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Original Research Article

ANATOMICAL AND HISTOPATHOLOGICAL EFFECTS OF ZINC OXIDE NANOPARTICLES ON LUNGS IN ADULT MALE ALBINO RATS

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ABSTRACT

Background: Zinc oxide nanoparticles (ZnO-NPs) are frequently used in many fields, including food industry for their antimicrobial activity. Acute exposure to high doses of such particles was found to be toxic to many organs. However, the lung toxicity resulting from chronic exposure to oral doses of ZnO-NPs was not adequately assessed before.

Aim of the work: to detect the anatomical and histopathological effects of chronic exposure to ingested ZnO-NPs on the lung of normal adult male albino rat.

Material and methods: It was carried out on 30 adult male Swiss albino rats with an average weight of 150-200 gm. They were divided into two groups: Group I: 10 rats serving as control group; Group II: 20 rats serving as experimental groups, divided into 2 subgroups (a&b) receiving oral ingestion by orogastric tube of a single daily dose (125mg/ kg) of average 20 nm sized ZnO-NPs for different durations: Group IIa (n=10): for 120 days; Group IIb (n=10): for 180 days. Histopathology and immunohistochemistry of the lungs in the three groups was performed to detect the possible effect of such exposure.

Results: Oral administration of ZnO-NPs induced lung damage manifested by congested blood vessels, interstitial inflammation, infiltration with macrophages& lymphocytes, supurative granuloma, thickened interalveolar septa. These changes were more evident with longer exposure for 180 days ($P \leq 0.5$). This substantial damage to the lungs is caused by oxidative stress and chronic inflammation.

Conclusion: Caution should be considered when using these particles in food packaging and food additives, and for those who are in close contact with these particles especially in factories.

KEY WORDS: ZnO-NPs, Oral ingestion, Immunohistochemistry, lungs.

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INTRODUCTION

The use of nanomaterials has a wide range of medical applications which are constantly growing. They are currently being engineered and tailored according to the requirements of the scientists. Nanoparticles have been developed for the food industry such as food storage, and also as a nutritional ingredient [1-3].

Zinc oxide nanoparticles (ZnO-NPs) are amongst the most widely used NPs. They are added into different materials and products and especially used in food industries for their antimicrobial activity [4].

In vitro studies had reported that among the

metal oxide nanoparticles, ZnO-NPs induced the higher toxicity [5].

ZnO-NPs can enter the human body through inhalation, ingestion or injection, causing adverse biological and toxic reactions in many organs including spleen, stomach, pancreas, kidney, brain, eye, heart, and liver [6-10].

The lungs are particularly susceptible to ZnO-NPs because of its high vascularity and ability to concentrate toxins [11, 12].

Up to our knowledge, the vast majority of the previous studies on lung toxicity of ZnO-NPs were addressing the short term exposure to it, and mostly the exposure was through inhalation and intratracheal instillation [13-15].

The toxic effect of short duration exposure to orally ingested ZnO-NPs on lungs was assessed in previous studies [10, 12].

However, the observation period is important when assessing the persistence of inflammation in an animal model. Accordingly, the present study was conducted to detect the anatomical and histopathological effects of chronic exposure to ingested zinc oxide nanoparticles on the lung of normal adult male albino rat. Unlike the previous research in this field, the current study is characterized by a longer period of chronic exposure to repeated oral administration of ZnO-NPs up to 180 days. The oral route of administration was selected because these particles are frequently used in packaging of food. There is also a risk of ingestion even when dealing with other forms of products containing these particles.

MATERIALS AND METHODS

Preparation and characterization of ZnO-NPs: ZnO-NPs were prepared using chemical methods (Hydrothermal) from initial precursors [16]. These precursors were purchased from ElSafwa chemical company.

The prepared ZnO-NPs were analyzed using X-ray diffractometer (XRD), transmission electron microscopy (TEM) and zeta potential analyzer [17-21].

Experimental animals and treatment: The present study was carried out on 30 adult male Swiss albino rats obtained from faculty of agriculture, Alexandria University; each of an average weight ranging from 150-200 gm and of age about 6-8 weeks.

The animals were kept under standard laboratory conditions in a 12-hour light/dark cycle, at a temperature of 22°C ± 2°C with relative humidity 50% ± 20%. The animals were handled according to the code of ethics for experimental research adopted by Alexandria, Faculty of Medicine.

The animals were randomly divided into 2 groups:

Group I: (control group) 10 rats, received only the vehicle (distilled water) by oral gavage tube.

Group II: (experimental group): 20 rats, receiving oral ingestion by orogastric tube of a single daily dose (125mg/kg) of ZnO-NPs in an average size of 20 nm sized for two different durations:

They are divided into2 subgroups

Group IIa (n=10): treated with ZnO-NP for 120 days

Group IIb (n=10): treated with ZnO-NP for 180 days [22].

Histopathologic study: At the end of each duration for administration of ZnO-NPs, the animals were anesthetized and sacrificed. The lungs were immediately dissected out, fixed in formalin then embedded into paraffin tissue blocks. The blocks were:

a. Cut into five microns thick sections.

b. Stained with standard Hematoxylin & Eosin (H&E) stain and examined for evaluation of parenchymal architecture, the presence or absence of inflammatory cell infiltrates, evidence of lung injury, lymphoid agglomerates or fibrosis.

Immunohistochemistry staining: Evaluation of the degree of pulmonary damage was performed via two immunohistochemical markers. The first being CD68 a marker of macrophages to quantify the number of macrophage infiltrates within the lung parenchyma. The pro-inflammatory mediator COX-2 was used to elucidate the intensity of inflammation within the lung tissue. Four microns thick tissue sections of rat lung paraffin blocks were cut and mounted onto positively charged slides. Heat induced antigen

retrieval using citrate buffer at pH 6 was performed. Immunostaining using monoclonal CD68 (Invitrogen # MA5-13324) primary antibody, at a dilution (1:200) and monoclonal COX-2 (Invitrogen #35-8200) primary antibody, at a dilution (1:50) , was performed, followed by Streptavidin-HRP conjugate (Thermoscientific # D22187) and developed using DAB chromogen with counterstaining using Meyer's Hematoxylin.

Image analysis for CD68 and COX-2 immunostaining:

Sections were analyzed and images were captured with a microscope digital camera. The image analyser (ImageJ version 5, National Institutes of Health, MD, USA) was used to count the number of macrophages positive for CD68 in ten random high power fields in the lung sections for each animal. The results were expressed as a mean total count for the number of macrophages for each animal.

The mean area and area percent of COX-2 expression was measured in ten high power fields in each specimen of all animal groups using the image analyzer computer system. The results were expressed as mean density of COX-2 expression in each animal. The mean values of the different groups were calculated and statistically compared using the ANOVA test using SPSS program version 19.

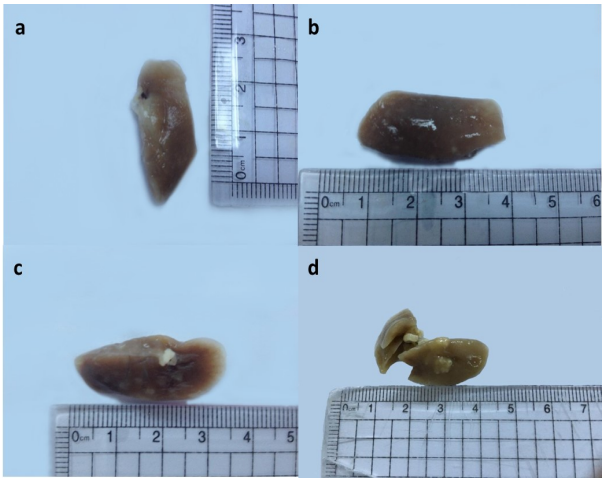
RESULTS

Particle characterization: The average diameter of the prepared ZnO-NPs was 20 nm with a positive surface charge.

Mortality rate during the experiment: In the present study there were three mortalities reported during ZnO-NPs administration period: one rat from subgroup IIa, and two rats from subgroup II b.

Gross features: The naked eye appearance of the rat lungs in the control group (I) showed normal smooth surface and normal cut section with unremarkable pathological changes. However, lungs from group IIa & IIb showed micronodularity of the outer surface with multiple small greyish-white nodules scattered throughout the whole lung, some of them were remarkably large (Figure 1).

Fig. 1: A photograph of naked eye appearance of the rat lungs



a) control lung showing unremarkable pathological changes, b) lung from group IIa showing vague micronodularity of the outer surface, c) cut surface of the lung at photo (b) showing multiple small greyish-white nodules scattered throughout the whole lung, d) lung from group IIb showing a single large central greyish-white nodule.

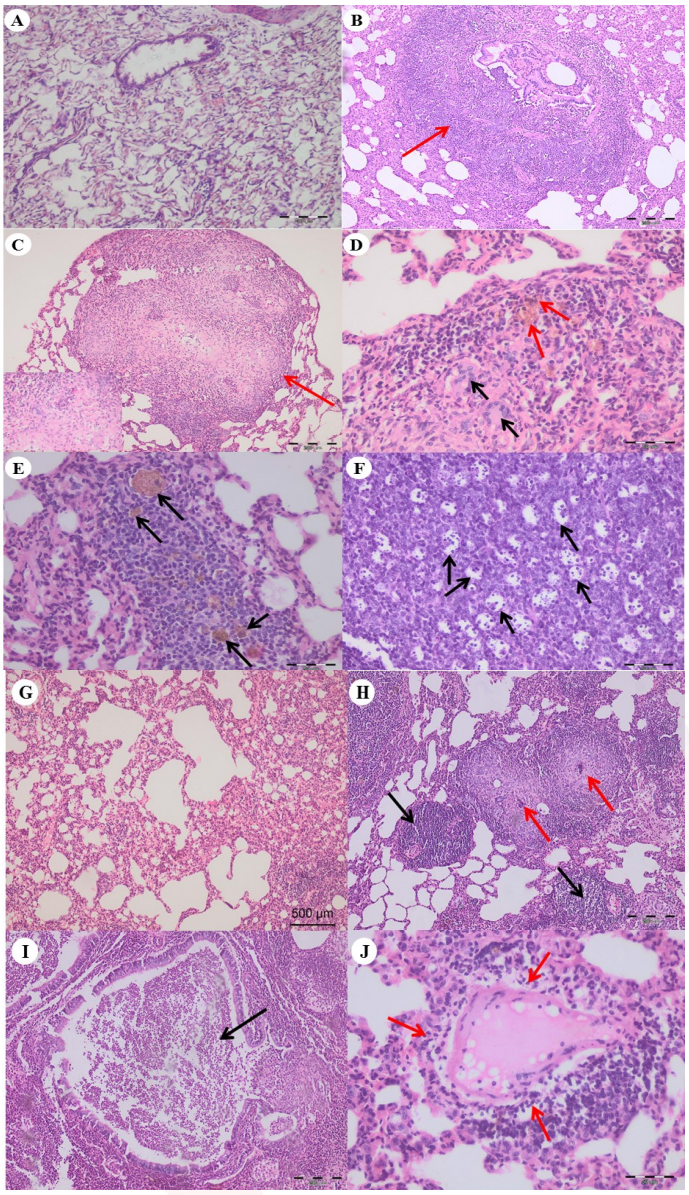
Lung Histopathology (H & E):

Control Group (I): Light microscopic examination of the paraffin sections of rat lung of the control group revealed normal architecture of the alveoli as evident by distended alveoli separated by thin interalveolar septa. The alveoli were lined by thin flat type I pneumocyte and few cuboidal type II pneumocyte that appeared bulging into alveolar lumen (Figure 2, a).

Test groups (II): ZnO-NPs induced diverse chronic pathological lung lesions when rats were treated for both durations, 120 & 180 days.

Group IIa (120 days' exposure): Microscopic examination revealed disturbed pulmonary architecture with focal honey-combed appearance of the lung parenchyma. Intense interstitial inflammation involving the alveolar walls and bronchovascular sheaths is present. The alveoli are lined by hyperplastic type II pneumocytes and show cystic dilation in areas. The interstitium shows heavy infiltration by macrophages, small mature lymphocytes and plasma cells with occasional lymphoid follicle formation (Figure 2, b& c). The lymphoid follicles show numerous apoptotic bodies within (Figure 2, f). Multiple granulomata are seen composed of epithelioid cells, multinucleated giant cells as well as a collar of lymphocytes and plasma cells. Macrophages within and outside the

Fig. 2: A photomicrograph of rat lung in different groups (H&E):



A- lung of control group showing patent alveoli and a small bronchiole; note the absence of interstitial and peribronchial inflammatory cellular infiltrates (200X).
B- lung of group IIa showing evidence of follicular bronchiolitis, with lymphoid follicle formation around the terminal bronchioles (arrow). The surrounding lung parenchyma shows honey-combing with interstitial inflammatory infiltrates (100X).
C- lung of group IIa showing granuloma formation (arrow) composed of aggregates of epithelioid cells surrounded by lymphoplasmacytic infiltrates and compressing the surrounding alveoli (100X). Inset: A high power view of the granuloma (400X).
D- lung of group IIa showing aggregates of macrophages engulfing a brown-stained particulate matter (NPs) (red arrows). Multinucleated giant cells within the granuloma are noted (black arrows) (400X).
E- lung of group IIa showing aggregates of macrophages engulfing a brown-stained particulate matter (NPs)within a lymphoid follicle (black arrows) (400X).
F- lung of group IIa showing a lymphoid follicle within the alveolar walls showing numerous apoptotic bodies (arrows) (400X).
G- lung of group IIb showing intense interstitial inflammation with honey-combed appearance as well as cystic-dilation of the alveoli (40X).
H- lung of group IIb showing multiple intrstitial granulomata (red arrows) as well as follicular bronchiolitis (black arrows) (100X).
I- lung of group IIb showing a dilated bronchiole with intraluminal inflammatory exudates (arrow) and intense inflammation of the submucosa (100X).
J- lung of group IIb showing a blood vessel with evidence of inflammatory infiltrates within its wall (arrows) (400X).

granulomata are seen to engulf brown-stained particulate material which most probably represent the ZnO-NPs (Figure 2, d&e). Congested vascular spaces along with focal evidence of vasculitis within the interstitium are appreciated. Evidence of follicular bronchitis in the form of lymphoid follicles and plasma cells surrounding the distal bronchi and bronchioles, infiltrating the fibromuscular wall and occasionally compressing their lumina is noted. However, the bronchial epithelium shows no evidence of hyperplasia, reactive changes, metaplasia, or neoplasia.

Group IIb (180 days' exposure): Microscopic examination revealed similar pathological changes to the previously mentioned group IIa, however with greater severity. These changes are appreciated in the greater number and coalescence of the formed granulomata. In addition a few bronchopneumonic patches are recognized within the lungs in the form of acute inflammatory exudates filling the alveolar spaces as well as inflammatory infiltrates surrounding the bronchioles. Honey-combed interstitial inflammation occupies wider areas of the lungs. Of note, is the dominance of chronic inflammatory cellular infiltrates over the presence of fibroblastic lesions within the interstitium. More frequent macrophages are noted within the interstitium engulfing the foreign particulate matter (Figure 2,g-j). An appreciable reduction in the vascular congestion was noted in this group.

Immunohistochemical results:

Macrophage immunostaining using CD68 and image analysis: Microscopic examination of the lung specimens of all of the groups revealed positive cytoplasmic staining within the macrophages which were found to reside within the lung interstitium and the alveolar septae (Figure 3).

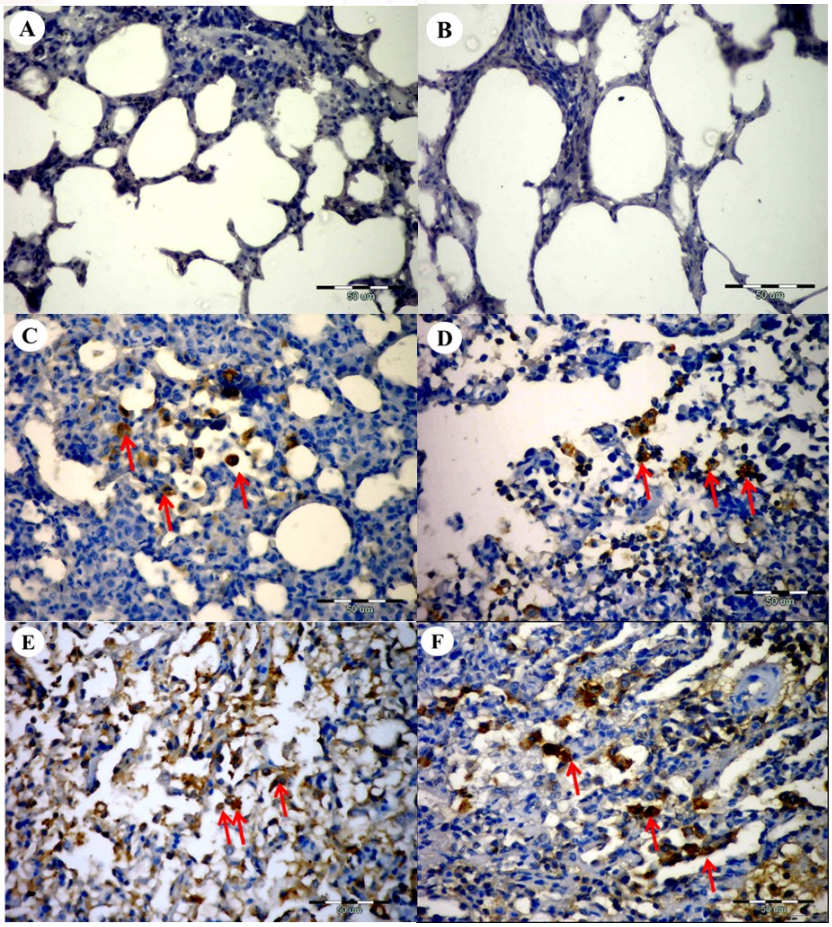
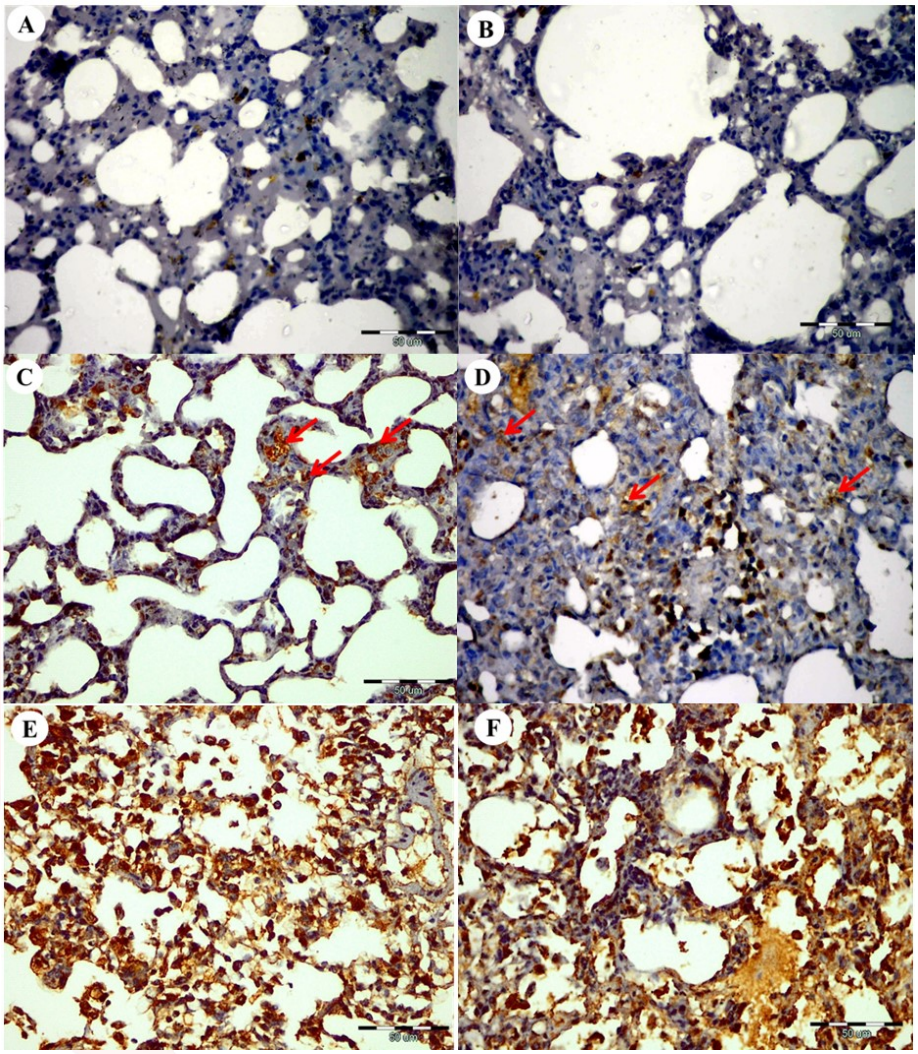


Fig. 3: Immunostaining using CD68:

A&B: lungs of control group showing absent CD68 stained macrophages within the alveolar septae and interstitium (100X).
C&D: lungs of group IIa showing few positively stained macrophages within the interstitium and alveolar septae (arrows) (100X).
E&F: lungs of group IIb showing numerous positively stained macrophages within the interstitium and alveolar septae (arrows) (100X).

COX-2 expression and image analysis: The immunohistochemical staining with COX-2 antibodies appeared as positive cytoplasmic staining within the cellular infiltrates of the lung interstitium and alveolar septae (Figure 4).

Fig. 4: COX-2 expression:



A& B: lungs of control group showing minimal COX-2 cytoplasmic expression in the interstitial cells (100X).

C&D: lungs of group IIa showing a moderate COX-2 cytoplasmic expression in the interstital cellular infiltrates (arrows) (100X).

E&F: lungs of group IIb showing intense COX-2 cytoplasmic expression within the interstitial and alveolar cellular infiltrates (100X).

The mean number of infiltrating macrophages was significantly higher in group IIb in comparison with the other groups ($p \leq 0.05$). (Table 1; Graph 1).

Table 1: Comparison between the three studied groups regarding the cell count.

Cell Count	Control group (I)	Group IIa	Group IIb
Range	6.0 – 8.0	15.0 - 33	46 - 103
Mean	7.13	24.08	76.7
S.D.	0.83	6.02	19.4
F	22.65		
P	0.001*		
P1	0.003*		
P2	0.0001*		
P3	0.001*		

Graph 1: Comparison between the three groups regarding the cell count.

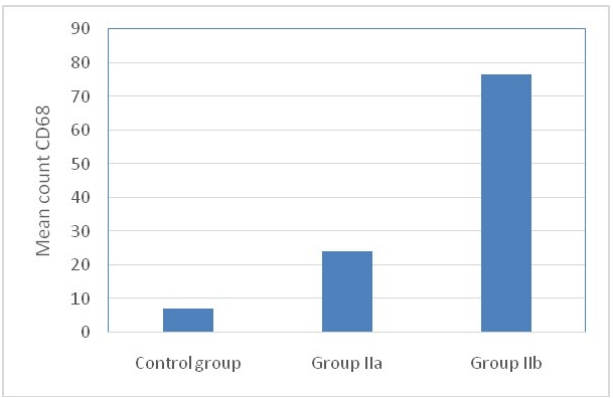


Image analysis of the staining density in the control group lungs showed the least density when compared to other studied groups. Group

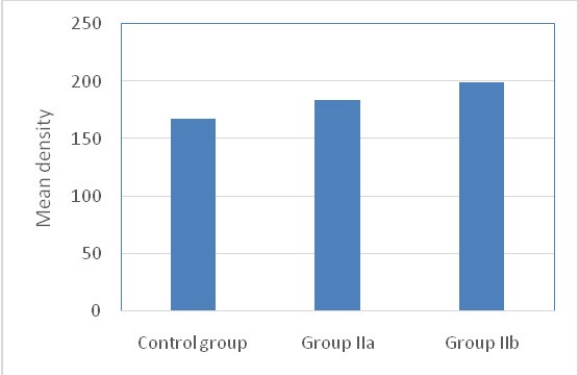
IIb showed the highest density of staining (COX-2 expression) when compared to other groups. There was a significant difference between the control group (I) and both of group IIa & IIb ($p \leq 0.05$), but the difference was insignificant between group IIa & group IIb (Table 2; Graph 2).

Table 2: Comparison between the three groups regarding COX-2 immune staining density.

density	Control group (I)	Group IIa	Group IIb
Range	163.5 - 172.5	173.085 - 203.942	192.787 - 208.739
Mean	167.55	183.43	199.01
S.D.	3.77	13.9	6.03
F	12.37		
P	0.013*		
P1	0.042*		
P2	0.011*		
P3	0.236		

F: ANOVA test
*= statistically significant at $p \leq 0.05$
P1 comparison between control group (I) and group (IIa)
P2 comparison between control group (I) and group (IIb)
P3 comparison between group IIa and IIb

Graph 2: Comparison between the three groups regarding COX-2 immune staining density.



DISCUSSION

The increasing uses of ZnO-NPs in different applications may increase the possibility of different organs' exposure to these particles.

The nanoparticles when ingested into the body could access different organs because of their small size. Previous studies on ZnO-NPs toxicity have revealed that mostly the target organs after oral exposure were the liver, kidney, stomach, and spleen [9, 10]. However, lung toxicity after exposure to prolonged oral ingestion of ZnO-NPs was not addressed enough in previous literature.

The current study was designed to determine the possible toxic effect of chronic oral administration of environmental-equivalent doses of ZnO-NPs on rats' lungs. The dose was selected according to the findings of Chung et al. who reported that repeated oral exposure to ZnO-NPs up to the dose of 125 mg/kg could accumulate in the systemic circulation; accordingly, they concluded that the NOAEL (no observed adverse effect level) values could be less than 125 mg/kg through oral ingestion [22].

The present study shows that prolonged oral exposure to ZnO-NPs leads to marked inflammatory lung injury; and this injury becomes more manifested with longer periods of exposure. These results were in agreement with the findings of a previous study of intratracheal instillation of two doses of ZnO-NPs (50 and 150 cm2/rat) into rats, with assessments made at 24 h, 1 wk, and 4 wks after instillation to evaluate dose- and time-course responses. The results revealed influx of eosinophills, fibrosis and marked injury of airway epithelium [11].

Shokouhian et al. assessed the effect of different oral doses of ZnO-NPs (100, 200, and 400 mg/ kg) on the rat's lung; however, the duration of exposure was for 2weeks only. They reported that this leads to increase in serum inflammatory markers as LDH, IgG, TNF- α and IL-6. Moreover, high concentration of ZnO caused irreversible damage to the lung manifested by interstitial fibrosis, and atelectasis which was evident in the gross features of lungs and histopathologically [12]. These findings were in agreement with the findings of the present study; however, the main difference is that Shokouhian et al. study was dose dependent while the present study was duration dependent and the dose was fixed at a much lower level.

Vandebriel and De Jong reported similar histopathological findings of lung damage as those of the current study although the way of exposure was through inhalation of ZnO-NPs. Signs included evidence of phagocytosis by macrophage, lymphocytic perivasculitis and pribronchiolitis and interstitial inflammation [7].

The findings of previous studies suggested that other routes of exposure to ZnO-NPs as intratracheal instillation could lead also to severe tissue damage and could progress to chronicpathological conditions [14, 15].

The oxidative stress is a known contributor to

pulmonary toxicity induced by ZnO- NPs. It was proved recently that, the mechanisms underlying the toxicity of ZnO-NPs depend on the induction of apoptosis [23, 24].

Significant increase in the levels of pro-inflammatory mediator (COX-2) in lung tissue indicated pulmonary damage. Moreover, alveolar and interstitial macrophages have been identified as important regulators of pathological lung processes [25, 26].

Qiao et al. provided evidence that the pulmonary inflammation induced by exposure to ZnO-NPs is through modulation of miRNA expression [27].

Fukui et al. reported that the inflammatory response and also the oxidative stress induced by ZnO-NPs were prevented by co-treatment with ascorbic acid in human lung carcinoma A549 cells [24]. However, further studies are recommended to investigate this protective effect on chronic lung inflammation induced by long term exposure to oral ingestion of ZnO-NPs.

CONCLUSION AND RECOMMENDATIONS

Exposure to chronic oral administration of ZnO-NPs showed substantial damage to rat' lung; accordingly, caution should be considered for those who are in close contact with these particles especially in factories. Moreover, these hazards should be considered when using these particles in food packaging and food additives.

Conflicts of Interests: None

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