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Original Article

Multi-biomarker approach to assess oxidative stress and antioxidants profile in male albino rats exposed to ZnO nanoparticles

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Abstract

ZnO Nanoparticles (ZnO NPs) have wide applications in many fields of life ranging from health, food, agriculture, veterinary medicine, biotechnology, public health, textile and cosmetics. However, exposure to these NPs poses risks to public health, non-target living organisms and the environment. Hence, this study assessed toxicological impacts of ZnO NPs on hematopoietic tissues (bone marrow) and different visceral organs like lungs, intestine and muscles of male Wistar albino rats. Twenty male (20) Wistar albino rats were placed in four groups such as T0 (control group), T1 (50 mg/kg/day ZnO NPs), T2 (75 mg/kg/day ZnO NPs), and T3 (100 mg/kg ZnO NPs). Treated rats exhibited different signs of toxicity like depression and anxiety at higher doses of ZnO NPs. The bone marrow and other visceral organs/tissues were removed and analyzed to know the status of oxidative stress and antioxidant biomarkers. Results revealed notable increase ($P \leq 0.05$) in contents of oxidative stress biomarkers (ROS and TBARS) and significant decrease ($P \leq 0.05$) in antioxidant enzymes (POD, SOD, CAT, and GSH) in bone marrow as well as lungs, intestine, and muscles (gums) tissues. Histopathological examination indicated degeneration of muscle fibers, atrophied cells and presence of inflammatory materials in muscles (gums) of treated rats. Histologically, lungs were edematous, hemorrhagic and showed severe interstitial pneumonia while necrosis of epithelium of villi along with degeneration of villi in intestine of rats were observed at higher doses of nanoparticles. In conclusion, it can be suggested that ZnO-NPs may induce oxidative stress in multiple visceral organs of albino rats at higher concentrations highlighting disruption of physiological mechanisms.

Keywords: Nanoparticles, Zinc oxide, Nanoparticle toxicity, Oxidative stress biomarkers

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Introduction

Nanoparticles (NPs) own unique physico-chemical properties and have wide-ranging applications in the fields of biomedicine (Saif et al., 2021; Elbehary et al., 2023), health, food, biotechnology, agriculture and textile industry as well as cosmetics (Bommakanti et al., 2022; Huang et al., 2023; Nazir et al., 2024). Recently, the researchers are using multi-biomarkers approach to unveil the exact mechanisms of impacts of the NPs on living organisms including mammals and plants particularly with respect to their safety and/or potential risks associated with the use of these particles on biological systems (Wu and Tang, 2018; Ali et al., 2024; Nazir et al., 2024). NPs can be obtained from a variety of precursors, dividing them into organic and inorganic forms. NPs are conjugated with various ligands via ionic, hydrophobic, and binding mechanisms a useful process known as bio-conjugation which serves as the foundation for many of their bio-medical applications (Saif et al., 2023; Yu et al., 2023; Hasan et al., 2024). Cancer diagnosis, drug administration, and other diagnostic and therapeutic applications of NPs have been bio-conjugated with antibodies (Abs), surface biomarkers, and medicines (Bisla et al., 2022; Saif et al., 2024). Albino rats have been frequently employed as animal model in toxicological studies owing to their physiological/functional similarity to humans as well as ease of handling (Khan et al., 2022; Khan et al., 2023; Saif et al., 2024). Male rats in particular are frequently employed to study the toxic effects of NPs on physiological parameters (Festing and Lutz, 2021; Saif et al., 2024).

ZnO nanoparticles (metal oxide) are considered as the second most frequently used nanoparticles after iron. Among different NPs, ZnO NPs are emerging metal oxide particles and their commercial demand has been increased in recently due to their versatile applications in broad range of fields (Raha and Ahmaruzzaman, 2022). ZnO NPs have better crystallinity and are much more stable. ZnO NPs have been used in medicinal field especially in the delivery of drugs owing to their unique characteristic of healing of wounds (Saghazadeh et al., 2018). ZnO and different other NPs are routinely and widely used in a variety of compounds such as paints, alloys, rubber, ceramics, cosmetics, food additives, sunscreens, and as well as in biological materials including medicines. The widespread usage of ZnO NPs draws attention because of the potential risks and toxicity levels of these NPs to the living organisms including environment. Different biological systems, such as bacteria, mammalian cells, and *in vivo* models have been used to assess the toxicity levels. ZnO NPs have been demonstrated to induce detrimental effects on mammalian cells leading to apoptosis, DNA damage, inflammatory response, and membrane injury (Yousef et al., 2019). The nanoparticles and other environmental contaminants may provoke oxidative stress which causes imbalance between ROS production and antioxidant defense mechanisms (Jabeen et al., 2021; Bello et al., 2023). ROS molecules which are oxygen free radicals are highly reactive and can lead to damage to different cellular components including DNA. The bone marrow and red blood cells are an integral part of the hematopoietic system and are highly prone to oxidative damage. Therefore, investigation of the toxic effects of ZnO NPs is vital in understanding potential risks involved with their usage (Samy et al., 2022).

The cytotoxic effects of these NPs have been reported in detailed investigations carried out using different animal models (Chong et al., 2021; Han et al., 2016). Studies have reported dose-dependent toxicity to cells while others found minimum to no cytotoxic effects of these NPs (Chen et al., 2019; Hanley et al., 2009; Namvar et al., 2015). In published literature, scanty information could be found regarding the investigation and monitoring of oxidative stress biomarkers as well as antioxidant enzymes (superoxide dismutase, SOD; catalase, CAT; peroxidase, POD) under different dosage levels of ZnO nanoparticles. The frequent and persistent use of ZnO nanoparticles causes threats to both environment and public health. Therefore, monitoring potential mechanisms of induction of injuries in exposed animals due to exposure to ZnO nanoparticles is crucial. Hence, this trial was executed to know the safety/toxicity of ZnO NPs.

Material and Methods

Experimental animals

For the experimental trial a total of 20 male Albino rats (*Rattus norvegicus*), weighing 100-150 gm were procured from laboratory animal house of the Islamia University of Bahawalpur. All the rats were active and free of any obvious clinical ailments. The rats were housed in clean wire cages and were provided

standard laboratory conditions throughout the experiment. Clean fresh water (tap water) and feed were provided ad-libitum to all the rats on daily basis. The rats underwent acclimatization period (10 days) before the start of experimental trial. All the rats were handled following the guidelines regarding the Use and Welfare of Laboratory Animals.

Experimental groups, dosage and observational parameters

After acclimatization period, the rats were randomly divided and placed in four different groups (T0, T1, T2, and T3) separately in wire cages equipped with feed and water. The rats in group T0 serve as control group while T1, T2 and T3 were treated (intraperitoneal route) with ZnO NPs @50mg/kg/day, 75mg/kg/day, 100mg/kg/day respectively for 15 days. The rats were observed for any obvious clinical disorders (behavior, posture, alertness, any apparent changes (skin and eyes) or secretions from the body.

Necropsy and tissue collection

All the rats including untreated control group and exposed to ZnO NPs were euthanized and necropsies were carried out for separation of various tissues. The tissues including intestine, lungs, muscle (gum) and bone marrow were removed and immersed in cold deionized water separately (Hussain et al., 2020). Approximately 0.1 gm of each visceral tissue was homogenized and processed for estimation of oxidative stress profile following the procedure (Akram et al., 2021; Iqbal et al., 1996) and various antioxidants (Akram et al., 2021; Kakkar et al., 1984).

The bone marrow was obtained from the femur bones and was centrifuged. The pellet containing cells was separated. After that, 10% lysate of bone marrow cells was prepared and was subjected to analyze various biomarkers including oxidative stress (reactive oxygen species; ROS) and thiobarbituric acid reactive substances; TBARS), and antioxidant enzyme (catalase; CAT), peroxidase; POD), and superoxide dismutase; SOD). A UV-Vis spectrophotometer was used to measure absorbance values for POD, SOD, CAT, ROS and TBARS taken at wavelengths 470 nm, 560 nm, 240 nm, 505 nm and 532 nm respectively.

Histopathological investigations

The collected visceral organs including intestine, lungs and muscle (gum) were fixed using 10% formalin solution for pathological lesions. After that, fixed specimens were processed using the traditional paraffin methods. Finally, 5-6 µm thick sections of study organs were cut with the rotary microtome. These sections were then dehydrated, embedded in paraffin wax, and stained using hematoxylin and eosin following standard histopathological procedures (Hussain et al., 2017; Anyogu et al., 2023). Tissue sections were examined for histopathological changes under light microscope.

Statistical analysis

The data on oxidative and antioxidant biomarker enzymes were statistically analyzed by applying ANOVA test using IBM statistical software package (SPSS). Tukey's test was used to compare the means of all studied parameters like oxidative and antioxidant enzymes to that of control group. $P \leq 0.05$ was used as the level of significance.

Results

Physical parameters

Various physical alterations like depression, anxiety, muscular weakness, lethargy and locomotory disorders were observed in rats administered high dose of ZnO NPs (T3).

Oxidative stress and antioxidant biomarkers in Lungs

The lungs of rats exposed to ZnO NPs in groups T2 and T3 exhibited significantly higher levels ($P \leq 0.05$) of ROS and TBARS compared to unexposed rats. The rats in group T3 showed significantly lower ($P \leq 0.05$) quantity of reduced GSH in lung tissue, along with notable decrease in various antioxidant enzymes (Fig. 1).

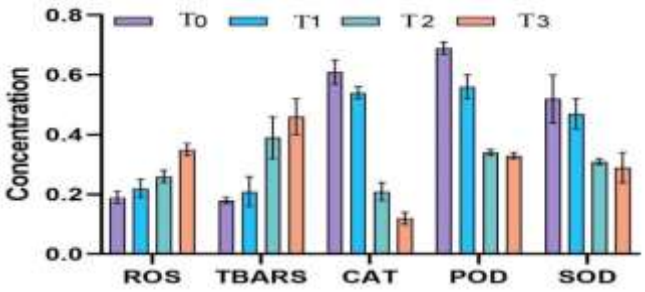

Figure-1. Photograph showing status of Oxidative stress and antioxidant profile in lungs of zinc oxide nanoparticles treated rats.

Oxidative stress and antioxidant biomarkers in intestine

The contents of ROS increased significantly (P≤0.05) in intestinal tissues of albino rats of treated group (T3). The contents of TBARS showed significant difference in the values of T2 and T3 groups treated with 75 mg/kg/day and 100 mg/kg/day of ZnO NPs respectively in comparison to healthy/untreated group. The antioxidant biomarkers (Fig. 2) including SOD, CAT and POD decreased significantly (P≤0.05) in the treated group (100 mg/kg/day).

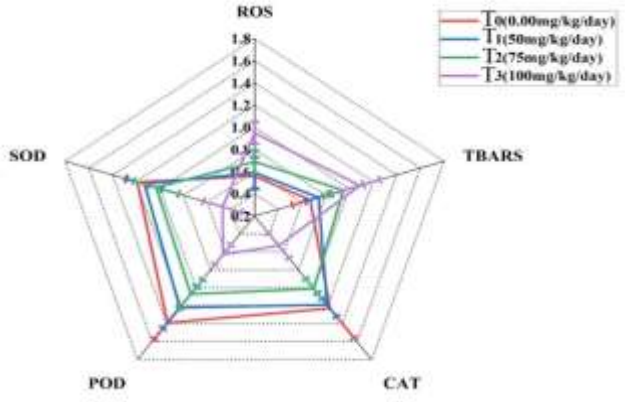

Figure-2. Oxidative and antioxidant profile of intestinal tissues of rats exposed to zinc oxide nanoparticles

Oxidative stress and antioxidant biomarkers in muscles (gums)

The ROS and TBARS values in the muscles (gum) increased noticeably in rats reared in group T3(100 mg/kg/day). The antioxidant enzymes decreased significantly at higher dosage (100mg/kg/day) level in gums of rats (Fig. 3).

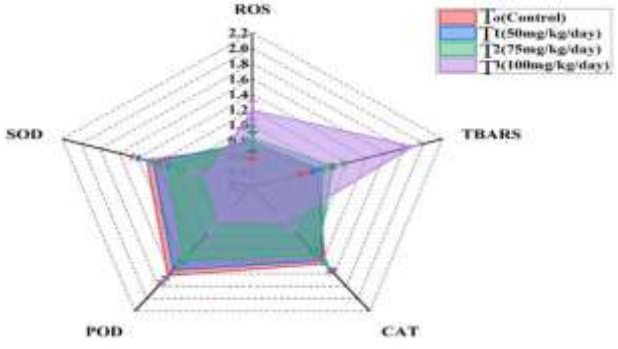

Figure-3. Oxidative and antioxidant profile in muscles (gums) of rats exposed to zinc oxide nanoparticles

Oxidative stress and antioxidant biomarkers in bone marrow

Figure 3 illustrates the effects of zinc oxide nanoparticles on the hematopoietic tissue (bone marrow cells) of rats specifically focusing on ROS and TBARS. The results recorded that the control group (T0) exhibited the lowest ROS levels at 0.17±0.07, suggesting a baseline for comparison. In contrast, the rats of treated group (100 mg/kg/day) demonstrated the highest ROS levels (0.45±0.04) indicating a potential dose-dependent increase in oxidative stress. Similarly, TBARS contents were notably elevated (0.92±0.05) in rats of group T3 compared to control group (0.33±0.08). The results on adverse effects of zinc oxide nanoparticles on bone marrow of rats measured in terms of antioxidant enzymes like CAT, POD, and SOD contents indicated significant changes. The contents of CAT in rats of control group (T0) displayed the highest value (0.62 ± 0.13) indicating robust antioxidant potential. Conversely, the treatment groups exhibited a notably lower contents of CAT suggesting a potential decrease in catalase functions in rats exposed to 100 mg/kg/day dosage. The contents of POD in rats of the control group (T0) had the highest value (0.45 ± 0.02) suggesting a baseline peroxidase functions. The rats of treated groups displayed decreased POD contents (0.41±0.13, 0.32±0.13, and 0.28±0.12) respectively signifying a potential diminishing trend in peroxidase functions. The contents of SOD were significantly lowered in rats of treated group in comparison to untreated rats (Fig 4).

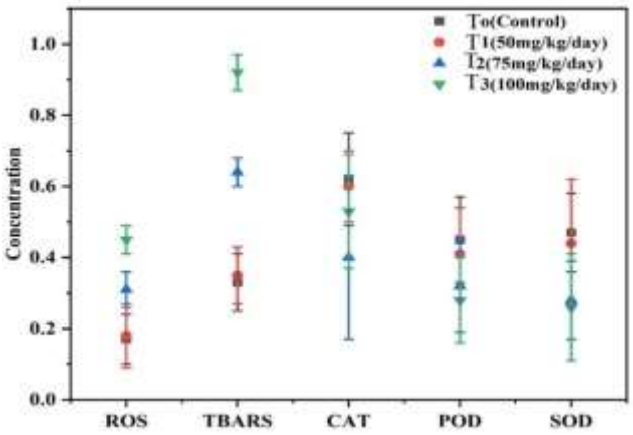

Figure-4. Effect of zinc oxide nanoparticles on Oxidative and antioxidant biomarkers on bone marrow of rats.

Histopathological studies

Lungs

The lungs of the unexposed T0 group displayed normal histological structures at microscopic level. However, various microscopic changes were evident in the lungs of rats exposed to ZnO-NPs. In groups T2 and T3, the lungs exhibited a spectrum of histopathological lesions, ranging from mild to moderate and severe changes including edema, hemorrhages, emphysemas, neutrophilic infiltration, interstitial pneumonia, cuffing of bronchioles, atelectasis, and degeneration of interstitial pneumonia. These findings are summarized in Table 1 and depicted in Figure 5.

Table-1. Assessment of histopathological lesions severity in lungs of albino rats exposed to different dosages of ZnO NPs

Parameter	Groups		
Lungs	**T1 50mg/kg /day**	**T2 75mg/kg/ day**	**T3 100mg/kg/ day**
Edema	+	++	+++
Hemorrhages	++	+++	+++
Emphysemas	++	+++	++++
Neutrophilic infiltration	+	++	+++
Interstial pneumonia	++	++	+++
Cuffing of bronchioles	++	++	+++
Atelectasis	++	+++	+++
Degeneration of Interstial pneumonia	++	+++	+++

Mild (+); moderate (++); severe (+++); very severe (+ +++)

Table-2. Severity of histopathological lesions in muscle (gum) of albino rats exposed to different dosages of ZnO NPs

Histopathological lesions	Groups/Treatment		
	T1 50mg/kg/ day	**T2 75mg/kg /day**	**T3 100mg/kg/ day**
Atrophy of myocytes	++	+++	+++
Necrosis of myocytes	+	++	+++
Inflammatory cell infiltration	+++	++	+++
Intrafiber necrosis	+	++	+++
Muscle mass loss	+	++	+++
Inflammatory cell infiltrate	+	++	++
Edema	+	++	+++
Degeneration of muscle fibers	++	+++	++++

Mild (+), Moderate (++), Severe (+++), Very severe (++++)

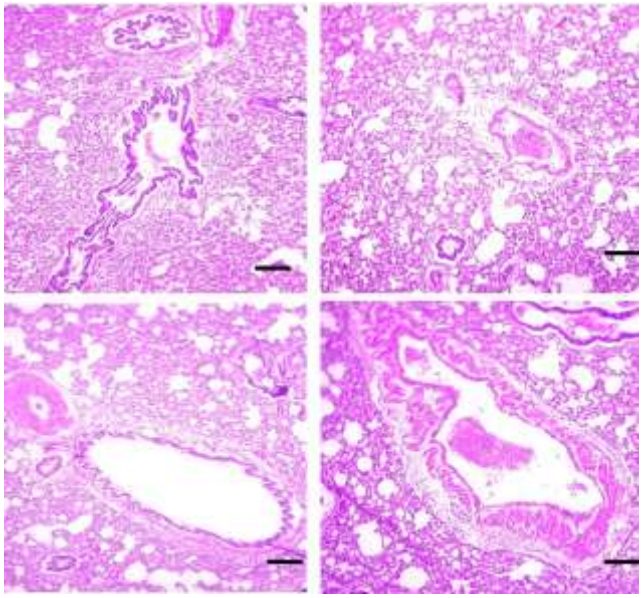

Figure-5. Photomicrograph showing different mild to moderate pathological alterations such as edema, hemorrhages, emphysemas, neutrophilic infiltration, and interstitial pneumonia in the lungs of ZnO NPs-treated rats in group T3. H & E stain; 400×.

Muscles

The mild to moderate histopathological alterations observed in this trial including, atrophy of myocytes, edema, inflammatory cell material, intra-fiber necrosis have been observed at low dosage levels. Higher dosage led to severe changes especially inflammatory cell infiltration, degeneration of muscle fibers (Fig 6; Table 2).

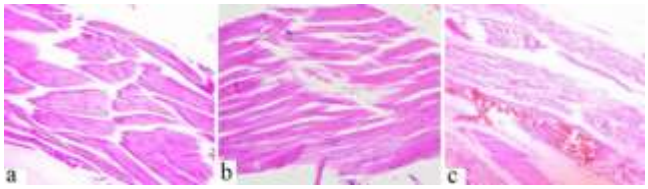

Figure-6: (a) Photomicrograph showing normal histological arrangement of muscles in the untreated control group of NPs. (B) Photomicrograph showing different pathological alterations (breakdown of muscle fibers and necrosis of myocytes) in the muscles of ZnO NPs-treated (@75mg/kg/day) rats. (C) Photomicrograph showing different pathological alterations (thin muscular fibers and hemorrhages) in the muscles of ZnO NPs-treated @100mg/kg/day) rats.

Discussion

Different behavioral alterations like anxiety, locomotory disorders, depression, muscular weakness and lethargy were observed in treated rats. These clinical alterations could be due to induction of oxidative stress in albino rats via generation of free radicals (Jarrar et al., 2021; Zhuo et al., 2024). The noticeable increased contents of oxidative stress biomarkers and lower contents of different antioxidant enzymes in bone marrow, muscles, lungs and intestinal tissues of ZnO NPs treated rats in this study could be related to induction of inflammatory process (Zhuo et al., 2024). Previously, different reports (Srisuvetha et al., 2020; Zhuo et al., 2024) described that exposure to NPs activates NLRP3 inflammasomes leading to mitochondrial damage in association with triggering of inflammatory reactions in the tissues causing oxidative stress. Hence the lower contents of various antioxidant biomarkers in multiple visceral organs of rats might be due to oxidative stress in terms of increased generation of free radicals resulting in demise of different cellular organelles. In earlier published literature, different studies have reported that injury in different cells occurs due to oxidative stress which can disrupt normal physiological functions leading to necrosis of tissues via apoptosis (Ali et al., 2024; Samim et al., 2023).

The histopathological alterations observed in muscles of rats in current study can be related to induction of myotoxicity by ZnO nanoparticles due to interaction with different organelles of myocytes such as lysosomes, mitochondria and myofibrillar proteins leading to induction of oxidative stress causing decreased mitochondrial membrane potential. The histopathological alterations in various visceral organs of ZnO NPs treated rats in our study can also be related to dysfunctions of ROS scavenger (N-acetyl-L-cysteine), inhibitors for NLRP3 inflammasomes (Glibenclamide), and inhibitor of mitochondrial depolarization (Cyclosporin A) resulting in release and activation of IL-1β. Furthermore, the histopathological alterations in lungs and muscles could also be due to modification of cellular proteins and lipids, mitochondrial damage resulting in damage to DNA due to increased release of intracellular Ca^{2+} as a consequence of oxidative (Zhang et al., 2017; Marchi et al., 2023; Zhuo et al., 2024). It has been recorded that different chemicals including drugs after entry in blood interact with organelles of muscles ultimately causing adverse effects such as disorders in electrolytes, immunological responses and inflammatory reactions which affects normal physiological functions of the muscles (Janssen et al., 2020). The histopathological alterations observed in this research study including, inflammatory materials, necrosis of myocytes, myofibrillosis can also be related to disruption of mechanisms of oxidative phosphorylation, inhibition of production of ATP, glycolysis and inhibition of acetylcholinesterase in muscles of treated rats (Raghupathy et al., 2010). Previously, it is reported that induction of oxidative stress in visceral organ enhances the quantity of aniline which damages lipids proteins, and DNA in spleen which can be related to DNA damage by over production of free radicals and initiation of DNA glycosylases expression (APE1, OGT1, NTH1, NEIL1/2 and PNK) in rats. Additionally, pathological alterations in different visceral organs of nanoparticle treated rats could also be related to up-regulation of different inflammatory mediator like cytokines, IL-6, TNF-α and IL- 1 through activation of nuclear factor-kappa B, redox-sensitive transcription mechanisms and AP-1 as a result of oxidative stress (Makhdoumi et al., 2019) .

Furthermore, increased oxidative stress profile and depletion of antioxidant enzymes in smooth muscles of rats due to nanoparticles have also been reported in published literature. Different histopathological ailments in smooth muscles including apoptotic cells due to induction of oxidative stress has been observed (Yaman et al., 2018). The muscle toxicity in nanoparticles treated rats in our study can also be related to enhanced molecular events such as phosphorylation of IκB kinases (IKKα and IKKβ) and mitogen-activated protein kinases (MAPKs) being the potential activators of NF-κB and AP-1 (Makhdoumi et al., 2019).

Moreover, these histopathological alterations in muscles of rats could also be related to nanoparticle induced apoptosis through changes in mitochondrial pathways (Du et al., 2019). Previously, increased in oxidative stress, 8-hydroxy-2′-deoxyguanosine, creatine kinase, p53, cytokines, norepinephrine, lipid profile and acetylcholine while decreased quantity of paraoxonase 1, antioxidant enzymes, neurotransmitters, total antioxidant capacity, reduced glutathione and acetylcholine esterase due to nanoparticles in different visceral organs of rats have been recorded (Yousef et al., 2022). The microscopic changes in skeletal muscles of rats exposed to

nanoparticles have not been reported in accessible published data. However, it has been recorded that nanoparticles in circulation induce myocardial congestion, injury to cardiac myocytes, via generation of free radicals, depletion of antioxidant enzymes and induction of inflammatory reactions in tissues (Yousef et al., 2022). The histopathological and oxidative ailments elicited by ZnO NPs in current study in hematopoietic tissue, intestine and lungs might be linked to over release of free radicals, depletion of antioxidants and activation of signaling cascades (Balkrishna et al., 2021; Venkatappa et al., 2022). It is recorded that free radicals interact directly with different micro and macromolecules including lipids, DNA and proteins of variety of cells causing oxidative stress related lesions. Remarkably increased contents of oxidative stress parameters (TBARS and ROS) multiple visceral organs of rats in this study suggests ZnO NPs-induced pathological changes in integrity of cell membranes responsible for lower contents of antioxidants (Moussaoui et al., 2021). Previously, lower contents of GSH in treated fish due to copper oxide NPs (Aziz and Abdullah, 2023; Naguib et al., 2023), metal nanoparticle (García-Medina et al., 2022) and zinc NPs (Ghafarifarsani et al., 2023) have also been reported. Furthermore, it is recorded that oxidative stress induced by nanomaterials reduces the contents of antioxidant enzymes a well-known secondary defense response (Ghaffar et al., 2021; Sanati et al., 2022; Wang et al., 2022). SOD and CAT enzymes are responsible for neutralization of superoxide free radicals and act as agents for detoxification of hydrogen peroxide while POD scavenge lipid peroxides (Hussain et al., 2017; Kumar et al., 2023). Hence depletion of these enzymes causes oxidative damages in different tissues.

Conclusion

In conclusion, it is recorded that ZnO-NPs can induce detrimental effects on exposed albino rats via induction of oxidative stress in different tissues in a dose and time dependent interval highlighting the disruption of normal physiological process. Our results suggest a strong foundation for further research to completely comprehend the effects of zinc oxide nanoparticles on biological systems.

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Conflict of Interest: None.
Source of Funding: None.

Data Availability Statement

The data/findings of the study are available upon request to the corresponding authors.

Ethical Approval

All applicable national, international, as well as institutional guidelines mentioning the care and use of animals were strictly followed.

Contribution of Authors

Mahmood Y: Conceived idea and designed experiment, collected and analyzed data
Ijaz N & Rajeh N: Reviewed and edited the final draft of manuscript
Maheen A & Masood N: Data collection and analysis
Mustafa G: Designed the experiment and reviewed literature
Bafail DA, Qamar MR & Ahsan MA: reviewed literature and prepared the initial draft of manuscript
Mohiuddin M: Supervised trial execution, data collection and analysis, critiqued and edited the final draft of manuscript

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