Research Article

Biochemical, Histological and Molecular Docking into the Impact of Polystyrene Nanoplastics on Antioxidant Enzymes and Testicular Health in Rats

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Abstract | Polystyrene nanoplastics (NPs) are widespread environmental pollutants that may pose health hazards. This study examined the impact of NPs exposure on antioxidant enzyme activity and testicular structure in rats. A total of 24 *Rattus norvegicus* (n=6) were allocated into four groups and administered varying NPs concentrations (0, 1, 2, and 4 µL/kg) via oral gavage for 35 days. After the exposure period, the rats were sacrificed to assess superoxide dismutase (SOD) and catalase (CAT) levels, as well as testicular histology. The findings indicated that NPs exposure did not significantly alter SOD levels but led to a significant decline in CAT levels at the highest concentration (4 µL/kg, $p<0.05$). Histological assessment showed reduced seminiferous tubule diameter and epithelial thickness, along with a significant reduction in spermatocyte and spermatid counts, indicating disruption in later stages of spermatogenesis. Complementary molecular docking analysis revealed that the polystyrene monomer had a stronger binding affinity for the catalase enzyme than for superoxide dismutase, supporting the observed reduction in CAT activity. These results highlight the potential adverse effects of NPs on reproductive health and raise concerns about human exposure risks.

Keywords | Antioxidant, Health, Histology, Polystyrene nanoplastics, Spermatogenesis, Testicles

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INTRODUCTION

Plastic pollution is a growing global environmental concern, with microplastics (MPs) and nanoplastics (NPs) now widespread in various ecosystems. NPs, in particular, have garnered attention for their extremely small size and their ability to penetrate biological systems at the cellular and molecular levels (Kihara *et al.*, 2021; Brandts *et al.*, 2020). Ranging from 1 to 1000 nanometers, NPs are primarily formed through the breakdown of larger plastics due to environmental factors like UV radiation, mechanical stress, and chemical degradation (Chamas *et al.*, 2020; Mikael *et al.*, 2025; Pfohl *et al.*, 2025). They have been detected in water, soil and even atmospheric dust, raising serious concerns about their impact on both wildlife and human health (Allen *et al.*, 2022; Cheng *et al.*, 2023; Khanna *et al.*, 2024).

Because of their nanoscale size, NPs can enter the body via ingestion, inhalation, or skin contact (Yee *et al.*, 2021;

Ramsperger *et al*., 2023). Once absorbed, they can cross biological barriers such as the intestinal lining and blood–brain barrier, allowing for systemic distribution and accumulation in key organs (Ali *et al*., 2024; Yee *et al*., 2021). Research indicates that NPs can accumulate in the liver, kidneys, and reproductive organs, where they may disrupt normal physiological functions (Hayati *et al*., 2024; Zhang *et al*., 2024). The male reproductive system is especially vulnerable due to the high sensitivity of testicular tissue to oxidative stress and inflammation (Wang *et al*., 2018).

At the cellular level, NPs can enter cells via endocytosis—a process by which nanoparticles are engulfed by the cell membrane and enclosed in vesicles called endosomes (Gao *et al*., 2023; Li *et al*., 2022). These endosomes later fuse with lysosomes, where the nanoparticles may undergo partial degradation or be released into the cytoplasm, potentially causing cytotoxic effects (Lu *et al*., 2022; Franco-Juárez *et al*., 2022). The presence of polystyrene NPs in the cytoplasm has been linked to disruptions in cellular homeostasis, oxidative stress induction, inflammation, and interference with critical metabolic pathways (Lee *et al*., 2022).

One of the main mechanisms of NPs toxicity is the induction of oxidative stress—an imbalance between the production of reactive oxygen species (ROS) and the capacity of antioxidant systems to neutralize them (Wang *et al*., 2023; Soepriandono *et al*., 2025). ROS, such as superoxide radicals (O_2^-) and hydrogen peroxide (H_2O_2), are normal by products of cellular metabolism, but in excess, they can damage lipids, proteins, and DNA, leading to cell dysfunction and death.

The body possesses antioxidant enzymes like superoxide dismutase (SOD) and catalase (CAT) to manage oxidative stress (Gao *et al*., 2023; Kadac-Czapska *et al*., 2024). SOD converts superoxide radicals into H_2O_2, which is then broken down into water and oxygen by CAT. Exposure to NPs can impair this defence system. For instance, high-dose NPs exposure (4 µL/kg) significantly reduces CAT activity, decreasing the ability to detoxify H_2O_2 and increasing susceptibility to oxidative damage.

In addition to biochemical disruptions, NPs also cause structural alterations in the male reproductive system, particularly the testes. The testicular environment is highly specialized for the process of spermatogenesis—the differentiation of spermatogonia into matures spermatozoa within the seminiferous tubules (Hayati *et al*., 2022). Disruptions in this process can seriously impact fertility. Histological analyses have shown that NPs exposure reduces the diameter and epithelial thickness of seminiferous tubules, especially at higher doses (Li *et al*., 2022), and decreases the number of spermatocytes and spermatids (Zhang *et al*., 2024). These disruptions are likely triggered by oxidative stress, which induces germ cell apoptosis and damages the testicular microenvironment.

Moreover, NPs can alter the surface charge of cell membranes, increasing their affinity for ROS (Chelsea and Phoebe, 2023; Holloczki and Gehrke, 2019). Smaller particles generate more ROS and elicit immune responses, including the activation of phagocytes and the innate immune system (Zhang *et al*., 2024). The combination of oxidative stress and autophagy disruption exacerbates testicular cell apoptosis and reproductive toxicity (Wang *et al*., 2023; Forrester *et al*., 2018; Juan *et al*., 2021).

Although growing evidence demonstrates the adverse effects of NPs on reproductive health, significant knowledge gaps remain regarding their influence on antioxidant enzyme activity and testicular histopathology (Hayati *et al*., 2022). Understanding how these nanoparticles affect male fertility at both biochemical and histological levels is essential for assessing the long-term consequences of NPs exposure (Li *et al*., 2022; Iftikhar *et al*., 2021). Therefore, this study aims to investigate the effects of NPs exposure on antioxidant enzyme activity and the structural integrity of the seminiferous tubules, including the number of spermatogenic cells, in rats. *In silico* predictions using molecular docking experiments of nanoplastic polystyrene monomers were also used to determine the type of chemical interactions that occur in the test ligand-receptor complexes in rats. By uncovering the mechanisms of NPs-induced reproductive toxicity, this research is expected to provide important insights into the potential health risks of NPs contamination and contribute to broader efforts to mitigate its impact on the environment and human health.

MATERIALS AND METHODS

Material and Chemical

Polystyrene nanoplastics (NPs) were sourced from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany). The SOD and CAT assay kits were obtained from Bioassay Technology Laboratory (Shanghai Korain, Shanghai, China). All chemicals used in this study were of analytical grade. Molecular docking experiments were carried out on a Dell Vostro 14 3000 laptop equipped with an Intel® AMD Ryzen 5 3500U processor and Radeon Vega Mobile Gfx (2.10 GHz), running Windows 10 Ultimate 64-bit. The styrene monomer of polystyrene was retrieved from the PubChem database (https://pubchem.ncbi.nlm.nih.gov), while the SOD (UniProt ID: P07632) and CAT (UniProt ID: P04762) protein structures from Rattus norvegicus were obtained via the UniProt database (www.uniprot.org). Receptor preparation, including ligand purification,

was conducted using PyMol v1.74 software (Schrödinger, LLC, USA). Molecular docking was performed with PyRx 0.8 software (SourceForge, San Diego, California, USA). The docking results were validated using the CABS-flex web server (https://biocomp.chem.uw.edu.pl/CABSflex2/index), and ligand–receptor interactions were visualized using BIOVIA Discovery Studio 2019 (Dassault Systèmes BIOVIA, San Diego, California, USA).

Animal

Eight-week-old male Winstar rats (Rattus norvegicus) weighing 150 ± 25 grams were procured from the Faculty of Veterinary, Universitas Airlangga, Surabaya, Indonesia. Each rat was accommodated in a 30 × 40 × 15 cm box with a spacious wire lid. The rats were housed in an environment maintained at approximately 20 °C and subjected to a12-h light/12-h dark cycle. Throughout the entire duration of the experiment, the rats had unrestricted availability to both food and water. All procedures for the maintenance and treatment of animals were approved by the Ethics Committee of the Faculty of Dental Medicine, Universitas Airlangga (No. 440/HRECC.FODM/VII/2022), as a commitment to ethical principles and safety standards in laboratory animal research.

Experimental Design

After a two-week acclimation period, twenty-four rats were randomly assigned to four groups: a control group receiving water (G0) and three treatment groups exposed to NPs at doses of 1 µL/kg (G1), 2 µL/kg (G2), and 4 µL/kg (G3), respectively. NPs was administered orally through gavage for 35 days. After the treatment period, the rats were euthanized. The research methods for experimental design adopted from Susilo *et al.* (2025), in this research modified the NP doses with 1, 2, and 4 µL/kg to know the effect of the lower doses of NP from the previous research (Susilo *et al.*, 2025).

Determination of Antioxidant Enzymes

Whole blood samples were collected and centrifuged at 3000 rpm for 10 minutes at 4 °C to separate the serum, as described by Daud *et al.* (2022). The concentrations of SOD and CAT enzymes were measured using a commercial ELISA kit (Bioassay Technology Laboratory, Shanghai, China) following the manufacturer's protocol. Absorbance was read at 450 nm using an ELISA reader, and the resulting optical density (OD) values were converted to enzyme concentrations using a pre-established standard curve.

Histological Assessment

Testicular tissue was fixed in 10% neutral buffered formalin to preserve histological structure, then processed through a graded dehydration series and embedded in paraffin. The paraffin blocks were sectioned using a microtome at a thickness of 4–5 µm, as described by Hayati *et al.* (2024). Tissue sections were stained with routine Haematoxylin and Eosin (H&E) to observe cellular structures. The preparations were examined under a light microscope (Olympus, Tokyo, Japan), and micrographs were documented using a digital camera (Optilab Advanced Plus, Miconos, Yogyakarta, Indonesia). Morphometric analysis was performed at 100× magnification, including the counting of spermatogenic cells and measurement of the diameter and epithelial thickness of seminiferous tubules using microscopic image processing software.

Molecular Docking Experiment

Molecular docking was carried out using PyRx 0.8 software (SourceForge, San Diego, California, USA) to assess the binding affinity between ligands and target receptors (Alifiansyah *et al.*, 2024). A more negative binding affinity indicates a stronger potential for interaction with the receptor, which may result in a physiological effect (Herdiansyah *et al.*, 2024).

Validation and Visualization of Docking Complexes

Docking results were validated using the CABS-flex web server (https://biocomp.chem.uw.edu.pl/CABSflex2/index), based on parameters such as protein rigidity, restraints, C-alpha and side-chain restraint weights, trajectory, temperature range, number of cycles, and RNG seed (Shivanika *et al.*, 2020). Visualization of ligand–receptor interactions and identification of the bond types involved were performed using BIOVIA Discovery Studio 2019 (Dassault Systèmes BIOVIA, San Diego, California, USA).

RESULTS AND DISCUSSIONS

Levels of Antioxidant Enzymes

NPs particles can induce toxicity by increasing ROS levels, which trigger oxidative stress within cells. This increase in ROS can disrupt various metabolic processes and enzyme functions, ultimately potentially leading to cell death (Gopinath *et al.*, 2021). ROS induce damage to cellular components such as lipids, proteins, and DNA, causing molecular fragmentation and reducing the structural integrity of cells (Huerta-Garcia *et al.*, 2020). Lipid peroxidation triggered by ROS disrupts cell membranes, particularly those rich in polyunsaturated fatty acids (PUFAs), making them highly vulnerable to continued damage, which leads to apoptosis signalling and inflammatory responses (Gao *et al.*, 2013).

In this study, although ROS levels increased due to NP exposure, SOD activity remained stable, while CAT activity declined. ROS generated in the form of superoxide anions

(O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (•OH) stimulate the cellular antioxidant defence system through the activation of antioxidant enzymes such as SOD and CAT. SOD functions to convert O_2^- into H_2O_2, and despite the increase in ROS, the activity of this enzyme did not show a significant decrease due to its structural resistance to oxidation and its role as the first line of defence against oxidative stress (Gopinath *et al.*, 2021). In fact, some studies have shown that SOD can be transcriptionally induced in response to mild to moderate oxidative stress (Gopinath *et al.*, 2021; Huerta-García *et al.*, 2020). Conversely, CAT activity, which is responsible for neutralizing H_2O_2, is more susceptible to increased ROS. Accumulation of H_2O_2 produced by SOD leads to oxidation of the heme group or active residues of CAT, thus reducing its effectiveness in decomposing H_2O_2 (Wang *et al.*, 2023). In addition, damage to the peroxisomal membrane, where CAT activity occurs, due to lipid peroxidation also affects the performance of this enzyme.

The measured levels of SOD and CAT enzymes are shown in Figure 1. It displays the enzyme activity levels of SOD and CAT in rats after exposure to various NP concentrations at G0, G1, G2, and G3 group. For SOD, the control group showed the highest SOD activity, while the NP-exposed groups (G1, G2, and G3) experienced a decrease in SOD levels, although the difference appeared statistically insignificant. However, for CAT, G0, G1, and G2 groups exhibited relatively stable CAT activity, while the G3 group showed a significant decrease, indicated by an asterisk (*), signifying a statistically significant difference.

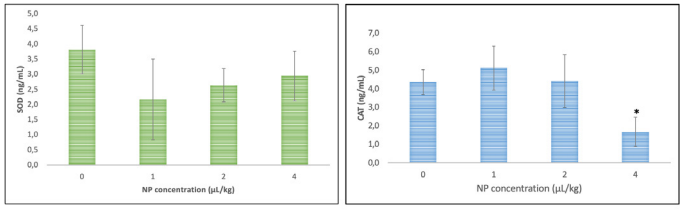

Figure 1: SOD and CAT levels after exposure to variations in NP concentrations.

SOD and CAT work in tandem to protect against oxidative stress. SOD converts superoxide radicals to hydrogen peroxide, which is then decomposed by CAT into water and oxygen. A decline in their activity signals oxidative imbalance, with CAT particularly sensitive to ROS accumulation. However, since no statistically significant reduction in SOD levels was found, this suggests that although the antioxidant function is affected by NP exposure, the impact may be dose-dependent or require longer exposure to become critical. In contrast, the significant reduction in CAT levels at the highest NP dose (4 µL/kg) from G3 group indicates a threshold effect, where high NP concentrations seriously impair the enzyme's ability to neutralize hydrogen peroxide. This finding is consistent with research indicating that excessive ROS accumulation due to impaired CAT function can lead to oxidative damage, lipid peroxidation, and mitochondrial dysfunction (Zheng *et al.*, 2020). These results suggest that high-dose NP exposure can overwhelm the endogenous antioxidant defence system, increasing oxidative stress and potentially causing cellular damage. Further research is needed to investigate the underlying mechanisms of NP-induced oxidative stress and explore protective strategies, such as antioxidant supplementation, to mitigate its negative effects.

Exposure to NP at a concentration of 4 µL/kg did not cause a statistically significant effect (P>0.05). This was also observed in testis weight and SOD levels. Except for the significant decrease in CAT levels at 4 µL/kg, no significant differences were found between the control and treatment groups. However, body weight, testis weight, SOD levels, and CAT levels all tended to decrease with increasing NP concentrations (Figure 2).

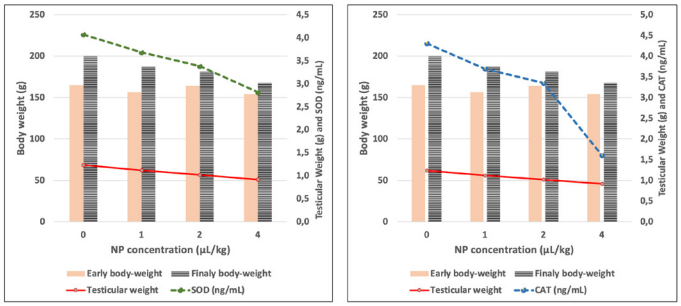

Figure 2: The correlation between the initial body weight, final weight, testicular weight, SOD levels, and CAT levels following exposure to different concentration of NP.

Figure 2 illustrates the relationship between NP concentrations and various physiological and biochemical parameters, including initial and final body weight, testis weight, as well as levels of the antioxidant enzymes SOD and CAT in male rats. In the left panel, although there is some fluctuation in body weight from the beginning to the end of the treatment, the differences are not particularly significant across all groups. However, testis weight shows a gradual decline with increasing NP concentrations. This trend suggests that NP exposure may not significantly affect overall body weight. However, testicular tissue appears more vulnerable to damage, particularly with prolonged exposure.

The decrease in testis weight correlates with a progressive decline in SOD levels, which is evident from the control group to the group receiving the highest dose (4 µL/kg). Although the reduction in SOD activity is not sharply pronounced, the trend supports the presence of oxidative stress as a response to NP exposure. Meanwhile, the

right panel shows a similar pattern for testis weight, but the most striking finding is the sharp reduction in CAT levels, particularly in the group exposed to the highest NP concentration. CAT activity drops significantly at the 4 µL/kg dose, suggesting that ROS accumulation, especially hydrogen peroxide (H_2O_2), has exceeded the neutralizing capacity of the antioxidant system. Excess H_2O_2 that is not neutralized by CAT may trigger lipid peroxidation and cellular damage, particularly in PUFA-rich tissues such as the testes (Wang *et al*., 2023; Gopinath *et al*., 2021).

Overall, this study revealed that NPs exposure has a negative impact on male reproductive health, as evidenced by reduced testis weight and disrupted antioxidant enzyme activity. The significant decline in CAT activity at the highest dose indicates that the antioxidant defence system is under substantial oxidative stress, which could directly affect spermatogenesis and the structural integrity of the testes.

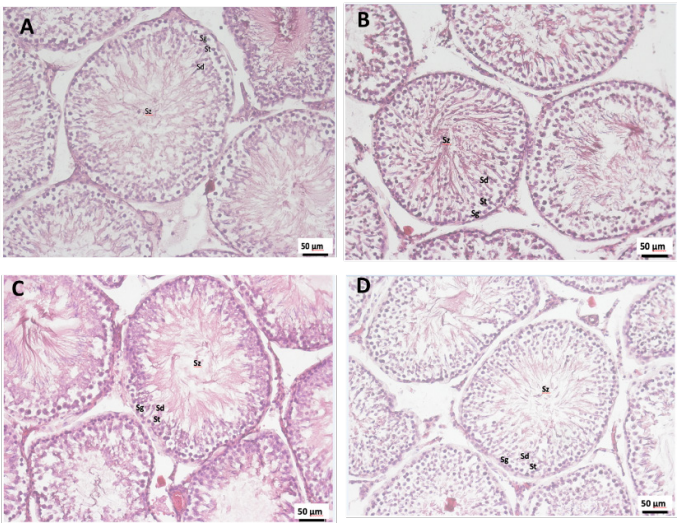

Figure 3: Testicular Histology of Rattus norvegicus at 100x magnification (A) Control = 0 µl/kg NP; (B, C and D) = 1, 2, and 4 µL/kg NP respectively.

Histological Assessment

The histological evaluation of the testes, which revealed no gross morphological changes, was conducted to analyze the effects of NP exposure on antioxidant levels and gonadal structure in rats, based on several considerations (Gao *et al*., 2023). Histological analysis showed structural changes in the testes, particularly in the group exposed to the highest NP dose (4 µL/kg). Although no macroscopic abnormalities were observed (Figure 3), quantitative measurements revealed a significant reduction in the size of seminiferous tubules, including decreased epithelial thickness and diameter (Figure 4). These reductions correspond to decreased CAT activity, suggesting oxidative stress plays a central role in testicular damage.

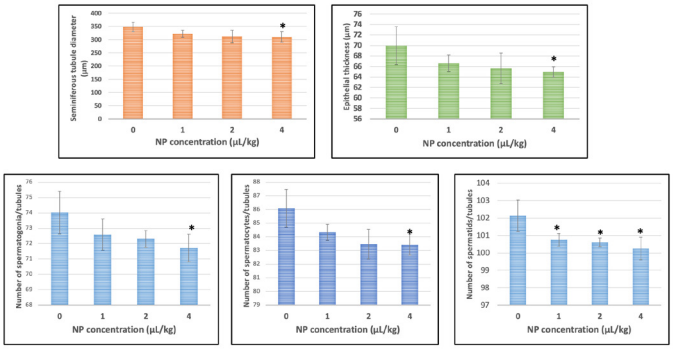

Figure 4: Thickness and diameter of the epithelium of testicular seminiferous tubule and the number of spermatogenic cells after subjected to different NP concentrations.

However, when measurements and counts of spermatogenic cells were conducted, the observations showed that variations in NP concentration did not significantly affect the number of spermatogonia between the control and treatment groups (G0, G1, G2, and G3). This may be attributed to the complex nature of the spermatogenesis process, in which spermatogonia differentiate into spermatocytes, undergo meiosis to form spermatids, and eventually mature into spermatozoa. Each of these developmental stages may have differing sensitivities to NP exposure (Gao *et al*., 2023).

This study also demonstrated that the number of spermatocytes and the size of seminiferous tubules (epithelial thickness and diameter) decreased in response to the highest NP exposure (4 µL/kg NP), while the number of spermatid cells significantly declined following NP exposure (Figure 4). These findings suggest that NP may disrupt meiosis and spermatocyte development. The reduction in seminiferous tubule size is likely associated with the loss of spermatocytes, as these cells contribute to the overall structure and size of the tubules (Liang *et al*., 2024; Triwahyudi *et al*., 2023).

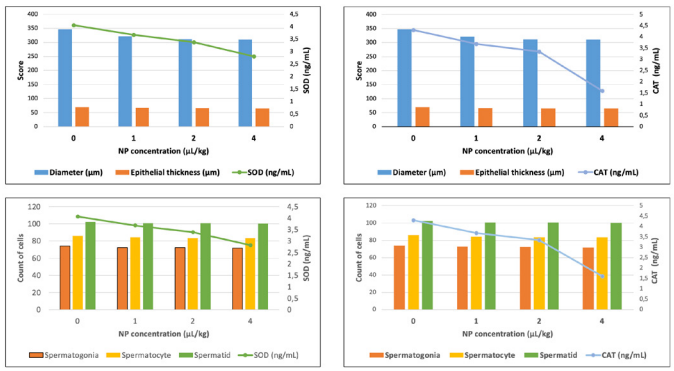

Figure 5: Relationship between size and number of spermatogenic cells on SOD and CAT levels exposed to NP.

Figure 5 illustrates the relationship between the size and number of spermatogenic cells with SOD and CAT lev-

els following NP exposure. It shows a trend of decreasing SOD and CAT levels, accompanied by a reduction in the size of seminiferous tubules in the testes. This also applies to the number of spermatogenic cells (spermatogonia, spermatocytes, and spermatids), which declined in parallel with the decrease in SOD and CAT levels as NP concentration increased. Therefore, it can be inferred that NP exposure induces oxidative stress (Zhang *et al*., 2024; Ferrante *et al*., 2022; Arif *et al*., 2022).

The mechanisms of oxidative stress and testicular apoptosis induced by NP in male rats demonstrate that NP exposure leads to decreased expression of antioxidant enzymes, particularly CAT, accompanied by increased apoptosis in testicular tissue. The tolerance of spermatogonia to oxidative stress associated with high NP levels indicates that spermatogonia possess greater resilience to reactive oxygen species. Taken together, the findings support that oxidative stress from NP exposure impairs testicular structure and function, particularly during spermatogenesis.

Molecular Docking Experiment Results of Polystirene Monomer Againts SOD and CAT Receptors

The molecular docking experiment of polystyrene monomer in the form of styrene shows that the binding value between the ligand complex and the receptor tends to bind to the active side of chain A in each test receptor. In the supeorxide dismutase receptor, the active binding side is at chain A coordinates (X: 2.317; Y: 2.330, and; Z: 1.514). Binding affinity of the styrene-SOD complex (-4.1 kcal/mol) has a smaller value than that of the styrene-CAT complex (-5.3 kcal/mol). The styrene-SOD complex binds by Van der Walls to the amino acids Glu78(A), Asp77(A), Arg80(A), Val80(A), His68(A), and His111(A). The bonding contact between each atom in the Van der Walls bond involves the force of attraction between molecules. This bond occurs due to the weak intermolecular polarization of the receptor and test ligand (Yunta, 2016). In addition to the Van der Walls bond, the styrene-SOD complex also binds in Pi-sigma at amino acid Val104 (A) and Pi cation at amino acid Lys70 (A). Meanwhile, the styrene-CAT complex has Van der Walls bonds at amino acids Phe266(A), Ala79(A), Gly80(A), Ala81(A), and Asn321(A). The active binding site of styrene-CAT complex is at chain A coordinates (X: -6.681; Y: 2.361; Z: -1.795). Just like the styrene-SOD complex, the styrene-CAT complex also binds actively to chain A. The main difference in these two complexes is seen in the presence of binding to the amino acids Leu265(A), Pro322(A), and Leu262(A) which are hydrophobically bound to the receptor. Hydrophobic bonds can increase the binding affinity value of the complex and strengthen the chemical bond (Herdiansyah *et al*., 2024) (Table 1 and Figure 6).

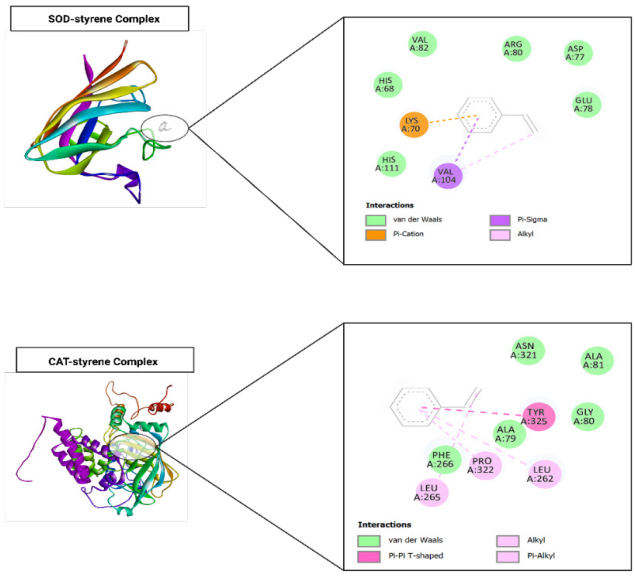

Figure 6: Interaction of ligand-receptor complexes.

The molecular dynamics results using CABSFlex webserver show that the interaction of styrene and receptor complexes both have RMSF values <3 Å (Figure 7). The SOD-styrene complex tends to be more stable than the CAT-styrene complex with an RMSF value of <2 Å. Fluctuation stability in the form of good atomic constituent ligand and protein interactions has an RMSF value of <3 Å (Wijaya *et al*., 2021).

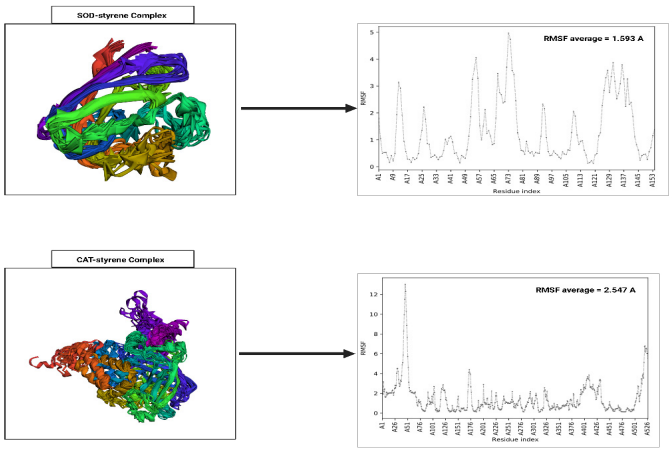

Figure 7: Molecular dynamics results of each complex.

While the findings provide valuable insights into the oxidative and histological alterations induced by polystyrene nanoplastics in male rats, some important limitations must be acknowledged. This study has several limitations, including the relatively short duration of exposure and a lack of direct fertility outcome assessments such as sperm motility or hormonal levels. Moreover, the sample size, although appropriate for a preliminary study, limits the generalizability of the findings. Future studies should investigate the long-term effects of NP exposure, explore different types of nanoplastics, assess the role of dietary antioxidant interventions,

Table 1: Molecular docking experiment results.

Compound	Receptor	Docking Coordinates			Complex Binding Affinity (kcal/mol)	Type of Interaction	Amino Acids Involved in Interaction
		X	Y	Z			
Styrene	Superoxide dismutase (SOD)	2.317	2.330	1.514	-4.1	Van der Walls	Glu78(A), Asp77(A), Arg80(A), Val82(A), His68(A), His111(A)
						Pi-sigma	Val104(A)
						Pi-cation	Lys70(A)
	Catalase (CAT)	-6.681	2.361	-1.795	-5.3	Van der Walls	Phe266(A), Ala79(A), Gly80(A), Ala81(A), Asn321(A)
						Pi-Pi T-shaped	Tyr325(A)
						Alkyl	Leu265(A), Pro322(A), eu262(A)

and evaluate fertility outcomes including sperm quality and mating success. Such approaches would provide a more comprehensive understanding of the reproductive toxicity of NPs. Addressing these limitations in future research will not only strengthen the scientific evidence but also support the development of preventive strategies to mitigate nanoplastic-induced reproductive risks.

CONCLUSIONS AND RECOMMENDATIONS

Exposure to NP at a dose of 4 µL/kg resulted in a decline in CAT levels and modifications in testicular histology, characterized by a reduction in seminiferous tubule size and a decreased number of spermatogenic cells. Beside of that, the molecular docking experiment also revealed that the monomer of polystyrene has the best binding affinity on catalase enzyme rather than on superoxide dismutase enzyme. These indicate that NP exposure may interfere with spermatogenesis and contribute to reproductive toxicity associated with oxidative stress.

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NOVELITY STATEMENT

This study is among the first to evaluate the effects of polystyrene nanoplastics (NPs) on the antioxidant defense system and histopathological changes in testicular tissue in *Rattus norvegicus*. Additionally, we employed molecular docking to investigate potential binding interactions between styrene monomer and antioxidant enzymes (SOD and CAT), offering mechanistic insight. This dual approach highlights the mechanistic link between oxidative stress and male reproductive toxicity due to NP exposure.

AUTHOR'S CONTRIBUTIONS

AH and MP conceptualized and supervised the study. AH, MP, and HS designed the experiment and methodology. FRA, LP, and SM were responsible for data collection and laboratory analysis. WA, FPD, and RM performed data analysis, figure preparation, and literature review. All authors contributed to writing, reviewing, and approving the final manuscript.

Conflict of Interest

The authors have declared no conflict of interest.

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