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Protective role of *Moringa oleifera* leaves extract against zinc oxide nanoparticles induced oxidative stress and infertility in adult male rats

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Abstract

Background: Nowadays, zinc oxide nanoparticles (ZnO-NP) are one of the most commonly used nanoparticles. They are utilized in different fields, such as agriculture, industry, and biomedicine, and their release into the environment has a negative impact on humans. *Moringa oleifera* is a medicinal plant with a high nutritional value and a potential antioxidant property. Despite this, research on the impact of *M. oleifera* on the male reproductive system has been limited. **Aim:** The aim of the present study was to evaluate the ameliorative role of *Moringa oleifera* leaf extract on ZnO-NP induced oxidative stress and testicular toxicity. **Materials and methods:** zinc oxide nanoparticles were synthesized by the sol-gel approach and characterized by X-ray diffraction (XRD), field emission scanning electron microscopy (FE-SEM), and energy dispersive spectroscopy (EDS). *Moringa oleifera* leaves were extracted by the maceration method. Thirty-six adult male albino rats were randomly divided into six experimental groups (n = 6), Group (I) control received 1ml/day of 1% dimethyl sulfoxide (DMSO) as a vehicle for 4 weeks and 0.5 ml normal saline injected intraperitonially once a week for 2 weeks, group (II) and group (III) received an extract of *M. oleifera* leaves by oral gavage at a dosage of 400 and 600 mg/kg.bw/day respectively for 4 weeks, group (IV) received 150 mg/kg.bw ZnO-NP injected intraperitonially once a week for 2 weeks, group (V) and group (VI) received 150 mg/kg.bw ZnO-NP injected intraperitonially once a week for 2 weeks co-administrated with an extract of *M. oleifera* leaves at a dosage of 400 and 600 mg/kg.bw/day respectively for 4 weeks. At the end of the experiment, the rats were sacrificed, and blood samples were collected for estimation the level of serum testosterone. The epididymis was used for semen collection and analysis. The testes were dissected out from each rat and homogenized for the measurement of oxidative stress biomarkers. **Results:** The levels of serum testosterone, sperm count, normal sperm morphology, testicular weight, catalase, and glutathione peroxidase were significantly decreased ($p<0.01$) in the ZnO NP-treated group. While the level of Malondialdehyde (MDA), and abnormal morphology were significantly increased ($p<0.0001$) in the ZnO-NP treated group. *M. oleifera* leaf extract caused significant increase ($p<0.01$) in the levels of testosterone, sperm count, normal morphology, and catalase, and significantly reduced ($p<0.01$) Malondialdehyde (MDA) levels and abnormal morphology in a dose-dependent manner in the groups (II) and (III) compared to the control group (I). ZnO-NP co-administrated with an extract of *M. oleifera* leaves of groups (V) and (VI) extract significantly increased ($p<0.01$) the levels of testosterone, sperm count, normal morphology, testicular weight, catalase, and glutathione peroxidase, and, significantly

reduced ($p<0.01$) MDA and abnormal morphology in a dose-dependent manner when compared with the ZnO-NP treated group. **Conclusion:** ZnO-NP caused oxidative stress in the rats, while the oral administration of *Moringa oleifera* leaf extract significantly mitigated the testicular toxicity induced by ZnO-NP in a dose-dependent manner.

Introduction

Nanotechnology is a new field of modern science and technology that has the potential to revolutionize every scientific field (1). In recent decades, nanotechnology has developed rapidly not only in the industrial or electronic sectors but also in the fields of medicine and biology for example, using nanoemulsion as a vehicle for increasing the absorption of vitamin D in rats (2). As a result of the rapid development of nanotechnology, the possibility of human exposure to nanoparticles has emerged (3) and, these nanoparticles accumulate in the vital organs and cause histological changes (4). The size range of nanoparticles is approximately 1 to 100 nanometers. Due to their large surface area and nanoscale dimension, they possess distinctive chemical and physical properties compared to their corresponding bulk materials (5, 6). ZnO NPs, which are semiconductor metal oxide nanoparticles, are utilized in a broad spectrum of industrial items, such as sunscreen, toothpaste, ointments, food additives, plastics, paints, and electrical components (7, 8). ZnO-NPs have been found to accumulate in a number of tissues, including the brain and testicles, which demonstrates that they can easily pass through the blood-brain barrier and the blood-testis barrier (9, 10).

Male infertility is a popular issue and described as the inability of an adult male to produce and transmit effectively functioning sperm. The World Health Organization (WHO) defines the infertility as the inability to become pregnant after one year of sexual activity without contraception (11). Due to the negative impact of reactive oxygen species and free radicals on sperm function and DNA integrity, oxidative stress has been identified as one of the most common causes of male infertility (12). Because of their low antioxidant enzyme capacity and high polyunsaturated fatty acid (PUFA) content in the plasma membrane, spermatozoa are highly vulnerable to oxidative stress (13). A number of studies have shown that large dosages of ZnO NPs, such as 50, 150, 300, and 350 mg/kg in mice, have adverse impacts on the testicles (14). Recent research indicates that ZnO NPs can accumulate in the testes and epididymis (15). Male rats administered intraperitoneally with ZnO nanoparticles had a decline in sperm count, motility, and increased abnormal sperm morphology (16).

Moringa oleifera, which belongs to the family Moringaceae and is also known as the drumstick tree or horseradish tree, is considered to be one of the most medicinally essential plants. It originated in the Himalayas. However, it is currently grown in a number of tropical and subtropical countries across the world (17). *Moringa oleifera's* leaves, flowers, fruits, roots, bark, and seed oil provide nutritional and medicinal advantages (18). It possesses several biological activity such as antioxidant, hepatoprotective, anti-inflammatory, anticancer, anti-diabetic, hypocholesterolemic, antibacterial, and wound-healing(19, 20). Previous investigations demonstrated that aqueous and ethanol extracts of the *Moringa oleifera* seed enhanced normal sperm morphology, viability, motility, and sperm count in male rats (21, 22). Furthermore, *Moringa oleifera* leaf extract has been proven to have aphrodisiac characteristics and improve the function of testicles and sexual activity in stressed rats (23). Additionally, *Moringa oleifera* leaf extract reduced chromium-induced testicular toxicity (24). The aim of the current study is to investigate the protective role of *Moringa oleifera* leaf extract in zinc oxide nanoparticles-induced oxidative stress and testicular toxicity in male Wistar rats.

Materials and methods

Plant collection and identification

The collection of *M. oleifera* leaves was conducted between November 2021 to January 2022. The leaves were obtained from a botanical garden (greenhouse) close to Bakrajo municipality in Sulaimani Province. The leaves were identified and authenticated by Assistant Professor Dr. Karzan O. Qadir, a plant taxonomist affiliated with the department of biology at the university of Sulaimani. the leaves underwent a washing process using tap water in order to eliminate all of the dust and debris. After that, the leaves underwent a drying process in a shaded environment, ensuring they were not exposed to direct sunshine, and were kept at ambient temperature for a duration of three weeks. using an electronic blender, the dried leaves were pulverized into a tiny powdered form, which was then kept at 4 °C for later usage.

Preparation of Moringa oleifera leaf extract

Fifty grams of the fine powder of *M. oleifera* leaves were macerated with 1000 ml of 70% ethanol (at a ratio of 1:20, weight to volume) in a dark bottle, sealed, and incubated in the shaker incubator (LabTech-Korea) for 72 hours, 150 rpm at 37 °C. To remove the solid parts completely, filter paper (Whatman No. 1) was used to filter the extract solution. A rotary evaporator (Büchi Rotavapor R-200) was used to remove the ethanol from the filtrate and concentrate it at 45°C to prevent denaturing the bioactive compounds. Finally, the residual aqueous part of the filtrate was lyophilized by running it through a freeze dryer (Christ LCG-Germany) for 48 hours to get rid of all water molecules and form the powder. The resultant crude powder was stored at -20°C in a dark, tightly closed container until usage (25).

Synthesis and characterizations of Zinc oxide nanoparticles

The synthesis of zinc oxide nanoparticles was conducted using the Sol-gel auto-combustion approach. The zinc nitrate Zn (NO3)2.6H2O and citric acid C6H8O7 were measured in stoichiometric quantities and completely dissolved in a sufficient volume of deionized water to provide a clear homogeneous solution. after stirring, the ammonia solution was added drop by drop finally adjusting the pH of the solution to 7 (26). After two hours of constant stirring at a temperature of 90 °C, the final solution was transformed into a viscous gel phase. the solution changed into a very viscous gel during the evaporation process. to initiate an auto-combustion process and produce as-burnt ZnO powder, a viscous gel has been heated to 250°C in an oven after all the water molecules have been removed from the mixture. in order to achieve a fine powder, the rough powder was collected and subjected to gentle grinding in a mortar with agitation (27). After combustion, the as-burned powder was calcined for three hours at 400, 500, and 600 °C in a programmed furnace to remove waste organic material and improve homogeneity. After that, their structural and morphological properties were investigated using field emission scanning electron microscopy (FE-SEM), X-ray diffraction (XRD), and energy dispersive spectroscopy (EDS).

Experimental animals

In this study, 36 adult male albino rats (Rattus norvegicus) weighing about 250–270 g and aged 10–12 weeks have been used. the animals were housed in the animal house of the Biology Department at the College of Education, University of Sulaimani. The rats were housed in polypropylene plastic cages bedded with wooden chips, with three rats per cage. The cages were washed and sterilized twice weekly with 70% alcohol, and the wooden chips were replaced twice a week during the period of the study. The animals were kept according to the typical laboratory environments including a 12-hour light and 12-hour dark light period, a relative humidity ranging from 60% to 70%, and a temperature of 23 ± 2°C. Prior to the start of the study, the animals undergo a one-week acclimatization period. During the period of the study, the rats were provided with typical pellet food and free access to water.

Preparation of ZnO nanoparticles suspension

A suspension of zinc oxide nanoparticle powder was prepared by dispersing it in deionized water at a concentration of 50 mg/ml using ultrasonic vibration (Qsonica sonicator Q700, USA, 30 kHz) for 30 min to avoid particle aggregation. ZnO NPs suspension was vortexed for 1 min before use to break down agglomerates and ensure a uniform suspension (28).

Preparation of moringa oleifera solution

To produce a homogenous solution, the extract of Moringa oleifera was dissolved in 1% dimethyl sulfoxide (DMSO) at a concentration of 75 mg/ml and vigorously mixed. The suspension was prepared freshly every day and vortexed for one minute before administration for each rat.

Experimental design

The animals were allocated into six experimental groups using a randomized method, with each group consisting of six rats, as follows:

- **Group I (Control):** The animals of which were administered orally with 1% DMSO (1ml/rat/day) for 4 weeks by oral gavage and injected intraperitoneally with normal saline (0.5 ml/rat/week) for two weeks. The1% DMSO (1 ml DMSO and 99 ml distilled water) was given as a vehicle.
- **Group II (Moringa 400 mg):** Animals of which were administered orally with the *M. oleifera* leaves extract at a dosage of 400 mg/kg.bw/day (low dose) for 4 weeks by oral gavage and injected intraperitoneally with normal saline (0.5 ml/rat/week) for two weeks.
- **Group III (Moringa 600 mg):** Animals of which were administered orally with the *M. oleifera* leaves extract at a dosage of 600 mg/kg.bw/day (high dose) for 4 weeks by oral gavage, and injected intraperitoneally with normal saline (0.5 ml/rat/week) for two weeks.
- **Group IV (ZnO-NP 150 mg):** Animals of which were injected intraperitoneally with the ZnO-NP at a dosage of 150 mg/kg.bw/week for two weeks**.**
- **Group V (ZnO-NP 150 mg + Moringa 400 mg):** Animals of which were injected intraperitoneally with the ZnO-NP at a dosage of 150 mg/kg.bw/week for two weeks + CO-administered orally with the *M. oleifera* leaves extract at a dosage of 400 mg/kg.bw/day (low dose) for 4 weeks by oral gavage.
- **Group VI (ZnO-NP 150 mg + Moringa 600 mg):** Animals of which were injected intraperitoneally with the ZnO-NP at a dosage of 150 mg/kg.bw/week for two weeks + CO-administered orally with the *M. oleifera* leaves extract at a dosage of 600 mg/kg.bw/day (high dose) for 4 weeks by oral gavage.

Collection of blood sample and dissection

After the completion of the experiment (14 successive days for the ZnO-NP treated group and 28 successive days for the other groups), the animals passed through an overnight fasting period and then anesthetized with chloroform inhalation in a special glass container of desiccator. Blood samples have been collected through a heart puncture with a syringe, and the blood sample was slowly poured into a gel tube with a clot activator and allowed to clot for approximately 15 to 20 minutes at room temperature. After 10 minutes of centrifugation at 3000 rpm, the serum was collected and transferred into six Eppendorf tubes (1.5 ml) for each rat and kept in a deep freeze (-80 °C) until further investigation. The animals were sacrificed, the abdominal cavity was opened with an incision, and the testes were dissected out immediately from each rat, washed with normal saline (0.9%), and the adhering fat and connective tissues removed, then weighed. After that, the epididymis was carefully removed from the testes and used for semen analysis, and the testis was homogenized for the measurement of oxidative stress biomarkers.

Testicular homogenization

The left testis was separated and washed with phosphate buffer saline. To prepare testicular homogenates, 1 g of testicular tissue was homogenized in 9 ml of ice-cold phosphate buffer saline (PBS) with a pH of 7.4. The homogenization process was carried out on ice using an electrical tissue homogenizer (29). Centrifuging the homogenate at 5000× g for 5 min at 4 °C. The homogenate supernatant was carefully transferred into Eppendorf tubes and stored in a deep freeze (-80 °C) until the levels of catalase (CAT), glutathione peroxidase (GPX), and malondialdehyde (MDA) were measured (30).

Sperm count

To prepare sperm suspension and semen analysis, the caudal epididymis was separated carefully from the testes. 200 milligrams of caudal epididymis have been placed in 2 ml of warm normal physiological saline at 37°C in a sterilized Petri dish and then cut into 3 to 4 pieces with a scissor. The tissue was allowed to incubate for a duration of 30-60 seconds to facilitate the release of sperm from the epididymal tubules. The obtained solution exhibited a whitish/grayish color, resembling diluted semen. After that, the sperm suspension was collected in a 1.5-ml Eppendorf tube for microscopic analysis of sperm count and morphology (31). The sperm count was conducted using an improved Neubauer hemocytometer chamber according to World Health Organization (WHO) guidelines. Tissue is diluted 10 times with normal saline, and 20 µl of sperm suspension is diluted with 180 µl of semen diluent (5 g sodium bicarbonate, 1 ml formalin, and 99.0 ml distilled water) and thoroughly mixed. About 10 µl of the diluted sperm suspension has been loaded into each counting chamber of the hemocytometer, and left to stand for a duration of five minutes in a moist condition to avoid drying. During this time, the spermatozoa were settled down and counted at 400x using a light microscope (32). In five-16-celled secondary squares, the number of spermatozoa was counted. The result is calculated, multiplied by 10^6 and represented as (N) × 10^6 /ml, where N is the count of spermatozoa in a five-16-celled secondary squares (33).

Sperm morphology

One drop of epididymal sperm suspension was spread on a pre-heated slide to perform the analysis of sperm morphology. the smear was thereafter subjected to an air-drying process, followed by staining with a 1% eosin solution for a duration of 10 minutes. It was then rinsed with tap water and allowed to dry at an ambient temperature. Afterward, the smear was stained with hematoxylin for a period of 15 minutes, rinsed with tap water, and again dried at room temperature (34). Observation of smears was done by using an oil-immersion objective at ×100. To assess morphological abnormalities, 400 sperm cells were screened in each animal. Any abnormalities in the structural and morphological features of either the tail, head, or both have been identified as abnormal. The ratio of abnormal sperm cells has been calculated as a percentage of the total number of spermatozoa (35). The identification of headless sperm, tailless sperm, abnormal heads, abnormal hooks, and abnormal necks was evaluated. Ten various fields have been assessed in each sample.

Biochemical study

Determination of testicular Malondialdehyde (MDA)

The final result of the lipid peroxidation process is malondialdehyde (MDA). the reaction between MDA and thiobarbituric acid (TBA) is improved by the addition of heat and acidic conditions, which leads to the formation of a clearly visible pink-colored compound (36). The level of MDA was estimated by using the following calculation method:

$$\text{MDA (µmol/L)} = \frac{\text{Absorbance at 532 nm}}{\text{L x Eo}} \text{ x D x } 10^6$$

E_0: Extinction coefficient = 1.56x105 M^{-1} .cm^{-1}

D: Dilution factor = 1 ml Vol. Used in ref./0.15 = 6.7

L: light path (1cm)

Assessment of testicular catalase and glutathione peroxidase

The activity of catalase (CAT) and glutathione peroxidase (GPX) has been measured in testicular homogenate via rat-specific enzyme-linked immunosorbent assay (ELISA) kits obtained from (BT LAB, UK) following the guidelines provided by the company that manufactured the kits.

Measurement of serum testosterone

The level of testosterone was determined through rat-specific enzyme-linked immunosorbent assay (ELISA) kits obtained from (BT LAB, UK) following the guidelines provided by the company that manufactured the kit.

Statistical analysis

The statistical analysis has been done using the GraphPad Prism program (version 9.0). All data were represented as the mean ± standard error of the mean (SEM) and subsequently analyzed via one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests for analysis. A significance level of $P<0.05$ was considered to be statistically significant.

Results

ZnO-NPs characterization

XRD studies

The sharpness of the XRD peaks exhibited in (Figure 1) revealed the characteristic patterns, strong crystallinity, and confirmed the formation of crystalline ZnO samples at calcination temperatures of 400, 500, and 600 °C for 3 hours. The three strongest diffraction peaks at an angle of 2θ are indexed with miller indices (100), (002), and (101) illustrating high phase crystallinity and corresponding to the hexagonal wurtzite phase of the ZnO nanostructure. The increased intensity of the distinctive peaks reveals the crystalline nature of the products. No peaks corresponding to impurities were identified, showing that the end product is purely ZnO nanostructures. According to the Scherrer formula, the average crystallite size was (20.95, 24.23, and 44.71) for the temperatures of 400, 500, and 600 °C, respectively.

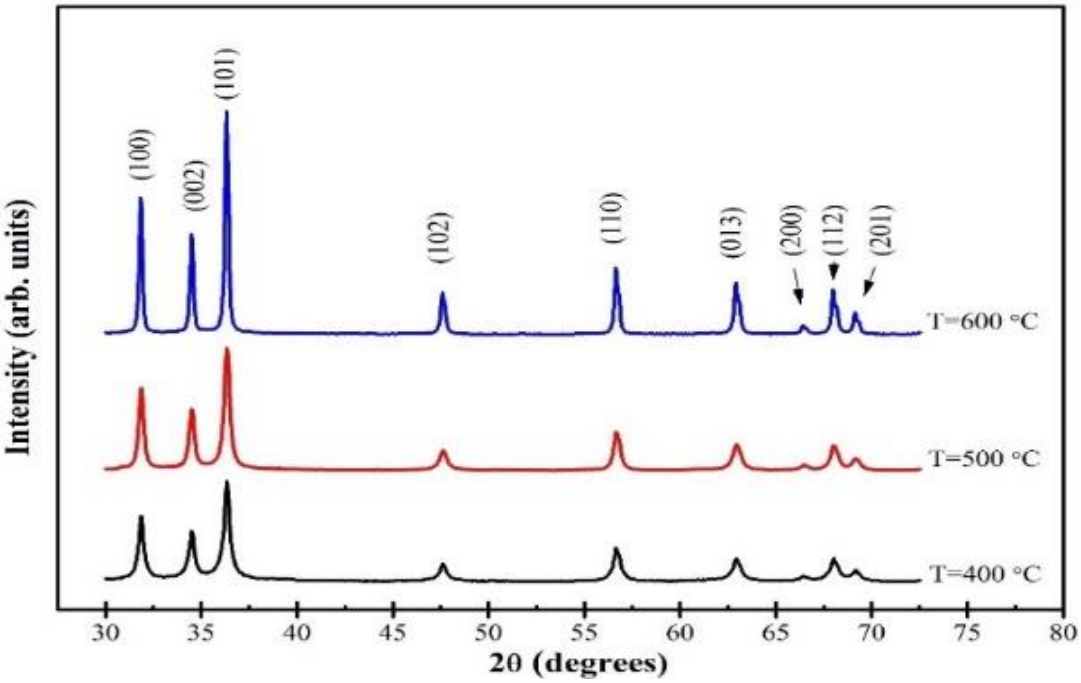
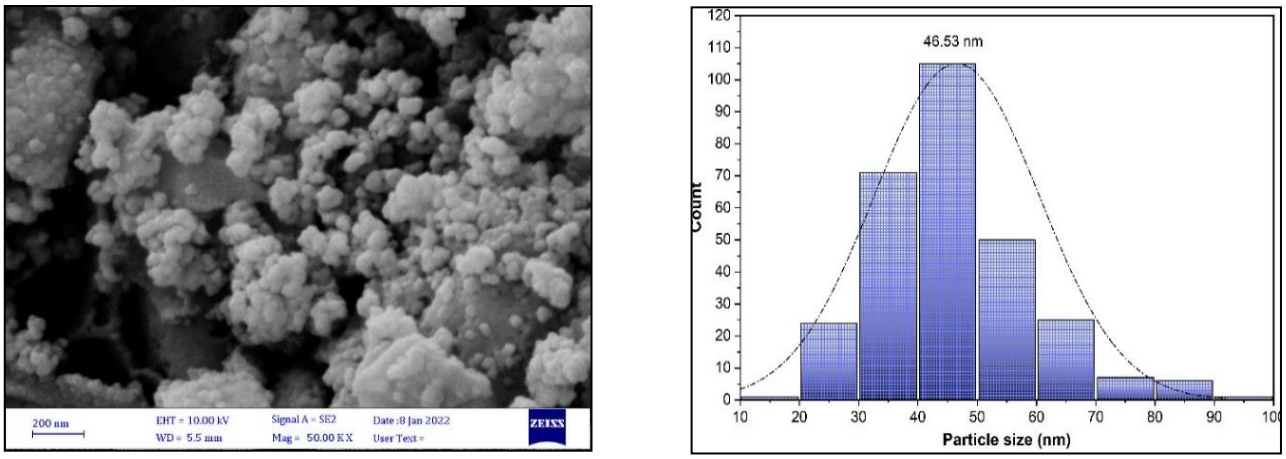


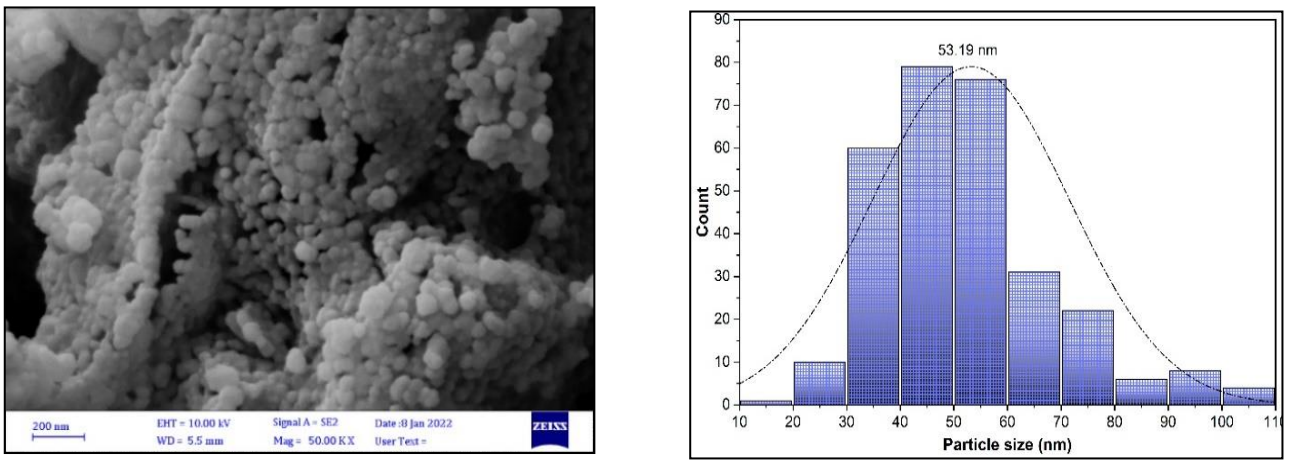
Figure 1. XRD patterns of ZnO NPs samples calcined at 400, 500, and 600 °C.

FE-SEM and EDS studies

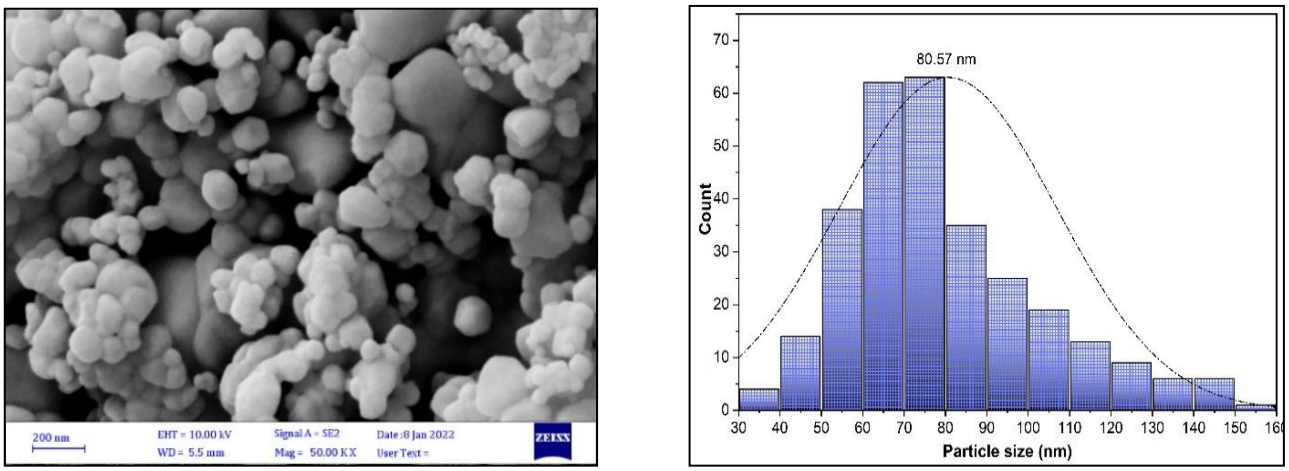
The investigation of surface morphology and EDS spectra was conducted using a field emission-scanning electron microscopy (FE-SEM) model (Mira3-XMU, TESCAN, Japan). The analysis of surface morphology and histogram distribution of ZnO NPs samples calcined at different temperatures has been conducted at room temperature, as shown in (Figures 2. A–C). The findings from the FE-SEM image suggest that the particles of interest exhibit a composition consisting of many nano-crystals, as confirmed by X-ray diffraction (XRD) analysis. The average particle size measured at temperatures of 400, 500, and 600 °C was found to be (46.53, 53.19, and 80.57 nm), respectively. The use of energy dispersive spectroscopy (EDS) has shown the absence of undesirable precursors, including nitrate ions, in the final product. As a result, the main components of the product have been identified as zinc (Zn) and oxygen (O).



(A)



(B)



(C)

Figure 2. FE-SEM images and frequency distribution of histogram of ZnO nanoparticles (A) calcined sample at 400ºC, (B) calcined sample at 500 ºC, and (C) calcined at 600 ºC

Effect of M. oleifera, ZnO-NP, and their combination on sperm count

The group treated with ZnO-NP exhibited a significant decline in sperm count ($p<0.01$), whereas the group received an extract of *M. oleifera* leaves revealed a significant elevation in sperm count ($p<0.01$) when compared with a group serving as a control. In contrast, the co-administration of ZnO-NP with *M. oleifera* leaf extract demonstrated a significant rise ($p<0.05$) in sperm count when compared with a group treated with ZnO-NP alone (Figure 3-A).

Effect of M. oleifera, ZnO-NP, and their combination on sperm morphology

The group received ZnO-NP revealed a significant drop ($p<0.001$) in the percentage of normal sperm morphology, whereas, the group received *M. oleifera* leaf extract displayed a significant rise ($p<0.01$) in normal sperm morphology when compared with a group serving as a control. On the other hand, the co-administration of ZnO nanoparticles with an extract of *M. oleifera* leaves displayed a significant rise ($p<0.01$) in the percentage of normal sperm morphology as compared with a group treated with ZnO-NP (figure 3-B).

The group received ZnO-NP revealed a significant elevation in the percentage of abnormal sperm morphology ($p<0.001$) when compared with a group serving as a control. whereas, the treatment with an extract of *M. oleifera* leaves at a high dosage by oral route revealed a significant reduction ($p<0.01$) in abnormal sperm morphology in comparison to the group serving as a control. In contrast, the co-administration of ZnO-NP with *M. oleifera* leaf extract revealed a significant drop ($p<0.01$) in abnormal sperm morphology as compared to the ZnO nanoparticles treated group (figure 3-C).

Effect of M. oleifera, ZnO-NP, and their combination on testosterone

The experimental group treated with ZnO-NP revealed a significant drop ($p<0.01$) in the level of serum testosterone as compared to the group serving as a control. In contrast, the administration of an extract of *M. oleifera* leaves at a high dosage by oral route exhibited a significant elevation ($p < 0.01$) in serum testosterone levels as compared to the group serving as a control. In contrast, the co-administration of ZnO-NP with *M. oleifera leaf* extract showed a significant rise ($p<0.01$) in testosterone levels as compared with a group treated with ZnO-NP alone (figure 3-D).

Effect of M. oleifera, ZnO-NP, and their combination on testicular weight

The group received ZnO-NP showed a statistically significant decline ($p<0.05$) in testicular weight when compared with a group serving as a control. In contrast, the administration of an extract of *M. oleifera* leaves by oral route did not bring about a statistically significant change in testicular weight as compared to a group serving as a control. In contrast, the co-administration of ZnO-NP with an extract of *M. oleifera* leaves at a high dosage revealed a statistically significant increase ($p<0.05$) in testicular weight in comparison to the ZnO-NP group (figure 5-A).

Effect of M. oleifera, ZnO-NP, and their combination on catalase activity

The group treated with ZnO-NP showed a significant decline ($p<0.05$) in the activity of testicular catalase. whereas the administration of an extract of *M. oleifera* leaves by oral route at a high dosage displayed a significant rise ($p < 0.01$) in the level of catalase when compared to the group serving as the control. In contrast, the co-administration of ZnO-NP with an extract of *M. oleifera* leaves at a high dosage demonstrated a significant increase ($p<0.001$) in the level of catalase as compared with ZnO-NP group (figure 5-B).

Effect of M. oleifera, ZnO-NP, and their combination on Glutathione peroxidase activity

The group received ZnO-NP displayed a significant decline in glutathione peroxidase activity ($p<0.05$) when compared with group serving as the control. whereas, the co-administration of ZnO-NP with an extract of *M. oleifera* leaves in a high dosage revealed a significant increase ($p<0.05$) in glutathione peroxidase activity when compared with ZnO-NP group (figure 5-C).

Effect of M. oleifera, ZnO-NP, and their combination on MDA

The group received ZnO nanoparticles displayed a significant elevation in the level of testicular MDA ($p<0.0001$) when compared with a group serving as a control. However, the group that received an extract of *Moringa oleifera* leaves by oral route at a high dosage exhibited a significant decline ($p < 0.05$) in the concentration of testicular MDA as compared with a group serving as the control. In contrast, the co-administration of ZnO-NP with an extract of *M. oleifera* leaves at a high dosage exhibited a significant drop ($p<0.05$) in MDA concentration as compared to the ZnO-NP group. However, the co-administration of ZnO-NP with an extract of *M. oleifera* leaves at a low dosage displayed a significant elevation ($p<0.01$) in MDA levels as compared with a group serving as a control (Figure 5-D).

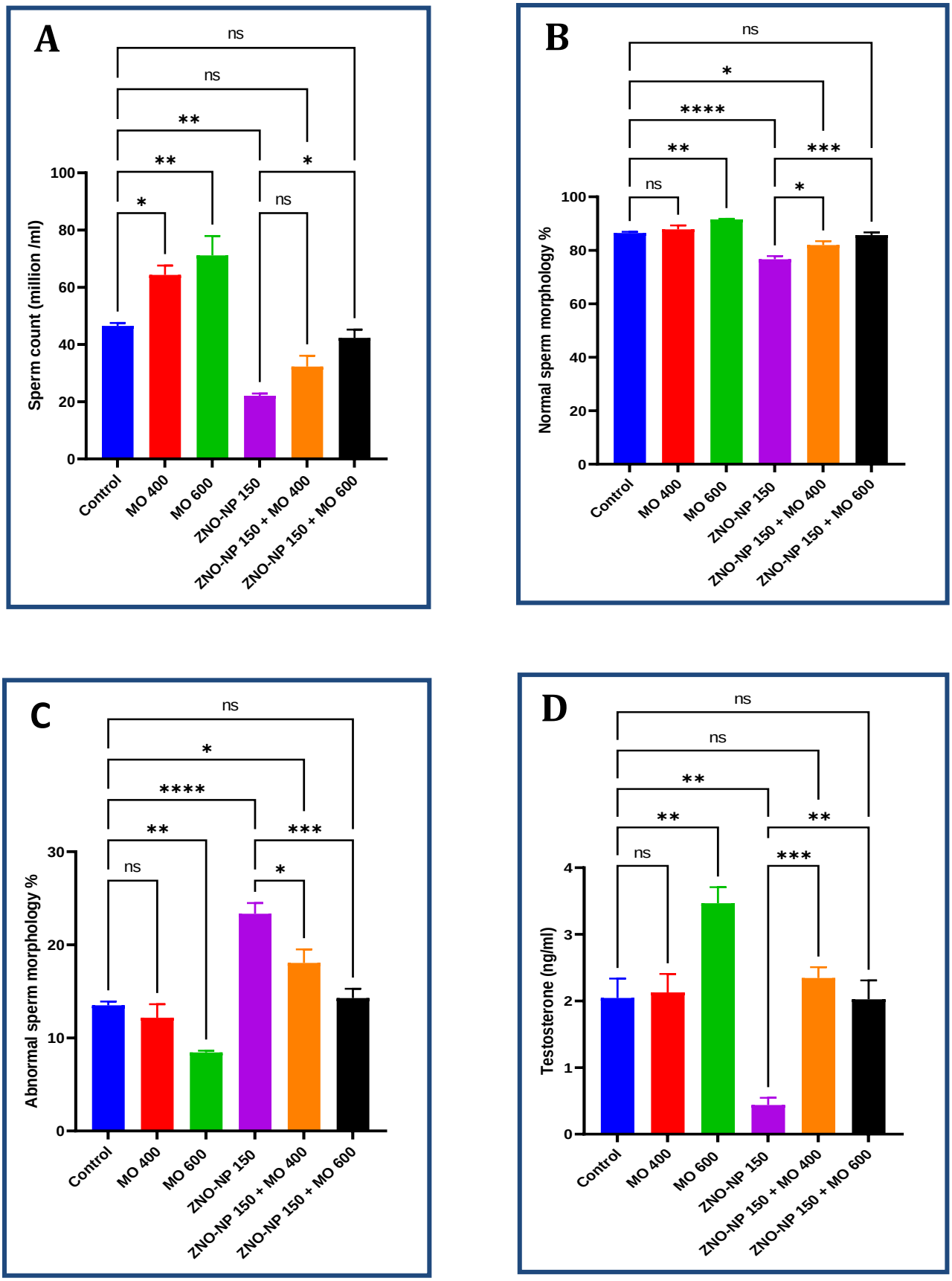


Figure 3. Effect of *M. oleifera*, ZnO-NP, and their combination on **(A)**: sperm count (mean ± S.E) $\times 10^6$ /ml, **(B)**: normal sperm morphology (mean ± S.E) %, **(C)**: abnormal sperm morphology (mean ± S.E) %, **(D)**: testosterone (mean ± S.E) ng/ml. * =p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001

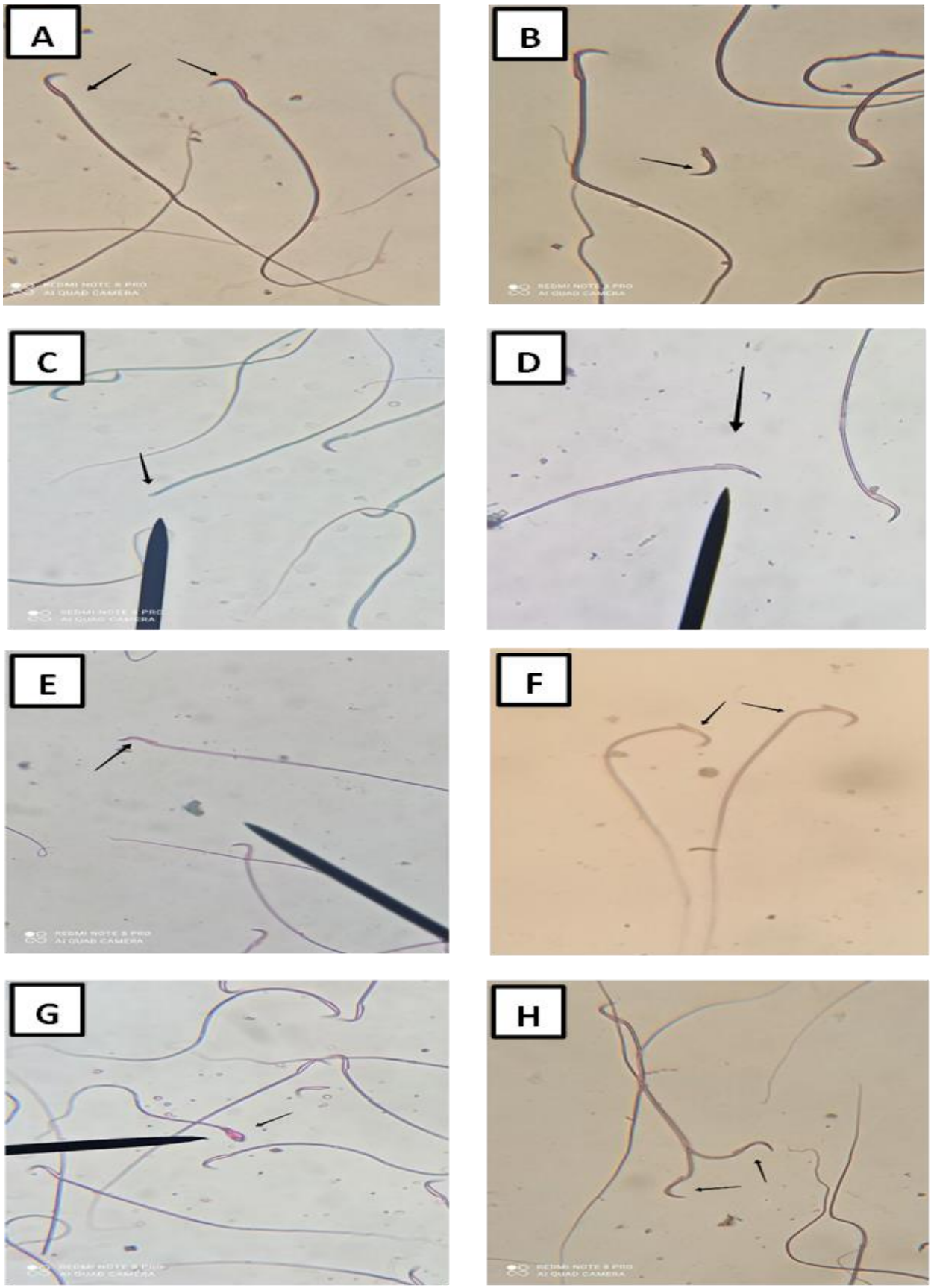


Figure 4. A photomicrograph of epididymal spermatozoa from adult male albino rats. (A) normal sperm, (B) sperm without tail, (C) sperm without head, (D) sperm without hook, (E) sperm with defective hook, (F) sperm with abnormal head neck attachment point, (G) sperm with defective head, (H) sperm with double head (eosin & hematoxylin x400)

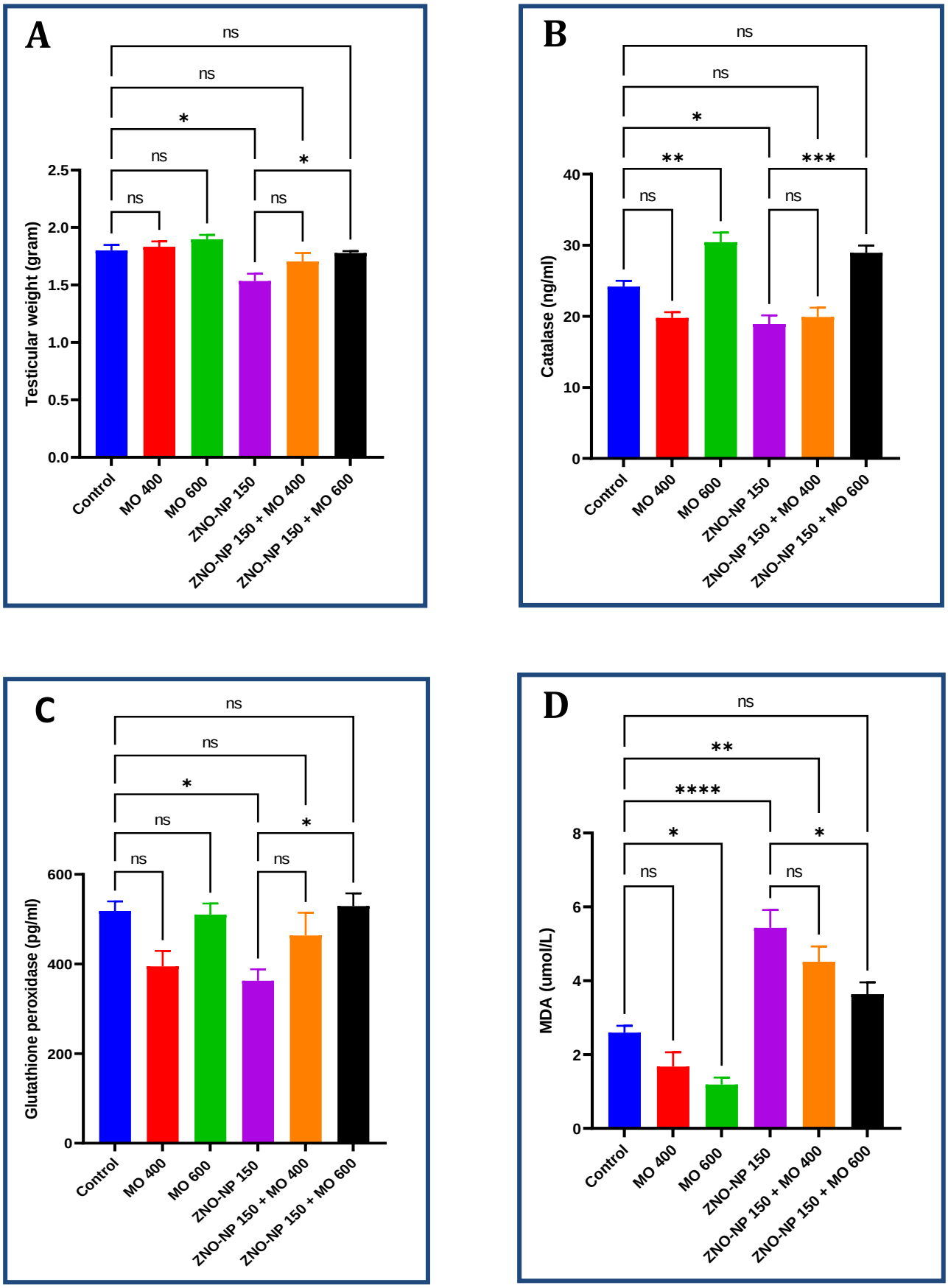


Figure 5. Effect of *M. oleifera,* ZnO-NP, and their combination on **(A)**: testicular weight (mean ± S.E) gram, **(B):** catalase activity (mean ± S.E) ng/ml, **(C):** glutathione peroxidase activity (mean ± S.E) pg/ml and, **(D):** MDA (mean ± S.E) µmol/L. * =p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001

Discussion

ZnO NPs have become essential metal oxide nanomaterials due to their various medical applications and widespread usage in daily life. However, concerns about their potential effects on the human body, especially the male reproductive system, have become increasingly prevalent (37, 38). Hence, in the current study, we evaluated the toxicity of ZnO nanoparticles on certain physiological reproductive parameters as well as the potential protective role of *Moringa olifera* leaf extract in minimizing ZnO nanoparticle toxicity in the reproductive system of adult male rats. Adult albino rats have been chosen based on their suitability for investigating reproductive toxicity due to their relatively long lifespan and ease of handling(39).

The XRD analysis revealed that the intensity of XRD peaks displayed distinctive patterns, indicating the synthesis of crystalline ZnO NPs samples for 3 hours at 400, 500, and 600 °C. The distinctive peaks got higher and sharper as the calcination temperature was raised, indicating an improvement in crystallinity due to agglomeration and an increase in crystallite size (27, 40). The diameter of ZnO nanoparticles as revealed by FE-SEM pictures is substantially larger than the crystallite size as determined by XRD (41). In addition, because of the fusion of adjacent small particles and the high surface energy of the ZnO nanoparticle (42), increasing the calcination temperature impacts the porosity structure, high polyhedral particle aggregation, and increased particle size (41). The spectrum of EDS revealed that the purity of ZnO was sufficient. The size, shape, and chemical structure of nanoparticles have been proven to have a significant impact on their cytotoxicity (43).

Semen quality, including sperm quantity, motility, and abnormalities, is the most important determinant of male fertility (44). The evaluation of sperm parameters in the current study revealed a substantial decrease in sperm count, normal morphology, and an increase in sperm abnormalities in the ZnO NPs-treated group. These findings are consistent with those of Talebi and colleagues (45), who found that administering zinc oxide nanoparticles at concentrations of 50 and 300 mg/kg dramatically reduced sperm count, motility, and abnormalities in mice. In this direction, Han et al. (46) discovered that ZnO NPs could reduce the quality of mice's sperm. Another study conducted by Abbasalipourkabir and colleagues (16) observed that ZnO NP causes substantial alterations in both sperm quality and quantity in mature male Wistar rats. As a consequence, these variations in epididymal sperm characteristics could be correlated to the synthesis of reactive oxygen species by ZnO NPs (47). Furthermore, polyunsaturated fatty acids are prevalent in mammalian spermatozoa, and an insufficient amount of antioxidant makes spermatozoa more susceptible to reactive oxygen species leading to membrane lipid oxidation by free radicals (48). A physiologically minimal amount of reactive oxygen species is required for normal sperm activity. Subsequently, increase that occurs in the concentration of ROS regarding that the normal range is correlated with a decrease in sperm efficiency (49). Alvarez and his colleagues (50), as well as Saradha and Mathur (51), shown that reactive oxygen species (ROSs) can initiate fatty acid peroxidation in sperm cells, ultimately leading to lipid peroxidation. As a result, phosphatide impairment occurs within the cell membrane, resulting in abnormal sperm morphology and a decrease in sperm count. On the other hand, in the present investigation, supplementation of *M. oleifera* leaf extract increased sperm count, normal morphology, and lowered sperm abnormalities, particularly among the high dose administered groups. These findings agree with those of Dafalla et al. (22), They found that providing male rats with an extract of *M. oleifera* leaves increased sperm count, normal morphology, sperm motility, and viability. On the same side, Priyadarshani and colleagues (52) discovered that treatment with 200 mg/kg.bw powder of *M. oleifera* leaves for 21 days significantly increased sperm count and morphology. Also, Afolabi and his colleagues (53), showed that a methanolic extract of *M. oleifera* leaves improved spermatozoa and biochemical markers in cryptorchidism rats, further confirm the results we have discovered. Furthermore, additional research found that *Moringa oleifera* leaf extract, which is rich in powerful antioxidants that are naturally occurring, like vitamin C, vitamin E, beta-carotene, polyphenols, and flavonoids, preserved spermatozoa membranes and protected against sperm damage associated with free radicals, which led to a

higher quantity of sperm and enhancement of spermatozoa morphology (54, 55). The results presented here are in agreement with the findings published by D'cruz and Mathur (56), who found that the cytoplasm of sperm contained very small amounts of free radical-scavenging enzymes; thus, increasing the antioxidant enzyme system levels through *Moringa oleifera* treatment might enhance the process of reproduction as well as spermatogenesis.

The hormone of testosterone is an important androgenic hormone that contributes to a substantial part of spermatogenesis, sertoli-spermatid adhesion, blood-testis barrier integrity, and spermatozoa elimination into the lumen (57). The current investigation found that in the ZnO NPs-treated group, the serum testosterone levels significantly diminished. This result is in accordance with Moridian and his colleagues (14), who examined the impact of different doses of ZnO NPs on the testicular tissue of mice for a 35-day period at concentrations of 5, 50, and 300 mg/kg/day. When compared to the control group, it demonstrated a substantial drop in testosterone levels at 50 mg/kg and a highly significant drop at 300 mg/kg. Furthermore, the results we obtained are consistent with those of Tang and his colleagues (58), who investigated the change in the level of testosterone in the serum of male mice after 14 days of oral administration with 50, 150, and 450 mg/kg/day ZnO NPs. The researchers discovered that increasing the dosage of ZnO NPs was associated with a more significant decrease in testosterone concentrations in the serum. In addition, a significant decrease in serum testosterone levels demonstrates that ZnO NPs can influence androgen production by Leydig cells. Han and his colleagues (46), examined the accumulation of ZnO NPs in Sertoli cells and Leydig cells, which resulted in apoptosis in these types of cells. In the current study, however, oral administration of *Moringa oleifera* leaf extract substantially elevated testosterone levels, particularly in the high-dose treatment groups. Our result is similar to that of Syarifuddin and his colleagues (59), who concluded that increased levels of testosterone were found in male rats after treatment with *M. oleifera*. Also, our findings agree with Khalifa and his colleagues (60), who observed that using *Moringa oleifera* improved testosterone, LH, and FSH levels. On the same side, Gouda and his colleagues (61) showed that using *Moringa oleifera* considerably enhanced the level of the testosterone hormone in rabbit blood. Furthermore, previous research projects indicated that administering chloroform and hydro-alcoholic *Moringa oleifera* leaf extract to experimental animals improved their levels of testosterone (30, 62). Moreover, the significant concentrations of phenolic compounds, carotenoids, flavonoids, tannins, saponins, and isothiocyanate in *Moringa oleifera* might have influenced hormone synthesis (63). The presence of flavonoids in Moringa oleifera extract might impact the level of androgen through reducing the aromatase enzyme, which catalyzes the change of androgens to estrogens, which subsequently promotes the production of testosterone (64). In regards to this study (65), saponin has the property to improve testosterone levels within the male reproductive system.

Animal toxicity research study use the body and organ weights as sensitive and crucial markers (66). The findings of the current investigation indicated that testicular weight decreased significantly in the ZnO NPs-treated group. this result agrees with a study by Talebi and his colleagues (45), who determined that daily administration of zinc oxide nanoparticles to mice for a duration of 35 days at doses of 5, 50, and 300 mg/kg demonstrated that the testicular weight at dosage 5, 50, was slightly decreased compared to the control groups, while dose 300 exhibited a substantial reduction in the weight of the testes. In addition, Kuang and his colleagues (67) observed a decrease in the absolute testis weight of mice after the administration of ZnO nanoparticles. On the same side, our finding is similar to Shirvani et al.(68), who revealed that male rats were given ZnO-NPs at various dosages (25, 50, and 100 mg/kg) for a duration of 15 days observed a decrease in testicular weight as compared with the group that served as a control. The decrease in testicular weight also suggests that ZnO-NPs may induce the loss of testicular cells, including germ and somatic cells. On the other hand, co-administration of ZnO NPs with a large dosage of *M. oleifera* leaf extract in the current research preserved testicular weight. Our findings are consistent with the results published by Priyadarshani and colleagues (52), which found the administration of Moringa oleifera leaf powder increased testicular weight in diabetic mice. Also, our finding is similar to that of Abarikwu and his colleagues (69), who identified that

Moringa has the ability to prevent testicular injury, as testicular toxicity produced by mercury in male rats can be mitigated by orally administering 2 mL/kg of moringa seed oil, which has a beneficial effect on testicular weight. Thus, the raised testicular weight may be attributed to the potent phytochemicals found in Moringa oleifera including kaempferol, quercetin, vitamins C, E, A, and rutin, that are responsible for preserving both the testicular and body weights (70).

Reactive oxygen species (ROS) may cause damage, which is referred to as oxidative stress (71). Reactive oxygen species (ROS) have a high level of reactivity and are generated in organs with high metabolic activity, such as the testis. Therefore, the antioxidant capacity of the testis is decreased, which causes the development of oxidative stress (53). In the current research, the assessment of oxidative stress indicators in testicular tissues exhibited a significant elevation in malondialdehyde (MDA) levels and a reduction in the enzymatic activity of glutathione peroxidase and catalase in the ZnO NPs-treated group. These results agree with Hussein and his colleagues (72), who reported that the administration of ZnO NPs at dosages of 100 and 400 mg/kg/day caused a substantial elevation in MDA and reduced the levels of SOD, GPx, and CAT in the testicular tissue of the groups treated with ZnO NPs. Also, Rafiee and his colleagues (73) demonstrated that administration of ZnO nanoparticles (NPs) causes the generation of oxidative stress inside the testes of mice. This was indicated by the reduction in the levels of catalase and superoxide dismutase enzymes as well as an elevation in malondialdehyde levels. On the same side, Yousef and his colleagues (74) investigated the reproductive toxic effects of ZnO nanoparticles in rat males. They showed significant reductions in the levels of SOD, CAT, and GPx in both plasma and testis, whereas there was a substantial elevation in MDA levels as compared to the group serving as a control. Furthermore, Anan et al. (75) revealed that the administration of ZnO nanoparticles at a dose of 5 mg/kg caused an elevation in the levels of SOD, CAT, and GPx and a decline in the level of MDA in the testicular tissue of rats. Various factors, including particle size, morphology, concentration, and surface area, determine the effects of ZnO nanoparticles. At lower levels, ZnO nanoparticles (NPs) have antioxidant properties, but at higher levels, ROS is produced and causes apoptosis (38). On the other hand, in the present research, oral administration of *M. oleifera* for a duration of 28 days significantly elevated GPx and CAT and declined MDA in the homogenized testicular tissue. This effect was more significant in the groups that received higher doses of *M. oleifera*. These results are similar to those of Nayak and his colleagues (76), who reported that the use of *Moringa oleifera* leaf extract at a dosage of 25 mg/kg BW before cyclophosphamide treatment elevated the levels of CAT and SOD with a concurrent reduction in the level of lipid peroxidation in the tissue of the testes in rats. Also, Uma and his colleagues (77) demonstrated that giving hydro-ethanolic *M. oleifera* leaf extract to rats decreased the liver toxicity caused by paracetamol by reducing the level of lipid peroxidation and increasing the levels of glutathione reductase, glutathione S-transferase, and glutathione peroxidase. Moreover, previous research has shown that *Moringa oleifera* leaf extract has a powerful ability to mitigate cellular oxidative stress via the elevation of potent antioxidant enzymes like SOD, CAT, GSH, and GPx (24, 78). Hence, the antioxidant activities and suppression of lipid peroxidation in *Moringa oleifera* leaf extract might be related to the existence of several bioactive compounds such as beta-carotene, polyphenols, flavonoids, quercetin, kaempferol, rutin, as well as vitamin C and vitamin E (70).

Conclusions

In conclusion, our findings suggest that ZnO-NP had negative impacts on the testes of the Wistar rat, induced oxidative stress and testicular damage by decreasing the levels of CAT and GPx, increased MDA in testicular tissue. However, oral administration of *M. oleifera* leaf extract significantly mitigates the toxicity of the testes induced by ZnO-NP. This positive impact might be related to the existence of powerful phytochemical compounds that are capable of improving the activity of antioxidant enzymes in the testes and also improving sperm parameters. This finding provides evidence to support the traditional usage of *M. oleifera* in the treatment of male infertility. Further studies are required to isolate and purify the bioactive compounds responsible for improving testicular toxicity.

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