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Immunotoxicity of Zinc Oxide Nanoparticles on Spleen of Male Albino Rats with Ameliorative Role of *Moringa olifera*

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Abstract

THIS study aimed to investigate the immunotoxicity of oral zinc oxide nanoparticles (ZnO NPs) on spleen of Albino rats with the ameliorative effect of *Moringa olifera* leaves extract. For 28 days, rats were oral gavaged ZnO NPs at concentrations of 200 and 400 mg/kg, either with or without Moringa extract. Serum levels of antioxidants, Inerleukin-1 Beta (IL-1β), Interleukin-6 (IL-6), and Caspase-1 were examined, with the different leukocytic count. Histopathological examination of spleen was performed. Additionally, the comet test was used to assess DNA damage. The findings of this study showed that, in comparison to the control group, the mean percentage of lymphocytes increased from ~59% up to ~71% and the mean percentage of neutrophils dropped from ~38% down to ~22% following exposure to 400 mg ZnO NPs. There was a significant rise ($P<0.001$) in nitric oxide (NO) and malondialdehyde (MDA) levels and fall in glutathione (GST) and superoxide dismutase (SOD) levels. Furthermore, compared to the control group, there was a significant increase in the blood levels of IL-1β (~78 pg/ml), IL-6 (~79 pg/ml), and Caspase-1 activity. The toxicity of ZnO NPs was confirmed by histological evaluation of the spleen, which showed that ZnO NPs cause a variety of pathological lesions. Significant increases in tail length and tail moment relative to control indicate DNA damage caused by ZnO NPs. This discovery may provide a health risk to those who are exposed to ZnO nanoparticles on a regular basis. Moringa leaves showed the protective effect through its anti-inflammatory, anti-tumor, and antioxidant pathways. Numerous bioactive substances, macro and micronutrients are being found in moringa. In conclusion, enhancing hematological and immunological markers allows moringa leaves extract to reduce the toxicity of ZnO NPs and brought them back to normal levels.

Keywords: Zinc oxide, Nanoparticles, Spleen, antioxidants, DNA damage, Moringa Olifera leaves.

Introduction

Nanomaterials are incredibly tiny structures that come in a variety of forms and sizes between 0.1 and 100 nm. [1]. Because of their enormous surface area, high reactivity, and tiny size, nanoparticles (NPs) exhibit unique physiochemical characteristics. Because of these characteristics, they are widely used in the food and non-food sectors, as well as in consumer goods, coating materials, medicinal procedures, and fluorescent labeling [2-4]. In the biomedical industry, zinc oxide nanoparticles, are widely employed as anticancer medications, gene delivery medications, and cosmetics. They are employed in agriculture and the food business because of their fungicidal and antibacterial qualities. Understanding the toxicity of nanoparticles is crucial to prevent their detrimental effects on the human body since their interactions with biological systems can have unanticipated outcomes [5,6].

The antigen-specific immune responses were boosted by ZnO NPs in mice. They improved serum production of antigen-specific antibodies, particularly immunoglobulin E (IgE) and immunoglobulin G (IgG), and they may also improve T cell activity. Additionally, ZnO NPs were found to have an adjuvant effect on the Th2 response, boosting cytokine synthesis and activation [7].

It is thought that the release of Zn^{++} ions from ZnO NPs is what causes their hazardous action. Thus, even for brief periods, a high concentration of ZnO NPs in diets can make animals poisonous to

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zinc [8]**.** Furthermore, ZnO NPs resulted in hemolysis, lowering erythrocyte, platelet counts, serum haptoglobin concentrations, also cause liver histopathological abnormalities [9].

Plant-derived products have been exploited as medicinal treatments with minimal detrimental effects [10]. The Moringa (Moringa oleifera Lam.), sometimes referred to as the drumstick tree, is a member of the Moringaceae family and is found throughout North and South America [11]. Crude protein and important amino acids including methionine, cysteine, tryptophan, and lysine are abundant in moringa [12]. Along with phytochemicals like terpenoids, flavonoids, tannins, anthocyanins, and proanthocyanidins, it also contains ginseng and ginseng saponins [13]. The idea that Moringa may be involved in immune modulation is supported by its several characteristics, which include anti-inflammatory, anti-apoptotic, antioxidant, and immuno-stimulant qualities [14]. The anti-inflammatory activities of Moringa oleifera compounds and their mechanisms of action were characterized as it was believed that these compounds functioned through a variety of pathways [15-17]:

(a) Cyclo-oxygenase (COX) and lipoxygenase (LOX), two crucial enzymes involved in the synthesis of inflammatory mediators such as prostaglandins and leukotrienes, are inhibited by M. oleifera compounds quercetin and kaempferol; **(b)** control of cytokine production: isothiocyanates modulates signaling pathways that are implicated in inflammation, such as the nuclear factor-kappa B (NF-kappa B) pathway; this ensures that signaling pathways are modulated. Furthermore, isothiocyanates decrease the production of pro-inflammatory cytokines like tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) while stimulating the production of anti-inflammatory cytokines like interleukin-10 (IL-10); and **(c)** antioxidant activity: polyphenols and flavonoids help in reducing oxidative stress and inflammation. These compounds may also decrease the action of pro-inflammatory enzymes and change cytokine production. Rats received oral administration of 100 mg/Kg ZnO NPs for sixty days exhibited a substantial rise in white blood cell count (WBCs), whereas co-adminstration of 100 mg/Kg ZnO NPs and 300 mg/kg of Moringa extract produced a significant recovery impact on hematological disorders [18]. Despite the extensive use of ZnO NPs, their immunotoxic effects on the spleen remain underexplored. Moreover, natural products such as Moringa oleifera may offer protective effects due to their antioxidant and immunomodulatory properties. Therefore, this study aimed to assess the extract from Moringa olifera's ability to prevent the immunotoxicity of ZnO NPs in rats as well as its mechanism.

Material and Methods

Chemicals and reagents

Zinc oxide nanoparticles (ZnO NPs)

ZnO NPs were obtained from Sigma-Aldrich (CAS No. 1314-13-2) were a white, odorless powder with a Zn content of approximately 80%, a surface area of 10–25 m^2, a purity of >99%, a band gap of 3.37 eV, and a particle size of less than 100 nm by dynamic light scattering (DLS) and less than 35 nm by aerodynamic particle sizer. Distilled water from SEDICO Company in Egypt was used to dissolve it.

Reagents: Sodium Chloride [Natco, Egypt], Sodium monobasic Phosphate [El-Naser Co., Egypt] , Sodium dibasic Phosphate [El-Naser Co., Egypt], Potassium chloride (KCl) [Natco, Egypt], Potassium monobasic phosphate (KH_2PO_4) (El-Naser Co., Egypt), Formalin 40 percent (Natco, Egypt), Hematoxyline and Eosin [Biodiagnostic, Egypt], Methylene Blue stain [Biodiagnostic, Egypt], Ethylenediamine tetraacetic acid (EDTA), Dimethyl sulfoxide (DMSO), Phosphate buffer saline (PBS), Neutral buffered formalin were also used in our study.

Preparation of Moringa Olifera leaves extract

Under typical environmental circumstances, the plant's fresh leaves were collected and allowed to air dry. Prior to extraction, the air-dried leaves were crushed and steeped in distilled water for 24 hours at a weight-to-volume ratio of 1:2. The mixture was then filtered through Whatman's filter paper to separate the filtrate from the debris. After collecting the filtrate, the solvent was extracted using a rotary evaporator, and the residue was weighed [19]. 500 g of the residue was dissolved in one liter of sterile distilled water to create the concentrated stock solution of Moringa leaf extract, which was then stored at 4°C. Throughout the research period, the rats received daily oral administration of the concentrated extract at determined dosages via stomach tube.

Characterization of ZnO NPs

To assess size and shape, the dried powdered particles were suspended in distilled water (1 g/L) and sonicated at room temperature for 10 min until a homogenous suspension was formed. Thereafter the samples were produced by drop coating of the stock solution on to carbon-coated copper grids. At the Electron Microscope Research Unit, Faculty of Agriculture, Mansoura University, Egypt, the films on the grids were left to dry for few hours to ensure the complete removal of solvents and precursors to produce a uniform and stable film before their size and shape were assessed using transmission electron microscopy (TEM) at an accelerating voltage of 80 kV (Model JEM-1400, JEOL, Tokyo, Japan). Cellular toxicity of ZnO NPs is based on various

physiochemical parameters such; particle size (the smaller the particle size, the greater the cytotoxicity), surface charge, shape, dissolution kinetics, aggregation rate, and surface chemistry [20].

Experimental animals and design

In all, 48 healthy male albino rats (150–200 g; 50–60 days old, were acquired from Zagazig University's Department of Lab Animals, Faculty of Veterinary Medicine. The rats were kept in plastic cages with wood shavings for bedding and under controlled conditions (23°C ± 1°C, 12 hours of light and 12 hours of dark cycles). Lab. Animals were fed a standard laboratory pelleted diet with water provided ad libitum. The rats were adapted for the laboratory conditions for two weeks before starting the experiment. According to the general guidelines for the care and use of laboratory animals published by the National Institutes of Health (NIH), this protocol was approved the Institutional Animal Care and Use Committee (IACUC) of Mansoura University with approval no. (Ph.D/62).

There were six groups of rats, each with eight rats.

- Group1: control group (Cntr), rats orally administered 1ml distilled water.
- Group 2: Moringa Olifera extract group (MO), rats were administered 100 mg/kg body weight of aqueous extract of Moringa Olifera leaves for 28 days by oral gavage was selected on the basis of literature [19].
- Group 3: ZnO NPs 200 mg/kg group, rats received ZnO NPs at a dose of 200 mg/kg body weight (1/40 LD50) for 28 days by oral gavage. dose adjusted according to [21].
- Group 4: ZnO NPs 200 mg + MO group, rats received a combination of ZnO NPs and Moringa extract (200 mg/kg + 100 mg/kg aqueous extract, respectively) for 28 days via oral gavage.
- Group 5: ZnO NPs 400 mg/kg group, rats received ZnO NPs at a dose of 400 mg/kg body weight (1/20 LD50) for 28 days by oral gavage.
- Group 6: ZnO NPs 400 mg + MO group, rats received a combination of ZnO NPs and Moringa extract (400 mg/kg + 100 mg/kg aqueous extract, respectively) for 28 days via oral gavage.

All rats in the various groups had their blood samples taken within 24 hours of the previous treatment being administered. The samples were split into two sections, one of which was taken in tubes containing EDTA for hematological analysis [differential leucocytes' count (neutrophils, lymphocytes)]. To generate clear blood samples, the remaining portion was centrifuged for five minutes at 3000 RPM. It was then stored at -20°C until it was needed for further oxidant stress indicators and immunological testing. Rats were put to sleep by cervical dislocation, and their spleens were taken out and kept in 10% neutral buffered formalin for comet assay and histopathology.

Measurements

Measurment of differential leucocytic count

To measure the hematological parameter (differential leucocytic count) (neutrophils and lymphocytes), about 1 milliliter of blood samples were taken in test tubes containing EDTA (an anticoagulant) [22].

Analysis of oxidant stress markers

Serum was separated by centrifuging blood at 3000 rpm for 15 minutes after 2 mL of blood samples were taken in sterile test tubes devoid of anticoagulant and allowed to clot for 30 minutes at 25°C. Using fine-tipped automated pipettes, the supernatant sera were pipetted off and kept at -20°C until they were needed for cytokine and biochemical tests. Serum concentrations of glutathione S-transferase (GST), malondialdehyde (MDA), superoxide dismutase (SOD), and nitric oxide (NO) were measured using [23].

Measurment of pro-inflammatory markers

Proinflammatory cytokine levels in serum: interleukin-1 beta (IL-1β) (Cat. No. SEA563Ra) [24], Using rat ELISA kits (CUSABIO Life Sciences, China), the enzyme-linked immunosorbent assay (ELISA) technique was used to detect interleukin-6 (IL-6) (Cat. No. SEA079Ra) [25] and caspase-1 (Casp-1) (Cat. No. E4594-100) in accordance with the manufacturer's instructions.

Comet assay of splenocytes

To find any potential DNA damage following different treatments, the comet assay—also known as single cell gel electrophoresis, or SCGE—was employed. It measures the movement of DNA from immobilized nuclear DNA to identify alkali labile spots and breaks in DNA strands [26]. A little portion of spleen tissue was first crushed, moved to 1 milliliter of ice-cold PBS, then homogenized at 500 rpm in ice to create the spleen homogenate. 100 µL of spleen homogenate and 600 µL of 0.8% low-melting agarose were combined for the experiment. The resulting mixture was then spread out on slides that had been coated with 300 µL of normal melting agarose and left in lysis buffer (0.045 M trisborate-EDTA (TBE), pH 8.4) for 15 minutes. To allow DNA to unwind, slides were then placed in a horizontal electrophoresis apparatus with an alkaline pH (1 mM Na2EDTA and 300 mM NaOH, pH 13) for 20 minutes. The electrophoresis conditions were 25 V, 300 mA, and 30 cm between electrodes for 20 minutes at room temperature in the alkaline solution (pH 13) that was previously mentioned. The slides

were stained with ethidium bromide (20 µg/mL) at 4 ◦C. After that, they were sealed and kept in airtight containers until the images were examined. To stop further UV damage to DNA, all processes were carried out under dim light. Kinetic Imaging, Ltd. (Liverpool, UK) developed the Comet 5 image analysis software, which is linked to a charge-coupled device (CCD) camera. This software measures the tail length (TL = distance of DNA migration from the center of the body of the nuclear core) and the percentage of migrated DNA in order to determine the quantitative and qualitative extent of DNA damage in the cells. The software then computes the tail moment. Each sample typically consists of 50–100 randomly chosen cells. The formula used to calculate the tail moment (TMOM) was TMOM = DNA in tail as a percentage of total DNA × tail length (TL).

Histopathological examinations

Splenic tissue samples were collected, preserved in 10% neutral buffered formalin (pH 7.0), and then processed using standard techniques to produce paraffin sections. For histopathological analysis, the embedded paraffin sections were cut at 4.5 µm and stained with H&E stain [27].

Statistical analysis

The findings were presented in the form of mean ± SD. The one-way analysis of variance (ANOVA) test was used to compare the means between groups. To assess the differences between the experimental and control groups, the Least Significant Difference (LSD) test was employed. For every test, the threshold for statistical significance was established at $p < 0.001$ and $\eta 2 = 0.32$. Using SPSS software (version 16: SPSS), the correlation coefficient (r) was utilized to examine the relationship between two continuous variables.

Results

Zinc Oxide Nanoparticle Characterization

Size and shape of ZnO NPs were assessed using transmission electron microscopy (TEM), as shown in fig.1, the nanoparticles were semi-spherical in shape with diameter less than 100 nm.

Effects of ZnO NPs and extract of Moringa Olifera leaves on hematological parameters

Fig.2 illustrates how oral ZnO NPs delivery at 200 or 400 mg/kg considerably reduced the mean percent of neutrophils when compared to the control group, with no discernible difference between the two groups. Compared to administering ZnO NPs alone, co-administration of Moringa and ZnO NPs in the groups (ZnO NPs 200+Moringa) and (ZnO NPs 400+Moringa) showed changes with $p < 0.001$. At 200 or 400 mg/kg, ZnO NPs considerably raised the mean percentage of lymphocytes in comparison to the control group, but the change with $p < 0.001$ between the two groups showing the insignificance. In groups administered ZnO NPs, lymphocytosis may indicate an inflammatory response. When 200 mg/kg of ZnO NPs and Moringa were administered together, the mean percentage of lymphocytes was considerably lower than when 200 mg/kg was administered alone. However, the difference between the 400 mg/kg dosage of ZnO NPs and Moringa when administered together as opposed to when administered alone showed insignificance with $p < 0.001$. These findings demonstrate the immunological dysfunction and inflammatory response brought on by ZnO NPs, as well as the anti-inflammatory properties of Moringa olifera extract.

Effects of ZnO NPs and extract of Moringa Olifera leaves on antioxidant markers:

Fig. 3 illustrates how oral treatment of both ZnO NP dosages significantly reduced Glutathione-S-Transferase blood levels as compared to the control group. When ZnO NPs and Moringa extract were administered together in the ZnO NPs 200+Moringa group, the blood levels of GST increased non-significantly in comparison to when 200 mg of ZnO NPs was administered alone. As opposed to administering 400 mg of ZnO NPs alone, the ZnO NPs 400+Moringa group's serum levels of GST significantly increased when ZnO NPs and Moringa extract were administered together.

Both dosages of ZnO NPs administered orally demonstrated a considerable rise in serum Nitric Oxide levels as compared to the control group, with $p<0.001$ between the two groups showing the insignificant difference. When ZnO NPs and Moringa are co-adminstrated, the serum NO levels in the ZnO NPs 200+Moringa group and the ZnO NPs 400+Moringa group are dramatically reduced without a discernible difference.

When ZnO NPs were administered orally, the blood levels of MDA at both dosages significantly increased in comparison to the control group. Serum MDA levels are considerably reduced when ZnO NPs and Moringa are co-adminstrated in the ZnO NPs 200+Moringa group and the ZnO NPs 400+Moringa group.

Superoxide dismutase serum levels were significantly lower at both dosages when ZnO NPs were administered orally as compared to the control group. The blood levels of SOD are dramatically increased when ZnO NPs and Moringa are co-adminstrated in the ZnO NPs 200+Moringa group and the ZnO NPs 400+Moringa group. These findings demonstrate the oxidative stress linked to ZnO NPs exposure and the antioxidant properties of Moringa olifera extract.

Oral ZnO NPs treatment significantly increased Caspase-1 activity and blood levels of IL-1β and IL-

6 at both dosages when compared to the control group as shown in fig.4.

When ZnO NPs and Moringa extract were administered together, the activity of Caspase-1 and the blood levels of IL-1β and IL-6 were significantly reduced in the ZnO NPs 200+Moringa and ZnO NPs 400+Moringa groups. These findings demonstrate how ZnO NPs trigger an inflammatory response by activating the inflammasome, Casp-1, and IL-1β production.

Comet assay of splenocytes:

Oral administration of ZnO NPs at both dosages dramatically raised all comet assay indices (comet percentage, tail length, tailed DNA percentage, tail moment, and olive moment) in comparison to the control group as shown in figure 5. In contrast to single administration of ZnO NPs, co-administration of ZnO NPs with Moringa extract significantly reduced all comet test indices in the ZnO NPs200+Moringa and ZnO NPs 400+Moringa groups. These findings supported the beneficial effects of moringa extract and suggested that ZnO NPs caused DNA damage as shown in fig. 6.

Histopathological findings

Both the Moringa extract group and the control group had normal splenic histological structure, which includes the splenic parenchyma and the white and red pulps (Fig. 7a). With an eccentric central arteriole encircled by a peri-arterial lymphatic sheath (PALS), the white pulp was made up of lymphoid follicles. The lymphoid follicles seemed to consist of a germinal core (only present in secondary follicles) encircled by a mantle zone, or ring of lymphocytes, which in turn was encircled by the lymphocyte-containing border zone. The red pulp and the splenic lymphoid follicle were separated by a marginal zone (MZ). Blood sinusoids and branching splenic cords (Billroth cords) seemed to make up the red pulp (Fig. 7b). Red pulp grew and the lymphoid tissue surrounding the central vein significantly atrophy in the group that received 200 mg/kg of ZnO NPs (Fig. 7c). Lymphoid cells with comparatively typical lymphoid follicles were restored in the group that received ZnO NPs 200+ Moringa (Fig. 7d). The ZnO NPs 400 group had significant congestion in the red pulp and atrophy of the lymphoid tissue surrounding the central vein with bleeding (Fig. 7e). Around the central vein, the ZnO NPs 400 + Moringa group had normal lymphoid follicles and lymphocytic aggregation (Fig. 7f). It is very advised to do this splenic histopathology analysis in order to assess the immune system.

Discussion

Because zinc oxide nanoparticles (ZnO NPs) are widely used in many different sectors, they have attracted a lot of interest. Ingestion, inhalation, injection, and cutaneous penetration were among the several ways that humans were exposed to them [28]. When ZnO NPs are administered orally every day and deposited in various organs over a 28-day period, the quantity of zinc ions in plasma increases [6], making oral exposure the most common route of exposure [29]. According to recent research, ZnO NPs can have a harmful impact on a wide range of biological systems, including microbes, plants, invertebrates, and vertebrates [30].

Since the spleen is crucial to the immune response [31]**,** any change in the number of T cells or their cytokines, as well as any DNA damage following ZnO NP ingestion, may indicate immunotoxicity. According to [32], white blood cells support the body's immunity and defense against antigen invasion. The body typically produces them in reaction to the invasion of any foreign substance [33].

According to [34], it has a number of biological qualities, including anti-inflammatory, antioxidant, antibacterial, antifertility, anticancer, antihepatotoxic, and antiulcer effects.

While the number of neutrophils fell in all exposed groups with no discernible difference, the ZnO NPs group's hematological examination showed a substantial rise in lymphocytes. Our results concur with those of [18], who found that the ZnONPs 100 mg/kg + M. Oleifera 300 mg/kg group had significantly higher hematological parameters than the ZnO NPs group. The inflammatory response brought on by ZnO NPs was blamed for the elevated WBC count, which did not go down after a two-week recuperation period [35,36]. Additionally, our results are in line with [37]'s observation of a decrease in neutrophilic count following oral administration of 10 mg/kg B.W. for five days in a row. The lower neutrophil and higher lymphocyte count in our research are indicative of the inflammatory response and immunological dysfunction caused by ZnO NPS. These findings are explained by the presence of vitamins, minerals, and phytochemical components in the extract. The generation of blood cells in the bone marrow is directly impacted by these well-known hemopoietic components, which include phytochemicals [38].

Two distinct pathways, including oxidative stress and inflammation, were examined in order to look into the molecular processes behind the immune-toxicity caused by ZnO NPs and the protective effect of Moringa olifera extract. ZnO NPs increased lipid peroxidation, which was shown by higher MDA as a secondary product, in splenic tissues (Fig.2), causing an imbalance between oxidants and antioxidants and oxidative stress. Additionally, ZnO NPs decreased the activity of the antioxidant enzyme SOD and the amount of GST, the most significant cellular antioxidant. The nuclear factor erythroid 2-related factor 2 (Nrf2), which is crucial to the anti-oxidative

process, regulates the synthesis of the antioxidant enzymes. Nrf2 releases Kaap1 transcripted in the nucleus when oxidative stress occurs, which in turn promotes the synthesis of antioxidant enzymes like SOD [39]. The histological analysis of spleen tissues (Fig. 7) confirms that oxidative stress causes several pathogenic and cellular abnormalities. Oxidative stress has also been linked to reactive nitrogen species, which are nitric oxide (NO) derivatives. Rats given 200 or 400 mg/kg/28 days in the current investigation had significantly higher blood levels of nitrite, a stable metabolite of NO (Figure 3b), which may indicate enhanced NO synthesis due to NO synthase (NOS) activation. In splenic tissues, ZnO NPs are thought to have the ability to prime and/or activate neutrophils to create ROS and to stimulate NOS to release additional NO.

It is well known that Moringa Olifera extract possesses antioxidant properties [40]. According to our findings, Moringa corrected the lipid peroxidation (MDA), GST level, and SOD activity alterations brought on by ZnO NPs in splenic tissues. As a result, Moringa improved free radical scavenging while lowering reactive oxygen species (ROS) levels. These findings demonstrated that moringa has an antioxidant effect against oxidative stress caused by ZnO NPs. These findings are consistent with those of [41], who found that when rats were given a 25 mg/kg BW dose of Moringa oleifera leaf extract prior to cyclophosphamide treatment, their levels of SOD increased and their levels of lipid peroxidation decreased in the testes' tissue. Additionally, [42] showed that by raising glutathione S-transferase levels and decreasing lipid peroxidation, administering rats hydro-ethanolic M. oleifera leaf extract reduced the liver damage brought on by paracetamol. Furthermore, prior studies have demonstrated that Moringa oleifera leaf extract effectively reduces cellular oxidative stress by increasing strong antioxidant enzymes like SOD and GST [43,44]. The presence of various bioactive substances, including beta-carotene, polyphenols, flavonoids, quercetin, kaempferol, rutin, as well as vitamins C and E, may be linked to the antioxidant properties and inhibition of lipid peroxidation in Moringa oleifera leaf extract [45].

Regarding the second potential mechanism of the toxicity of ZnO NPs on the spleen, the inflammatory response, our results showed that ZnO NPs significantly increased Caspase-1 activity and serum levels of IL-1β and IL-6 at both doses, while ZnO NPs co-administration with Moringa extract resulted in significant decreases in Caspase-1 activity and serum levels of IL-1β and IL-6 in the ZnO NPs 200 + Moringa and ZnO NPs 400 + Moringa groups. This suggests that ZnO NPs can activate the NLRP3 inflammasome. There have also been reports of indirect cellular processes during inflammasome activation in response to particles [46,47]. Since various particles have been shown to activate NLRP3 inflammasomes by a variety of routes or mechanisms, enhanced ROS production is essential for NLRP3 inflammasome activation. When it is active, the Caspase-1 enzyme is triggered, which results in the release of inflammatory cytokines such as IL-1β and IL-18. This investigation revealed that these signals result in significant inflammation and damage to immune cells. According to [48], the inflammasome has been shown to play a significant role in the development of inflammation by cleaving caspase-1 and producing IL-1β downstream. One potent pro-inflammatory cytokine that has both local and systemic effects is IL-1β. Through the production of adhesion molecules and chemo-attractants, IL-1β promotes the recruitment of immune cells to the site of inflammation. IL-1β promotes dendritic cells, neutrophils, and macrophage activation and effector activities [49].

However, in response to the toxicity of ZnO NPs, Moringa Olifera extract increased inflammatory markers in the rats' serum. However, with MO treatment, these cytokines significantly reduced, demonstrating MO's effectiveness as an anti-inflammatory drug [50,51]. According to [52], MO has an increased quantity of monounsaturated fatty acids (oleic acid) and saturated fatty acids (palmitic and stearic acid), which may contribute to an elevated immunological response to changes in cell membranes. Furthermore, the presence of high concentrations of vitamins A, C, and K, which boost the immune system, may be linked to MO's protective impact [53].

According to [54], ZnO NPs' toxicity was ascribed to their tiny size, which allowed them to pass through the blood-brain barrier. They have the ability to directly harm cells and trigger cell death once they enter the cytoplasm of the cell. According to [55], it was proposed that the intracellular release of ionic Zn^{2+} from the particles, followed by an increase in the cytosolic Zn^{2+} concentration, was the cause of cell membrane damage, inflammatory reactions, DNA damage, and cell death.

One of the main harmful impacts of ROS produced by nanoparticles is DNA damage, and the hydroxyl radical (•OH) is one of the main species responsible for this damage. These free radicals damage DNA by reacting with it, causing single-stranded and double-stranded breaks that may potentially start the carcinogenesis process [56,57]. Both the 200 and 400 mg/kg dosages of ZnO NPs administered orally caused DNA damage in the current investigation. According to the results of the comet assay, the higher dose of nano-zinc particles caused DNA damage. These findings are consistent with those of [58], who investigated the effects of intermittent intraperitoneal exposure of 50 and 250 mg/kg body weight ZnO NPs on the spleen of male rats. Similarly, oral treatment of 50 and 300 mg/kg

of ZnO NPs after 14 days in a row caused DNA damage in the mouse liver, as assessed by the Comet test, which showed a significant rise in liver lesions specific to Fpg, a marker of oxidative stress [6]. Additionally, ZnO NPs caused genotoxicity in the bone marrow and liver after a single intraperitoneal dose [59].

Despite reports that ZnO NPs have genotoxic potential [60,61], however, nothing is known about the DNA damage caused by oral ZnO NPs. According to the current findings, the comet assay revealed DNA damage in every group that received oral ZnO NPs treatment when compared to the normal control (Figure 6). Higher rates of DNA strand breaks are indicated by longer DNA tail lengths. In our investigation, ZnO NPs groups exhibited a substantial increase in tail length and tail moment in both dosage and time-dependent comparison to the normal control. DNA damage can be caused by intracellular ROS production brought on by ZnO NPs or by direct contact between ZnO NPs and the DNA upon nuclear absorption [60]. Lipid peroxidation products have the ability to interact with DNA, resulting in genetic damage and disruption of cell signaling [62]. Our findings are consistent with research done on cell lines that showed oxidative DNA damage following ZnO NP exposure [63,64].

It was suggested that the oxidative characteristics of ZnO NPs caused their toxicity by releasing Zn^{2+} during particle disintegration and producing oxidants upon contact with other common cellular components. The physico-chemical characteristics are also important in the toxicity caused by ZnO NPs [65].

It is very advised to examine the spleen histopathologically in order to assess the immune system. Thickened stroma, atrophy of white pulps with the loss of germinal centers, increased red pulp, and vacuolation and death of some splenocytes were seen in the group treated with nanozinc. Rats exposed to ZnO NPs at dosages of 5, 50, and 300 mg/kg for 14 days showed similar effects on splenocytes and hepatocytes [66]. Additionally, following 14 days of mice were exposed to 20- and 120-nm ZnO powder at dosages of 1, 2, 3, 4, and 5 g/kg body weight [21].

The toxicity of ZnO NPs in rats was confirmed in the current investigation by histological evaluation of spleen tissue. According to our findings, ZnO NPs caused a variety of pathological splenic lesions. Blood infiltration into the interstitial tissue and inflammatory production are two possible classifications for the represented pathological lesions. Actually, there was a clear necrosis in the region, along with neutrophil and lymphocyte infiltration. These findings demonstrate that ZnO NPs cause inflammation and are in line with research on their cytotoxicity [67-69].

Male Wistar rats subjected to high doses of ZnO NPs for four weeks experienced tissue damage as a result of prolonged intermittent exposure to 50 and 250 mg/kg of ZnO NPs thrice a week [70]. Male Wistar rats exposed daily intramuscularly to 100, 200, and 400 mg/kg ZnO NPs for 10 days showed similar histopathological changes in their splenic tissue [71]. Additionally, male rats given oral dosages of 100, 200, and 300 mg/kg of ZnO NPs showed atrophy of white pulps with the loss of germinal centers, an expansion of red pulp, thicker stroma, vacuolation, and partial splenocyte apoptosis [72].

Conclusion

Overall, this study shows that Moringa Olifera (MO) extract has a positive impact on reducing ZnO NPs-induced immunotoxicity by enhancing hematological and immunological parameters through its anti-oxidative and anti-inflammatory properties, as well as by modulating DNA damage and histopathological alterations in the rat spleen. Regarding to the Sustainable Development Goals (**SDGs**), this study contributes to **SDG 3** (Good Health and Well-being) by investigating the health risks associated with zinc oxide nanoparticles and to **SDG 12** (Responsible Consumption and Production) by promoting the safe use of nanomaterials.

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Conflicts of interest

There are no conflicts to declare.

Data availability

The authors declare that all data supporting the findings of this study are available within the paper.

Ethical of approval

This protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Mansoura University with approval no. (Ph.D/62).

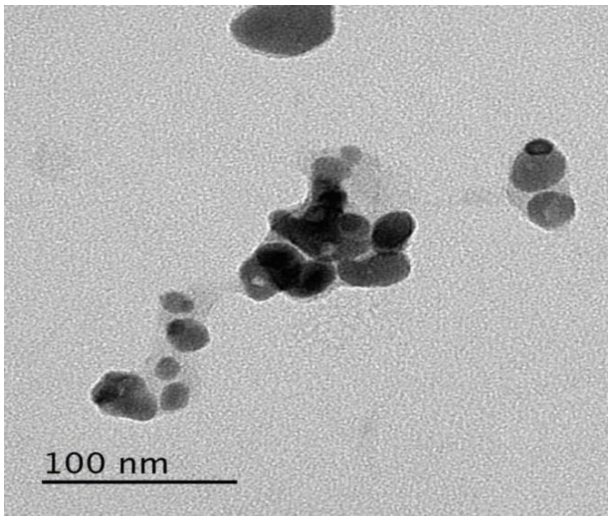

Fig. 1. Examining zinc oxide nanoparticles with a transmission electron microscope showed that they were less than 100 nm.

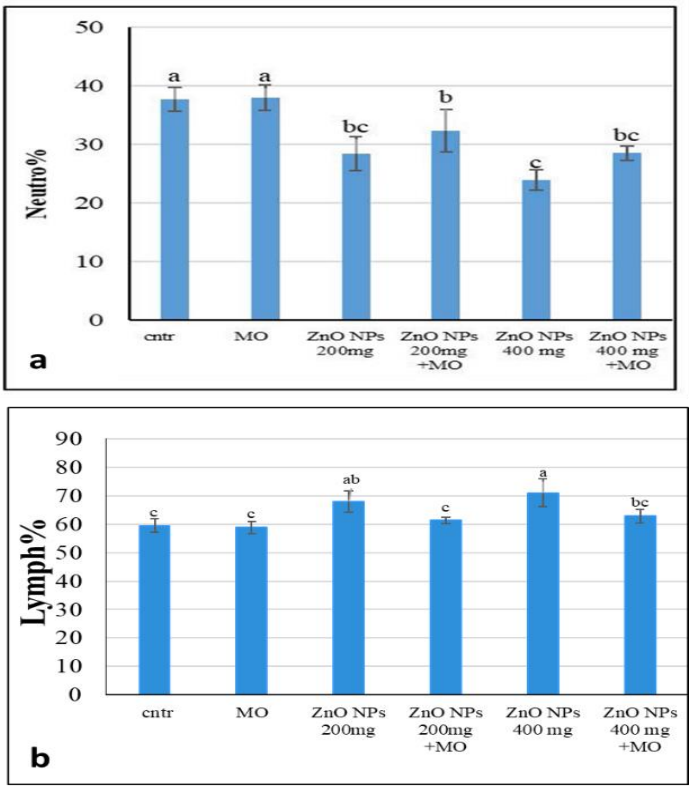

Fig. 2. Effects of oral ZnO NPs and/or Moringa extract on male albino rat serum hematological parameters (a) neutrophils (b) lymphocytes. The information is displayed as Mean±SD (n=8). At P<0.001, bars with the distinct letters a, b, c, and d are substantially different.

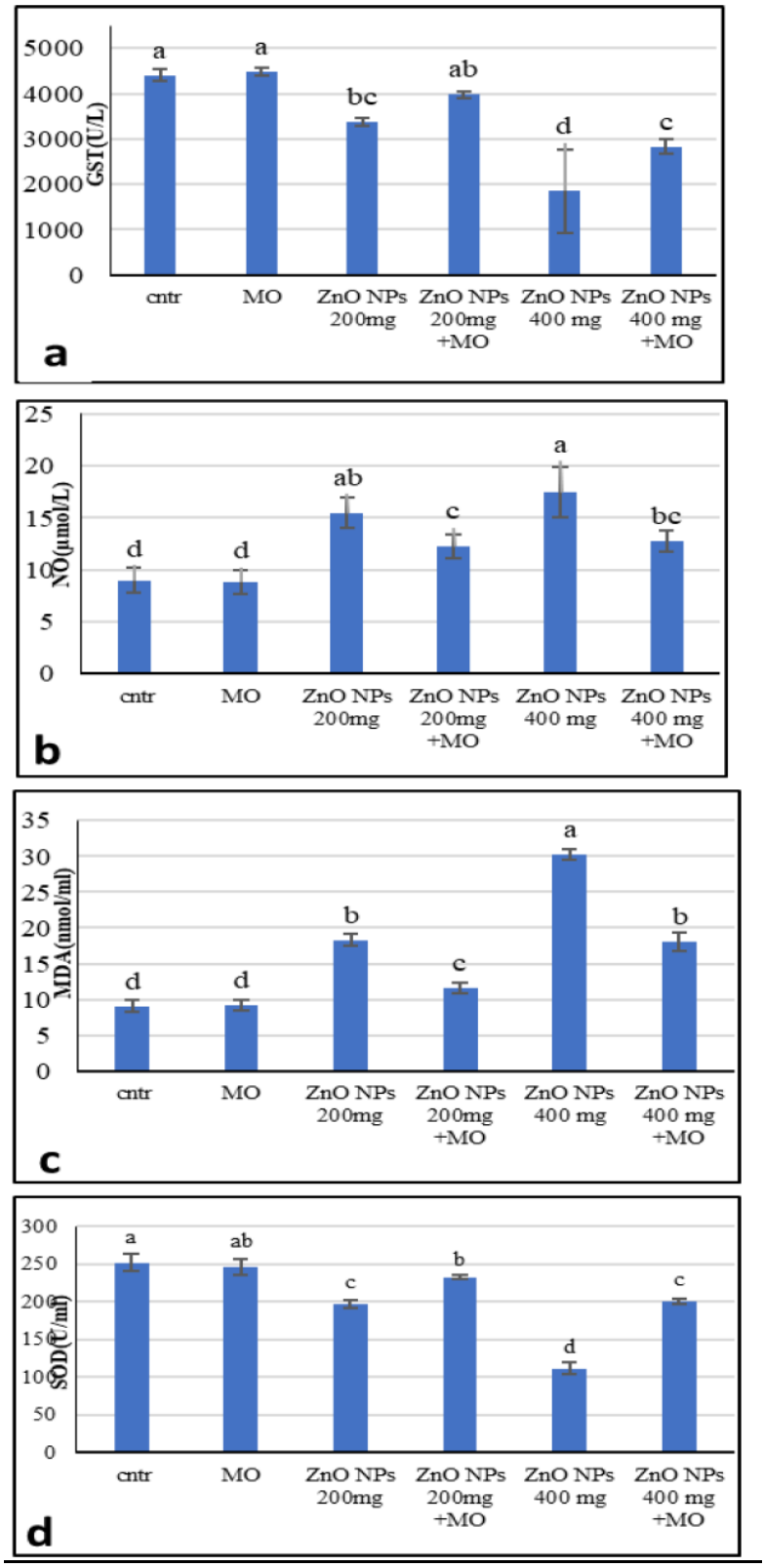

Fig. 3. Oxidative stress indicators in the blood of male albino rats (28 days) after oral treatment of ZnO NPs and/or Moringa extract. GST (a), NO (b), MDA (c), and SOD (d). Data are expressed as mean ± SD, n = 8 for each group. At P<0.001, bars with the distinct letters a, b, c, and d are substantially different.

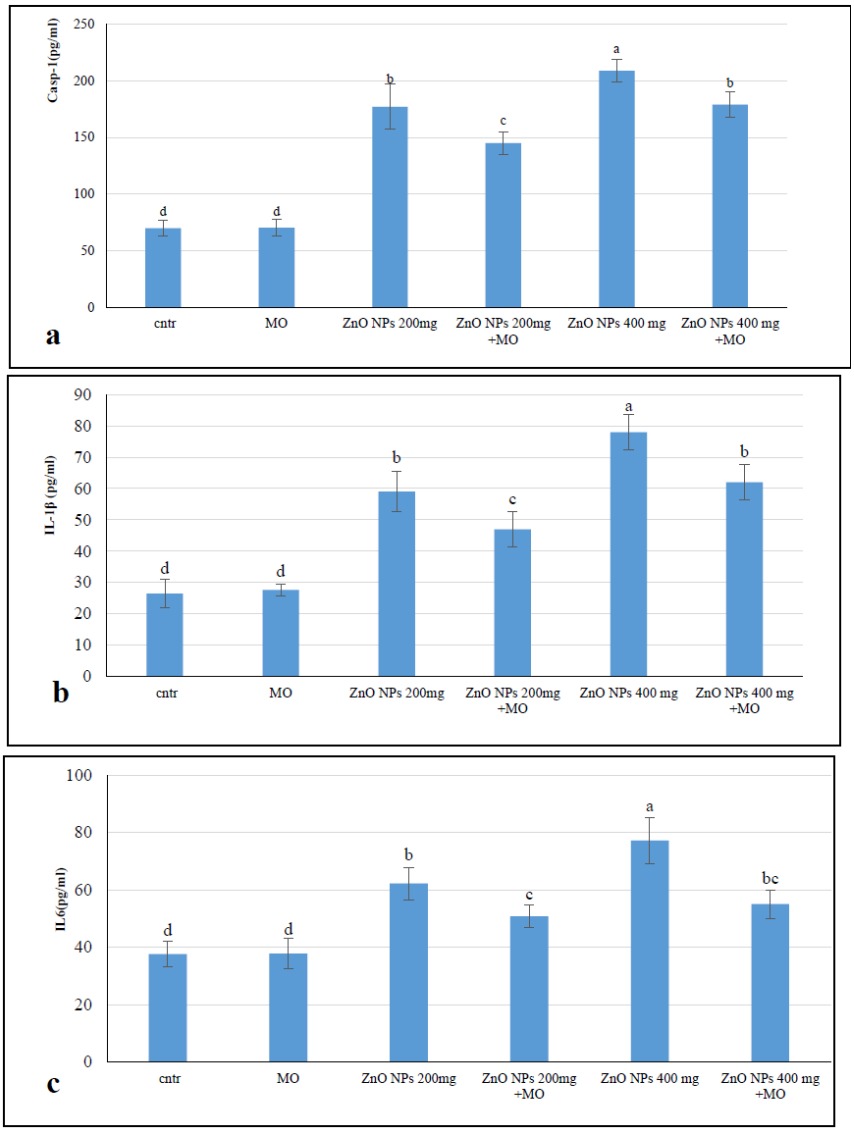

Fig. 4. impact of oral ZnO NPs and/or Moringa extract on male albino rat blood levels of inflammatory cytokines. (a) Casp-1 activity, (b) IL-1β, and (c) IL-6. For each group, the data are presented as mean ± SD, with n = 8. At P<0.001, bars with the distinct letters a, b, c, and d are substantially different.

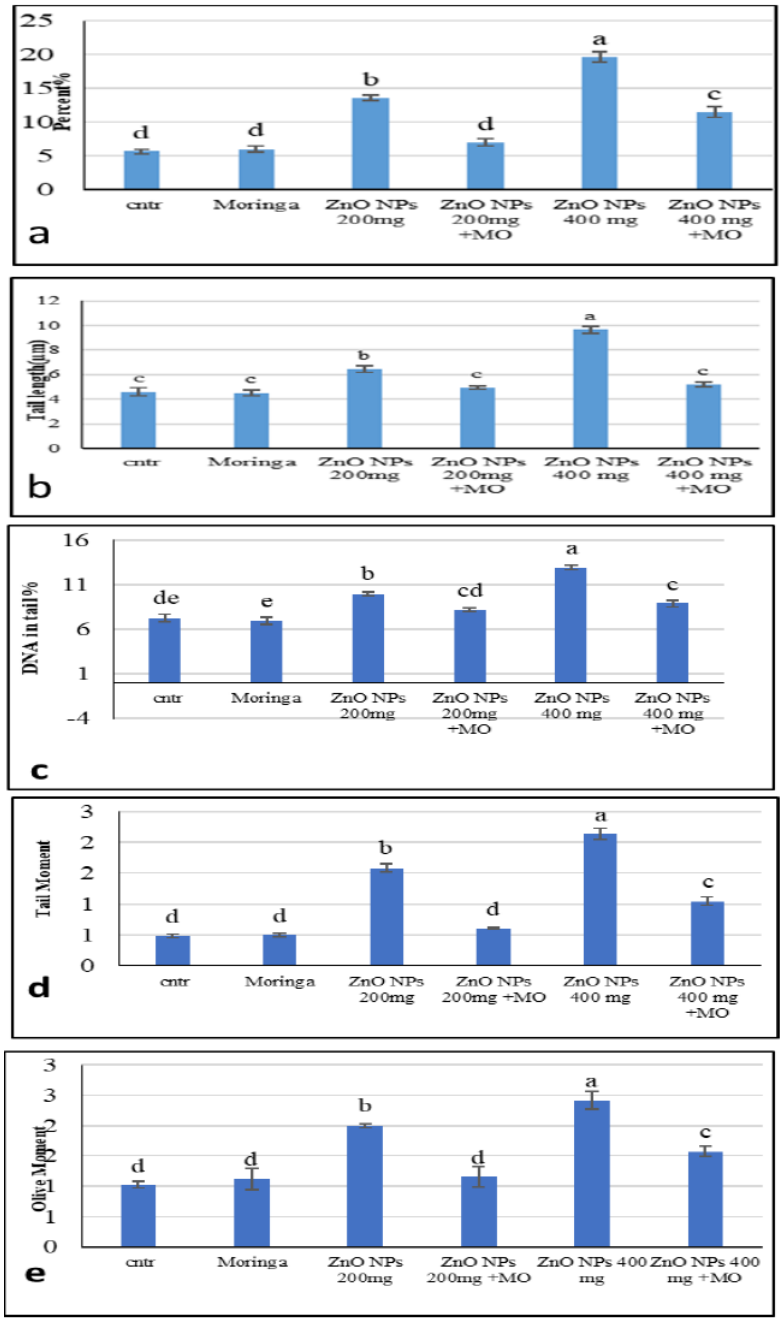

Fig. 5. Impact of ZnO NPs and/or Moringa extract given orally on the Comet parameters in rat spleen tissue (28 days). (a) DNA damage percentage, (b) length of the tail, (c) tail DNA percentage, (d) tail moment, and (e) olive moment. For each group, the data are presented as mean ± SD, with n = 8. At $p<0.001$, bars with the different letters a, b, c, and d are substantially different.

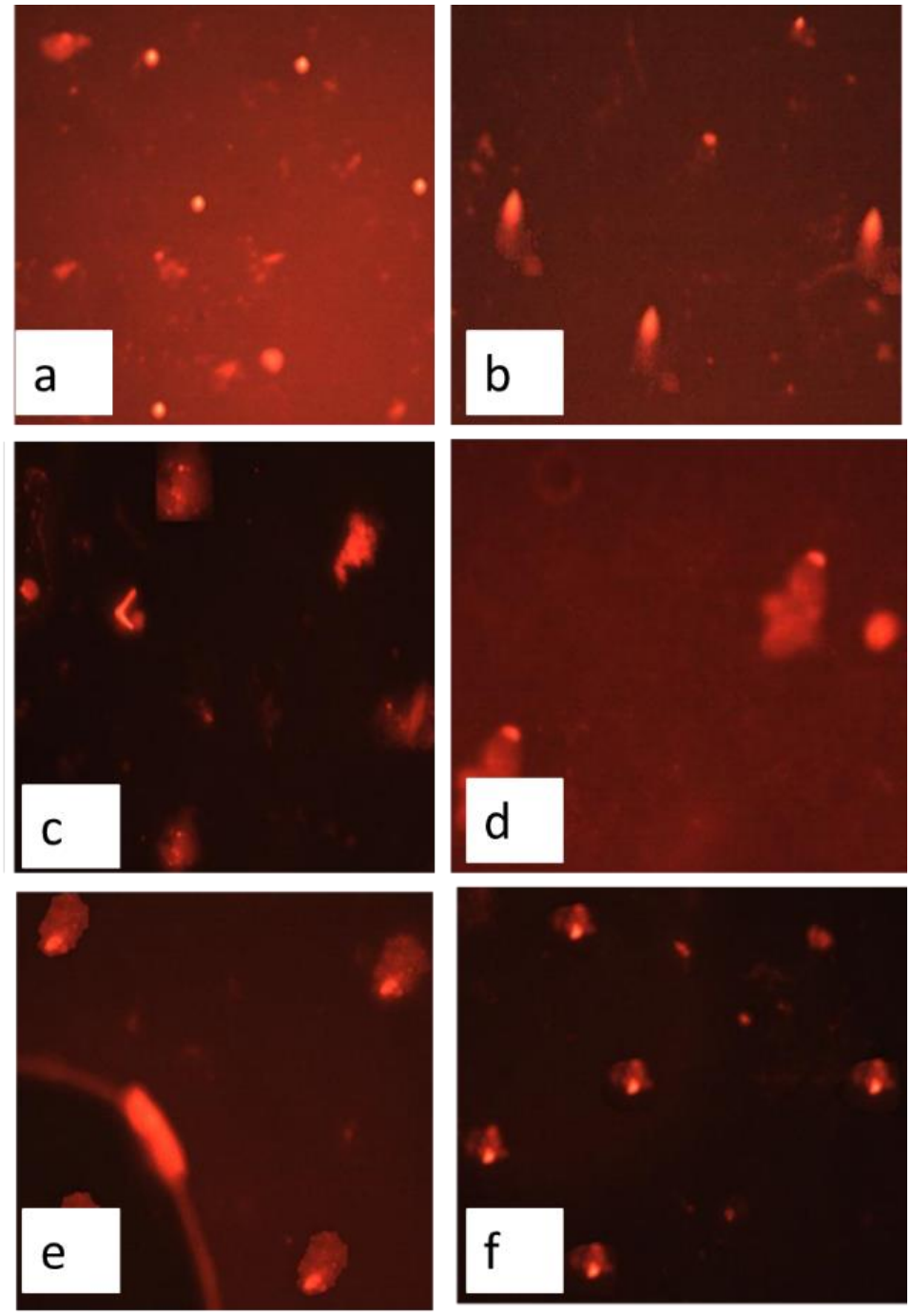

Fig. 6. Comet assay DNA epifluorescence images: (a) control samples demonstrating a subject free of DNA damage. (b) Moringa olifera displaying a person with intact DNA. (b) ZnO NPs 200 mg/kg demonstrated varying degrees of splenic DNA damage. (d) 200 mg/kg of ZnO NPs with Moringa demonstrates a reduction in damaged cells. (e) ZnO NPs 400 mg/kg exhibit varying degrees of splenic DNA damage. (f) Moringa + ZnO NPs 400 mg/kg demonstrates a reduction in damaged cells.

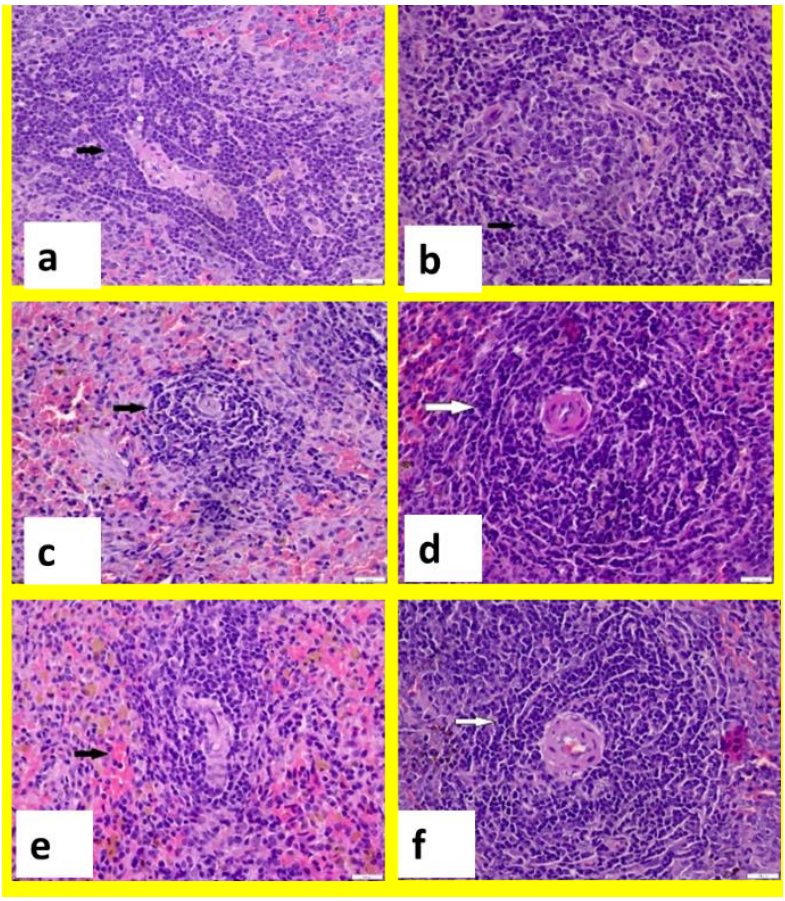

Fig. 7. A photomicrograph of a splenic slice from the (a) control group demonstrates typical lymphoid cell aggregations surrounding the central arteriole. (b) The Moringa group has typical lymphoid and plasma cell aggregations. (c) Red pulp enlargement and significant lymphoid tissue atrophy surrounding the central vein are observed with ZnO NPs 200 mg/kg. (d) Restoration of lymphoid cells with comparatively normal lymphoid follicles with ZnO NPs 200 mg/kg + Moringa. (e) ZnO NPs 400 mg/kg exhibit significant congestion in the red pulp and atrophy of the lymphoid tissue surrounding the central vein with bleeding. (f) Normal lymphoid follicles and lymphocytic aggregation surrounding the central vein are seen in ZnO NPs 400 mg/kg + Moringa (arrow). [H&E, X 400].

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السمية المناعية المحدثة بجزيئات اكسيد الزنك النانووية على طحال ذكورالجرذان البيضاء والدور التحسينى لمستخلص اوراق المورينجا

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الملخص

الهدف من هذا العمل هو دراسة و استكشاف تاثير سمية اكسيد الزنك النانووية علي الجهاز المناعى و دراسة الدور التحسينى لمستخلص اوراق الموربنجا.

اجريت هذه الدراسة علي عدد 48 من ذكور الجرذان البيضاء مقسمة الي 6 مجموعات كالاتى:

المجموعة الاولى (المجموعة الضابطة): (8جرذ) تم اعطاء كل جرذ (1 مل) من الماء المقطر عن طريق الفم مرة واحدة يوميا لمدة 28 يوم.

المجموعة الثانية (مجموعة مستخلص اوراق المورينجا): (8جرذ) تم اعطاء كل جرذ (100 مجم/كجم) من مستخلص اوراق المورينجا عن طريق الفم مرة واحدةًيومًيا لمدة 28 يوم.

المجموعة الثالثة (مجموعة أكسيد الزنك):(8 جرذ) تم اعطاء كل جرذ (200مجم/كجم) من أكسيد الزنك ذو الجزيئات متناهيه الصغر) النانووية عن طريق الفم مرة واحدة يوميا لمده 28 يوم.

المجموعة الرابعة (مجموعة أكسيد الزنك و مستخلص اوراق المورينجا): (8 جرذ) تم اعطاء كل جرذ 200 مجم/كجم من اكسيد الزنك ذي الجزيئات متناهيه الصغر النانووية ثم تم اعطائه100 مجم/ كجم من مستخلص اوراق المورينجا عن طريق الفم مرة واحدة يوميا لمدة 28 يوم.

المجموعة الخامسة (مجموعة أكسيد الزنك):(8 جرذ) تم اعطاء كل جرذ (400 مجم/كجم) من أكسيد الزنك ذو الجزيئات متناهيه الصغر) النانووية عن طرٌق الفم مرة واحدة يوميا لمده 28 يوم.

المجموعة السادسة (مجموعة أكسيد الزنك و مستخلص اوراق المورينجا): (8 جرذ) تم اعطاء كل جرذ 400 مجم/كجم من اكسيد الزنك ذي الجزيئات متناهيه الصغر النانووية ثم تم اعطائه 100 مجم/ كجم من مستخلص اوراق المورينجا عن طريق الفم مرة واحدة يوميا لمدة 28 يوم.

استمرت الدراسة لمدة 28 يوم وفى نهاية الدراسة تم تخدير 8 جرذان من كل مجموعة لاخذ عينات الدم لعمل الفحوصات المعملية (مضادات الاكسدة والسيتوكينات و تحليل كريات الدم البيضاء التفريقى) بعدها تم تخٌدير و ذبح الجرذان وأخذت عٌينات من الطحال لاجراء دراسة ميكروسكوبية ضوئية لتحديد التغيرات الهستوباثولوجيه التى حدثت بها ، وكذلك تم اجراء فحص الهالم الكهربائى لخالايا الطحال لتحديد مدى الضرر الذي حدث فى الحمض النووي. تم تجميع النتائج و تحليلها بطرق احصائية مناسبة و تجميعها فًى جداول ومناقشتها.

النتائج: أظهرت النتائج ارتفاعا ذا دلالة إحصائية فى متوسط نسبة الليمفاويات كما أسفرت النتائج عن وجود انخفاضا ذى دلالة احصائيه فى متوسط نسبة العدلات بعد 28 يوم مقارنة بالمجموعة الضابطة. كما أظهرت النتائج أيضا ارتفاعا ذا دلالة إحصائية فى مستويات اكسيد النيتريك و المالوندای الديهايد بعد 28 يوم مقارنه بالمجموعه الضابطه. كما أسفرت النتائج عن وجود انخفاضا ذى دلالة احصائيه في مستويات جلوتاثيون اس-ترانسفيراز و سوبر اكسيد ديسميوتاز. وايضا اسفرت النتائج عن ارتفاعا ذا دلالة احصائية في مستويات السيتوكينات مقارنة بالمجموعة الضابطة. كما أسفر فحص الهالم الكهربائى للحامض النووي المستخلص من الطحال لجرذان مجموعة أكسيد الزنك متناهية الدقة عن وجود تجزئة فى الحامض النووي بعد 28 يوم مقارنة بالمجموعة الضابطة. كما أظهر الفحص المجهرى الضوئى لنسيج الطحال باستخدام صبغة الهيماتوكسلين وجود تغيرات هستوباثولوجية واضحة بانسجه الطحال بعد 28 يوم مقارنة بالمجموعة الضابطة. إن إعطاء مستخلص اوراق المورينجا مع أكسيد الزنك ، وفر حماية ضد الاثار السميه الناتجه عنه وأدى إلى تحسن فى نسبة الليمفاويات و العدلات مقارنة بمجموعة أكسيد الزنك متناهية الدقة بعد 28 يوم. كما أسفرفحص الهالم الكهربائى للحامض النووي المستخلص من الطحال عن وجود التئام ملحوظ فى الحامض النووي مقارنة بمجموعة أكسيد الزنك متناهية الدقة بعد 28 يوم. كما أظهر الفحص المجهرى الضوئى لنسيج الطحال باستخدام صبغة الهيماتوكسلين وجود تحسن واضح فى التغيرات الهستوباثولوجية المحدثه بانسجه الطحال مقارنة بمجموعة أكسيد الزنك متناهية الدقة بعد 28 يوم.

الخلاصة: اثبتت الدراسة الحالية أن التعرض لجزيئات أكسيد الزنك متناهية الدقة أدى إلى تاثير سمى على الجهاز المناعى من قبل الخلايا المبرمجٌة للطحال، وتقليل وظيفة المناعة أيضا ، تلف فى وظائف وأنسجة الطحال وإحداث تدمير فى الحمض النووي فى خلايا الطحال لذكور الجرذان البيضاء البالغة . وأن إعطاء مستخلص اوراق المورينجا مع اكسيد الزنك يوفر الحمايه ضده ويحدث تحسن اللاثار السلبية الناجمه عنه..

الكلمات الدالة: جزيئات اكسيد الزنك، النانووية، الطحال، السمية المناعية.