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Nephron ultrastructural alterations induced by zinc oxide nanoparticles: an electron microscopic study

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Abstract: Due to their unique properties, zinc oxide nanoparticles (ZnO NPs) are invested in many industries, commercial products, and nanomedicine with potential risk for human health and the environment. The present study aims to focus on alterations that might be induced by ZnO NPs in the nephron ultrastructure. Male Wister Albino rats were subjected to ZnO NPs at a daily dose of 2 mg/kg for 21 days. Kidney biopsies were processed to transmission electron microscopy (TEM) and ultrastructural pathology examinations. Exposure to ZnO NPs-induced ultrastructural alterations in the proximal convoluted tubules (PCTs) and to lesser extent in the distal ones (DCTs), while the loops of Henle were almost not affected. The glomeruli demonstrated dilatation, partial mesangial cells loss, matrix ballooning, slits filtration widening, and basement membrane thickening. Moreover, PCT revealed cytoplasmic necrosis, vacuolation, erosion, and disorganisation of the apical microvilli together with mitochondrial swelling and cristae destruction. The nuclei of the renal cells exhibited nuclear deformity, heterochromatin accumulation, and apoptotic activities. The findings indicate that ZnO nanomaterial have the potential to affect the nephron ultrastructure suggesting alteration in the kidney functions. More work is needed for better understanding the toxicity and pathogenesis of ZnO oxide nanomaterial.

1 Introduction

Zinc oxide NPs (ZnO NPs) are being produced in large scales and invested in various industrial and commercial sectors [1, 2]. These nanomaterial are used in electronic devices, paints, building materials, cosmetics, makeup products, tooth paste, textile material, polymeric matrices, packaging materials, and food systems [3–5]. In addition, ZnO nanomaterial have recently received much attention due to their possible applications in nanomedicine especially cancer therapy, and in protection coaters from UV radiation [6]. Moreover, ZnO nanomaterial have antimicrobial activity against different types of pathogenic bacteria and fungal spores resistant to high pressure and high temperature [7, 8]. In addition, ZnO nanosized semiconductor crystals improved the catalytic activity of enzymes in photo-controlled enzyme-based biosensors [3]. ZnO nanomaterials also exhibit various traits of biosensing for catalytic efficacy, strong adsorption capability and high isoelectric point based on enzymic reaction, immunoreactions, and molecular computation [9]. The majority of invested ZnO NPs biosensors are used in biochemical applications such as magnetic resonance, nano-optical devices, emission tomography, dual-modality imaging, gene delivery, and biosensing [10]. Fast, simple, sensitive, and eco-friendly novel electrochemical glucose biosensors and urea biosensors were developed by depositing ZnO NPs [11]. Moreover, an electrochemical H_2O_2 biosensor was fabricated by modification of a glassy carbon electrode using ZnO NPs [12].

ZnO NPs have unique in vivo behaviour such as easier clearance from the site of injection, longer circulating residue, and slower passage to the interstitial spaces than their parent compounds due to their small size and larger surface area to volume ratio [13–16]. Moreover, several reports indicated that ZnO nanomaterial could induce oxidative stress and free radicals causing damage to tissues, cells, and macromolecules [17, 18]. Other studies showed that ZnO NPs could exhibit selective apoptosis in some malignant cells via p53 pathway activation [18–23]. Moreover, these particles are able to cross the blood vital organs barrier and to pass the cell membrane easily. In addition, some reports indicated that ZnO nanomaterial could exhibit cytotoxicity, genotoxicity, neurotoxicity, mitochondrial damage, and apoptosis [16, 17, 22–24]. Other studies indicated potential risks of ZnO nanomaterial on the vital organs especially liver, kidney, spleen, heart, and lung as target organs with smaller particles were more toxic than the larger ones and ZnO nanorods were more toxic than the nanospherical ones [25–27]. In vivo investigations demonstrated that inhalation of ZnO NPs could induce alveolar inflammation together with damage in the kidney and liver tissues [15, 27]. Other studies reported that these nanomaterial could induce potent but reversible inflammation and granulomatous one [28, 29]. In addition, some studies indicated that ZnO NPs could induce epithelial cells proliferation, pulmonary fibrosis, and goblet cells hyperplasia [30]. Moreover, ZnO nanomaterial could significantly alter the serum levels of total protein and some enzymes such as creatine kinase and lactate dehydrogenase [15].

Exposure to ZnO NPs is becoming part of our environment with questions about their possible potential cytotoxicity remains unanswered. These concerns need to be considered before determination if their benefits outweigh the potential risks with special attention is needed towards the cellular toxicity of these nanomaterials. To the best of our knowledge, limited information is available on the ultrastructural alterations induced by ZnO NPs on the vital organs [31]. The present work was conducted to explore the ultrastructural pathology that might be induced by these particles in the nephron structure.

2 Materials and methods

2.1 Nanoparticles

ZnO NPs used in the current study were purchased from Sigma (USA, Aldrich). According to the manufacturer, the nanoparticles dispersion had the following characterisation: average particle size 35 nm; concentration 50 wt% in H_2O; pH 5.5 ± 0.1; density 1.7 ± 0.1 g/ml. To evaluate the veracity of the manufacturer's specification, the particle size was assessed by using Jeol

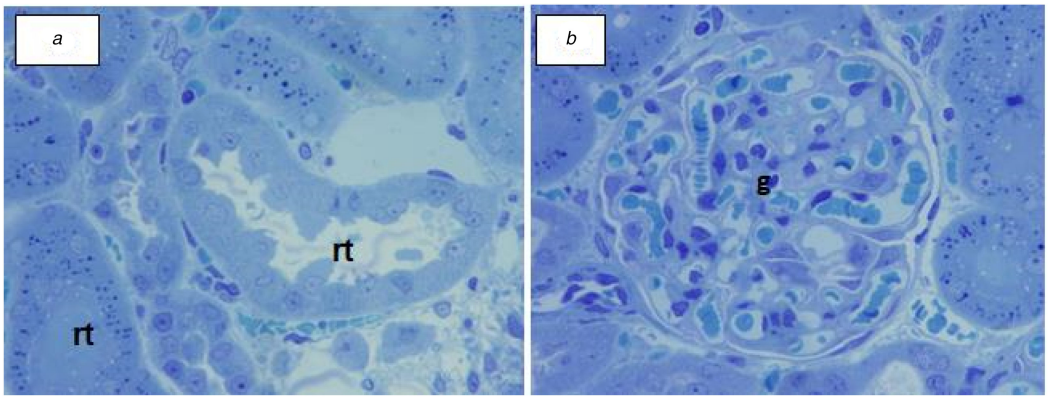

Fig. 1 *Light and electron micrographs of semi-thin and ultrathin sections of control rats kidney demonstrating*
(a) Light micrograph of normal renal tubules (rt). Toluidine blue stained semi-thin section, 10,000×, ***(b)*** Light micrograph of normal glomerulus (g). Toluidine blue stained semi-thin section, 1000×

transmission electron microscope (TEM) at 80 Kv (JEM-1011, Japan). The chosen dose was based on previous studies [19–34].

ZnO nanomaterials have rapid dissolution in acidic condition (pH 5.5). Accordingly, nanoparticles dispersion was disaggregated by ultrasonication before being diluted with sterile acidic distilled water (pH 5.5) at 37°C immediately before use. Nanoparticles solution was prepared so that the necessary dose could be administered in a volume of 400 µl.

2.2 Animals and conditions

Test group and a control one (10 rats each) of male Wistar albino rats of the same age weighing 220–235 g were used in the present study. The animals were housed at 24 ± 1°C, on 12 h dark/light cycle.

2.3 Experimental protocol

Control rats received daily single i.p. injection of 400 µl of the distilled water (pH 5.5, 37°C), while the test group members received a daily i.p. injection dose of 2 mg/kg bw of 35 nm ZnO NPs (dissolved in acidic distilled water, pH 5.5, 37°C) for 21 days. Animals handling and experimentation were carried out in accordance with King Saud University protocols.

2.4 Electron microscopic investigation

Kidney pieces including cortex and medulla from each rat were minced into small cubes of 1 mm in length and fixed in glutaraldehyde fixative (2.5%) in phosphate buffer (0.1 M, pH 7.4) for 24 h at 4°C. Tissue cubes were then post fixed in 2% osmium tetroxide (OsO_4) in cacodylate buffer for 90 min at room temperature. The samples were then washed in the buffer and dehydrated at 4°C through a gradual bath of ethanol (20, 40, 70, 90, 95 and double changes of 100% ethanol), cleared in propylene oxide (1,2 epoxy oxide) and embedded in Epon-araldite epoxy resin mixture [32]. Semi-thin (500–1000 nm) and ultrathin (60 nm thick) sections were obtained on Leica EM UC6 ultramicrotome and mounted on 200-mesh hexagonal copper grids, stained with toluidine blue (1%) and uranyl acetate-lead citrate double stain, respectively[33]. Ultrasections examination and structural abnormalities evaluation were carried out by using Jeol transmission electron microscope at 80 Kv (JEM-1011, Japan).

3 Results

Examination of the semi-thin and ultrathin sections of the control kidneys and the treated ones revealed the followings:

3.1 Control kidneys

The semi-thin sections of the control kidneys demonstrated normal nephron ultrastructure (Figs. 1*a* and *b*). The glomeruli showed normal capillary tufts, basement membrane, and mesangial cells together with intact primary and secondary podocytes processes separated from each other by slight pores (Fig. 2*a*). The primary processes trabiculae and the secondary foot processes pediculae of the podocyte showed typical appearance (Fig. 2*b*). In addition, the control glomeruli demonstrated fenestrated endothelial cells and visceral epithelial cells (podocytes) on the urine side of the capillaries. The glomerular basement membrane showed trilaminar structure while the mesangium demonstrated normal mesangial cells in contact and amorphous mesangial matrix (Fig. 2*c*).

The epithelial lining of the proximal convoluted tubules (PCTs) demonstrated spherical nuclei with euchromasia, normal profuse apical microvilli, together with basal cell membrane enfolding and elongated mitochondria. The cytoplasm of the renal cells demonstrated a considerable number of mitochondria, with some lodged at parallel between the basal enfolding of the cell membrane (Fig. 2*d*).

3.2 Rats exposed to 35 nm ZnO NPs

The renal tissues of ZnO NPs-treated rats demonstrated the following remarkable ultrastructural alterations:

3.2.1 Glomerular alterations: The glomeruli of rats exposed to ZnO nanomaterial demonstrated the following ultrastructural alterations:

3.2.2 Urinary spaces widening: The urinary spaces of the ZnO NPs treated rats appeared wider as compared with the control ones (Fig. 3*a*). This was accompanied with vascular alterations in the capillaries of the glomeruli.

3.2.3 Mesangial cells proliferation: Mild vacuolation, mesangial hyperplasia, and mesangiolysis were seen in nephrons of rats treated with ZnO nanomaterial (Fig. 3*b*).

3.2.4 Podocytes hypertrophy: The podocytes manifested hypertrophy together with fusion, swelling, and elongation of their primary and secondary processes (Fig. 3*c*). Some foot processes demonstrated disorganisation and discretion, while others were diffused together. In addition, the podocytes demonstrated degenerative changes, basement membrane thickening, granulation and shrinkage of nuclei together with reduction in the number and widening of podocyte pedicels. Lysosomal accumulation was also demonstrated by some podocytes.

3.2.5 Endothelial cells enlargement: The glomerular capillaries of the treated rats demonstrated hypertrophoid endothelial cells, while foci of the visceral epithelial cells showed protrusion (Fig. 3*d*).

3.2.6 Glomerular filtration slit widening: Filtration slit membrane structure demonstrated deterioration and degeneration. The pores size were larger than sizes observed in the control rats (Fig. 4*a*).

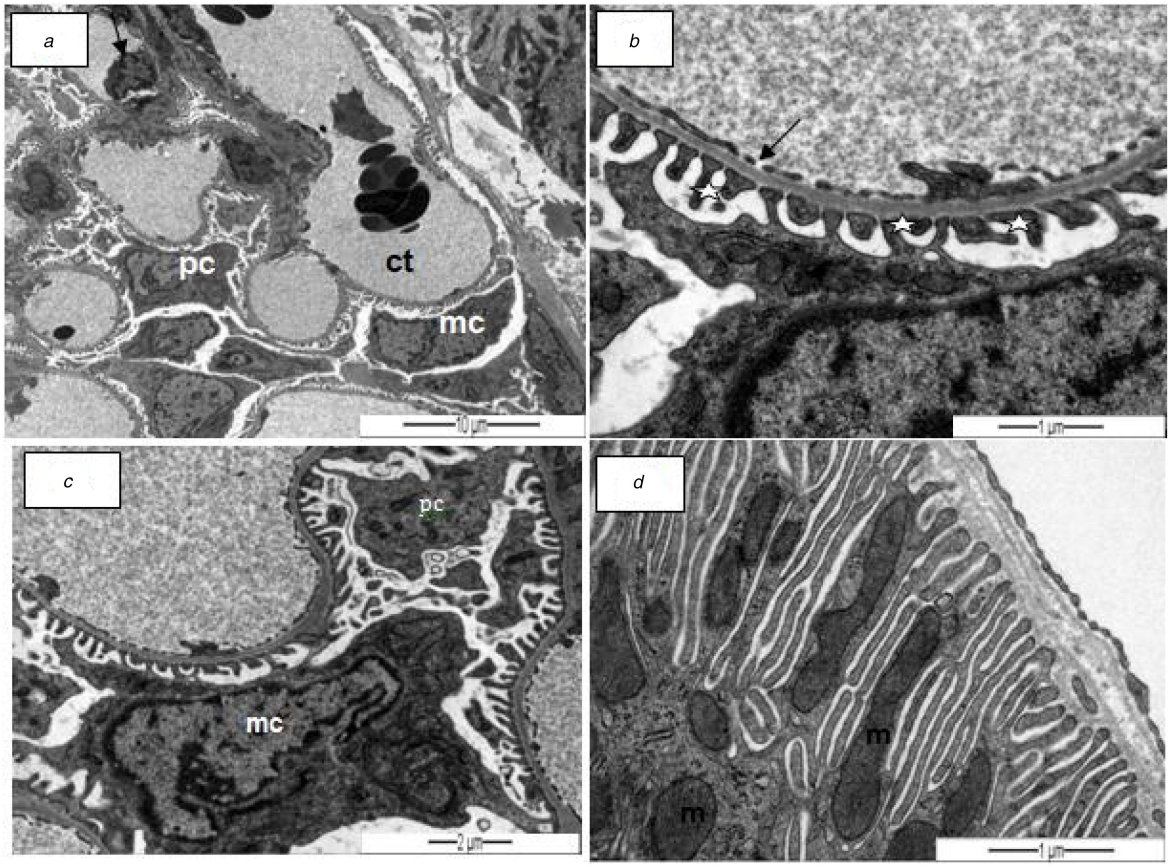

Fig. 2 *Electron micrographs of ultrathin sections of control rats kidney stained with uranyl acetate-lead citrate double stain demonstrating*
(a) Normal capillary tuft (ct), mesangial cells (mc) and podocytes (pc), ***(b)*** Typical appearance of primary and the secondary foot processes of podocyte pediculae (stars) separated from each other by slight pores (arrow), ***(c)*** Contact mesangial cell (mc) and podocyte (pc) embedded in amorphous matrix, ***(d)*** Mitochondria (m) lodged at parallel between the basal enfolding of the cell membrane

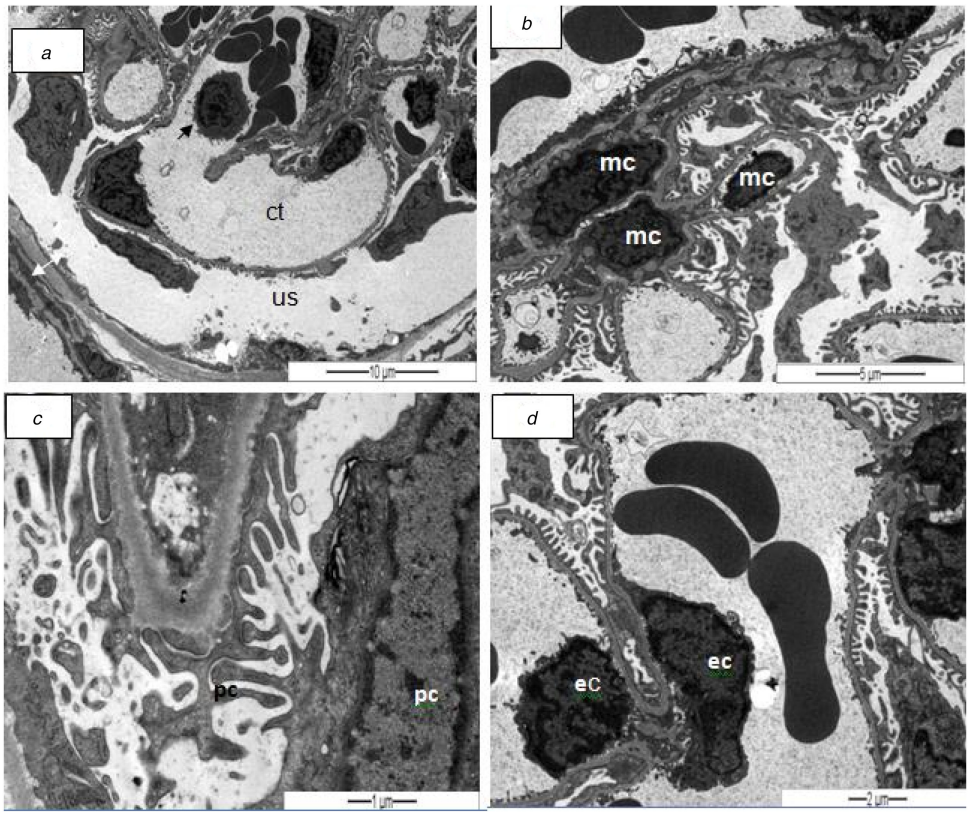

Fig. 3 *Transmission electron micrograph of ZnO NPs-treated rat glomeruli stained with uranyl acetate-lead citrate double stain demonstrating*
(a) Glomerular capillary (ct) tufts dilatation with urinary space (us) widening. Note the detached endothelial cells (arrow), ***(b)*** Mesangial cells (mc) proliferation, ***(c)*** Podocyte (pc) hypertrophy and swelling with numerous long primary and secondary processes. Pedicels fusion, disorganisation and discretion are also seen, ***(d)*** Endothelial cells (ec) hypertrophy

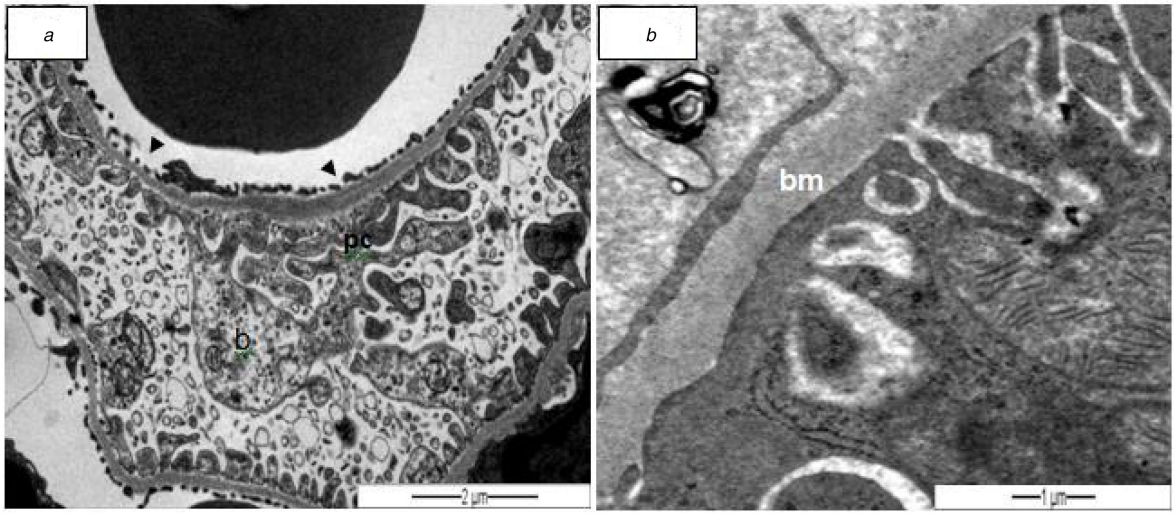

Fig. 4 *Transmission electron micrograph of ZnO NPs-treated rat glomeruli stained with uranyl acetate-lead citrate double stain demonstrating* ***(a)*** Glomerular filtration slit widening (arrow heads) with podocyte process bubbling (b), ***(b)*** Glomerular basement membrane (bm) thickening

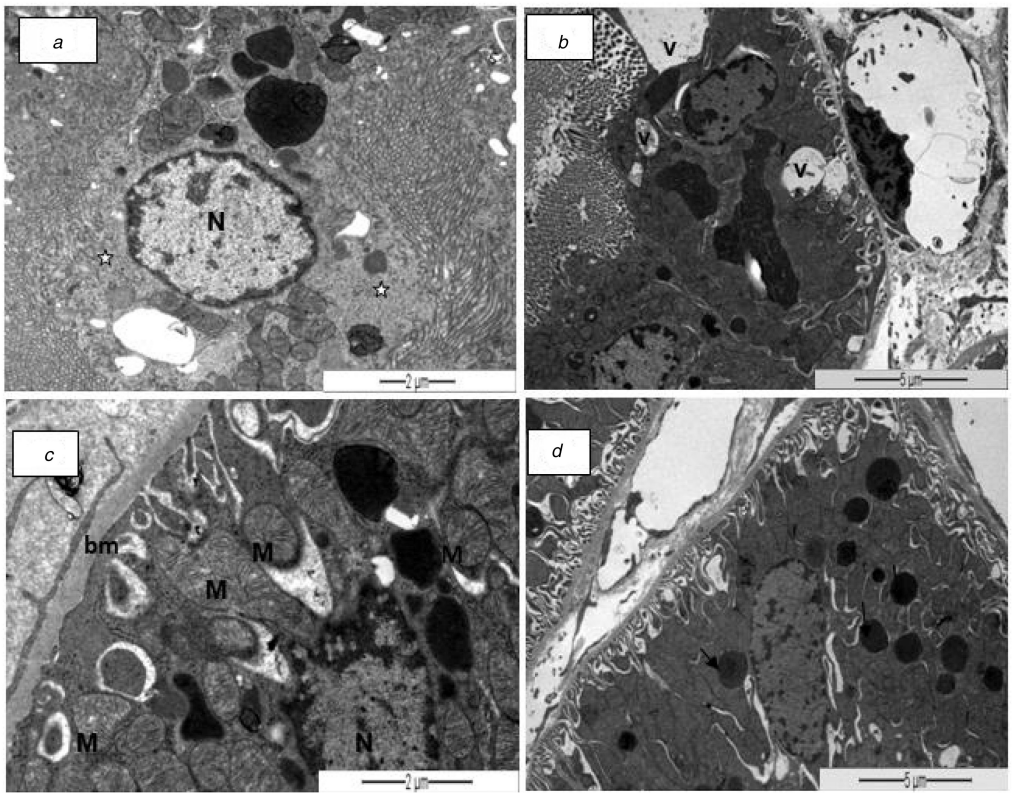

Fig. 5 *Transmission electron micrograph of ZnO NPs-treated rats renal tubules stained with uranyl acetate-lead citrate double stain demonstrating* ***(a)*** Degenerative changes (star), ***(b)*** Vacuolation (v), ***(c)*** Pleomorphic mitochondria (m) with swelling and crystolysis, ***(d)*** Lysosomal accumulation (arrows)

3.2.7 Glomerular basement membrane thickening: Some glomeruli demonstrated destructed and focal thickening of their basement membrane (GBM) (Fig. 4*b*).

3.3 Renal tubules alterations

The following ultrastructural alterations were demonstrated in the renal cells of rats treated with ZnO nanomaterial:

3.3.1 Degenerative changes: Some PCT cells demonstrated lyses and loss of organelles and accumulation of electron dense deposits (Fig. 5*a*). Moreover, some cells were extended and showed cytoplasmic bulges towards the lumen from the apical cytoplasm causing lumen narrowing.

3.3.2 Cytoplasmic vacuolisation: The PCTs epithelial lining exhibited basal and apical vacuolation of different sizes (Fig. 5*b*). Some renal cells demonstrated large vacuoles with pushed nuclei at one side of the cell. Autophages vacuoles and primary lysosomes were also seen.

3.3.3 Mitochondrial alterations: The nephrocytes of the PCTs demonstrated pleomorphic mitochondria with rarefied matrix. Misshaped mitochondria with swelling, elongation, angulations, fragmentation, and cristolysis were also seen (Fig. 5*c*).

3.3.4 Lysosomal accumulation: Some renal cells showed numerous variable sizes lysosomal-related structures filled with minute electron-dense ZnO NPs (Fig. 5*d*).

3.3.5 Renal cells nuclear alterations: Some renal cells exhibited deformity of the nuclei mainly irregularity and swelling of the nuclear envelope (Fig. 6*a*). In addition, irregular heterochromatin

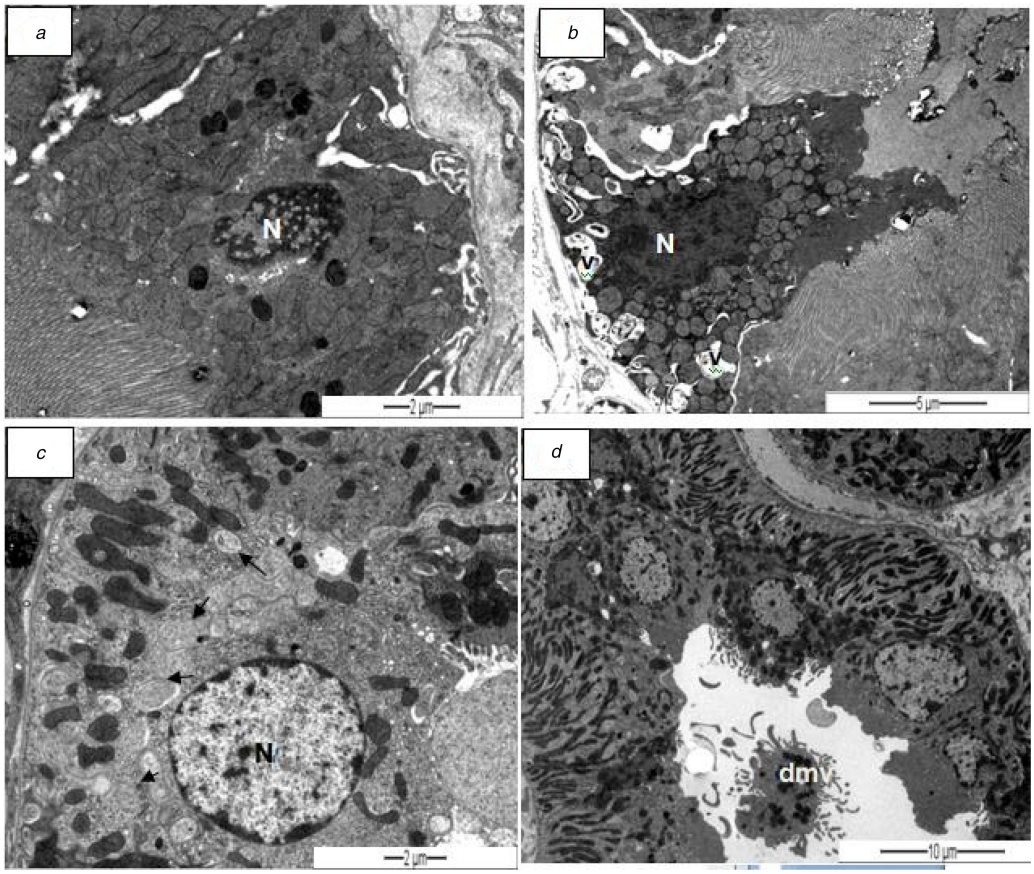

Fig. 6 *Transmission electron micrograph of ZnO NPs-treated rat renal tubules stained with uranyl acetate-lead citrate double stain demonstrating* ***(a)*** Nuclear irregularity and heterochromasia, ***(b)*** Apoptotic renal cell, ***(c)*** Autophagosomes (arrows), ***(d)*** Apical microvilli detachment (dmv)

accumulations in the nuclear membrane as well as in the nucleoplasm together with pyknosis were also observed.

3.3.6 Apoptotic activity: Some renal cells of the PCTs demonstrated nuclei shrinkage, condensed chromatin together with proapoptotic and apoptotic bodies (Fig. 6*b*).

3.3.7 Autophagosome formation: Some injured renal cells demonstrated autophagosomes with variable sizes. This alteration targeted various intracellular organelles for degradation (Fig. 6*c*).

3.3.8 PCTs microvilli alterations: The microvilli of the PCTs cells revealed swelling, disorganisation, compression, erosion, and partial detachment of the apical microvilli (Fig. 6*d*). In addition, some renal cells exhibited irregularity, focal loss, and reduction in number and size of the apical microvilli.

The distal renal tubules cells showed little morphological changes with occasional electron latency and vacuolation of cytoplasm as well as occasional mitochondrial swelling. However, cytoplasmic bulges were seen in the distal convoluted tubules. The epithelial lining of the Henle's loop showed unchanged organelles while the collecting tubules showed minor and focal degenerative changes. In addition, no alterations were seen in the juxtaglomerular apparatus of rats exposed to ZnO nanomaterial. Moreover, the basolateral plasma membrane enfolding of the renal cells of this group of rats were not as deep as seen in the control ones.

4 Discussion

ZnO nanomaterials are invested widely in industry and nanomedicine with potential risk on human health and the environment. Recent studies indicated that these nanomaterials have potential oxidative stress in the hepatic tissues that may affect the function of the liver [34]. Some reports revealed that the kidney is one of the target organs of ZnO NPs and could cause damage in renal tissues [15, 35]. Reports indicated epithelial cells necrosis and gene toxicity resulted by nano-size ZnO with low mitochondrial membrane potential and loss of membrane integrity [36, 37]. In addition, ZnO nanomaterials were reported to affect monocytes and macrophages by initiating production of interferon tumour necrosis factor by the peripheral blood monocytes [38].

The findings of the present work pointed out that ZnO NPs could induce variable ultrastructural changes in the glomeruli and the renal tubules. The glomerial alterations include urinary space widening, basement membrane thickening, podocytes hypertrophy, endothelial enlargement, and mesangial cells proliferation. Widening of the urinary space may indicate accumulation of urine in this space. This might be resulted from the induced glomerular basement membrane thickening that may decrease the glomerular functional rate (GFR) and by then accumulate the urine in the urine space. It was reported that any change in the GFR leads to proteinurea [39]. The mesangial cells have contractile properties and contribute in regulating the filtration rate by altering resistance in the capillaries of the glomeruli. The thickening of GBM might be correlated with the proliferation of the mesangial matrix and considered a feature in many glomerular diseases. On the other hand, the swelling and detachment of the glomerular endothelial cells induced by ZnO nanomaterial might be a sign of nephrotoxicity and disturbance in reabsorption of sodium and water. Swelling and fusion of podocytes may facilitate widening of the glomerular filtration slits that may justify escape of proteins through the glomerular barrier.

The degenerative effect on the renal cells induced by ZnO nanomaterial may indicate interaction of these nanomaterials with cellular proteins. ZnO NPs have affinity towards hydrophobic proteins while nanomaterials always interact with cellular proteins forming corona [40]. In addition, the results of the current work pointed out PCTs cells vacuolation resulted from exposure to ZnO NPs. This ultrastructural injury may indicate changes in the permeability of the cell membrane of these cells that may inhibit proper cellular excretion of excess fluids leading to fluid accumulation in vacuolation form.

Our results showed that the mitochondria of the renal cell especially that line the proximal tubules were affected by the toxicity of ZnO NPs. The mitochondrial damage was demonstrated in the form of swelling and crystolysis. The induced swelling might be resulted from alterations in the osmolarity of the renal cells causing zinc ions influx to the mitochondrial matrix and affecting the integrity of the inner membrane. The access of ZnO NPs into the mitochondria may stimulate cytotoxicity by ROS generation that could cause damage and depolarize in the membranes of this organelle. Mitochondrial cristae play an important role in the function of this organelle [41–43]. The induced crystolysis by ZnO NPs may impair oxidative phosphorylation, ATP production, and reduce the efficiency of the electron transport system and lipid peroxidation [44].

The results of the present study showed that ZnO NPs exposure increased the number of lysosomes which may indicate the need to autophacocytosis of the affected renal cells to degrade the foreign materials and damaged organelles due to ZnO nanomaterial toxicity. This finding is online with some nano-studies indicated lysosomal autophagy induced by nanomaterials [45]. The increase in the number of lysosomal bodies in the PCT cells may indicate the need of the injured renal cells to sequester the foreign materials and damaged organelles.

The present work indicates that ZnO nanomaterial could target the renal cells nuclei inducing irregularity, pyknosis, and apoptotic activity. Apoptosis is a programmed cell death induced by oxidative stress [46]. This alteration might be resulted from the reduction of ATP production due to the mitochondrial damage induced by ZnO NPs. Furthermore, some toxicological studies explained that ZnO NPs exposure could induce necrosis and gene alterations [47]. The detected apoptotic activity in the renal cells of rats treated with ZnO NPs might be related to the intercellular stress induced by these fine particles. ZnO NPs were reported to induce apoptosis, p53 upregulation and increased cell cycle progression [40]. Oxidative dissolution of ZnO NPs and releasing of Zn^{2+} in the renal tissues could deplete tissues dissolved oxygen leading to ROS generation [41]. Zinc is a component of many enzymes and transcription factors [42]. The entrance of ZnO NPs into the cell increases Zn^{+2} content in the cytosol leading to disruption of cellular zinc homeostasis [22, 43]. This may indicate that the induced alterations by ZnO NPs may increase intracellular zinc ions, inducing oxidative stress, and interaction with the renal cells components [5].

The findings pointed out that DCTs were less affected than the PCTs. This may indicate that DCTs are more protected due to zinc ions absorption by the PCTs reducing the distal tubules exposure to the nanotoxicity.

5 Conclusions

One may conclude that exposure to ZnO NPs could induce renal ultrastructural alterations that may cause nephron damage and affecting the function of the kidney. The findings may raise the concerns about the potential risk on human health that might be related with exposure to ZnO NPs. More work is needed to elucidate probable pathophysiological alterations and renal toxicity that might be resulted from ZnO NPs exposure.

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