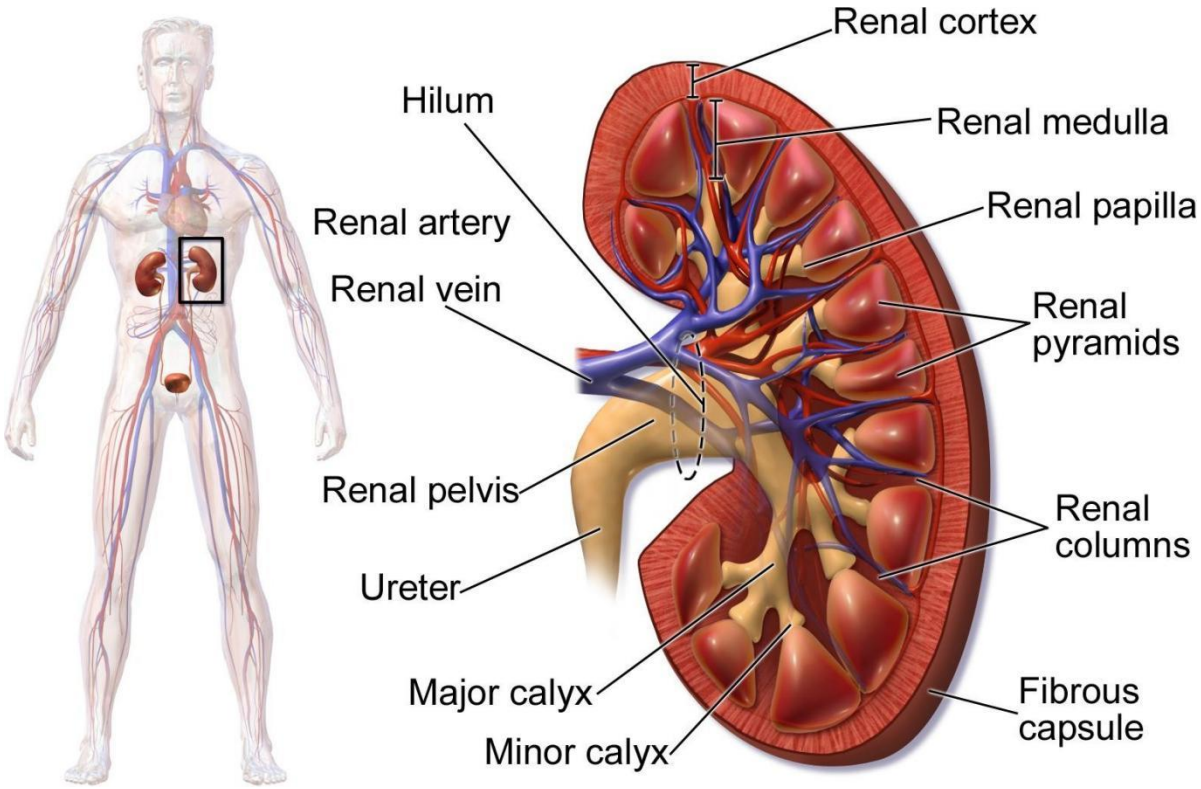

Figure: 1 Structural Components of the Kidney (https://en.wikipedia.org/wiki/)

The Kidney play a crucial role in maintaining overall health and homeostasis in the human body through several essential functions. These functions include:

1. Filtration of Blood: The primary function of the Kidneys is to filter waste products and excess substances, such as urea, creatinine, and electrolytes, from the blood to form urine [1].

2. Regulation of Fluid Balance: The Kidneys help regulate the body's fluid balance by adjusting urine concentration and volume in response to changes in hydration status and blood pressure [2].

3. Electrolyte Balance: They maintain the proper balance of electrolytes (sodium, potassium, chloride, etc.) in the blood and tissues, crucial for nerve function, muscle contraction, and overall cellular health [1].

4. Acid-Base Balance: The Kidneys maintain the body's acid-base balance by eliminating hydrogen ions and reabsorbing bicarbonate ions, keeping the pH within an optimal range.

5. Blood Pressure Regulation: Through the Renin-Angiotensin-Aldosterone System (RAAS), the Kidneys regulate blood pressure by adjusting blood volume and systemic vascular resistance [1].

6. Erythropoiesis: They generate erythropoietin, a hormone that promotes the formation of red blood cells in the bone marrow, which enhances oxygen transport within the body [2].

7. Detoxification: The Kidneys help eliminate various metabolic waste products, drugs, and toxins from the body, contributing to overall detoxification processes [1].

The importance of Kidney function in maintaining health is underscored by the severe consequences of Kidney dysfunction or failure, which can lead to fluid and electrolyte imbalances, hypertension, anaemia, bone disease, and accumulation of toxic waste products in the body [5]. Kidney toxicity, or nephrotoxicity, is a significant clinical and pathological concern that can result from exposure to various environmental toxins (such as radionuclides, plasticizers, heavy metals etc.), pharmaceuticals, and other harmful substances[6,7]. The Kidneys are essential for homeostasis, as they filter waste products, balance electrolytes, and regulate fluid levels in the body [2]. However, their high perfusion rate and complex functional architecture make them particularly susceptible to toxic insults.

Plastics are predominantly used in our daily lives due to their affordability, durability, lightness, and ability to be easily shaped. Global plastic production has surged dramatically, exceeding an annual output of 320 million tons. It is projected that by 2050, a staggering 33 billion tons of plastic will have been produced, underscoring the immense challenge of plastic pollution and the urgent need for solutions [8]. Only 9% of plastic waste is recycled, while 12% is incinerated [9, 10, 11].In 2019, the World Health Organization (WHO) called for global researchers to intensify their efforts in studying the impact of Microplastics on human health [12]. The kidneys play a crucial role in filtering and excreting toxins from the body. Styrene is metabolized in the liver to styrene oxide, a more reactive compound, which is then further metabolized and excreted through the Kidneys. The presence of these metabolites can potentially cause oxidative stress and damage to Kidney tissues [13]. Plastics are categorized based on their chemical compositions, polar properties, and intended uses. Key synthetic

polymer classes widely used in industry encompass Polyethylene (PE), Polypropylene (PP), Polystyrene (PS), Polyethylene Terephthalate (PET), and Polyvinyl Chloride (PVC).In the field of ecotoxicology, numerous studies have focused on various types of Microplastics (MPs), including PP, PES/PET, PVC, Polyamide, Acrylic, Polyether, Cellophane, and Polyurethane. Polyethylene (PE) and Polystyrene (PS) have specifically garnered attention in aquatic environments [14]. Polystyrene, a synthetic polymer used in various products, releases harmful styrene monomers during production and degradation. Polystyrene Microplastics, found in personal care and biomedical products, pose environmental and health risks by entering the food chain and potentially disrupting human endocrine systems.

Histopathology, the microscopic examination of tissue to study the manifestations of disease, is a critical tool in the assessment of Kidney toxicity [15]. Through histopathological analysis, structural and cellular changes in the Kidney tissue can be identified, providing valuable insights into the extent and nature of the toxic damage [16]. This method allows for the detection of early signs of toxicity, which may not be evident through biochemical markers alone, and can reveal specific pathological alterations such as tubular necrosis, glomerular damage, interstitial fibrosis, and inflammatory cell infiltration [5].

The assessment of Kidney toxicity through histopathology involves a systematic examination of Kidney tissue sections, typically stained with Haematoxylin and Eosin (H&E) to highlight cellular and structural details [17]. Other specialized stains like Periodic Acid Schiff staining, Picro-Sirius Red staining and Immunohistochemical techniques may be employed to identify specific cell types, proteins, and pathological processes [18].

Review of Literature:

Review of Kidney

The Kidneys are vital organs in the human body responsible for maintaining homeostasis by filtering blood, removing waste products, regulating electrolyte balance, and controlling blood pressure. Located in the retroperitoneal space of the abdominal cavity, each Kidney receives blood from the renal artery, filters it through nephrons—the functional units of the Kidney—and returns purified blood to circulation via the renal vein. This filtration process eliminates waste products like urea and maintains the body's fluid and electrolyte balance through mechanisms such as reabsorption and secretion.

Structure of the Kidneys

The Kidneys are bean-shaped organs, roughly 10-12 cm long, 5-7 cm wide, and 3 cm thick. They are located on either side of the spine, between the T12 and L3 vertebrae. Each Kidney is composed of several distinct regions and structures that collaborate to fulfil its various functions:

Renal Cortex: The outer region of the Kidney, the renal cortex, contains the renal corpuscles and convoluted tubules of the nephrons. It houses the glomeruli, which are networks of capillaries where blood filtration begins.

Renal Medulla: The renal medulla lies beneath the cortex and consists of renal pyramids, each containing nephrons and collecting ducts. These structures are crucial for concentrating urine and transporting it towards the renal pelvis.

Renal Pelvis: The renal pelvis is a funnel-shaped structure within the Kidney that collects urine from the collecting ducts and funnels it into the ureter, which leads to the urinary bladder.

Nephrons: Nephrons are the functional units of the Kidney, each consisting of a renal corpuscle (Glomerulus and Bowman's capsule) and a renal tubule (proximal convoluted tubule, loop of Henle, distal convoluted tubule, and collecting duct). These structures perform filtration, re-absorption, and secretion processes essential for maintaining fluid and electrolyte balance.

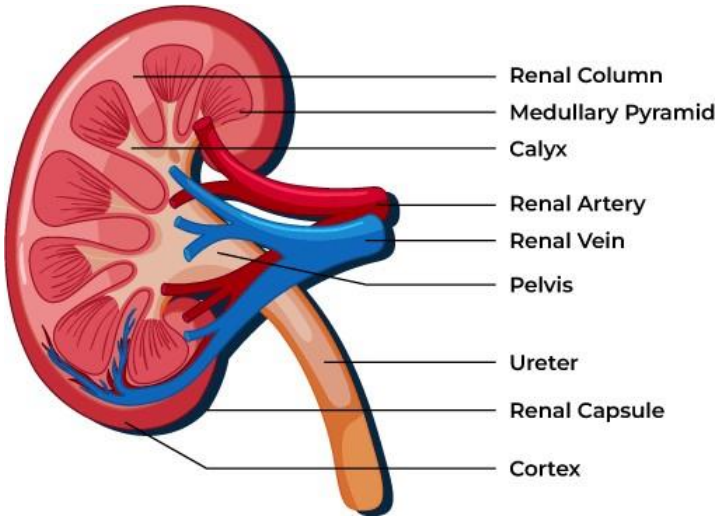

Figure: 2 Structure of the Kidneys (https://www.geeksforgeeks.org/)

Overview Polystyrene (PSMPs):

Polystyrene Microplastics (PSMPs) are frequently detected in various studies [22]. Polystyrene, a versatile material, is commonly found in both solid and foamed forms, created by linking together styrene monomers. These Microplastics, which are smaller than five millimetres in diameter, originate from the breakdown of larger Polystyrene items such as packaging and disposable goods. Polystyrene is known for its high resistance to light-induced degradation compared to other plastics, meaning it takes longer to break down when exposed to sunlight. Previous research has shown that PSMPs make up a significant portion (29.41%) of Microplastics found in surface sediments of urban water areas [20]. In these sediment samples, most Microplastics (58.31%) were smaller than 1 mm, with average concentrations. Additionally, research indicates that small polystyrene Microplastics (~2 µm) may pose biological risks due to their relatively higher bio-concentration factor. Consequently, further

studies are necessary to assess the potential toxicity of Polystyrene Microplastics to public health [21].

Figure: 3 Household Items Made from Polystyrene (https://collegedunia.com)

Polystyrene impacts on mice organs:

Studies investigating the impact of polystyrene Microplastics on mice organs have shown varied effects depending on the experimental conditions and exposure levels. Research typically focuses on assessing changes in organ morphology, function, and biochemical markers following exposure to different doses of Polystyrene Microplastics. Results indicate that medium doses of Polystyrene Microplastics can lead to alterations in organ weights, particularly affecting adipose tissue by reducing fat levels. However, findings regarding other vital organs such as the Kidneys, liver, and lungs have been inconsistent, with some studies reporting minimal to no significant changes.

The specific mechanisms by which Polystyrene Microplastics may affect organs are not fully elucidated but could involve pathways related to inflammation, oxidative stress, and disrupted cellular signalling. These impacts underscore the potential complexities and systemic responses within biological systems to Microplastic exposure. Further research is needed to comprehensively understand the long-term implications of Polystyrene

Microplastics on organ health, including potential cumulative effects and underlying mechanisms of toxicity.

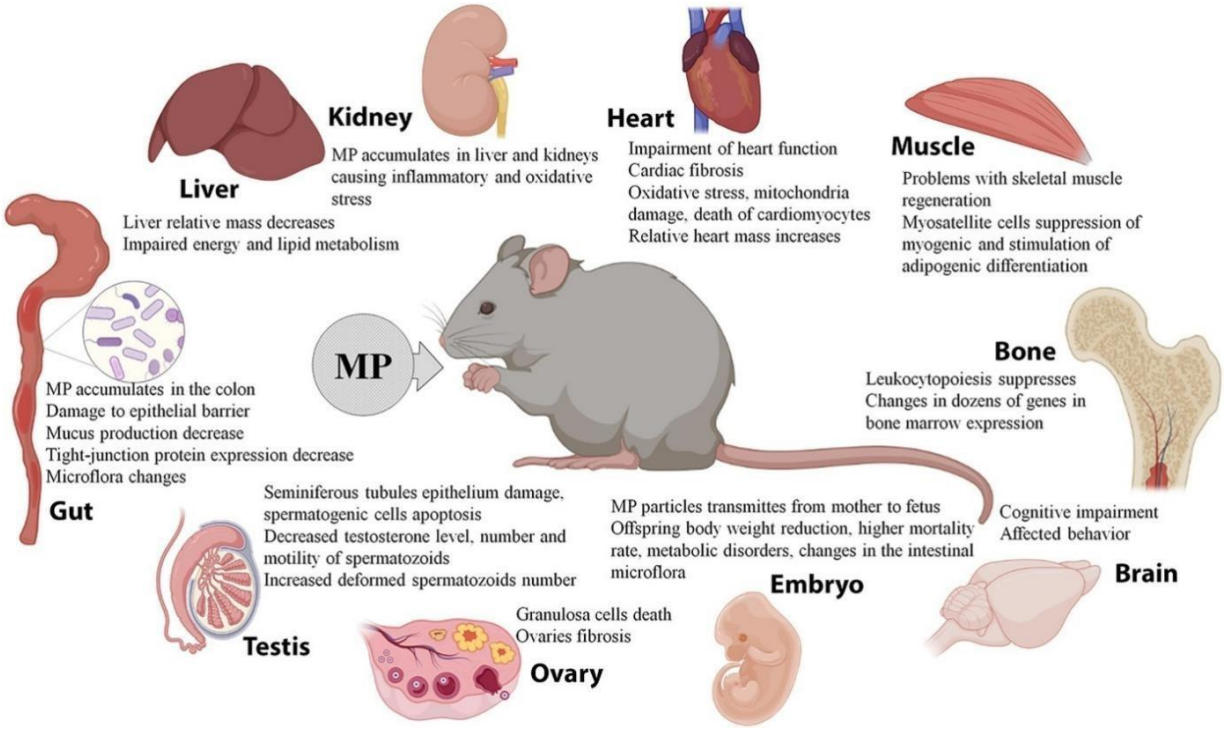

Figure:4 Microplastic Accumulation in Mice Organs:(https://peerj.com/articles/13503/)

Impact of Polystyrene on the Human Kidney:

Increasing concerns within the medical community about the potential impact of Microplastics (MPs) on human health have driven a significant rise in research investigating their effects on various human tissues, including the Kidneys. Microplastics are tiny plastic fragments that are smaller than 5 millimetres in size, resulting from the degradation of various plastic products due to environmental factors [12]. Researchers have identified varying levels of Microplastics in a range of food items, raising concerns about human exposure through ingestion. Studies indicate that Microplastics can infiltrate the food chain through plastic-packaged products (such as water bottles and processed foods), ingestion during seafood consumption, and pollution of groundwater [23]. It remains uncertain whether Polystyrene Microplastics (PS-MPs) are risk factors for Chronic Kidney Disease of unknown etiology (CKDu) [24]. Modelling the behaviour of polystyrene estimates human threshold

concentrations to range from 5.1 to 53.3 mg per gram of body weight [25]. Such exposure levels are unlikely to occur [26]. In this context, the harmful effects of Microplastics on human health are still debated and not well understood. Many important questions remain unanswered, such as whether Microplastics contribute to cancer in marine animals and potentially humans and what the long-term effects of human exposure to Microplastics are, considering the multiple routes of exposure [27]. Additionally, 1 μm Polystyrene Microplastic (PS-MP) particles have been detected in the environment and are relevant to human exposure. A deeper understanding of the causes of CKDu is crucial for developing effective health policies and public health responses. Previous studies have shown that Microplastics and Nano plastics accumulate in the gut, liver, and Kidneys of mice [28]. Researchers examined the impact of Microplastics on human kidney and liver cells using the Human Embryonic Kidney 293 (HEK 293) cell line and the human Hepatocellular carcinoma (Hep G2) cell line. Both cell lines have a history of use in toxicological studies[23]. We hypothesize that HEK293 cells can ingest PSMPs, leading to adverse Kidney effects such as cytotoxicity and renal barrier dysfunction[21]. However, the absorption of these Microplastics is expected to be minimal (≤0.3%). Only Microplastics smaller than or equal to 20 μm could enter organs, with the smallest fraction (0.1 > 10 μm) able to reach all organs, cross cell membranes, the blood-brain barrier, and the placenta. Microplastics might spread to other tissues such as the liver, muscle, and brain [20].

Routes of Exposure to Polystyrene Microplastics:

Microplastics, tiny plastic particles less than 5 millimetres in size, can enter the human body through various exposure routes. Understanding these routes is crucial to assessing the potential health risks associated with Microplastic exposure. The main routes of exposure include ingestion, inhalation, and dermal contact.

Ingestion: Microplastics enter the human body primarily through food and water. Marine organisms ingest Microplastics, which then enter the human food chain when these organisms are consumed as seafood [29]. Sea salt can also be contaminated with Microplastics. Processed foods may become contaminated during production, packaging, and storage. Additionally, fruits and vegetables can carry Microplastics due to contamination from soil, water, or agricultural practices [30]. In terms of water, Microplastics have been detected in both bottled and tap water [31], and other beverages such as beer, tea, and soft drinks are not immune to contamination.

Inhalation: Microplastics can be present in the air, especially in urban environments and indoor spaces. These airborne particles can be inhaled and later ingested through mucociliary clearance. Indoor activities, such as vacuuming and using synthetic textiles, can release Microplastics into the air, contributing to indoor air contamination [32].

Dermal Contact: Some cosmetics and personal care products contain Microplastics, such as micro beads, that can be absorbed through the skin [33]. Synthetic fabrics can shed Microplastic fibres that come into contact with the skin during wear and use. Additionally, household dust often contains Microplastics, which can settle on the skin and be absorbed, particularly when in contact with sweat or moisture.

Polystyrene Microplastics can infiltrate the human respiratory system through multiple pathways. Occupational exposure poses a substantial risk for workers in industries engaged in manufacturing, handling, or recycling Polystyrene products, where airborne Polystyrene particles are often prevalent. Environmental exposure is also worrisome, as Polystyrene Microplastics have been detected in urban air due to the breakdown of larger Polystyrene items and the wear of products containing Polystyrene.

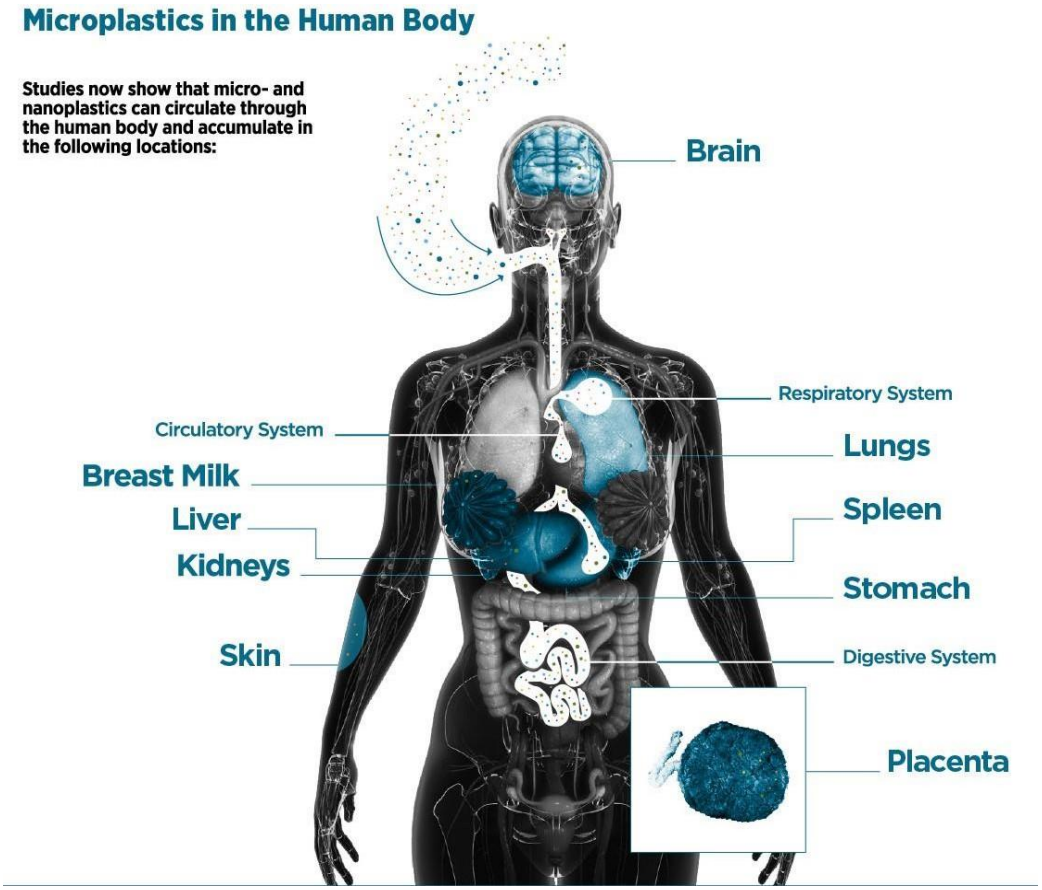

Figure: 5 Mechanisms of Microplastic Entry and Accumulation (www.ciel.org)

Material and Methodology

Animal Handling:

BALB/c mice were obtained from the GLP-certified animal house facility located at CSIR-Indian Institute of Toxicology Research in Lucknow, Uttar Pradesh, India. The experimental procedures conducted during this study were ethically approved by the Institute's ethical committee (IEAC reference no. IITR/IEAC/27/19). Upon their arrival, the mice underwent a quarantine period of one week before being housed in Polystyrene cages, ensuring access to food and water without restriction. These mice were kept in standard environmental conditions, maintaining a temperature of 23°C ± 2 and a relative humidity range of 30-70%. After the acclimatization phase of one week, the mice were weighed and then randomly divided into four groups, each containing 7 mice with nearly identical body weights. These groups were labeled Control, low, medium, and high dose of Polystyrene. Over a period of 28 days, the mice were exposed to Polystyrene and their body weights were recorded before the intervention and on the 1st, 8th, 15th, 22nd, and 28th days of exposure. The administration of Polystyrene was controlled by oral gavage through a cannula, ensuring accurate delivery into the stomach. Throughout the polystyrene exposure period, careful monitoring of food intake was conducted, and the food pellets were weighed regularly to ensure an equal amount of food intake across all four groups. Body weight measurements were taken weekly for all groups. At the culmination of the 28-day exposure period, the mice were anesthetized using ketamine- xylazine and subsequently sacrificed. Blood samples were collected and centrifuged to obtain serum samples. Kidney, liver, and fat tissues were preserved using a 10% formaldehyde solution. The remaining samples were flash-frozen in liquid nitrogen and stored at -80°C for subsequent analyses.

Histopathology

Haematoxylin & Eosin Staining (H&E):

Haematoxylin and Eosin (H&E) staining is a foundational technique in renal pathology, crucial for examining the complex structures and pathological changes within the kidney. This staining method uses Haematoxylin to selectively colour cell nuclei blue or purple, and Eosin to stain cytoplasm and extracellular matrix pink or red, providing detailed insights into renal morphology at a microscopic level.

The Kidney's intricate architecture, which includes nephrons, tubules, glomeruli, and interstitial tissue, requires meticulous histological examination to comprehend its function and detect abnormalities. H&E staining allows for the visualization of various renal components, such as the renal corpuscle within the glomerulus, tubular epithelial cells, and the interstitium, thereby facilitating the assessment of structural integrity and pathological changes.

In the field of renal pathology, H&E staining is indispensable for diagnosing a wide range of Kidney diseases, including glomerular disorders (e.g., glomerulonephritis), tubulointerstitial diseases (such as acute tubular necrosis), and renal tumours. Its capability to distinguish between normal and abnormal tissue morphology helps characterize disease processes, guiding treatment strategies and prognostic evaluations.

Procedure:

The staining procedure begins by immersing the slides in xylene 1 for 10 minutes, followed by another 10-minute immersion in xylene 2. Afterward, the slides are placed in a mixture of xylene and alcohol for 5 minutes. They then undergo a series of alcohol immersions: 100% ethyl alcohol for 5 minutes, 90% ethyl alcohol for 5 minutes, and three consecutive immersions in 70% ethyl alcohol, each for 5 minutes. Following the alcohol series, the slides are rinsed in distilled water for 2 minutes. Next, Haematoxylin staining is carried out for 1.5 minutes, followed by washing in tap water for 8-10 minutes. A brief dip in 90% ethanol for 10-15 seconds prepares the slides for Eosin staining, which lasts 30-45 seconds. Subsequently, the slides are sequentially immersed in 50% ethyl alcohol for 2 minutes, 70% ethyl alcohol for 2 minutes, 90% ethyl alcohol for 2 minutes, and finally 100% ethyl alcohol for 2 minutes.

To complete the process, the slides are placed in a xylene and alcohol mixture for 5 minutes, followed by two additional immersions in xylene, each lasting 10 minutes. The final step involves mounting the slides with DPX mounting media. Once mounted, examine the slides under a microscope.

Periodic Acid Schiff (PAS) Staining:

Periodic Acid-Schiff (PAS) staining is a crucial histological technique extensively used in renal pathology to examine the microscopic structure and pathological alterations within the kidney. This staining method, named after its components—Periodic Acid and Schiff's

reagent—is renowned for its ability to selectively stain polysaccharides, glycoproteins, and mucosubstances found in various renal structures.

The Kidney's complex architecture, comprising glomeruli, tubules, interstitium, and blood vessels, requires meticulous histopathological examination to comprehend its function and identify abnormalities. PAS staining plays a pivotal role in visualizing specific renal components such as the glomerular basement membrane, mesangial matrix, and tubular basement membranes. It provides critical insights into structural integrity, glycogen storage, basement membrane changes, and the presence of pathological deposits, thereby aiding in the diagnosis and classification of renal diseases. In renal pathology, PAS staining is essential for diagnosing a range of conditions, including glomerulopathies (like membranous nephropathy and diabetic nephropathy), tubulointerstitial diseases, and specific renal tumours. Its ability to highlight pathological changes and distinguish them from normal tissue morphology is crucial for accurate disease characterization, guiding treatment strategies and prognostic evaluations.

Procedure:

The staining process begins with immersing the slides in xylene 1 for 5 minutes, followed by xylene 2 for another 5 minutes, and then in xylene ethanol for 3 minutes. Next, the slides are sequentially dipped in 100% ethyl alcohol for 3 minutes, 90% ethyl alcohol for 3 minutes, and 70% ethyl alcohol for 3 minutes, followed by a 3-minute rinse in distilled water.

Afterwards, the slides are treated with periodic acid for 8 minutes, followed by rinsing in distilled water once or twice. Schiff reagent is applied for 15 minutes, followed by a rinse in lukewarm distilled water for 6 minutes. Haematoxylin staining is performed for 1 minute, followed by washing in tap water for 2 to 3 dips.

Subsequently, the slides are sequentially immersed in 70% ethyl alcohol and 90% ethyl alcohol for 2 minutes each, and finally in 100% ethyl alcohol for 2 minutes. Clear the slides in xylene for 2 minutes, followed by a second xylene treatment for another 2 minutes.

The staining process is completed by mounting the slides using DPX mounting media and covering them with a coverslip before examination under a microscope.

Picrosirius Red Stain (PSR):

Picrosirius Red Stain (PSR) is a specialized histological technique used extensively in renal pathology to examine and characterize collagen fibres within Kidney tissues. This staining method, named after its constituent dye Picrosirius Red, offers unique advantages in visualizing and quantifying collagen types based on their birefringence properties under polarized light microscopy.

The Kidney's structural integrity relies significantly on collagen, which forms the framework supporting various renal components such as glomeruli, tubules, and interstitial spaces. PSR staining highlights collagen fibres, providing insights into their distribution, organization, and pathological changes. The method distinguishes between collagen types, particularly type I (thick, mature fibres) and type III (thin, immature fibres), crucial for understanding fibrotic processes in renal diseases.

In renal pathology, PSR staining plays a critical role in assessing fibrosis, a common feature in Chronic Kidney Diseases. It helps quantify collagen deposition and evaluate fibrotic changes associated with conditions like diabetic nephropathy, glomerulonephritis, and renal fibrosis. The ability to visualize collagen alterations aids in disease diagnosis, progression monitoring, and therapeutic planning.

Procedure:

The staining protocol using Picrosirius Red (PSR) involves several sequential steps to prepare and stain Kidney tissue sections for collagen visualization. Initially, the tissue is deparaffinized with xylene for 3 minutes followed by a second xylene treatment of the same duration to remove paraffin wax. This is followed by dehydration in a descending series of ethanol concentrations: 100% ethyl alcohol for 2 minutes, 90% ethyl alcohol for 2 minutes, and 70% ethyl alcohol for 2 minutes, ensuring the tissue is adequately prepared. Subsequently, the sections are briefly rinsed in distilled water for 2 minutes to further facilitate staining penetration. The tissue is then immersed in Picrosirius Red solution (0.1% Sirius Red F3B in saturated aqueous picric acid) for 1 hour, allowing the dye to selectively bind to collagen fibres. After staining, the slides undergo thorough washing in distilled water three times (10 dips each) to remove excess dye. The sections are dehydrated again in ascending ethanol concentrations: 70% ethyl alcohol for 2 minutes, 90% ethyl alcohol for 2 minutes, and 100% ethyl alcohol for 2 minutes. Clearing is achieved with two 3-minute treatments in xylene to remove the alcohol and prepare the tissue for mounting. Finally, the tissue sections are mounted using DPX mounting media and examined under a microscope to

observe and analyse the stained collagen fibres, providing insights into the fibrotic changes and structural integrity of Kidney tissues.

Others technique

Immunoblotting

Protein Extractions Protocol:

1. Place cell-containing flask on ice for 5 minutes, then wash cells with PBS.
2. Add protein lysis buffer, let it interact for 10-15 minutes.
3. Detach cells using a scraper, collect into a tube, and keep on ice for 20-30 minutes.
4. Centrifuge at 12000rpm for 20 minutes at 4°C.
5. Transfer the supernatant containing proteins to an Eppendorf tube.
6. Measure total protein content: mix 5 µL of each standard and sample with BCA reagent (1:8 ratio) in a 96-well microplate.
7. Shake plate for 30 seconds, incubate at 37°C for 30 minutes.
8. Measure absorbance at 562 nm using a Varioskan Plate Reader.
9. Calculate protein concentration using the standard curve and graph data with a linear equation.

Protein Estimation Protocol:

The Bicinchoninic acid (BCA) assay, or Smith assay, quantifies total protein content using a colorimetric method with copper. In an alkaline environment, Cu^{2+} ions react with proteins, reducing to Cu^{+}. Each Cu^{+} ion binds two BCA molecules, causing a colour change to purple, absorbable at 562 nm. This complex formation depends on peptide bonds and amino acids like cysteine, cystine, tyrosine, and tryptophan. Performing the assay at 60°C enhances exposure of these amino acids, minimizing variations in protein samples. Standard and unknown samples (5 µL each) were mixed with BCA reagent (1:8 ratio) in triplicate in a 96-

well plate, shaken, and incubated at 37°C for 30 minutes. Absorbance at 562 nm was measured using a Varioskan Plate Reader to calculate protein concentration via a standard curve.

Western Blot:

Western blotting, or immunoblotting, detects and analyses specific proteins based on molecular weight. It includes three steps: size separation, transfer to a solid support, and antibody-based protein detection for visualization.

Principle of Western Blotting:

Western blotting identifies specific proteins by ensuring equal loading, separating by molecular weight, transferring to a membrane, and probing with labelled antibodies. This method is highly specific, using antibodies that bind exclusively to the desired protein, and is also known as immunoblotting.

Protocols of buffers:

For preparing a 10% SDS solution, dissolve 10g of SDS in 90ml of distilled water.

The Transfer Buffer consists of 14.4g of glycine, 3.03g of Tris-base, an appropriate amount of SDS, and sufficient distilled water to make up 1000ml.

To make 1X PBST solution, mix 100ml of 10X PBS with 900ml of distilled water, and add 1ml of Tween-20.

The Running Buffer is composed of 14.4g of glycine, 3.03g of Tris-base, 200ml of methanol, and 800ml of distilled water.

Resolving Gel Composition:

1. **7% Gel:** 4.35ml water, 2.02ml acrylamide, 2.18ml Tris-base (pH 8.8), 100ul SDS, 100ul APS, 15ul TEMED.
2. **8% Gel:** 4.06ml water, 2.32ml acrylamide, 2.18ml Tris-base (pH 8.8), 100ul SDS, 100ul APS, 15ul TEMED.

3. **10% Gel:** 3.75ml water, 3ml acrylamide, 2.25ml Tris-base (pH 8.8), 100ul SDS, 100ul APS, 15ul TEMED.

Stacking Gel Composition:

1. **5% Gel:** 3.4ml water, 830ul acrylamide, 630ul Tris-base (pH 6.8), 50ul SDS, 80ul APS, 20ul TEMED.
2. **4% Gel:** 3.07ml water, 670ul acrylamide, 630ul Tris-base (pH 6.8), 50ul SDS, 80ul APS, 20ul TEMED.

These compositions vary in acrylamide concentration and include components like Tris-base, SDS, APS, and TEMED, essential for gel electrophoresis in protein analysis.

Buffer and Sample Preparation:

Tris-base [1M] (pH 8.8 and pH 6.8):

- Tris-base (1M): 12.11g dissolved in 180ml water, adjusted with HCl to pH 8.8 or pH 6.8, and made up to 200ml.

10% APS (Ammonium Persulfate):

- APS: 1g dissolved in 10ml distilled water.

RIPA Buffer:

- Components include 1M Tris-HCl (pH 7.4), 0.25M EGTA, 1M NaCl, 0.5M NaF, 100mM Sodium orthovanadate, 10% IGEPAL/NP-40, and 10X Protease Inhibitor Cocktail (PIC) in Milli-Q water.

Sample Preparation:

- Mix 100µl protein supernatant with 16.6 or 20µl loading dye (1X Bromophenol Blue).
- Heat in a new tube at 95°C for 15 minutes, then store at -20°C.

10X PBS Composition:

Dissolve Na2HPO4 (44.4g), KH2PO4 (42.4g), NaCl (80.1g), and KCl (2g) in 1000ml of distilled water.

Polyacrylamide Gel Electrophoresis (SDS-PAGE) Overview:

In SDS-PAGE, proteins are loaded into gel wells with molecular weight markers. The gel matrix, made of polyacrylamide, allows proteins to migrate under an electric field. Starting at low voltage (around 60V for 30 minutes), proteins uniformly enter the gel. Voltage is then increased (typically to 120V) to separate proteins based on molecular weight. SDS denatures proteins and imparts a negative charge, causing them to migrate towards the positive pole. Smaller proteins move faster through the gel pores, while larger ones move more slowly. SDS-PAGE primarily separates proteins by molecular weight, with other factors like charge and isoelectric point also influencing migration. Bands of separated proteins are visualized and analysed for purity, quantification, or identification purposes.

Protein Transfer (Blotting):

In Western blotting, choosing between nitrocellulose and PVDF membranes depends on experimental needs. PVDF membranes offer durability and high protein absorption, suitable for applications like Coomassie Brilliant Blue staining and detection with substrates for alkaline phosphatase. Before use, PVDF membranes are activated by methanol. During transfer, the PVDF membrane is aligned carefully atop the gel in a sandwich with filter papers, ensuring no air bubbles. The setup is placed in a transfer tank with ice to maintain cold temperatures, crucial for protein integrity. Transfer occurs at 300mA for approximately 1 hour and 30 minutes in a cold room to prevent protein denaturation.

Blocking in Western Blotting:

After transferring proteins to the membrane in Western blotting, excess Ponceau stain is rinsed off with water. The membrane is then washed with PBST to remove residual stain and prepare for blocking. Blocking is crucial to prevent non-specific antibody binding, which can cause background noise and interfere with target protein detection. Common blocking agents like 3% BSA or skim milk create a barrier on the membrane surface, reducing non-specific interactions during antibody probing. This step typically lasts about 2 hours at room temperature, ensuring thorough coverage of the membrane. While blocking reduces non-

specific binding, it doesn't eliminate cross-reactivity entirely, where antibodies may bind to non-target proteins with similar epitopes. However, effective blocking significantly minimizes cross-reactivity, enhancing the specificity and reliability of Western blot results. Both 3% BSA in PBS and 3% skim milk in PBS are widely used for blocking, with skim milk often preferred for its superior blocking capacity.

Primary Antibody Incubation in Western Blotting:

Following blocking in Western blotting, the membrane undergoes three PBST rinses to remove residual blocking solution. Each rinse lasts 10 minutes to ensure thorough cleaning. The primary antibody, specific to the target protein, is diluted per manufacturer's instructions in PBST or PBS to optimize binding affinity and minimize non-specific interactions. The membrane is then incubated with the primary antibody solution for either 4 hours at room temperature or overnight at 4°C, with gentle agitation to enhance binding. Proper adherence to antibody dilution and incubation conditions is critical for achieving reliable Western blot results, tailored to experimental needs and ensuring sensitivity and specificity.

Secondary Antibody Incubation in Western Blotting:

After washing with PBST to remove excess primary antibody, the membrane is incubated with a secondary antibody conjugated to an enzyme like horseradish peroxidase (HRP). This enzyme-conjugated antibody specifically binds to the primary antibody-antigen complex on the membrane surface, enhancing signal amplification and sensitivity. Incubation typically lasts around 3 hours at room temperature, allowing the secondary antibody to bind and form a sandwich structure, facilitating precise detection of the target protein. Optimization of secondary antibody dilution and incubation conditions is critical for reliable and sensitive detection in Western blotting experiments.

Visualization in Western Blotting:

After washing with PBST to remove unbound secondary antibody, a substrate is applied that reacts with the enzyme-conjugated secondary antibody. For chemiluminescent detection, this produces light captured by an imaging system, reflecting the target protein's presence and amount. Alternatively, chromogenic substrates create visible colour changes at the protein site, quantified by densitometry or visual intensity assessment. Incubation times vary and

optimize based on substrate type and manufacturer guidelines, crucial for accurate protein detection and analysis in Western blot experiments.

Data Analysis in Western Blotting:

The Western blot image is analysed to quantify target protein bands using software or densitometry. Comparing results with controls or experimental conditions helps draw conclusions about protein expression levels or changes. This analysis provides insights into the protein's function, regulation, or response in specific experimental or biological context.

Results

Food Intake and Body Weight Changes in Male Mice and Female Mice Exposed to Polystyrene Microplastics Over 28 Days:

Male and female mice exposed to Polystyrene Microplastics showed a gradual decline in body weight over the study period of 5 weeks following exposure to both high and low doses. This decrease was statistically significant compared to the control group ($p < 0.05$), suggesting a potential link between Polystyrene Microplastic exposure and body weight changes (Figure 6). Specifically, male mice exhibited a significant decrease in body weight during the fifth week of exposure to both high and low doses, while female mice did not show statistically significant changes. These gender-specific differences highlight the possibility of sex-dependent responses to Polystyrene exposure, prompting further investigation into the underlying factors influencing these effects. In terms of food intake patterns, male mice exposed to medium doses showed changes that were not statistically significant compared to the control group (n=7). Conversely, female mice demonstrated an increase in body weight in response to dose concentration during the fifth week of exposure, but these changes were not statistically significant.

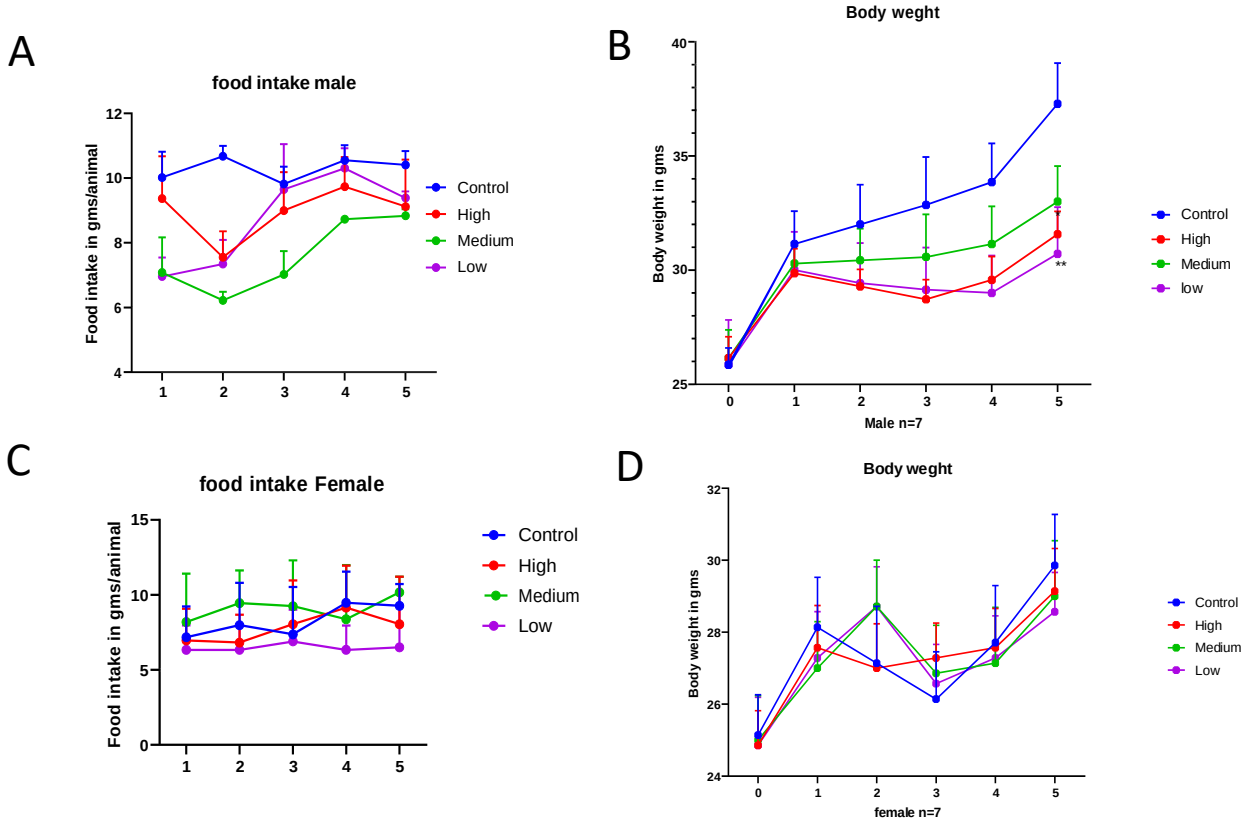

Figure: 6 Body weight, food intake after male and female mice exposed to Polystyrene MPs from 28days; (A) Food intake patterns in male mice demonstrated changes in response to medium dose exposure; however, these changes were not statistically significant compared to the control group (n=7). (B) Male mice exhibited a significant decrease in body weight during the fifth week of exposure to both high and low doses of Polystyrene (n=7) (C) Female mice displayed alterations in food intake, with an increase observed in the high-dose groupand a decrease in the low-dose group; however, these changes were not statistically significant.(D) In contrast, the body weight of female mice increased according to dose concentration in the fifth week of exposure, but these changes were not found to be statistically significant.

Impact of Polystyrene Microplastics on Organ Weight in Male Mice:

The findings suggest that exposure to a low dose of Polystyrene Microplastics affected the organ weights of male mice. Notably, there was an enlargement of the spleen and a visible reduction in adipose tissue in mice exposed to this low dose. However, there were no statistically significant changes observed in other vital organs such as the Kidney, liver, and

lungs. These results emphasize how low-dose exposure to Polystyrene can selectively impact specific organs. Additionally, reducing fat levels has been associated with improvements in metabolic parameters like insulin sensitivity and glucose regulation, potentially leading to a healthier metabolic profile in mice.

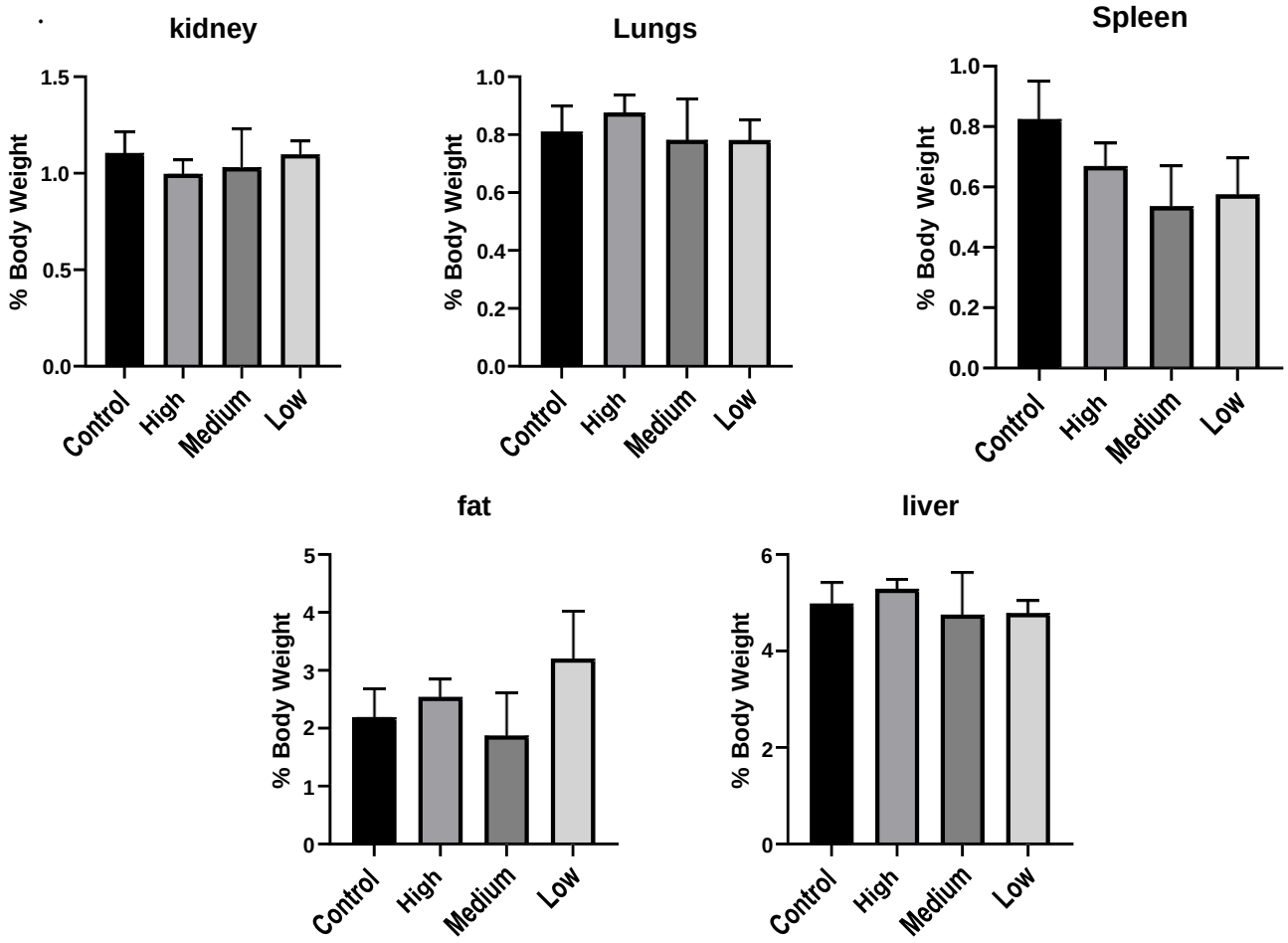

FIGURE: 7 Polystyrene Microplastics induced spleen enlargement and decreased fat content in male mice, while no significant changes were observed in the weights of the kidney, lungs, and liver.

Effects of Polystyrene Micro plastics on Organ Weight in Female Mice:

Exposure to medium doses of Polystyrene Microplastics reduced adipose tissue in female mice without significant effects on Kidney, liver, or lung weights. This suggests specific organ targeting and potential metabolic benefits related to improved insulin sensitivity and glucose regulation.

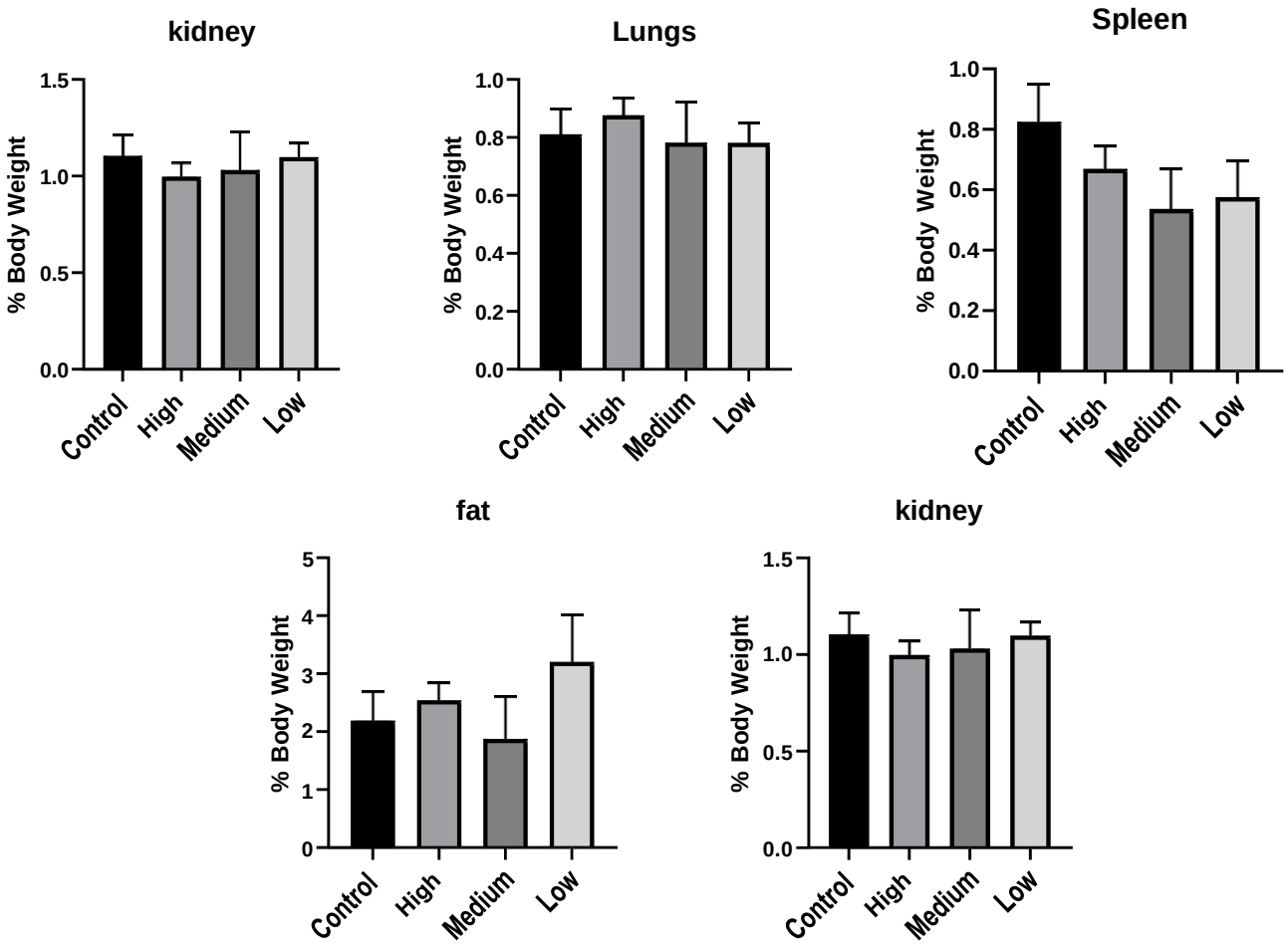

Figure: 8 Polystyrene Microplastics induced decreased fat content in female mice, while no significant changes were observed in the weights of the kidney, lungs, and liver.

Picrosirius Red (PSR) staining:

Picrosirius staining Representative photographs of Picrosirius Red staining on sections of renal tissues from control mice and mice treated with Polystyrene Microplastics. (A) The control group showed normal Kidney architecture and histology. (B) Treated groups (High Medium, low) showed stained fibrotic areas (arrowheads) and yellow non-collagen structures, with significantly higher collagen deposition observed in the high or medium group.

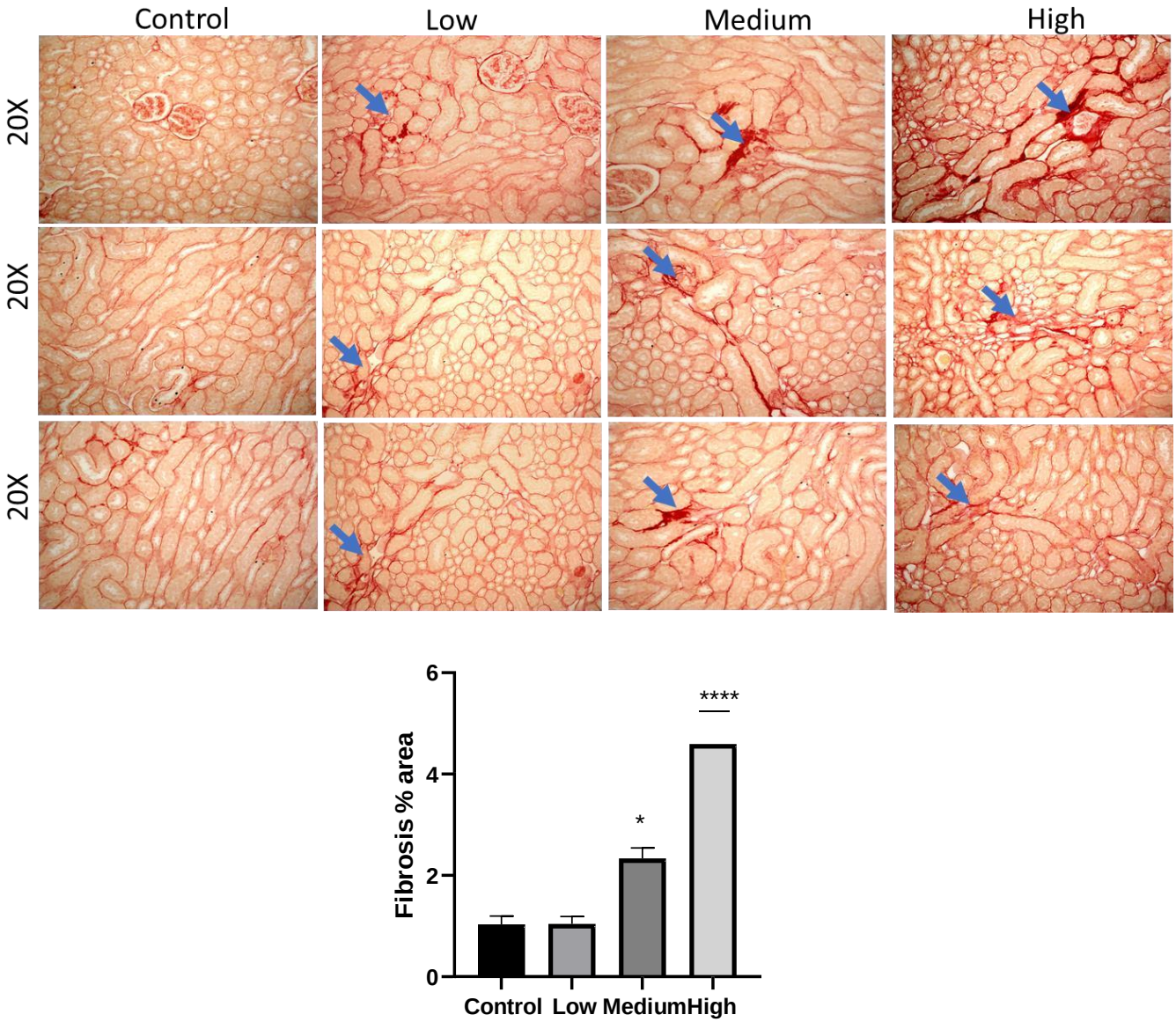

Figure: 9 Picrosirius Red staining showed normal Kidney histology in controls and increased fibrosis with significant collagen deposition in the high and medium dose treated group.

Periodic Acid-Schiff (PAS) staining:

Representative Periodic Acid-Schiff (PAS) staining in renal tissues of control and Polystyrene Microplastic-treated groups revealed more severe glomerular lesions in the treated group, including glomerular hypertrophy, global mesangial Constriction (white arrow), and basement membrane thickening. (black arrow). Magnification: 20X.

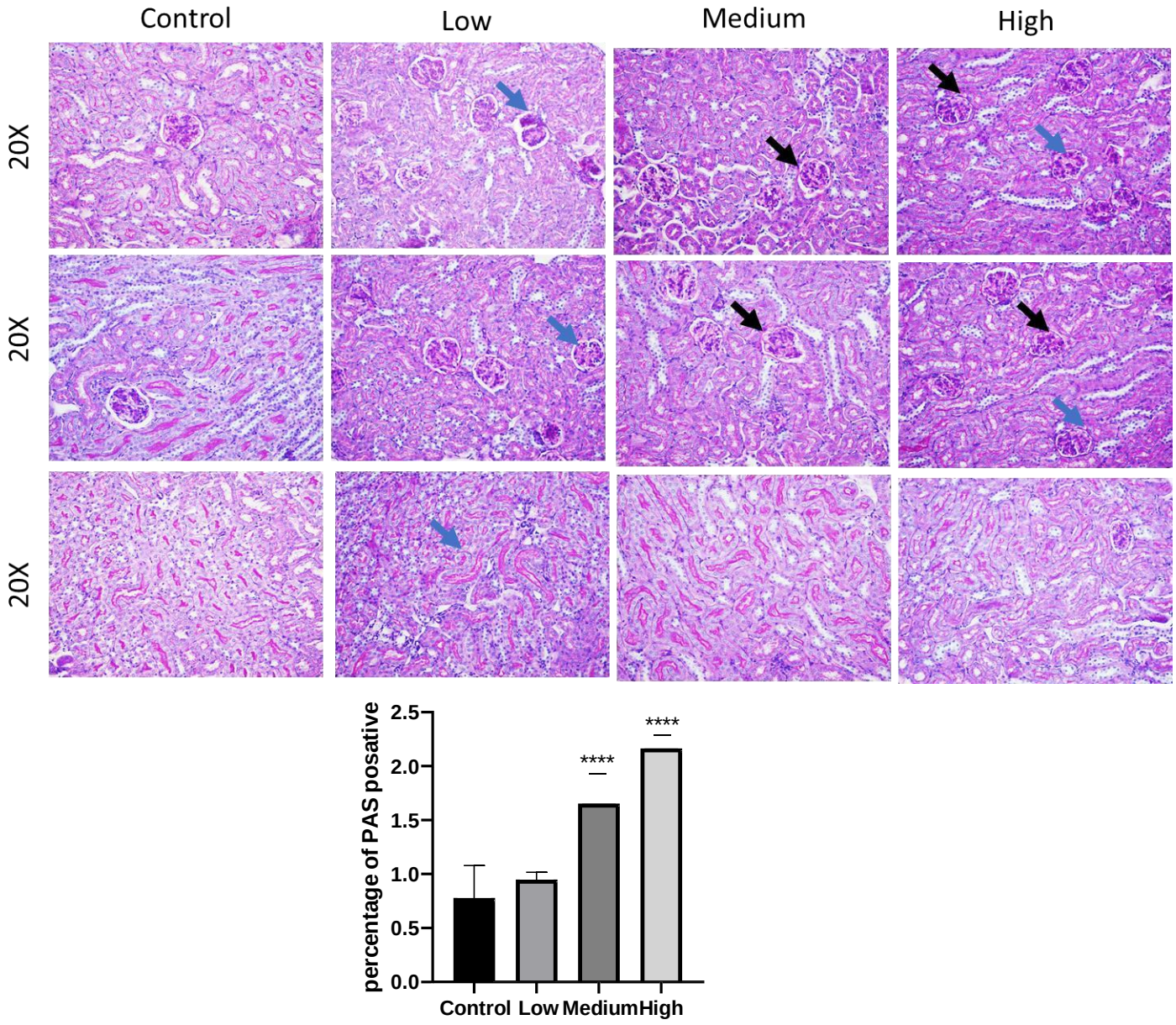

Figure: 10 Periodic Acid-Schiff (PAS) staining revealed normal kidney histology in controls and significantly increased glomerular lesions in the treated group, characterized by glomerular hypertrophy, global mesangial suppression (blue arrow), and basement membrane thickening (black arrow). Magnification:20X.

Haematoxylin and Eosin (H&E) staining:

The histopathological examination of Kidney tissue sections stained with Haematoxylin and Eosin (H&E) revealed significant findings across different experimental groups. The control group displayed normal glomeruli and tubules without any abnormalities, indicating healthy kidney architecture. In contrast, the high medium and low dose of Polystyrene Microplastic group exhibited substantial glomerular and tubular damage accompanied by inflammation, characteristic of nephritis or similar Kidney disease.

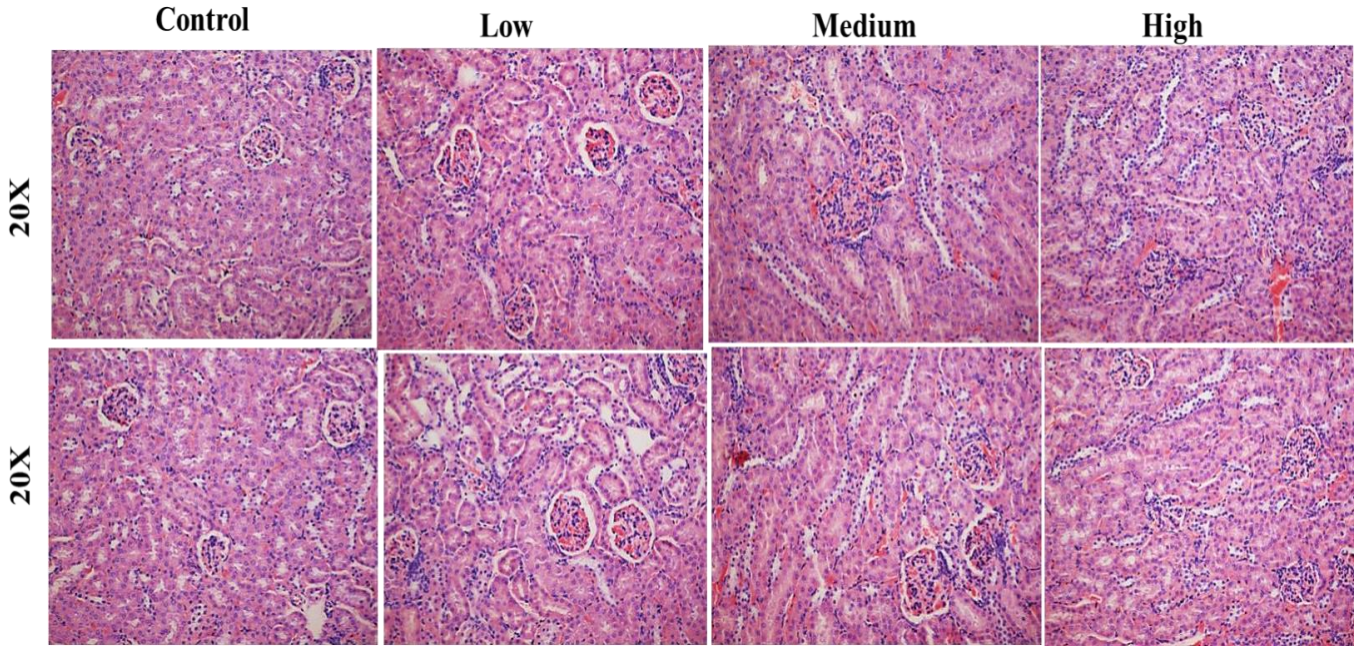

Figure: 11 Histopathological analysis of Kidney tissue sections (H&E staining) show normal glomeruli and tubules in the control group, mesangial expansion in the low dose treatment group (black arrow), mesangial constriction (black arrow) and tubular damage (blue arrow) in the medium and high dose treatment groups.

Discussion

The present study aimed to investigate the histopathological changes in the Kidneys induced by Polystyrene exposure, providing insights into the potential nephrotoxic effects of this widely used synthetic polymer. Our findings revealed significant alterations in renal architecture, suggesting that Polystyrene exposure could lead to considerable Kidney damage.

The histopathological examination of Kidney tissues from Polystyrene-exposed groups demonstrated notable pathological changes, including glomerular atrophy, tubular degeneration, and interstitial fibrosis. These findings are consistent with previous studies that have reported similar nephrotoxic effects induced by other environmental pollutants and synthetic materials. The severity of these changes was dose-dependent, with higher concentrations of Polystyrene resulting in more pronounced damage.

Glomerular atrophy observed in this study is indicative of impaired glomerular function and reduced filtration capacity. This can lead to proteinuria and a subsequent decline in Kidney function. The disruption of glomerular integrity could be attributed to the oxidative stress and inflammatory responses triggered by Polystyrene particles, as supported by increased levels of biomarkers for oxidative stress and inflammation in the treated groups.

Tubular degeneration and vacuolization were prominent in the Kidney sections of Polystyrene-treated animals. These changes suggest tubular cell injury and apoptosis, which can compromise renal reabsorption and secretion functions. The presence of tubular casts and dilatation further corroborates the nephrotoxic potential of Polystyrene. Previous studies have also highlighted the susceptibility of tubular cells to toxic insults, which can precipitate Acute Kidney Injury (AKI) and Chronic Kidney Disease (CKD).

Interstitial fibrosis, characterized by the accumulation of extracellular matrix components, was evident in the Kidneys of Polystyrene-exposed animals. This fibrotic response is a hallmark of Chronic Kidney injury and is often associated with irreversible loss of renal function. The fibro genic process may be driven by the activation of fibroblasts and the upregulation of profibrotic cytokines in response to sustained injury and inflammation.

The underlying mechanisms of Polystyrene-induced nephrotoxicity are likely multifactorial. Oxidative stress appears to play a central role, as evidenced by elevated levels of Reactive Oxygen Species (ROS) and lipid peroxidation products in the Kidney tissues. Polystyrene

particles may disrupt cellular homeostasis by generating ROS, leading to oxidative damage to cellular components such as lipids, proteins, and DNA. Additionally, the inflammatory response triggered by Polystyrene exposure, marked by increased infiltration of inflammatory cells and upregulation of pro-inflammatory cytokines, may exacerbate renal injury.

The findings of this study have significant clinical implications, particularly in the context of increasing environmental exposure to Microplastics and Nanoplastics. Polystyrene, being a major component of plastic pollution, poses a potential risk to renal health, especially in populations with pre-existing Kidney conditions or compromised renal function. The identification of specific histopathological changes provides valuable diagnostic markers for early detection and monitoring of Polystyrene-induced nephrotoxicity. While this study provides important insights into the nephrotoxic effects of Polystyrene, several limitations should be acknowledged. The study was conducted in an animal model, and the relevance of these findings to human health requires further investigation. Additionally, the long-term effects of chronic low-dose exposure to Polystyrene were not addressed and warrant further exploration. Future studies should focus on elucidating the molecular pathways involved in Polystyrene-induced Kidney injury and exploring potential therapeutic interventions to mitigate its adverse effects.

Conclusion

In conclusion, this study highlights the nephrotoxic potential of Polystyrene, evidenced by significant histopathological changes in the Kidneys. The observed Glomerular mesangial expansion, tubular degeneration, and interstitial fibrosis underscore the need for increased awareness of the potential health risks associated with plastic pollution. Further research is essential to fully understand the implications of Polystyrene exposure on renal health and to develop strategies to protect against its harmful effects.

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