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RESEARCH ARTICLE

Calcium Nanoparticles Induce Oxidative Stress in Erythrocytes, Neurotoxicity and Testicular Toxicity in Albino Rats *(Rattus norvegicus)*

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A B S T R A C T

Calcium nanoparticles (Ca-NPs) are emerging as promising materials and are frequently used in different areas of medical science including treatment therapy. In current study, different unwanted impacts of Ca-NPs were observed on various tissues including teeth, testes, brain and erythrocytes of male albino rats. A total of twelve active and sexually mature albino rats free from any obvious clinical ailments were procured from local market and were placed in wire cages in three groups. The rats in groups B and C were administered Ca-NPs at two dosage levels (25 and 50 mg/kg body weight) while the rats in control group received no treatments. No physical changes in terms of mortality, morbidity, behavioral and neurological signs were observed in rats of all groups. Different pathophysiological alterations such as significantly increased contents of oxidative parameters (reactive oxygen species and thiobarbituric acid reactive substance) while reduced contents of antioxidant enzymes (reduced glutathione, peroxidase, catalase and superoxide) in red blood cells (RBC) of albino rats of group B and group C were recorded. Results on various histo-architecture changes in different visceral organs like the testes, brain, and teeth exhibited various microscopic alterations in albino rats treated with high dosages of Ca-NPs as compared to the control group. Therefore, the current study showed that the prepared Ca-NPs had adverse physiological effects on different tissues in the albino rats.

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INTRODUCTION

Currently, there is an increasing tendency towards producing, developing, and broadening the application fields of innovative materials that comprise of nanosized particles along with the methods used to create them (Bovina *et al.,* 2019; Samy *et al.,* 2022). Nanomaterials are predicted to have a significant impact on various sectors of human economic activities, potentially leading to improvements and even revolutionary changes in several industries and technologies (Batool *et al.,* 2023; Umair *et al.,* 2022). This prediction is primarily due to the exceptional physical and chemical properties of nanomaterials which arise from their small size, high surface area, unique shape, surface charge, enhanced accumulative potency and other characteristics that set them apart from bulk materials (Ramachandraiah *et al.,* 2015; Sores *et al.,* 2018). The high penetrating ability of nanomaterials which stems from their inherent properties, can cause potential risks by increasing their toxic properties upon entering the human body at any stage of production or consumption of products (Sukhanova *et al.,* 2018; El-Dawy *et al.,* 2022). The nanomaterial industry has integrated these materials into a vast majority of products used by humans, including food, clothing, and numerous other applications. Nanomaterials have made

significant inroads into the medical and dental fields, where they are used in various applications, such as dental fillings made of nanomaterials (Al-Shaibani *et al.,* 2022). In vitro studies have been increasing constantly; in vivo studies of nanoparticles have not established a unified system until now.

The male reproductive system is widely recognized as being more susceptible and sensitive to various types of stressors including heavy metals, xenobiotic compounds, microwaves, and nanoparticles compared to other tissues (Meena and Kajal, 2015; Ban *et al.,* 2018). Exposure to NPs can induce cytotoxic effects that can alter the physical and chemical properties of various cells like erythrocytes. The changes in erythrocyte morphology can disrupt their elastic properties and deformability resulting in abnormal rheological and respiratory functions (Muravyov *et al.,* 2013; Sergeeva *et al.,* 2016). Hence, this makes erythrocytes a unique model for evaluating the effects of both exogenous and endogenous factors on the entire organism.

Moreover, it is of vital importance to investigate the exposure of animals and humans to multiple types of nanoparticles and their various entry routes resulting from environmental, therapeutic, diagnostic, and cosmetic uses. Nanoparticles such as Au and TiO_2 can transfer to the fetus, leading to varying toxic effects on different organs such as the brain, kidneys, liver, and reproductive system (Leonardo *et al.,* 2006; Brohi *et al.,* 2017). Calcium (Ca) is a vital component of bones and teeth in the body and it plays a crucial role in the proper functioning of different living cells including osteoblasts, ameloblasts, and odontoblasts. These cells are responsible for the formation of calcified tissues such as bone, enamel, and dentin (Goldberg *et al.,* 2011; Cheng *et al.,* 2015). Apart from its role in the formation of bones and teeth, Ca is also essential for various cellular functions, immune responses, hormone secretion, enzyme activation, and blood clotting. Numerous studies have recommended the use of Ca-based nanoparticles in medicine and dentistry due to their ability to release calcium ions, which are the main elements of human bones and teeth and play a vital role in the functioning of living cells. Ca-NPs is considered an essential mineral for all organisms (Blair *et al.,* 2011; Paula *et al.,* 2019). Ca-NPs have great biodegradability and biocompatibility making them ideal transporters for biomolecules in therapeutic applications. These NPs have exceptional bioavailability and tolerable biodegradability, setting them apart from other biominerals and making them a good choice for drug delivery in nanomedicine, dentistry, and orthopedics or as additives in various vaccines (Khalifehzadeh and Arami, 2020). Studies have investigated that high doses of Ca-NPs can lead to toxic effects in animals particularly in the liver and kidneys. The accumulation of Ca-NPs in these organs may cause inflammation and cell damage leading to impaired organ functions (Al-Shaibani, 2018).

Based on current literature, there is a lack of conclusive evidence regarding the direct influence of Ca-NPs on teeth and other visceral tissues. To the best of our knowledge, there are no other studies in the literature that have examined the impact of intravenous administration of Ca-NPs on tooth structure and visceral tissues in albino rats. Therefore, this study is intended to investigate the toxic effect of the synthesized Ca-NPs on erythrocytes, teeth, brain and testes of albino rats.

MATERIALS AND METHODS

Experimental species and management: A total of 12 male Albino rats having approximately two months of age were procured from the local animal house. The rats used in current study weighed between 160-220g were observed to be healthy and free from any diseases. The rats were kept under standard conditions like temperature (23±1°C) and humidity (65±5%) under a 12h light/dark cycle. All the rats had free access to standard food (commercially available poultry feed containing approximately 22% proteins) and normal fresh water. All the study protocols including use of laboratory animals were conducted in accordance with the guidelines set forth by the National Institute of Health's "Guide for the Care and Use of Laboratory Animals" (NIH publication no. 85-23, 1985).

Toxicant and chemicals: Ca-NPs and all the other chemicals used for estimation of oxidative stress and antioxidant enzymes in erythrocytes and histopathology were purchased from Sigma Aldrich (USA) and Merck (Germany).

Exposure of Ca-NPs: For intravenous administration, Ca-NPs nanoparticles were mixed with saline solution for injection. After two weeks of acclimatization, the rats were randomly divided into three groups (A-C) and were kept in wire cages each containing four rats. Group A served as the control group and received a dose of normal saline (0.9% NaCl). Group B was exposed to a dose of 25 mg/kg of Ca-NPs, while Group C was exposed to a dose of 50 mg/kg of Ca-NPs. These NPs were diluted and administered intravenously (IV) (mg/kg weight of the rats) to each rat with a one-day interval, over a period of 24 days, following the protocol that described earlier (Al-Maula *et al*., 2021).

Histopathological studies: After the completion of the trial, different visceral organs like brain, testes and teeth of rats from each group were separated after dissection, weighed, washed and fixed in 10% neutral buffered formaldehyde solution. Approximately, 4-5µm thick sections of brain, testes and teeth were cut by using rotary microtome. Subsequently, these sections were then dehydrated and were stained with Hematoxylin and Eosin (H&E). The microscopic observations were recorded using a light microscope (Nikon Eclipse 80i, Nikon Co., and Tokyo, Japan) to observe any alteration in the visceral organs of rats (Ghaffar *et al.*, 2021).

Tissue preparation and biochemical analyses: After 24 days of the trial period, the blood (2.5ml) was collected from the jugular vein of each rat in tubes without EDTA. Initially, blood samples were centrifuged at 5000rpm for 5min to separate the serum and the pellets containing RBC. Erythrocytes were collected and 10% hemolysate of RBC was prepared from both the NPs-treated and untreated Albino rats to determine oxidative and

antioxidant enzymes. Oxidative stress biomarkers such as ROS (Hayashi *et al.*, 2007), TBARS (Iqbal *et al.*, 1996), and reduced GSH (Jollow *et al.*, 1974) in erythrocytes of both the NPs-exposed and unexposed rats were determined at 505, 532 and 412nm, respectively using a UV- spectrophotometer. Additionally, different antioxidant parameters such as POD (Kakkar *et al.*, 1984; Akram *et al.*, 2021), SOD, and CAT (Maehly and Chance, 1954) were also measured in erythrocytes of both the Ca-NPs exposed and unexposed rats at 470, 560 and 240nm.

Statistical analysis: The collected data during the trial were presented as mean±SE. Statistical analysis was conducted using IBM SPSS statistics (version-20), where all the collected data in each group were found to be normally distributed. One-way analysis of variance (ANOVA) was used for the statistical analysis. To determine the difference in mean values (mean±SE) of oxidative and antioxidant parameters in erythrocytes of control and NPs exposed rats, post hoc Tukey's test was conducted at a significance level of $p<0.05$.

RESULTS

Physical parameters: No mortality, morbidity, behavioral, neurological and clinical alterations in control and treated rats were observed during the trial.

Histopathological analysis of teeth: The results showed that the percentage of osteoclasts in teeth of group A was normal. Severe to very severe various microscopic alterations such as dentin bridge thickness, pulp calcifications, deposition of new dentin, inflammatory reactions, periodontal ligament thicknesses, increased predentin thicknesses, decrease in vascularization in the pulp tissue, increase in thickness of the periodontal ligament, increase in the height of inner enamel epithelial cells, cellularity of enamel organ at the base of the root, enamel hypoplasia, delayed increase of the mineral content, decreased alveolar bone volume, increased osteoclast cell numbers and elevated percentage of osteoclasts in teeth of rats exposed to 50mg/kg dose of Ca-NPs treated albino rats were recorded (Table 1 and Fig. 1). Mild to moderate histological changes were observed in teeth of albino rats exposed to 25mg/kg dose of Ca-NPs.

Histopathological analysis of brain: The microscopic observations of different sections of brain of control rats indicated normal histological arrangements. Mild to moderate histological changes including inflammatory reaction, atrophy of neurons, vacuolar degeneration and neuronal degeneration were observed in various sections of brain of albino rats exposed to 25mg/kg dose of Ca-NPs (Fig. 2). Severe to very severe histopathological lesions including edema, necrosis of neurons, microgliosis, neuronal degeneration, vacuolation of neuron were observed in various sections of brain of albino rats exposed to 50mg /kg dose of Ca-NPs (Fig. 2). All microscopic alterations in group B and group C are shown in Table 1.

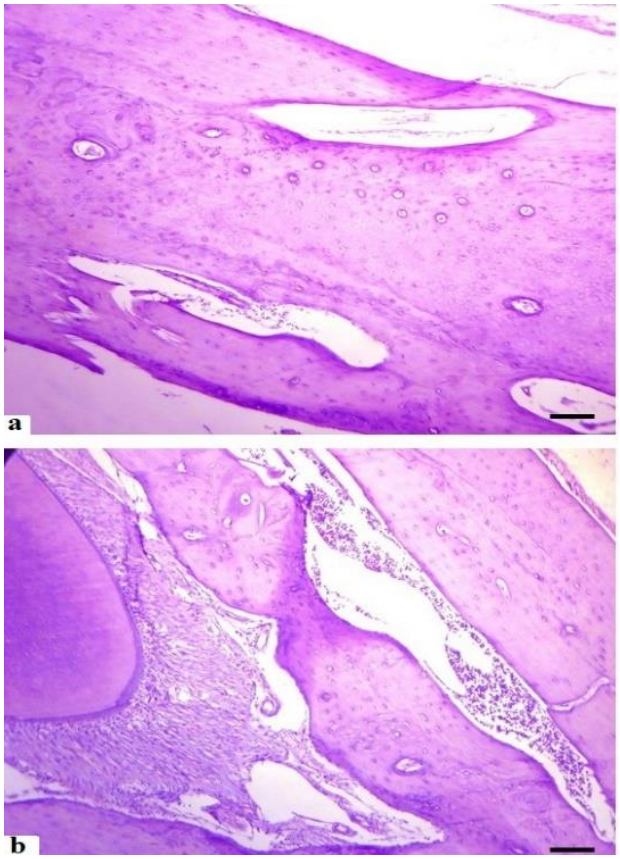

Fig. 1: Photomicrograph of teeth (a) from albino rats in group B (25 mg/kg) demonstrating mild to moderate while (b) showing severe to very severe microscopic changes such as increased percentage of osteoclasts, increased dentin bridge thickness, increase in thickness of periodontal ligament, increased deposition of new dentin, inflammatory reactions, pulp calcifications, increase height of inner enamel epithelial cells, and increase in cellularity of enamel and group A (50 mg/kg) exposed to Ca-NPs. H & E stain; 400X.

Table 1: Severity of various histopathological alteration in teeth of Ca-NPs exposed and unexposed groups male albino rats.

Parameters	Groups/Treatments		
Histopathological lesions	A (0.0 mg/kg)	C (25 mg/kg)	D (50 mg/kg)
Elevated percentage of osteoclasts	-	++	+++
Increased dentin bridge thickness	-	+	+++
Increase in thickness of periodontal ligament	-	++	+++
Increased deposition of new dentin	-	++	++
Inflammatory reactions	-	++	+++
Pulp calcifications	-	++	+++
Increase height of inner enamel epithelial cells	-	++	+++
Increase in cellularity of enamel	-	++	++++
Increased periodontal ligament thicknesses	-	+++	+++
Decrease in vascularization in pulp tissue	-	+++	+++
Enamel hypoplasias	-	++	+++
Decreased alveolar bone volume	-	+++	+++
Decrease of the mineral content	-	++	+++
Increased osteoclast cell numbers	-	++	++++
Brain			
Inflammatory reactions	-	+++	++++
Microgliosis	-	+++	++++
Atrophy of neuron	-	++	+++
Necrosis of neuron	-	+++	+++
Vacuolation of neuron	-	++	+++
Edema	-	+++	+++
Neuronal degeneration	-	+++	++++

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

Histopathological analysis of testes: The results on severity of various microscopic alterations such as inflammatory processes, sloughed cells, disorganization of spermatogonia and Sertoli cells, degeneration and damage of spermatogonia, increase cellular debris in lumen of seminiferous tubules, hypospermatogenesis, decrease in frequency of normal seminiferous tubules, arrest of process of spermatogenesis, necrosis of spermatids, increased germ cell depletion in seminiferous epithelium, necrosis of spermatogonia and Sertoli cells, reduction in diameter of seminiferous tubules, partial germ cell arrest and vacuolation of epithelia of seminiferous tubules observed in male albino rats of control group and treated groups are presented in Table 2. The severity of microscopic alterations was high in albino rats exposed to higher concentrations of Ca-NPs at a dose of 50mg/kg in comparison to that of group B exposed to Ca-NPs at a dose of 25mg/kg (Table 2 and Fig. 3).

Oxidative and antioxidant profile: The results on oxidative biomarkers and various antioxidant enzymes determined in red blood cells of albino rats given different doses of Ca-NPs are recorded in Table 3 and Fig. 4a-b. The results showed significantly elevated levels of ROS, and TBARS in the red blood cells of male albino rats that were administered a 50mg/kg dose of Ca-NPs. In contrast, in rats in group B given a 25mg/kg dose of Ca-NPs, the levels of ROS were significantly increased, while the levels of TBARS were non-significantly increased as compared to the control group. The contents of different antioxidant enzymes like POD, SOD, GSH and CAT were significantly decreased in the red blood cells of albino rats of group C that were exposed to a high dose of Ca-NPs. When compared to unexposed rats, the level of SOD was significantly decreased, while the levels of POD and CAT were non-significantly reduced in the red blood cells of group C.

DISCUSSION

The increased applications of nanoparticles across diverse fields has raised concerns regarding their potential effects on human health, specifically in consumer products and medical applications (Roy *et al*., 2013; Komiyama *et al*., 2019). Consequently, ensuring the safe implementation of nanotechnology in healthcare has become a critical concern. Nanoparticles have found applications in the pharmaceutical industry and sensor technology, enhancing the activity of catalysts used in the detection of chronic diseases. Additionally, Ca-NPs are widely used in biomedicine for drug delivery, treatment therapy and as antibacterial agents (Abraham and Sarathy, 2018). However, various animal studies reported in the literature (Al-Shaibani, 2018) have shown that the injection of Ca-NPs has significant effects on the functioning of various body components, including the liver, brain, heart, and body weight. The growing utilization of fabricated NPs in diverse fields indicates an increasing exposure of individuals to these materials in their daily lives. While nanomaterials have numerous scientific applications, their widespread use also poses potential risks to the environment and living organisms.

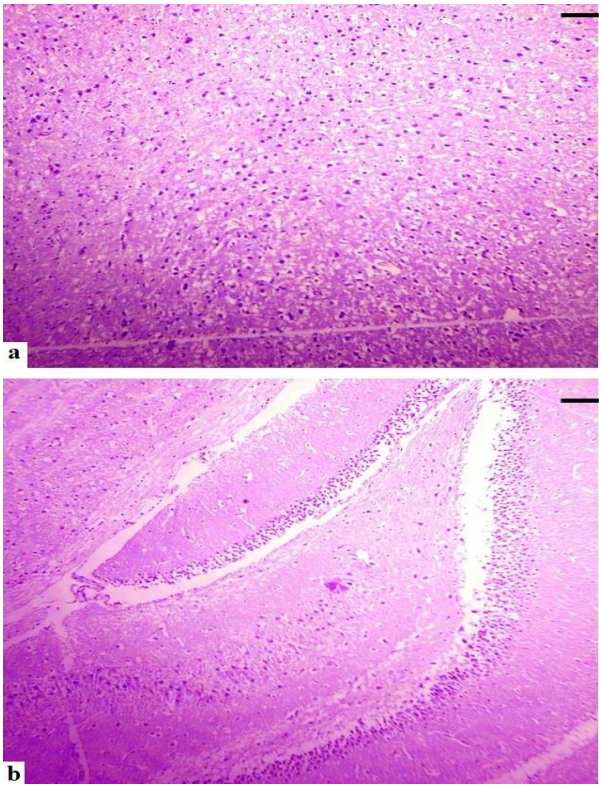

Fig. 2: Photomicrograph of brain (a) from albino rats in group B (25mg/kg showing mild to severe while (b) in group A (50mg/kg showing severe to very severe histopathological changes such as inflammatory reactions, microgliosis, atrophy of neuron, necrosis of neuron, vacuolation of neuron, edema, and neuronal degeneration in albino rats exposed to Ca-NPs. H & E stain; 400X.

Table 2: Severity of various histopathological alteration in testes of Ca-NPs exposed and unexposed groups male albino rats.

Parameters	Groups/Treatments		
Histopathological lesions	A (0.0 mg/kg)	C (25 mg/kg)	D (50 mg/kg)
Inflammatory processes	-	+++	+++
Sloughed cells	-	++	+++
Disorganization of spermatogonia and Sertoli cells	-	++++	++++
Degeneration and damage of spermatogonia	-	++	+++
Increase cellular debris in lumen of seminiferous tubules	-	++	+++
Hypospermatogenesis	-	++	++
Decrease in frequency of normal seminiferous tubules	-	++	+++
Arrest of process of spermatogenesis	-	+++	++++
Necrosis of spermatids	-	++	+++
Increased germ cell depletion in seminiferous epithelium	-	++	+++
Necrosis of spermatogonia and Sertoli cells	-	++	+++
Reduction in diameter of seminiferous tubules	-	+++	++++
Partial germ cell arrest	-	+++	+++
vacuolation of epithelia of seminiferous tubules	-	+++	+++

Normal (-), Mild (+), Moderate (++), Severe (+++), Very severe (++++).

Therefore, the aim of the current study was to assess the potential adverse effects of Ca-NPs on the histopathology of teeth, testes, brain, and oxidative and antioxidant enzymes in erythrocytes. This investigation could provide valuable insights into the safety and potential risks associated with the use of Ca-NPs in biomedical applications.

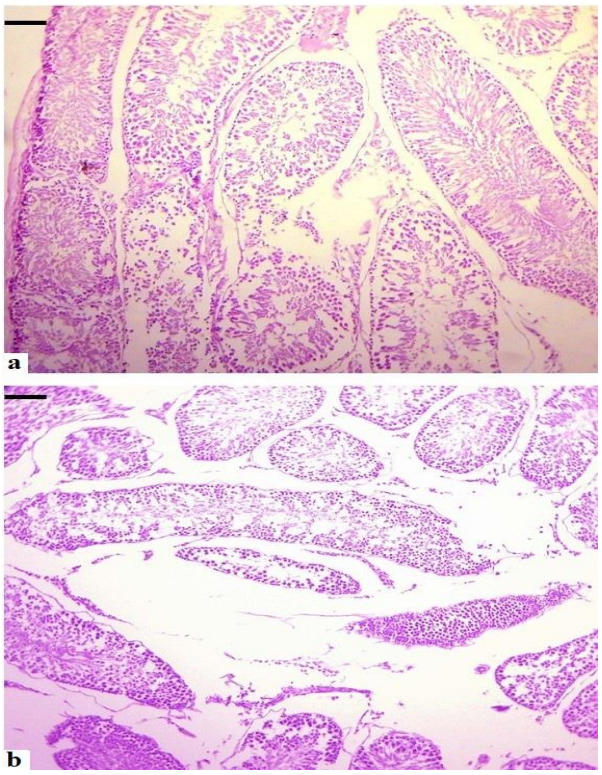

Fig. 3: Photomicrograph of testes (a) from albino rats in group B (25mg/kg) showing mild to moderate while (b) from group A (50mg/kg) showing sever to very serve microscopic changes like degeneration and damage of spermatogonia, hypospermatogenesis, increase cellular debris in lumen of seminiferous tubules, increased germ cell depletion in seminiferous epithelium, necrosis of spermatids, decrease in frequency of normal seminiferous tubules, necrosis of spermatogonia and arrest of process of spermatogenesis in male albino rats exposed to Ca-NPs. H & E stain; 400X.

The study findings suggest that IV administration of 25 and 50mg/kg Ca-NPs did not result in mortality among albino rats, indicating that these doses could be considered as the maximum tolerated dose for rats. The teeth of all rats in the control group appeared normal, with no observed increase in osteoclasts, dentin bridge thickness, periodontal ligament thickness, or new dentin deposition, as determined by histopathological analysis. However, histological examination revealed that treatment with Ca-NPs at a dose of 50 mg/kg resulted in the highest predentin thickness. It is worth noting that a decrease in calcium levels has been linked to impaired tooth mineralization (Botelho *et al.*, 2020). Previous studies have shown that calcium ions are transported to the mineral formation site in the dentin matrix through odontoblasts, which are responsible for dentin formation (Lundquist, 2002). Additionally, calcium ions can reduce capillary permeability, which inhibits the flow of serum and promotes mineralization by reducing the levels of inhibitory pyrophosphates (Sonarkar and Purba, 2015). Our study also found that administration of Ca-NPs resulted in an increase in predentin formation activity and periodontal ligament thickness. The optimal concentration of Ca-NPs was found to be 50mg/kg, which significantly increased predentin thickness and decreased vascularization compared to the unexposed group. These findings are consistent with previous studies (Al-Maula *et al.*, 2021).

The results of our research indicate that the presence of NPs in the testes increases the expression of pro-inflammatory and apoptotic markers, resulting in testicular inflammation, apoptosis, and oxidative stress. Elevated levels of ROS can damage the plasma membrane, thereby reducing sperm function (Kobayashi and Suda, 2012). Additionally, these pathological alterations could also be related to changes in hemato-biochemical biomarkers and oxidative stress profile (Ismail, 2022). It is also observed that NPs can reduce sperm motility by interfering with mitochondrial function. Our study suggests that inducing Ca-NPs in the testes of rats can lead to reproductive toxicity, with high doses causing edema and weight gain in the testicular tissue, and a noticeable decrease in testosterone levels. Studies showed the toxicity can cause histological changes in the testes of male rats, agreeing with previous reports (Butt *et al.*, 2015; Shaibani, 2018). A previous study also reported that the CaO- NPs in rats can cause similar toxicity (Al-Shaibani *et al.*, 2022). Other studies agree with a report with different NPs (Adebayo *et al.*, 2018). Decreased sperm count, tissue degeneration in the testes, and a

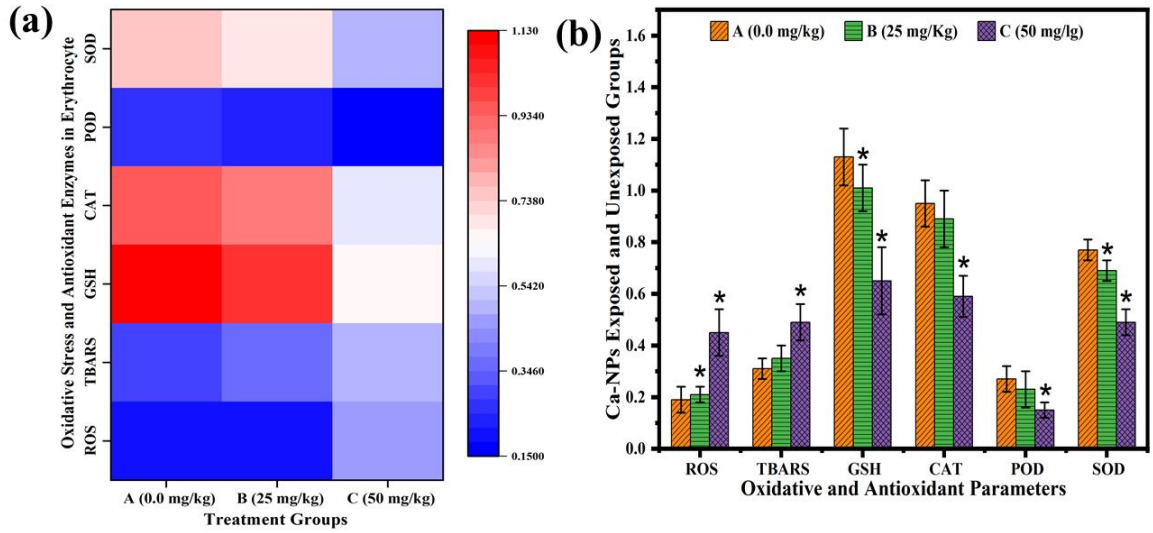

Fig. 4: (a) Heat map showing Effect of Ca-NPs on oxidative biomarkers and antioxidant enzymes (b) Comparison between oxidative and antioxidant enzymes of exposed and unexposed groups.

Table 3: Status of oxidative biomarkers and various antioxidant enzymes in red blood cells of control and calcium nanoparticle treated albino rats

Parameters	Groups/Treatments		
	A (0.0 mg/kg)	C (25 mg/kg)	D (50 mg/kg)
Oxidative biomarkers			
ROS (optical density)	0.19±0.05	0.21±0.03*	0.45±0.09*
TBARS (nmol/TBARS formed/mg protein/min)	0.31±0.04	0.35±0.05	0.49±0.07*
GSH (mmol-g^{-1} tissue)	1.13±0.11	1.01±0.09*	0.65±0.13*
Antioxidant Enzymes			
Catalase (units/min)	0.95±0.09	0.89±0.11	0.59±0.08*
Peroxidase (units/min)	0.27±0.05	0.23±0.07	0.15±0.03*
Superoxide (units/mg protein)	0.77±0.04	0.69±0.04*	0.49±0.05*

"*" shows a significant decrease and increase.

reduced number of sperms in the seminiferous tubules were observed in male rabbits subjected to poisoning, consistent with the results reported in this study (Amin *et al.,* 2015). Therefore, we recommend using medium or low doses (25mg/kg) of NPs as a testicular tonic to stimulate testosterone production, as this outcome was not previously reported in research on the effects of nanocomposites.

This study found that high doses of Ca-NPs can cause significant oxidative stress in the brain tissues of rats, potentially leading to neurotoxicity. Excessive ROS production has been linked to several diseases, including neurodegenerative disorders, cancer, and ageing. Our results also revealed a pathomorphological change in the form of subarachnoid hemorrhage in the brain of exposed animals, consistent with earlier findings (Butt *et al.,* 2015). Although the presence of calcium in brain vessels was not established in this experiment, subarachnoid hemorrhage may be related to the possible disruption of tight contacts (Kim *et al.,* 2006) in the blood-brain barrier. Further research is necessary to determine the precise mechanism by which Ca-NPs affect the brain. Our findings are supported by previous studies (Zaitseva *et al.,* 2019).

The alteration in blood biomarkers observed in the exposed groups may be attributed to the toxic effect induced by the high dose of Ca-NPs. Previous research has demonstrated that blood parameters serve as reliable biomarker for evaluating the pathophysiological status of living organisms exposed to toxicants (Hussain *et al.,* 2012). Erythrocytes, in particular are susceptible to redox imbalance due to the presence of fatty acids in their membranes, high levels of oxygen, and the presence of hemoglobin. These cells are natural targets for free radicals (Ben Saad *et al.,* 2014) and can suffer from toxicity, leading to deformation, hemolysis, and mitochondrial dysfunction. Our findings are consistent with previous research (Zemlyanova *et al.,* 2021).

This current study suggests that an imbalance between radical-generating and radical-scavenging systems leads to oxidative stress, resulting in the impairment of cell membranes and DNA damage. We observed a reduction in the levels of antioxidant enzymes (POD, SOD and CAT) and oxidative enzymes (GSH), along with an increase in oxidative biomarkers (TBARS and ROS), indicating the occurrence of an imbalance in oxidant enzymes that induces an inflammatory response. These results suggest that exposure to NPs induces oxidative stress by increasing the amount of ROS and TBARS in the erythrocytes of rats. However, exposure of rats to different toxicants can cause an increase in the formation of ROS and subsequently induce oxidative stress. This production initiates the LPO process, causing in cellular membrane irregularities and the formation of TBARS (Akram *et al.,* 2021). This increase in oxidative stress may be attributed to a reduction in antioxidant enzyme activities and an imbalance in the detoxifying systems of the rats. The decrease in CAT activity observed in the study may indicate its consumption during the breakdown of free radicals, which suggests that CAT plays a crucial role in cellular defense against oxidative stress (Santana *et al.,* 2022). These findings highlight the importance of maintaining a balance between radical-generating and radical-scavenging systems to prevent oxidative stress-induced damage to cellular membranes and DNA.

Conclusions: The increasing use of nanoparticles (NPs) has led to their release into the environment, exposing living organisms to their potential hazards. This study demonstrates that exposure to high doses (50mg/kg) of Ca-NPs can induce significant oxidative stress in brain tissues, alter blood biomarkers, and cause histopathological changes in teeth, brain, and testes. The observed imbalance between radical-generating and radical-scavenging systems in the erythrocytes of NPs-exposed rats, resulting in oxidative stress and cellular damage, emphasizes the need for antioxidant-based therapies to mitigate the toxic effects of Ca-NPs and other nanoparticles. The findings highlight the importance of safe disposal and handling protocols for organic nanoparticles to prevent their harmful effects. Further research is necessary to understand the mechanisms underlying the toxic effects of NPs and to develop effective strategies for their safe use and disposal.

Authors contribution: RH, FAA, AMA, AZA designed the experiment. NA, AU, AS, and AA conducted research and collected the data. RH, FAA, AMA, MSM, AZA analyzed the data and finalized the write up of this manuscript. All authors approved and finalized the manuscript.

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