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Histopathological and Ultrastructural Effect of Zinc Oxide Nanoparticles on Male Wistar Rats Submandibular Glands

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Abstract: *Nanotechnology applications have occurred rapidly. Zinc oxide nanoparticles (ZnO NPs) have useful effects for their less hazards on the environment and for their application usages: paints, cosmetics, biomedicine and food as zinc supplements. ZnO NPs toxic effect is due to their tissue accumulation ability. Four ZnO NPs concentration levels effect was investigated on 75 male Wistar rats submandibular gland for 8 weeks. Treated groups histopathological sections showed progressive atrophy and degenerative changes in acini and striated ducts dilatation. Treated groups ultrathin sections showed small irregular nucleus, degenerated mitochondria, few zymogen granules, dilated rough endoplasmic reticulum and plenty of phagocytosed apoptotic bodies and lysozomes. Study results proved ZnO NPs toxicity as their doses are one of critical factors which are influencing their toxicity.*
Key words: *histopathology, ultrastructure, degeneration, atrophy, Zinc oxide nanoparticles.*
Abbreviations: NPs*: nanoparticles,* ***ZnO:*** *zinc oxide,* ***ZnO NPs:*** *zinc oxide nanoparticles.*

I. Introduction

Recently, rapid advances in nanotechnology have contributed to engineered nanoparticles manufacture, which are generally defined as particles with size range of 1–100 nm in one dimension [1]. Nanoparticles have two particular properties including their large surface area that dominates contributions of the material small bulks and their quantum effects. Atom fraction at their surface is increased and have high particle number per unit mass compared to microparticles or bulk [1].

In order to prevent nanoparticles toxicology, an important role in the development of safe and sustainable nanotechnology is required as there is currently little information available regarding their toxicological effects [2]. Physical and chemical properties of nanomaterials are expected to translocated to blood and interact with biological components of the cell of several organs which are considered to be the secondary major sites or sites of interaction, then induce significant effects on the cell structure [3]. Researchers suggest clearly that nanoparticles have a greater risk of toxicity than larger particles [4].

Zinc has focused on its essential roles in the body and its deficient state on growth, development and health [2]. Zinc oxide is one of the most commonly utilized materials in diverse industrial fields, medical diagnosis, sunscreens, cosmetics, personal care products and food additives. Relatively little attention has been directed toward toxic properties of ZnO NPs usages [5]. Along with extensive application of ZnO NPs in the industrial field, it is conceivable that the human body may be intentionally or unintentionally exposed to nanoparticles via several possible routes, including oral ingestion, inhalation, intravenous injection and dermal penetration, but gastrointestinal tract is one of the most important routes [6]. There is increasing evidence that food supplements usages included ZnO NPs by humans, even at fairly modest concentrations, may have adverse consequences [7]. It is suggested that ZnO NPs should be applied with more precautions in relevant industries and also occupational health surveillance should be necessarily considered [8]. The aim of this study was to investigate the destructive effects of ZnO NPs with four different concentration levels with size of 20 nm on submandibular gland of male Wistar rats as it is important to determine whether and how far the oral route exposures by ZnO NPs affect the tissue structure of submandibular gland.

II. Materials AND Methods

Materials: Zinc acetate $Zn(CH3COO)_2$, 2-propanol and sodium hydroxide were used in ZnO NPs synthesis and were obtained from King Abdullah institute for nanotechnology-King Saud University-KSA.

Animals and Experimental Design: experiment was carried out on 75 male Wistar rats (aged 75:90 days) with initial body weight 250:300 g. Rats were housed in clean and ventilated cages with constant controlled climate (at Faculty of Pharmacy, King Saud University, KSA). All groups, received filtered tap water ad libitum and standard rodents chow diet (19.80% protein, 39.25% carbohydrate, 4.41% fat, 13.25% fibre, and 2.76 kcal/g of metabolizable energy). Negative control group [**group1 (n=15)]:** received drinking water only. Treated groups received ZnO NPs in aqueous solutions by gavage for 8 weeks (according to Organization for Economic Cooperation and Development (OECD) guideline), as: **group2 (n=15)**: received 40 mg ZnO NPs/mL daily,

group3 (n=15): received 70 mg ZnO NPs/mL daily, **group4 (n=15):** received 100 mg ZnO NPs/mL daily, **group5 (n=15):** received 150 mg ZnO NPs /mL daily.

Methods: synthesis, characterization and dosage preparation of ZnO NPs (at King Abdullah institute for nanotechnology, KSA): ZnO NPs were prepared by precipitation of ZnO NPs by adding drop wise of 0.6 M NaOH solution to 0.2 M $Zn(CH3COO)_2$ which is dissolved in 2-propanol solution with heating (35:37 C^o) [9]. ZnO NPs morphology was examined by transmission electron microscopy [10]. ZnO NPs were suspended in normal saline buffer, then dispersed by vortexing for one minute to prepare a stock solution with a concentration of 500 mg ZnO NPs /mL as the final suspension pH was 7.3. The required doses of ZnO nanoparticles were then prepared with concentrations (40, 70, 100 and 150 mg ZnO NPs/mL normal saline).

Histopathological and Ultrastructural Investigations (Faculty of Sciences, King Saud University, KSA)**:** At 8^{th} week end, animals were anaesthetized (0.1 mL i.p. of 1% sodium barbiturate), then, immediately, submandibular glands of 10 animals from each group were dissected. Histopathological investigations occurred as samples (2-3 cm^3) were fixed in 10% formalin neutral buffered for 24 h, washed, dehydrated, cleared, embedded in paraffin and processed into 5 µm sections for light microscopic examinations by hematoxylin and eosin stain (H&E) [11]. Ultrastructural investigations occurred as samples (1-2 mm^3) were fixed in 3.6% glutaraldehyde at 4 °C for 2 h, postfixed in 2% osmium tetroxide, dehydrated, cleared and embedded in Epon 812. Ultrathin sections were contrasted with uranyl acetate and lead citrate for a transmission electron microscope investigations [12].

III. Results

Characterization of ZnO NPs: ZnO NPs prepared sample morphology was investigated by transmission electron microscopy. ZnO NPs sample appeared as aggregated hexagonal shape particles with mean diameter 20 nm (Figure 1).

Histopathological and Ultrastructural Investigations: Hx & E sections from control group submandibular glands showed the gland composition of several lobules separated by connective tissue septa. Lobules were composed of seromucoas acini (predominantly serous acini), striated (intralobular) and granular (intercalating) convoluted ducts. Intercalating (granular) convoluted duct were lined by basal round nucleus cuboidal cells as their striation were less obvious. Striated intralobular ductal cells were columnar with rounded central nucleus with basal striation (Figure 2A). Acinar cells were pyramidal in shape, surrounded by numerous basket (myoepithelial) cells and possess secretory granules in cytoplasm apical part as nucleus was pushed to cell base (Figure 2B). After 40 mg ZnO NPs/mL induction for 8 weeks in group 2, intralobular ducts and acini showed atrophic changes. Affected acini became small and their lining cells had vacuolated cytoplasm. Striated (intralobular) duct lost their striation and had dilated luminae (Figure 2C). After 70 mg ZnO NPs/mL induction for 8 weeks in group 3, degenerative changes and apoptotic bodies were observed. Acinar cells were shrunken with atrophic vacuolated cytoplasm. Intralobular duct had a wide lumen, lost striation and lined with atrophic epithelial cells with pyknotic nuceli (Figure 2D). After 100 mg ZnO NPs/mL and 150 mg ZnO NPs/mL induction for 8 weeks in group 4 and 5 respectively, there were severely increasing of submandibular gland alterations which were previously appeared in group 2 and 3. Also, connective tissue appeared with obvious fibrosis around striated (intralobular) ducts, granular ducts and acini (Figures 2E, 2F). Submandibular gland ultrastructure of negative control group showed acinar cells with oval nuclei, parallel channels of rough endoplasmic reticulum, Golgi Apparatus, oval/elongated mitochondria with numerous crests, moderate electron density matrix and plenty of dark spherical secretory (zymogen) granules in the apical of cytoplasm. Cellular membranes of adjacent cells were joined together by desmosal junctions (Figures 3A, 3B). After 40 mg ZnO NPs/mL induction for 8 weeks in group 2, acinar cells were observed with irrgular nucleus, heterogenic chromatin condensation, degenerated mitochondria with loss of their cristae, few zymogen granules and phagocytosed apoptotic bodies appeared (Figure 3C). After 70 mg ZnO NPs/mL induction for 8 weeks in group 3, in addition to observed alterations in group 2, dilatation of rough endoplasmic reticulum and plenty of cytoplasmic secretory vacuoles were noticed (Figure 3D)**.** After 100 mg ZnO NPs/mL induction for 8 weeks in group 4, previous alterations were observed besides excessive vacuolation in degenerated cytoplasm with high electron density and lysozomes appearance (Figure 3E). After 150 mg ZnO NPs/mL induction for 8 weeks in group 5, there were excessive appearance of all last alterations which were observed in groups 2,3, 4 and 5 (Figure 3F).

IV. Discussion

Nanotoxicology is a field of study centered on trying to understand how nanomaterials may affect cellular function and their degree of toxicity [13]. Bibliometric indicators suggest that research into environmental concern of NPs has increased since the first paper on nanoecotoxicology was published in 2006 [14]. Retention of ZnO NPs in the environment and food chain is high and continuous exposure to them may affect human health [7]. ZnO NPs toxicity in mice occurred when exposed via digestive tract [15]. Pathological

changes and cytotoxicity induced by ZnO NPs were both size, time and dose dependent. ZnO NPs can produce irreversible damage to cells by oxidative stress or/and organelle injury [16]. Researchers investigated that human/rodent cells died after exposure to ZnO NPs concentrations above 15 ppm [17].

In recent study, we investigated effect of four concentration levels of ZnO NPs on submandibular gland cell structures as histopathological and ultrastructural changes in secretory cells of rats submandibular salivary gland were ZnO NPs concentration-dependent. Histopathological investigations of treated groups showed that there were intralobular ducts and acini atrophic changes, degenerative changes and apoptotic bodies. **However,** ultrastructure sections of treated groups showed that there were heterogenic chromatin condensation, degenerated mitochondria, few zymogen granules. These results agreed with [18] and [15]. Toxic effects of ZnO NPs are due to their solubility, resulting in increased intracellular [Zn^{2+}] [19] and this results in cytotoxicity, oxidative stress and mitochondrial dysfunction [20]. ZnO NPs are expected to increase inflammation in lymph nodes and cells involved in the inflammatory reaction as ZnO NPs are taken up inside the cells. ZnO NPs with high activity and influence their target tissues by their penetration, then, immediately, a thick coating of NPs that cannot be absorbed by phagocytes, entered lymph flow, accumulated in lymph nodes and ultimately to body tissues and organs [21]. According to this ability and resulting from their small size which allows them to penetrate physiological barriers and travel within the circulatory systems [22]. Once NPs are located in the cytoplasm, coarse grain materials will present which can cause direct damage and cell death as are caused by many interactions [23]. Understanding the specific mechanisms of NPs and their interaction require very extensive researches in this field.

V. Conclusion

We conclude that the development of nanotechnology and nanotoxicology study have increased our awareness of environmental particulate pollution generated from natural and anthropogenic sources. We hope that this new awareness will lead to significant reductions in human exposure to these potentially toxic materials. We recommended to increase knowledge and ongoing study to find cures for diseases associated with nanoparticles exposure.

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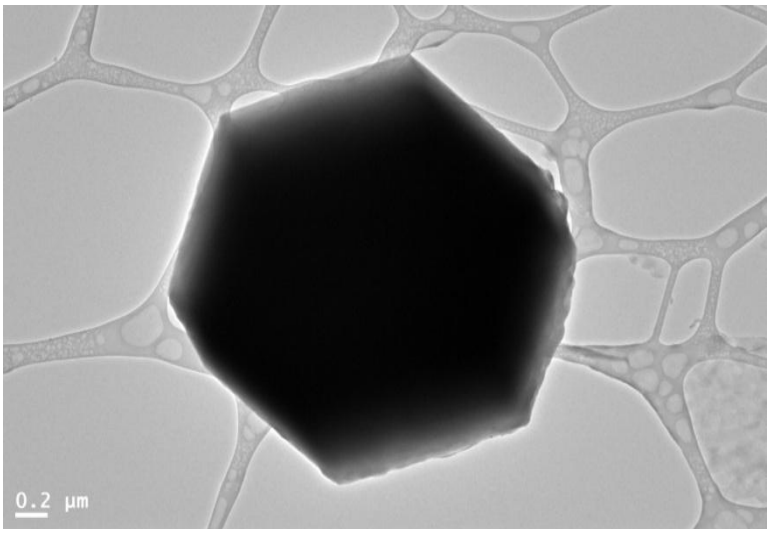

Figure 1: Transsimistion electron microscope (TEM) images of ZnO nanoparticles sample. Images was captured in 0.2 micrometer scale bar.

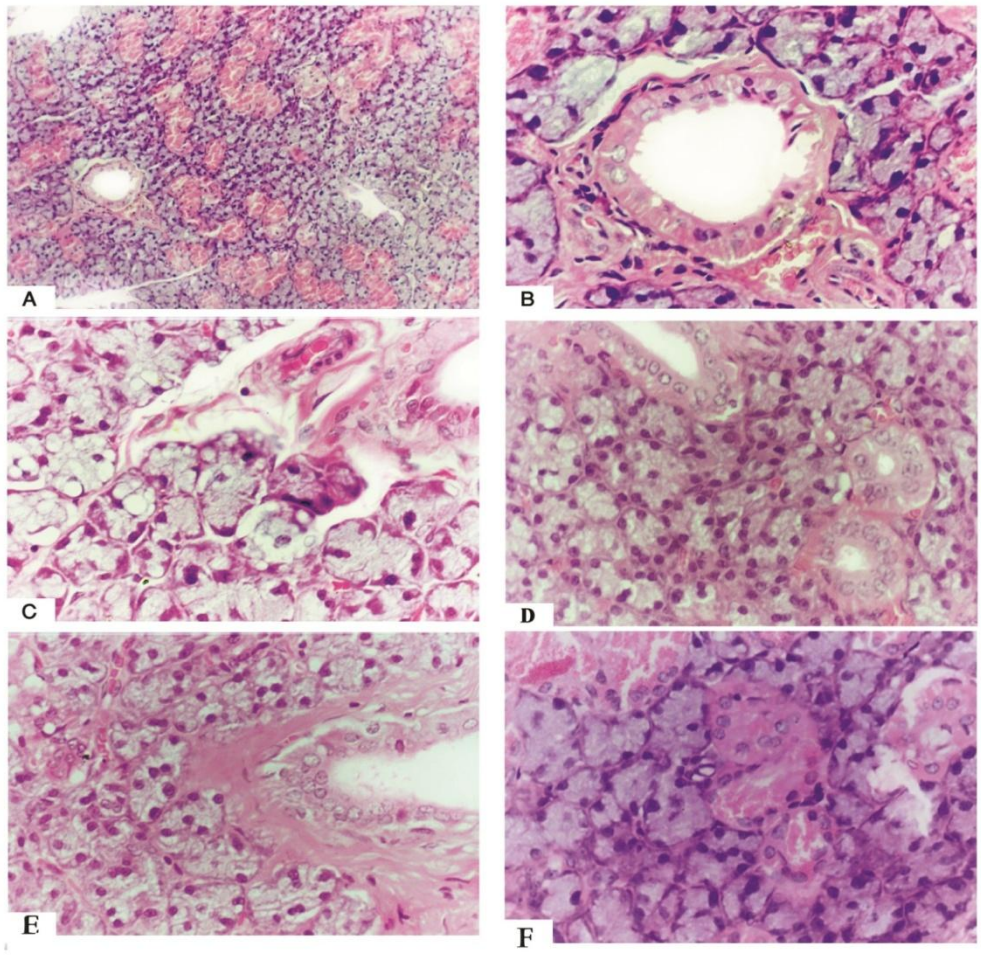

Figure 2: Histopathological findings of male Wistar rats submandibular gland (transitional section) by H&E stain. **[A (X100), B (X400)]** showed normal histological findings in the control group as: seromucoas acini,

striated (intralobular) lined by columnar cells, granular (intercalating) convoluted ducts lined by cuboidal cells, numerous basket (myoepithelial) cells appeared. **[C (X400)]** showed alterations after 40 mg ZnO NPs/mL induction as: atrophic and vacuolated intralobular ducts and acini cells appeared. Striated duct had dilated luminae. **[D (X400)]** showed alterations after 70 mg ZnO NPs/mL induction as: degenerative changes and apoptotic bodies were observed. Acinar cells were shrunken with atrophic vacuolated cytoplasm. Intralobular duct had a wide lumen, lost striation and lined with atrophic epithelial cells with pyknotic nuclei. **[E (X400)]** showed alterations after 100 mg ZnO NPs/mL induction as: connective tissue appeared around striated ducts, granular ducts and acini with sever atrophic vacuolated cytoplasmand obvious dilatation. **[F (X400)]** showed alterations after 150 mg ZnO NPs/mL induction as: increasing connective tissue with obvious fibrosis around striated ducts, granular ducts and acini appeared.

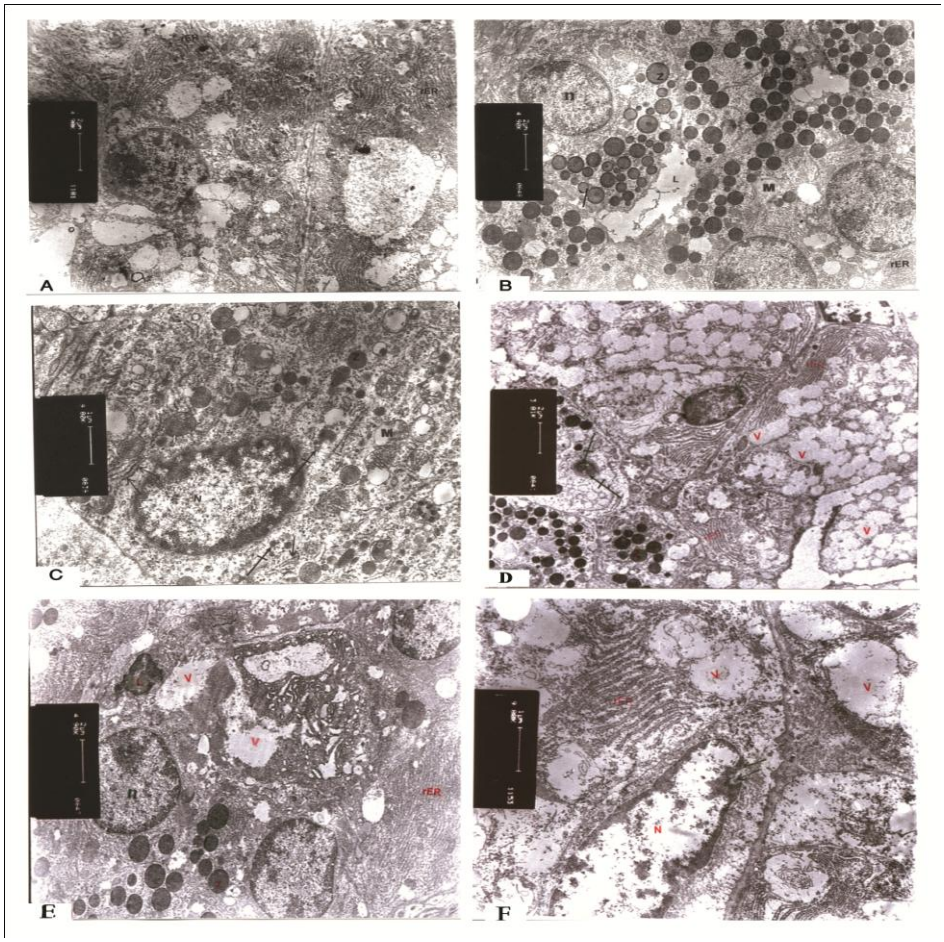

Figure 3: Ultrastructural findings of male Wistar rats submandibular gland by transsimistion electron microscope (TEM). **[A (X9800), B (X400)]** showed normal findings in the control group as: nucleus (n), abundant amount of rough endoplasmic reticulum (rER), plenty of dark spherical zymogen granules (Z), acini lymen (L) and mitochondria which clearly are visible (M). **[C (X19600)]** showed alterations after 40 mg ZnO NPs/mL induction as: secretory cell processes (arrow), irregular nucleus (N) with heterogenic chromatin condensation and degenerated mitochondria (M), few zymogen granules (Z), presence of phagocytosed apoptotic bodies (cross arrow) were presented. **[D (X19600)]** showed alterations after 70 mg ZnO NPs/mL induction as: dilatation of rough endoplasmic reticulum (rER) and plenty of cytoplasmic secretory vacuoles (V), few zymogen granules (Z), irregular nucleus (arrow) with heterogenic chromatin condensation presence of phagocytosed apoptotic bodies (cross arrow) were presented. **[E (X19600)]** showed alterations after 100 mg ZnO NPs/mL induction as: excessive vacuolation in degenerated cytoplasm (V) with high electron density and lysozomes appearance, irregular nucleus (N) and dilated rough endoplasmic reticulum (rER) were presented. **[F (X19600)]** showed alterations after 150 mg ZnO NPs/mL induction as: irregular pyknotic nucleus (N), dilated rough endoplasmic reticulum (rER), excessive vacoulation of degenerative cytoplasm (V).