# Zinc oxide nanoparticles induce oxidative stress and histopathological toxicity in the thyroid gland and liver of rats

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**Abstract**

Zinc oxide nanoparticles are incorporated into cosmetics and sunscreens, and are widely used in biomedical applications and food industry. The increasing use of zinc oxide nanoparticles raises concern about their safety. This aim of the study was to assess the effects of zinc oxide nanoparticles on oxidative and genotoxic parameters in thyroid gland and liver of adult albino rats. Rats were divided into three groups; control, vehicle, and zinc oxide nanoparticles (200 mg/kg) and where subjected to treatment for 30 days. Oxidative stress parameters and genotoxicity was determined. Histopathological examination of both organs was undertaken. A significant reduction in triiodothyronine, thyroxine, and thyroid-stimulating hormone was noted, whereas aspartate aminotransferase and alanine aminotransferase levels were significantly elevated. Increased malondialdehyde and decreased reduced glutathione levels were indicative of oxidative stress response in both organs. Additionally, elevated serum 8-hydroxydeoxyguanosine was noted which was supported by results of the comet assay. Histopathological examination revealed alterations in thyroid gland and liver. Sub-chronic exposure resulted in oxidative stress mediated toxicity and genetic perturbations in both organs. Caution is warranted with repeated usage of products containing zinc oxide nanoparticles.

## 1. Introduction

Zinc oxide nanoparticles (ZnONPs) are most commonly utilized in consumer products. They are incorporated in commercial paints, cosmetics, textiles, and personal hygiene products (Saman et al. 2013). ZnONPs provide protection against ultraviolet A and B radiation. Accordingly, they are frequently encountered in sunscreens and moisturizers. Additionally,

ZnONPs have proven antimicrobial properties and they are therefore used as additives in the food industry and for packaging thereof (Kim et al. 2020). The high degree of selectivity of ZnONPs towards cancerous cells has drawn attention to their promise as new anticancer agents (Saliani, Jalal, and Goharshadi 2016).

Exposure to ZnONPs takes place via ingestion, inhalation, and parenteral injection (Liao et al. 2020), with little evidence for skin penetration (Borm et al. 2006). Due to the tiny size of ZnONPs, they enter cells by means of passive diffusion, whereafter they are transported through the cytoskeletal filaments to mitochondria and nuclei (Condello et al. 2016). ZnONPs can also be up taken into cells by endocytosis and become encapsulated in vesicles, where the reduced pH in these vesicles accelerates the dissolution rate of ZnONPs and increases release of soluble $Zn^{2+}$ ions (Mittag et al. 2021). The high concentration of $Zn^{2+}$ ions within the cell disrupts the ionic homeostasis, cell metabolism, and redox state of mitochondria (Saliani, Jalal, and Goharshadi 2016).

Mitochondria seem to be the core target of NPs, which, once inside the organelle, can alter the balance of proteins causing membrane damage, or impair functionality via altering electron transport chain or activating the Nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase enzymes (Giordo et al. 2020). The altered mitochondrial respiration results in excessive generation of reactive oxygen species (ROS) (Yu et al. 2020). The ROS-induced oxidative stress has been proposed as the most probable mechanism whereby ZnONPs mediate toxicity (Saliani, Jalal, and Goharshadi 2016).

The ZnONPs have been reported to enhance genotoxicity along with cytotoxicity (Singh, Cheng, and Singh 2020). Nucleic acids such as DNA and RNA are extremely vulnerable to the detrimental effects of ROS because of their low redox potential. ROS can directly interact with their nucleobases and modify them resulting in DNA damage. The genotoxic potential of NPs together with ROS generation, enable them to produce single and double-strand DNA breaks, chromosome damage, and aneuploid genetic events (Yu et al. 2020).

Damage to DNA should be carefully assessed for all NPs encountered by humans, given that this damage correlates with the development of oncogenesis (Yousef, Mutar, and Kamel 2019), mutagenesis, and may eventually lead to cell death (Yu et al. 2020). Previous *in vitro* studies have shed the light on the potential genotoxicity of ZnONPs (Demir, Creus, and Marcos 2014; Ng et al. 2017; Singh, Cheng, and Singh 2020). However, the negative results where ZnONPs showed no or weak genotoxicity (Kwon et al. 2014; Alaraby et al. 2015; Carmona et al. 2016), along with the limited number of genotoxic studies in *vivo*, suggest that the genotoxicity of ZnONPs remains controversial and necessitates further exploration (Ng et al. 2017).

The thyroid gland plays a critical role in the regulation of several physiological functions including metabolic processes, neuronal growth, bone remodelling, and cardiac functions (Shirband et al. 2014). In addition to the synthesis of thyroid hormones, it is a site where oxidative processes occur. Under normal conditions, a balance between the generated free radicals and their detoxification is maintained (Lewińska and Bilska 2012). Homeostasis is mediated by the presence of enzymatic antioxidants such as superoxide dismutase (SOD), glutathione (GSH) peroxidase (GSH-Px) and catalase (CAT), and non-enzymatic antioxidants such as α- and γ-tocopherols, coenzyme Q and ascorbic acid (Kovacic and Edwards 2010).

However, when excessive oxidation occurs; damage to macromolecules results (Lewińska and Bilska 2012). Unfortunately, approximately 10% of the population suffer from thyroid gland disorders which represent a major health problem. Possible contribution of NPs in thyroid disorders has been suggested (Sulaiman, Luaibi, and Qassim 2018).

The liver is the primary metabolizing organ (Yousef, Mutar, and Kamel 2019). Integrity of the structure, morphology, and enzymatic system of the liver are essential to maintain its detoxification function (Mohammad et al. 2019). Liver represents the main accumulator and the foremost target of NPs regardless the route of exposure (Guo et al. 2020). As the liver possesses abundant macrophages, it can engulf NPs by phagocytosis, resulting in trapping of particulate materials within the organ (Kuang et al. 2016). Cells performing phagocytosis produce excess ROS, increasing the occurrence of toxicity (Valentini et al. 2019). A heavy build-up of ZnONPs in liver results in oxidative stress that can trigger DNA damage (Sharma et al. 2012). Previous studies have revealed that ZnONPs-enhanced oxidation is associated with low antioxidant status and inhibited activities of the antioxidant enzymes in the liver of treated rats (Mansouri et al. 2015; Almansour et al. 2017; Yousef, Mutar, and Kamel 2019; Hosseini et al. 2020). However, the role of ROS associated gene toxicity is still not fully understood (Ng et al. 2017).

Thyroid hormones regulate calorigenesis of hepatocytes and thereby modulate the hepatic function (Manjula et al. 2013). The liver in turn, activates and deactivates thyroid hormones via deiodination and regulates the transport and metabolism of thyroid hormones (Malik and Hodgson 2002). Thus, while the thyroid gland is important for maintaining the integrity of liver

cells, the integrity of liver cells is essential for maintaining the homeostasis of thyroid function (Ambiger and Chincholikar 2019).

Only a few in *vivo* studies have evaluated the genotoxicity of ZnONPs in liver (Sharma et al. 2012; Yousef, Mutar, and Kamel 2019), whereas information on the effects of NPs on the thyroid gland is very scarce. Liver and thyroid hormones are closely connected, and dysfunction of one organ disturbs functionality of the other (Malik and Hodgson 2002). The aim of this study was to assess the *in vivo* effects of ZnONPs on oxidative and genotoxic parameters in the thyroid gland and liver of adult male albino rats. To the authors' knowledge, this paper is the first to study the oxidative and genotoxic potential of ZnONPs in thyroid gland *in vivo.*

## 2. Materials and Methods

### 2.1. ZnONPs

The ZnONPs used in this study was an odourless, white powder with a particle size < 100 nm by dynamic light scattering (DLS), < 35 nm by aerodynamic particle sizer, surface area 10-25 m$^2$, purity > 99%, band gap of 3.37 eV, and Zn content of ~ 80%, ZnONP (CAS No. 1314-13-2, Sigma-Aldrich, St. Louis, MO, USA).

To determine size and morphology, the dried powdered particles were suspended in deionized (DI) water (1 g/L) and sonicated at room temperature for 10 min until a homogeneous suspension was obtained. Thereafter the samples were prepared by drop coating of the stock suspension on to carbon-coated copper grids. The films on the grids were allowed to dry after

which the morphology and size was determined using transmission electron microscopy (TEM) at an accelerating voltage of 80 kV (Model JEM-1400, JEOL, Tokyo, Japan).

## 2.2. Experimental animals

Animal experiments were conducted in accordance with the regulations of the Institutional Animal Care and Use Committee of Zagazig University, Egypt (Ethics approval number ZU-IACUC/3/F/174/2019) which are in accordance with the National Institutes of Health Guidelines for Animal Care. Before commencing experimentation, rats were acclimatized for two weeks under standard laboratory conditions. The rats were fed a standard pellet diet containing all nutritive elements (fabricated by an Egyptian company that manufactures oil and cleaning soap). Food and fresh tap water were available *ad libitum*. The room temperature was maintained at $23 \pm 2$ °C, relative humidity between 40 and 60 % and the light–dark cycles were set at 12 h. The bedding and wood shavings were kept in galvanized iron-mesh cages with solid bottoms that were changed frequently to ensure the cages were clean.

Adult male albino rats (twenty-four; weighing 150-200 g; aged 16-18 weeks) were used. The rats were divided randomly into three groups of 8 rats each; the control group received no treatment, the vehicle group received 1 mL deionized (DI) water, and the NPs treated group received 1 mL (200 mg/kg b.wt.) ZnONPs. The ZnONPs were dispersed in DI water to form a freshly prepared aqueous suspension that was sonicated for 10 min and vortexed for 5 min prior to administration to avoid aggregation. The ZnONPs dose (200 mg/kg) selected was based on the study of Abbasalipourkabir et al. (2015). All treatments were given by gavage daily for 30 days using a metallic tube specialized for gastric intubation of rats. Rats were weighed every week and the dose was changed according to the changes in weight.

## 2.3. Specimen collection

Following the last dose, animals were anaesthetized by means of an intra-peritoneal injection with pentobarbital (50 mg/kg), after which blood (3-5 mL) was collected by cardiac puncture. The samples were transferred into non-heparinized glass tubes and allowed to clot for 30 min at 25 ºC after which serum was separated by centrifugation (1000 g for 10 min) and stored at -80 ºC until biochemical analyses were performed.

After blood samples were collected, rats were sacrificed by cervical dislocation. The thyroid gland and liver were excised and washed with ice cold saline, weighed and inspected for gross anomalies. Each organ was bisected; the left lobes were fixed in 10 % formalin for histopathological examination, while the right lobes were covered in aluminium foil and stored at -20 ºC until DNA extraction and determination of malondialdehyde (MDA) and reduced glutathione (GSH) levels.

## 2.4. Biochemistry

### 2.4.1 Hormonal assays

Triiodothyronine (T3), thyroxine (T4) and thyroid-stimulating hormone (TSH) levels were determined using enzyme-linked immunosorbent assay (ELISA) kits according to Klee (1996), Nelson and Wilcox (1996), and Hood et al. (1999). The kits were purchased from Mybiosource (Cat. No. MBS700273 and MBS704309; San Diego, USA) for T3 and T4, and from ELISA Genie (Cat. No. RTFI01195; Dublin, Ireland) for TSH level determination.

Liver enzyme levels were determined using BT29 4QY kits (Randox Laboratories; Crumlin, UK) for aspartate aminotransferase (AST) according to Rej and Horder (1984), whereas alanine aminotransferase (ALT) levels were determined using the GTP (ALT)-LQ Spinreact kit (Sant Esteve de Bas, Spain) according to Murray (1984).

*2.4.2 Oxidative stress markers*

The right lobes of the excised tissues were homogenized in 10 times (10% w/v) ice cold phosphate buffer (0.12 mol/L, pH 7.2) and centrifuged at 10 000 x g for 30 min at 4 °C. The supernatants were collected as tissue homogenates and were used to determine oxidative stress markers.

Malondialdehyde levels were determined as described by Ohkawa, Ohishi, and Yagi (1979) with kits obtained from Elabscience (Cat. No. E-BC-K025-S; Texas, USA). An aliquot of 0.2 mL tissue supernatant was mixed with 1 mL trichloroacetic acid (20%) and 2 mL aqueous solution of thiobarbituric acid (0.67 %), after which the mixture was heated for 1 h at 100 °C. Thereafter the mixture was allowed to cool and the supernatant was collected by centrifugation at 3000 x g for 10 min. The absorbance of the sample was measured spectrophotometrically at 532 nm, using a blank containing all the reagents excluding the sample.

Reduced glutathione was measured according to Sedlak and Lindsay (1968) using kits obtained from Myobiosource (Cat. No. MBS265966; San Diego, USA). An aliquot of 0.5 mL tissue homogenate was mixed with 0.2 mol/L Tris buffer (pH of 8.2), after which contents were mixed with 0.1 mL of 0.01 mol/L Ellman's reagent, (5,5 - dithiobis-(2-nitro-benzoic acid), followed

by centrifugation (3000 x g) for 15 min. The reaction between the thiol groups and 5-5-dithiobis-(2-nitrobenzoic acid) produces a chromophore absorbing light at 412 nm. The GSH concentration was obtained from the absorbance value using a standard curve.

8-Hydroxydeoxyguanosine (8-OHdG) DNA adducts in serum samples were measured with the use of an ELISA kit obtained from Biovision (Cat. No. E4442-100; Milpitas, USA), the assay being performed according to the manufacturer's instructions. Briefly, horseradish peroxidase conjugated antibody (100 μL) specific for 8-OHdG was added to each micro ELISA strip plate well except for the blank control well. After incubation, all unbound components were washed off with phosphate buffered saline (PBS) diluted (1:10) with distilled water. To each well 90 μL 3,3′,5,5′-tetramethylbenzidine substrate solution (0.4 g/) was added. The optical density was measured spectrophotometrically at a wave length of 450 nm and the concentrations of 8-OHdG in the samples delineated from a standard curve.

## 2.5. Genotoxicity

The level of DNA damage was determined using the method described by Singh et al. (1988). Tissue (0.1 g) was transferred to 1 mL ice-cold PBS and homogenized. Thereafter, 600 μL of 0.8 % low-melting agarose was mixed with 100 μL thyroid or liver homogenate. An aliquot of 100 μL was spread onto microscopic slides which were pre-coated with 300 μL of melted agarose and immersed in lysis buffer (45 mol/L trisborate-EDTA containing 2.5 % sodium dodecane sulphate, pH 8.4) for 15 min. Slides were then placed in an electrophoresis chamber containing TBE buffer devoid of sodium dodecane sulphate for 20 min. The power supply was set at 2 V/cm (between electrodes) and the current was adjusted to 100 mA by raising or lowering the buffer level. Total run time was 20 min. The slides were stained using a solution

of 20 mg/L ethidium bromide in sealed boxes at 4 °C until image analysis was carried out. The DNA fragment migration patterns of 100 cells for each dose level were determined using a fluorescence microscope (with excitation filter 420-490 nm) linked to a CCD camera and Komet 5 image analysis software (Kinetic Imaging, Liverpool, UK).

## 2.6. Histopathology

Tissue sections were stained with haematoxylin and eosin according to Bancroft and Stevens (1996). Briefly, sections were de-waxed in xylene, rehydrated by submersion into descending grades of alcohol (100 %, 96 %, and 70 %), stained with Ehrlich haematoxylin for 20 min and washed under running tap water. This was followed by differentiation in 1 % acid alcohol (1 % HCl in 70 % alcohol) for 5-10 sec, after which it was washed again under tap water till a blue colour was noted (5 min) and stained in 1 % eosin for 10 min. A third washing step was performed after which the sections were dehydrated using alcohol and cleared in Xylol. Mounting was done with Canada balsam and slides were examined under a light microscope.

## 2.7. Statistics

Data was captured and analysed using SPSS version 19 (SPSS Inc., Chicago, IL, USA). Results are expressed as means ± standard deviations (SD) of at least three repeats carried out in triplicate. One-way analysis of variance was used to compare the means of the groups. The least significant difference method was used to compare the means between groups. A p-value of $< 0.05$ was considered statistically significant.
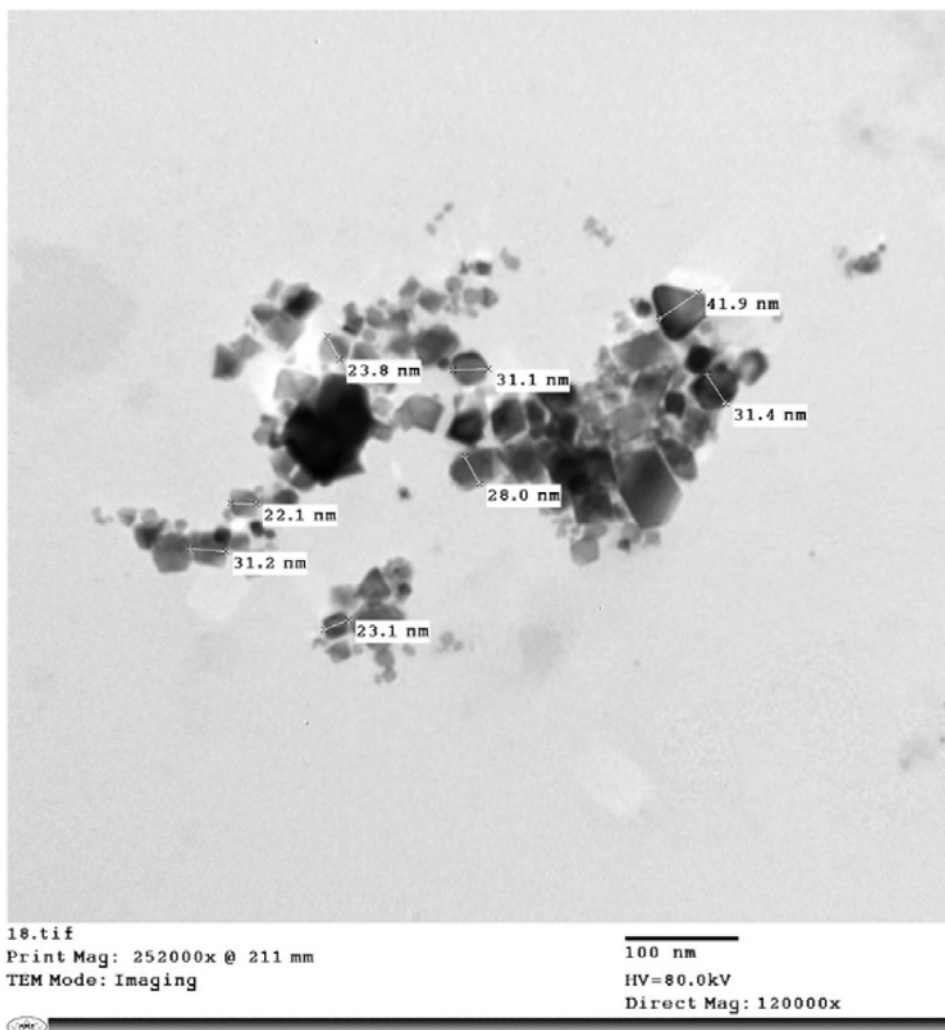
**Figure 1.** Transmission electron microscopy image of zinc oxide nanoparticles.

## 3. Results and Discussion

### 3.1. Characterization of ZnONPs

Cellular toxicity of ZnONPs is dependent on several physiochemical properties including; size of the particle (the smaller the particle size, the greater the cytotoxicity), surface charge, shape, dissolution kinetics, aggregation rate, and surface chemistry (Sivakumar et al. 2018). Characterization of ZnONPs by TEM revealed an average size of 31.2 nm (range 14.8–41.4

nm). Variation in the shape of the ZnONPs was noted which included triangular, circular and polyhedral shapes (Figure 1).

Solubility, re-dispersity, and stability of NPs are a key step before introducing the NP into the biological system. For any exposure media, the size of NPs, the ionic strength, and the pH of media determine the rate of dissolution and aggregation of NPs (Preedia Babu et al. 2017). By comparing the dissolution rate of ZnONPs of different diameters, it was found that the smallest nanoparticles dissolve more readily than larger ones (Bian et al. 2011). In media of high ionic strength such as saline and PBS, the presence of chloride and phosphate ions compresses the electric double layer of NPs and decreases the repulsive force between them leading to aggregation of NPs (Preedia Babu et al. 2017). This has been reported for ZnONPs, where large aggregates are formed in sea water when compared to fresh water due to a higher ionic strength (Keller et al. 2010). The stability of ZnONPs in water follows the Derjaguin-Landau-Verwey-Overbeek theory. According to this theory, aggregation of ZnONPs depends on the sum of Van der Waals attraction and electric double layer repulsion. In the case of water, the electric double layer is higher, leading to repulsion of NPs and uniform dispersion, making them stable in water (Preedia Babu et al. 2017). Also, solubility of ZnONPs is inversely proportional to pH, while the size of agglomeration is proportional to the pH of medium. The isoelectric point of ZnONPs at which the electrostatic repulsive interaction between particles decreases, is 8.7 to-10.3. Accordingly, in medium with a pH > 8, agglomeration increases. Furthermore, a higher pH (> 7) is associated with a higher concentration of hydroxide ($OH^-$) ions being present in the medium, which enhances formation of $Zn(OH)_2$, resulting in lower concentrations of free $Zn^{2+}$ ions in the medium (Lee et al. 2020).

*3.2. Hormone levels*

There was no significant difference in hormonal levels between the control and vehicle treated group. In the group administered ZnONPs, a significant ($p < 0.001$) reduction in serum T3, T4 and TSH levels was noted when compared to the control and vehicle treated groups (Table 1). The reduction in these hormone levels may be ascribed to the direct damage of thyroid follicular and para-follicular cells inflicted by the NPs' mediated cytotoxicity (Pani and Pani 2017). During the oxidative state, NPs impair the mitochondrial function of thyroid follicular cells resulting in decreased energy required for the synthesis and release of thyroid hormones (Sulaiman, Luaibi, and Qassim 2018). Also, as liver is concerned with most of the peripheral production of T3, so, the reduction in serum T3 can be partly related to the impaired hepatic function (Jatwa and Kar 2008; Alkaladi et al. 2020). Unlike other metal oxide NPs, such as cerium oxide, iron oxide, and titanium dioxide, ZnONPs are relatively unstable and tend to dissolve in aqueous solutions, releasing $Zn^{2+}$ from the particles (Li et al. 2017). Although zinc generally has a stimulatory effect on thyroid gland, excess zinc has demonstrated dose dependent inhibition of the production and secretion of thyroid hormones (Baltaci et al. 2004).

The decreased TSH levels may be attributed to the diminished regulatory effect mediated by the pituitary gland on the thyroid gland secondary to excess zinc (Dean, Hargis, and Hargis 1991). The current findings are corroborated by Cheric and Rafieirad (2015), where administration of ZnONPs (1.25, 2.5, 5 mg/kg intraperitoneally) in rats led to a decrease in these hormones. Similarly, in fish receiving doses of half and a third of the $LC_{50}$ of ZnONPs for 4 consecutive days, a significant reduction in hormonal levels of T3, T4, and TSH was detected (Alkaladi et al. 2020). Furthermore, Luaibi and Zayed (2020) reported a significant reduction in T3 and T4 levels after administration of ZnONPs (30 and 60 mg/kg) to male rats

for 7, 14 and 28 days. Similar results have been obtained in *in vivo* studies conducted with other metal NPs. Thyroid hormone levels decreased after rats were injected intramuscularly with copper NPs (2.0 mg/kg b.wt) (Polyakova et al. 2015). Similarly, mice who were administered 100 mg/L of titanium dioxide for 14 days via oral gavage had reduced levels of both T4 and TSH (Mahdieh et al. 2015).

**Table 1.** Serum biochemistry of experimental groups.

| Parameter | Treatment group (n = 8) | | |
|---|---|---|---|
| | **Control** | **Vehicle** | **ZnONPs** |
| T3 (µg/L) | $4.80 \pm 0.20$ | $4.75 \pm 0.20$ | $0.86 \pm 0.02$* |
| T4 (µg/L) | $56.7 \pm 2.38$ | $56.8 \pm 0.20$ | $48.07 \pm 1.21$* |
| TSH (µg/L) | $21.00 \pm 0.88$ | $20.77 \pm 0.87$ | $3.20 \pm 0.08$* |
| AST (U/L) | $38.44 \pm 1.62$ | $38.02 \pm 1.60$ | $80.11 \pm 2.01$* |
| ALT (U/L) | $27.46 \pm 1.15$ | $27.16 \pm 1.16$ | $50.07 \pm 1.26$* |

**p<0.001 when values are compared to control group.*

*Values are presented as mean ± SD.*

T3 = triiodothyronine; T4 = thyroxine; TSH = thyroid-stimulating hormone; AST = aspartate aminotransferase; ALT = alanine aminotransferase.

### 3.3 Liver enzymes

AST for the most part is a mitochondrial enzyme which is mainly used to diagnose and verify the course of hepatotoxicity in conjunction with other enzymes (Vozarova et al. 2002). ALT on the other hand is located primarily in the cytosol of hepatocytes, and is an early indicator of hepatotoxicity and marker of cellular necrosis (Ramaiah 2007). In the group treated with

ZnONPs, there was a significant ($p < 0.001$) increase in liver transaminases compared to the control and vehicle treated groups (Table 1). The latter is indicative of the detrimental effects these compounds have on the liver. An increase in AST and ALT enzymes has been reported after sub-acute oral administration of ZnONPs (10 mg/kg) to rats for 5 consecutive days (Ben-Slama et al. 2015). Similarly, a significant elevation in hepatic transaminases was found in mice who received ZnONPs (300 mg/kg) for 14 days (Sharma et al. 2012) and rats which were repeatedly administered 100 mg/kg ZnONPs (orally) for 75 days (Yousef, Mutar, and Kamel 2019) and 100 mg/kg ZnONPs (intraperitoneal) every second day for 10 days (Moatamed et al. 2019). Corroborative results have been reported for other metal NPs including silver nanoparticles (Ramadi et al. 2016) and copper oxide NPs (Mohammadyari et al. 2014). Contradictory, ALT and AST levels were reduced in chickens which received a dietary supplement of ZnONPs (Fathi 2016). The opposing effects may be ascribed to the difference in animal species.

### 3.4. Oxidative stress

With respect to oxidative stress, the ability of ZnONPs to induce oxidative stress is related to: i) the oxidative properties of the NPs themselves (attributed to their physicochemical properties), ii) the dissolution of ZnONPs and release of $Zn^{2+}$ ions which results in ROS-mediated cytotoxicity and altered mitochondrial function, iii) the generation of oxidants upon interaction of ZnONPs with cellular material or activation of mitochondrial NADPH-oxidase enzymes (Saliani, Jalal, and Goharshadi 2016), and iii) the crystal defects which contribute to the formation of electron-hole pairs at the surface of ZnONPs with subsequent ROS generation (Rasmussen et al. 2010).

The ZnONPs-mediated generation of ROS has been attributed to their nano-level and semiconductor characteristics (Mir et al. 2020). Semiconductors have a band gap between the electrons containing a lower valence band and the upper unoccupied conduction band. When electrons move from the valence band to the conduction band, holes are left in the valence band. The quality of ZnO nanocrystals declines with the decreased NP size leading to increased interstitial $Zn^{2+}$ and development of crystal defects (Sharma et al. 2009). These crystal defects produce high amount of electron–hole pairs which in turn react with $O^{2-}$ and $OH^{-}$ (in aqueous suspension) leading to formation of highly reactive superoxide and hydroxyl radicals (Singh, Cheng, and Singh 2020).

In the liver, ZnONPs generate excess ROS (hydrogen peroxide, hydroxyl radical species and nitric oxide or superoxide anion) which results in oxidative stress mediated cytotoxicity. The Zn ions lost from NPs can directly interact with NADPH oxidases on the plasma membrane or mitochondria of hepatocytes causing disruption of the electron transport chain with generation of superoxide anion. Moreover, ZnONPs can enhance damage of cell membrane directly upon interaction with the external membrane surface of hepatocyte leading to generation of ROS (Yousef, Mutar, and Kamel 2019). The cell membranes in the liver are sensitive to free radicals and lipid peroxidation, and results in injury of the membranes and subsequently decreased fluidity and increased permeability. Changes in fluidity of the liver membrane leads to impairment in the activity of liver enzymes and inhibition of hepatic function (Yao et al. 2019). Oxidative stress mediated by ZnONPs was evident from the elevated levels of MDA (lipid peroxidation marker) and the reduced glutathione levels in both the thyroid glands and livers of treated rats (Table 2). The reduction in GSH levels may be attributed to its consumption during the elimination of the generated ROS. Thyroid hormones play a crucial role as antioxidants by increasing ATP needed for oxidative phosphorylation, and in so doing reduce

ROS, and enhance production of NADPH required for regeneration of reduced GSH (Moustafa et al. 2009). Ultimately the altered thyroid hormones increase oxidative stress. The excess ROS generation enhances lipid peroxidation by converting polyunsaturated fatty acids in the cell membrane into toxic lipid peroxides destroying the membrane and causing cell injury (Attia, Nounou, and Shalaby 2018).  Similar findings have been reported where cytotoxicity of ZnONPs was mediated by oxidative stress and led to the depletion of antioxidants as was evident from the high levels of lipid peroxidation end products and low antioxidant status in the liver (Almansour et al. 2017; Moatamed et al. 2019), kidney and liver (Yousef, Mutar, and Kamel 2019), brain (Attia, Nounou, and Shalaby 2018), testes (Hussein et al. 2016) and lung (Huang et al. 2010) of ZnONPs treated rats.

**Table 2.** Oxidative stress parameters of experimental groups.

| Parameter | Treatment group (n = 8) | | |
|---|---|---|---|
| | **Control** | **Vehicle** | **ZnONPs** |
| MDA thyroid (mmol/g) | 21.84 ± 0.92 | 21.60 ± 0.91 | 57.86 ± 1.45* |
| MDA liver (mmol/g) | 27.30 ± 1.15 | 27.00 ±1.13 | 64.09 ± 1.61* |
| GSH thyroid (mmol/g) | 3.28 ± 0.14 | 3.24 ± 0.14 | 1.73 ± 0.04* |
| GSH liver (mmol/g) | 5.24 ± 0.22 | 5.18 ± 0.22 | 1.57 ± 0.04* |
| 8-OHdG serum (pg/mL) | 162.65 ± 1.54 | 160.86 ± 1.52 | 282.61 ± 1.94* |

*$*p<0.001$ when values are compared to control group.*

*Values are presented as mean ± SD.*

MDA = malondialdehyde; GSH = reduced glutathione; 8-OHdG = 8-hydroxydeoxyguanosine.

8-Hydroxydeoxyguanosine is a nucleoside of DNA which acts as a reliable and sensitive biomarker of oxidative stress mediated DNA damage (Ng et al. 2017). The elevated 8-OHdG

is indicative of oxidative DNA damage and genotoxicity (Table 2). These findings are supported by Ng et al. (2017) where exposure of Drosophila cells to ZnONPs resulted in ROS production, leading to the accumulation of 8-OHdG in the cells. Furthermore, increased 8-OHdG levels were reported in the lungs of rats one month after intratracheal instillation and three months after inhalation of ZnONPs (Li et al. 2018). Also, increased formation of DNA adducts (8-OHdG) were found in mouse liver tissue homogenate after being exposed to 50 $\mu$g/mL ZnONPs (Syama et al. 2013). Similar to the current findings, excess 8-OHdG generation was detected in lungs of rats treated with NiO and $CeO_2$ NPs (Li et al. 2018) and in livers of mice treated with $TiO_2$ NPs (Trouiller et al. 2009).

### 3.5. Genotoxicity

Zink oxide NPs resulted in noteworthy DNA damage in both the thyroid and liver as compared to the control and vehicle treated groups. This DNA damage was confirmed by the significant ($p < 0.001$) increase in comet parameters; tail length, tail DNA, and unit tail moment (Table 3). Genotoxicity of ZnONPs has been attributed to the oxidative stress mediated mechanisms (Sharma et al. 2012; and Attia, Nounou, and Shalaby 2018). ROS can react with the nucleophilic centers of the cells and thereby bind covalently to the DNA molecules resulting in a single strand break, double strand break, and formation of DNA adduct and thus induce DNA damage (Kang 2002). Sharma et al. (2012) reported a significant increase in the comet parameters and Fpg-specific DNA lesions in liver of ZnONPs treated rats. Additionally, ZnONPs create an oxidant environment that enhances DNA damage and also impairs the repair pathways in cells via depletion of antioxidants. This has been noted in Caco-2 cells where there was failure to repair the oxidative stress mediated DNA damage induced by ZnONPs, albeit this repair was afforded after $TiO_2$-NPs administration (Zijno, De Angelis, and De Berardis

2015). Similarly, genes related to DNA repair pathways (mismatch repair, base excision repair) and DNA synthesis (DNA polymerase and DNA ligase) were found to be down regulated in *Deinococcus radiodurans* bacterial cells treated with ZnONPs, while the response genes to DNA damage were markedly up regulated (as much as 20-fold) (Singh, Cheng, and Singh 2020). Also, fragmentation of DNA occurred in the brains of rats orally treated by 40 and 100 mg/kg/day ZnONPs for 7 days (Attia, Nounou, and Shalaby 2018). ZnONPs have been reported to reduce the mitochondrial membrane potential, result in a G0/G1 cell cycle arrest, and induce chromosomal aberrations as well as micronuclei formation in bone marrow cells of Albino mice (Ghosh et al. 2016). Furthermore, ZnONPs have been found to induce DNA damage in the kidneys of rats (Faddah et al. 2012). Additionally, migration of DNA away from nucleus and the formation of comet, evident from the significant increase in the tail length and tail DNA moment, have been reported in Drosophila cells treated with ZnONPs (Ng et al. 2017).

**Table 3.** Comet assay parameters of experimental groups.

| Parameter | Treatment group (n = 8) | | |
|---|---|---|---|
| | **Control** | **Vehicle** | **ZnONPs** |
| Tail length thyroid (µm) | $1.51 \pm 0.06$ | $1.48 \pm 0.06$ | $2.55 \pm 0.05*$ |
| Tail length liver (µm) | $2.80 \pm 0.11$ | $2.77 \pm 0.10$ | $3.57 \pm 0.08*$ |
| Tail DNA thyroid (%) | $1.71 \pm 0.07$ | $1.69 \pm 0.07$ | $2.52 \pm 0.05*$ |
| Tail DNA liver (%) | $2.52 \pm 0.10$ | $2.49 \pm 0.10$ | $3.57 \pm 0.08*$ |
| Unit DNA moment thyroid (%) | $1.68 \pm 0.07$ | $1.66 \pm 0.06$ | $4.07 \pm 0.09*$ |
| Unit DNA moment liver (%) | $1.98 \pm 0.08$ | $1.95 \pm 0.08$ | $5.08 \pm 0.11*$ |

*$p<0.001$ when values are compared to control group.*

*Values are presented as mean $\pm$ SD.*

## 3.6. Histopathology

No gross abnormalities were noted for the liver and thyroid glands of any of the treatment groups. An increase in thyroid weight was noted in the group that was administered ZnONPs (Table 4). The weight increase of the thyroid gland may be attributed to the induction of hypothyroidism and the compensatory hypertrophy (Soukup et al. 2001). Histological examination of the thyroid glands of control and vehicle groups (Figure 2) showed normal organization with different sized thyroid follicles that were filled with vacuolated acidophilic homogenous colloid. Follicles were lined by cuboidal follicular cells with round vesicular nuclei. Furthermore, inter follicular cells were separated by thin collagen fibres. In the ZnONPs treated group (Figure 2), thyroid follicles were distended due to excess colloid formation. The epithelium that lined the follicles was flattened with dark flattened nuclei, denoting hypothyroidism. During a resting state, the colloid content increases and follicular cells become flattened in the thyroid (Park et al. 2017). Some areas showed a reduction in the number of follicles, inflammatory cell infiltration and vascular congestion. The enhancement of inflammation is ascribed to the ZnONPs entering the cells (Vandebriel and De Jong 2012). Rats administered silver NPs have been reported to present with altered thyroid gland structure, visible as distended follicles filled with colloidal fluid (Pani and Pani 2017).

**Table 4.** Organ weight of experimental groups.

| Parameter | Treatment group (n = 8) | | |
|---|---|---|---|
| | **Control** | **Vehicle** | **ZnONPs** |
| Thyroid weight (mg) | 31.78 ± 1.24 | 31.43 ± 1.22 | 38.22 ± 0.81* |
| Liver weight (g) | 5.77 ± 0.24 | 5.70 ± 0.24 | 5.51 ± 0.14* |

*$p<0.001$ when values are compared to control group.*

*Values are presented as mean ± SD.*
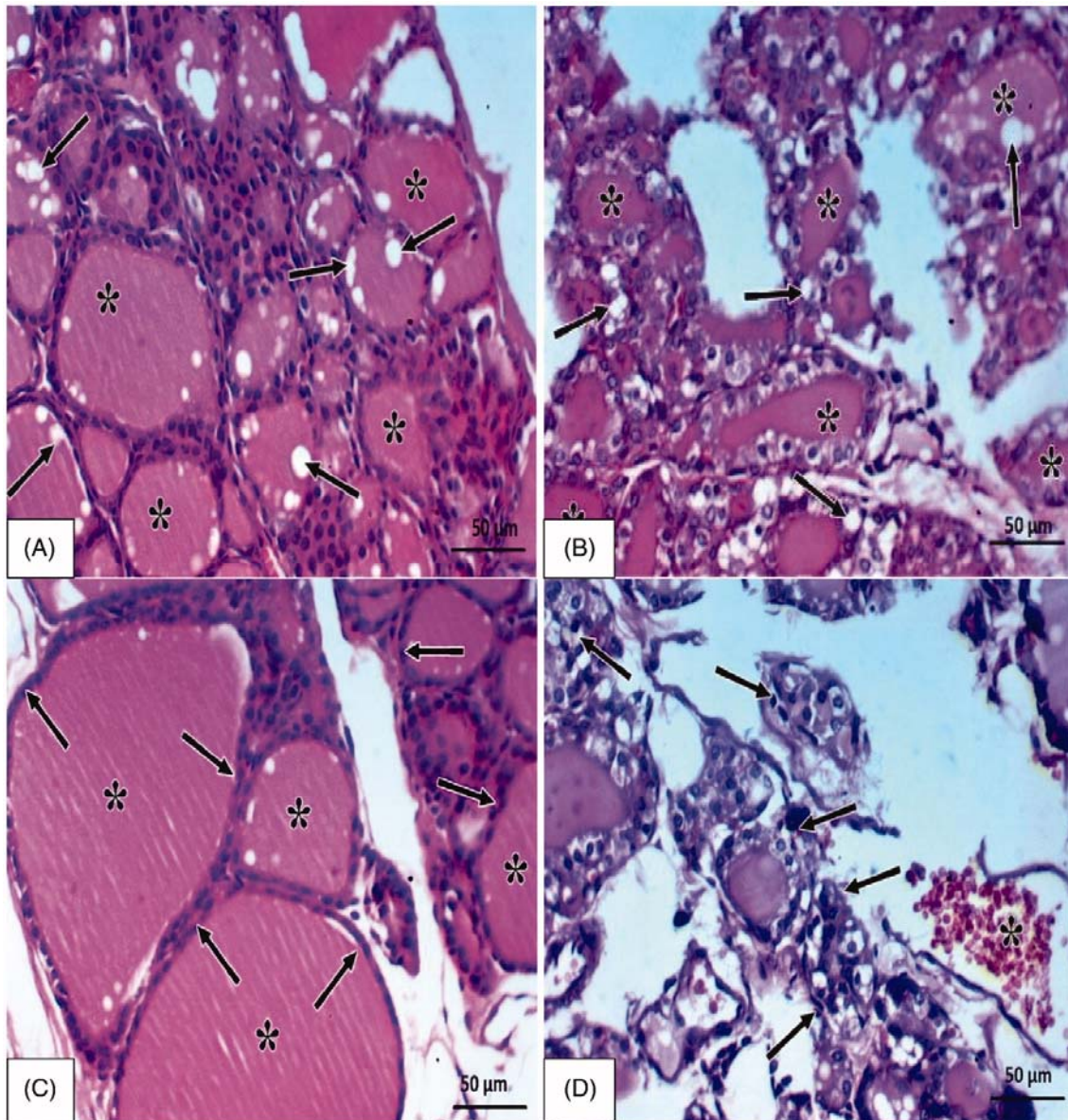
**Figure 2**. Photomicrograph of the thyroid gland of (A) control group, (B) vehicle group where the normal architecture of follicles, lined with cuboidal epithelium and filled with colloid material (star) and multiple peripheral colloidal vacuolizations (scalloping) is shown (arrow), (C) ZnONPs treated group showing dilated follicles filled with excess colloidal material (star) which is lined with flattened epithelium (arrow) and (D) damaged follicles with inflammatory infiltration (arrow) and dilated congested vascular spaces (star) (magnification x400).

Contrary to the thyroid glands, the weight of the livers of the ZnONPs treated group decreased in comparison to the control and vehicle treated groups (Table 4). These results are corroborated by Yousef, Mutar, and Kamel (2019) in rats orally treated with 100 mg/kg ZnONPs for 75 consecutive days. The livers of control and vehicle groups (Figure 3) showed normal sized central veins surrounded by tightly packed cords of hepatocytes with vesicular nuclei and acidophilic cytoplasm radiating from the central vein. Sections of the liver of rats treated with ZnONPs revealed altered architecture with markedly dilated and congested central veins (sinusoidal congestion), hydropic degeneration and vacuolization of hepatocytes, lymphocytic infiltration, as well as focal areas of hepatic necrosis (Figure 3). Similar findings have been reported for rats treated by ZnONPs at a dose of 300 mg/kg b.wt. for 14 days (Mansouri et al. 2015), 1 g/kg b.wt. for 5 consecutive days (Hegazy et al. 2018), and 100 mg/kg b.wt. every second day for 10 days (Moatamed et al. 2019). The hydropic degeneration of hepatocytes that was noted indicates that ZnONPs may affect ion and fluid homeostasis leading to an increase in intracellular water permeability with massive influx of water and Na+ (Schrand et al. 2010). Moreover, hepatocyte degeneration might be accompanied by leakage of lysosomal hydrolytic enzymes that results in increased cytoplasmic degeneration (Almansour et al. 2017). The cause of necrotic foci may be ascribed to lysis of the cells and formation of cell debris, which could initiate phagocytic infiltration (Edinger and Thompson 2004). ROS generation induced by NPs is known to result in cell death and hepatocyte damage via necrosis (Yao et al. 2019). Furthermore, vacuolization of hepatocytes has been associated with the necrotic effects of ZnONPs (Hegazy et al. 2018).
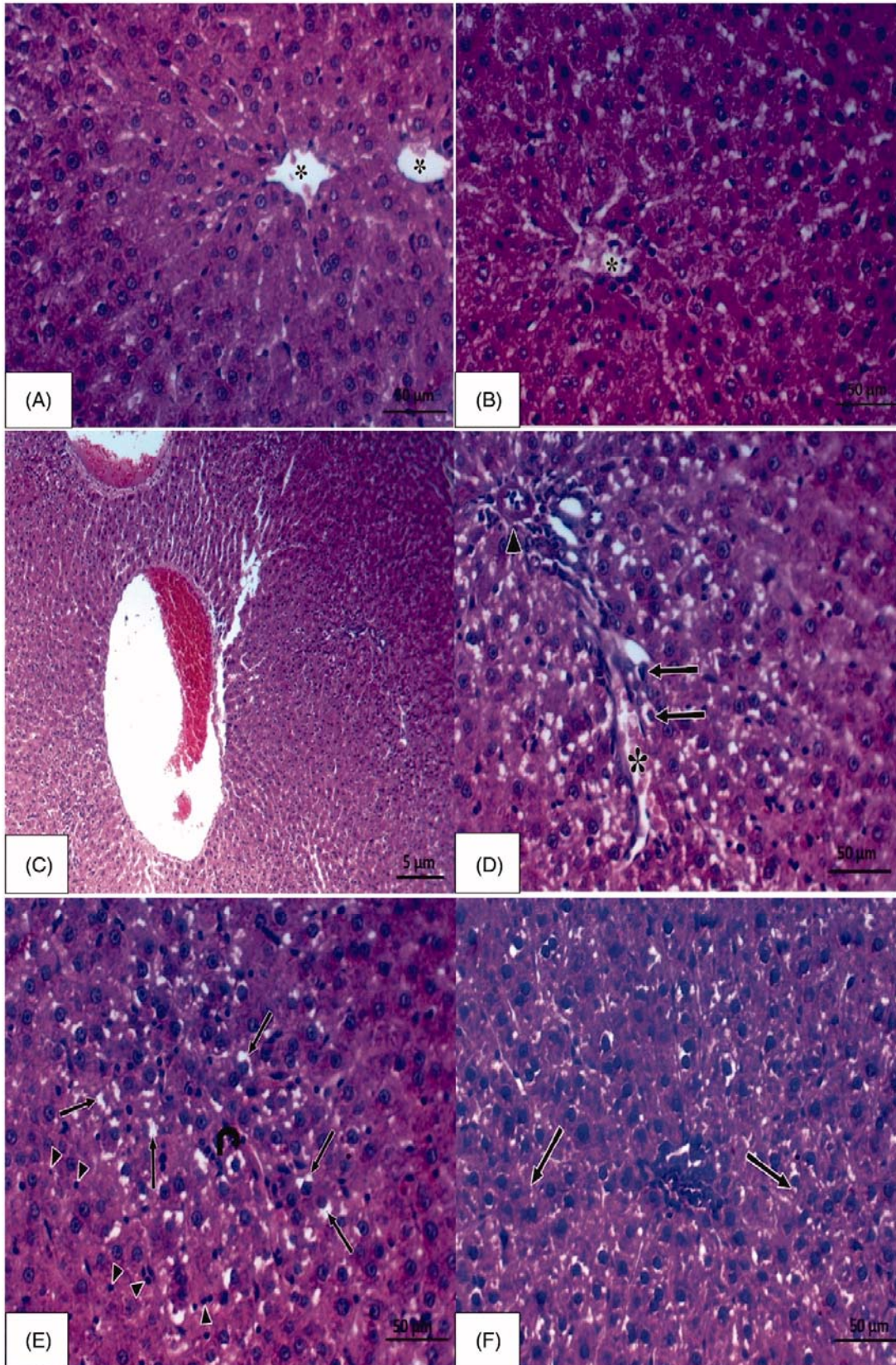
**Figure 3.** Photomicrograph of sections of the liver of (A) control group, (B) vehicle group showing the normal hepatic architecture of tightly packed cords of hepatocyte with vesicular nuclei and acidophilic cytoplasm radiating from the central vein (star), (C) ZnONPs treated group showing the markedly dilated and congested central vein (magnification ×200), (D) indicating the congested central vein (star) surrounded by aggregates of lymphocytes (arrow) with the invasion of the portal tract by inflammatory cells (triangle), (E) hydropic degeneration of hepatocytes (arrow) with vacuolated cytoplasm (curved arrow) and lymphocytic infiltration (triangle), and (F) focal areas of hepatic necrosis (arrow) (magnification ×400).

## 4. Conclusions

Sub-chronic exposure of rats to ZnONPs exerts cytotoxicity in both the thyroid gland and liver as was evidenced by the biochemical evaluations and histopathological examinations. Oxidative stress is proposed as the primary mechanism of inducing toxicity which was confirmed by the increase in lipid peroxidation, depletion of glutathione, elevation in 8-OHdG and the noted DNA damage in both organs. Caution is warranted with repeated usage of products containing ZnONPs.

**Author contributions**

Both authors were involved in the design of the study, data analysis and write up of the article.

Samar Sakr additionally carried out the experimentation.

**Disclosure statement**

The authors declare no conflict of interest.

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