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Histopathological Assessment of Kidney Toxicity Induced by Polystyrene

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Abstract

The increasing environmental prevalence of microplastics, particularly Polystyrene Microplastics (PS-MPs), has raised significant health concerns due to their potential toxicity in vital organs. This study investigates the nephrotoxic effects of Polystyrene Microplastics in male and female mice following oral exposure over a period of 28 days. Mice were administered varying concentrations (low, medium, and high doses) of PS-MPs, and their physiological, histological, and metabolic parameters were closely monitored.

A significant decline in body weight was observed in male mice, especially at high and low doses, while female mice showed non-significant changes. Food intake patterns were minimally affected. Organ weight analysis revealed adipose tissue reduction in both sexes, with spleen enlargement noted in low-dose male mice. Histopathological evaluation using Hematoxylin & Eosin (H&E), Picrosirius Red (PSR), and Periodic Acid-Schiff (PAS) staining revealed dose-dependent renal damage characterized by glomerular atrophy, tubular degeneration, mesangial expansion, interstitial fibrosis, and basement membrane thickening.

The study suggests that the renal toxicity of PS-MPs may be mediated by oxidative stress and inflammatory responses, contributing to structural and functional impairments in kidney tissues. These findings underscore the growing threat of microplastic pollution to kidney health and highlight the need for further studies on the long-term effects and underlying mechanisms of PS-MP exposure.

1. Introduction:

The kidneys are vital organs essential for maintaining homeostasis within the human body through a range of complex physiological processes. Located bilaterally in the retroperitoneal space, these bean-shaped structures play a central role in filtering metabolic waste from the blood, regulating electrolyte and fluid balance, controlling blood pressure, and supporting red blood cell production through the synthesis of erythropoietin [1,2].

Each kidney is composed of an outer renal cortex and an inner renal medulla, containing approximately one million nephrons—the fundamental functional units responsible for blood filtration and urine formation [3]. A nephron consists of a renal corpuscle (comprising the glomerulus and Bowman's capsule) and a renal tubule, which includes the proximal convoluted tubule, loop of Henle, distal convoluted tubule, and collecting duct [4]. The glomerulus initiates the filtration of blood plasma, while selective reabsorption and secretion along the tubule regulate urine composition and volume [3,4].

This intricate architecture enables the kidneys to carry out essential functions necessary for maintaining internal physiological stability. Consequently, any structural or functional impairment can result in significant health consequences, including various renal diseases and disorders [5]. A thorough understanding of the kidneys' anatomy and physiology is therefore crucial for recognizing their vulnerability to pathological conditions and the effects of nephrotoxic agents.

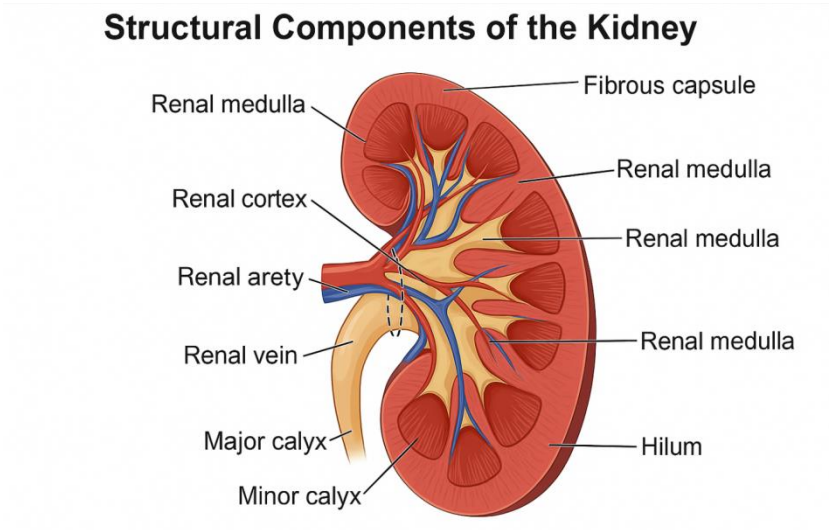


Figure: 1 Structural Components of the Kidney

The kidneys play a crucial role in maintaining overall health and homeostasis in the human body by performing a variety of essential physiological functions, including:

1. **Filtration of Blood:** The primary function of the kidneys is to filter metabolic waste products and excess substances—such as urea, creatinine, and electrolytes—from the blood, leading to the formation of urine [1].
2. **Regulation of Fluid Balance:** The kidneys regulate the body's fluid balance by adjusting urine volume and concentration in response to hydration status and blood pressure fluctuations [2].
3. **Electrolyte Balance:** They maintain the correct levels of key electrolytes (e.g., sodium, potassium, chloride), which are critical for nerve conduction, muscle function, and cellular processes [1].
4. **Acid-Base Balance:** The kidneys preserve systemic pH by excreting hydrogen ions and reabsorbing bicarbonate ions, thereby maintaining acid-base equilibrium.
5. **Blood Pressure Regulation:** Through the Renin-Angiotensin-Aldosterone System (RAAS), the kidneys regulate blood pressure by modulating blood volume and vascular resistance [1].
6. **Erythropoiesis:** The kidneys synthesize erythropoietin, a hormone that stimulates red blood cell production in the bone marrow, thus enhancing oxygen delivery throughout the body [2].
7. **Detoxification:** They also contribute to detoxification by excreting metabolic waste, xenobiotics, and various drugs and toxins [1].

Given their complex and vital roles, kidney dysfunction or failure can result in serious systemic complications, including fluid and electrolyte imbalances, hypertension, anemia, bone disorders, and accumulation of toxic metabolites [5]. Nephrotoxicity, or kidney toxicity, is a major concern in both clinical and environmental contexts and may arise from exposure to pharmaceuticals, heavy metals, radionuclides, plasticizers, and other environmental contaminants [6,7].

The kidneys' high perfusion rate and intricate functional structure make them particularly susceptible to toxic insults. Among emerging environmental threats, **plastics and microplastics** have gained significant attention due to their widespread use and persistence.

Plastics are favored in modern life for their durability, light weight, and affordability. However, global plastic production has exceeded 320 million tons annually and is projected to reach 33 billion tons by 2050, highlighting the scale of the plastic pollution crisis [8]. Alarmingly, only 9% of plastic waste is recycled, with 12% incinerated, leaving the vast majority to accumulate in the environment [9–11]. In 2019, the World Health Organization (WHO) called for intensified global research into the health effects of microplastics [12].

The kidneys play a central role in filtering and excreting toxic compounds from the body. **Styrene**, a monomer used in the production of **Polystyrene (PS)**, is metabolized in the liver to the more reactive compound **styrene oxide**, which is subsequently processed and eliminated via the kidneys. However, these reactive intermediates may induce **oxidative stress** and cause structural damage to renal tissues [13].

Plastics are categorized by their chemical properties and intended uses. Common synthetic polymers include **Polyethylene (PE), Polypropylene (PP), Polystyrene (PS), Polyethylene Terephthalate (PET),** and **Polyvinyl Chloride (PVC)**. In ecotoxicology, studies have increasingly focused on microplastics (MPs) derived from PP, PET/PES, PVC, Polyamide, Polyether, Cellophane, and Polyurethane. PE and PS, in particular, have received attention due to their widespread presence in aquatic ecosystems [14].

Polystyrene microplastics, used in personal care and biomedical products, pose a significant health risk. These microplastics can enter the food chain and potentially disrupt human endocrine systems. The degradation of polystyrene can release **styrene monomers**, which are harmful to both environmental and human health.

To assess kidney damage caused by such toxins, **histopathology**—the microscopic study of diseased tissues—is a critical investigative tool [15]. Through histological examination, specific

structural and cellular alterations in renal tissues can be detected, providing valuable insights into the nature and extent of kidney injury [16]. Histopathology is especially useful in identifying early toxic effects that may not be evident through biochemical tests alone. Observed pathological changes may include **tubular necrosis, glomerular injury, interstitial fibrosis**, and **inflammatory cell infiltration** [5].

The process typically involves staining tissue sections with **Hematoxylin and Eosin (H&E)** to highlight cellular and structural features [17]. Additional techniques, such as **Periodic Acid-Schiff (PAS)** staining, **Picro-Sirius Red** staining, and **immunohistochemical** labeling, are used to identify specific cell types, proteins, or pathological changes [18].

2. Review of Literature:

2.1 Review of Kidney

The kidneys are vital organs responsible for maintaining homeostasis in the human body. They perform several essential physiological functions, including filtering the blood, removing metabolic waste products, regulating electrolyte and fluid balance, and controlling blood pressure. Located in the retroperitoneal space of the abdominal cavity, each kidney receives blood via the renal artery, processes it through specialized filtration units called nephrons, and returns the filtered blood to circulation through the renal vein. Through mechanisms such as reabsorption and secretion, the kidneys ensure the elimination of waste products like urea while preserving necessary substances, thus maintaining internal balance.

Structure of the Kidneys

The kidneys are bean-shaped organs, each measuring approximately 10–12 cm in length, 5–7 cm in width, and about 3 cm in thickness. They are situated on either side of the vertebral column, between the T12 and L3 vertebrae. Each kidney comprises several anatomically and functionally distinct regions:

- **Renal Cortex:**

  The outermost region of the kidney, the renal cortex contains renal corpuscles and the convoluted tubules of nephrons. This area houses the **glomeruli**, which are capillary networks where the filtration of blood plasma begins.

- **Renal Medulla:**

  Located beneath the cortex, the renal medulla consists of renal pyramids. These pyramids contain loops of Henle and collecting ducts essential for the concentration of urine and its movement toward the renal pelvis.

- **Renal Pelvis:**

  The renal pelvis is a funnel-shaped cavity that collects urine from the collecting ducts and channels it into the ureter, which subsequently transports it to the urinary bladder for excretion.

- **Nephrons:**

  Nephrons are the microscopic functional units of the kidney, with each kidney containing approximately one million nephrons. Each nephron consists of a **renal corpuscle** (composed of the glomerulus and Bowman's capsule) and a **renal tubule** (comprising the proximal convoluted tubule, loop of Henle, distal convoluted tubule, and collecting duct). Nephrons carry out the key processes of **filtration**, **reabsorption**, and **secretion**, which are essential for maintaining the body's fluid, electrolyte, and acid-base balance.

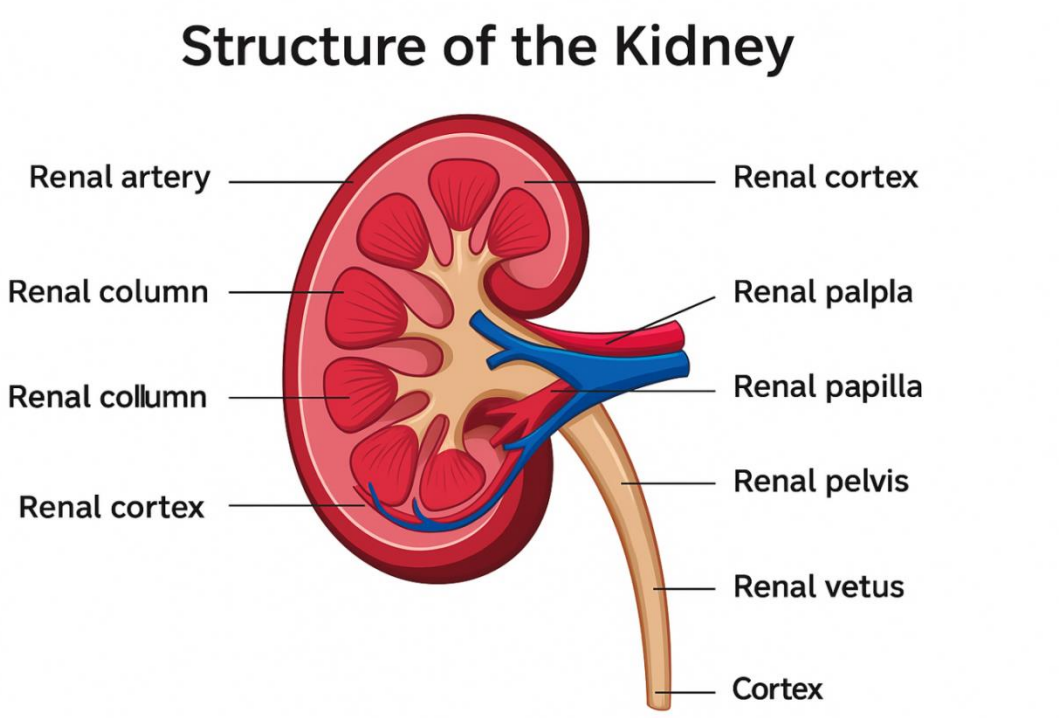


Figure: 2 Structure of the Kidneys

2.2 Overview of Polystyrene Microplastics (PSMPs):

Polystyrene microplastics (PSMPs) have been frequently detected in various environmental studies [22]. Polystyrene is a versatile plastic material that exists in both solid and foamed forms, produced by polymerizing styrene monomers. These microplastics, defined as plastic particles smaller than 5 millimeters, are primarily generated through the breakdown of larger polystyrene products such as packaging materials and disposable items.

Compared to other plastics, polystyrene exhibits high resistance to photodegradation, meaning it takes longer to break down under sunlight exposure. Previous studies have reported that PSMPs constitute a significant portion (29.41%) of the microplastics found in surface sediments of urban water bodies [20]. Among these samples, a majority (58.31%) of microplastic particles were smaller than 1 mm in size.

Additionally, research suggests that smaller polystyrene microplastics (around 2 µm) may pose biological risks due to their relatively high bio-concentration factors. Therefore, further investigation is essential to evaluate the potential toxicity of polystyrene microplastics and their implications for public health [21].

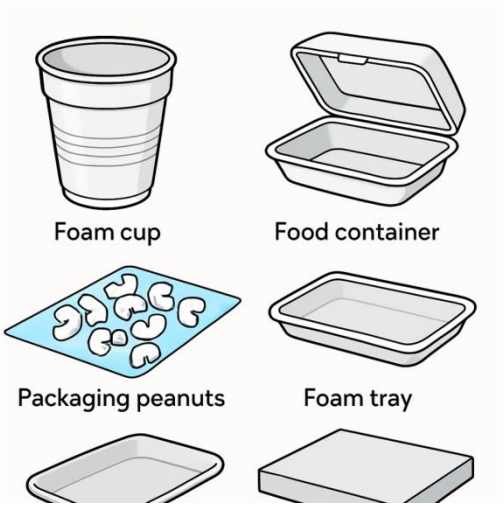


Figure: 3 Household Items Made from Polystyrene

2.3 Impact of Polystyrene Microplastics on Mice Organs:

Studies examining the effects of polystyrene microplastics (PSMPs) on mice organs have revealed varying outcomes depending on the experimental conditions and levels of exposure. Most research has focused on evaluating changes in organ morphology, function, and biochemical markers following exposure to different concentrations of PSMPs. Findings suggest that moderate doses of polystyrene microplastics can result in changes in organ weights, with a notable reduction in adipose tissue mass.

However, results related to other vital organs—such as the kidneys, liver, and lungs—have been inconsistent, with some studies reporting minimal or no significant alterations. The precise biological mechanisms through which polystyrene microplastics exert their effects remain

unclear, but they are believed to involve processes such as inflammation, oxidative stress, and disrupted cellular signaling.

These findings highlight the complexity of systemic biological responses to microplastic exposure. Therefore, further research is essential to fully understand the long-term health impacts of polystyrene microplastics on organ systems, including their potential cumulative effects and the underlying mechanisms of toxicity.

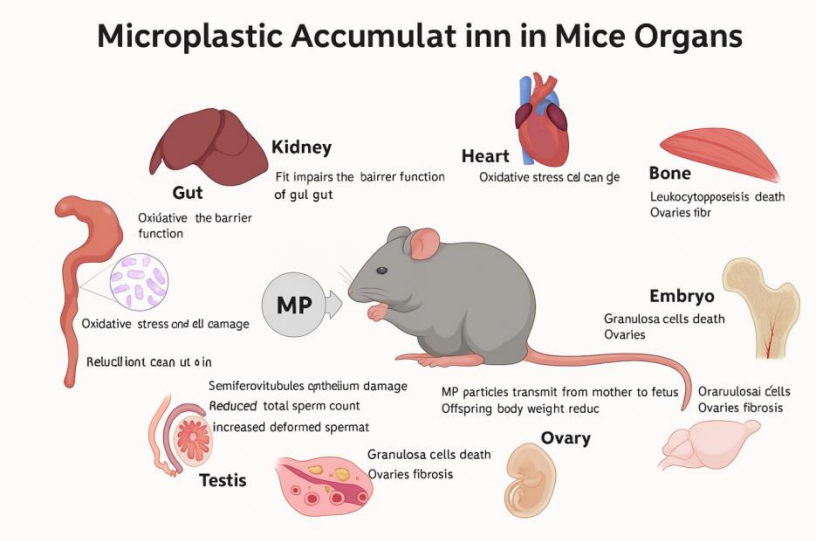


Figure: 4 Microplastic Accumulation in Mice Organs

2.4 Impact of Polystyrene Microplastics on the Human Kidney

Growing concern within the medical and scientific communities regarding the potential health effects of microplastics (MPs) has led to increased research into their impact on various human tissues, including the kidneys. Microplastics are defined as plastic particles smaller than 5 millimetres, typically formed from the environmental degradation of larger plastic items [12]. Their presence has been detected in numerous food sources, raising concerns about human exposure through ingestion.

Polystyrene microplastics (PS-MPs), in particular, can enter the human body via the consumption of plastic-packaged products (e.g., bottled water, processed foods), seafood, and contaminated groundwater [23]. Although it remains uncertain whether PS-MPs are contributing factors to chronic kidney disease of unknown etiology (CKDu), some studies have begun exploring this possible link [24]. Modelling studies estimate the human threshold concentrations for polystyrene microplastics to range from 5.1 to 53.3 mg per gram of body weight [25]; however, such exposure levels are considered unlikely to occur under normal environmental conditions [26].

Despite this, the potential health risks associated with microplastic exposure remain under debate. Several unanswered questions persist, such as whether microplastics contribute to carcinogenesis in marine animals and humans, and what the long-term effects of chronic exposure may be given the multiple possible entry routes [27]. PS-MP particles as small as 1 µm have been detected in the environment and are particularly relevant for human exposure scenarios.

A clearer understanding of CKDu's underlying causes is critical for guiding effective public health policies. Animal studies have shown that microplastics and nanoplastics can accumulate in the gastrointestinal tract, liver, and kidneys of mice [28]. In vitro studies using human kidney (HEK 293) and liver (HepG2) cell lines—both widely used in toxicological research—have explored the cellular-level impacts of microplastics [23]. It has been hypothesized that HEK 293 cells can internalize PS-MPs, leading to adverse kidney effects such as cytotoxicity and renal barrier dysfunction [21].

However, the absorption of PS-MPs by human tissues is estimated to be low (≤0.3%), and only particles ≤20 µm in size are considered capable of entering internal organs. Of these, a small fraction (0.1–10 µm) may penetrate cellular membranes, cross the blood-brain barrier and placenta, and distribute to other tissues such as the liver, muscles, and brain [20].

2.5 Routes of Exposure to Polystyrene Microplastics

Microplastics—defined as plastic particles smaller than 5 millimetres—can enter the human body through various exposure routes. Understanding these pathways is essential for assessing the potential health risks associated with microplastic contamination. The three primary routes of exposure include ingestion, inhalation, and dermal contact.

Ingestion:

The most common route of exposure is through the consumption of contaminated food and water. Marine organisms that ingest microplastics can transfer these particles through the food chain when consumed as seafood [29]. Sea salt is another known source of contamination. Processed foods may be exposed to microplastics during manufacturing, packaging, and storage processes. Fruits and vegetables can also carry microplastics due to exposure to contaminated soil, irrigation water, or agrochemicals [30]. Furthermore, microplastics have been detected in both bottled and tap water [31], as well as in other beverages such as beer, tea, and soft drinks.

Inhalation:

Airborne microplastics pose a respiratory risk, especially in urban areas and indoor environments. These particles can be inhaled directly or ingested indirectly through mucociliary clearance. Common indoor activities, such as vacuuming, dusting, and wearing synthetic textiles, can release microplastic fibres into the air, contributing to indoor pollution [32].

Dermal Contact:

Certain personal care products, such as exfoliants and cosmetics, contain microplastics like microbeads that may be absorbed through the skin [33]. Additionally, synthetic clothing can shed microplastic fibres during wear, which may come into contact with the skin. Household dust, which often contains microplastic particles, may also contribute to dermal exposure, particularly when combined with sweat or moisture.

Polystyrene-Specific Exposure:

Polystyrene microplastics can infiltrate the human respiratory system through multiple pathways. Occupational exposure is of particular concern for individuals working in industries involved in the manufacturing, handling, or recycling of polystyrene products, where airborne polystyrene

particles are prevalent. Environmental exposure is also a growing concern, as polystyrene microplastics are increasingly being detected in urban air due to the degradation of larger plastic items and the wear and tear of products containing polystyrene.

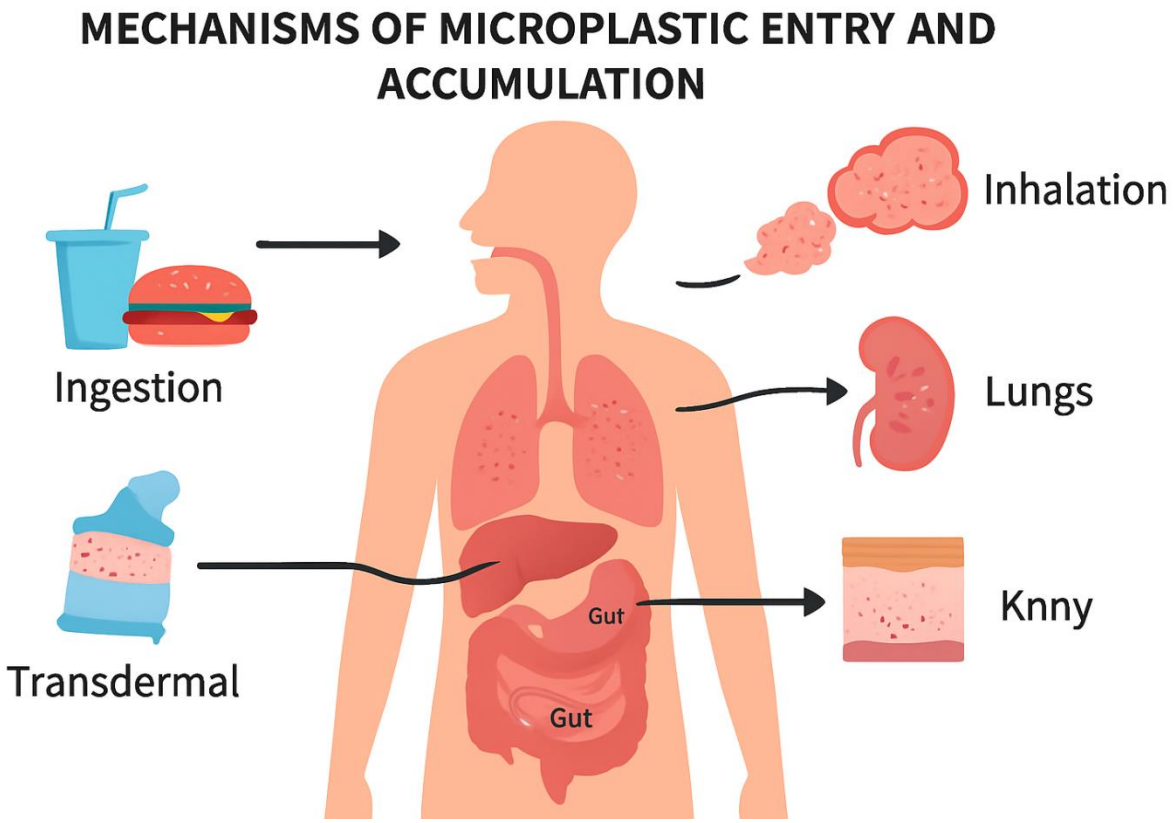


Figure: 5 Mechanisms of Microplastic Entry and Accumulation

3. Material and Methodology

3.1 Animal Handling:

BALB/c mice were procured from the GLP-certified animal facility at the CSIR–Indian Institute of Toxicology Research (IITR), located in Lucknow, Uttar Pradesh, India. All experimental procedures carried out in this study were ethically approved by the institute's ethical committee (IEAC reference no. IITR/IEAC/27/19).

Upon arrival, the mice underwent a one-week quarantine period before being housed in polystyrene cages, with unrestricted access to food and water. They were maintained under standard environmental conditions: a temperature of 23°C ± 2 and a relative humidity range of 30–70%. After a one-week acclimatization period, the mice were weighed and randomly assigned to four groups, each comprising seven mice with nearly identical body weights. These groups were categorized as: Control, and low, medium, and high doses of polystyrene.

Over a duration of 28 days, the mice were administered polystyrene, and their body weights were recorded before the intervention and subsequently on the 1st, 8th, 15th, 22nd, and 28th days of exposure. Polystyrene was administered by oral gavage using a cannula to ensure precise delivery to the stomach. Throughout the exposure period, food intake was closely monitored, and food pellets were regularly weighed to ensure consistent intake across all groups. Body weight measurements were taken weekly for all groups.

At the end of the 28-day exposure period, the mice were anesthetized using a ketamine-xylazine combination and then sacrificed. Blood samples were collected and centrifuged to separate the serum. Kidney, liver, and fat tissues were preserved in a 10% formalin solution. The remaining samples were flash-frozen in liquid nitrogen and stored at –80°C for further analysis.

3.2 Histopathology

3.2.1 Hematoxylin & Eosin Staining (H&E):

Hematoxylin and Eosin (H&E) staining is a standard technique in renal pathology used to examine the complex structures and pathological alterations in the kidney. Hematoxylin selectively stains cell nuclei blue or purple, while eosin stains the cytoplasm and extracellular matrix pink or red, allowing detailed microscopic observation of renal morphology.

The kidney's intricate structure — including nephrons, tubules, glomeruli, and interstitial tissues — requires careful histological analysis to assess function and identify abnormalities. H&E staining enables visualization of renal components such as the glomerular corpuscle, tubular

epithelium, and interstitium, making it valuable for evaluating structural integrity and detecting pathological changes.

This method is critical in diagnosing various kidney diseases, including glomerular disorders (e.g., glomerulonephritis), tubulointerstitial diseases (e.g., acute tubular necrosis), and renal tumors. Its ability to differentiate between normal and abnormal morphology helps guide both diagnosis and treatment strategies.

Procedure:

The staining process begins with immersion of the slides in xylene 1 for 10 minutes, followed by xylene 2 for another 10 minutes. The slides are then placed in a xylene-alcohol mixture for 5 minutes. Subsequently, the slides undergo sequential immersion in 100% ethanol (5 minutes), 90% ethanol (5 minutes), and three rounds of 70% ethanol (5 minutes each). After rinsing in distilled water for 2 minutes, the slides are stained with hematoxylin for 1.5 minutes and washed under tap water for 8–10 minutes.

A brief dip in 90% ethanol for 10–15 seconds is done before eosin staining for 30–45 seconds. The slides are then dehydrated using a graded ethanol series: 50%, 70%, 90%, and 100% ethanol, each for 2 minutes. After this, they are cleared in a xylene-alcohol mixture for 5 minutes, followed by two immersions in xylene for 10 minutes each. Finally, slides are mounted with DPX and examined under a microscope.

3.2.2 Periodic Acid–Schiff (PAS) Staining:

Periodic Acid–Schiff (PAS) staining is a widely used histological method in renal pathology, designed to highlight microscopic structures and pathological changes in the kidney. It selectively stains polysaccharides, glycoproteins, and mucosubstances in renal tissues.

Due to the complex architecture of the kidney, including the glomeruli, tubules, interstitial tissue, and blood vessels, PAS staining is instrumental in visualizing essential structures such as the glomerular basement membrane, mesangial matrix, and tubular basement membranes. It allows

for the assessment of structural changes, glycogen content, and the presence of pathological deposits.

PAS staining is crucial in diagnosing diseases such as membranous nephropathy, diabetic nephropathy, and tubulointerstitial disorders. Its effectiveness in differentiating pathological from normal tissue plays an important role in disease classification, treatment planning, and prognosis.

Procedure:

The slides are first immersed in xylene 1 and xylene 2 for 5 minutes each, followed by a xylene-ethanol mixture for 3 minutes. Next, they are passed through a series of ethanol concentrations — 100%, 90%, and 70% — for 3 minutes each, and then rinsed in distilled water for 3 minutes.

The slides are then treated with periodic acid for 8 minutes and rinsed with distilled water once or twice. Schiff's reagent is applied for 15 minutes, followed by a 6-minute rinse in lukewarm distilled water. Hematoxylin staining is performed for 1 minute, followed by a quick rinse in tap water (2–3 dips). The slides are then dehydrated using 70%, 90%, and 100% ethanol for 2 minutes each. Clearing is done using xylene twice for 2 minutes each. Finally, the slides are mounted with DPX and examined under a microscope.

3.2.3 Picrosirius Red (PSR) Staining:

Picrosirius Red (PSR) staining is a specialized method used to visualize and analyze collagen fibers in kidney tissues. The dye binds specifically to collagen and is visualized under polarized light microscopy due to its birefringence properties.

Collagen is vital for the structural integrity of renal tissues, supporting glomeruli, tubules, and interstitial areas. PSR staining reveals the distribution and organization of collagen types, especially type I (thicker fibers) and type III (thinner fibers), providing insight into fibrotic processes in kidney disease.

PSR staining is widely used to assess fibrosis in chronic kidney diseases such as diabetic nephropathy, glomerulonephritis, and renal fibrosis. It helps monitor disease progression and evaluate therapeutic interventions.

Procedure:

Slides are deparaffinized with two rounds of xylene (3 minutes each), followed by dehydration in descending ethanol concentrations: 100%, 90%, and 70%, each for 2 minutes. The slides are then rinsed in distilled water for 2 minutes and stained with 0.1% Sirius Red F3B in saturated aqueous picric acid for 1 hour.

After staining, the slides are rinsed in distilled water three times (10 dips each) and dehydrated in ascending concentrations of ethanol (70%, 90%, and 100%, each for 2 minutes). Finally, slides are cleared in xylene (2 × 3 minutes), mounted with DPX, and observed under a microscope.

3.3 Other Techniques: Immunoblotting

Protein Extraction Protocol

Protein extraction was performed under cold conditions to preserve protein integrity. Initially, cell-containing culture flasks were placed on ice for 5 minutes, followed by washing with phosphate-buffered saline (PBS) to remove residual media. Subsequently, protein lysis buffer was added to the cells and allowed to interact for 10–15 minutes. Cells were then detached using a cell scraper and collected into pre-chilled microcentrifuge tubes. The lysates were incubated on ice for 20–30 minutes and centrifuged at 12,000 rpm for 20 minutes at 4°C. The supernatant, containing the total protein, was transferred to Eppendorf tubes and kept on ice or stored at –20°C until further analysis.

Protein Estimation Protocol

Total protein concentration was quantified using the **Bicinchoninic Acid (BCA) Assay**, a colorimetric method that relies on the reduction of Cu^{2+} to Cu^{+} ions by proteins in an alkaline

environment, followed by complexation of Cu^{+} with bicinchoninic acid to form a purple chromophore, measurable at 562 nm. The assay is particularly responsive to peptide bonds and amino acids such as cysteine, cystine, tyrosine, and tryptophan. To enhance sensitivity and uniformity, the assay was performed at 37°C for 30 minutes.

For estimation, 5 µL of each protein sample and standard were mixed with BCA reagent (prepared in a ratio of 1:8) in a 96-well microplate in triplicates. After brief shaking (30 seconds), the plate was incubated at 37°C. Absorbance was recorded at 562 nm using a Varioskan Plate Reader. Protein concentrations were calculated using a standard curve derived from known protein concentrations plotted against absorbance values using a linear regression equation.

Western Blotting (Immunoblotting)

Western blotting, also known as immunoblotting, was used for the detection and analysis of specific proteins based on molecular weight. The method consists of three main steps: (1) separation of proteins by SDS-PAGE, (2) transfer of proteins onto a membrane (PVDF or nitrocellulose), and (3) immunodetection using specific antibodies.

Principle

Western blotting enables the identification of target proteins by separating them based on size via SDS-PAGE, transferring them onto a solid membrane, and probing with specific primary and secondary antibodies. Antibody binding is highly specific, ensuring accurate detection of proteins even within complex mixtures.

Buffer Preparation Protocols

- **10% SDS**: Prepared by dissolving 10 g of SDS in 90 mL of distilled water.
- **Transfer Buffer**: 14.4 g glycine, 3.03 g Tris-base, appropriate amount of SDS, and distilled water to a final volume of 1 L.

- **Running Buffer**: 14.4 g glycine, 3.03 g Tris-base, 200 mL methanol, and 800 mL distilled water.
- **1X PBST**: Dilution of 100 mL 10X PBS with 900 mL distilled water and addition of 1 mL Tween-20.
- **10X PBS Composition**: Na_2HPO_4 (44.4 g), KH_2PO_4 (42.4 g), NaCl (80.1 g), KCl (2 g) in 1 L of distilled water.

Gel Preparation for SDS-PAGE

Resolving Gel Composition:

% Gel	Water (mL)	Acrylamide (mL)	Tris-base (pH 8.8) (mL)	SDS (µL)	APS (µL)	TEMED (µL)
7%	4.35	2.02	2.18	100	100	15
8%	4.06	2.32	2.18	100	100	15
10%	3.75	3.00	2.25	100	100	15

Stacking Gel Composition:

% Gel	Water (mL)	Acrylamide (µL)	Tris-base (pH 6.8) (µL)	SDS (µL)	APS (µL)	TEMED (µL)
5%	3.40	830	630	50	80	20
4%	3.07	670	630	50	80	20

Additional Reagents:

- **1M Tris-base (pH 8.8 or 6.8)**: 12.11 g Tris dissolved in 180 mL distilled water, adjusted with HCl, and made up to 200 mL.
- **10% APS**: 1 g ammonium persulfate in 10 mL distilled water.

- **RIPA Buffer**: Contains 1M Tris-HCl (pH 7.4), 0.25M EGTA, 1M NaCl, 0.5M NaF, 100 mM sodium orthovanadate, 10% IGEPAL/NP-40, and 10X protease inhibitor cocktail in Milli-Q water.

Sample Preparation

Protein samples were prepared by mixing 100 µL of supernatant with 16.6–20 µL of loading dye (1X Bromophenol Blue), followed by heating at 95°C for 15 minutes. Samples were then stored at –20°C until use.

SDS-PAGE and Protein Transfer

Proteins were resolved via SDS-PAGE, using a stacking and resolving gel system. Initially, electrophoresis was conducted at 60V for 30 minutes, then increased to 120V for optimal protein separation. Proteins were then transferred onto PVDF membranes (activated with methanol) using a semi-dry transfer system at 300 mA for 90 minutes in a cold environment (4°C) to preserve protein structure.

Membrane Blocking

Post-transfer, membranes were briefly stained with Ponceau S and rinsed with distilled water. To prevent non-specific antibody binding, membranes were blocked with 3% BSA or 3% skimmed milk in PBS for 2 hours at room temperature. Blocking reduces background noise and enhances signal specificity by saturating non-target protein binding sites.

Antibody Incubation

Following three 10-minute washes with PBST, membranes were incubated with primary antibodies diluted in PBS or PBST as per manufacturer's recommendation. Incubation was performed for 4 hours at room temperature or overnight at 4°C with gentle agitation. After primary antibody incubation, membranes were washed again with PBST, then incubated with HRP-conjugated secondary antibodies for 3 hours at room temperature.

Visualization and Data Analysis

Detection was carried out using chemiluminescent substrates for HRP, producing a signal captured via a digital imaging system. Alternatively, chromogenic substrates were used for colorimetric detection. Band intensities were analyzed using densitometry software. Protein expression levels were quantified relative to control or reference bands, providing insights into protein regulation and expression under experimental conditions.

4. Result:

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

Male and female mice exposed to polystyrene microplastics exhibited a gradual decline in body weight over the 28-day exposure period. This reduction was statistically significant in comparison to the control group ($\mathbf{p < 0.05}$), indicating a potential association between polystyrene microplastic exposure and changes in body weight (Figure 6).

In particular, male mice exposed to both high and low doses of polystyrene microplastics showed a significant decrease in body weight during the fifth week of exposure. In contrast, female mice did not exhibit statistically significant weight changes at any dose level, suggesting a possible **sex-dependent difference** in physiological response to polystyrene microplastics. This gender-specific variation warrants further investigation into the underlying biological mechanisms.

Regarding food intake, male mice exposed to medium doses showed non-significant alterations when compared to the control group ($\mathbf{n = 7}$). Interestingly, female mice displayed a trend toward increased body weight in relation to dose concentration during the fifth week of exposure; however, this increase was also not statistically significant.

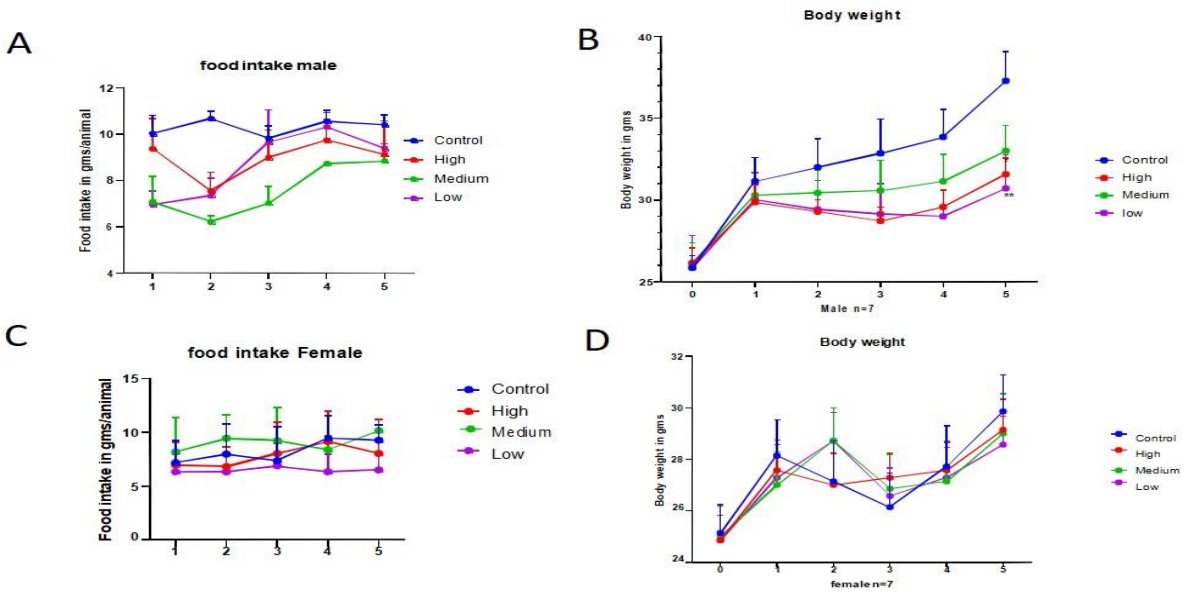


Figure: 6 Body weight, food intake after male and female mice exposed to PolystyreneMPs from 28days; (A) Food intake patterns in male mice demonstrated changes inresponsetomediumdoseexposure;however,thesechangeswerenotstatisticallysignificant compared to the control group (n=7). (B) Male mice exhibited a significantdecrease in body weight during the fifth week of exposure to both high and low doses ofPolystyrene (n=7) (C) Female mice displayed alterations in food intake, with an increaseobserved in the high-dose groupand a decrease in the low-dose group; however, thesechanges were not statistically significant.(D) In contrast, the body weight of female miceincreasedaccordingtodose concentrationin the fifthweek ofexposure,butthesechangeswerenotfoundtobestatisticallysignificant.

4.2 Impact of Polystyrene Microplastics on Organ Weights in Male Mice

The results indicate that exposure to a low dose of polystyrene microplastics (PSMPs) led to notable changes in the organ weights of male mice. Specifically, a **significant enlargement of the spleen** and a **visible reduction in adipose tissue** were observed in the low-dose exposure group. These alterations suggest a selective organ-specific response to polystyrene microplastic exposure.

In contrast, **no statistically significant changes** were detected in the weights of other major organs, including the **kidneys, liver, and lungs**, across the treatment groups. The observed reduction in adipose tissue is particularly noteworthy, as it has been linked in prior studies to **improved metabolic parameters**, such as enhanced insulin sensitivity and better glucose regulation. This may indicate a potential shift toward a more favorable metabolic profile in the exposed male mice.

Overall, these findings highlight that even **low-dose exposure to polystyrene microplastics** can selectively influence organ systems, underscoring the importance of dose-specific and tissue-specific toxicological evaluations.

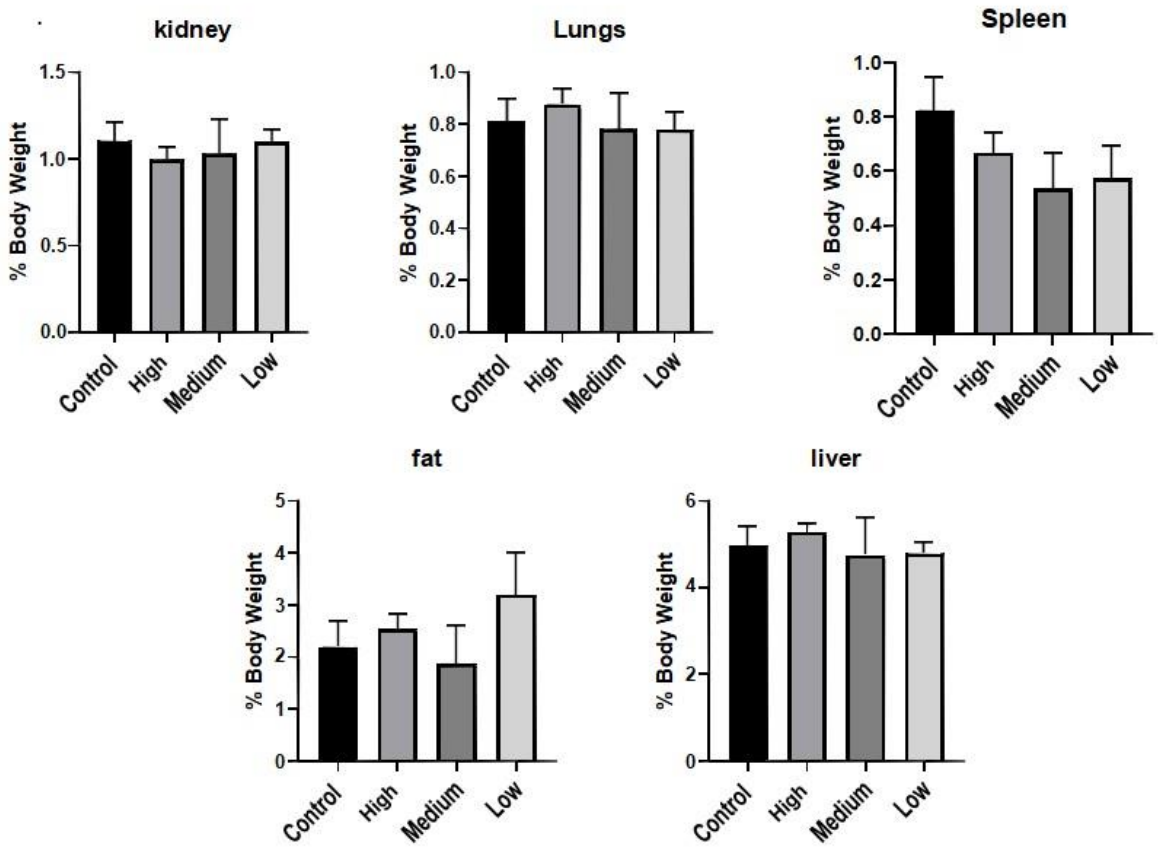


FIGURE: 7 Polystyrene Microplastics induced spleen enlargement and decreased fatcontent in male mice, while no significant changes were observed in the weights of thekidney,lungs,andliver.

4.3 Effects of Polystyrene Microplastics on Organ Weights in Female Mice

In female mice, exposure to medium doses of polystyrene microplastics (PSMPs) resulted in a **notable reduction in adipose tissue**, while the weights of other major organs—including the **kidneys, liver, and lungs**—remained **largely unaffected** and showed **no statistically significant changes**.

The selective reduction in adipose tissue suggests **targeted organ-specific effects** of PSMP exposure, which may have implications for metabolic function. This decrease in fat mass is potentially associated with **enhanced insulin sensitivity and improved glucose regulation**, pointing toward possible **metabolic benefits** following moderate PSMP exposure.

These findings underscore the need for further investigation into **dose-dependent and sex-specific responses** to microplastic exposure, particularly in relation to energy metabolism and systemic health outcomes.

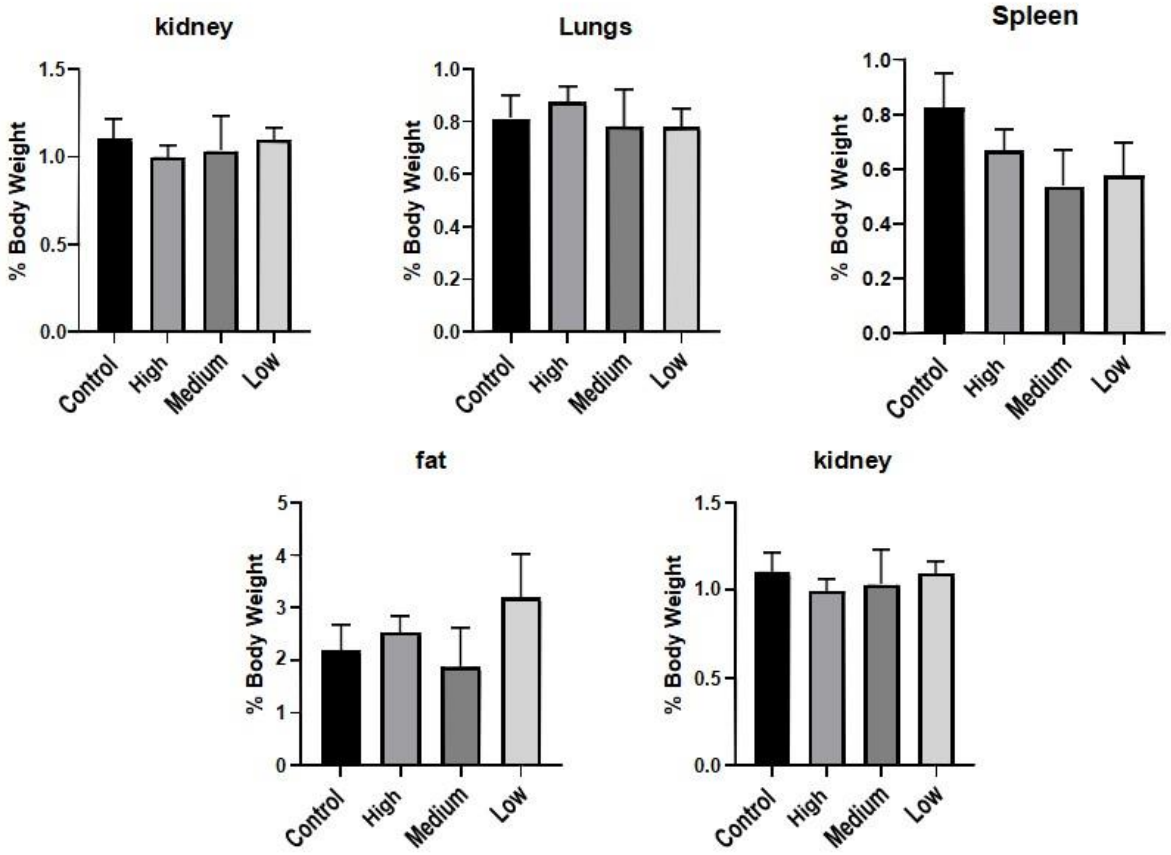


Figure:8 PolystyreneMicroplastics induceddecreasedfatcontentinfemalemice,whileno significantchangeswereobservedintheweightsofthekidney,lungs,andliver.

4.4 Picrosirius Red (PSR) Staining

Representative images of **Picrosirius Red (PSR) staining** of renal tissue sections revealed notable differences between control and polystyrene microplastic (PSMP)-treated groups.

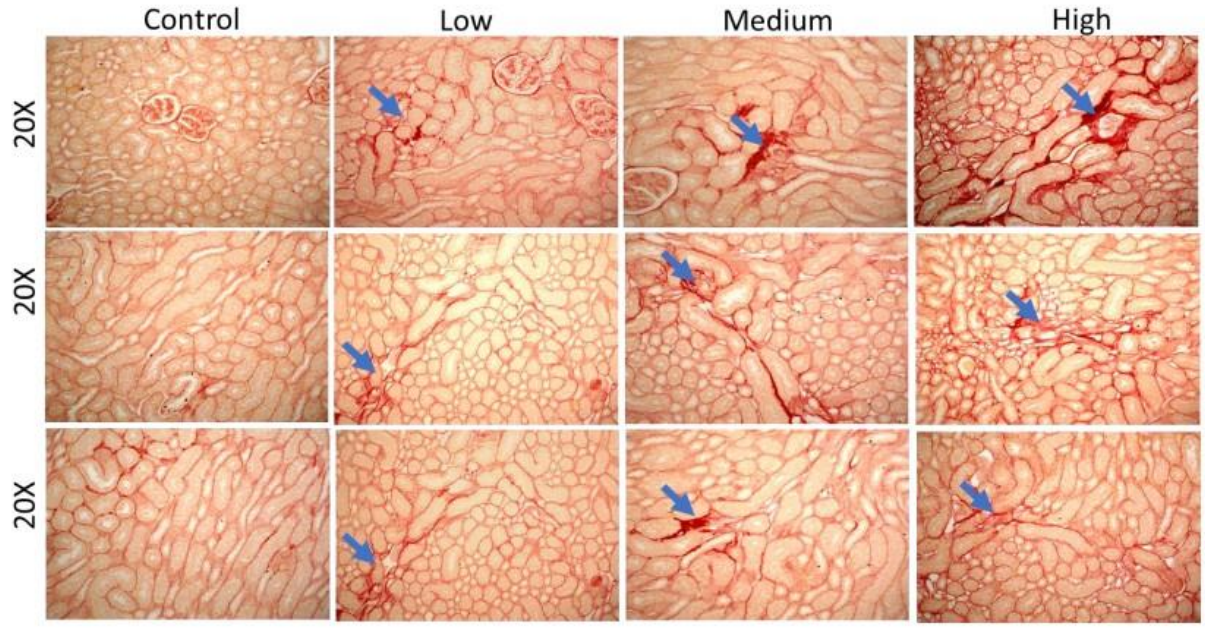
(A) In the **control group**, kidney sections exhibited **normal renal architecture**, with no evidence of fibrotic changes or abnormal collagen deposition.

(B) In contrast, the **PSMP-treated groups** (low, medium, and high dose) demonstrated **clear signs of collagen accumulation**, particularly in the **medium and high dose groups**, where **stained fibrotic regions** were prominently visible. These fibrotic areas appeared as **intensely**

birefringent red and orange fibers under polarized light microscopy, indicating **significant collagen deposition**.

Additionally, **yellow-stained non-collagenous structures** were also observed, which further differentiate pathological changes in the treated tissues. The increase in collagen fiber content in the **medium and high dose groups** suggests **early signs of renal fibrosis**, potentially driven by sustained microplastic exposure.

These findings imply that polystyrene microplastic exposure may initiate **fibrogenic responses** in renal tissue, particularly at **higher exposure levels**, thereby contributing to **kidney structural remodeling and potential dysfunction**.



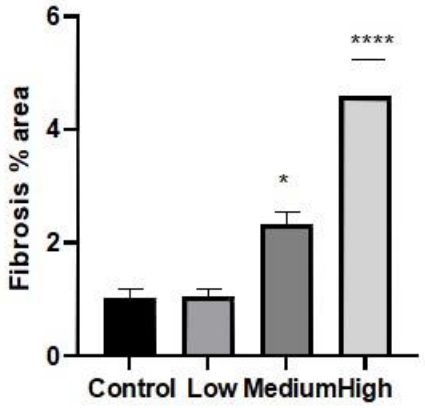


Figure: 9 Picrosirius Red staining showed normal Kidney histology in controls andincreased fibrosis with significant collagen deposition in the high and medium dosetreatedgroup.

4.5 Periodic Acid-Schiff (PAS) Staining

Representative PAS-stained renal tissue sections revealed distinct histopathological differences between the **control** and **Polystyrene Microplastic (PSMP)-treated groups**.

In the **control group**, renal tissue exhibited **normal glomerular morphology**, with well-defined capillary loops, intact basement membranes, and minimal mesangial expansion.

In contrast, **PSMP-treated groups** displayed clear signs of **glomerular injury**. Notable observations included:

- **Glomerular hypertrophy**, indicating enlargement of the glomerular tufts,
- **Global mesangial constriction** (indicated by white arrows), suggesting alterations in the mesangial matrix, and
- **Thickening of the glomerular basement membrane** (black arrows), a hallmark of progressive glomerulopathy.

These structural alterations were most prominent in the **medium and high dose groups**, suggesting a **dose-dependent renal response** to microplastic exposure.

The findings highlight that chronic exposure to PSMPs can induce **early pathological changes** in the kidney, potentially compromising glomerular filtration and overall renal function.

Magnification: 20X

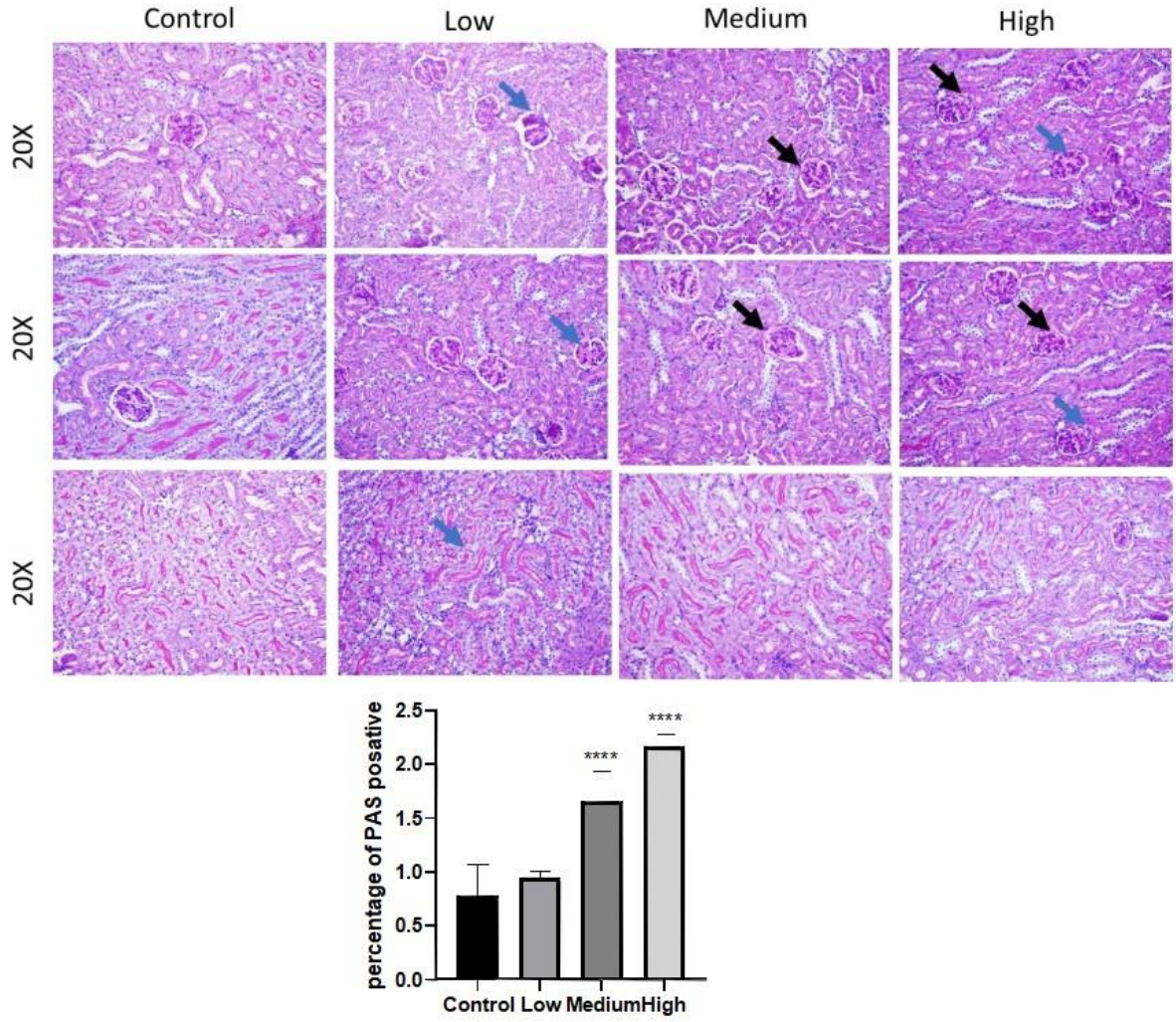


Figure:10 Periodic Acid-Schiff (PAS) staining revealed normal kidney histology incontrolsandsignificantlyincreasedglomerularlesionsinthetreatedgroup,characterized by glomerular hypertrophy, global mesangial suppression (blue arrow),andbasementmembranethickening(blackarrow).Magnification:20X.

4.6 Haematoxylin and Eosin (H&E) Staining

Histopathological analysis of kidney sections stained with **Haematoxylin and Eosin (H&E)** demonstrated marked differences between the **control** and **Polystyrene Microplastic (PSMP)-exposed groups**.

In the **control group**, renal tissue exhibited **normal histological architecture**, including well-defined **glomeruli**, intact **renal tubules**, and **minimal interstitial infiltration**, indicating a healthy kidney morphology.

However, mice treated with **low, medium, and high doses** of **Polystyrene Microplastics** showed **progressive histological damage**. Observations included:

- **Glomerular degeneration and shrinkage**,
- **Tubular dilation and epithelial disruption**, and
- **Interstitial inflammation**, suggestive of **nephritic changes**.

The severity of these alterations appeared to correlate with the **dose of PSMPs**, with the **high-dose group** exhibiting the most pronounced renal damage. These pathological changes are consistent with the onset of **nephritis or early-stage chronic kidney disease**, indicating that **prolonged exposure to PSMPs may compromise renal structure and function**.

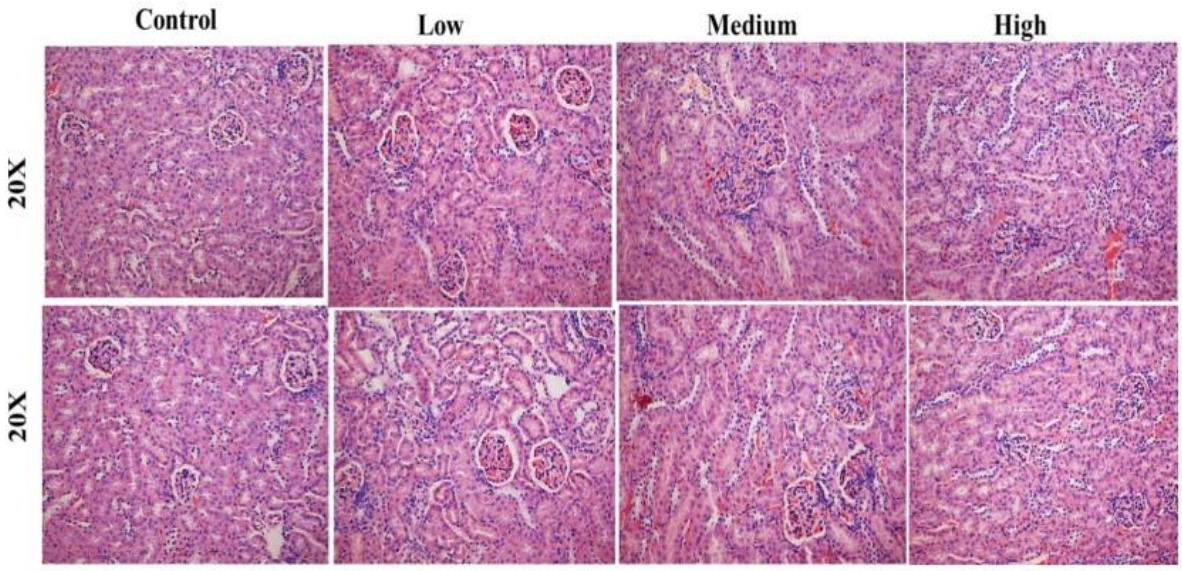


Figure: 11 Histopathological analysis of Kidney tissue sections (H&E staining) shownormal glomeruli and tubules in the control group, mesangial expansion in the low dosetreatmentgroup(blackarrow),mesangialconstriction(blackarrow)andtubularda mage (blue arrow)inthe mediumandhighdosetreatmentgroups.

5. Discussion

The present study investigated the **histopathological changes in renal tissue** resulting from **Polystyrene Microplastic (PSMP) exposure**, aiming to elucidate its potential **nephrotoxic effects**. The findings indicate that PSMPs can cause **significant structural and functional kidney alterations**, suggesting a substantial risk to renal health following environmental or dietary exposure.

Histological evaluation revealed **dose-dependent renal damage**, including **glomerular atrophy**, **tubular degeneration**, and **interstitial fibrosis**. These pathological alterations mirror those observed in response to various environmental pollutants and synthetic materials, reinforcing the notion that **PSMPs may be equally deleterious**.

The **glomerular atrophy** observed suggests **reduced filtration capacity** and **compromised glomerular function**, potentially leading to **proteinuria** and progressive **renal insufficiency**. This damage may be linked to **oxidative stress and inflammation** induced by PSMPs, as supported by elevated markers of oxidative damage and pro-inflammatory mediators in exposed groups.

Furthermore, **tubular degeneration and vacuolization** were prominent features in exposed animals. These changes imply **apoptotic injury of tubular epithelial cells**, reducing their capacity for **reabsorption and secretion**, which are vital for maintaining fluid and electrolyte balance. The presence of **tubular casts and dilatation** further substantiates the **toxic impact on tubular structures**, with implications for both **acute kidney injury (AKI)** and **chronic kidney disease (CKD)** development.

A particularly concerning finding was **interstitial fibrosis**, marked by excessive extracellular matrix deposition. As a key indicator of **chronic renal damage**, fibrosis reflects **irreversible tissue remodeling** and diminished renal function. The **fibrogenic process** may be driven by **chronic inflammation**, fibroblast activation, and **upregulation of profibrotic cytokines**, all of which were likely triggered by sustained exposure to Polystyrene particles.

Mechanistically, the **nephrotoxicity of PSMPs** appears to be **multifactorial**. Central to this process is **oxidative stress**, as evidenced by increased levels of **reactive oxygen species (ROS)** and lipid peroxidation products. ROS can disrupt cellular homeostasis, damaging lipids, proteins, and DNA, ultimately leading to **cell death and tissue dysfunction**. In parallel, a robust **inflammatory response**, characterized by immune cell infiltration and cytokine production, likely exacerbates the extent of renal injury.

These findings carry **important public health implications**, especially in light of the **increasing environmental and dietary exposure to microplastics and nanoplastics**. Polystyrene, as a prevalent plastic pollutant, may pose a hidden but significant **risk to kidney health**, particularly in individuals with existing renal impairments.

However, several limitations should be acknowledged. This study employed a **murine model**, and although mice provide useful insights into mammalian toxicology, the direct **translation of findings to human physiology** remains to be fully validated. Furthermore, this investigation focused on **short-term exposure (28 days)**; the **long-term effects of chronic, low-dose exposure** were not examined and should be the subject of future research.

To fully understand the renal impact of PSMPs, future studies should:

- Elucidate the **molecular pathways** underlying PSMP-induced kidney damage;
- Investigate **sex-specific differences** in response to exposure;
- Explore **therapeutic or preventive interventions**;
- And assess **chronic exposure scenarios** that better mimic real-world environmental conditions.

6. Conclusion

In conclusion, this study underscores the **nephrotoxic potential of Polystyrene Microplastics**, as demonstrated by significant **histopathological alterations**, including **glomerular mesangial expansion**, **tubular degeneration**, and **interstitial fibrosis**. These findings raise serious concerns about the **health risks posed by plastic pollution**, particularly in the context of renal health. As microplastics continue to accumulate in our environment and food chain, it is imperative to expand research into their **biological impacts** and develop strategies to **minimize exposure and mitigate harm**. Greater public awareness and policy action are also essential to address the broader implications of **plastic contamination in ecosystems and human health**.

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